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Synthesis and evaluation of vitamin-drug conjugate for its anticancer activity

Cancer is the uncontrolled growth of cells in the human body that has the ability to spread. The purpose of the study is to explore that vitamins can be used as a targeting moiety for new anticancer drugs to address issues like non-selectivity, systemic toxicity. 5-Fluorouracil acetic acid–Vitamin D3 (5FUAC-Vit.D3) conjugate has been synthesized, characterized, and evaluated for its anticancer activity. 5-FUAC-Vit.D3 conjugate was synthesized via esterification mechanism in the presence of N-Hydroxy succinimide (NHS) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) by using HCL as coupling agent. Formation of 5-FUAC-Vit.D3 conjugate via esteric bond and the structure of the compounds were confirmed by spectroscopic data, i.e., IR, NMR, and mass spectra. The docking studies showed that 5-FUAC-Vit.D3 conjugate interacted at Arg-215 and Lys-47 of the human thymidylate synthase proteins, through hydrogen bonding and ionic bonds respectively with a binding score of -8.614 which is higher than only 5-FU (-3.475). So, it was proved that forming 5-FUAC-Vit.D3 conjugate shows greater binding to the target protein.

Keywords: synthesis, molecular modeling, molecular docking, vitamin-drug conjugate, 5-fluorouracil acetic acid, vitamin D3.

Introduction

Following cardiovascular diseases, cancer is the world's second leading cause of death [1]. Cancer is described as the uncontrolled growth of cells in the human body that are able to spread to other parts of the body [2]. If this spread is not controlled, cancer may lead to severe death [3]. There are several strategies for treating cancer but chemotherapy is the most widely used method for treating cancer, however it has the drawback of being non-selective. Since cytotoxic chemotherapy does not discriminate between tumor and non-tumor cells, it causes serious, frequently life-threatening side effects in susceptible healthy cells [4]. The most difficult step in conventional chemotherapy is the delivery of a cytotoxic agent that kills proliferating tumor cells [5–6]. Since anticancer therapy should last longer, and that longer therapy may be the cause of its side-effect profile. The other side of the coin, i.e., side effect minimization, also has to focus to get effective drug. Although the side effect profile is widespread, it has generally been shown to inhibit the rapid growth required to maintain normal cells such as hair follicles, bone marrow and gastrointestinal tract cells. This leads to the potential undesirable side effects observed in cancer treatment. Low aqueous solubility, short biological half-life, multidrug tolerance, and non-specificity or dose-limiting cellular toxicity are other limitations of free chemotherapeutic agents in cancer treatment [7]. Consequently, it is still difficult to achieve selectivity of anticancer drugs for cancer cells and to reach breakthrough in cancer research, which may spare healthy tissue and help to overcome the intrinsic limitations of conventional anticancer drugs.

To achieve successful tumor-targeting drug delivery it should include a tumor-recognizing moiety and a chemotherapeutic agent that is linked directly by a linker. As a result, a conjugate acting 'prodrug' is produced, which, once integrated into a cancer cell, readily splits and regenerates the cytotoxic agent's activity. In these conditions the so-called vitamin-mediated drug targeting has recently emerged as a new concept for carrying anticancer drugs particular to tumors [8–10]. Since cancerous cells grow quickly, they demand more vitamins as well as other nutrients than healthy cells, and the receptors involved in the cellular internalization of vitamins are abundantly expressed on the surface of growing cancer cells. As a result, it is thought to be worthwhile to develop “Vitamin-Drug Conjugate” that could be able to target tumor cells [11–13]. Vitamin drug conjugates will be nontoxic, it will specifically be internalized into cancerous cells and release the anticancer drugs without loss of potency, it will minimize the systemic toxicity by being stable in blood circulation, and provide a target-specific activity by sparing the normal cell that will minimize the side effects [14–15].

Recently, cancer being widely increasing, there has been a greater need for formulating targeted drug delivery with minimum side effects. These needs can be fulfilled by “Vitamin-Drug Conjugate” (targeted)

