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Sitagliptin enhances the effects of 5-fluorouracil in cancer colon through inhibition of thymidylate synthase

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Abstract
5-fluorouracil (5-FU) is a regularly used treatment for colon cancer because of its low cost. It functions by interfering with DNA replication in cancer cells. However, its effectiveness is often limited by the development of chemoresistance. This study evaluates the cytotoxic effects of combined 5-fluorouracil (5-FU) and Sitagliptin (Sita), a medication primarily used for diabetes but also possessing potential cancer-modulating properties on Caco-2 cells and explores the molecular interactions between sitagliptin and thymidylate synthase (TS) through *in vitro* and in silico analyses. Treatments for Caco-2 cells included different doses of 5-FU, sitagliptin, and their combinations. Using MTT assays, the cytotoxic effects were evaluated. The binding affinities of sitagliptin, 5-FU, and raltitrexed (a recognized TS inhibitor) to the TS protein were examined using molecular docking experiments. Results of the MTT assay indicated a dose-dependent reduction in Caco-2 cell viability for both 5-FU and sitagliptin, with the combination treatment showing a substantially greater reduction in cell viability. The molecular docking study revealed that sitagliptin binds strongly to the TS active site. In summary, the combined administration of 5-FU and sitagliptin enhances cytotoxicity against Caco-2 cells, suggesting that sitagliptin may potentiate the efficacy of 5-FU in colon cancer treatment. The strong binding affinity of sitagliptin to TS supports its potential role in improving chemosensitivity. To confirm the therapeutic effects of this combination in patients with colon cancer, our findings call for more molecular and clinical research.

Keywords: Sitagliptin, chemoresistance, 5-flourouracil, molecular docking

1. Introduction
As the third most common cancer globally, colon cancer is a serious public health concern. This particular kind of cancer affects the colon and the rectum, which can cause serious problems with the absorption of nutrients and the removal of waste, both of which can be fatal [1]. Abdominal pain, blood in the stool, altered bowel habits, and unexplained weight loss are common signs of colon cancer [2]. The disease arises due to the growth of abnormal cells that have lost the normal control over cell division, causing these cells to expand, invade nearby tissues, and metastasize to other organs [3].

Several factors contribute to the development of colon cancer, such as age, family history, diet, and a sedentary lifestyle. However, genetic and epigenetic changes are particularly significant in transforming normal colon cells into carcinoma. Depending on the patient's general condition and the disease's stage, treatment options for colon cancer might vary and include targeted therapy and surgery. Unfortunately, these treatments can be invasive and costly, especially in developing countries [4].

For colorectal cancer, 5-fluorouracil (5-FU) is a frequently used and reasonably priced chemotherapy medication. With a fluorine atom in place of a hydrogen atom at the C-5 position, this antimetabolite chemotherapeutic shares structural similarities with uracil (Figure 1). 5-FU converts into cytotoxic metabolites, such as 5-fluorouridine-5-triphosphate (FUTP) and 5-fluoro-2-deoxyuridine-5-triphosphate (FdUTP), which are effective against cancer cells. As a result of these compounds' incorporation into RNA and DNA during synthesis, cell proliferation is stopped and apoptosis is triggered [5]. Furthermore, 5-FU becomes 5-fluoro-2-deoxyuridine-5-monophosphate (FdUMP), which inhibits the enzyme thymidylate synthase (TS), which is required for the replication of nucleic acids [6].

Despite its effectiveness and affordability, the use of 5-FU is limited due to the development of chemoresistance, which results in dose-dependent toxicity and severe adverse effects [7, 8]. Key factors contributing to 5-FU chemoresistance include increased drug metabolism, enhanced DNA repair mechanisms, and certain epigenetic regulators [9, 10, 11].

To overcome chemotherapy resistance, various strategies have been proposed, including combination therapy. This approach involves using one or more compounds with anti-cancer activities in conjunction with traditional chemotherapy to target different molecular pathways in cancer cells synergistically [12, 13]. Our research has identified that dipeptidyl peptidase-4 (DPP-4) inhibitors, commonly used as diabetic medications, exhibit efficacy in targeting colon cancer *in vitro* [14]. Notably, sitagliptin (Sita) is the only DPP-4 inhibitor that belongs to the organofluorine class of heterocyclic compounds, similar to 5-FU. Sitagliptin contains a trifluoromethylated triazolopyrazine moiety and a 2, 4, 5-trifluorophenyl group (Figure 1).

