Inhibition of Fibroblast Activation Protein alpha (FAP-α) in Colorectal Cancer

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

The Department of Pharmacy
Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

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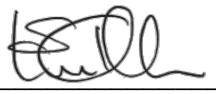

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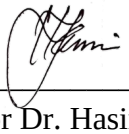

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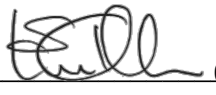

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Ethics Statement

This study does not involve any kind of human or animal trial.

Abstract

Being one of the leading cause of death worldwide, the fatality of colorectal cancer is increasing day by day causing approximately 862,000 deaths annually, according to IARC, 2020. As most of the conventional chemotherapeutics are becoming resistant, an *in silico* study was done using synthetic and natural small molecules to identify possible drug that may be proposed in the treatment of colorectal cancer. Because of having a significant role in the tumor progression of colorectal cancer, FAP-α was taken as the macromolecule. Molecular docking was performed and binding affinities were evaluated. Furthermore, the results were visualized by Discovery Studio to find out non-bonded ligand-protein interaction. At the end, admetSAR property of the drugs were assessed and compared with the standard drug. Therefore, analyzing all the *in silico* results, the selected drugs having the property of inhibiting FAP-α were proposed for the treatment of colorectal cancer.

Keywords: *In silico;* Molecular docking; FAP-α; Binding affinity.

Dedication

Dedicated to my sister.

Acknowledgement

First and foremost, praises and thanks to the Almighty Allah, for His showers of blessings throughout my research work to complete the research successfully.

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List of Acronyms

FAP-α	Fibroblast Activation Protein alpha.
CRC	Colorectal Cancer.
VEGF-A	Vascular Endothelial Growth Factor-A
p-AKT	Phosphorylated protein kinase B
p-ERK	Phosphorylated extracellular-signal-regulated kinase
SW1116	Human colorectal cancer cell line
HT29	Human colorectal adenocarcinoma cell line
CM	Conditioned medium
BMP	Bone morphogenetic protein
TIMP3	Metalloproteinase inhibitor 3
IGF-1R	Insulin-like growth factor 1 receptor
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA

Chapter 1

Introduction

1.1 Rationale

Because of the persistent rise of global population as well as burden of disease over the previous years, the necessity of drugs has increased tremendously (Kaplan et al., 2011). In traditional drug development, drug discovery is a high-risk, high-investment and time-consuming process (Xue, Li, Xie, & Wang, 2018) (*Figure 1*). Thus it has become a necessity to embrace new approaches for designing new drugs. A popular strategy in recent years is repositioning the drugs. It is different from conventional drug development strategies and the strategy is efficient, inexpensive and safe (Xue et al., 2018). For instance, the total research and development (R&D) expenses for the drug discovery increased 10 times from 1975 (US $4 billion) to 2009 ($40 billion) on the contrary the number of new molecular entity's approved has remained largely flat (In 1976, only 26 new drugs approved and in 2013, 27 new drugs approved) (Shim & Liu, 2014).

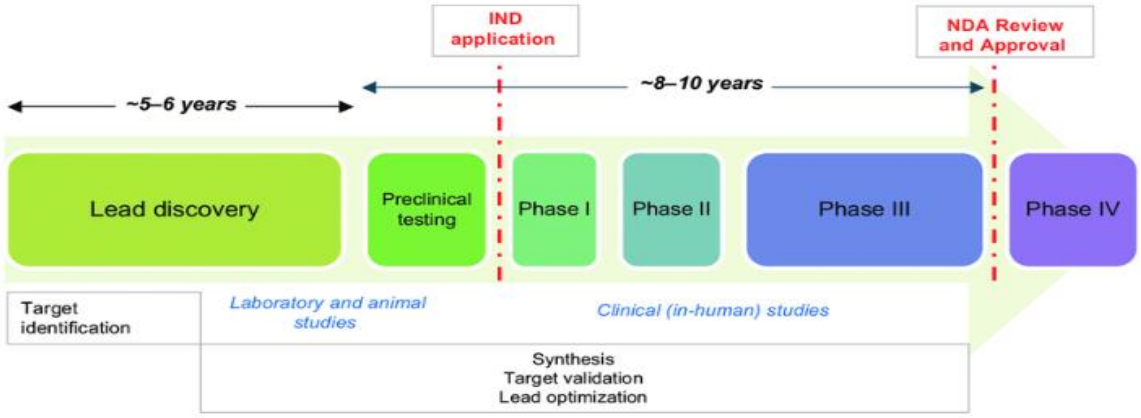

Figure 1: Overview of traditional drug development process (Coloma, 2012).

Cancer is a disease which is responsible for causing numerous deaths all over the world and no satisfactory treatment or solution is available yet. In cancer, resistance of drug has become a common phenomenon. Amongst the most common cancers, colorectal cancer (CRC) is ranked the third affecting both men and women worldwide however resistance to drugs remain one of the barrier for the low survival rates of CRC patients (Van Der Jeught, Xu, Li, Lu, & Ji, 2018).

One of the most prescribed and earliest drugs for colorectal cancer was methotrexate. Unfortunately, due to growth of resistance in colorectal cancer cells, effectiveness of this chemotherapeutic agent is compromised (Wu et al., 2017). To overcome this issue, synthetic and natural small molecules can be addressed to inhibit potential proteins that are responsible for the growth of the tumor in colorectal cancer and among those proteins fibroblast activation protein alpha (FAP-α) have been used in this study. Through molecular docking and computational approaches, effects of synthetic and natural small molecules were identified in the inhibition of FAP-α in colorectal cancer.

1.2 Cancer

Cancer causes due to the abnormal growth of cells. The principle cause of death in cancer is because of the spreading (metastasis) of cancer cells to distant organs and growing into the surrounding tissues. Almost 1,688,780 cancer cases were addressed in 2017 according to "National Centre for Health Statistics" in USA. Identification and treatment of cancer in the initial stage can build the opportunity for restoring the cancer cell growth and dropping the mortality rate significantly (Kurniawan et al., 2018).

Worldwide, cancer has become a crucial health issue. In recent decade, the incidents of cancer has kept up an upward pattern due to an increasing prevalence of several established

risk factors, for instance, diabetes, air pollution, physical inactivity, being overweight, smoking, lifestyle changes and particularly, the aging of the people (Luo & Liu, 2019a).

The protocol of cancer treatment is mainly surgery by removing cancerous cells from the healthy tissue. However, surgery is only effective for the treatment of local cancer. Handling metastatic cancer is difficult by this method. Other treatment protocols are chemotherapy and radiation therapy. Individually these methods can only eradicate a fraction of the malignant cells, thus, surgery, chemotherapy and radiation therapy are complementary. Immune-based therapy (immunotherapy) has recently been applied in the treatment of cancer. Though this method can reduce the rapid development of different cancers, but is not functional for all cancer-types (Kurniawan et al., 2018).

In cancer, genetic changes occur in cell due to chronic irritations. Because of genetic changes, uncontrolled rapid proliferation occurs in cancerous cell. The healthy cells supplies nutrients to assist tumor growth by tumor microenvironment. And thus the tumor enlarges. The tumor grows rapidly with the continuous production of oncometabolites. Because of the growth of the tumor, the healthy cells face metabolic imbalance. In certain cases, where the patient is suffering from other health related issues such as obesity, aging, high fat diet or diabetes, the healthy cell fails to provide proper supplications to the tumor cells. And thus, the tumor cells undergo invasion and metastasis to other parts and causes necrosis (Luo & Liu, 2019a) (*Figure 2).*

