# Role of Statins as Antagonists of 1DHF in Cancer

A Project

Submitted By

Nashrah Mustafa

ID: 14146044

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Department of Pharmacy

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*Dedicated to my Supervisor, Professor Dr. Eva Rahman Kabir*

# Certification Statement

This is to certify that the project titled “Role of Statins as Antagonists of 1DHF in Cancer” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Professor Dr Eva Rahman Kabir, Chairperson, Department of Pharmacy, BRAC University. Throughout the project, I have given appropriate credit where I have used the language, ideas or writings of another.

Signed,

_____

Countersigned by the Supervisor

_____

## Acknowledgement

I would like to begin by thanking the Almighty by enabling me to undertake this research in good health and frame-of-mind. Firstly, I would like to express my gratitude to Professor Dr. Eva Rahman Kabir, my project supervisor, for allowing me the opportunity to finish my thesis at my own pace. This project wouldn't come to light without the help of numerous individuals who are recognized here.

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## Abstract

Cancer involves the uncontrollable and abnormal proliferation of cells in the body and is among the leading causes of deaths worldwide. The enzyme, dihydrofolate reductase (DHFR) referred to as 1DHF functions as a precursor for the biosynthesis of purine and pyrimidine bases and is critical for the de novo synthesis of DNA. Maintenance of the normal levels of folate is extremely important, since disruption in the folate metabolism pathway has a lethal effect on rapidly proliferating cells, such as the bone marrow and red blood cells particularly because the normal cell division process gets hampered. A key feature of cancer cells is abnormal cell division or cell mitosis. 1DHF is highly expressed in cancer cells, which makes it a potential target for anti-cancer drug development. A positive correlation between cholesterol accumulation and cancer provide insights into further investigation of anti-cancer indication of statin drugs. In this study, data science, drug repurposing, and other *in silico* computational techniques have been employed to find a potential statin antagonist of 1DHF that would not only lower serum cholesterol levels but also successfully inhibit the folic acid biosynthetic pathway. The merits of drug repurposing include a safer, cost-effective, productive and more efficient drug. Molecular docking of statin drugs with the proteins 1DHF was carried out using PyRx and the drug-protein interactions were identified using Discovery Studio. The structure of the protein was validated using Ramachandran plot, z-scores, Errat and Verify 3D, and a z-score of -9.09 for 1DHF was obtained. The potential statin small molecules chosen were Pravastatin, Pitavastatin, Rosuvastatin and Simvastatin. Amongst these small molecules, Pitavastatin showed the highest binding affinity (-12.7Kcal/Mol) which indicates that Pitavastatin may possibly reduce the risk of cancer development. It may be given as an adjunct drug to cancer patients, with high levels of LDL, with their anti-cancer therapy. The main aim of this study was to find a statin drug that targeted 1DHF in the treatment of cancer and the statin drug Pitavastatin demonstrated such effect; however, they are required in doses much higher than that of patients receiving the usual statin treatment for hypercholesterolemia. Due to the advantages associated with drug repositioning, it is widely used. The study shows that the statin drug Pitavastatin, may have significant anti-cancer property as they bind with 1DHF with a high binding affinity. Thus,

drug repositioning may be used in combination with other oncology therapeutics to obtain a credible drug treatment by causing necrosis of malignant cells.

**Keywords:** 1DHF, data science, drug-protein interaction, drug treatment

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# Abbreviations

| | |
|---|---|
| ACS | Acute coronary syndrome |
| CHD | Coronary Heart Disease |
| DHFR | Dihydrofolate Reductase |
| DTI | Drug target interactions |
| EBV | Epstein-Barr virus |
| HDL | High density lipoprotein |
| LCL | Lymphoblastoid cell lines |
| LDL | Low density lipoprotein |
| LFA-1 | Lymphocyte function-associated antigen 1 |
| MAPK | Mitogen-activated protein kinase |
| MI | Myocardial infarction |
| MTHFR | Methylenetetrahydrofolate reductase |
| VLDL | Very low density lipoprotein |

# 1 Introduction

The immensely increasing incidence and mortality rates from cancer have prompted researchers and scientists to search for anti-cancer medications, and drug repurposing is a promising method to find such drugs. The process of novel drug discovery and development is extremely expensive as well as time-consuming; it takes about ten years for a new canditate drug to be finally accepted in the community after many phases of clinical trials (Roder & Thomson, 2015). In drug repurposing, a drug that has been widely accepted and had undergone many clinical activities are looked upon to find new indications (Kabir, Siam, Kabir, Khan, & Rajib, 2017). Due to its low cost and greater efficiency, it is more evident that the approach of drug repositioning is becoming more prevalent to find anti-cancer use of the available and already established drugs. In this study, anticancer properties of statins were investigated using 1DHF as the molecular target.

## 1.1 Dihydrofolate Reductase, its variants and its relation to Cancer

Dihydrofolate reductase (1DHF) is a member of the oxidoreductase class of enzymes; it catalyzes the reduction of dihydrofolate to tetrahydrofolate (the active biological form) in folate metabolism, aided by the cofactor, NADPH (Lin & Gerson, 2014). The enzyme is encoded by the human dihydrofolate reductase gene (Chen et al., 1984) and is located between the q11 and q22 region (Funanage, *et. al.* 1984) of chromosome 5 (Gao, Cui, Du, & Ji, 2013) The reduction is carried out by the transfer of a hydride from NADPH to dihydrofolate with an accompanying protonation to produce tetrahydrofolate. As a result of the reduction, NADPH is oxidized to NADP+ (Wan et al., 2014). Tetrahydrofolate is source of methyl group in synthesis of purine, amino acid and thymidylate (Pekamwar & Wadher, 2013). The enzyme is a relatively small water-soluble protein with a molecular weight in the range of 18-25 KDa for most of the species of the enzyme.

The protein has eight β-sheets, which form a rather rigid skeleton of the enzyme molecule. All forms of the enzyme, 1DHF contain at least four α-helices one of which makes up the substrate binding site and two other helices compose the coenzyme binding

site (Polshakov, 2001). 1DHF is a critical enzyme in de novo DNA synthesis and cell proliferation and is thus becoming a target for drug development and cancer therapy.

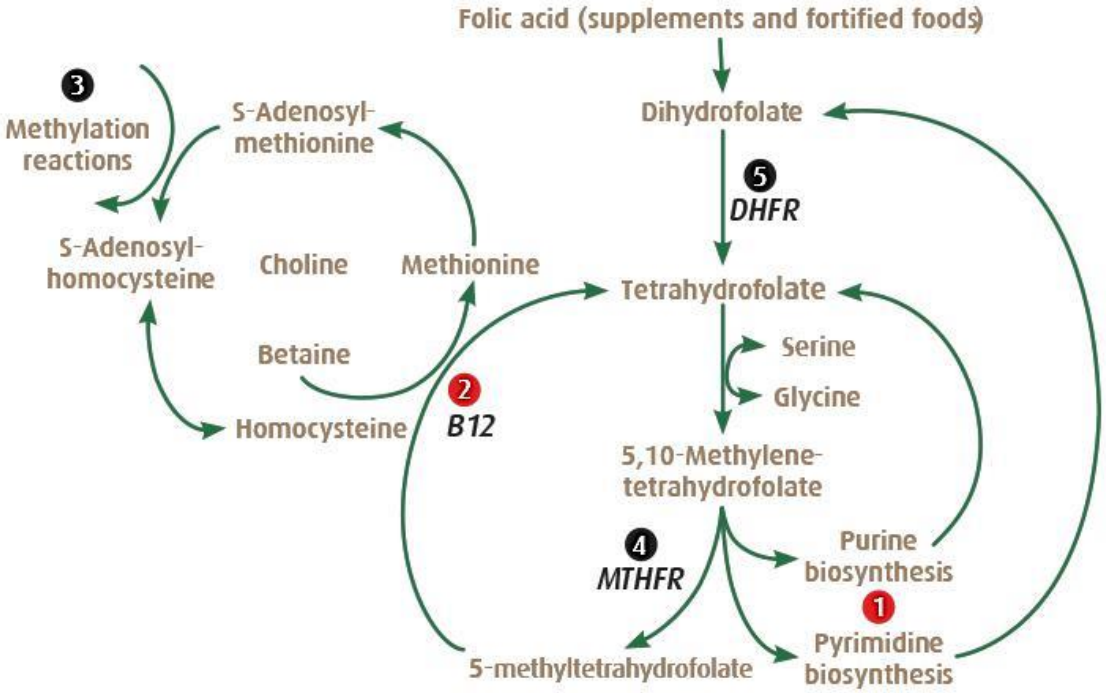



**Figure 1** 1DHF Biosynthetic Pathway.

Folic acid is first converted to dihydrofolate. 1DHF catalyzes the reduction of dihydrofolate to tetrahydrofolate, which is further used in the biosynthesis of purine and pyrimidine bases and thus the synthesis of DNA. Overproduction of these bases lead to abnormal cell proliferation.

## Main Components of the Folate Biosynthetic Pathway Reactions:

Pathway 1 - Biosynthesis of purine and pyrimidine bases for incorporation into RNA and DNA;

Pathway 2 - Remethylation of homocysteine leading to formation of methionine (vitamin B12 serves as a coenzyme in this reaction);

Pathway 3 - Methylation of substrates, together with RNA, DNA, proteins and phospholipids;

Pathway 4 - MTHFR, which catalyzes the production of 5-methylfolate required for methylation reactions;

Pathway 5 – 1DHF, Dihydrofolate reductase enzyme . (Sharif, Hossain, Kabir, & Rajib, 2017)

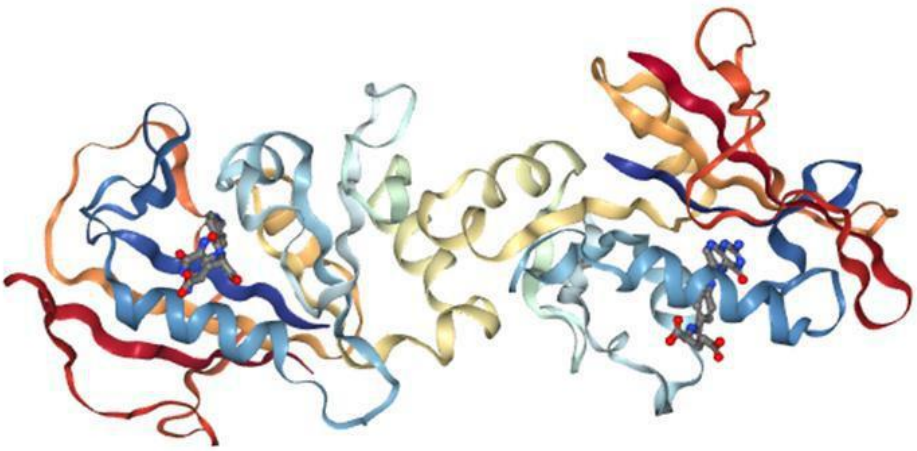


**Figure 1.1** Crystal Structure of Human DHFR, 1DHF: Chains A and B are shown.

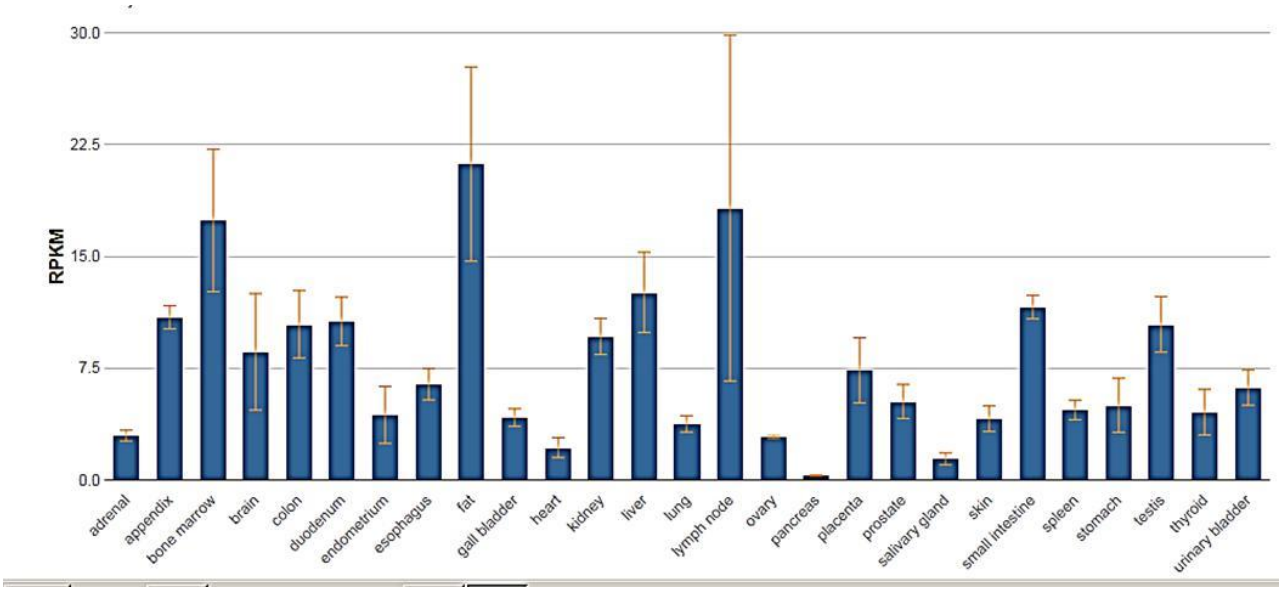


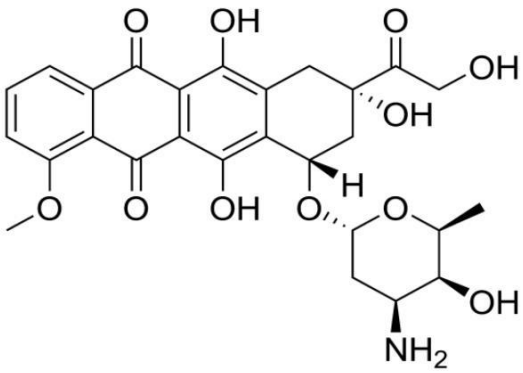

**Figure 1.1.1** The results of RNA-seq performed on tissue samples.

The graph shows tissue specific expression of genes across human tissues and organs. 1DHF is highly expressed in bone marrow, fat tissues and lymph nodes (Fagerberg et al., 2014).

