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R.P. Bhole[1*], M.R. Swamy[1], R. Wavhale[1], C.G. Bonde[2], R. Chikhale[3]

[1]Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, India;
[2]NMIMS School of Pharmacy, Shirpur, Dist: Dhule, India;
[3]UCL School of Pharmacy, London, United Kingdom
([*]Corresponding author's e-mail: ritesh.bhole@dypvp.edu.in)

Design of potential vitamin-drug conjugate for enhanced anticancer activity

5-Fluorouracil, a primary molecule widely used in the treatment of various cancer stages, is rapidly metabolized to an inactive form, namely 5,6-dihydro-5-FU and various mutational changes in chemotherapy. We utilized a carbodiimide catalyst to form a conjugate with folic acid. As folic acid receptors are over-expressed in cancerous tissues, it increases the bioavailability of 5-FU. This work represents design and synthesis of the new vitamin-drug conjugate, possibly enhancing anticancer activity. 5-Fluorouracil has potent action on breast, colorectal, stomach, and skin cancer tissues. Folic acid aided in targeting FRα receptors of cancer cells selectively. 5-FUFA was subjected to spectral characterization to confirm successful conjugation. The molecular dynamics simulation was studied in the Schrodinger suite and validated by molecular trajectory in CPPTRAJ software. This conjugate was further studied for molecular modeling studies and the docking score of the conjugate represented a higher binding score than 5-FU, i.e., –8.0 Kcal/mol. The drug-receptor interaction was further validated using molecular dynamics simulation in the Schrodinger suite and molecular trajectory CPPTRAJ software for 100 ns. The molecular dynamics simulation results showed stability with slight conformational change at 25 ns from 2–4 Å.

Keywords: 5-Fluorouracil, folic acid, synthetic conjugate, molecular docking, molecular dynamics, drug design, chemotherapeutic agent, human thymidylate synthase.

Introduction

5-Fluorouracil (5-FU) has been 3rd most commonly prescribed antimetabolite cytotoxic drug in treatment of various malignant forms of tumors, namely breast, head, and neck, as well as pancreas, stomach, and skin since 1957. It is an aromatic water soluble heterocyclic compound structurally common to pyrimidine nitrogenous base pairing found in DNA and RNA. 5-FU is a prodrug, which is bio activated by nucleotides, namely 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-FdUMP), 5-fluoro-2'-deoxyuridine 5'-triphosphate (5-FdUTP) and 5-fluorouridine triphosphate (5-FUTP) [1–5]. 5-FU administered IV for tumors located within body tissues has several issues. It is rapidly metabolized into inactive form i.e., 5,6-dihydro-5-FU (80–90 %). It has low bioavailability and non-specificity to cancer cells. Large doses, namely 400–600 mg/m^2 are needed for optimum therapeutic effect. Another primary reason of resistance to 5-FU was observed due to overproduction of Bcl-2, Bcl-XL, Mcl-1 proteins, augmentation of drug inactivation and mutation of target leading to anomalous conformation [6]. In a study by Yu & co-workers [7] it is stated that Rosmaric acid increases sensitivity to gastric carcinoma SGC7901 cells by downregulating miR-6785-5p and miR-642a-3p leading to increase in expression of FOXO4 in 5-FU treatment regime. In another study by Zheng & co-workers [8] Oridion was proposed to enhance cytotoxicity of 5-FU in renal carcinoma cells by inducing necroptosis.

The study carried out in France showed that among 76,200 patients who received fluoropyrimidine as chemotherapy 1,200 suffered from life threatening toxicity and 150 (0.197 %) died each year might be due to the lack of dihydropyrimidine dehydrogenase (DPD) activity [9].