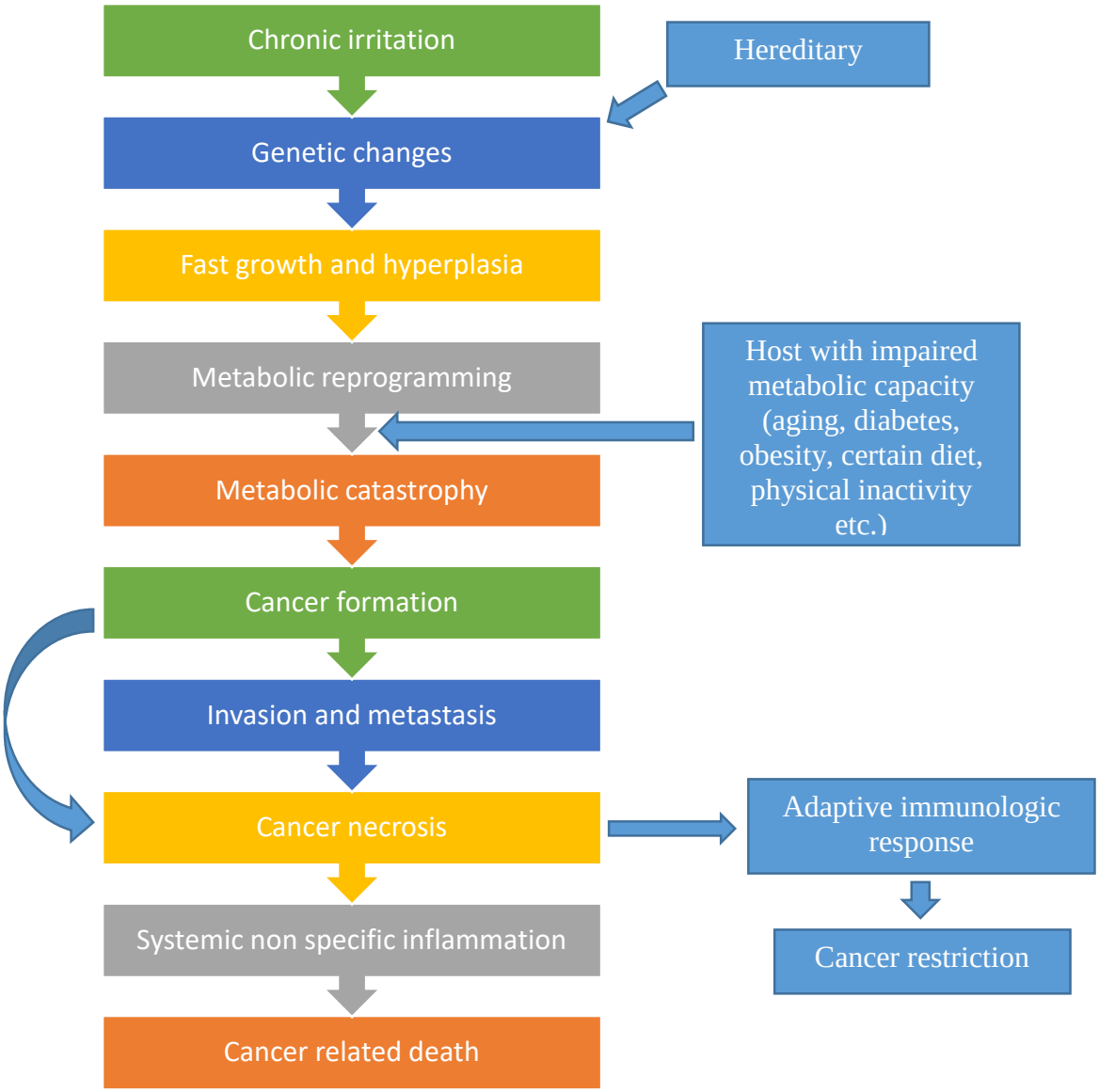

Figure 2: The integrative theory of cancer (Luo & Liu, 2019).

1.3 Colorectal Cancer

For its high mortality rate, colorectal cancer (CRC) categorizes as the top three frequently diagnosed cancers in the world (Lee & Song, 2013). The incidence of colorectal cancer is increasing every year and most cases are found in Western countries. The possibility of suffering from colorectal cancer is approximately 4%–5% and the risk factors of developing CRC is related to habits or personal features which includes age, lifestyle and chronic disease history (Mármol, Sánchez-de-Diego, Dieste, Cerrada, & Yoldi, 2017).

One of the significant risk factors of colorectal carcinoma is increasing age. Other than age specific incidents, lifestyle or environmental factors or both have greatest impact on causing colorectal cancer. Because of increasing obesity and consumption of processed foods, colorectal cancer is more frequent in developed countries rather than in underdeveloped or 3rd world countries (Siegel et al., 2017).

With the phage of tumor, area of tumor as well as patient's usual features, management and treatment of colorectal cancer differs. For instance, while planning for curative treatment protocol, surgical resection is not sufficient for advanced metastatic colorectal cancer rather chemotherapy is useful and thus it is introduced to the patients to reduce the size of tumor before surgery. 5-flurouracil (5-FU) was the mainstay of chemotherapy in colorectal cancer. But now-a-days, alongside 5-FU, various other drugs are found and administered (Fong & To, 2019).

Almost half of the patients with advanced colorectal cancer are forming resistance towards 5-FU based chemotherapeutics. To overcome this situation, it is important to diagnose the disease at an early stage, discover predictive biomarkers as well as developing novel drugs for CRC.

1.4 Expression of FAP-α in colorectal cancer

The formation of new blood vessels is known as angiogenesis and it plays an important role in progression, development and prognosis of tumor (Cao, Wang, Wang, & Tang, 2018a). Due to angiogenesis, the vessels can grow inside the tumor and provide nutrients inside the tumor. Through angiogenesis, tumors can exchange wastage materials as well. To improve patient outcome in treating colorectal cancer, finding an effective target is necessary to inhibit angiogenesis.

In more than 90% of epithelial tumor stromal cells, fibroblast activation protein alpha is present (FAP-α). FAP-α serves an important roles in angiogenesis, proliferation, invasion and metastasis, immune escape and tissue remolding (Cao et al., 2018a).

1.4.1 The characteristic of FAP-α

FAP-α belongs to the dipeptidyl peptidase (DPP) subfamily. It is a "type II integral membrane serine protease" which cleaves the bond between proline and any other amino acids (Jiang et al., 2016).

In activated stromal fibroblasts, human fibroblast activation protein alpha (FAP-α) is expressed which is a "type II cell-surface-bound trans membrane glycoprotein" with molecular weight 95,000. The structure is composed of a large extracellular domain of 736 amino acids, an 18 amino acid trans-membrane domain and a short 6 amino acid cytoplasmic domain which combines to 760 amino acids. With the presence of aspartate (Asp702), serine (Ser624) and histidine (His734) the critical structure of catalytic trial is formed *(Figure 4).* For enzymatic activity, Ser624 is important. The proteolytic activity of fibroblast activation protein alpha (FAP-α) is terminated while this serine is changed to alanine (Jiang et al., 2016) *(Figure 3).*

According to the crystal structure, FAP-α exists as a homodimer. FAP-α is overexpressed by Cancer Associated Fibroblasts (CAFs) in 85-90% of primary and metastatic colorectal cancers. High levels of FAP-α in human colon tumors promote tumor growth, progression, metastasis, and recurrence. Moreover, the level of FAP-α in rectal carcinomas, which have received preoperative chemo or radiotherapy, is a negative prognostic factor. Not only the level of FAP-α, but also the location of FAP-α, is related to poor prognosis of colon cancer patients. All of these findings provide rationale for the development of FAP-α directed therapy (Jiang et al., 2016).

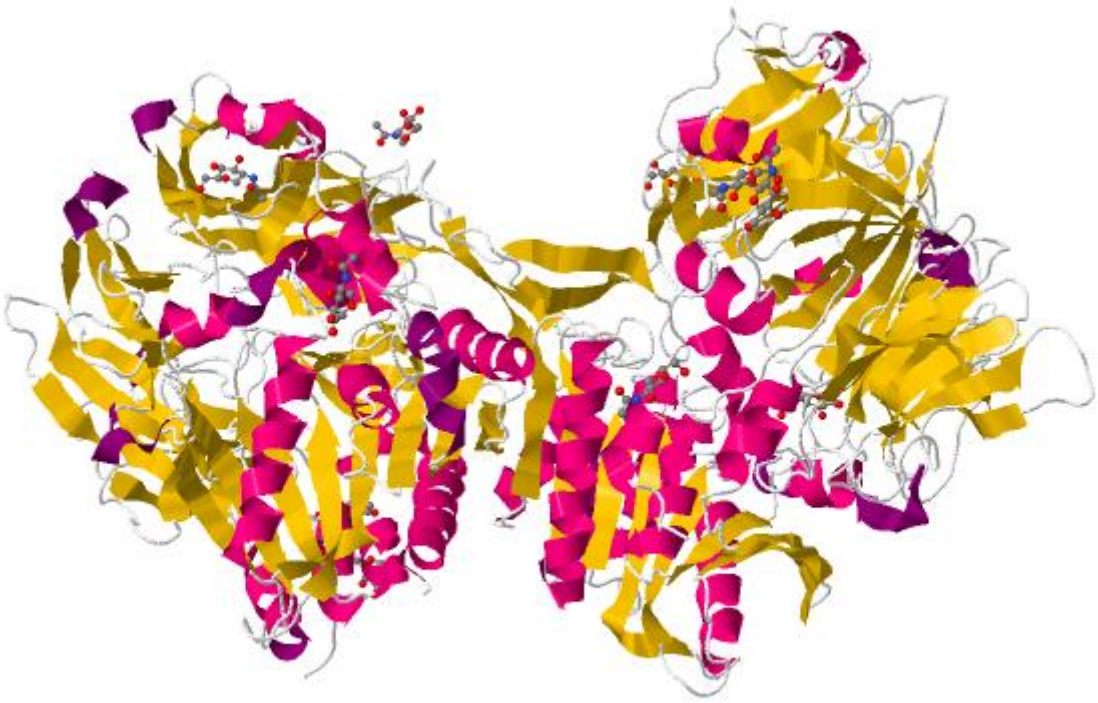

Figure 3: Structure of Fibroblast Activation Protein alpha (FAP-α) homodimer.

(The critical structure of the catalytic triad is formed by serine (Ser624), aspartate (Asp702), and histidine (His734), and Ser624 is essential for enzymatic activity. The figure was generated using JSmol (PDB ID 1Z68). The structure represents two same subunit of FAP-α which contains helixes and β-sheets (Jiang et al., 2016).

1.4.2 FAP-α regulates VEGF-A expression via Akt and ERK signaling pathways

p-ERK and p-Akt are the potential molecular pathways which is responsible for the proangiogenic characteristic of FAP-α (Cao et al., 2018a).

VEGF-A which stands for "vascular endothelial growth factor" is remarkably associated with the expression of FAP-α in colorectal cancer cell (Cao et al., 2018a).

SW1116 stands for human colorectal cancer cell line. By performing western blotting, FAP-α is identified in SW1116 cell line. Significant up regulation of VEGF-A expression is responsible for the overexpression of FAP-α in SW1116 cells and inhibition of VEGF-A

expression is markedly noticed by the silencing of FAP-α in HT29. HT29 cells stands for human colorectal adenocarcinoma cell line. (Cao et al., 2018a) (*Figure 4*).

Patients having higher expression of VEGF-A and FAP-α had shortest survival time. From colorectal cancer cell line, conditioned medium (CM) is collected to identify the effects of fibroblast activation protein alpha (FAP-α) on human umbilical vein endothelial cells (HUVECs). The outcome of the experiment revealed that with overexpressed FAP-α, conditioned medium (CM) collected from SW1116 cells exhibit significant increase of VEGF-R2, p-RAC-α serine/threonine-protein kinase (Akt) in HUVECs and phosphorylated extracellular signal-regulated kinase (p-ERK) along with increased proliferation rate. On the contrary, with silenced FAP-α, conditioned medium (CM) collected from HT29 cells exhibits remarkably inhibited proliferation rate. Analysis of molecular mechanism revealed that p-Akt and p-ERK in HT29 and SW1116 cells were affected with the overexpression and silencing FAP-α. The condition of VEGF-A up regulation initiated by FAP-α overexpression is improved by treating with appropriate inhibitors. The presence of FAP-α in colorectal cancer cells are validated through western blotting of conditioned medium of SW1116 and HT29 cell line and confirmed that angiogenesis in colorectal cancer cell is significantly promoted by FAP-α through the Akt and ERK signaling pathways (Cao, Wang, Wang, & Tang, 2018b) (*Figure 4*).

