Prediction of Thymidylate Synthase Inhibitors: An *In Silico* Study

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ABSTRAC: Human Thymidylate synthase (hTS) is among the most exploited enzymes in the modern structure based drug design of new anticancer agents due to its key role in the biosynthesis of DNA. Therefore, inhibition of hTS is considered to be a potent approach to cure many types of cancers. The most well-known hTS inhibitor is 5-fluorouracil which has been marketed as an anticancer drug, but more optimization is needed for its biological activity. In this work, six potential hTS inhibitors have been designed based on structure of 5-FU as a lead compound. The designed compounds were in silico investigated for their potency to inhibit hTS by using autodock vina. The results were showed that the designed compounds have binding modes similar to that of 5-FU, and most of them have robust binding affinities to hTs. Further molecular docking study was performed against dihydropyrimidine dehydrogenase to investigate whether the designed compounds are susceptible for degradation by this enzyme. The molecular docking study indicated that compounds 1-5 are the most potent ones.

Keywords: 5-FU, Molecular docking, hTS, In silico, DPD.

1. Introduction

Cancer disease is a major health problem presented worldwide, particularly in developed countries, where it ranks the second cause of death after cardiovascular diseases, and it is mainly correlated with ageing of the population and lifestyle [1]. Currently, millions of people worldwide are diagnosed with cancer, and more than half of them ultimately succumb to the disease [2]. According to the World Health Organization, there has been a dramatic increase worldwide, and it is expected that there will be almost 22.2 million new patients diagnosed with cancer annually by 2030 [3]. Recently, various therapeutic strategies to stop or cure this disease have been suggested and evaluated by researchers, however, there remains a necessity to discover more efficient approaches [3]. 5-fluorouracil (5-FU) was developed by Heidelberger over 50 years ago which is up to now the drug of choice for systemic therapy against colorectal, head and neck cancer. The anticancer activity of 5-FU is exerted by different mechanisms of action, where, after administrating the drug, it is activated through intracellular conversion into various nucleotide forms such as 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) which is considered to be a critical nucleotide metabolite, that covalently binds to the Thymidylate synthase, resulting in inhibition of this enzyme, while the other nucleotide metabolites such as 5-fluorodeoxyuridine-5'-

triphosphate is incorporated into DNA, leading to stopping of DNA synthesis and function [4]. However, this drug has been faced drug resistance and toxicity as side effect [5]. Moreover, previous studies were demonstrated that approximately 85% of the administered 5-fluorouracil is degraded in the liver by an enzyme called Dihydropyrimidine dehydrogenase (DPD) [6-7]. The present study aims to overcome the problem of 5-FU degradation and toxicity through virtual screening of 5-FU analogues with potential anticancer activity.

2. Materials and methods

2.1. Protein preparation

The 3D structures of hTS (PDB code: 1hvy) and Dihydropyrimidine dehydrogenase (PDB code: 1GT8) were selected from the protein data bank (https://www.rcsb.org/). The typical PDB structure file may contain heavy atoms, a co-crystallized molecule, water molecules, cofactors and metal ions. Therefore, the obtained PDB files of proteins from the protein data bank are not suitable for direct use. To perform the molecular docking study, all the undesired molecules and ions were removed via using autodock tools 1.5.6 (ADT) [8]. Polar hydrogen atoms and partial charges, assigned using the Gasteiger method, were added to the proteins via ADT.

2.2. Library Preparation

Pubchem was used to download the ligands that have high structure similarity with the prodrug 5-fluoro-2'-deoxyuridine. Based on the literatures, we excluded the compounds that were previously screened as hTS inhibitors, thereby, a small library of 55 organic compounds was built and subjected for molecular docking. 2.3. *Virtual screening*

The Virtual screening process was carried out using Mcule server (https://mcule.com/). The active binding sites of the two target enzymes have been previously characterized [9], The conformation with the lowest binding energy was considered the most favorable. Discovery Studio Visualizer (v4.5) was used to analyze and visualize the intermolecular interactions between the receptors and the ligands. [10].

3. Results

3.1. Target enzymes

Thymidylate synthase is an essential enzyme involved in DNA biosynthesis, where, it's involved in the synthesis of DNA precursor 2'-deoxythymidine-5'-monophosphate.

Consequently, this enzyme has been gained a great consideration in cancer chemotherapy [11]. 5-fluorouracil was the first human Thymidylate synthase inhibitor that clinically used for the treatment of some cancer types such as breast, pancreatic, colorectal, stomach and ovarian cancers [12]. Previous studies were utilized to recognize the nature and the function of the residues located in the binding site of Thymidylate synthase. Previous investigations of different PDB crystal structures of TS which is available in complex

with its natural substrates or control drugs were identified the key amino acid residues involved in ligand–protein interactions, the active residues are Arg50, Ile108, Trp109, His196, Arg215, Ser216, Asp218, Gly222 Tyr258 [13].