Sitagliptin has shown potential in influencing the response of cancer cells to chemotherapy, although the exact mechanisms remain unclear [15].

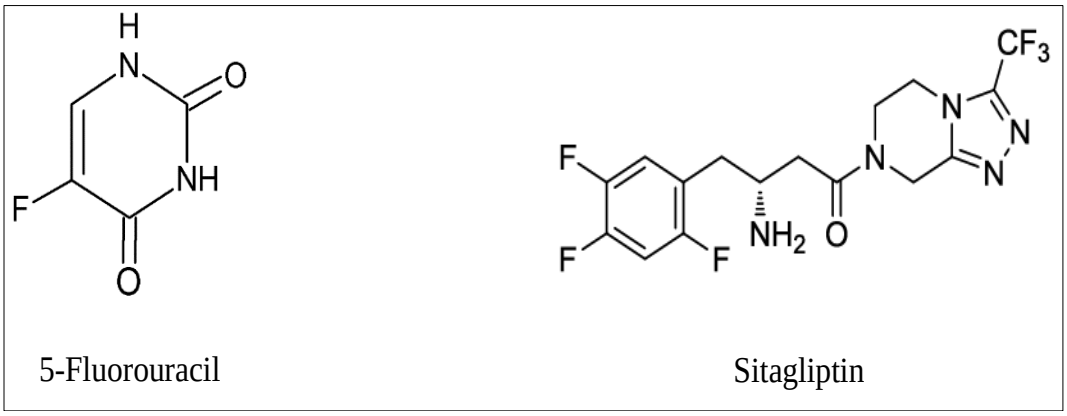


Fig 1: Structures of 5-fluorouracil and Sitagliptin

Thus, using both *in vitro* and in silico analyses, our aim was to examine how sitagliptin influences the susceptibility of colon cancer cells to 5-FU and the possible pathways involved. Our *in vitro* studies showed that the administration of sitagliptin in addition to 5-FU reduces the proliferative activity of caco-2 cells as compared to treatment with 5-FU alone. Both sitagliptin and 5-FU have been shown to bind to TS protein through molecular docking experiments, which implies that sitagliptin may increase the susceptibility of caco-2 cells to 5-FU.

All things considered, our results demonstrate how sitagliptin may make colon cancer cells more sensitive to 5-FU, providing a viable means of boosting the effectiveness of chemotherapy and overcoming drug resistance.

2. Materials and Methods

2.1. *In vitro* assays

Cell lines

The CaCo-2 cell line was kindly donated by VACSERA in Giza, Egypt. It is produced from epithelial cells associated with colon cancer. The growth medium used for these cells was Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat-treated bovine serum albumin (BSA), 4 mM L-glutamine, 25 mM HEPS, and 4 mM sodium pyruvate. The CaCo-2 cells were propagated in a CO2 incubator with 37 °C and 95% relative humidity [16, 17]. Using Zeiss A-Plan 10X objective lens inverted microscope, the cultivated cells were examined and captured on film.

Preparation of drugs

DNase-RNase free water was used to prepare the 5-FU (F6627 Sigma) and sitagliptin (SML3205 Sigma) stocks at a concentration of 5 mg/mL. Final concentrations of 0.6 mg/mL, 1.2 mg/mL, 2.5 mg/mL, and 5 mg/mL were obtained by dissolving various quantities of 5-fluorouracil and sitagliptin in distilled and sterilized water. Before they were used, these solutions were gathered in sterile tubes and kept at 4 °C.

Proliferation assay

An inverted microscope was used to examine the morphology of the cells. Cells were planted in triplicate at a density of 10x104 cells per well in 96-well plates in order to determine cell growth. Moreover, 10x104 cells were put into each of the duplicate wells in a 6-well plate.