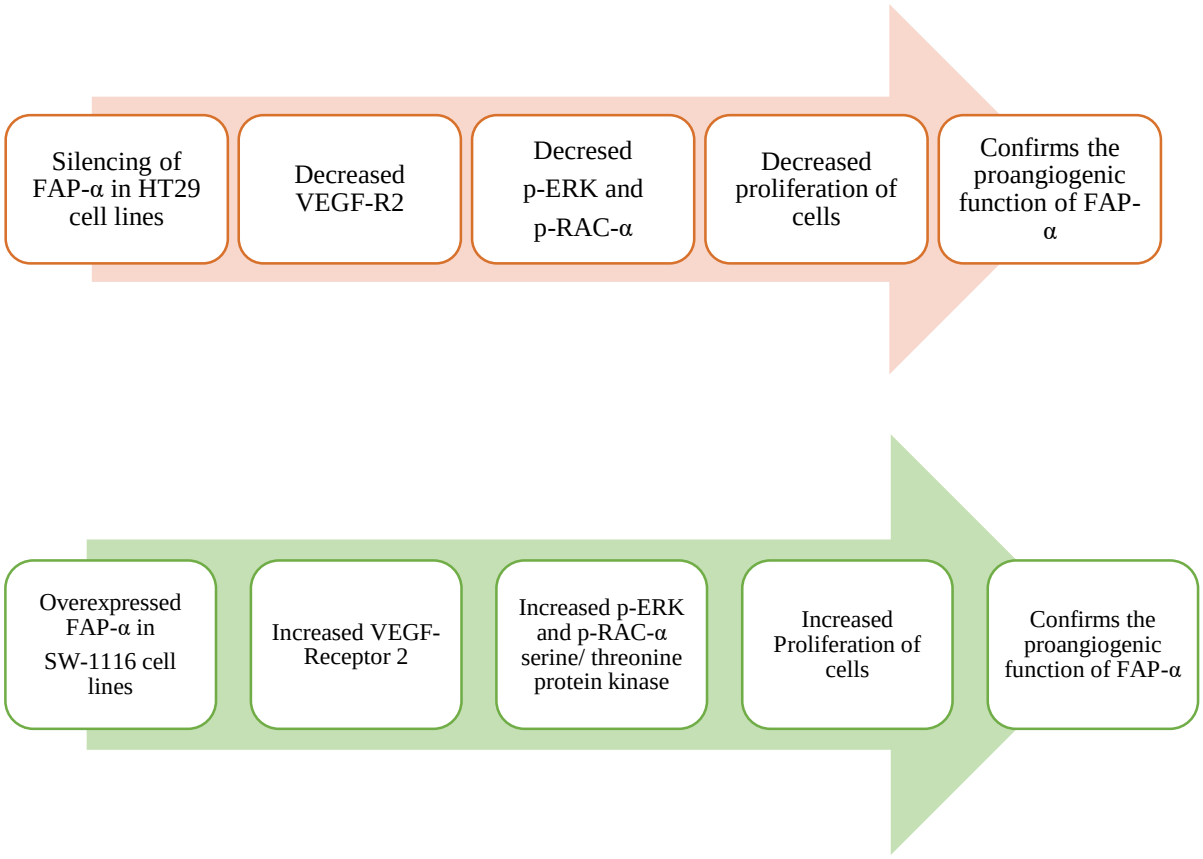

Figure 4: Flow diagram of the progression of Fibroblast Activation Protein alpha (FAP-α) in colorectal cancer (Cao et al., 2018). SW1116 and HT29 are two cell lines of colon.

VEGFR-2 stands for vascular endothelial growth factor receptor 2. In endothelial cells, it regulates multiple signaling pathways. It is a key mediator of VEGF-initiated angiogenesis. VEGFR-2 plays important role in regulating angiogenesis and core angiogenic responses which includes migration, prolifiration and tube formation abilities. VEGF is considered as the “most potent proangiogenic growth factor”, which plays a crucial role in the formation of tumor stroma when up-regulated in several tumors (Patsouras, Papaxoinis, & Kostakis, 2015).

1.5 Statins as repositioned drugs

Statins are prescribed for the treatment of hypercholesteremia by the inhibition of HMG-CoA reductase, which is known as “the rate-limiting enzyme” of the “endogenous cholesterol synthetic pathway” (Fong & To, 2019).

Statins are claimed to have anticancer property. Moreover, statins have positive influence to the clinical outcomes of patients with colorectal cancer by reducing in the metastatic properties as well as invasiveness in CRC (Ferreira, Santos, Oliva, & Andricopulo, 2015).

In many preclinical studies, statins were suggested for overcoming chemotherapy resistance. As an example, use of statins with chemotherapy has shown a synergistic anticancer effect on colorectal cancer (CRC) cell lines which were previously resistant. Statin is assumed to turn on BMP (tumor-suppressive bone morphogenetic protein) signaling pathway. Statin inhibits DNMT (DNA methyltransferase) and demethylates H1C1, BMP and TIMP3 promoter region reactivating the expression of BMP. While activated, inhibition of cancer cell stemness and promotion of cell differentiation is achieved which resensitizes colorectal cancer cells to 5-FU treatment (Fong & To, 2019).

Statin also inhibits IGF-1R (insulin-like growth factor1 receptor) signaling pathway. The principle of IGF-1R pathway is to promote proliferation and survival of cells. Overexpression of IGF-1R inhibits apoptosis in cell and causes up regulation of ATP-binding cassette transporter of proteins, contributing to resistance towards chemotherapy. This resistance can be reduced by statins through the down regulation of IGF-1R pathway as well as the inhibition of antiapoptotic ERK/Akt activation induced by IGF-1R in HT 29 cell line of CRC. With the study of all the accepted preclinical assessments, clinical trials have been evaluated to notice the effectiveness and efficacy of adding statins to the chemotherapeutic treatment protocol in treating CRC patient (Fong & To, 2019) *(Figure 5)* .

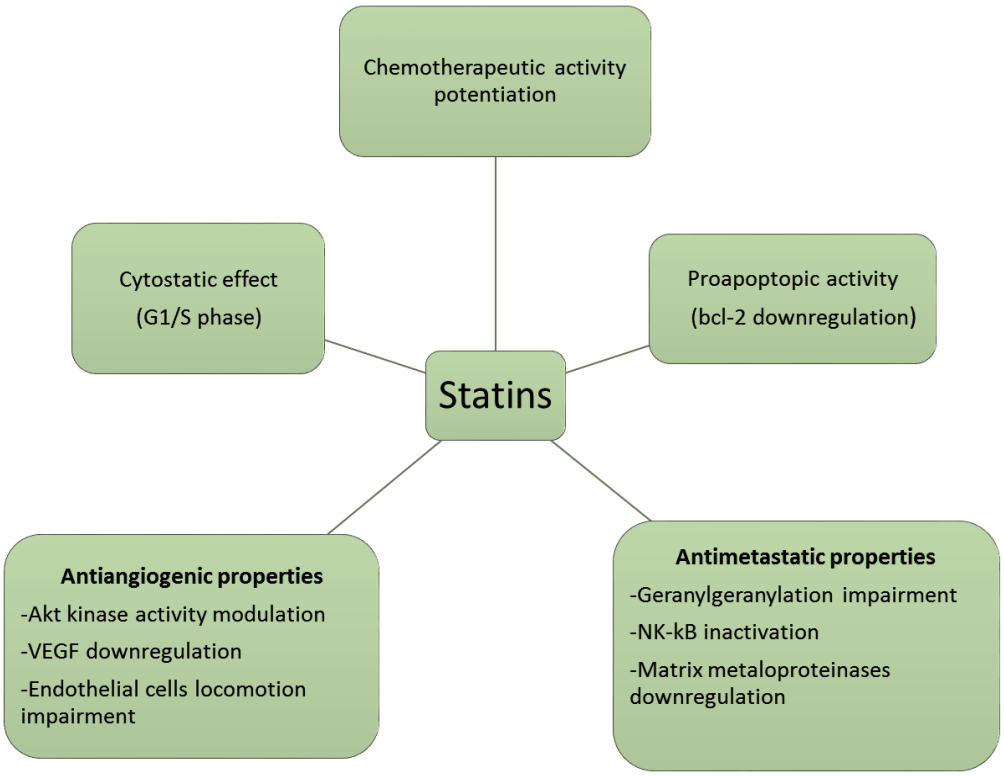

Figure 5: The schematic presentation of anticancer properties of statins (Zaleska, Mozenska & Bill, 2018).

1.6 Anti-inflammatory drugs for repositioning

Anti-inflammatory drugs are used in the treatment of inflammatory diseases which provides relief from inflammation, fever and pain. There are two classes of anti-inflammatory drugs which are used for inflammatory disease: steroidal anti-inflammatory drugs and non-steroidal anti-inflammatory drugs (NSAID). Example of some steroidal anti-inflammatory drug includes flumethasone, medryasone and prednicarbate and some non-steroidal anti-inflammatory drugs are droxicam and balsalazide.

Cancer and inflammation are interlinked. For these reason the effect of anti-inflammatory drugs in cancer is being observed and evidence of positive outcome has been found through clinical trials (Nowak-sliwinska, Scapozza, & Ruiz, 2019).