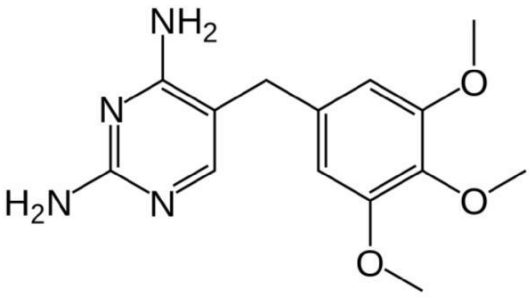
### 1.1.1 Folate Antagonists as Anti-Cancer Drugs

Folate is an important component of the diet and is used for normal homeostasis and cell division. It is produced with the help of 1DHF. A normal level of folic acid has to be maintained for normal metabolism. Intracellular levels of folic acid, if not optimum could be detrimental. Antifolate drugs, such as methotrexate, results in megaloblastic anemia. Studies show that patients with cancer usually have high levels of folic acid being produced which results in abnormal mitosis. Folate antagonists or antifolate drugs are cytotoxic medications that inhibit cells to use folic acid to synthesize DNA. They may be used in the necrosis of cancer cells (Kamen, Cole, & Bertino, 2003; Lambie & Johnson, 1985).

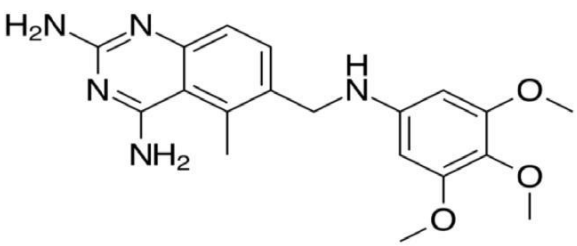
Established anticancer drugs include methotrexate, doxorubicin, curcumin, afatinib, trimetrexate and trimethoprim, which are all antagonists of the enzyme. These drugs competitively target the enzyme, thereby inhibiting the production of tetrahydrofolate. As a result, the purine and pyrimidine synthesis is hampered and DNA production slows down, thereby, inhibiting abnormal cell mitosis which is the main feature of cancer cells (Sharif, Hossain, Kabir, & Rajib, 2017).



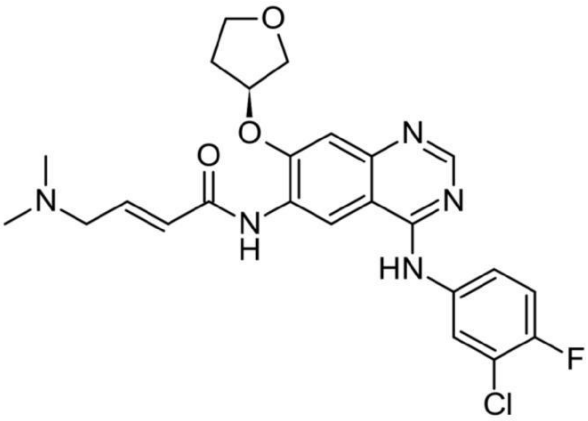

**Doxorubicin**



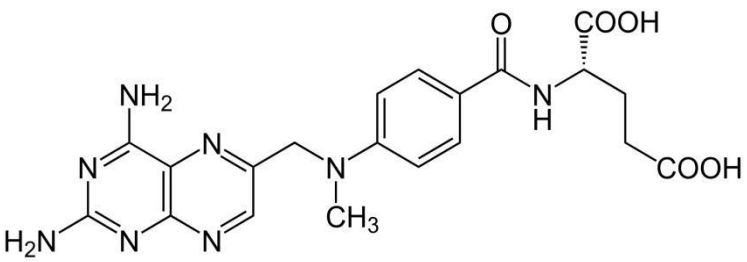

**Trimethoprim**



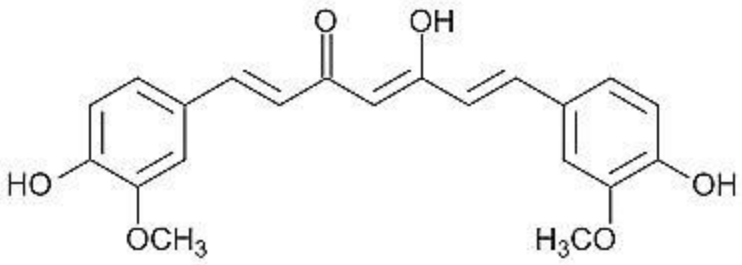
**Trimextrexate**



**Afatinib**



**Circumin**



**Methotrexate**

**Figure. 1.2** Structures of Established Anticancer Drugs (Obtained from NCBI)

## 1.2 Role of Cholesterol in Cancer

Cancer is among the leading causes of deaths worldwide ("WHO | Cancer," 2018). Its treatments include chemotherapy or radiotherapy or may require surgery (Gangaraju &

Lin, 2009). In case of chemotherapy, lower molecular weight molecules are used to target and inhibit the abnormal growth and proliferation of tumor cells. These cytotoxic agents are associated with a number of adverse effects including hair loss, nausea, bone marrow suppression, gastrointestinal tract lesions and the development of clinical resistance as these cytotoxic agents act on both healthy cells and tumour cells (Kubzansky & Thurston, 2007). The use of chemotherapy dates back to 1940 when nitrogen mustards were used because of their strong antimetabolite and alkylating properties. Early success of these agents prompted the development of new anticancer drugs (Gangaraju & Lin, 2009).

Based on their mechanism of action, anticancer drugs are classified into several groups, such as anti-tubulin agents, antimetabolites, agents that interact with DNA, monoclonal antibodies, hormones, molecular targeting and other biological agents (Kubzansky & Thurston, 2007).

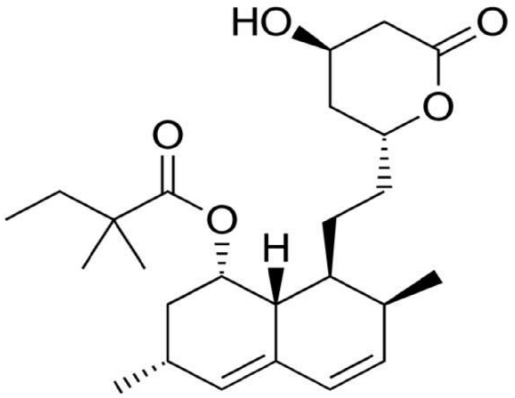
Anticancer drugs mainly bind to 1DHF disrupting or inhibiting the cell mitotic process of cell. Cholesterol plays a vital role in the organization of cell membrane, essentially their function and dynamics. It is also vital for cell proliferation, differentiation and membrane biogenesis. Cholesterol is mainly obtained from diet but principally it is synthesized in the liver and is extensively distributed in the body through HDL and LDL transporters (Silvente-Poirot & Poirot, 2014). For the normal growth of eukaryotic cells, maintenance of cholesterol homeostasis is a basic requirement.

Recent studies demonstrate that cholesterol plays a significant role in cancer progression including those of prostrate, breast and colorectal cancers. It has been recently observed that tumor formation and progression of cancer is associated with cholesterol biosynthesis pathway dysregulation (Murai, 2015).

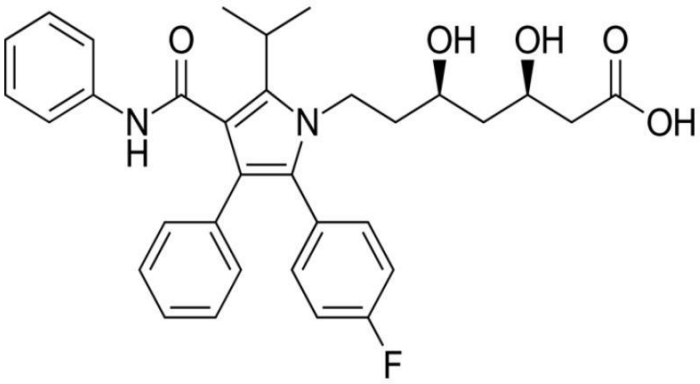
## 1.3 Statin Drugs

Statins are a class of lipid lowering medications, molecules of fungal origin predominantly used to treat cardiovascular complications and also to reduce the risk of

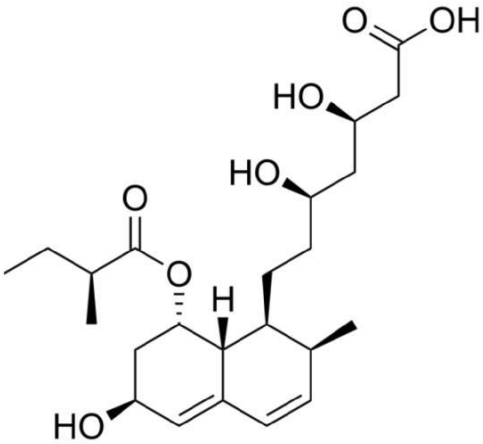
mortality in patients with cardiovascular conditions. Statin drug was first isolated from the fungus *Penicillium citrinum* (A Endo, 1988; Akira Endo, 2010)



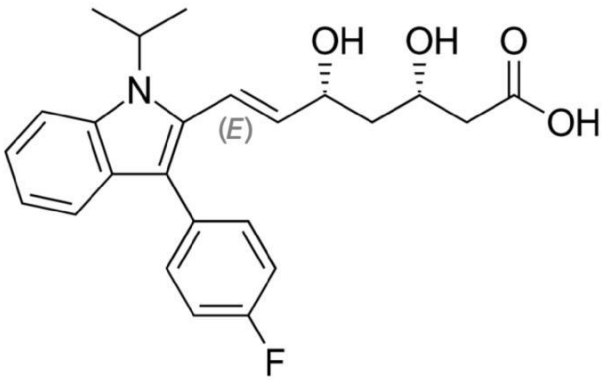
**Simvastatin**



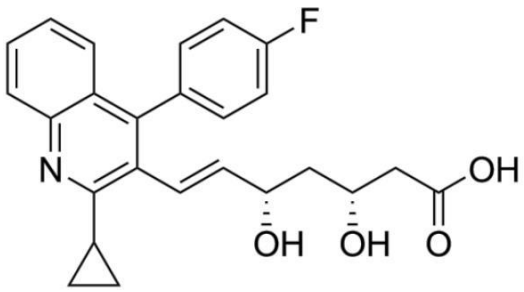
**Atorvastatin**



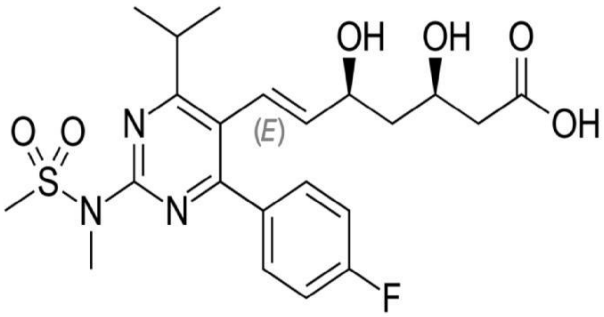
**Pravastatin**



**Fluvastatin**



**Pitavastatin**



**Rosuvastatin**

**Figure 1.3** Some structures of Statin Drugs (Obtained from NCBI)

Statins function by competitively inhibiting the enzyme HMG-CoA reductase. Previously it was known that HMG-CoA reductase (Figure 1.4) is the first acting and the main rate-limiting enzyme of the biosynthetic pathway of cholesterol. Statins basically mimic HMG-CoA, which is the natural substrate molecule, and compete to bind to the HMGCR enzyme. Consequently, the rate at which mevalonate is produced slows down and so does the series of steps that follow for the production of cholesterol thus ultimately blocking the pathway (Sirtori, 2014).

Though statins are generally well tolerated, muscular adverse effects appear to be the most common obstacle, thus limiting their use. Myalgia commonly associated with statin therapy.

Statins are associated with myopathy when given at high doses. To address this problem, some drugs were identified that could be used in combination with statins to reduce the dose requirement and basically to reduce the occurrence of adverse effects (Andréjak, Gras, Massy, & Caron, 2003; Pedro-Botet, Millán Núñez-Cortés, Chillarón, Flores-Le Roux, & Rius, 2016)

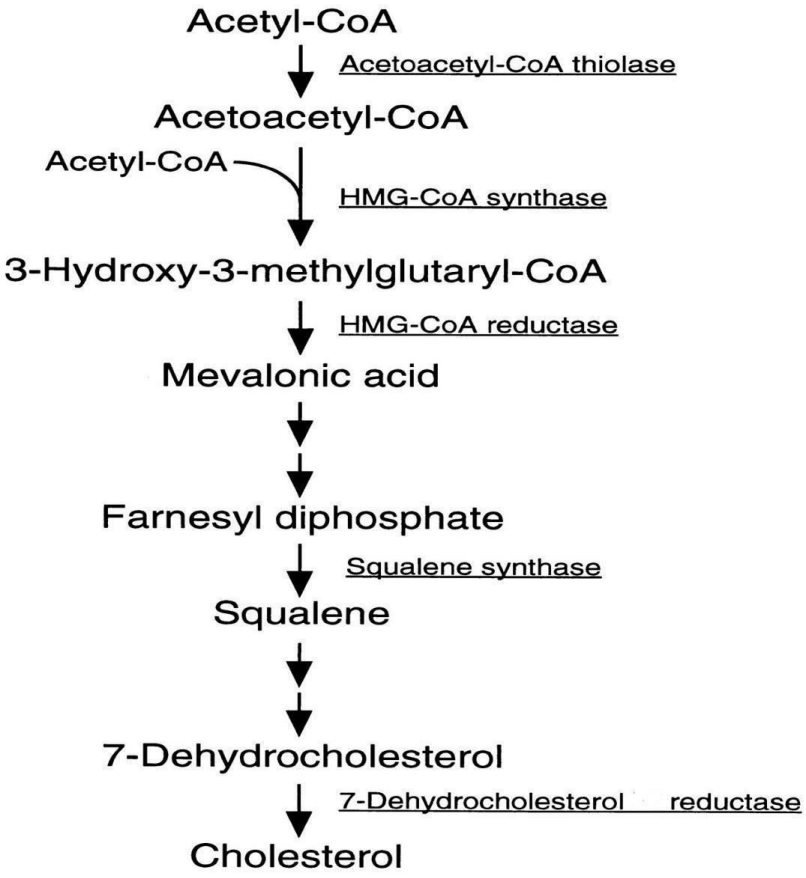



**Figure 1.4** Cholesterol Biosynthetic Pathway. (Vance, 2012).

Acetyl CoA is converted to Acetoacetyl CoA, with the help of the enzyme, Acetoacetyl CoA thiolase. HMG-CoA synthase causes its conversion into 3-hydroxy-3-methyl-glutaryl-coenzyme A(HMG-CoA). The enzyme is reduced to mevalonic acid with the assistance of the enzyme HMG-CoA reductase. A series of reactions take place and finally cholesterol is produced. Statins target HMG-CoA reductase to diminish mevalonic acid production which further decreases cholesterol levels

### 1.3.1 Atorvastatin

Atorvastatin is a lipid lowering agent which belongs to the statin class of drugs. The drug may be used as primary prevention as well as secondary prevention of Coronary Heart Disease (CHD). It reduces the risk of angina, myocardial infarction (MI), revascularization procedures and risk of cardiovascular events in people with acute coronary syndrome (ACS); it is further used in the treatment of dyslipidemia,

hypercholesterolemia and hypertriglyceridemia. It functions as a competitive inhibitor of HMG-CoA reductase, reducing mevalonate levels. It primarily carries out its function in the liver. Decreased levels of cholesterol in the liver, upregulates the hepatic LDL receptors; this in turn, increases the uptake of cholesterol from the liver, causing a decrease in the plasma LDL levels (Chauvin, Drouot, Barrail-Tran, & Taburet, 2013). A study showed that the anti-proliferative effect of Atorvastatin in ovarian cancer was due to the induction of autophagy, cellular stress, apoptosis and G1 arrest in the cell cycle through inhibition of mTOR and also by the stimulation of MAPK pathways (Jones et al., 2017). No study concerning 1DHF pathway to inhibit cell proliferation had been carried out previously, so the drug was chosen for this study.