The hemocytometer was used to find the cell survival rate. The cells were first cleaned twice using phosphate-buffered saline (PBS), discarding the old media. The cells were then treated with trypsin and incubated for three minutes at 37 ºC. After trypsinization, the cells were treated to 8 ml of full RPMI medium, and the number of cells was assessed by looking at 1 µl of the cell suspension under an inverted microscope [18, 19].

Cytotoxic concentration 50% (CC_{50})

CaCo-2 cells were used to test the anticancer properties, toxic effects, and cytotoxic concentration 50% (CC50) of the given concentrations of each medication and their combination. At a density of 10X103 cells per well, the cells were seeded in triplicate in 96-well plates, and they were then incubated at 37 ºC in a humid environment for the entire night. Following this, the cells were cultured for an additional night at varying concentrations of each component (0.6 to 5 mg/ml). Using an MTT colorimetric test kit, the cell viability rate and CC50 were ascertained (Sigma-Aldrich, Germany). In short, the treated cells' medium was thrown away, and PBS was used to wash them. Subsequently, each well received 100 µl of full RPMI media. Following the addition of 10 µl of MTT solution to each well, the plate was incubated at 37 ºC for two hours. Following incubation, each well received 100 µl of SDS-HCl solution, and the mixture was incubated for a further 4

hours at 37 ºC. The optical density at 570 nm was used to measure the vitality of the cells. This was done by converting the water-soluble MTT into an insoluble formazan, which was then solubilized and quantified [20].

2.2. Molecular docking analyses

Using MOE software (Molecular Operating Environment software ver. 2014.9), a molecular docking analysis study was conducted to evaluate the binding interactions between sitagliptin and 5-FU with the proliferative protein human thymidylate synthase (TS). Under code 1HVY, the TS protein was taken out of the X-ray structures that are accessible at the Protein Data Bank (http://www.rcsb.org). A protein called 1HVY stands for TS linked to dUMP and the inhibitor raltitrexed. Next, the MOE software's default preparation module was used to prepare the imported protein [21]. On the other hand, the co-crystallized ligand Raltitrexed, sitagliptin, and 5-FU's chemical structures were obtained from the PubChem chemical database (Http://pubchem.ncbi.nih.gov). After that, the imported ligand was ready for docking using MOE software. To do this, the MMFF94 forcefield was applied to minimize energy until a 0.01 Kcal/mol gradient of convergence was reached. Prior to docking calculations, the synthesized molecules were then imported into the MDB file of the analysis database. The protein's ligand was docked inside the produced MDB file, and the created poses were assessed based on the type of interaction and the binding score (S-value) with particular amino acids inside the target protein's active site, as previously mentioned [22].

Statistical Analysis

The data is presented as means ± standard deviation (SD). ANOVA, or one-way analysis of variance, was used to compare different parameters. According to Tukey's ANOVA post-hoc test, a significant statistical difference is indicated by a p-value of 0.05 or less. SPSS version 20.0 was used to conduct the statistical analysis (SPSS Inc.).

3. Results

Combination 5-Fluorouracil and Sitagliptin's Cytotoxic Effects on Caco-2 Cells: Evaluation Using the MTT Assay

CaCo-2 cells were pre-treated with varied dosages of either 5-fluorouracil, sitagliptin, or their mixtures. MTT assays were then performed on the CaCo-2 cells to evaluate the potential cytotoxic impact of these compounds on the viability of the cells. The data presented in (Figure 2) demonstrated that the mean absorbance values suggested a dose-dependent decrease in the Caco-2 cells' viability rate in response to sitagliptin and 5-fluorouracil. However, their mixtures significantly decreased the Caco-2 cells' survivability rate in a dose-dependent manner. The cumulative findings indicate that the co-administration of 5-fluorouracil and sitagliptin exhibits a heightened cytotoxic response against Caco-2 cells as compared to the individual drugs. The combination treatment appears to have a synergistic effect.