DPD is a vital enzyme in catabolism of pyrimidine bases uracil and thymidine. It is highly expressed in liver and blood. It is found in monocytes followed by lymphocytes, granulocytes and platelets. In contrast, no effect on erythrocytes is detected. Wörmann & et al. [10] state that activity of DPD prior administration of 5-FU, capecitabine and tegafur is blocked by folinic acid, increasing intracellular concentration leading to increase in active metabolite rate. Thus, pharmacologically blocking of DPD increases intracellular concentration, increasing active metabolite rate.

Folic acid is a water-soluble vitamin consisting of pteridine, *p*-aminobenzoic acid and glutamic acid. It is a fully oxidized folate. Folates play an essential role in DNA synthesis and methylation, responsible for maintaining DNA integrity, metabolizing amino acid, gene expression, and remethylation of homocysteine to methionine [11–12].

Folates obtained from food intake have presence of extra glutamate residues that are hydrolyzed to single glutamate within gut before absorption by active transport across intestinal mucosa. Whereas passive diffusion is observed in doses of pharmacological administration. Monoglutamate is converted to tetrahydrofolate, further converted to methyl or formyl forms. Folates are in 5-methyl-THF forms in blood. Some folates are also circulating in blood unaltered, i.e., unmetabolized folic acid [13].

Folates are transported by Reduced Folate Carrier (RFC), Proton-Coupled Folate Transporter (PCFT), and Folate receptors α, β, respectively [14–16]. FRα and FRβ are glycosylphosphatidylinositol (GPI)-anchored cell surface glycoproteins and FRγ is a secretory protein [17]. The uptake of folates by FRα and FRβ is receptor-mediated endocytosis [18]. FRα is mostly expressed in epithelial cells of uterus, placenta, choroid plexus, retina, and kidney. It is also expressed in cancers of epithelial origin, namely adenocarcinoma of breast, ovary, cervix, uterus, kidney, lung, bladder, and pancreas [19–22].

Folate transporters, PCFT are active in acidic pH, while RFC has low binding to non-reduced FA. Hence, we estimate that the hypothesized folate receptor α (FRα) may take up the 5-FUFA scaffold, as it is upregulated in many primary and metastatic cancer, namely epithelial and over 90% of non-mucinous OCs also platinum-resistant ovarian cancer. Thus, targeting cancer cells selectively, associated side effects (non-specificity, resistance, toxicity, high doses, etc.) are overcome. The bioavailability of 5-FU within cancer cells is improvised with either cleavage by amidase enzyme or showing action as a whole, e.g., CT900. At concentration as low as 100–250 nM, i.e. in nanomolar range, it is possible to overcome drug resistance issues by not affecting levels of HTS and having allosteric action [23–29].

The present study aims to design a new vitamin-drug conjugate by utilizing folic acid and 5-fluorouracil as a primary compound to form 5-fluorouracil folic acid (5-FUFA). The conjugate is expected to have the targeted drug activity with minimal side effects [30–33].

Experimental

Chemicals: 5-Fluorouracil was a kindly gifted sample from Intas Pharmaceuticals, Ahmedabad. Folic acid (FA) was purchased from Sigma Aldrich Chemicals Pvt. Ltd. Dimethylformamide (DMF). 4-dimethylaminopyridine (DMAP), isopropanol, and N-hydroxysuccinamide (NHS) were purchased from SD Fine Chem Ltd. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDC. HCl) was purchased from AVRA Laboratories Pvt. Ltd. Ethyl acetate and methanol were purchased from HiMedia Laboratories Pvt. Ltd.

Instruments used: Melting-boiling point apparatus (Veego) was used to determine the melting point. FTIR analysis was carried out to obtain the FTIR spectra on FTIR spectrophotometer Shimadzu FTIR 8400S. Mass spectra were recorded on a mass spectrometer, Shimadzu LCMS-8040. MD simulation was executed on Nvidia V100-SXM2-16GB Graphic Processing Unit using the PMEMD.CUDA module installed on the Computational Shared Facility (CSF3), UCL School of Pharmacy, UK.