Dihydropyrimidine dehydrogenase (DPD): The human DPD has recently considered as an important target for anticancer agents design, where, DPD is responsible for degrading approximately 85% of the administered dose of 5-fluorouracil (5FU) and converting it into toxic fluorinated products and therapeutically inactive metabolites. Therefore, the catabolism of 5-FU by DPD is considered as a major obstacle of its pharmacokinetics [14]. In order to overcome this obstacle, the screened compounds mustn't behave as a good substrate for DPD, thus, we were motived to screen a potential hTS inhibitors which are expected to be non-degradable by DPD.

3.2. Library preparation

A wide variety of 5-fluorouracil analogues have been examined as anticancer agents by many researchers, and they were used different docking softwares to simulate the binding ability and to understand the inhibition mechanism, in the current study, we were motived to screen a small library of some 5-fluorouracil analogues and evaluate (*in silico)* their ability in inhibiting thymidylate synthase. The selected compounds have a sufficient structural resemblance with the prodrug 5-FU or with the drug (5-fluoro-2'-deoxyuridine-5'-monophosphate) as shown in Table 1, this structural resemblance is quite enough to ensure a convenient inhibition activity, on the same time the selected compounds are structurally-different from the substrate of DPD, which indicates that they are expected to be escaped from the enzymatic degradation by this enzyme.

3.3. Molecular docking-based virtual screening

Molecular docking is a valuable computational approach for predicting the binding affinities between ligands and proteins, providing deeper insight into the inhibitory potential of the ligands and their possible mechanisms of action. The constructed small library was docked using Mcule server (https://mcule.com/), to estimate the ligand binding affinity against human Thymidylate synthase (hTS). The obtained docking scores for compounds were compared with the control inhibitor (5-FdUMP). As mentioned in previous studies that the formed complex is stabilized with lowest binding energy [15], the ligand-protein complex with the lowest binding energy value was selected.

The control prodrug (5-FU) inhibits hTS after intracellular conversion into its nucleotide (5-FdUM), therefore, the substrate of hTS is the nucleotide of uracil (dUMP), and hence the selected compounds were docked in the form of their nucleotides to ensure precise simulation. Noteworthly that the phosphate group of the nucleotide dissociates at the physiological pH, thus, the selected

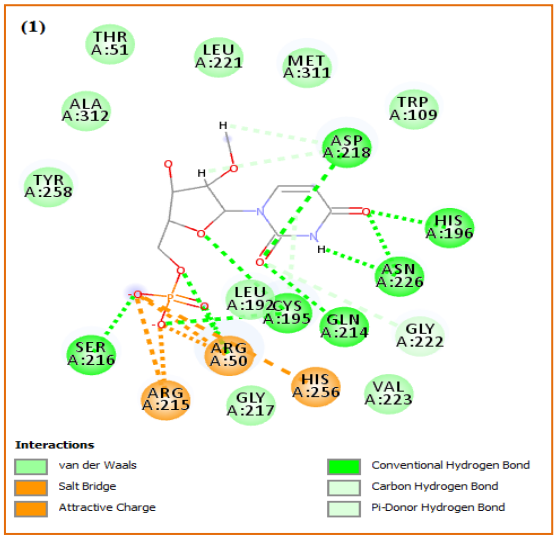
compounds were docked in the form of negatively charged nucleotides. The results were showed that the highlighted candidates were positioned in the active loop of hTS and stabilized by a combination of different interactions as shown in Fig 1. Among the 55 screened compounds, the top five were selected and visualized to illustrate their molecular interactions (Fig.1). The top binding score towards hTS was -8.5 kcal/mol as shown in Table 1, all five compounds exhibit binding affinities superior to those of the reference drug (5-FdUMP). The Cys195 which is located in the active loop of hTS has been classified as a critical amino acid for the binding with the substrate, via a covalent bond. In case of our compounds the observed interactions of the active residue Cys195 with the docked compounds were included hydrogen bonds, π-sulfur interactions and hydrophobic interactions. Also, the hydrogen bonding were observed between the docked molecules and various key residues, such as Arg50, His196, Arg215, Ser216, Asp218, Gly222, Asn226, Tyr258 as illustrated in Fig 1.