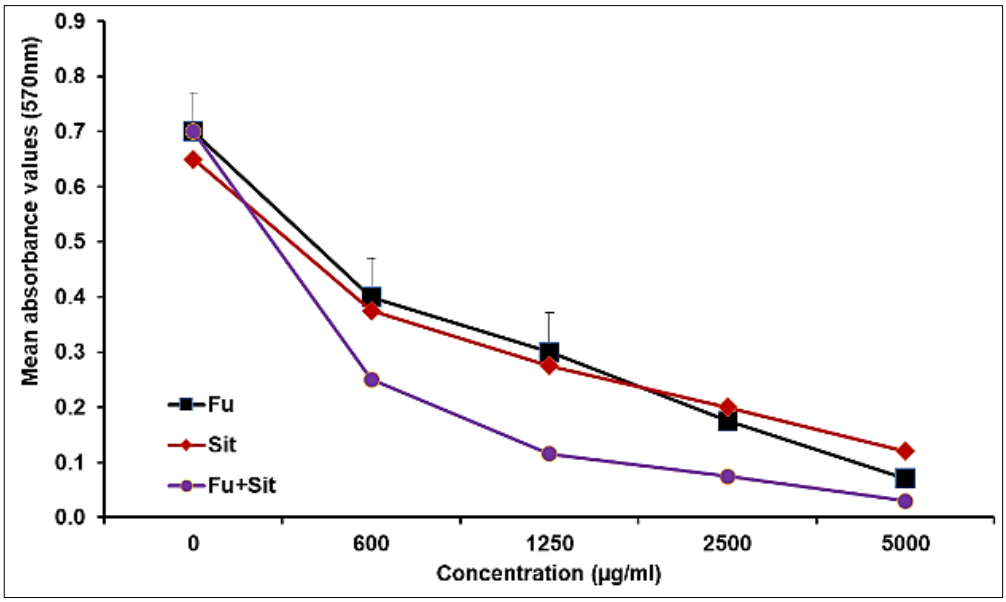


Fig 2: Cytotoxicity and Influence of 5-flourouracil, sitagliptin and their combinations on CaCo-2 cell viability; Absorbance values at 570nm in relation to concentration (mg/ml) of 5-flourouracil, sitagliptin and their combinations on Caco-2 cells.

Results of the Molecular docking study

Sitagliptin strongly docked in the active site of the human thymidylate synthase (TS) protein

A study using molecular docking was conducted to assess how sitagliptin binds to TS at the 1HVY inhibitor binding site, which may explain its potential to inhibit cell proliferation in the CaCo-2 cell line. Initially, the original co-crystallized TS inhibitor (Raltitrexed) was redocked in the inhibitor binding site as a way to confirm the reliability and precision of the docking process. The findings showed that the docking conditions we used successfully placed our compound in an alignment that closely resembled the original co-crystallized ligand (Figure 3), and -7.705 kcal/mol was its binding free energy. The binding affinity of sitagliptin was -6.389 kcal/mol, which was marginally greater than that of the native ligand (raltitrexed = -7.705 kcal/mol).

Conversely, the binding energy of 5-FU was -4.125 kcal/mol, suggesting a much weaker binding compared to Sitagliptin (Figure 4). The amino acid residues responsible for the binding of the protein to raltitrexed, 5-FU, and sitagliptin can be found in table 1.