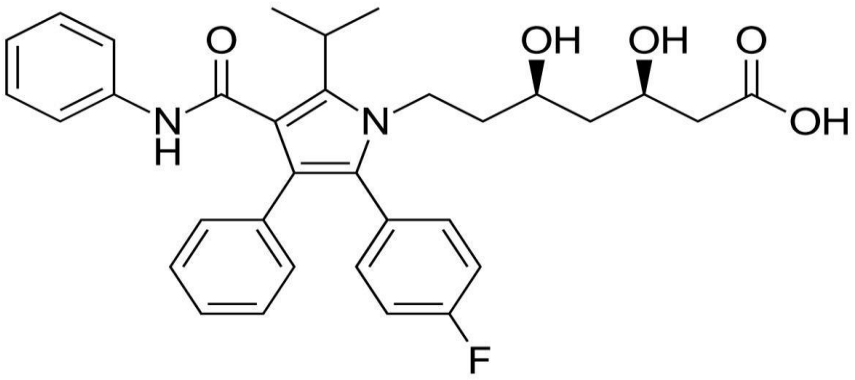



**Figure 1.4.1** Structure of Atorvastatin (Obtained from NCBI) (Sieczkowski, Lehner, Ambros, & Hohenegger, 2009)

### 1.3.2 Pravastatin

Pravastatin is a cholesterol-lowering drug that belongs to a class of statins. It was obtained from the microbial transformation of the first statin discovered, mevastatin. It has a ring-opened dihydroxyacid and a 6'-hydroxyl group which does not need *in vivo* activation. Pravastatin is among the lower potency statins; however, due to its increased hydrophilicity it is thought to have advantages, such as less pronounced side effects than Lovastatin and Simvastatin and greater selectivity for liver tissue ("Pravastatin: an evidence-based statin?," 2008; Sharma, Holmes, Elsby, Lambert, & Surry, 2010).

Structurally, the drug is similar to HMG which is a substituent of HMG-CoA reductase. It does not require to be activated in vivo unlike its parent compounds. Its hydrolyzed lactone ring allows the drug to bind with a higher affinity than its normal substrate. Prvastatin sodium lowers cholesterol in two ways.

1. It reversibly inhibits HMG-CoA reductase action, thereby causing a modest decrease in the intracellular levels of cholesterol. As a result, the number of LDL receptors increase on the cell surface thereby leading to enhanced LDL catabolism and diminished circulating LDL.

2. It inhibits the production of LDL by inhibiting the hepatic synthesis of the LDL precursor, VLDL (Morisawa & Takikawa, 2009).

The link between cholesterol and cancer forces scientists to look into the relation between statins and cancer and numerous clinical trials have revealed anti-tumor activities of statins. However, previous studies have not yet shown Pravastatin's role in cancer treatment and it remains controversial. A study was conducted to determine the role of the lipid lowering agent in patients with stage III and stage IV (advanced) gastric cancer. No significant improvements were observed (Bujanda et al., 2016). A possible explanation of this may be the agent was administered at a very advanced stage. Results would have been more prominent if it had been given at an earlier stage. Or Pravastatin therapy to treat high LDL levels could possibly reduce the risk of acquiring cancer. In this study, the anti-cancer property of the lipid lowering agent was investigated by targeting the enzyme 1DHF.

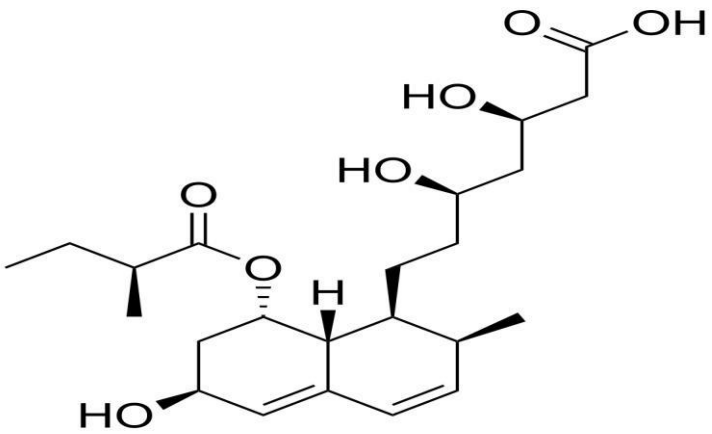



**Figure 1.4.2** Structure of Pravastatin (Obtained from NCBI) (Costantine et al., 2016)

### 1.3.3 Simvastatin

Simvastatin is a synthetic cholesterol- lowering drug used to treat hypercholesterolemia and for reducing significantly the risk of cardiovascular events and mortality from cardiac disease. It is produced from the fermentation of of Aspergillus terreus. It acts as a potent inhibitor of the rate-limiting enzyme of cholesterol biosynthesis, HMG-CoA reductase. The prodrug has a tendency of interfering with steroid hormone synthesis. It induces hepatic LDL receptors which further increases LDL catabolism (Becker et al., 2013).

A study conducted addressed that Simvastatin induced apoptosis of EBV-transformed LCLs.This effect of Simvastatin mainly depends on its binding to the domain of LFA-1. It was observed in the study that when Simvastatin was given to mice with compromised immunity following inoculation with LCLs, the development of B cell lymphoma was delayed and it resulted in prolonged survival in mice (Katano, Pesnicak, & Cohen, 2004).However, this study aims to target 1DHF to explore anti-cancer properties of Simvastatin.

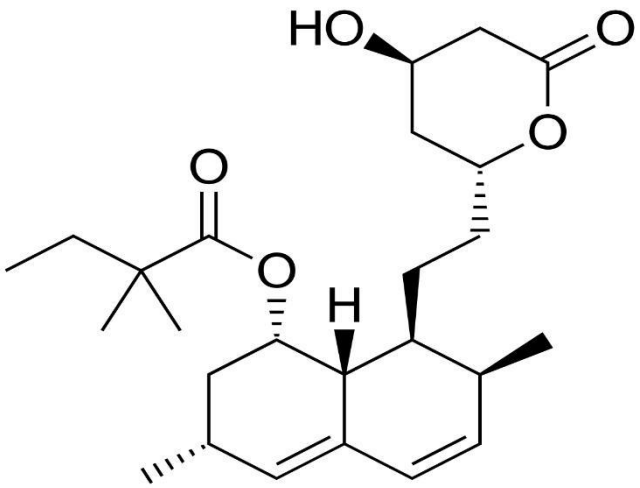



**Figure 1.4.3** Structure of Simvastatin (Obtained from NCBI) (Sieczkowski et al., 2009)

### 1.3.4 Rosuvastatin

Rosuvastatin is a synthetic antilipemic statin drug that inhibits cholesterol biosynthesis via the mevalonic acid pathway. It is indicated in patients with hypercholestemia and to prevent cardiovascular complications. Although the structure is similar to other statin drugs, the structure has been modified in such a way that it binds with a higher affinity to HMG-CoA.

Several studies proved that the drug reduces the risks of ischemic heart disease and other related cardiovascular complications. In addition, Rosuvastatin helps circumvent endothelial conditions and play a role in stabilizing the plaques that are formed during atherosclerosis.

Rosuvastatin significantly reduces the risk of inflammation and thrombosis in the arterial walls which is why it is used in the prevention of cardiovascular conditions. The anti-cancer property of the drug has been reported recently (Luvai, Mbagaya, Hall, & Barth, 2012). This drug is safe and considering its other beneficial effects, the drug was chosen for this study that targeted 1DHF.

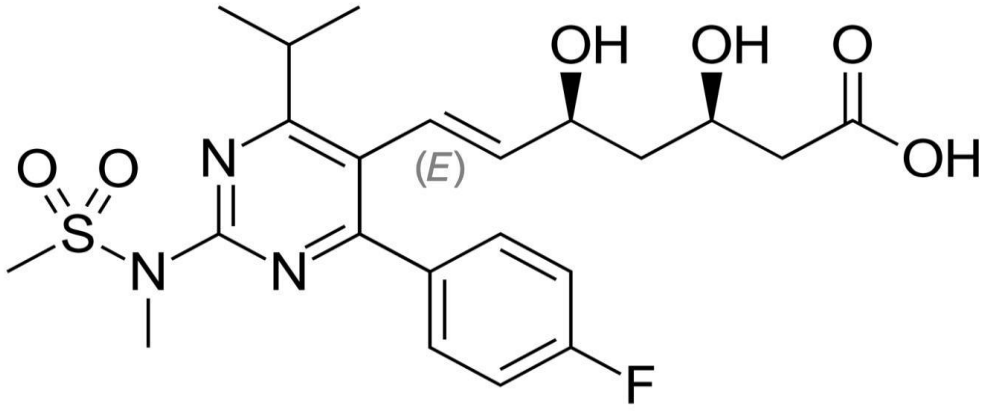



**Figure 1.4.4** Structure of Rosuvastatin (obtained from NCBI) (Everett, Glynn, MacFadyen, & Ridker, 2010)

### 1.3.5 Pitavastatin

Pitavastatin is a drug belonging to the statin class of drugs, prescribed to patients with dyslipidemia. It can also be used in prevention of cardiovascular disease as it acts as a lipid lowering agent. Recent studies have shown that the drug shows significant anti-cancer property (Morgan, Campbell, Yu, Sponseller, & Muster, 2012).

A study demonstrated pitavatatin when combined with phosphatidylinositol 3-kinase inhibitor pictilisib, and BH3 mimetic ABT-737, in cell growth assays using Igrov-1 and Ovcar-3 cells, were more effective than when Pitavastatin was used alone. These anticancer drugs with Pitavastatin synergystically increased cell necrosis induced by Pitavastatin in several types of ovarian cancer (De Wolf, De Wolf, & Richardson, 2017). The lipid-lowering medication functions to control cholesterol synthesis through competitive inhibition of the liver enzyme, HMG-CoA reductase. A compensatory rise in LDL-receptor expression leads to increased breakdown of LDL, thus causing serum LDL levels to fall (Jung et al., 2012). This study investigates the anti-cancer effects of Pitavastatin via the 1DHF pathway.

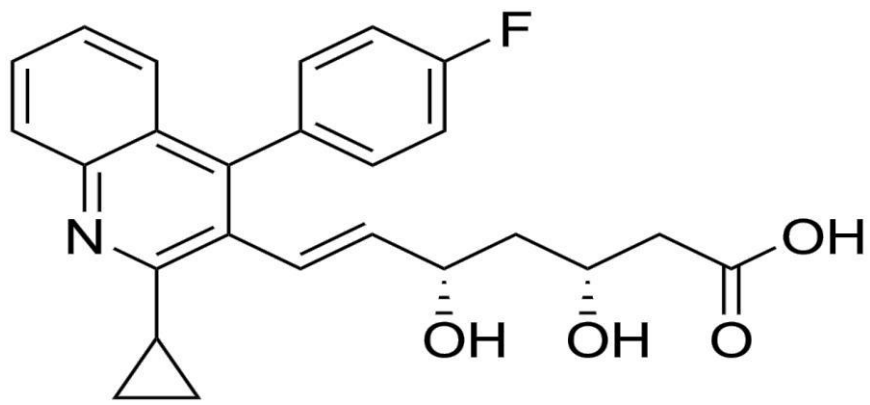



**Figure 1.4.5** Structure of Pitavastatin (Obtained from NCBI) (Morgan et al., 2012)

## 1.4 Drug Repurposing

Drug repurposing aims to identify multiple targets of an already existing drug (Ashburn & Thor, 2004; Novac, 2013). An example of such repurposing is thalidomide drug which was initially used to mitigate morning sickness (McBride, 1977) but has presently been identified with anticancer properties (D'Amato, Loughnan, Flynn, & Folkman, 1994) such as in case of multiple myeloma cancers (Lengauer & Rarey, 1996; Singhal et al., 2010). Itraconazole is another such example of drug repurposing which was initially intended as anti-fungal drug but has been shown to have anti-cancerous activity against non-squamous non-small cell lung cancer and prostate cancer. (Huijgens et al., 1999).

Drug repurposing is considered an important branch in drug discovery as it helps to circumvent optimization issues related to drug discovery and development and preclinical development (O'Connor & Roth, 2005). Due to the pharmacological and toxicological data of existing drugs, repositioning of already approved drugs can be cheaper and faster (Morgan et al., 2012). It not only capitalizes on previous investments it also derisks clinical activities, thus reducing expenses, time efforts and failures that are typically related to the drug discovery process. Furthermore, it provides an understanding of the drug-target interaction matrix (March-Vila et al., 2017)

Various approaches for drug repositioning have been developed in the past few years, and these approaches include –

a) Using drugs that are chemically identical for the treatment of identical disease (Keiser et al., 2009)
b) Using drug molecules with similar side effects (Campillos, Kuhn, Gavin, Jensen, & Bork, 2008)
c) Targeting drug molecules having similarity in their molecular activity (Li & Agarwal, 2009)
d) Targeting drug molecules having similar or identical molecular pathology (Roder & Thomson, 2015)

The idea of using both direct and indirect structural information on necessary anti-targets has gained increasing popularity to improve selectivity of ligands as well as to reduce off-target interactions to enhance safety and to thus to improve the pharmacokinetic profile (Gore & Jagtap, 2018).