Methodology:

Synthesis of 5-Fluorouracil Folic acid (5-FUFA)

1. Folic acid activation

3 g of FA were activated by EDC and NHS in 15 mL of DCM (molar ratio of FA:EDC:NHS =1:1:1) at room temperature under nitrogen gas for 24 h.

2. Conjugation

The 2° amine group of 5-FU (1 g) was conjugated to the carboxylic group of FA through the amide bond. 5-FU dissolved in 5 mL of DMF was added to the activated FA in DMF then EDC & DMAP were added at room temperature under nitrogen gas for 24 h.

Marverti et al. [34] performed peptide conjugates of folic acid that would selectively enter cancer cells by thymidylate synthase dimer. Presented research work utilized their synthetic scheme by incorporating various synthetic procedures with variations in catalyst to form an amide linkage between folic acid and 5-fluorouracil, respectively. Initially, 3 g of FA were activated in the presence of an equimolar concentration of EDC.HCl, NHS, (4 mL) DMF, inert atmosphere for 24 h followed by adding 1 g of 5-FU dissolved in 1 mL of DMF and equimolar concentration of DMAP in the same reaction mixture continued to stir for another 24 h (Fig. 1). EDC is a water-soluble carbodiimide that creates a zero-length linker between carboxyl and amine groups. EDC coupled with NHS reacts with the carboxyl group of FA to form a semi-stable NHS-ester intermediate. The secondary amine of 5-FU reacts with the ester intermediate to form an amide bond. Resultant was treated with 10 mL of ethyl acetate and 10 mL of methanol. Unreacted FA & 5-FU were separated. FA is insoluble in methanol, precipitates out, and 5-FU is soluble in ethyl acetate. Thus, the product formed is present in insoluble methanol form. It was checked for purity and homogeneity by TLC. It was concentrated and dried to evaporate. This was further utilized for spectral analysis to confirm amide linkage formation.

The melting point of 5-FUFA was found to be 155^0C. Rf was 0.5.

Physical and spectral data for 5-FUFA: IR (KBr, cm^{-1}): Amide I bond at 1687.77 (C=O stretching), amide II bond at 1591.33 (N-H bending), O-H of carboxylic acid at 3244.66, (N-C stretching) at 1379.15 & (N-H wag) at 802.41, 3163.23 (C-H stretching). Ms: m/z (%) = 554.15 (M+1).

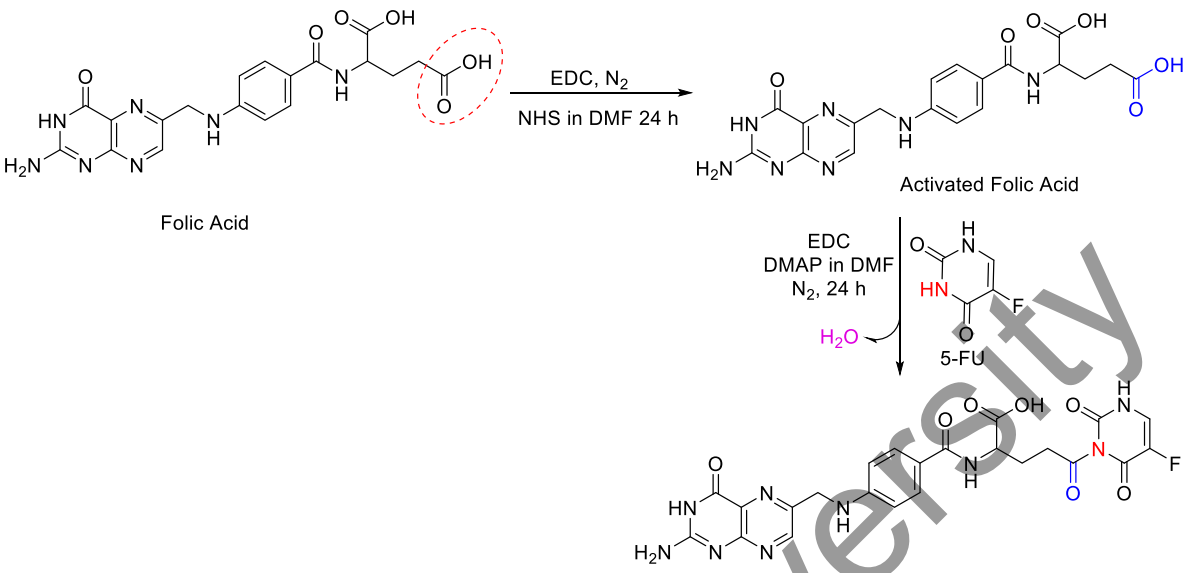
Protein structure preparation: The protein crystal structure of human Thymidylate Synthase (TS) enzyme (PDB ID- 1HVY) was retrieved from Protein Data Bank with the resolution of 1.90 A°. The enzyme structures were checked for missing atoms, bonds and contacts. The hydrogen atoms were added to the enzyme structure. The water molecules and bound ligands were deleted manually. The parameters during protein preparation were set with ionization and tautomerization using the Epik module for a pH range of 7 to 9.