Among the steroidal anti-inflammatory drugs, corticosteroids are classified as glucocorticoids. Glucocorticoids help in suppressing inflammation. The cancer cell

promoting ability of cancer associated fibroblasts (CAFs) can be neutralized by glucocorticoids in colorectal cancer. The condition medium of cancer associated fibroblasts (CAFs) collected from glucocorticoid receptor deficient HCT8/E11 cell line of colon cancer showed enhanced motility and higher proliferation rate. Whereas, the proliferation and motility of HCT8/E11 cell line of colorectal cancer has been impaired while the collected conditioned medium was treated with glucocorticosteroids (dextramethasone). Moreover, potential activity and expression of matrix metallopeptidase-2 (MMP-2) which induces metastases in tumor, is found at a decreased amount in the controlled media treated with glucocorticoids rather than the controlled media treated with solvent. The combined results explains that glucocorticoid treatment have a great impact on the colorectal cancer cells by affecting the cancer associated fibroblasts (CAFs) (Drebert et al., 2018).

Among the non-steroidal anti-inflammatory drugs (NSAIDs), the COX-2 inhibitors are found mostly effective in case of colorectal cancer. They are effective mainly due to the reduced production of prostaglandin by the inhibition of cyclooxygenase-2 (COX-2). Prostaglandin-2 is the mostly expressed in colorectal cancer cell line. In colorectal cancer cell progression and inflammatory effect mediation, prostaglandin E2 plays a vital role. It has been demonstrated in the preclinical studies that regression and formation of tumor can be prohibited successfully by inhibiting cyclooxygenase-2-prostaglandin-E2 signaling pathway (COX-2-PGE2) (Wang & DuBois, 2013).

1.7 Natural small molecules in drug repurposing

The beneficial effect of introducing natural small molecules in the treatment protocol of patients with colorectal cancer have been discussed in numerous preclinical studies and it has also been demonstrated that, this candidates can be served as an alternative of chemotherapeutics in colorectal (Huang et al., 2019).

One of the most effective natural small molecules is theaflavin which is originated from black tea leaves and are categorized as polyphenols. Theaflavin shows great impact in the inhibition of several cancer cell lines. It has the ability to significantly stop migration, invasion and proliferation of tumor cell (Shao, Meng, & Li, 2016). Moreover, theaflavin induces apoptosis in the colorectal cancer cell line. By culturing the conditioned media of COLO 205 cell line of colorectal cancer, it has been showed that, theaflavin and derivatives of theaflavin initiates apoptosis as well as growth inhibitory effect by fragmentation of DNA in tumor cells. Thus natural molecules are offered to introduce in the treatment of colorectal cancer (Hibasami, Jin, Yoshioka, Ina, & Ohnishi, 2003).

1.8 Aim of the study

The aim of the study is to find potential inhibitors of FAP-α in colorectal cancer from approved synthetic and natural small molecules.

Chapter 2

Materials and method

2.1 Molecules used for the study

In this *in silico* study, natural and synthetic small molecules such as statins, anti-diabetic, anti-inflammatory and natural small molecules are investigated for colorectal cancer therapy by using molecular docking and many other tools of computational biology.

2.2 Methodology

The methodology consists of three different parts. The first part consists of the thorough study of journal articles and literatures containing enormous information about colorectal cancer, its pathogenesis, prognosis and treatment. The second part contained the molecular docking of ligand and protein and obtaining the binding affinities. The last part contains the screening of protein drug orientation according to the best outcome. To complete the study, 3-dimensional structure of the protein (macromolecule) and ligand were needed.

2.3 Software and online tools for docking and visualization

To complete the *in silico* study, several software were used to screen and visualize the model. Moreover, to obtain the structure of protein, structure of ligand and results, multiple databases were used. The quality and acceptability has increased by using these software and databases *(Table 1).* This software and databases helped to obtain the best possible result in this study.

Table 1: List of software and online tools used for the study.

Sl no	Software and online tools	Function	Version	References
1	PyMOL	Molecular visualization software	1.7.4	(Yuan, Chan, & Hu, 2017)
2	Open Babel GUI	Chemical object software	2.3.1	(O'boyle et al., 2011)
3	Autodock Tools	Molecular Docking Software	1.5.6	(Trott & Olson, 2010)
4	Autodock Vina	Molecular modelling simulation software	1.5.6	(Trott & Olson, 2010)
5	Discovery Studio	Molecular Visualizer	16.1.0.15350	(Client, Studio, Discovery, & Client, 2020)
6	admetSAR	Pharmacokinetics and toxicity checking	1	(Cheng et al., 2012)

PyMOL (version 1.7.4) is open-source software for molecular visualization. It creates high quality images of macromolecules. Moreover, macromolecules are projected in different representations comprising ribbons, dots, cartoons, lines, surfaces, sticks and spheres. At the present time, one of the most popular macromolecular visualization tool is PyMOL (Yuan et al., 2017).

OpenBabel GUI (version 2.3.1) is developed by the Open Babel project. It is an open chemical toolbox having all the features. It is also capable of expressing various representations of chemical data. It allows to convert, search, store data from molecular modeling, analyze, solid-state materials, biochemistry, chemistry, or related areas (Boyle et al., 2011).

AutoDock Vina (version 1.5.6) is a program used for virtual screening and molecular docking (Trott & Olson, 2010).

Non bond interaction of protein and small molecules are investigated using Discovery Studio (version 16.1.0.15350). The amino acid sequence of standard drug and small molecules are matched after visualization is completed by Discovery Studio.

admetSAR (version 1) is a database showing ADMET structure activity relationship. It is associated with absorption, distribution, metabolism, excretion and toxicity property of molecules. admetSAR is an open source database, providing searchable text and structure and curating, collecting and managing available ADMET related data from the already published literature. From multiple literature works, admetSAR collects proteins, ADMET-associated properties, organisms or species (Cheng et al., 2012).

2.4 Molecular docking and visualization

In molecular docking, several steps are included such as screening, validation, visualization etc. the first step is to choose the protein of interest for the study. For choosing the desired class of protein, literatures were meticulously studied and gene responsible for causing colorectal cancer is chosen. The desired gene is FAP and the protein is FAP alpha (PDB ID: 1Z68). The protein is chosen by evaluating the mutation, resolution, organism and expression system and publication year using RCSB PDB. RCSB PDB is a protein data bank where numerous 3D structure of various proteins are available (Rose et al., 2017). The RCSB PDB

presents both the official PDB Validation Report and the validation sliders are available on the structural summary page (Rose et al., 2017). For this reason, protein 3D structure obtained directly from RCSB PDB need not be validated. Some important parameters are measured while choosing and finalizing the protein. Those criteria's are mentioned below:

1. The protein should not contain any mutation.

2. The organism must have to be from *Homo sapiens.*

3. The resolution and the publication year are important parameters. In case of selecting proteins, the recent publications with better resolution should be prioritized. Proteins having resolution value of about 1Å are known as high-resolution structures. They are highly ordered and thus it is easy to see every atom in the "electron density map". Structures having 3Å or higher are indicated as lower resolution structures. This protein structures shows basic diagram of the chain only. Most of the proteins having crystallographic structures fall between these two extremes (Berman, 2000).

Maintaining all the criteria above, the 3D structure of FAP alpha downloaded from the RCSB PDB is 1z68 which is "Crystal Structure of Human Fibroblast Activation Protein alpha".

2.4.1 Macromolecule preparation

The PDB structure of the protein was retrieved from RCSB PDB and opened using PyMOL. For curation, all the ligands and oxygen molecules were removed. Because of having similar amino acid sequences in chain B as chain A, chain B was deleted (Kabir, Mustafa, Kawsar, Siam, & Kabir, 2018).

Molecular docking was used to obtain the binding affinity values between ligand and active site or binding site of the protein. So the protein crystallographic structure with two binding site or dimer structure was minimized to a single chain with one binding site. Before docking, the crystallographic structure was prepared and chain B was deleted.

2.4.2 Ligand preparation

The structures of different classes of drugs include statins, anti-diabetic, anti-inflammatory, anti-rheumatic and anti-hypertensive as well as natural small molecules was obtained from PubChem (chemical molecule database). Then the SDF form of the small molecules were downloaded and then then were converted to PDB form by using OpenBabel GUI (O'boyle et al., 2011).

2.4.3 Steps in molecular docking

After completing the above stated steps, the curated FAP-α structure was docked with different classes of drugs. With the help of a flowchart, steps that are performed in molecular docking process are illustrated *(Figure 6).* Some classes of drugs showed better binding affinities than other classes of drugs. In this study, statins, anti-inflammatory and natural small molecules showed better binding affinities compared to anti-rheumatic and anti-hypertensive drugs.

AutoDock Vina was used for docking. For preparing the protein, firstly the protein PDB structure was opened in AutoDock as macromolecule. Then the polarity of the protein was changed by the addition of hydrogen to it. Then the grid box was selected and x, y and z dimensions were changed. The grid box was resized till it covered the whole curated protein structure and not a single amino acid chain is left outside the box. After completing the grid box, the protein structure was saved as "Ligand.pdbqt" as a pdbqt file. The values of the grid box were saved as text in a text box and then the file was named Conf.txt.

To prepare the ligand, the selected structure was opened as ligand. The number of torsion was chosen according to docking preference. Rigid docking was carried out. As for rigid docking, by clicking "Make all active bonds non rotatable" all the torsions were made inactive. For choosing this option, the number of rotable bonds was set to 0. Then the file was saved as

“Ligand.pdbqt”. After preparing the protein, ligand and the conf file, three of the files were taken to the vina folder in Local drive C. After preparing the vina folder in the Local disk C, the windows button and R was pressed. Then a folder opened named “Run”. In the blank box “cmd” was written and clicked. Windows opened the coding file and coding for binding affinity was done. After completion, CMD provided with results. The outfile.pdbqt and the log file is saved for further study.

Binding affinity value is an indicator of how well the drug or ligand bind to the protein. The more negative the value, the better the binding affinity (Kabir et al., 2018). The binding affinities of different molecules were obtained and the highest affinities were chosen for further steps.