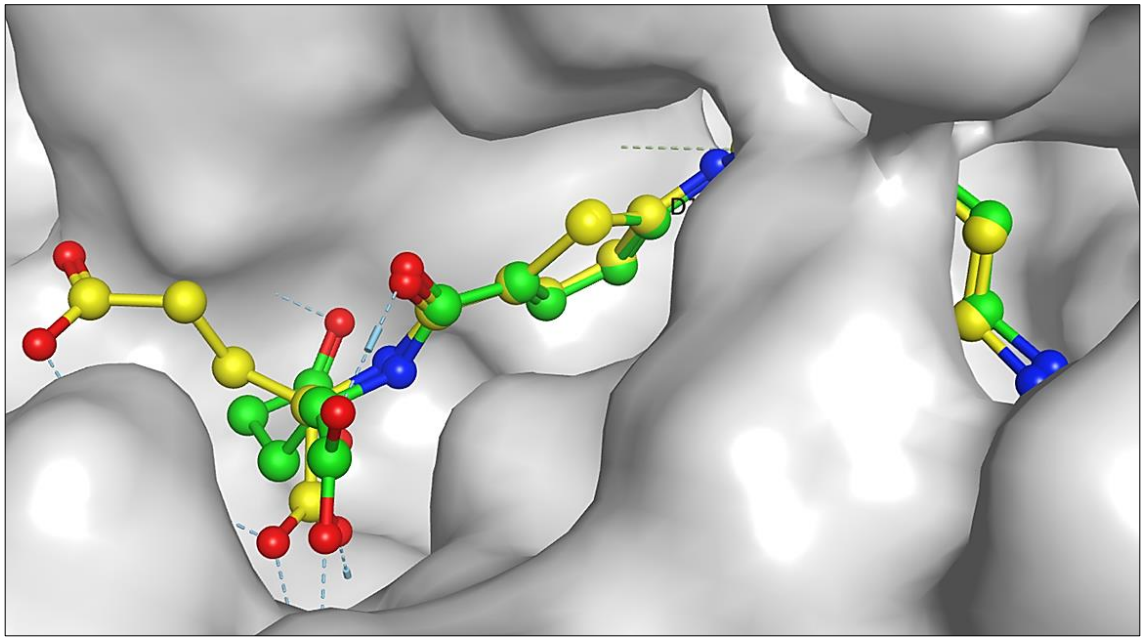
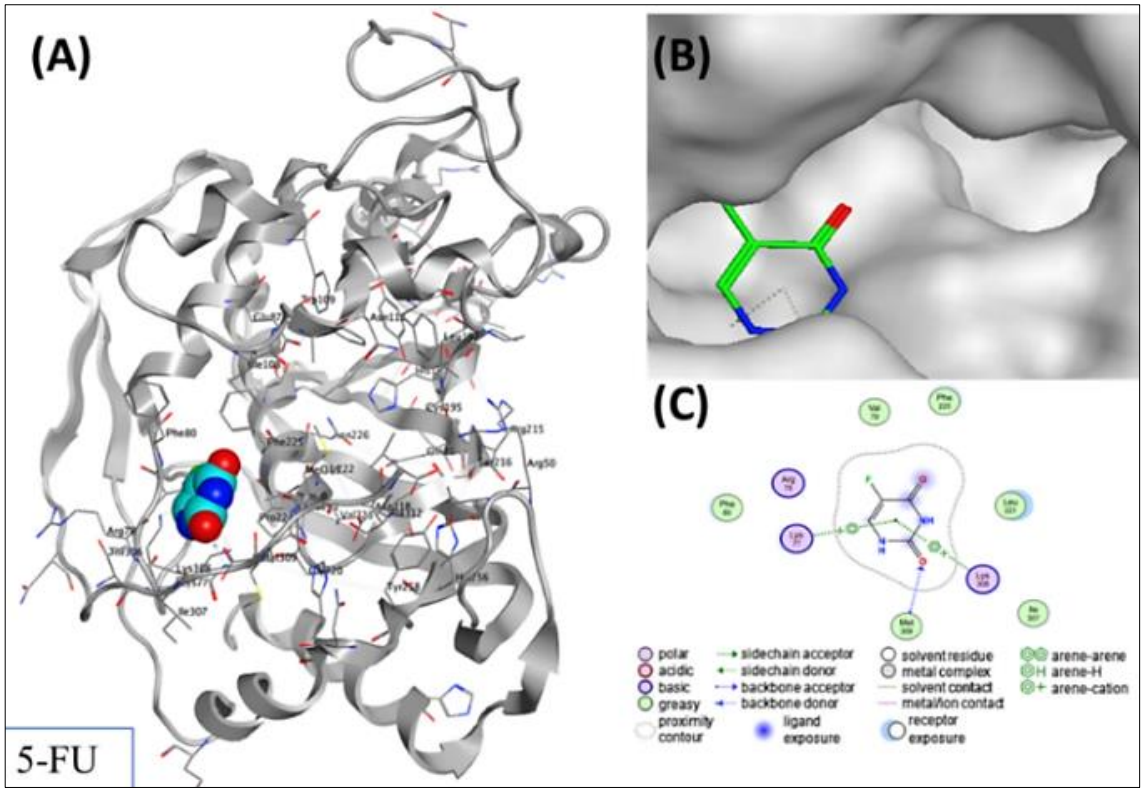


Fig 3: The re-docked ligand raltitrexed in the human thymidylate synthase protein's active region is depicted in a three-dimensional schematic (PDB ID: 1HVY). The colours yellow and green, respectively, represent the native and re-docked compounds. Between the re-docked ligands and the crystal structure, the root mean square deviation (RMSD) was 1.235 Å.