formation. In this study 5-Fluorouracil and cholecalciferol are used as raw materials to synthesize vitamin drug conjugates. 5-Fluorouracil is an anticancer drug belonging to the antimetabolite class. It is used in the treatment of the large number of malignant tumors, some of which include breast, colon, rectal, ovarian, and bladder cancer. 5-Fluorouracil is acted as an antitumor agent by converting intracellularly into some active metabolites. These active metabolites interfere with RNA production and thymidylate synthase action, resulting in anti-cancer activity. Recently, the ability of vitamin D3 to enhance the anti-tumor activity of chemotherapeutic drugs by activating apoptosis was reported. Vitamin D3 is selected as targeting moiety to cancerous cells as cancerous cells have an unquenchable appetite for vitamins. When Vitamin D3 is combined with 5-Fluorouracil, the anticancer activity of 5-Fluorouracil is increased compared to 5-Fluorouracil administered alone. 5-Fluorouracil shows various undesirable effects during cancer treatment if 5-Fluorouracil is given alone. So, by conjugating vitamin D3 with 5-Fluorouracil via an acid liable spacer, this conjugate acts as a prodrug, which has the potential to overcome the non-specificity and toxicity issue of 5-Fluorouracil. This approach not only overcomes associated toxic effects but also improved the desired bioavailability with a reduction in dose and dosage frequency. This combination shows a synergistic effect with targeted drug delivery of 5-Fluorouracil in the tumor tissues. Here we present high yield synthetic procedures for conjugate preparation as well as complete characterization results. Molecular modeling studies were also performed for the given synthetic conjugate for comparing the binding affinity of conjugate with 5-Fluorouracil.

Experimental

¹H NMR spectra of the compounds were recorded on Bruker Avance III HD NMR 500 MHz spectrophotometer using CDCl₃ as a solvent and operating at 500 MHz at room temperature with tetramethyl silane (TMS) as the internal reference. FTIR analysis was carried out to get the FTIR spectra on the FTIR spectrophotometer, Shimadzu FT-IR 8400S. Mass spectra were recorded on the mass spectrometer, Shimadzu LC-MS 8040. TLC was used to monitor the reaction progress and to check the purity of the compound, using Silica Gel plates F254 on Aluminum sheets. Docking studies were carried out using MOE-Dock, Chemical Computing Group Inc. on a machine having Pentium 1.6 GHz workstation, 512 MB memory using the Windows operating system.

Methodology

Synthesis of 5-Fluorouracil — Vitamin D3 conjugates

1. Synthesis of 5-Fluorouracil-acetic Acid (5-FUAC)

5-FUAC was synthesized according to the previously described method [16-17] with some modifications. 5 FU (24g) dissolved completely in 152 ml KOH (0.3 g, 5.3 mmol) aqueous solution (9.12 g) then stir the reaction mixture at 100 °C for 70 mins. After that prepare 40 ml of chloroacetic acid solution in aqueous KOH and add it to the above mixture slowly and stir using a magnetic stirrer under 60 °C for 6 hours. Then acidified the product to pH 2 with concentrated hydrochloric acid, followed by cooling at 4 °C for 12 hours. Then extract the mixture using a separating funnel as, take 50 ml of the above reaction mixture in separating funnel, add 30ml of ethyl acetate to it and shake for 10 mins. Collect the supernatant (i.e., ethyl acetate layer/organic layer) in a beaker separately, and pour the aqueous layer again in the separating funnel and add fresh ethyl acetate 30 ml to it and shake it for 10 min. Repeat the procedure for 3 times for each of the 50 ml of solution. Repeat the same procedure for the whole of the above reaction mixture. Then take the collected supernatant layer (120 ml) and add 1–2 spoons of sodium sulphate to it and keep it for 30 mins. Then filter the solution, evaporate the filtrate using Rota evaporator at 60 °C. Dry the product, and record the yield and R_f value of the synthesized product. The product yield was found to be 62 % and the R_f value of the conjugate was found to be 0.35 using mobile phase (Chloroform:Methanol:Triethylamine = 7:1:2 in proportion) and silica plate as stationary phase.

Physical and spectral data for 5-FUAC (Intermediate product): IR (KBr, 4500–500 cm⁻¹) γ = 1690 (C=O acid), 3194 (N–H), 2835 (C–H), 1211 (C–N), 1404 (C–F), 1479 (C–C); ¹H NMR (CDCl₃, 500 MHz): δ = 4.60 (2H, s), 8.65 (1H, s), 11.46 (1H, s) ppm; Ms: m/z (%) = 189.4 (M⁺)

2. Synthesis of 5-Fluorouracil-acetic Acid- Vitamin D3 conjugate

First, a solution of 5-FUAC (0.496 g) in 1 ml of dimethylformamide was prepared, then a solution of Vitamin D3 (0.5 g) in 20 ml of dimethylformamide and 2–3 drops of dichloromethane were added dropwise. Thereafter, 0.196 g (1.7 mmol) of N-Hydroxy succinimide (NHS) and 0.4 g (2.09 mmol) of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDC·HCl) were added subsequently. The above solution was incubated for 16 hrs away from light. Then the reaction mixture was precipitated with 150 ml of isopropanol,

filtered and dried. The yield and Rf value of the conjugate were recorded. The product yield was found to be 48 % and Rf value of the conjugate was found to be 0.5 using mobile phase (Chloroform:Methanol:Triethylamine = 7:1:2 in proportion) and silica plate as stationary phase.

