Use of Combination of Statins as Antagonists of SPARC in Stomach Cancer: An *in silico* Study

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
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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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Of Spring 15, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 29th May, 2019.

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Ethics Statement

The study does not involve any kind of animal trial and human trial.

Abstract

SPARC is a protein found in gastric cell line. It has a dual role of tumor suppression and tumor progression. Over expression of SPARC in gastric cell line can lead to Stomach / Gastric cancer which is one of the leading cause of death worldwide. Over the past few years, drug repurposing and other *in silico* computational techniques have been considered as an ideal approach to discover newer therapeutic alternatives to treat cancer like disease. The application of drug repurposing and molecular docking can play a major role to identify options in the treatment of Stomach / Gastric cancer. Over secretion of SPARC is responsible for Stomach / Gastric cancer. Antagonists of SPARC can be considered as a treatment of choice. In this study, various combination drugs were investigated by applying several in silico approaches. Different combinations of statin drugs were made and among them combination of (Atorvastatin + Pitavastatin) proved to have better antagonistic activity towards the targeted protein SPARC. The combination of (Atorvastatin + Pitavastatin) showed properties that make it a viable option to be considered in Stomach / Gastric cancer therapy with a binding affinity of -9.2 kcal/ mol.

Keywords: SPARC; Statins; Stomach / Gastric cancer; Drug repurposing; Molecular docking; Protein-ligand interactions.

Dedication

Dedicated to my supervisors, Professor Dr. Eva Rahman Kabir and

Mohammad Kawsar Sharif Siam

Acknowledgement

In the beginning, I would like to thank Almighty Allah who has blessed me with immense strength. The gratefulness and assistance that Almighty Allah has provided throughout the journey of accomplishing this project was beyond mentioned. I am really grateful to some people for their constant guidance and supervision without which this project seemed very difficult to finish. That why, I am recognizing them here to convey my gratitude.

Firstly, I would like to signify my respect, gratefulness and honor to my most esteemed supervisor Professor Dr. Eva Rahman Kabir, Chairperson and Professor, Department of Pharmacy, BRAC University, It was a life changing opportunity for me to work under her supervision and guidance. Her motivational words have inspired me to take new challenges while doing research. The constant direction and encouragement she provided was enough to complete this project in the shortest possible time. Her character, dedication and enthusiasm towards research is noteworthy.

I would like to express my gratitude and gratefulness to a very dedicated & supportive faculty member, Mohammad Kawsar Sharif Siam, Senior Lecturer, Department of Pharmacy, Brac University. Without his scientific inputs, direction and appreciation this project would not come to light. I am indebted to him for the suggestions and support I received throughout the project. .

At last, I am thankful towards my family and friends for the constant support and encouragement that they have provided me in all stages of my life. Today I am here because of their inspirations, prayers and unconditional love.

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List of Acronyms

SPARC	Secreted Protein Acidic and Rich in Cystine
FDA	Food and Drug Administration
HMG- CoA	Hydroxymethylglutaryl-CoA
CHD	Coronary Heart Disease
ACS	Acute Coronary Syndrome
LDL	Low Density Lipoprotein
MI	Myocardial Infraction
NAG	N-Acetyl-D-Glucosamine

Chapter 1

Introduction

Cancer is a term given to define a large group of diseases that may affect any part of the body. It generally refers to the abnormal and uncontrolled growth of cells in the body (Tohme et al., 2017). Cancer is usually initiated when normal cells transform into tumor cells originating from a pre-cancerous lesion. This eventually transforms into malignant cancer cells. These changes possibly occur due to a person's genetic factor or external agents. The external agents consist of biological carcinogens, physical carcinogens and chemical carcinogens (Plummer et al., 2016). There will be an estimated 18.1 million new cancer cases (17.0 million excluding nonmelanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding nonmelanoma skin cancer) had been predicted in 2018. In both male and female combined, lung cancer is the most commonly diagnosed cancer, with 11.6% of the total cases of cancer as well as being the leading cause of cancer death (recorded at an 18.4% of total cancer deaths). This has been closely followed by female breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) for incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality (Bray et al., 2018). The most frequent cancer and the leading cause of cancer death among males is lung cancer. This is closely followed by prostate and colorectal cancer (for incidence) and liver and stomach cancer (for mortality). Breast cancer is the most commonly diagnosed cancer among females and the leading cause of cancer death. This is followed by colorectal and lung cancer (for incidence), and vice versa (for mortality). Cervical cancer ranks fourth for both incidence and mortality (Bray et al., 2018).