Molecular docking studies: The compounds were subjected to docking with extra precision (XP) molecular docking using the Glide module of the Schrodinger suite. Before performing the docking studies, a grid generation protocol was performed to determine the binding site. The already bound ligand was used as a reference site for the grid generation with the Glide grid module. The generated grid was further used for the docking experiment. For the docking method, the van der Waals radii and scaling factor were set to 0.80 and 0.15, respectively, to soften the potential of nonpolar parts on drug molecules. No restraints were applied to the ligands during the entire docking protocol. Post-docking minimization was allowed to provide minimized docked structures with a maximum of five best poses per ligand to include in the docking output file. The drug molecules were allowed to be flexible during the docking process. The RMSD, docking score, Glide score, and binding energy were recorded for each molecule.

Molecular Dynamics Simulations: To explore the docked complex's structural, energetic status, and steric refinement, an all-atom MD simulation of 100 ns was performed using the AMBER18 software package. The docked complexes were immersed in a truncated octahedron of TIP3P water, giving 24,515 water molecules to the system. A sufficient number of Na^+ and Cl^- counter ions were added to neutralize the system and achieve an ionic strength of 0.1M to mimic the physiological pH. The ff14SB force field was used for protein topology generation. The entire experiment of MD simulation was executed on Nvidia V100-SXM2-16GB Graphic Processing Unit using the PMEMD.CUDA module installed on the Computational Shared Facility (CSF3), University of Manchester, UK. Simulations were run at 300 K using the Langevin thermostat with a collision frequency of 2 ps-1; at 1 atm using a Monte Carlo barostat with volume exchange attempts every 100 fs. A 2-fs integration step was employed. Covalent bonds involving hydrogen constrained using SHAKE algorithm. A cut-off of 8 Å was used for short-range non-bonded interactions, while long-range electrostatics were treated using the particle mesh Ewald method. Equilibration comprised rounds of NVT and NPT equilibration for 10 ns in total. Production MD run was performed for 100 ns. Root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and other interactions were analyzed using CPPTRAJ over full trajectory, taking configuration every 4 ps.

Results and Discussion

5-FUFA conjugate was synthesized. The melting point and TLC were performed to ascertain its purity and homogeneity. Spectral analytical data confirmed formation of conjugate.



5-FU — 5-Fluorouracil; EDC — 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide HCl;
NHS — N-hydroxysuccinamide; DMAP — 4-Dimethylaminopyridine; DMF — Dimethylformamide;
N_2 — Nitrogen gas

Figure 1. Proposed scheme for synthesis of 5-Fluorouracil folic acid (5-FUFA)

FT-IR

There are observed peaks at 1687.77 (C=O stretching) for an amide I bond and 1591.33 (N-H bending) for an amide II bond, which are characteristic of an amide C=O bond. These peaks in the IR spectra of 5-fluorouracil folic acid demonstrate the formation of 5-fluorouracil folic acid conjugate via the formation of an amide linkage.