## 1.5 *In silico* Drug Designing and Repurposing

*In silico* methods, a relatively new approach, has the advantage of using high-performance computers for a safe virtual screening. Virtual screening can be vital in large-scale production of drugs and working with a lot of data sets, however, the validation and accuracy of *in silico* methods used for prediction drug-protein interactions can be a limiting factor. Docking, which is a part of computational biology or *in silico* method is used for drug repurposing. Docking predicts the preferred orientation of one molecule to a second one, when binding with each other to form a stable complex. Knowing the preferred orientation can be used to predict the binding affinity between the two molecules in the complex, for example, scoring functions (Kabir et al., 2017).

Molecular docking is a recognized technique utilizing three-dimensional modeling and computer simulation to achieve the binding of a candidate drug into a protein binding pocket in order to attain the favourable energy for a high probability of the pair's interaction. The benefit of this approach is the strong structure-based insight it provides to improve the understanding of the interaction. However, the docking process relies on the presence of an appropriate 3-dimensional model of the protein. With regard to certain

target classes (such as membrane-bound proteins) experimental limitations prevent the existence of the model. The approach lacks feasibility on a large scale, especially in terms of the DTI prediction tasks, due to its high computational requirements. Alternatively, the compound can be evaluated via a construction of an abstract "pseudodrug representation" named as pharmacophore model. This approach is more ligand-based and less target-based, and since it allows compounds to be aligned and scored against the model, it is relatively less computationally demanding.

New therapeutic indications for a drug that has a therapeutic activity for a disease can be discovered with the help of this technique. This method also helps find the gaps in the drug-target interaction matrix and has available safety and efficacy data at some stages in medical studies (Schuster, Laggner, & Langer, 2005).

Drug repurposing is used in combination with other oncology therapeutics to obtain a synergistic effect to cause cell death or necrosis of malignant cells. Statins, as single agents have been reported to show activity in patients with ovarian cancer; however they are required in doses much higher than that of patients receiving normal statin treatment for hypercholesterolemia (De Wolf et al., 2017).

## 1.6 Rationale of the Study

Tremendous resources are being invested in the search for a solution to cancer treatment and prevention, yet cancer still remains among the leading causes of deaths all over the world. The method of drug repurposing is used in this study is because of the need to an alternative to the complex approaches of drug discovery. Due to its increasing failure rates, financial unfeasibility, limited efficacy, poor safety and bioavailability issues, lengthy testing procedure and design in the development of anti-cancer drugs, finding anti-cancer properties of established non-anti-cancer drugs can be useful (Gupta, Sung, Prasad, Webb, & Aggarwal, n.d.) that would help evade clinical activities and save costs significantly.

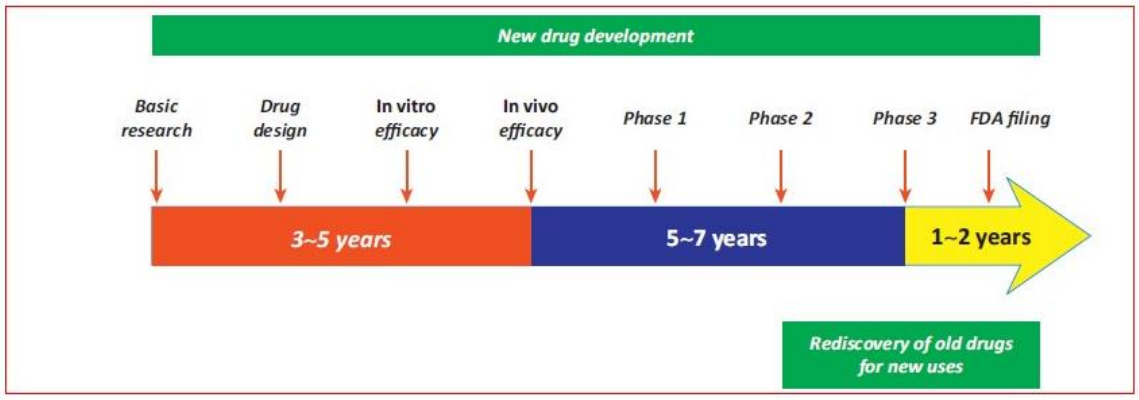



**Figure 1.5.** New Drug Development Process.

The process time-consuming and expensive compared to rediscovery of old drugs for new uses (Gupta et al., n.d.)The process of drug repositioning is considered very efficient in identifying new indications because the current drugs have-

1. well established and accepted formulations in addition to their well-known process of manufacture
2. well established pharmacokinetic data (ADMET information)
3. passed phase III or later stages of clinical activities and trials and therefore they have lower chances of safety-issue related failure
4. availability of data of phase IV clinical trial (post-marketing surveillance) which ensures drug safety

Studies show that over 46 different drugs have been repositioned till now and many more are in the process of being repurposed (Deotarse P. P., Jain A. S., Baile. M. B., Kolhe N. S., 2015).

Considering the correlation between cholesterol and cancer development (Hindler, Cleeland, Rivera, & Collard, 2006), the statin class of drugs was chosen. The protein 1DHF was targeted mainly because most established anticancer drugs such as trimethoprim, doxarubicn and circumin target 1DHF to inhibit cancer progression.

# 2 Methodology

## 2.1 Aim of the Study

In this *in silico* study, the anticancer use of statin drugs using molecular docking and computational techniques was determined.

The first part of the methodology consisted of a thorough review of past literature on the specific topic, followed by establishing the binding affinities between receptors and ligands, which were determined by utilizing molecular docking. Three dimensional structures of the macromolecule and ligands or small molecules were necessary to carry out computational docking.

## 2.2 Software and Online tools for Docking, Visualization and Validation

Multiple software were used for the computational methods specifically during this *in silico* study which included, AutoDock, PyRx, Discovery Studio, Clustal Omega, Open Babel, Vina, Swiss PDB Viewer, PyMOL, MEGA 6, MGL tools and POCASA.

It is also worth highlighting that a small number of databases were used to accumulate data which would enhance this study and this included RCSB-PDB (Protein Data Bank), Swiss Model, Drug Bank, PubChem Project, EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute), CASTp (Computed atlas of surface topography of proteins), Errat, Verify 3D, ProSA Web Server.

**Table 2.1** Software used in the Computational Study

| SI No. | Softwares and Online Tools Used | Versions |
|---|---|---|
| **1** | Open Babel | 2.4.0 |

| | | |
|---|---|---|
| **2** | PyMol | 1.8.4.0 |
| **3** | PyRx | 0.8 |
| **4** | POCASA | 1.1 |
| **5** | Discovery Studio | 17.2.0.16349 |
| **6** | Mega 6 | 6.06 |
| **7** | ConSurf | 2010 |
| **8** | Castp | 3.0 |
| **9** | Clustal Omega | 1.2.4 |
| **11** | ProtParam | 2005 |
| **12** | ERRAT | V3.0 |

## 2.3 Steps Involved in Molecular Docking

The steps involved are illustrated with the help of a flowchart (Figure 2.0). As depicted in Figure 2.0, the drugs and proteins that could be of potential use were all downloaded from the numerous choices of databases that included PubChem and RCSB PDB (Protein Data Bank). RCSB PDB contains the 3D structures of proteins and macromolecules, such as nucleic acids and proteins. It is a databank which contains the structures of the molecules of life, starting from bacteria to humans. A thorough knowledge about the 3D structure of the protein was obtained through journals. PubChem provided the files of small molecules in the sdf format and DrugBank provided the files in pdb format. As with the sdf files, conversion software, known as OpenBabel was employed. OpenBabel was used to convert the irregular formats into

the format that is required. MEGA6 was useful in determining similarities in sequences; it also shows the regions that are conserved of that protein sequence with the sequence of reference, which in this case was folic acid. In order to find the site of binding within the protein for the drug, the softwares CASTp (Binkowski, Naghibzadeh, & Liang, 2003) and POCASA (Yu, Zhou, Tanaka, & Yao, 2010) were used. CASTp handles information that is more detailed with regards to the binding pocket where POCASA gives a more accurate position of the amino acids which deal with the binding of drug with the protein of use.

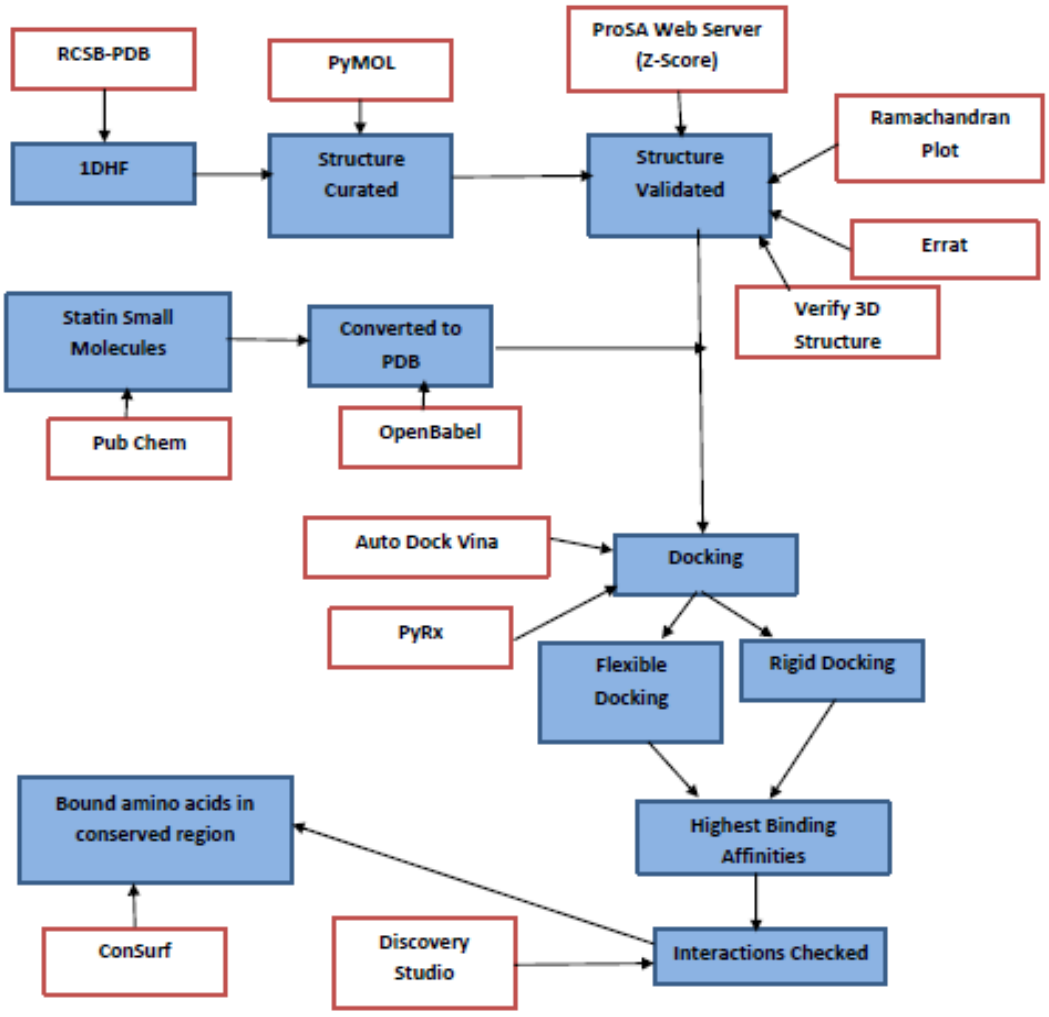



**Figure 2** Flow Chart showing the Steps involved in the Selection of Drugs binding to the Protein, 1DHF

In the *in silico* study, the chosen ligand for this particular experiment was made by curation of the protein for successive runs of docking using PyMOL. Simultaneously, a

database of ligands or small molecules was created for docking with the protein. After establishing the binding site with the assistance of grid box, the ligand and receptor were chosen (Delano, n.d.). In order to run the specific software from the line of command, materials were inputted to run the docking. Two softwares namely, AutoDock Vina and PyRx (Dallakyan & Olson, 2015) were used to predefine the flexible side chains during the docking process. Here, the selection of the flexible side chains was simplified by the plugin. The side chains in the docking box could be clearly visualized and the selections using PyMol were converted into a more flexible receptor. In order to curate the protein, Human DHFR 1DHF was opened with PyMol. Protein molecules consisted of numerous different hetero atoms (water molecules) and ligands (Folic acids) between them. The hetero atoms were all deleted and the structure of the protein was protected. 1DHF, the Human DFHR protein was downloaded from RCSB-PDB. Chains A and B had the same amino acid sequences; thus Chain B was deleted for simplification of work. Simultaneously drugs and small molecules belonging to the statin class of drugs were downloaded from PubChem Project and Drug Bank respectively. Small molecules and drugs were to be docked using PyRx with the protein of interest. In order to be adaptable, PyRx was run in a default manner without any changes made where 1DHF was selected as macromolecule and small molecules and drugs as ligands.

There were various results that were generated with binding affinities that showed a negative sign, as this is an exothermic reaction. We already know that the more negative the value for affinity, the greater is the binding affinity. The drugs and small molecules were run again through PyRx using rigid docking. The binding affinities were better this time compared to when the flexible approach was used. The suitable drugs and small molecules that were derived from rigid docking of the small molecules were again docked in AutoDock Tools. The suitable drugs were selected based on their binding affinities; highest binding affinities (-10 to - 12.8kCal/Mol) got preference. The protein molecule was opened using AutoDock Tools after which the grid box was positioned on the protein in the different sections of it. This was how the most suitable binding site was discovered for 1DHF. The next step was to obtain the best binding sites using Vina v1.1.2. The results were fruitful and seemingly produced a total of nine very suitable binding affinities; the pdbqt obtained was opened with PYMOL along with the protein. The complexes were then opened using Discovery Studio to provide a strong

representation of the actual distance between the ligands and the protein along with their bonding types, categories and amino acids of the protein. A few parameters were also set to meet the requirements of the protein and the ligand before the interaction information was received pertaining to the ligands and the drug.