The standard drug and out.pdbqt file of the selected drugs were superimposed by using PyMOL. The superimposed drugs were taken for studying further binding interaction using Discovery Studio and the non-superimposed ones are discarded. Thus, the drugs were narrowed down in this study.

The superimposed drugs were taken and then they were screened by using BIOVIA Discovery Studio. By this Discovery Studio the 3D structural visualization was achieved as well as the non- bond bonding interaction was observed by observing the distance between the ligands and protein, bond type, category of bond and amino acid sequence was also checked. The drugs having similar properties as the standard drug was selected for final study.

After completing the Discovery Studio study, the ligands were checked by admetSAR using the SMILES form of the SDF file of the small molecules. Then BBB, Human Ether-a-go-go, CYP450 and AMES toxicity were compared to the standard drug.

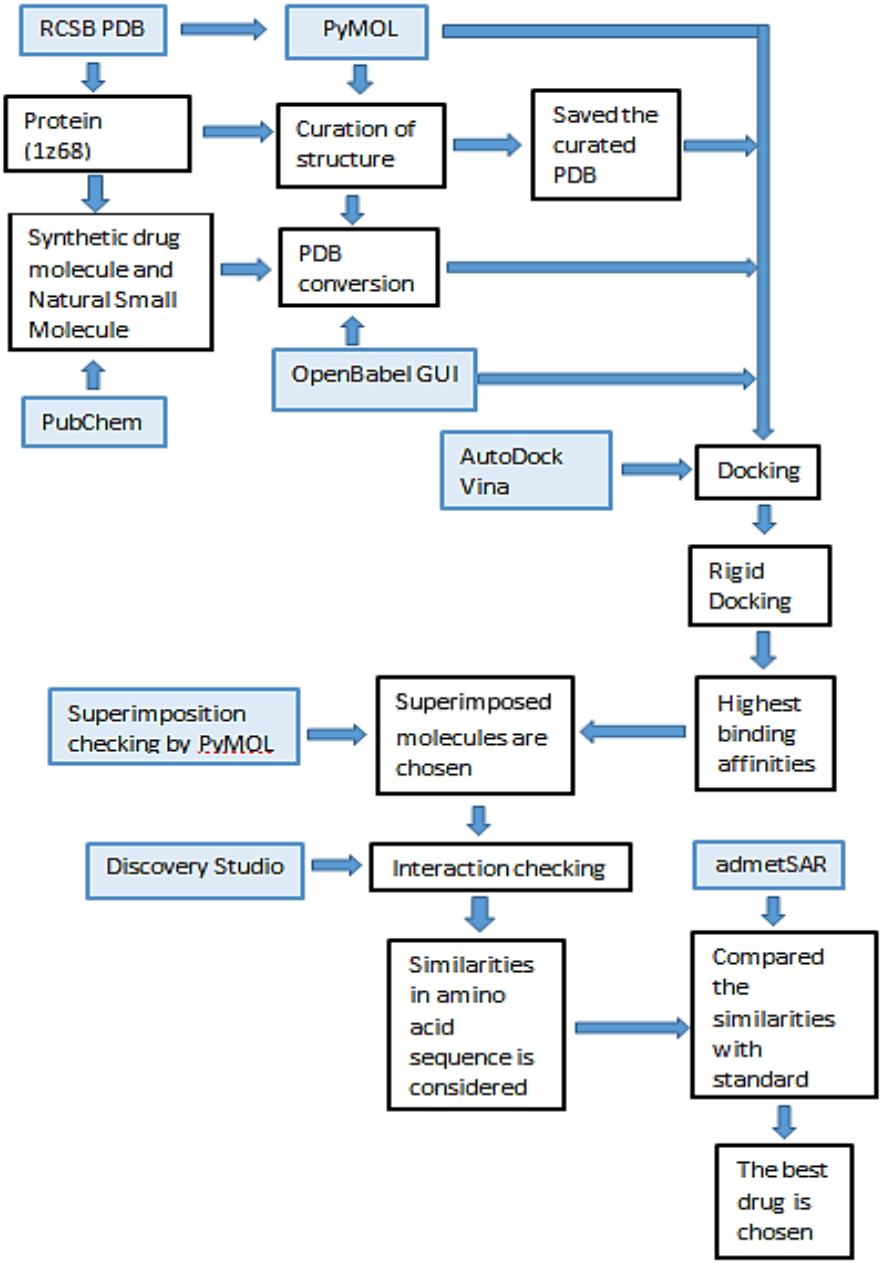

Figure 6: Flowchart showing molecular docking and screening steps.

Chapter 3

Result

3.1 Protein structure

Table 2: Basic information about Fibroblast Activation Protein alpha (FAP-α).

Protein	Mutation	Chain	Organism	Resolution	Publication year
1z68	0	A,B (Homodimer)	*Homo sapiens*	2.6 Å	2005

Figure 7: Crystal structure of Human Fibroblast Activation protein alpha (Jiang et al., 2016).

3.2 *In silico* molecular docking

The protein was docked with approximately hundreds of drugs from anti-inflammatory drugs, anti-diabetic drugs, statins and anti-hypertensive and natural small molecules. Among them 4 classes of drugs were finally chosen based on their binding affinity values with the protein.

The four classes of drugs are statins, anti-inflammatory drugs, anti-diabetic drugs and natural small molecules.

As standard drug, trifluridine has been chosen which is an FDA approved drug for treating colorectal cancer (Kish & Uppal, 2016). It is an antimetabolites and phosphorylase inhibitor which is prepared as an oral supplement. In the anticancer therapy, trifluridine acts as a thymidine-based nucleoside metabolic inhibitor that gets incorporated into DNA of cancer cells following cell uptake to deviate DNA function during cell replication. Thus, it was chosen as a standard drug for the study.

3.3 Docking results of small molecules

Table 3: Docking result with FAP-α using AutoDock Vina.

Class of dugs	**Drug**	**Binding affinity with FAP-α (kcal/mol)**
Anticancer Drug	• Trifluridine (Standard Drug)	-7.5
Statins	• Atorvastatin • Rosuvastatin • Pitavastatin • Fluvastatin	-10.6 -9.4 -9.4 -9.5

Anti-diabetic drug	• APD668 • Larmustine • Toresamide	-8.8 -5.5 -7.9
Anti-inflammatory drugs	• Lornoxicam • Olsalazine • Prednicarbate • Amcinonide	-7.4 -8.1 -10.4 -10.1
Natural Small Molecules	• Theflavin	-12.1

The binding affinity's fall under the range of -5.5 to -12.1.the binding affinity of standard drug (trifluridine) is -7.5 kcal/mol.

3.4 Superimposition of different classes of drugs with established anti-cancer drug used in colorectal cancer

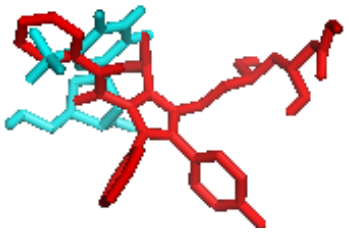

Figure 8: Superimposition of atorvastatin with trifluridine.

Cyan= trifluridine, Red=atorvastatin

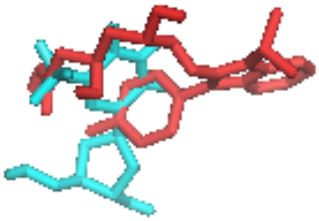

Figure 9: Superimposition of fluvastatin with trifluridine.

Cyan= trifluridine, Red=fluvastatin

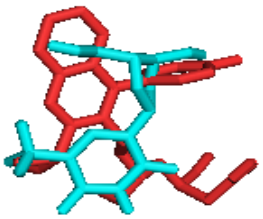

Figure 10: Superimposition of pitavastatin with trifluridine.

Cyan= trifluridine, Red=pitavastatin

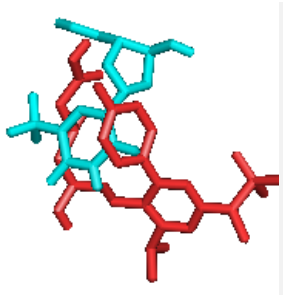

Figure 11: Superimposition of rosuvastatin with trifluridine.

Cyan= trifluridine, Red=rosuvastatin

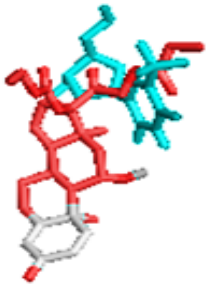

Figure 12: Superimposition of prednicarbate and trifluridine.

Cyan= trifluridine, Red= prednicarbate

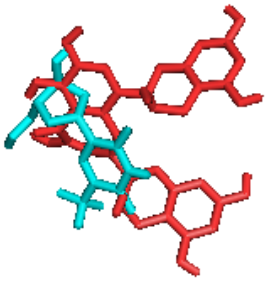

Figure 13: Superimposition of theaflavin and trifluridine.

Cyan= trifluridine, Red= theaflavin

3.5 Visualization using Discovery Studio

The interaction of standard anti-cancer drug used for colorectal cancer (trifluridine) and the fibroblast activation protein alpha (FAP-α) (curated 1z68 pdb) was observed by BIOVIA Discovery Studio Visualizer.

After that, the interactions of other classes of drugs were seen as they had shown higher binding affinity values and superimposition.

3.5.1 Non bond interaction between FAP-α and trifluridine

Figure 14 and *Figure 15* shows the amino acids of protein, FAP-α with which the ligand, trifluridine was bound. These amino acids were LEU105 (aa leucine), TYR113 (aa tyrosine),

SER156 (aa serine), ILE62 (aa isoleucine), PRO107 (aa proline), TRP 155 (aa tryptophan), TRP61 (aa tryptophan) which was used in validation.