Mass Spectroscopy

Mass spectra of 5-FUFA conjugate were recorded for its structural confirmation. The mass spectra of 5-FUFA conjugate showed the molecular ion peak at 554.15 m/z, confirming the conjugation of 5-FUFA by forming an amidase linkage and forming the final product, i.e., 5-FUFA conjugate.

Molecular simulation:

The molecular docking study was conducted to understand the binding of 5-fluorouracil-folic acid conjugate (5-FUFA) on the human thymidylate synthase (PDB: 1HVY). The crystal structure of human thymidylate synthase is complexed with dUMP and Raltitrexed. During the processing of the target receptor, water molecules and other crystallographic solvents were removed. The protein was minimised in the Glide protein preparation protocol. The Grid generation was performed with Raltitrexed as a reference ligand and the 5-FUFA was docked with the extra precision (XP) method. The docking studies showed that 5-FUFA conjugate interacted at Asn226, Cyc195, Asn112, Asp49 and Lys47 of the human thymidylate synthase proteins through hydrogen bonding and arene interaction with the π stacking with the Asp218 and the phenyl ring from the conjugate respectively with a binding score of –8.0 Kcal/mol (Figures 2, 3), which was higher than 5-FU, i.e., –3.475. So, it was proved that forming 5-FUFA conjugate showed greater binding to the target protein.

Molecular docking studies were performed in the Schrodinger suite utilizing the Glide module with the extra precision method. 5-FUFA was bound on human thymidylate synthase, which was complexed with dUMP & Raltitrexed as a reference ligand. Followed by Molecular Dynamic simulation was carried on AMBER18 software for 100 ns, and later to validate further the resultant docking score analysis of full trajectory was done using CPPTRAJ, resulting in possible variations by a ligand and a receptor (Figure 4).

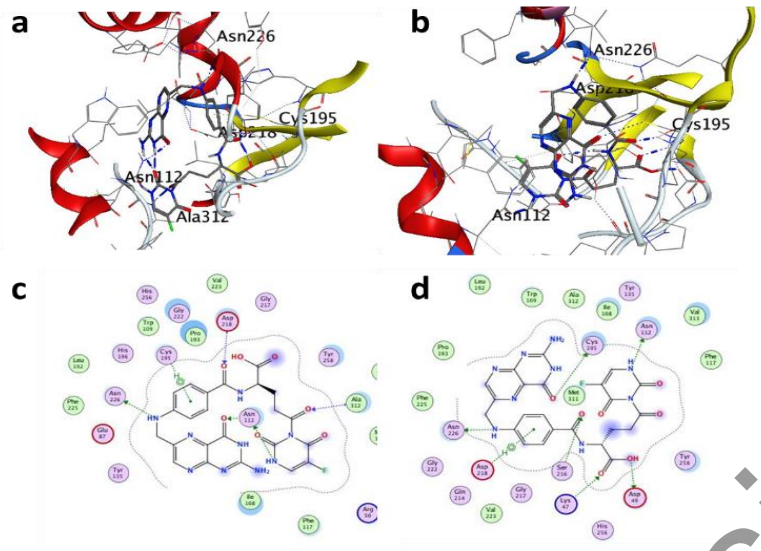


Figure 2. The figure 2a, c and 2b, d shows the initial and final conformation
from the simulation of the protein-ligand complex for 100 ns