Table 1: The hydrogen bond interactions and docking scores in the thymidylate synthase protein's binding site (PDB ID: 1HVY)

Molecule	Overall Binding Energy Kcal/mol	Ligands' interacting atoms	Receptors interacting atoms	Amino acid residues	H-Bond type	Interaction distance in Å	Single bond energy Kcal/mol
Raltitrexed	−7.705	O6	O	LEU221	H-donor	2.83	-3.5
		O4	NZ	LYS308	H-acceptor	3.12	-1.1
		O7	CE	LYS77	H-acceptor	3.19	-0.6
		C13	6-ring	TRP109	H-pi	4.3	-0.5
		C24	6-ring	TRP109	H-pi	3.84	-0.8
5-FU	−4.127	O3	N	MET309	H-acceptor	3.33	-1.5
		6-ring	NZ	LYS77	Pi-cation	4.1	-1.4
		6-ring	NZ	LYS308	Pi-cation	4.26	-1.1
Sitagliptin	−6.389	F1	NZ	LYS77	H-acceptor	3.09	-1.1
		F2	NZ	LYS 308	H-acceptor	3.18	-0.5



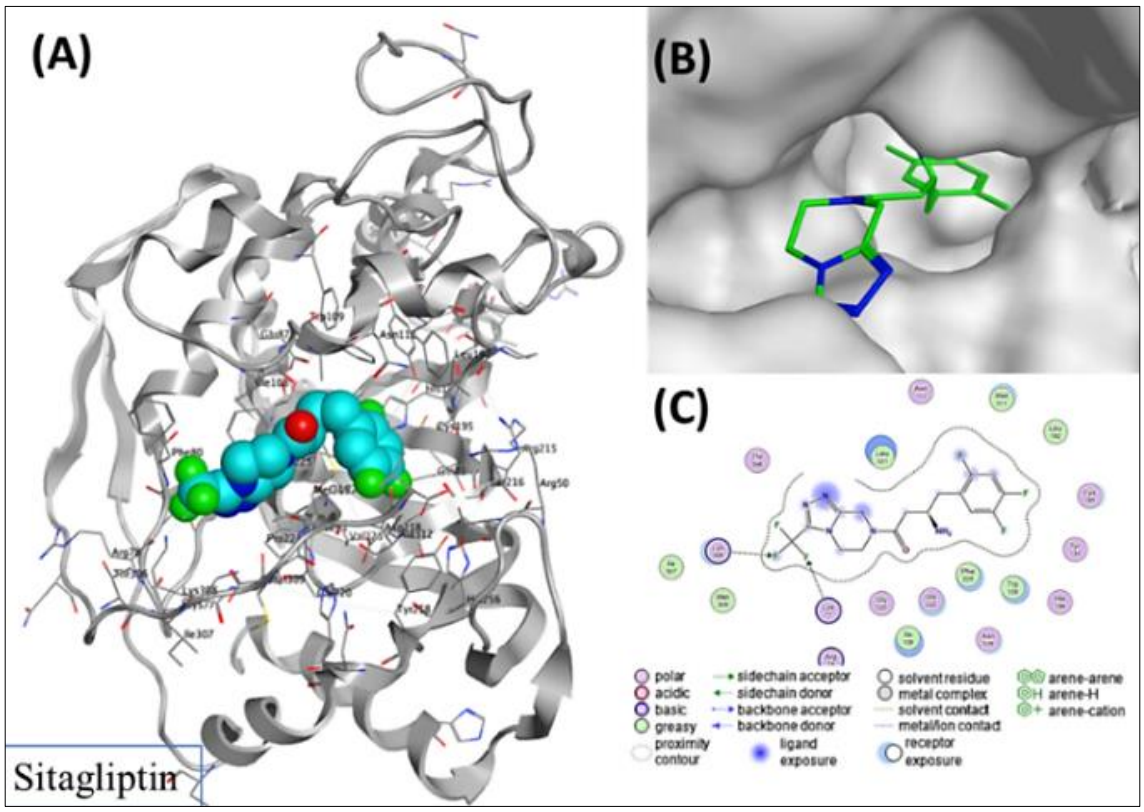


Fig 4: Molecular docking analysis was conducted *in silico* to examine how sitagliptin interacts with the human thymidylate synthase (TS) model protein (PDB ID: 1HVY). (A) The TS overall 3D structure, (B) 3D interaction (B) and (C) 2D interaction diagrams of the binding modes with Sitagliptin (upper diagram) and with 5-FU (lower diagram).

4. Discussion

5-FU chemoresistance presents a considerable obstacle in effectively treating patients with colon cancer and has a notable impact on their overall prognosis8. As colon cancer patients may exhibit an initial positive response to chemotherapy-based 5-fluorouracil. However, a substantial number of patients ultimately develop chemoresistance, leading to the recurrence of tumors within the first three to five years following treatment [23]. Many strategies have been put forth to lessen the negative effects of 5-FU while also addressing the issue of cancer cells' chemoresistance to it. One such approach involves utilizing drug combination therapy, which entails combining one or more compounds with anticancer properties alongside conventional chemotherapy to combat the development of chemoresistance. Therefore, our exploration has been focused on identifying compounds that possess anticancer attributes and the ability to affect chemotherapy medications' effectiveness. The results of our investigations have indicated that Sitagliptin satisfies both of these criteria. Sitagliptin is commonly utilized in the treatment of diabetes and is classified as an organofluorine heterocyclic compound, a group that also encompasses 5-FU [24, 25]. Interestingly, research has indicated that the use of sitagliptin has resulted in a reduced risk of cancer among individuals with diabetes [26, 27, 28]. Nevertheless, it is still unknown how precisely sitagliptin affects 5-FU's anticancer capabilities in colon cancer.