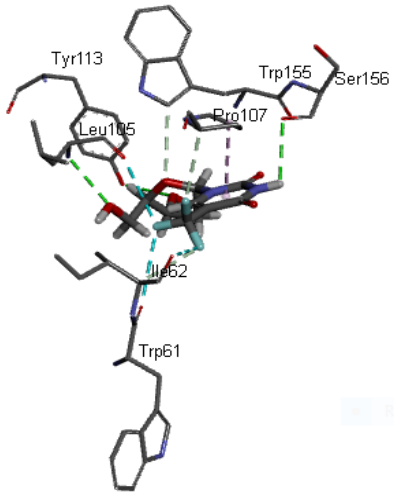

Figure 14: Non bond interaction of trifluridine with FAP-α (1z68) (3D)

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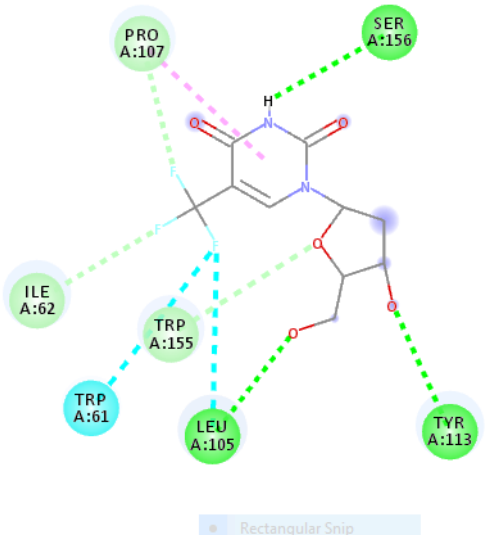

Figure 15: Non bond interaction of trifluridine with FAP-α (1z68) (2D.)

Table 4: Non bond interactions involved in the binding of trifluridine and FAP-α.

Amino acid, atom and ligand interaction	Type of bond	Distance (amino acid to ligand)	Category of bond
A:LEU105:N - :UNL1:O5*	Conventional Hydrogen Bond	2.91544	Hydrogen Bond
A:TYR113:OH - :UNL1:O3*	Conventional Hydrogen Bond	2.88459	Hydrogen Bond
:UNL1:H - A:SER156:O	Conventional Hydrogen Bond	2.97815	Hydrogen Bond
A:ILE62:CA - :UNL1:F	Carbon Hydrogen Bond;Halogen (Fluorine)	3.37688	Hydrogen Bond;Halogen
A:PRO107:CA - :UNL1:F	Carbon Hydrogen Bond;Halogen (Fluorine)	3.31665	Hydrogen Bond;Halogen
A:TRP155:CD1 - :UNL1:O4*	Carbon Hydrogen Bond	3.58828	Hydrogen Bond
A:TRP61:O - :UNL1:F	Halogen (Fluorine)	3.67461	Halogen
A:ILE62:O - :UNL1:F	Halogen (Fluorine)	3.62369	Halogen
A:LEU105:O - :UNL1:F	Halogen (Fluorine)	3.68701	Halogen
:UNL1 - A:PRO107	Pi-Alkyl	4.22684	Hydrophobic

Table 4 was obtained by using Discovery Studio which gave information about non-bond interactions involved in the binding of trifluridine to protein, FAP-α. The distance of the hydrogen bond ranged from 2.8- 3.5 Å (Table 4). The complex formed six hydrogen and three halogen bonds and one hydrophobic bond.

3.5.2 Non bond interaction between statins with FAP-α

3.5.2.1 Non bond interaction between pitavastatin and FAP-α

Figure 16 and *Figure 17* shows the amino acids of protein, FAP-α with which the ligand, pitavastatin was bound. These amino acids were TYR152 (aa tyrosine), PRO107 (aa proline) and CYS154 (cysteine 154). The complex also formed conventional hydrogen bond, alkyl bond and pi-alkyl bond.

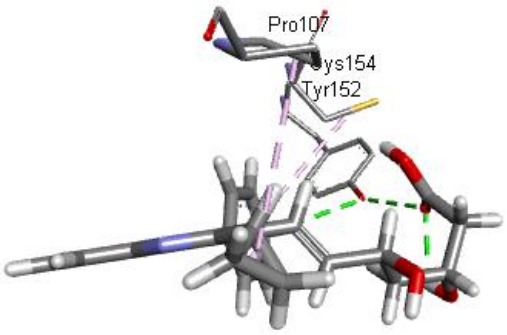

Figure 16: Non bond interaction of pitavastatin with FAP-α (1z68) (3D).

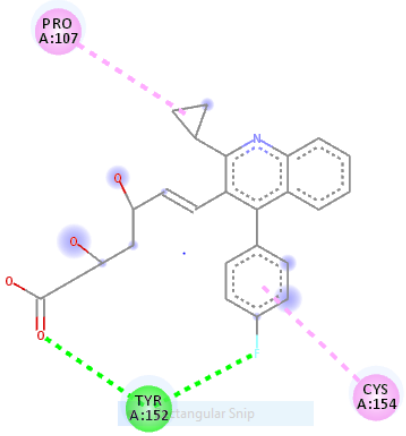

Figure 17: Non bond interaction of pitavastatin with FAP-α (1z68) (2D).

Table 5: Non bond interactions involved in the binding of pitavastatin and FAP-α.

Amino acid, atom and ligand interaction	Type of bond	Distance	Category of bond
A:PRO107 - :UNL1	Alkyl	4.79049	Hydrophobic

Table 5 was obtained by using Discovery Studio which gave information about non-bond interactions involved in the binding of pitavastatin to protein, FAP-α. The information such as bond length (4.7 Å), categories of bond (Hydrophobic) and bond types (Alkyl) of drug molecules with protein were present in the table.

3.5.2.2 Non bond interaction between rosuvastatin and FAP-α

Figure 18 and *Figure 19* shows eight complexes which are formed between rosuvastatin-FAP-α showing six amino acid containing TRP61 (aa tryptophane), TRP155 (aa tryptophan), LEU105 (aa leucine), SER156 (aa serine), PRO157 (aa proline), PRO 107 (aa proline).

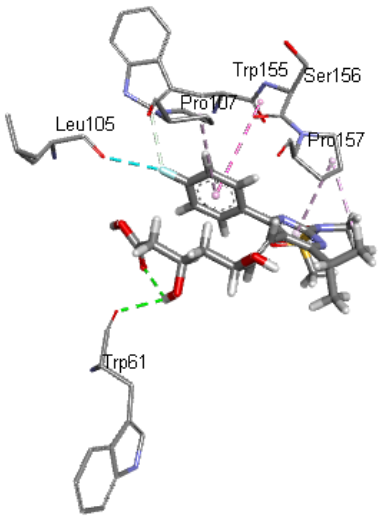

Figure 18: Non bond interaction of rosuvastatin with FAP-α (1z68) (3D).

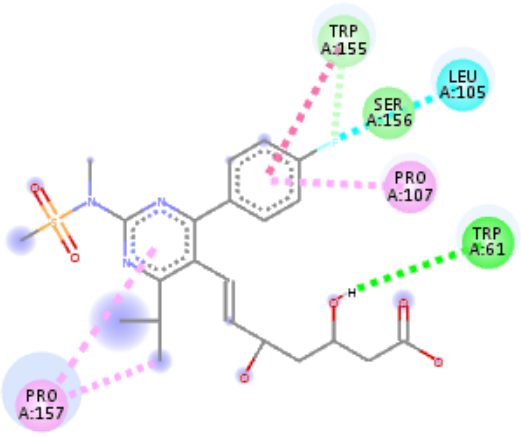

Figure 19: Non bond interaction of rosuvastatin with FAP-α (1z68) (2D).

Table 6: Non bond interactions involved in the binding of rosuvastatin and FAP-α.

Amino acid, atom and ligand interaction	Type of bond	Distance	Category of bond
:UNL1:H - A:TRP61:O	Conventional Hydrogen Bond	2.53763	Hydrogen Bond
A:TRP155:CD1 - :UNL1:F	Carbon Hydrogen Bond	3.10098	Hydrogen Bond
A:LEU105:O - :UNL1:F	Halogen (Fluorine)	3.21201	Halogen
A:TRP155:C,O;SER156:N - :UNL1	Amide-Pi Stacked	4.9352	Hydrophobic

In table 6, information about non-bond interactions involved in the binding of rosuvastatin to protein, FAP-α is shown. The information includes amino acid interactions, bond length, categories and bond types of drug molecules with protein were present in the table. The complex formed one hydrophobic, two hydrogen and one halogen bond which are similar to the standard drug trifluridine.

3.5.2.3 Non bond interaction between atorvastatin and FAP-α

Figure 20 and *Figure 21* shows nine complexes of atorvastatin and FAP-α showing eight amino acid containing TYR210 (aa tyrosine), TYR152 (aa tyrosine), CYS154 (aa cysteine), TRP213 (aa tryptophan), TRP298 (aa tryptophan), TRP155 (aa tryptophan), ILE162 (aa isoleucine), PRO107 (aa proline).

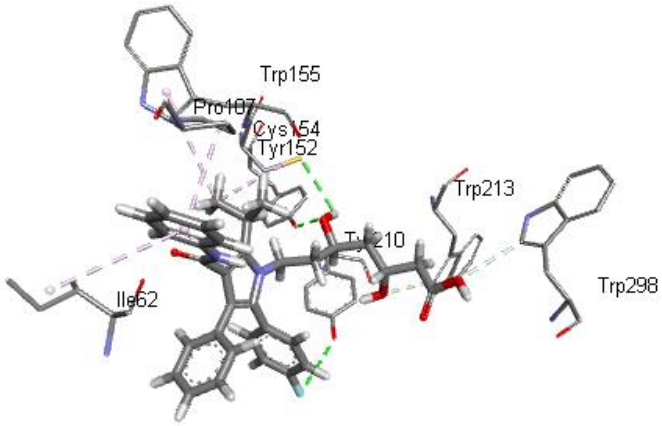

Figure 20: Non bond interaction of atorvastatin with FAP-α (1z68) (3D.)