Currently, stomach cancer is still the fourth most common cancer and the second most common cause of cancer death in the world (Brenner, Rothenbacher, & Arndt, 2009). Most anticancer drugs

possess a narrow therapeutic index, develop multidrug resistance (MDR) and present unspecific bio-distribution upon intravenous administration. This often leads to unacceptable side effects to healthy tissues, mainly bone marrow and gastrointestinal tract. These limitations of conventional chemotherapeutic strategies often result in suboptimal dosing, delay in treatment or discontinuation and reduced patient therapy compliance. Combination therapy has been recognized as the standard of care, especially in cancer treatment, due to it being a rationale strategy to raise response and tolerability and to decrease resistance. There is an increasing interest in the combination of anticancer drugs aimed at maximizing efficacy while minimizing systemic toxicity via the delivery of decreased drug doses (Catarina, Nuno, & Simoes, 2012). Current medical advancements have seen the emergence of novel approaches to drug rediscovery, the identification of opportunities to evaluate FDA-approved and abandoned drugs for new therapeutic uses. Patients obtain accessibility to promising new therapeutic strategies much rapidly by capitalizing on prior experience. This results in decreased drug development, registration cycle times and cost. Drug repurposing has emerged as the better substitute for such a situation, as it takes into consideration the subsiding of both expenses and time (Godwin et al., 2013). This study is focused on the numerous anti-cancer effects discerned due to amalgamation of statins and SPARC (Secreted Protein Acidic and Rich in Cysteine) which is expressed in stomach cancer as it was considered as the molecular target.

1.1 Combination Drug Therapy in Cancer

In the context of cancer treatment, chemotherapy is still rendered as the preliminary line of treatment. A combination chemotherapy is made merging several cytotoxic drugs possessing disparate mechanism. When subjected to diversification in terms of treating cancerous population, the intention is to impart improved therapeutic efficaciousness and diminishing the resistant cells. It was observed that combination drug administration showed improved response and diminishes toxicity in both cases when applied sequentially or in combination (Balázs Ligeti et al., 2017). Combination drug therapy is the ideal choice as it subside the tumor growth which is attributed to its improved therapeutic index, fastidious approach and numbing the cancer cells in their mitotic phase (Mokhtari, Homayouni, & Baluch, 2017).

1.2 Stomach/ Gastric Cancer

Although deemed as the fourth most leading type of cancer worldwide, Stomach cancer is associated with being the second most noteworthy reason of death related to cancer (Carcas, 2014). Higher rate of stomach cancer case has been observed in the developing countries mainly in the Asian Region in contrast to the developed countries. Stomach cancer is generally categorized as sporadic gastric cancer, early onset, gastric stump cancer, etc. Pathologically they can be divided as adenocarcinoma, undifferentiated carcinoma and signet ring-cell cancer (Sitarz et al., 2018). Intestinal subtypes have been observed at a higher frequency in comparison to the diffusive ones in terms of the pathological classification (LAURÉN, 2017). Factors related to stomach cancer include - age, sex, smoking, alcohol, lifestyle, etc. However, *H. Pylori* infection is liable for the formation of malignant lesion by causing serious changes in the stomach cell line (Kelley & Duggan, 2003). Although intestinal subtypes are less intrusive in comparison to the diffusive ones,

they are the most exophytic types with the highest frequency of occurrence (Henson, Dittus, Younes, Nguyen, & Albores-Saavedra, 2004). Again diffusive type stomach cancer which is more aggressive in nature might be the consequence of *H.Pylori* infection. This infection is seen on a mass scale in general population of various ages. The gene E- cadherin which exhibits anomalies in its expression inside the tumor might be the outstanding cause for the development of diffusive type stomach cancer (Norton et al., 2010).

1.3 Drug Repurposing

A strategic analysis recognized as drug repurposing (or repositioning, reprofiling or re-tasking) can be applied for the utilization of drugs in other diseases aside from the existing medical indications (Pushpakom et al., 2018). Data regarding the pharmacology, probable adverse reactions, and formulation development of the approved or abandoned drugs can be found in maximum cases in which this strategy of drug discovery uses to its full advantage (Astin & Hall, 2017). Drug repurposing methods like network-based approaches, network-based cluster approaches, network-based propagation approaches, text mining-based approaches, semantics-based approaches plays an important role in new treatment that can easily be achieved in little time along with minimal cost and labor compared to the conventional drug discovery methods (Xue, Li, Xie, & Wang, 2018). Substantial lowering of drug resistance along with decline in high individual dosing of drug can be achieved through establishment of combination therapy by utilizing drug repurposing strategy (Sun, Sanderson, & Zheng, 2017). Sildenafil can be considered as an outstanding example of drug repurposing, this was repurposed as medication to erectile dysfunction when it subsequently indicated erection as a side effect during trials (Slikker et al.,

2012). When Minoxidil was implemented as an anti-hypertensive agent substantial side effects triggered its repurposing for the treatment of alopecia (Azvolinsky, 2017).