Physical and Spectral data for 5-FUAC-Vitamin-D3 Conjugate: M.P. — 74–76 °C; IR (KBr, 4500–500 cm^{-1}) 1726 C=O (ester), 1200 C–O (ester), 1618 (C=O stretch in pyrimidine ring), 3337 (N–H stretch), 2932 (C–H stretch), 1199, 1101 (C–N), 1377 (C–F), 1431 (C–C), 3067, 3050 (C=CH₂), 1618 (C=C) cm^{-1} ; ¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 0.60–0.72 (q, 2H), 0.78–0.84 (8H, d), 0.94 (3H, s), 1.04 (3H, d), 1.16–1.32 (2H, 1.24 quint), 1.35–1.76 (12H, 1.65 m), 1.95 (1H, m), 2.18–2.52 (6H, 2.23, m), 2.59 (1H, dd, J = 14.2, 3.1 Hz), 4.58–4.59 (2H, s), 4.78 (1H, d), 4.94–5.09 (2H, m), 6.06 (1H, d), 6.32 (1H, d), 8.60 (1H, s) ppm; Ms: m/z (%) = 554.3 (M⁺).

Molecular Docking

5-FU and 5-FUAC-Vitamin D3 conjugate were docked in thymidylate synthase active site using MOE-Dock, Chemical Computing Group Inc.

Procedure: Protein (Code — 1HVY) was retrieved from PDB data resources with a resolution of 1.90 Å. The structure of 5-FU and Vitamin-D3 were retrieved from PubChem and conjugated. All the structures were energy minimized and their lower energy conformations were generated considering *pK_a* of ionizable groups and pH of the medium. The protein crystal structures of human thymidylate synthase were prepared and the missing residues were modeled. The parameters during protein preparation were set with ionization and tautomerization using the Epic module for a pH range of 7 to 9. The molecular docking program was run to evaluate ligand-protein binding energy and interactions.

Result and Discussion

5-FUAC-Vitamin D3 conjugate was synthesized, and the synthetic route is shown in Figure 1. The melting point and TLC were performed for the synthetic conjugate to confirm its purity and homogeneity. The structures of 5-FUAC and 5-FUAC-Vitamin D3 Conjugate were characterized by IR, NMR, and mass spectrometry. The data of IR, NMR, and Mass spectrometry was listed in the experimental section. Molecular modeling studies were performed for comparing the binding affinity of conjugate with 5-FU. Molecular docking studies of 5-FU and 5-FU- Vitamin-D3 Conjugate also were studied and results were shown in Table 1.

FTIR

The main peaks observed in the IR spectra of 5-fluorouracil acetic acid are 1690 cm^{-1} for C=O of carboxylic acid, 3194 cm^{-1} for N–H stretching, 1400 cm^{-1} for C–F, and 1211 cm^{-1} for C–N. The IR spectra of 5-fluorouracil acetic-acid — vitamin D3 conjugate are observed at 1726 cm^{-1} for C=O (ester), 1199 cm^{-1} for C–O (ester), and 1618 cm^{-1} for C=O stretch in pyrimidine ring. The peaks observed at 1726 cm^{-1} which is of C=O of ester and at 1199 cm^{-1} which is for C–O of ester. These peaks show the formation of 5-FUAC Vitamin D3 conjugate via formation of an esteric linkage. They are seen in the IR spectra of 5-FUAC-Vitamin D3 conjugate and absent in the IR spectra of other compounds.