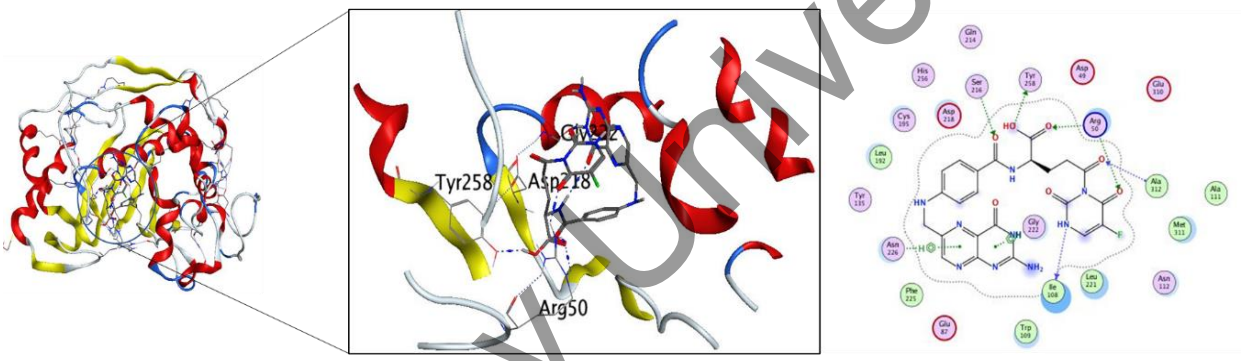
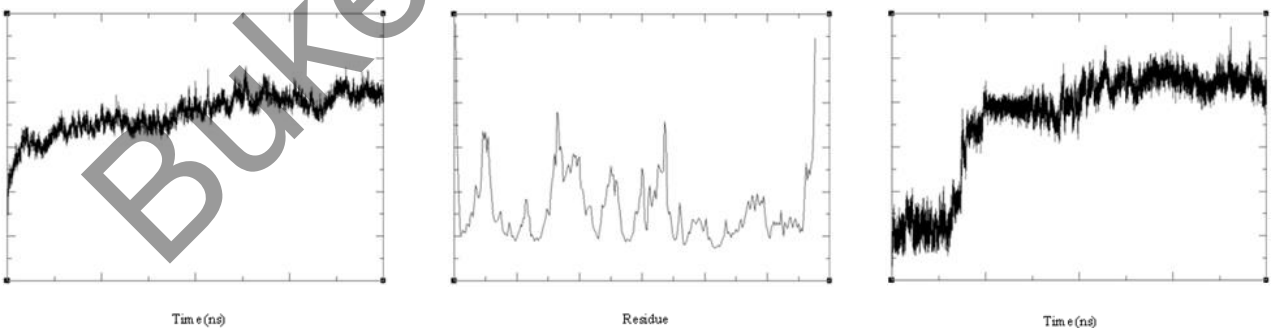


Figure 3. Docked complex of 5-FUFA with the human thymidylate synthase,
the highlighted region shows the 3D image of protein ligand interactions
and the 2D image shows the formation of various interaction between the ligand and receptor



a — Protein RMSD; *b* — Protein RMSF; *c* — Ligand RMSD

Figure 4. The trajectory analysis of the Protein-ligand complex

Molecular Dynamic simulation:

The molecular docking study was performed to understand the binding of 5- fluorouracil-folic acid conjugate (5-FUFA) on the human thymidylate synthase (PDB:1HVY). The crystal structure of human thymidylate synthase is complexed with dUMP and Raltitrexed. During the processing of the target receptor, water molecules and other crystallographic solvents were removed, and the protein was minimised in the Glide

protein preparation protocol. The Grid generation was performed with Raltitrexed as a reference ligand, and the 5-FUFA was docked with the extra precision (XP) method. The docking studies showed that 5-FUFA conjugate interacted at Asn226, Cyc195, Asn112, Asp49, and Lys47 of the human thymidylate synthase proteins through hydrogen bonding and an arene interaction with the π stacking with the Asp218 and the phenyl ring from the conjugate respectively with a binding score of -8.0 Kcal/mol, which was higher than 5-FU, i.e., –3.475. So, it was proved that forming 5- FUFA conjugate showed greater binding to the target protein.