Primarily, our MTT proliferation experiment in caco-2 cells indicated that the combination of sitagliptin and 5-FU exhibited distinct anti-proliferative properties over single treatments. This implies that sitagliptin might increase colon cancer cells' susceptibility to 5-FU, while preserving its antiproliferative efficiency *in vitro*. The enhanced efficacy of the 5-FU + Sita combination suggests that lower doses of each compound might be used to achieve the desired therapeutic effect, potentially reducing side effects associated with higher doses of individual treatments. This is particularly relevant in clinical settings where multi-targeted approaches are increasingly being recognized for their potential to improve patient outcomes.

Remarkably, our *in-silico* analysis of docking sitagliptin into the thymidylate synthase active site revealed that sitagliptin has binding affinities similar to those of 5-FU. Both compounds engage with the amino acid residues LYS77 and LYS308 located inside the thymidylate synthase's active site. Sitagliptin, however, displays stronger binding affinity, as evidenced by its lower binding energy of -6.389 kcal/mol in contrast to 5-FU's -4.125 kcal/mol, which is comparable the co-crystallized inhibitor Raltitrexed's -7.705 kcal/mol. These findings may explain the potent antiproliferative effects of sitagliptin on cancer cells.

Together, the results of this research offer strong evidence in support of conducting additional studies on the effectiveness of using a combination of sitagliptin and 5-FU for treating colon cancer. Extensive molecular investigations are required to elucidate the precise mechanisms underlying sitagliptin impact on chemosensitivity. Clinical studies will validate these preclinical observations and potentially translate them into improved treatment strategies for colon cancer patients.

5. Conclusion

In conclusion, combining Sitagliptin and 5-FU showed increased effectiveness in inhibiting colon cancer cell growth. Cumulatively, our findings establish that Sitagliptin

could help counter 5-FU resistance and be considered for combination therapy in colon cancer treatment. Further research is needed before Sitagliptin can be used in clinical trials. Finally, this study highlights the importance of repurposing drugs and investigating novel uses for medications that have been used in clinical settings in the past.

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There was no support for this research from government, private, or nonprofit organizations.

7. A statement of conflicting interests
There are no disclosed competing interests for the writers.

8. Data accessibility
Upon request, the author will provide the study's data.

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