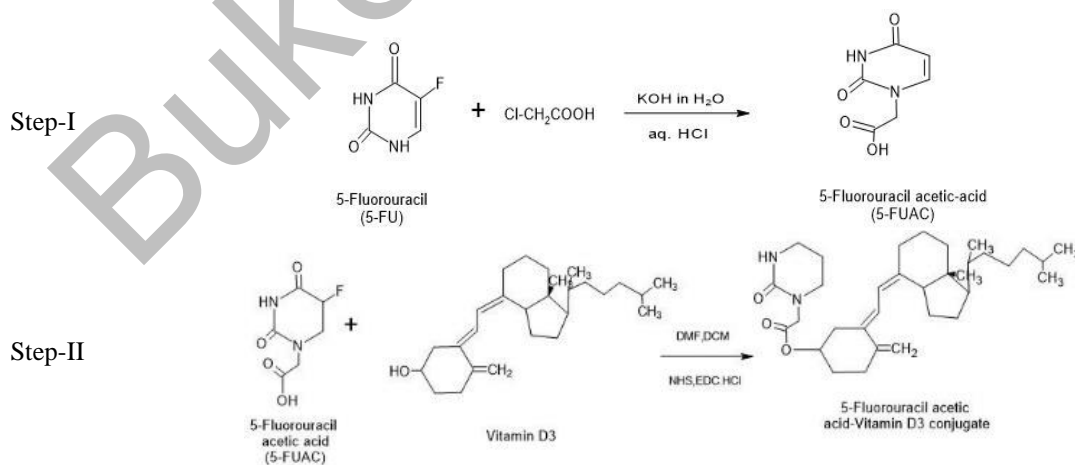


Figure 1. Synthetic scheme of 5-FUAC-Vitamin D3 conjugate

Docking Scores of 5-FU and 5-FUAC-Vit.D3 conjugate

Sr. No.	Molecule code/ structure	Docking score	Interactions with target protein
1	5-FU	-3.47	5-FU-C=O — ASN 226 (Hydrogen Bond)
2	5-FU-Vit D3 conjugate	-8.614	5-FU-Vit-D C=O — Arg-215(Hydrogen Bond) 5-FU-Vit-D C-O — Lys-47 (Ionic Bond)

NMR

The ¹H NMR spectra of 5-FUAC-Vitamin D3 are at δ value of 4.58–4.59 (2H, s), 4.78 (1H, d), 4.94–5.09 (2H, m). The NMR spectra of 5-FUAC-vitamin D3 showed the peaks at the above mentioned δ values, which confirm the structure of 5-FUAC-vitamin D3 conjugate. The peaks observed in the range of 3–5 ppm show the formation of esteric bond in the conjugate.

Mass Spectroscopy

Mass spectra of 5-FUAC-Vitamin D3 conjugate were recorded for its structural confirmation. The mass spectra of 5-FUAC-vitamin D3 conjugate showed the molecular ion peak at 554.3 m/z, which confirms the conjugation of 5-FUAC with Vitamin D3 by forming an esteric linkage and formation of the final product, i.e., 5-FUAC-Vitamin D3 conjugate.

Molecular Docking

The 5-FUAC-Vitamin D3 conjugate was evaluated by molecular modelling studies. The conjugate was docked on the thymidylate synthase (PDB Code — 1HVY) active site as shown in Figure 2.

The docking studies showed that 5-FUAC-Vitamin-D3 conjugate interacted at Arg-215 and Lys-47 of the human thymidylate synthase proteins, through hydrogen bonding and ionic bonds respectively with a binding score of -8.614 which is higher than 5-FU i.e., -3.475 as shown in Table 1. So, it was proved that forming 5-FUAC-Vitamin D3 conjugate shows greater binding to the target protein. The greater binding will also reveals that this conjugate will aid in the anticancer activity of the compound.

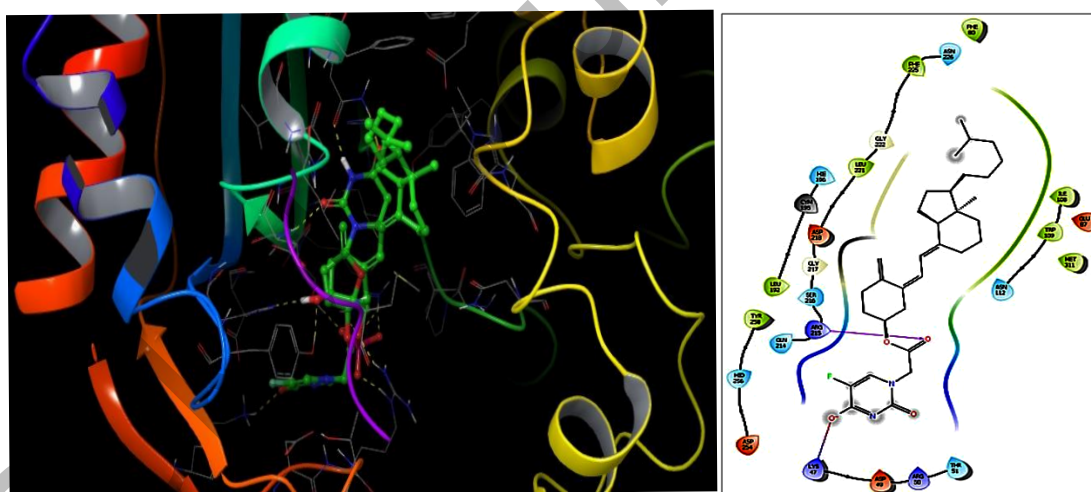


Figure 2. Binding interaction of 5-FU-Vit-D conjugate in the active site of human thymidylate synthase enzyme (Target of 5-FU, PDB Code — 1HVY)

Conclusion

5-Fluorouracil-Vitamin D conjugate was successfully synthesized, characterized, and evaluated. The characterization was carried out by performing IR, NMR, and Mass spectroscopy which suggested the formation of 5-FUAC-Vitamin D3 conjugate through an esteric linkage.