Screenshots were taken and the bond types, categories, distances, important amino acids in the non-bonded interactions of the proteins and small molecules were recorded respectively.

For the purpose of validation of the protein, Ramachandran plot (Lovell et al., 2003), ProSA WebServer (Sippl, 1993) (for z-score), Errat (Colovos & Yeates, 1993), Verfy 3D (Lüthy, Bowie, & Eisenberg, 1992) and Consurf (Celniker et al., 2013; Glaser et al., 2003; Landau et al., 2005) were used. Ramachandran plot was generated both before and after docking of the drugs with the protein. The main reason was to determine whether the drug caused any change in the psi and phi angles. 1DHF was then opened with ConSurf, an online tool which shows the conserved regions. To check whether the drugs bound to the amino acids of conserved regions or to the amino acids subjected to mutation, Consurf was used. ProtParam was used for checking the stability of the protein.

# 3 Results and Validation

The following sections show and describe the results obtained in this study.

## 3.1 1DHF Structure

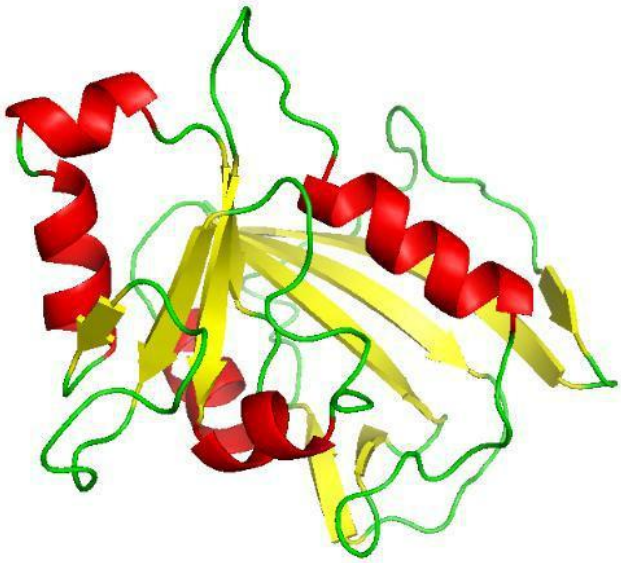
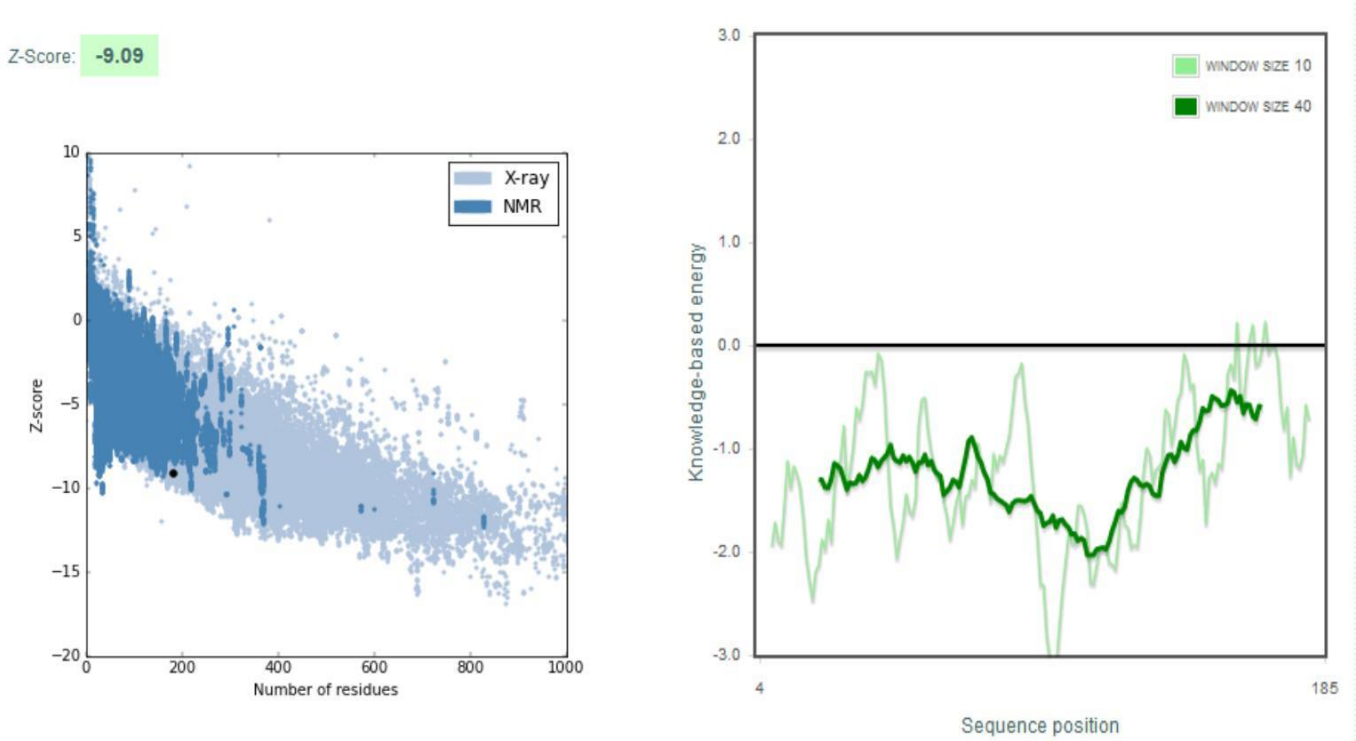


**Figure 3** Structure of 1DHF as vizualized in PyMOL

Red portions indicate alpha helices, yellow parts indicate beta sheets and green portions are coils. (The PyMOL Molecular Graphics System, Version 2.0.4, Schrödinger, LLC)

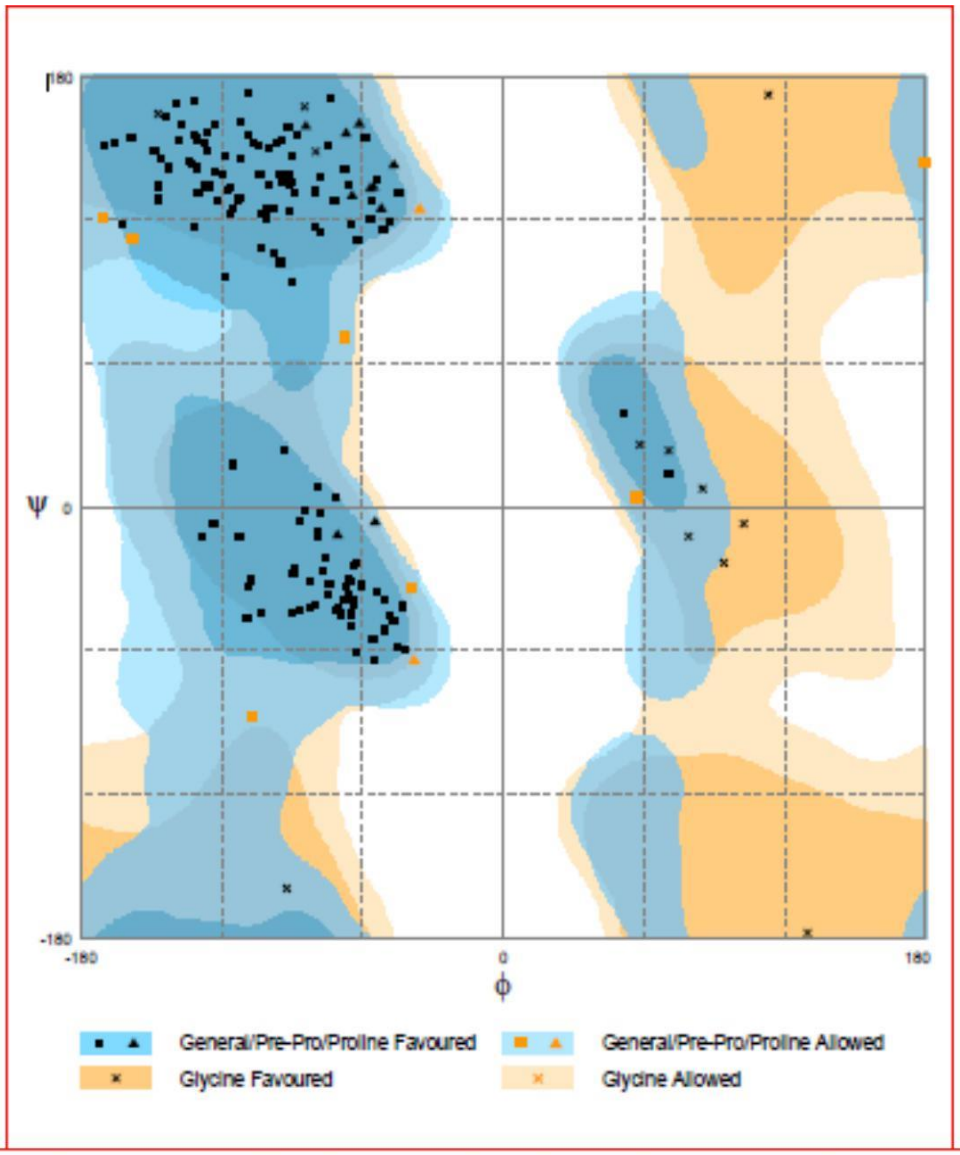



**(a)** The z-score of 1DHF

**(b)** The plot of residue score of 1DHF

**Figure 3.1 (a)** shows the z-score of 1DHF obtained from ProSA Web Server**. 3.1 (b)** shows the plot of residue score of 1DHF. The z-score of 1DHF, Human DHFR is -9.09, which is fairly a good value (Sippl, 1993).

Human DHFR, 1DHF was downloaded from RCSB-PDB (Protein Bank). It was opened with PyMOL for curation. The water molecules or heteroatoms and folic acid, the ligand within were removed. The z-score of the protein after curation was -9.09




| | | |
|---|---|---|
| Number of residues in favoured region (~98.0% expected) | : | 171 (95.0%) |
| Number of residues in allowed region (~2.0% expected) | : | 9 (5.0%) |
| Number of residues in outlier region | : | 0 (0.0%) |

**Figure 3.2** Ramachandran Plot showing the energy minimization of the whole protein, 1DHF

The Ramachandran plot helps to predict the conformation of the backbone of a given polypeptide chain more quantitatively starting from knowledge of its amino acid sequences (Lovell et al., 2003; "RAMPAGE: Ramachandran Plot Assessment," n.d.; Sheik, Sundararajan, Hussain, & Sekar, 2002). Number of amino acid residues in the

favored region is 95%. The number of residues in the allowed region is 5%. No residues are seen in the outlier region.

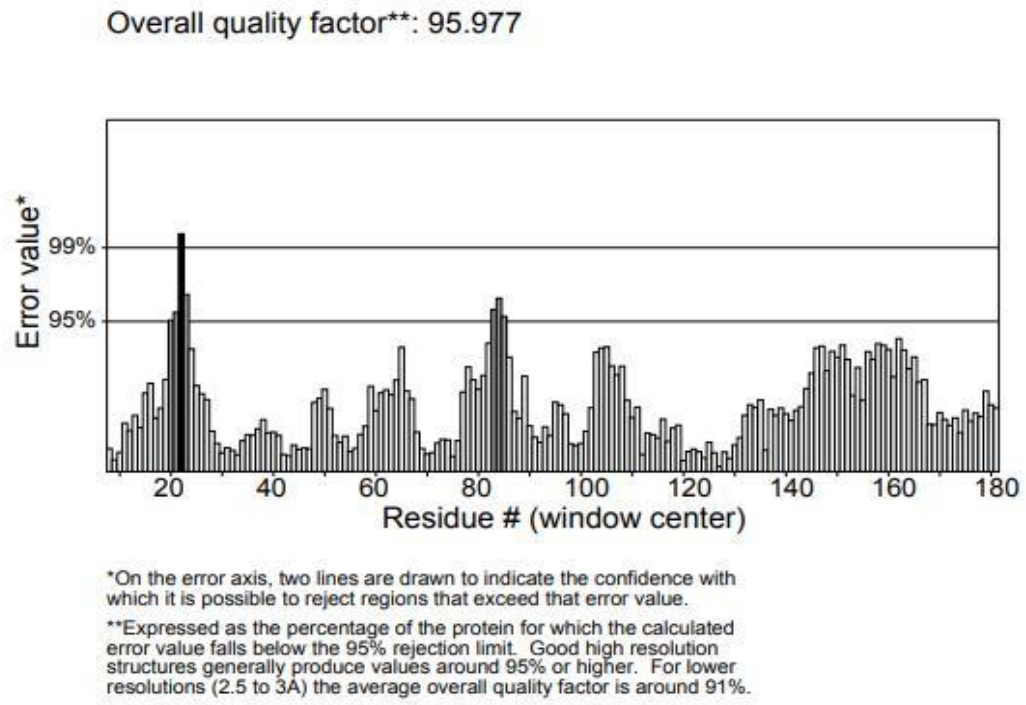



**Figure 3.3** Errat Results

Errat was used for the verification of 1DHF.(Colovos & Yeates, 1993) The results were quite good with 1DHF. No gaps were observed. The overall quality factor was 95.977.