The MD simulation suggests that the receptor residues form several new interactions during simulation with the formation of hydrogen bonds with Asn226, Cyc195, Asn112, Asp49, and Lys47. It forms the arene interaction with the π stacking with the Asp218 and the phenyl ring from the conjugate (Figure 3). The analysis of the MD trajectory was performed with the help of CPPTRAJ, and plots were prepared with XMGRACE software. The RMSD of the protein suggests a smooth transition and convergence between 1–2.5 Å. The RMSF for most of the residues was below 2.0 Å throughout the simulation. It was also found that the ligand underwent a conformational change, which was reflected in its RMSD (Figure 4*c*) around 25 ns. The ligand RMSD fluctuated from 2 to 4 Å and then remained stable for the remaining simulation time. All these findings suggest good docking and further good stability for most of the MD simulation time.

Conclusions

We conclude scaffold formed by simple carbodiimide catalyst (EDC.HCl) and 5-FUFA conjugate between 5-FU and folic acid was successfully synthesized. The characterization of the synthesized compounds was in line with the structure proposed. The drug design studies reveal that 5-FUFA forms hydrogen bonds with the residues in the active site of the receptor, i.e., Agr50 and Tyr258 form hydrogen bonds whereas the Gly222 and Asn226 form π-π stacking interactions with the best dock pose to present –8.0 Kcal/mol, which represents the highest dock score. By assessing the stability of the drug-receptor complex, the docked pose was subjected to molecular dynamics simulation in the Schrodinger suite and validated by molecular trajectory CRPPTRAJ software for 100 ns. It was found to be stable, with a slight conformational change at 25 ns from 2 to 4 Å.

Thus, the conjugate may be a potential molecule to increase the bio efficiency of 5-FU and decrease associated side effects by selectively targeting cancer cells, such as the uptake of folic acid by cancer cells during folate receptor endocytosis. However, this hypothesis may be further tested by performing detailed anticancer evaluation in the future.

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Р.П. Бхоле, М. Свами, Р. Вавхейл, К.Г. Бонде, Р. Чихэйл

Қатерліісікке қарсы белсенділікті арттыру үшін потенциалды витамин-дәрілік конъюгаттың дизайны

5-Фторурацил, қатерліісіктің әртүрлі сатыларын емдеуде кеңінен қолданылатын біріншілік молекула, белсенді емес түрге, атап айтқанда 5,6-дигидро-5-ФУ-ға дейін тез метаболизденеді және химиотерапия кезінде әртүрлі мутациялық өзгерістерге ұшырайды. Фолий қышқылы конъюгатын алу үшін карбодимидті катализатор қолданылған. Фолий қышқылының рецепторлары ісік тіндерінде шамадан тыс экспрессияланғандықтан, бұл 5-ФУ биожетімділігін арттырады. Бұл жұмыс ісіккеқарсы белсенділікті күшейтетін жаңа витамин–дәрілік конъюгатты алу мен синтездеу болып табылады. 5-Фторурацил сүт безі, тоқ ішек, асқазан және тері ісігі тіндеріне күшті әсер етеді. Фолий қышқылы рак клеткаларының FRα рецепторларына селективті әсер етуге көмектеседі. Конъюгацияны растау үшін 5-FUFA қосылысына спектрлік сипаттама жасалады. Молекулярлық динамикалық модельдеу Schrodinger пакетінде зерттелді және CRPPTRAJ бағдарламалық құралында молекулалық траекториямен расталды. Конъюгат одан әрі молекулярлық модельдеу әдістерімен зерттелді және конъюгаттың қондыру индексі 5-FU-пен салыстырғанда жоғары байланысу шамасын көрсетті, атап айтқанда -8,0 ккал/моль шамасына тең болды. Препараттың рецептормен өзара әрекеттесуі Schrodinger пакетіндегі молекулярлық динамикалық модельдеу және 100 нс молекулалық траекторияға арналған CRPPTRAJ бағдарламалық құралының көмегімен дәлелденді. Молекулалық динамикалық модельдеу нәтижелері 25 нс ішінде 2-ден 4 Å-ге дейін аздаған конформациялық өзгерістер болатынын, ал жалпы жағдайда тұрақты болатынын көрсетті.