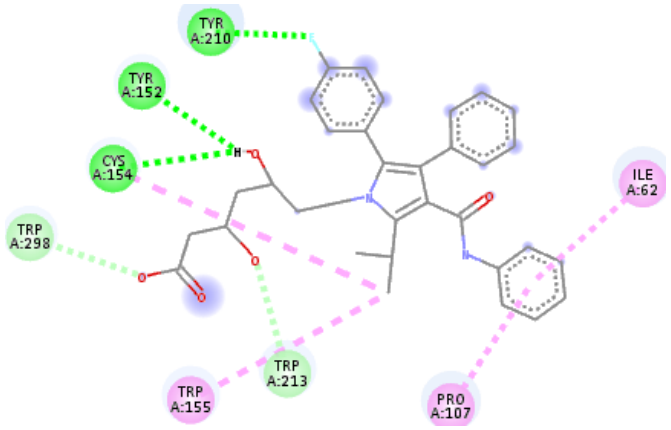

Figure 21: Non bond interaction of atorvastatin with FAP-α (1z68) (2D).

Table 7: Non bond interactions involved in the binding of atorvastatin and FAP-α.

Amino acid, atom and ligand interaction	Type of bond	Distance	Category of bond
A:TRP155 - :UNL1:C	Pi-Alkyl	5.00378	Hydrophobic
:UNL1 - A:ILE62	Pi-Alkyl	5.38046	Hydrophobic
:UNL1 - A:PRO107	Pi-Alkyl	4.0875	Hydrophobic

In table 7, information about non-bond interactions involved in the binding of Atorvastatin to protein, FAP-α is shown. Atorvastatin showed three similar non bonded interactions as standard drug trifluridine. The complexes formed three hydrophobic bond and the distance ranger between 4.08-5.3Å.

3.5.2.4 Non bond interaction between fluvastatin and FAP-α

Figure 22 and *Figure 23* shows eight complexes of fluvastatin and FAP-α containing five amino acid; TRP61 (aa tryptophan), LEU105 (aa leucine), PRO157 (aa proline), TRP155 (aa tryptophan), SER156 (aa serine).

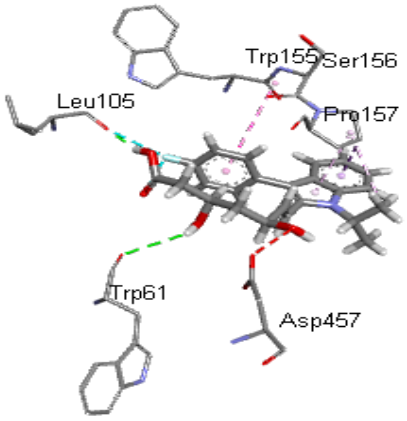

Figure 22: Non bond interaction of fluvastatin with FAP-α (1z68) (3D).

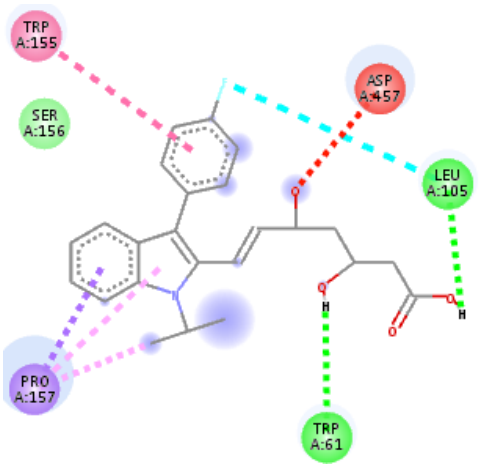

Figure 23: Non bond interaction of fluvastatin with FAP-α (1z68) (2D).

Table 8: Non bond interactions involved in the binding of fluvastatin and FAP-α.

Amino acid, atom and ligand interaction	Type of bond	Distance	Category of bond
:UNL1:H - A:TRP61:O	Conventional Hydrogen Bond	3.02251	Hydrogen Bond
:UNL1:H - A:LEU105:O	Conventional Hydrogen Bond	1.90861	Hydrogen Bond

A:LEU105:O - :UNL1:F	Halogen (Fluorine)	3.3901	Halogen
A:TRP155:C,O;SER156:N - :UNL1	Amide-Pi Stacked	5.53142	Hydrophobic

In this table information about non-bond interactions involved in the binding of fluvastatin to protein, FAP-α is shown. Fluvastatin formed four similar non bonded interactions as the standard drug trifluridine with protein FAP-α. The complexes also formed two hydrogen, one halogen and one hydrophobic bond. The hydrogen bond distance ranged between 1.9-3.0Å.

3.5.3 Non bond interaction of FAP-α and anti-Inflammatory drug

3.5.3.1 Non bond interaction between prednicarbate and FAP-α

Figure 24 and 25 shows complexes of prednicarbate with FAP alpha. Those are, TRP155 (aa tryptophan), TRP213 (aa tryptophan), TRP61 (aa tryptophan), CYS154 (aa cystine) ILE62 (aa isoleucin), LEU105 (aa leucin), PRO107 (aa prolin), TRP61 (aa tryptophan), TYR 210 (aa tyrosine) and TRP 214 (aa tryptophan). Six similar complex with the standard drug formed.

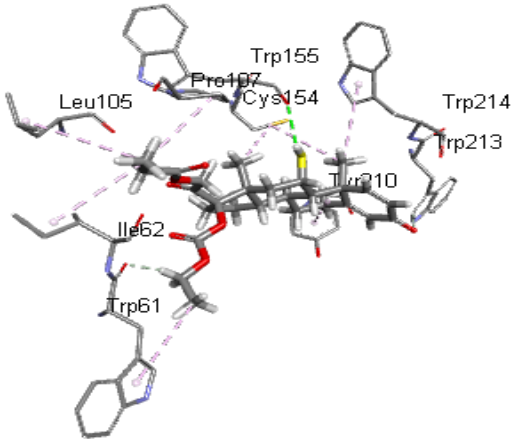

Figure 24: Non bond interaction of prednicarbate with FAP-α (1z68) (3D).

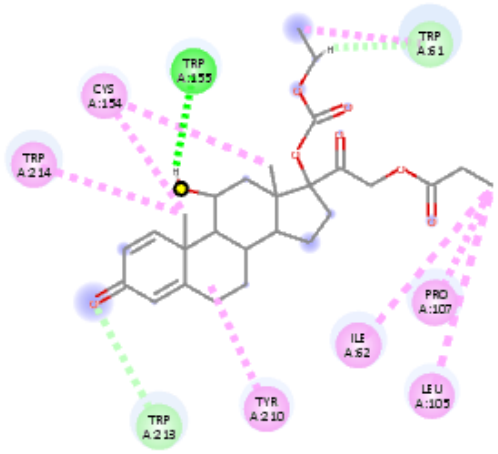

Figure 25: Non bond interaction of prednicarbate with FAP-α (1z68) (2D).

Table 9: Non bond interactions involved in the binding of prednicarbate and FAP-α.

Amino acid, atom and ligand interaction	Type of bond	Distance	Category of bond
:UNL1:H - A:TRP155:O	Conventional Hydrogen Bond	2.85707	Hydrogen Bond
:UNL1:H27 - A:TRP61:O	Carbon Hydrogen Bond	2.44281	Hydrogen Bond
:UNL1:C - A:ILE62	Alkyl	4.40102	Hydrophobic
:UNL1:C - A:LEU105	Alkyl	5.45554	Hydrophobic
:UNL1:C - A:PRO107	Alkyl	4.33182	Hydrophobic

In table nine, information about non-bond interactions involved in the binding of prednicarbate to protein, FAP-α is shown. Prednicarbate shows six similar complex as

standard drug trifluridine with FAP-α. The complexes also formed two hydrogen and four hydrophobic bonds. The distance between the hydrogen bonds ranges between 2.44-2.8Å and hydrophobic bond is 4.3-5.4Å.

3.5.4 Non bond interaction between FAP-α and natural small molecule

3.5.4.1 Non bond interaction between theaflavin and FAP-α

Figure 26 and *Figure 27* shows seven complexes of theaflavin with FAP alpha is formed. Those are TRP155 (aa tryptophan), TYR113 (aa tyrosine), TRP214 (aa tryptophan), TRP213 (aa tryptophan) and ASP457 (aa aspartic acid). Three similar complexes as the standard drug formed.

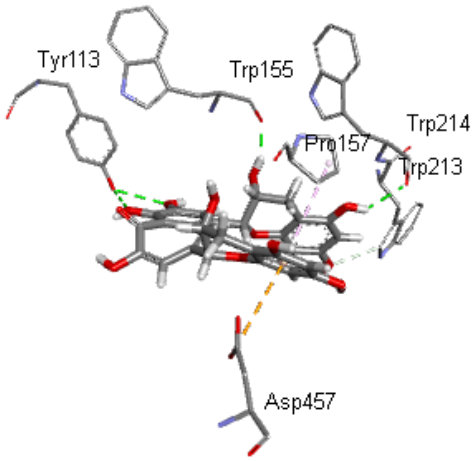

Figure 26: Non bond interaction of theaflavin with FAP-α (1z68) (3D).

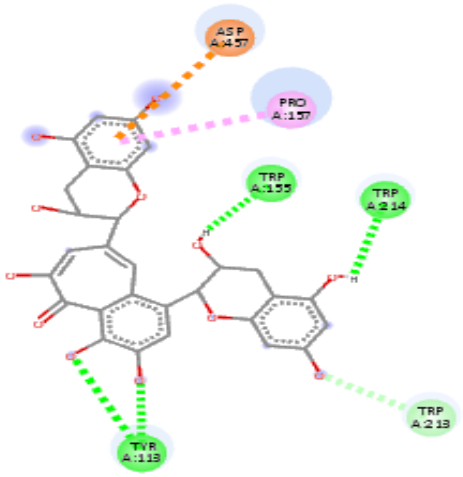

Figure 27: Non bond interaction of theaflavin with FAP-α (1z68) (2D).