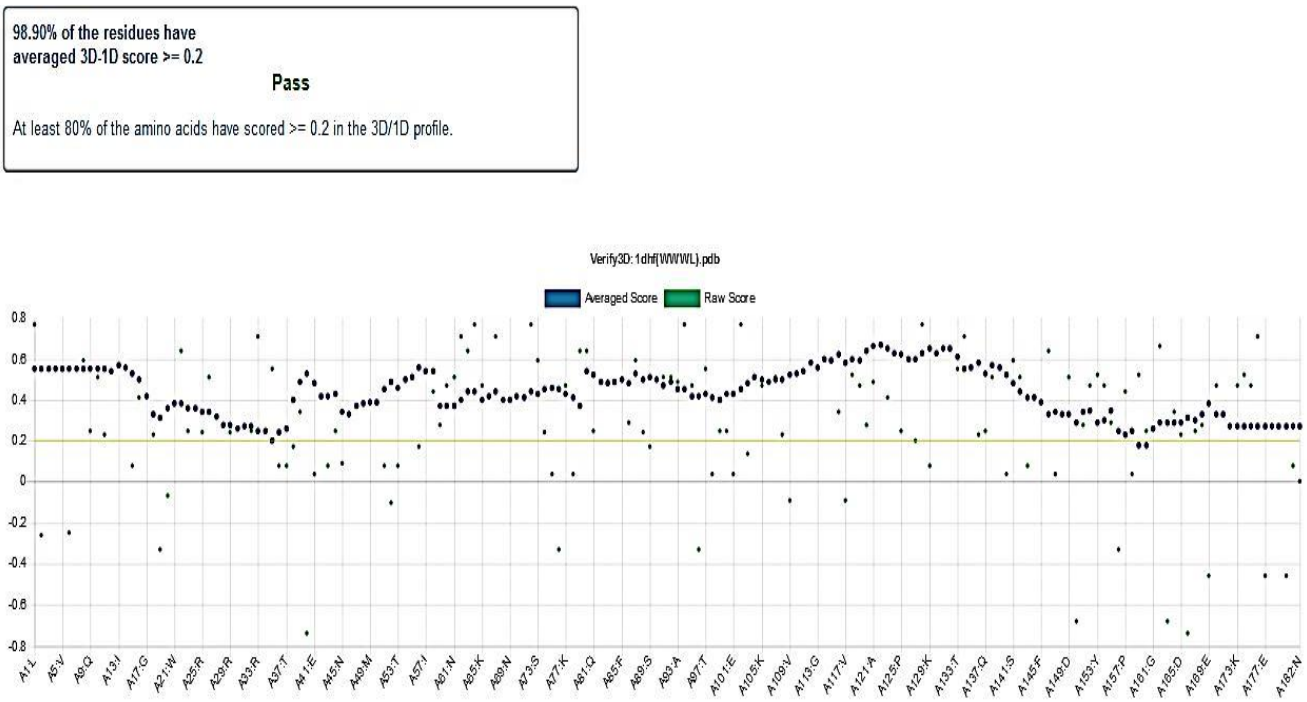



**Figure 3.4** Verify 3D Results

The results of Verify 3D for 1DHF (Lüthy, Bowie, & Eisenberg, 1992). The protein 1DHF passed this test, the percentages being 98.9%. Energy will further be minimized during docking. The lesser the energy, the little will be the vibrations and collisions.

## 3.2 *In silico* binding of 1DHF with Statins

Small molecules belonging to the statin class of drugs were downloaded simultaneously from PubChem and drugbank.ca respectively. Drugs downloaded from PubChem were in sdf format and thus were converted to pdb files using Open Babel. Molecular docking of 1DHF as the macromolecule and the statin drugs were carried out using PyRx. Both rigid and flexible docking were carried out. The drugs were selected based on their binding affinities; the more exothermic the binding affinity, the better it has a probability of binding to the protein, which in our case is 1DHF. High binding affinities were observed with cerivastatin, fluvastatin and Rosuvastatin (as in Table 3.1 and Table 3.2.), they were not considered. Cerivastatin was withdrawn from the market because of 52 deaths attributed to drug-related rhabdomyolysis that lead to kidney failure (Furberg & Pitt, 2001). The results of docking are summarized in Table 3.1.

**Table 3.1** Rigid and Flexible Docking Results of Statins with Human DHFR, 1DHF using PyRx, Version 0.8 (Dallakyan & Olson, 2015)

| Statin Drugs | Rigid Docking Affinity (kcal/mol) | Flexible Docking Affinity (kcal/mol) |
|---|---|---|
| Atorvastatin | -9.8 | -8.0 |
| Mevastatin | -12.5 | -9.2 |
| Pitavastatin | -12.7 | -9.2 |
| Pravastatin | -11.6 | -8.6 |
| Rosuvastatin | -10.6 | -8.4 |
| Simvastatin | -11.6 | -9.5 |

In rigid docking, both the protein and drugs are rigid in nature. During flexible docking of statin drugs with Human DHFR, 1DHF the protein is rigid and drugs are flexible in nature

### 3.2.1 Visualization using PyMol

Validation using PyMOL mainly involves visualization of the protein molecule to which the small molecules or ligands are bound. The protein molecule was at first loaded in PyMOL and then the output pdbqt files that were obtained from PyRx were visualized. The main ligand, folic acid was used as a reference to the used small molecule or ligand that was bound to the protein.

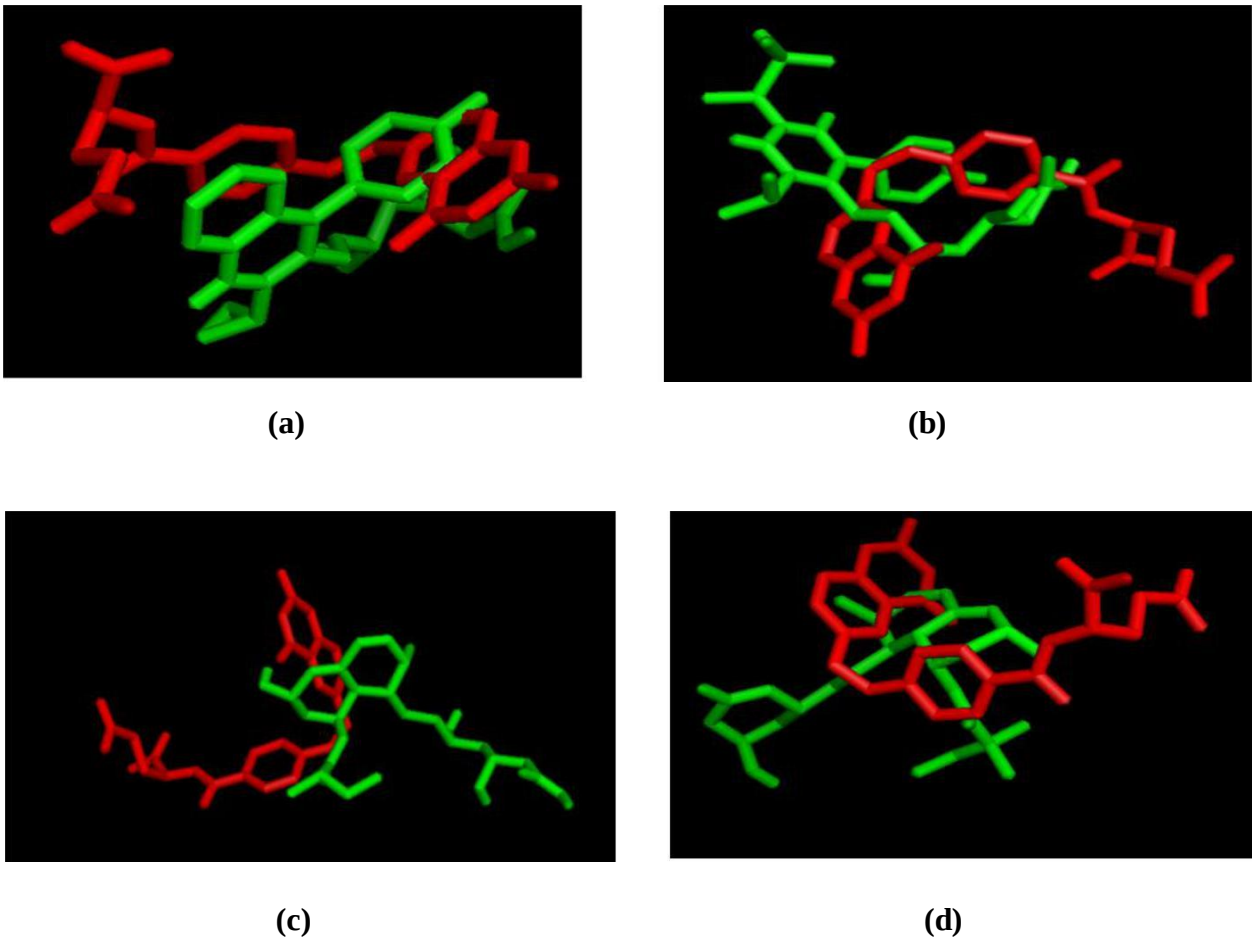



**Figure 3.5 (a)** Superimposition of Folic acid with Pitavastatin; **(b)** Superimposition of Folic acid with Rosuvastatin; **(c)** Superimposition of Folic acid with Pravastatin; **(d)** Superimposition of Folic acid with Simvastatin

Statins bind to the same pocket, the substrate binding pocket as the reference ligand, folic acid (Vizualization using PyMOL)

### 3.2.2 Identification of Binding Proteins

Discovery studio is used a validation tool which gives a clear and distinct idea about the interactions of proteins with the ligand. Bonds that exist between amino acids can be observed along with the bond types. It also allows evaluation of the distances between the proteins and small molecules. Bond lengths, bond types and amino acid interactions of the ligands with the molecules can be compared to the reference ligand. The pdb files of the protein-drug complexes were opened in discovery studio and the ligand interactions were observed. Most of the drugs interacted with the amino acids lining the binding pocket of the protein.

**Table 3.2** Non-bond Interactions that are involved in the Binding of Folic Acid to 1DHF (Visualized in Discovery Studio Version 17.2.0.16349)

| **Category of Bond** | **Amino acid..ligand and atom interaction** | **Distance in Angstroms (Amino acid-Ligand)** | **Type** |
|---|---|---|---|
| Hydrogen bond | ARG70 | 1.69996 | Salt Bridge |
| Hydrogen bond | GLN35 | 1.86376 | Conventional hydrogen bond |
| Hydrogen bond | ARG70 | 1.93525 | Conventional hydrogen bond |
| Hydrogen bond | GLN35 | 1.68847 | Conventional hydrogen bond |
| Hydrogen bond | THR56 | 2.86704 | Conventional hydrogen bond |
| Hydrogen bond | GLN35 | 1.62624 | Conventional hydrogen bond |
| Hydrogen bond | VAL8 | 2.12565 | Carbon hydrogen bond |

| | | | |
|---|---|---|---|
| Hydrophobic bond | ILE60 | 2.92565 | Pi-Sigma |
| Hydrophobic bond | PHE34 | 5.25609 | Pi-Pi T-Shaped |
| Hydrophobic bond | ILE7 | 5.22722 | Pi-Alkyl |
| Hydrophobic bond | ALA9 | 4.06742 | Pi-Alkyl |

Several hydrogen bonds were formed between Folic acid and 1DHF. Seven hydrogen bonds and four hydrophobic bonds were formed. The distances of the hydrogen bond ranged from 1.6-2.9 angstroms. The important amino acids in the binding of folic acid to 1DHF included GLN35 (aa Glutamine), ARG70 (aa Arginine), VAL8 (aa Valine), ILE60 (aa Isoleucine), ALA9 (aa Alanine and PHE34 (aa Phenylalanine) (Table 3.2.)

### 3.2.2.1 *In silico Binding of 1DHF with Simvastatin*

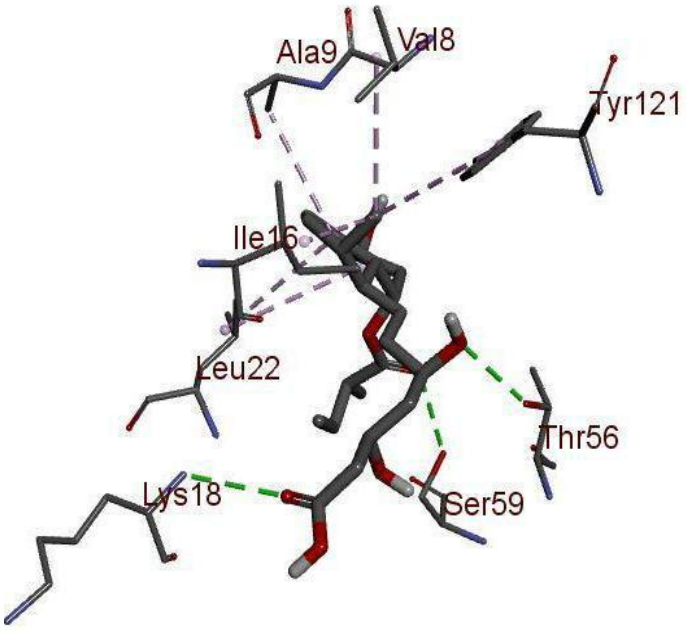



**Figure 3.5.1** Non-bond Interactions of Simvastatin with 1DHF

**Table 3.3** Non-bond Interactions and Binding Energy of 1DHF with Simvastatin (Discovery Studio Version 17.2.0.16349)

| **Ligand** | **Binding Affinity of Rigid docking (kCal/mol)** | **Category of Bond** | **Amino acid..ligand and atom interaction** | **Distance in angstroms (Amino acid-Ligand)** | **Type** |
|---|---|---|---|---|---|
| Simvastatin | -11.6 | Hydrogen Bond | A:THR56:OG1 - :UNL1:O | 2.70431 | Conventional Hydrogen Bond |
| | | Hydrogen Bond | A:GLY116:CA - :UNL1:O | 3.03638 | Carbon Hydrogen Bond |
| | | Hydrogen Bond | :UNL1:H34 - A:ILE16:O | 2.95732 | Carbon Hydrogen Bond |
| | | Hydrophobic | A:ALA9 - :UNL1 | 5.05025 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:LEU22 | 4.68259 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:PRO61 | 4.07293 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:LEU22 | 4.8295 | Alkyl |
| | | Hydrophobic | A:PHE31 - :UNL1 | 5.35665 | Pi-Alkyl |
| | | Hydrophobic | A:PHE31 - :UNL1:C | 4.1166 | Pi-Alkyl |
| | | Hydrophobic | A:PHE31 - :UNL1:C | 3.69022 | Pi-Alkyl |

| | | | | | |
|---|---|---|---|---|---|
| | | Hydrophobic | A:PHE34 - :UNL1 | 4.40262 | Pi-Alkyl |
| | | Hydrophobic | A:PHE34 - :UNL1 | 4.19186 | Pi-Alkyl |
| | | Hydrophobic | A:PHE34 - :UNL1:C | 5.08827 | Pi-Alkyl |

Several bonds were formed between Simvastatin and 1DHF. The types of bonds include hydrogen bonds and hydrophobic bonds. (Table 3.3). The first hydrogen bond formed between THR56 (aa Threonine) and oxygen atom of Simvastatin having bond length of 2.70 angstroms. The second hydrogen bond formed between GLY116 (aa glycine) and Simvastatin having a bond length of 3.03. The other hydrogen bond formed was between oxygen atom of ILE16 (aa isoleucine) and hydrogen of an unknown amnio acid with a bond length of 2.96 angstroms. The first hydrophobic bond formed had a bond length of 5.05 angstroms which was formed between ALA9 (aa alanine) and alkyl groups of Simvastatin. The other hydrobhobic bonds formed included LEU22 (aa leucine), an unknown amino acids hydrogen atom, PRO61 (aa proline), LEU22 (aa leucine), PHE31 (aa phenylalanine), PHE31(aa phenylalanine), PHE31 (aa phenylalanine), PHE34 (aa phenylalanine), PHE34(aa phenylalanine) and PHE34 (aa phenylalanine) with the alkyl groups of Simvastatin having bond lengths of4.68, 4.07,4.83, 5.36, 4.12, 3.69,4.40, 4.19 and 5.088. Hydrophobic interactions have a very significant role in nonpolar molecules. Davis and Teague found in their study that hydrophobic interactions greatly influence the affinity with which a ligand binds to its receptor molecule. They also revealed that hydrophobic bonds can increase the binding per methyl group by around 3.2 times (Davis & Teague, 1999).