Кілт сөздер: 5-фторурацил, фолий қышқылы, синтетикалық конъюгат, молекулалық қондыру, молекулалық динамика, дәрілік конструкция, химиотерапевтік агент, адамның тимидилатсинтазасы.

Р.П. Бхоле, М. Свами, Р. Вавхейл, К.Г. Бонде, Р. Чихэйл

Дизайн потенциального конъюгата «витамин–лекарство» для усиления противораковой активности

5-Фторурацил, первичная молекула, широко используемая при лечении различных стадий рака, быстро метаболизируется до неактивной формы, а именно до 5,6-дигидро-5-ФУ, и подвергается различным мутационным изменениям в ходе химиотерапии. Авторами статьи использован карбодиимидный катализатор для образования конъюгата с фолиевой кислотой. Поскольку рецепторы фолиевой кислоты сверхэкспрессированы в раковых тканях, это увеличивает биодоступность 5-ФУ. Данная работа представляет собой разработку и синтез нового конъюгата «витамин–лекарство», потенциально усиливающего противораковую активность. 5-Фторурацил обладает мощным действием на ткани рака молочной железы, толстой кишки, желудка и кожи. Фолиевая кислота помогает избирательно воздействовать на рецепторы FRα раковых клеток. 5-FUFA подвергали спектральной характеристике для подтверждения успешной конъюгации. Молекулярно-динамическое моделирование было изучено в пакете Schrodinger и подтверждено молекулярной траекторией в программном обеспечении CRPPTRAJ. Конъюгат был дополнительно изучен с помощью исследований молекулярного моделирования, и показатель стыковки конъюгата представлял собой более высокий показатель связывания, чем у 5-ФУ, а именно 8,0 ккал/моль. Взаимодействие лекарственного препарата с рецептором было дополнительно подтверждено с помощью молекулярно-динамического моделирования в пакете Schrodinger и программного обеспечения CRPPTRAJ для молекулярной траектории в течение 100 нс. Было обнаружено, что результаты молекулярно-динамического моделирования стабильны с небольшим конформационным изменением в течение 25 нс от 2 до 4 Å.

Ключевые слова: 5-Фторурацил, фолиевая кислота, синтетический конъюгат, молекулярный докинг, молекулярная динамика, дизайн препарата, химиотерапевтический агент, тимидилатсинтаза человека.

Information about authors

Bhole, Ritesh Prakash — PhD, Associate Professor, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Sant Tukaram Nagar, Pimpri, Pune-411018, India; e-mail ritesh.bhole@dypvp.edu.in; https://orcid.org/0000-0003-4088-7470;

Bonde, Chandrakant Ghansham — PhD, Professor, SPTM, NMIMS, School of Pharmacy, Shirpur, Dist: Dhule, India. e-mail: chandrakant.bonde@nmims.edu; https://orcid.org/0000-0001-5712-1119;

Swamy, Mahadevan Raju — M. Pharm, Research Scholar, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Sant Tukaram Nagar, Pimpri, Pune-411018, India; e-mail: mahadevan.swamy11@gmail.com; https://orcid.org/0000-0003-1399-1176;

Wavhale, Ravindra — PhD, Assistant Professor, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Sant Tukaram Nagar, Pimpri, Pune-411018; India; e-mail: ravindra.wavhale@dypvp.edu.in; https://orcid.org/0000-0003-1614-9742;

Chikhale, Rupesh — PhD, Research Fellow, UCL school of Pharmacy, London, UK; e-mail: R.Chikhale@ucl.ac.in, https://orcid.org/0000-0001-5622-3981