Table 10: Non bond interactions involved in the binding of theaflavin and FAP-α.

Amino acid, atom and ligand Interaction	Type of bond	Category of bond	Distance
A:TYR113:OH - :UNL1:O	Conventional Hydrogen Bond	Hydrogen Bond	3.1745
A:TYR113:OH - :UNL1:O	Conventional Hydrogen Bond	Hydrogen Bond	3.00366
:UNL1:H - A:TRP155:O	Conventional Hydrogen Bond	Hydrogen Bond	1.82905

In table ten, information about non-bond interactions involved in the binding of theaflavin to protein, FAP-α is shown. Theaflavin showed three similar complex as the standard drug trifluridine. The complexes formed three hydrogen bonds and they ranges from 1.8-3.1Å.

Tables are containing hydrogen, hydrophobic and halogen bonds in a significant number. In case of binding drugs to the protein, these three types of bonds are very crucial (Mendez,

Henriquez, Sirimulla, & Narayan, 2017). So as the hydrogen, hydrophobic and halogen bonds are present in the complexes of fluvastatin, rosuvastatin, atorvastatin, prednicarbate and theaflavin; they are considered for admetSAR property results.

3.6 admetSAR property

Lastly, admetSAR is checked to evaluate the pharmacokinetic properties of the drugs.

Table 11: ADMET property of standard drug trifluridine.

MODEL	RESULT	PROBABILITY
Blood-Brain Barrier	BBB+	0.8103
Human Intestinal Absorption	HIA+	0.7380
Caco-2 Permeability	Caco2+-	0.8154
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor Non-inhibition	0.9814 0.7308
AMES Toxicity	AMES toxic	0.7941

Table 11 shows that trifluridine crosses the blood brain barrier as it is BBB+. Moreover, it is absorbed through human intestine (HIA+). It is also a weak inhibitor of Human Ether-a-go-go Related Gene Inhibition. Also trifluridine is AMES toxic drug.

Table 12: ADMET property of rosuvastatin.

MODEL	RESULT	PROBABILITY
Blood-Brain Barrier	BBB-	0.6815
Human Intestinal Absorption	HIA+	0.9791
Caco-2 Permeability	Caco2-	0.5818
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	0.9856
	Non-inhibitor	0.8117
AMES Toxicity	Non AMES toxic	0.6620

Table 12 shows ADMET property of rosuvastatin which have similar parameters with trifluridine regarding HIA, Human ether-a-go-go-related gene inhibition. Rosuvastatin does not cross blood brain barrier (BBB-). It is human intestinal absorption positive (HIA+) also a weak inhibitor of Human Ether-a-go-go Related Gene Inhibitor. Rosuvastatin is not AMES toxic.

Table 13: ADMET property of fluvastatin.

MODEL	RESULT	PROBABILITY
Blood-Brain Barrier	BBB+	0.9382
Human Intestinal Absorption	HIA+	0.9943
Caco-2 Permeability	Caco2-	0.5053
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	0.9899
	Non-inhibitor	0.8480
AMES Toxicity	Non AMES toxic	0.9132

Table 13 shows the ADMET property of fluvastatin which have similar parameters with trifluridine including Blood brain barrier, HIA, Human ether-a-go-go-Related Gene Inhibition. Fluvastatin is absorbed through human intestine (HIA+). It is also a weak inhibitor of Human Ether-a-go-go-Related Gene Inhibitor. Fluvastatin is not AMES toxic like trifluridine.

Table 14: ADMET property of atorvastatin.

MODEL	RESULT	PROBABILITY
Blood-Brain Barrier	BBB-	0.7825
Human Intestinal Absorption	HIA+	0.8947
Caco-2 Permeability	Caco2-	0.8956

Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	0.9904
	Non-inhibitor	0.5101
AMES Toxicity	Non AMES toxic	0.9133

Table 14 shows the ADMET property of atorvastatin. It has negative blood brain barrier (BBB-) so it does not cross through blood brain barrier. Moreover, it is absorbed through human intestine (HIA+). Atorvastatin is a weak inhibitor of Human Ether-a-go-go Related Gene Inhibition. This drug is not AMES toxic.

Table 15: ADMET property of prednicarbate.

MODEL	RESULT	PROBABILITY
Blood-Brain Barrier	BBB+	0.9840
Human Intestinal Absorption	HIA+	0.9958
Caco-2 Permeability	Caco2-	0.5710
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	0.9899
	Non-inhibitor	0.7647
AMES Toxicity	Non AMES toxic	0.7817

Table 15 shows the ADMET property of prednicarbate. It has positive blood brain barrier (BBB+) so it crosses through blood brain barrier. Moreover, it is absorbed through human intestine (HIA+). Prednicarbate is a weak inhibitor of Human Ether-a-go-go Related Gene Inhibition. This drug is not AMES toxic.

Table 16: ADMET property of theaflavin.

MODEL	RESULT	PROBABILITY
Blood-Brain Barrier	BBB-	0.6153
Human Intestinal Absorption	HIA+	0.9661
Caco-2 Permeability	Caco2-	0.8970

Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	0.9899
	Non-inhibitor	0.7647
AMES Toxicity	Non AMES toxic	0.7817

Table 16 shows the ADMET property of theaflavin. It is blood brain barrier negative (BBB-) so it does not cross through blood brain barrier. Moreover, it is absorbed through human intestine (HIA+). Theaflavin is a weak inhibitor of Human Ether-a-go-go Related Gene Inhibition. This drug is not AMES toxic.

Significant difference is seen between standard drug trifluridine and rosuvastatin, atorvastatin, theaflavin and that is rosuvastatin, atorvastatin and theaflavin are BBB-, implicating that rosuvastatin, atorvastatin and theaflavin cannot cross the Blood Brain Barrier. In this case, BBB- can be an effective criterion over the established drug as the repurposed drug will not cause any neurotoxicity.

On the other hand, fluvastatin and prednicarbate crosses the blood brain barrier and similar to standard drug trifluridine.

Moreover, rosuvastatin, atorvastatin, fluvastatin, prednicarbate and theaflavin are AMES toxicity negative whereas the standard drug has AMES toxicity which can cause mutagenesis in future. Thus it is better to not be AMES positive.

Chapter 4

4.1 Discussion

The protein FAP-α (PDB ID-1z68) was obtained from RCSB PDB. From the structure, ligands and water molecules were deducted using the software PyMOL and the curated structure was saved for docking. Molecular docking was performed after preparing the protein and ligand pdbqt file. Rigid docking was performed as it showed higher result than flexible docking. Docking was completed by using AutoDock Vina. Classes of drugs were randomly chosen for docking. Among them, four classes of drugs showed better superimposition and binding affinity. They were anti-diabetic, anti-inflammatory, statins and natural small molecule. Statins showed better binding affinities as well as superimposition rather than other classes of drugs. Among all of the drugs, fluvastatin and prednicarbate matched with the standard drug trifluridine by amino acid chain sequence to admetSAR property. Other than fluvastatin and prednicarbate; rosuvastatin, atorvastatin and natural small molecule theaflavin showed high binding affinity, superimposition and non-bonded interaction in Discovery Studio Visualizer as well as admetSAR property.

Binding affinity values of fluvastatin was -9.5kcal/mol and prednicarbate was -10.4 kcal/mol which was greater than the binding affinity value of the standard drug trifluridine -7.5kcal/mol. Moreover, binding affinity values of atorvastatin, rosuvastatin and theaflavin were consecutively -10.6kcal/mol, -9.4kcal/mol and -12.1kcal/mol which were all greater than the standard trifluridine.

The natural small molecule theaflavin showed highest binding affinity among all the molecules (12.1 kcal/mol).

The 3 dimensional model of the small molecules were studied by using Discovery Studio for the visualization of amino acid and non-bonded ligand interaction, the distance, the category and the type of bond. The common amino acid and ligand interaction found in fluvastatin and trifluridine are TRP61 (aa tryptophan), LEU105(aa leucine), TRP155(aa tryptophan), SER156(aa serine). Six similar complexes between prednicarbate and standard drug were formed. Those are, TRP155(aa tryptophan), TRP61(aa tryptophan), ILE62(aa isoleucin), LEU105(aa leucin), PRO107(aa prolin), TRP61(aa tryptophan). The complexes also formed hydrogen, halogen and hydrophobic bonds. The distance of amino acid and ligand was seen to be in the range of 1.9-5.4.

Afterwards, admetSAR property was checked for all the molecules and drugs having the most similar characteristic with the standard drug were kept and other drugs were screened out. Thus fluvastatin and prednicarbate were proposed in the inhibition of FAP-α.

Chapter 5

5.1 Conclusion

Through this *in silico* study, the efficiency of both synthetic and natural small molecules in the inhibition of Fibroblast activation protein alpha (FAP-α) has been evaluated. Among them fluvastatin from statins and prednicarbate from anti-inflammatory class of drugs showed higher binding affinity than standard drug trifluridine. Previous studies had revealed that these classes of drugs have significant effect in colorectal cancer. Treating patients with CRC has become very challenging these days as conventional chemotherapeutics are growing resistance. To overcome this situation natural molecules and synthetic drugs should be taken under consideration in the treatment of CRC. Thus fluvastatin and prednicarbate can be considered to have anti-cancer property and as the inhibitor of colorectal cancer.

5.2 Future Direction

For this study, *in vitro* and *in vivo* experiments were not performed yet due to time constraint. A continued study could be carried out to confirm potential ligand protein (FAP-α) interaction. The selected drugs could be considered as a potent FAP-α inhibitor after passing *in vitro* tests. Then, *in vivo* tests could be performed for ensuring safety in clinical use for treating patient with colorectal cancer.

Chapter 6

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