### 3.2.2.2 *In silico Binding of 1DHF with Pravastatin*

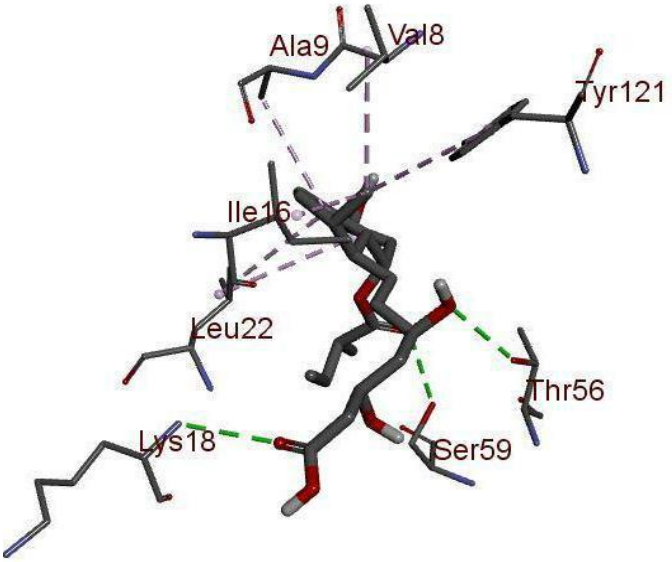



**Figure 3.5.2** Non-bond Interactions of Pravastatin with 1DHF

**Table 3.4** Non-bond Interactions and Binding Energy of 1DHF with Pravastatin (Discovery Studio Version 17.2.0.16349)

| **Ligand** | **Binding Affinity of Rigid docking (kCal/mol)** | **Category of Bond** | **Amino acid..ligand and atom interaction** | **Distance in angstroms (Amino acid-Ligand)** | **Type** |
|---|---|---|---|---|---|
| Pravastatin | -11.6 | Hydrogen Bond | A:LYS18:N -:UNL1:O | 3.10006 | Conventional Hydrogen Bond |
| | | Hydrogen Bond | A:THR56:OG1 - :UNL1:O | 2.70734 | Conventional Hydrogen Bond |
| | | Hydrogen Bond | A:SER59:OG -:UNL1:O | 3.08699 | Conventional Hydrogen Bond |

| | | | | | |
|---|---|---|---|---|---|
| | | Hydrophobic | A:ALA9 - :UNL1 | 4.29948 | Alkyl |
| | | Hydrophobic | A:LEU22 - :UNL1 | 5.10452 | Alkyl |
| | | Hydrophobic | :UNL1 - A:LEU22 | 4.86025 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:VAL8 | 4.55925 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:ILE16 | 4.13737 | Alkyl |
| | | Hydrophobic | A:TYR121 - :UNL1:C | 5.21935 | Pi-Alkyl |

Several bonds were formed between Pravastatin and 1DHF. Three hydrogen bonds and six hydrophobic bonds were formed during the binding of Pravastatin to 1DHF. The hydrogen bond formed between nitrogen atom of the LYS18 (aa lysine) and oxygen of Pravastatin had a distance of 3.1 angstroms. The distance between the hydrogen bond formed between THR56 ( aa threonine) and oxygen of Pravastatin was 2.7 angstroms. The other hygrogen bond formed between SER59 (aa Serine) and oxygen of Pravastatin was 3.09. The six hydrophobic bonds include bonds between the amino acids, ALA9 (aa alanine), LEU22(aa leucine), LEU22 (aa leucine), VAL9 (aa valine), ILE16 (aa isoleucine) and TYR121(aa tyrosine) and the ligand, Pravastatin; the distances are 4.3. 5.10, 4.86, 4.55, 4.13, 5.22 angstroms respectively. The types of bonds include conventional hydrogen bond, alkyl and pi alkyl bonds (Table 3.4).

### 3.2.2.3 *In silico Binding of 1DHF with Rosuvastatin*

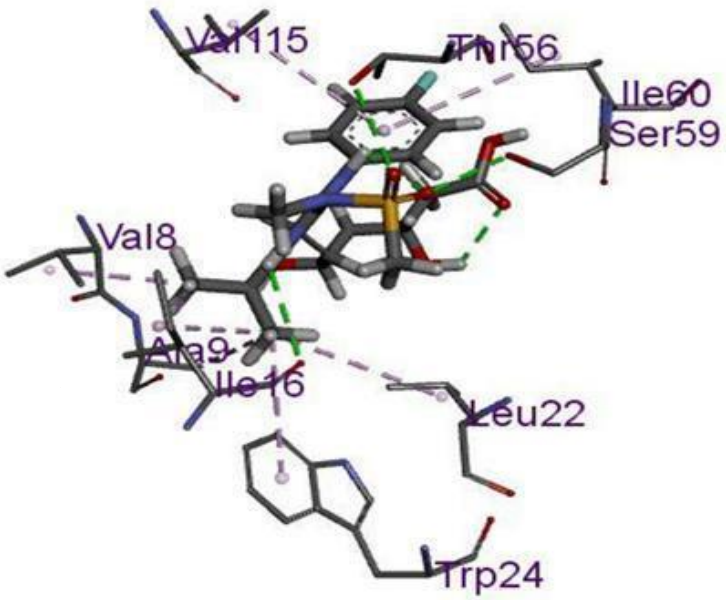



**Figure 3.5.3** Non-bond Interactions of Rosuvastatin with 1DHF

**Table 3.5** Non-bond Interactions and Binding energy of 1DHF with Rosuvastatin (Discovery Studio Version 17.2.0.16349)

| **Ligand** | **Binding Affinity of Rigid docking (kCal/mol)** | **Category of bond** | **Amino acid..ligand and atom interaction** | **Distance in angstroms (Amino acid-Ligand)** | **Type** |
|---|---|---|---|---|---|
| Rosuvastatin | -10.5 | Hydrogen Bond | A:THR56:OG1 - :UNL1:O | 3.15868 | Conventional Hydrogen Bond |
| | | Hydrogen Bond | A:SER59:OG - :UNL1:O | 2.71033 | Conventional Hydrogen Bond |
| | | Hydrogen Bond | :UNL1:HN - A:ILE16:O | 3.06196 | Conventional Hydrogen Bond |
| | | Hydrogen Bond | :UNL1:HN - :UNL1:O | 2.45841 | Conventional Hydrogen Bond |

| | | | | | |
|---|---|---|---|---|---|
| | | Hydrogen Bond | :UNL1:H - :UNL1:O | 2.33948 | Conventional Hydrogen Bond |
| | | Hydrophobic | A:ALA9 - :UNL1:C | 4.40419 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:VAL8 | 3.90611 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:ILE16 | 3.85503 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:ILE16 | 4.53478 | Alkyl |
| | | Hydrophobic | :UNL1:C - A:LEU22 | 5.08114 | Alkyl |
| | | Hydrophobic | A:TRP24 - :UNL1:C | 4.5681 | Pi-Alkyl |
| | | Hydrophobic | :UNL1 - A:ILE60 | 5.16782 | Pi-Alkyl |
| | | Hydrophobic | :UNL1 - A:VAL115 | 5.22051 | Pi-Alkyl |

Several bonds were formed between Rosuvastatin and 1DHF. Four hydrogen bonds and eight hydrophobic bonds were formed. The distance between the first hydrogen bond formed between THR56 (aa Threonine) and oxygen atom of Rosuvastatin was 3.16 angstroms. The hydrogen bond formed between SER59 (aa serine) and oxygen of Rosuvastatin was 2.7 angstroms; the hydrogen bond formed between the ligand and oxygen of ILE16 (aa isoleucine) had a distance of 3.06 Angstroms. The other two bonds had distances, 2.5 and 2,33 angstroms respectively. The hydrophobic bonds play a significant role in increasing the binding affinity of Rosuvastatin with 1DHF molecule.
The types of bonds include conventional hydrogen bond, alkyl and pi alkyl bonds (Table3.5)

### 3.2.2.4 *In silico Binding of 1DHF with Pitavastatin*

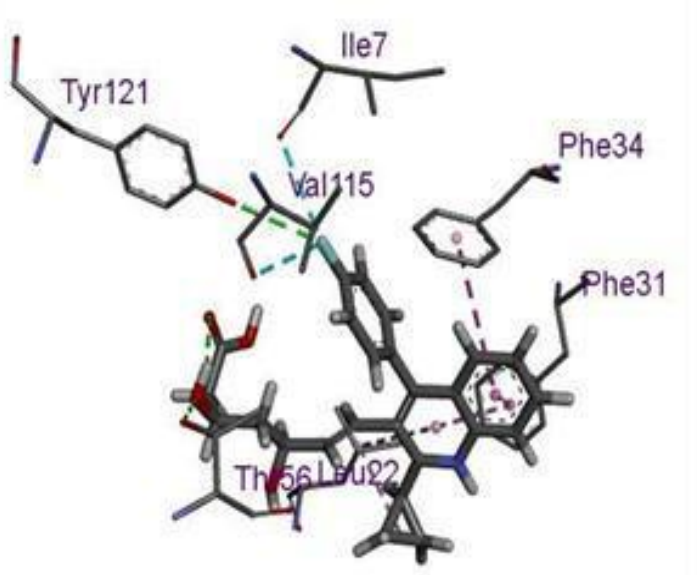



**Figure 3.5.4** Non-bond Interactions of Pitavastatin with 1DHF

**Table 3.6** Non-bond Interactions and Binding Energy 1DHF with Pitavastatin (Discovery Studio Version 17.2.0.16349

| Ligand | Binding Affinity of Rigid docking (kCal/mol) | Category of bond | Amino acid..ligand and atom interaction | Distance in angstroms (Amino acid-Ligand) | Type |
|---|---|---|---|---|---|
| Pitavastatin | -12.7 | Hydrogen Bond | A:THR56:OG1 - :UNL1:O | 2.69513 | Conventional Hydrogen Bond |
| | | Hydrogen Bond;Halogen | A:TYR121:OH - :UNL1:F | 3.18175 | Conventional Hydrogen Bond;Halogen (Fluorine) |
| | | Hydrogen Bond | :UNL1:H - :UNL1:O | 2.34284 | Conventional Hydrogen Bond |

| | | | | | |
|---|---|---|---|---|---|
| | | Halogen | A:ILE7:O - :UNL1:F | 3.34043 | Halogen (Fluorine) |
| | | Halogen | A:VAL115:O - :UNL1:F | 3.11747 | Halogen (Fluorine) |
| | | Hydrophobic | A:PHE31 - :UNL1 | 4.38008 | Pi-Pi Stacked |
| | | Hydrophobic | A:PHE31 - :UNL1 | 4.35338 | Pi-Pi Stacked |
| | | Hydrophobic | A:PHE34 - :UNL1 | 5.29595 | Pi-Pi T-shaped |
| | | Hydrophobic | :UNL1 - A:LEU22 | 4.66676 | Alkyl |
| | | Hydrophobic | :UNL1 - A:LEU22 | 5.33681 | Pi-Alkyl |

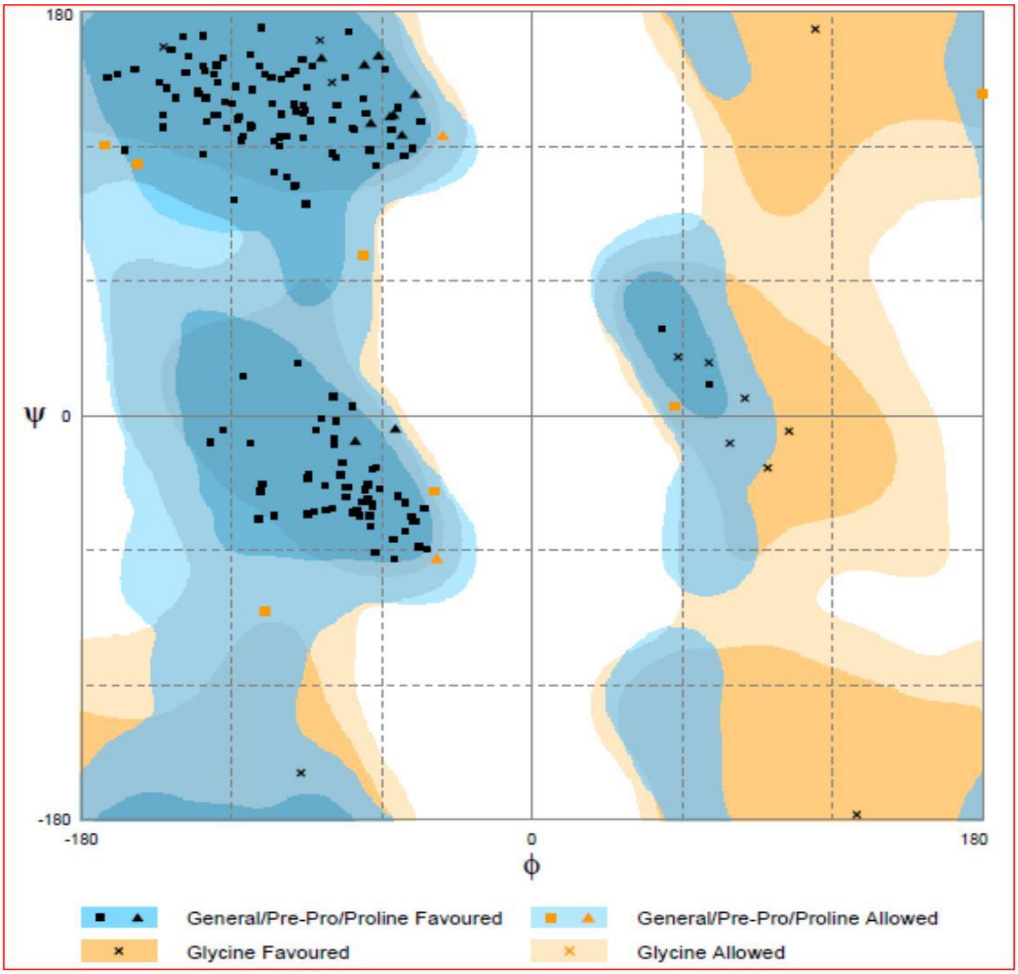
Several bonds were formed between Pitavastatin and 1DHF. The types of bonds include conventional hydrogen bond, halogen bond, pi-pi stacked, pi-pi T-shaped, alkyl and pi alkyl bonds (Table 3.6). Three hydrogen bonds, two halogen bonds and five hydrophobic bonds were formed between Pitavastatin and 1DHF. The first hydrogen bond was formed between THR56 (aa threonine) and Pitavastatin with a distance of 2.69 angstroms; The second hydrogen bond was formed between the OH of TYR121 (aa tyrosine) with the fluorine of Pitavastatin with a distance of 3.18 angstroms.