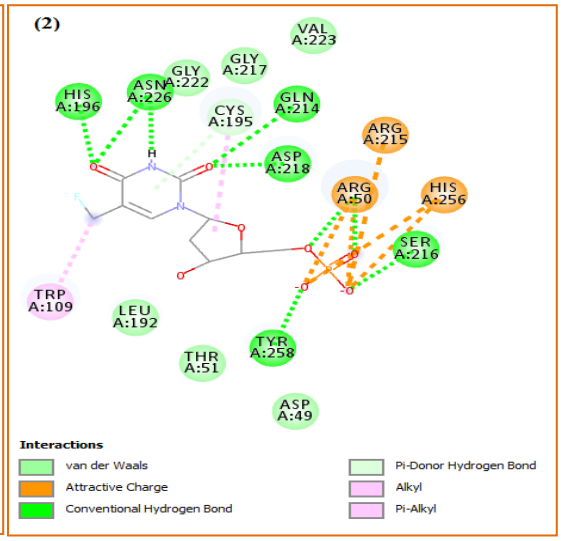
Furthermore, the binding modes of the docked compounds with DPD were analyzed to find out the molecular interactions with the key amino acids such as Asn609, Asn668, Cys671, Asn736 and Thr737 in DPD binding site. The results showed that the binding modes of compounds **1-5** were completely different from those of 5-FU (Fig 2), where, these compounds were positioned out of the active loop, indicating potential evasion of DPD-mediated enzymatic degradation.

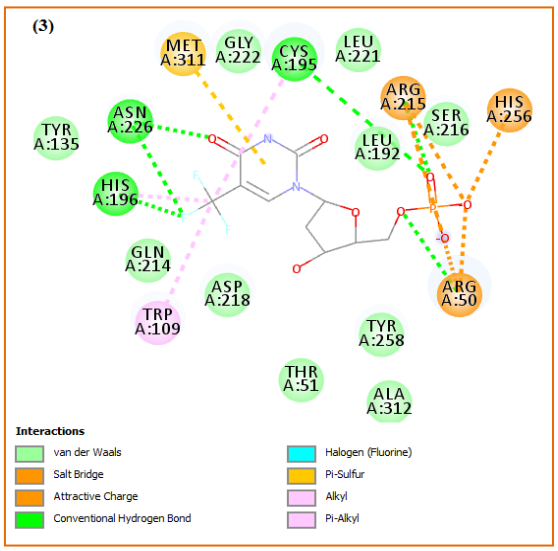
Table 1: Molecular docking scores of the top 5 compounds screened against hTS.

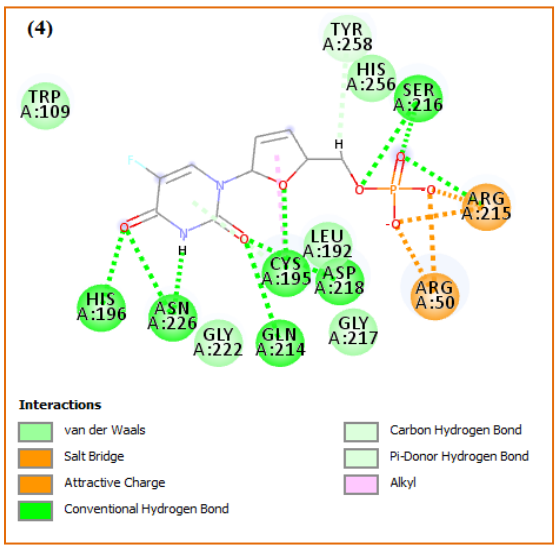
Comp.No.	Name	Docking score (kcal/mol)	Total Number of Interactions	Number of Hydrogen Bonds/ Interaction Residues
1	2'-O-Methyluridine	-8.5	16	6 (Cys195, His196, Gln214, Ser216, Asp218, Asn226)
2	5-(2-Fluoroethyl)-2'-deoxyuridine	-8.5	19	6 (His196, Gln214, Ser216, Asp218, Asn226, , Tyr258)
3	1-[(2S,4S,5S)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-5-	-8.5	17	3 (Cys195, His196, Asn226,)

	(trifluoromethyl)pyrimidine-2,4-dione			
4	5-fluoro-1-[(2S,5R)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]pyrimidine-2,4-dione	-8.4	18	6 (Cys195, His196, Gln214, Ser216, Asp218, Asn226)
5	1-[3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]pyrimidine-2,4-dithione	-8.2	19	3 (Arg215, Ser216, Asn226)
Ref.	5-flouro uracil	-7.49	7	4 (Gln214, Asp218, Asn226, Tyr258)