5FUAC-Vitamin D3 conjugate was designed and may be proven as a novel prodrug approach, that will have the potential to overcome non-specificity and toxicity issues of 5-FU. The present prodrug approach will also have the potential to improve the potency and bioavailability of the anticancer drug. Molecular docking analysis of 5-Fluorouracilacetic acid-Vitamin D3 conjugate with the human thymidylate synthase revealed excellent binding affinity compared to 5-FU. Moreover, this will also help in the accumulation of the drug in

more extend than the conventional therapies. The present study may be extended further for other anticancer drugs by forming conjugates with other vitamins.

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Витамин-дәрілік конъюгаттың қатерлі ісікке қарсы белсенділігін синтездеу және бағалау

Қатерлі ісік — адам ағзасында таралу қабілеті бар жасушалардың бақылаусыз өсуі. Зерттеудің мақсаты витаминдерді селективтілік пен жүйелік ұйғалық сияқты мәселелерді шешу үшін ісікке қарсы жаңа препараттардың негізгі компоненті ретінде қолдануға болатынын көрсету. 5-фторурацил сірке қышқылы-D3 витамині (5FUAC-Vit.D3) синтезделді, сипатталды және оның ісікке қарсы белсенділігі бағаланды. 5-FUAC-Vit.D3 конъюгациясы HCl байланыстырушы ретінде N-гидроксисукцинимид (NHS) және 1-(3-диметиламинопропил)-3-этилкарбодимид (EDC) қатысуымен этерификация механизмімен синтезделді. Эфир байланысы арқылы 5-FUAC-Vit.D3 конъюгатының түзілуі, сонымен қатар қосылыстардың құрылымы IR, NMR және масс-спектрометрия мәліметтерімен расталды. Докингітік зерттеулер 5-FUAC-Vit.D3 конъюгациясы сәйкесінше –8.614 байланыс индексі бар сутек пен иондық байланыстар арқылы адам тимидилатсинтазы ақуыздарының Arg-215 және Lys-47-мен өзара әрекеттесетінін көрсетті, бұл басындағы 5-ФУ (-3.475) препаратымен салыстырғанда жоғары болатыны байқалды. Сонымен, түзілетін 5-FUAC-Vit.D3 конъюгаты мақсатты ақуызбен көбірек әрекеттесетіні байқалды.

Кілт сөздер: синтез, молекулалық модельдеу, молекулалық кондыру, дәрілік конъюгат, 5-фторурацилсірке қышқылы, D3 витамині.

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Синтез и оценка конъюгата «витамин – лекарственный препарат» на предмет его противораковой активности

Рак — это неконтролируемый рост клеток человеческого тела, который имеет способность распространяться. Цель исследования — показать, что витамины могут применяться в качестве целевого компонента для новых противоопухолевых препаратов с целью решения таких проблем, как неселективность и системная токсичность. Конъюгат «5-фторурацил уксусная кислота – витамин D3» (5FUAC-Vit.D3) был синтезирован, охарактеризован и оценен на предмет его противораковой активности. Конъюгат 5-FUAC-Vit.D3 был синтезирован по механизму этерификации в присутствии N-гидроксиsuccинимида (NHS) и 1-(3-диметиламинопропил)-3-этилкарбодиимида (EDC) с использованием HCl в качестве связующего агента. Образование конъюгата 5-FUAC-Vit.D3 через сложно-эфирную связь, а также структура соединений были подтверждены данными ИК, ЯМР и масс-спектрометрии. Докинговые исследования показали, что конъюгат 5-FUAC-Vit.D3 взаимодействует с Arg-215 и Lys-47 белков тимидилат-синтазы человека посредством водородных и ионных связей с показателем связывания соответственно –8,614, что выше, чем для исходного 5-FU (–3,475). В целом, было отмечено, что образующийся конъюгат 5-FUAC-Vit.D3 показывает большее связывание с целевым белком.

Ключевые слова: синтез, молекулярное моделирование, молекулярный докинг, конъюгат «витамин–лекарство», 5-фторурацилуксусная кислота, витамин D3.

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