The other hydrogen bond was formed between an unknown amino acid, and the distance was 2.34 angstroms. The first halogen bond formed was between ILE7 (aa isoleucine) and the F of Pitavastatin; the other halogen bond was between VAL115 (aa valine) and F of Pitavastatin. Five hydrophobic bonds included bonds between the ligand and amino acids PHE31 (aa phenylalanine), PHE31 (aa phenylalanine), PHE34 (aa phenylalanine) and two LEU22 (aa leucine), the distances being 4.38, 4.35, 5.30, 4.67 and 5.33 angstroms. Hydrophobic bonds can strongly increase the binding affinities as a drug binds to its receptor. Thus, these allowed a greater binding affinity between 1DHF and Pitavastatin.

## 3.3 Validation of Protein bound to Ligands

For further validation, Ramachandran plots of protein bound to drugs were generated. Conserved regions were also identified using ConSurf.

### 3.3.1 Validation using Ramachandran plot




| | |
|---|---|
| Number of residues in favoured region (~98.0% expected) | : 171 (95.0%) |
| Number of residues in allowed region (~2.0% expected) | : 9 (5.0%) |
| Number of residues in outlier region | : 0 (0.0%) |

**Figure 3.6** Ramachandran Plot showing the energy minimization of the protein, 1DHF bound to statins.

The Ramachandran plot helps to predict the conformation of the backbone of a given polypeptide chain more quantitatively starting from knowledge of its amino acid sequences (Lovell et al., 2003; "RAMPAGE: Ramachandran Plot Assessment," n.d.; Sheik et al., 2002). Number of amino acid residues in the favored region is 95%. The number of residues in the allowed region is 5%. No residues are seen in the outlier region. No significant changes were observed, which indicates no notable changes have occurred in the conformation of psi and phi bonds after binding of drugs with 1DHF.

### 3.3.2 Validation using ConSurf

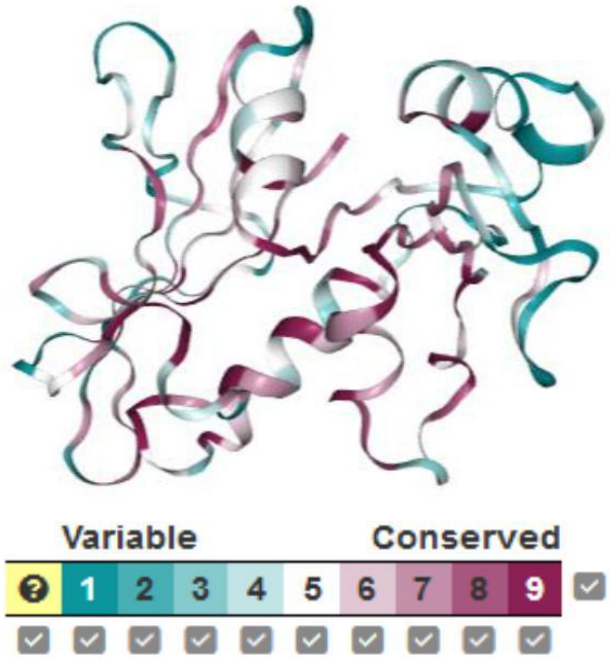



**Figure 3.7** Conserved regions and variable regions in 1DHF

1DHF was then uploaded in ConSurf, which is an online tool showing the conserved regions of the protein. A conserved region indicates that the part had remained unchanged for a very long time and is considered functionally important; they are not subjected to frequent mutations (Krishnan, Li, & Issac, 1998). Considering the non-bond interactions observed in Discovery Studio, the amino acids that were involved in drug binding, THR56 (aa threonine), TYR121(aa tyrosine), ILE7(aa isoleucine) , VAL115 (aa valine), PHE31 (aa phenylalanine), PHE34 (aa phenylalanine), LEU22 (aa leucine), ALA9 (aa alanine), SER59 (aa serine), VAL8 (aa valine), ILE16 (aa isoleucine) and

ILE60 (aa isoleucine) lie in the conserved regions. LYS 18 (aa lysine) and GLY116 (aa glycine) lie in the variable regions.

## 3.4 Selection of Statin Drugs

From the various results obtained, the drugs Pitavastatin and Rosuvastatin showed the best binding affinities and interactions with 1DHF. All the statin drugs chosen made no significant changes to the bond angles of psi and phi bonds. Although Pravastatin showed a high binding affinity, but by validation using ConSurf, it was observed that LYS18 (aa lysine) that binds to the drug falls in the variable region. Studies conducted previously showed Pravastatin's role in cancer remains controversial. (Bujanda et al., 2016; Costantine et al., 2016). Simvastatin also had the bound amino acid, GLY116 (aa glycine) in the variable region. Even though the binding affinity of Simvastatin with 1DHF was high (-11.6 kCal/Mol), the drug bound to the glycine of 1DHF. Thus in this study, we are hypothesizing Pitavastatin and Rosuvastatin could possibly inhibit abnormal cell mitosis via inhibition of the 1DHF biosynthetic pathway.

# 4 Discussion and Conclusion

## 4.1. Discussion

1DHF is an oxidoreductase enzyme responsible for the synthesis of purine and pyrimidine bases which are the building blocks of DNA and RNA. As it is important for the de novo synthesis of DNA and proliferation of cells, and because it is highly expressed in cancer cells, it primarily acts as a target for the development of anticancer drugs. It is being explicitly studied as it plays an important role in disease and health (McEntee et al., 2011; Polshakov, Birdsall, Frenkiel, Gargaro, & Feeney, 1999; Sharif, Hossain, Kabir, & Rajib, 2017).

Data from studies by Fagerberg et al., 2014 show that 1DHF is highly expressed in bone marrow, fat, lymph node and testis; finding inhibitors that could suppress 1DHF could be potentially useful anticancer drugs. Another possible explanation could be the subcellular location of the protein; 1DHF is found in the nucleus as well as in the mitochondria of cells. Mitochondria are signaling organelles which are biosynthetic and bioenergetic in nature, which play an integral role in sensing stress and thus allows for cellular adaptation to its environment. Hence, they are important mediators of tumorigenesis, as the process of tumor promotion requires flexibility to adapt to environmental and cellular alterations along with cancer treatments (Vyas, Zaganjor, & Haigis, 2016). A better understanding of the mechanisms mediating mitochondrial misregulation during tumor progression is thus vital for establishment of cancer therapeutics.

Reports have shown relationship between levels of cholesterol and cancer development. (Murai, 2015). Accumulation of cholesterol is a notable feature of cancer cells and studies highlight its role in breast, colorectal and prostate cancers. Disruption in the biosynthetic pathway of cholesterol may contribute to the formation of tumors and may also enhance cancer progression. 3 hydroxy 3 methyl glutaryl coenzyme A reductase is an important site in the biosynthesis of cholesterol. It is the primary target of cholesterol lowering drugs, referred to as statins. They essentially act as competitive inhibitors of

the enzyme HMG-CoA, blocking the pathway of cholesterol synthesis (Murai, 2015; Singh, Saxena, Srinivas, Pande, & Chattopadhyay, 2013).

Drug repurposing (Kabir et al., 2017; March-Vila et al., 2017) was used to validate anticancer properties or tumor suppressive activities of statin drugs. For this purpose, the protein explicitly expressed in cancer cells, 1DHF, was targeted. After removing the B-chain of the protein followed by elimination of the heteroatoms (water molecules) and folic acid, docking was carried out. The instability index of the protein 1DHF was determined using ProtParam the value being 27.25 which classifies the protein as stable. A value of less than 40 is considered stable (Gasteiger et al., n.d.). The protein was further validated by z score values, Ramachandran plot, Verify 3D as well as with the help of Errat. The z score of 1DHF was found to be -9.06 which is considered a satisfactory outcome. The greater the value is away from zero towards the negative, the better. The residues were seen on the X ray region. However it did not fall within the NMR region. Local quality of protein was determined using energy diagrams

Docking is a computational technique that helps determine the binding affinities of drugs with the protein. It can be carried out in two ways; both rigid and flexible docking were carried out. Better outcomes were obtained with rigid docking compared to flexible docking. The high binding affinities helped in the choice of the drugs chosen. A high binding affinity suggested a link between statin drugs and anticancer drugs, as they both bind to the same receptor but the extent was unknown. Pitavastatin had a binding affinity of -12.7kcal/mol and Rosuvastatin had a binding affinity of -10.6 kcal/mol. The affinities were higher than established anticancer drugs such as Trimetrexate (-10.5 kcal/mol) and trimethoprim (-8.7 kcal/mol) (Sharif, Hossain, Kabir, & Rajib, 2017). To find out the link more precisely, non-bonded interactions, bond distances and types were looked up in Discovery studio. Discovery studio was used as a validation tool which provided a more distinct idea about the interaction of the protein with the ligand.

With 1DHF, Pitavastatin was observed to interact with THR56 ( aa threonine), TYR121 (aa tyrosine), ILE7 (aa isoleucine), VAL115 (aa valine), PHE31(aa phenylalanine), PHE34 (aa phenylalanine) and LEU22 . Most of the bonds seen were hydrogen bonds and hydrophobic bonds; Halogen bonds were also observed. Hydrogen bonds included

conventional hydrogen bonds, and hydrophobic bonds included Pi-Pi stacked, Pi-Pi T shaped, alkyl and pi-alkyl bonds. All the hydrogen bonds ranged from 2-3 Å which was considered to be decent. Hydrophobic bonds help in enhancing the binding affinity.

Multiple bonds were formed between Rosuvastatin and 1DHF. Among them were four hydrogen bonds and eight hydrophobic bonds. The distance between the first hydrogen bond formed between THR56 (aa Threonine) and oxygen atom of Rosuvastatin was 3.16 angstroms. The hydrogen bond formed between SER59 (aa serine) and oxygen of Rosuvastatin was 2.7 angstroms; the hydrogen bond formed between the ligand and oxygen of ILE16 (aa isoleucine) had a distance of 3.06 Angstroms. The other two bonds had distances, 2.5 and 2.33 angstroms respectively. The hydrophobic bonds play a significant role in increasing the binding affinity of Rosuvastatin with 1DHF molecule.
The types of bonds included conventional hydrogen bond, alkyl and pi alkyl bonds.

Ramachandran plot was plotted to show the energy minimization of the entire protein. Ramachandran plot was another method that was used for validation of the protein as well as the 1DHF-drug complexes. The energy minimization plot showed same results with both the protein and the protein-drug complexes. The number of amino acid residues in the favored region was 95%, the number of amino acids in the allowed region was 5%. No residues were observed in the outlier region. The results were close to the expected results. Expected results for amino acid residues in the favored and allowed region are 98% and 2% respectively. In case of residues in outlier region, the percentage is expected to be as less as possible. Values over 90% are considered acceptable for residues on favored region and a deviation of ±5% is allowed in the allowed region. Same results were obtained with the 1DHF-ligand complexes; the results indicated that the amino acids were located in desirable locations after the protein complex was formed and no undesirable or harmful effects would be caused in the body. Previous studies have shown that Pitavastatin and Rosuvastatin exhibited anticancer properties. However, when the drug was administered at high doses, it showed side effects like rhabdomyolysis. It was highlighted in various reports that Pitavastatin demonstrated more effectiveness when used in combination with anticancer drugs than when used alone. It was revealed that it synergistically enhanced necrosis of tumor cells when it was used in combinations (Al-Qatati & Aliwaini, 2017; De Wolf, De Wolf, & Richardson, 2017).

Consurf (Glaser *et al.*, 2003; Landau *et al.*, 2005; Celniker *et al.*, 2013) results showed that the amino acids THR56, TYR121, VAL115, PHE31, PHE34, LEU22, SER59, ALA9, VAL8, ILE16, LEU22, TRP24, ILE60 AND VAL115 lie in highly conserved regions; this indicates that the drugs Rosuvastatin and Pitavastatin could possibly act as a potential 1DHF inhibitor to prevent tumor and cancer progression.

## 4.2. Conclusion and Future Work

From this *in silico* study it is apparent that the statin drugs, Pitavastatin and Rosuvastatin are both effective inhibitors of 1DHF. Thus this study suggests that Pitavastatin and Rosuvastatin may play significant roles through the inhibition of the folic acid pathway in cancer progression and tumor development.

High binding affinities were found with rigid docking, the values being -12.7 kCal/Mol and -10.5 kCal/Mol respectively. Pitavastatin, if given as a cholesterol lowering agent may possibly reduce the risk of development of cancer in the future. In addition, Pitavastatin would be a good choice of drug for a cancer patient with high levels of serum cholesterol as it significantly causes necrosis of cancer cells when given in combination with an anticancer drug. The statin drug, Rosuvastatin is a well-known INN drug which is indicated in hyperlipidemia. Previous studies stated its use as an anticancer drug through the mTOR pathway. However, no in vitro studies have been carried out yet. This study suggests is antitumorigenic property as an antagonist of 1DHF.

Further studies and *in vitro* tests could be performed to check the ligands potential interaction with 1DHF. If the *in vitro* tests prove the drugs to be effective, as potent antagonists of 1DHF, *in vivo* tests can then be performed.

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