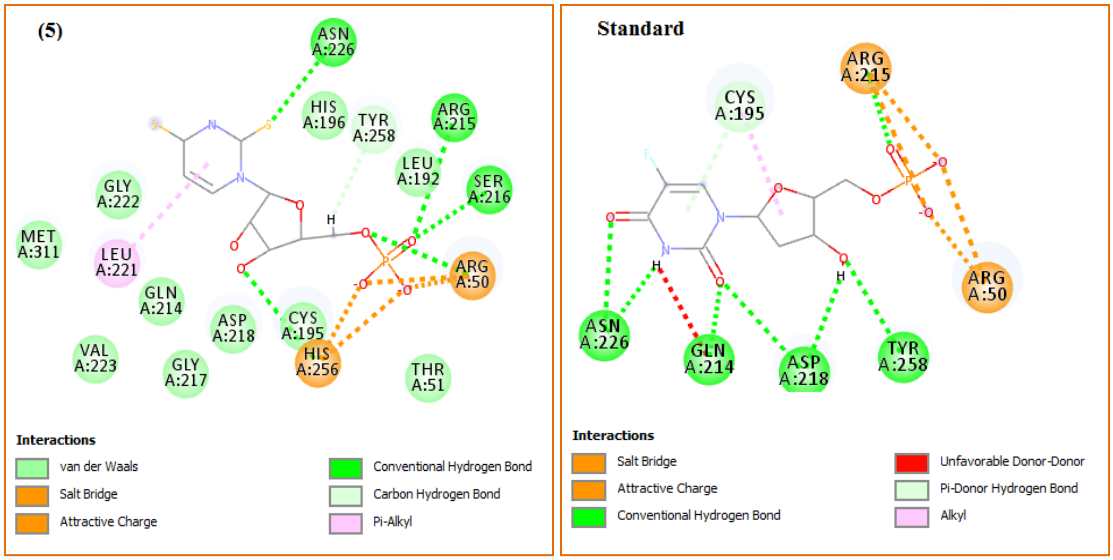


Figure 1: Interaction diagrams showing compounds 1-5 and standard drug in complex with hTS (PDB code: 1hvy).

4. Discussion

hTS is a primary target in cancer chemotherapy due to its indispensable role in the biosynthesis of DNA. It facilitates the production of 2'-deoxythymidine-5'-monophosphate (dTMP), a necessary precursor for DNA synthesis. By blocking the active site of hTS, specifically through interactions with key residues like Cys195, the enzyme's function is halted, effectively stopping DNA replication and cellular proliferation. Our results demonstrated that the top five candidate compounds (1–5) achieved binding affinities ranging from -8.2 to -8.5 kcal/mol, which are significantly more robust than the -7.49 kcal/mol observed for the standard drug, 5-FU. These compounds stabilized their presence in the active loop through various interactions, including hydrogen bonds and π-sulfur interactions with Cys195, and other key residues, suggesting a higher potential for effective enzyme inhibition compared to existing therapies.

A significant limitation of current 5-FU treatment is its poor pharmacokinetic profile; approximately 85% of the administered dose undergoes rapid degradation in the liver by dihydropyrimidine dehydrogenase (DPD). This catabolism not only renders the drug inactive but also produces toxic fluorinated metabolites, leading to severe side effects and requiring high administration doses to maintain therapeutic levels. Consequently, a successful 5-FU analogue must not only inhibit hTS but also evade DPD recognition. Our molecular docking studies against DPD revealed that compounds 1–5 were positioned outside the active loop of this enzyme, and away from the crucial Cys671 residue. This structural placement indicates that these candidates are likely poor substrates for DPD, potentially allowing them to remain active in

the system longer and with reduced systemic toxicity. What sets this research apart from similar studies is the integrated dual-screening approach. While many investigations focus solely on increasing the binding affinity of ligands to hTS, this study prioritized the selection of compounds that are structurally different from DPD substrates while maintaining high resemblance to hTS substrates. By building a library that specifically excludes previously screened hTS inhibitors and focusing on the physiological state of the compounds (docking them as negatively charged nucleotides to mimic physiological pH), we ensured a more precise and biologically relevant simulation. This strategic design addresses the two most prominent failures of 5-FU: drug resistance and metabolic degradation, offering a more comprehensive solution for next-generation anticancer drug discovery.

5. Conclusion

To elucidate the efficacy and binding affinity of the designed library, *an in silico* study was accomplished. The interactions between the screened compounds and hTS were also identified. Depending on the obtained results, compounds 1-5 demonstrated high inhibitory potential against Thymidylate synthase. An additional molecular docking study was conducted to evaluate the susceptibility of the screened compounds to enzymatic degradation by DPD. According to the findings, compounds 1–5 are predicted to be resistant to DPD-mediated degradation, in contrast to 5-FU, which is susceptible to DPD metabolism; this resistance may enhance their inhibitory efficacy against hTS. This work facilitates the drug discovery process of new anticancer drugs, however experimental screening of the candidated compounds is needed for further validation.

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