1.4 Molecular Docking

The process by which interaction and potent affinity towards a target is inspected with an established three dimension structure such as - binding of ligand with protein is deemed as molecular docking (Pagadala, Syed, & Tuszynski, 2017). The crucial aspects of molecular docking are algorithmic search and scoring functions. Determination of the most suitable ligand is done by sampling algorithm which is further checked by implementing free energy. For filtering out the wrong conformers in limited time and accuracy of chemical potential- scoring function plays a significant role (Oleg & Arthur J., 2010). Various software such as (FTDOCK, ZDOCK, FLOG) are implemented to execute rigid docking which is one of the most essential type of molecular docking that uses a search space which is usually small and also brings us a higher number of docking results of conformers (Pagadala et al., 2017). Furthermore, flexible docking which is recognized as standard docking protocol from every prospect is done through programs like Autodock Vina, MDock and DOCK (Huang, 2018).

1.5 SPARC Protein in Stomach Cancer

SPARC also known as BM-40/osteonectin was selected on the basis of poor diagnosis correlated with over expression for our further study (Wang et al., 2004). Regulation of apoptosis along with various level of SPARC in stomach cancer is a top feature. Lower expression levels produce inhibition of cancer cell growth (Xu et al., 2016).

1.6 SPARC and Its Structure

SPARC is identified as a glycoprotein or as non -collagenous matrix protein owing to its calcium binding capability (Brekken & Sage, 2002). The key feature of the structure of SPARC is its domains. Domain-I is acidic in nature, Domain II is follistatin (FS)-like and EF-hand related calcium binding EC domain (Hohenester et al., 1997).

1.7 Mechanism of Action of SPARC in Cancer Development

SPARC is a protein found in the bone and binds particularly to collagen and hydroxyapatite. A complex is formed due to attachment of SPARC to insolubilized type 1 collagen which then binds to free calcium ions and synthetic apatite crystals. The SPARC-Collagen complexes turn into mineral phase deposition from metastable balanced salt solutions. The protein is confined to mineralized bone trabeculae and higher levels of it is detected in the matrix. It is tissue specific in nature which commences active mineralization in normal skeletal tissue and also joins the collagen phases and bone mineral (Termine et al., 1981).

Although not being connected to death receptor stimulation, SPARC elevates the effect of apoptosis by amplifying the signaling cascade in a capase-8 dependent manner which eventually leads to downstream involvement of apoptosis (Tang & Tai, 2007). The Figure 2 depicts the pathways by which SPARC functions in apoptosis. SPARC functions in the cytoskeletal rearrangement, maintaining of cell adhesion, proliferation, tissue remodeling and matrix assembly (Bradshaw, 2016). SPARC also assists in wound healing, bone formation, tumor progression, fibrosis and angiogenesis. Angiogenesis appertains to endothelial cell proliferation, migration, extracellular matrix synthesis and degradation. It is observed that in some cases SPARC boosts

angiogenesis while in some cases it exhibits anti- angiogenetic activity (Rivera, Bradshaw, & Brekken, 2011).

Though there are developing proof that SPARC plays a significant role in some specific cancers but no accurate model has been established which describes its functions and how it assists in the progression and development of cancer. SPARC can serve both as tumor suppressor and tumor promoter based on some factors. It plays a versatile circumstantial role resolving around the type of cancer, the surrounding milieu and the type of cell (Neveen Said, 2016). Compared to normal tissue, SPARC is expressed in tumors also with its surrounding stroma in some cancers. Its expression pattern varies depending on the cancer. For example: A higher extent of SPARC expression has been identified in melanoma, breast cancer, glioblastomas and gastric cancer. This pattern shows a potential role of SPARC in tumor progression. On the other hand some cancers like colorectal cancer, ovarian cancer, pancreatic cancer and acute myelogenous leukemia indicates lower level of SPARC thus speculating the inhibitory role of SPARC in tumor formation (Neveen Said, Frierson, Sanchez-Carbayo, Brekken, & Theodorescu, 2013).

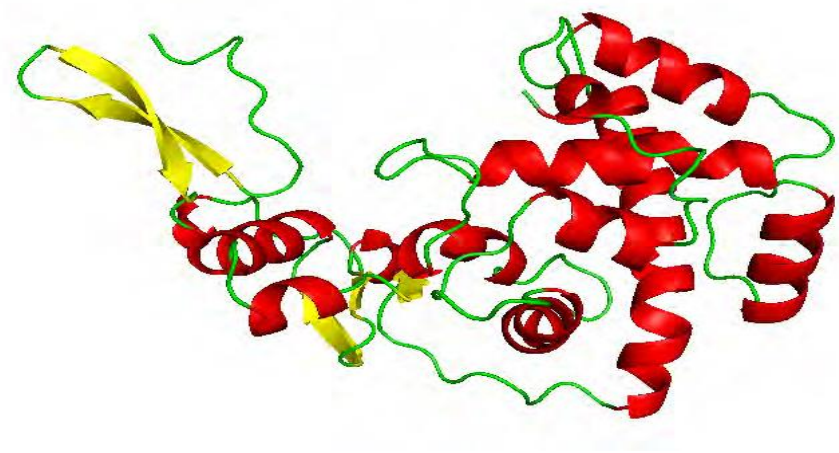
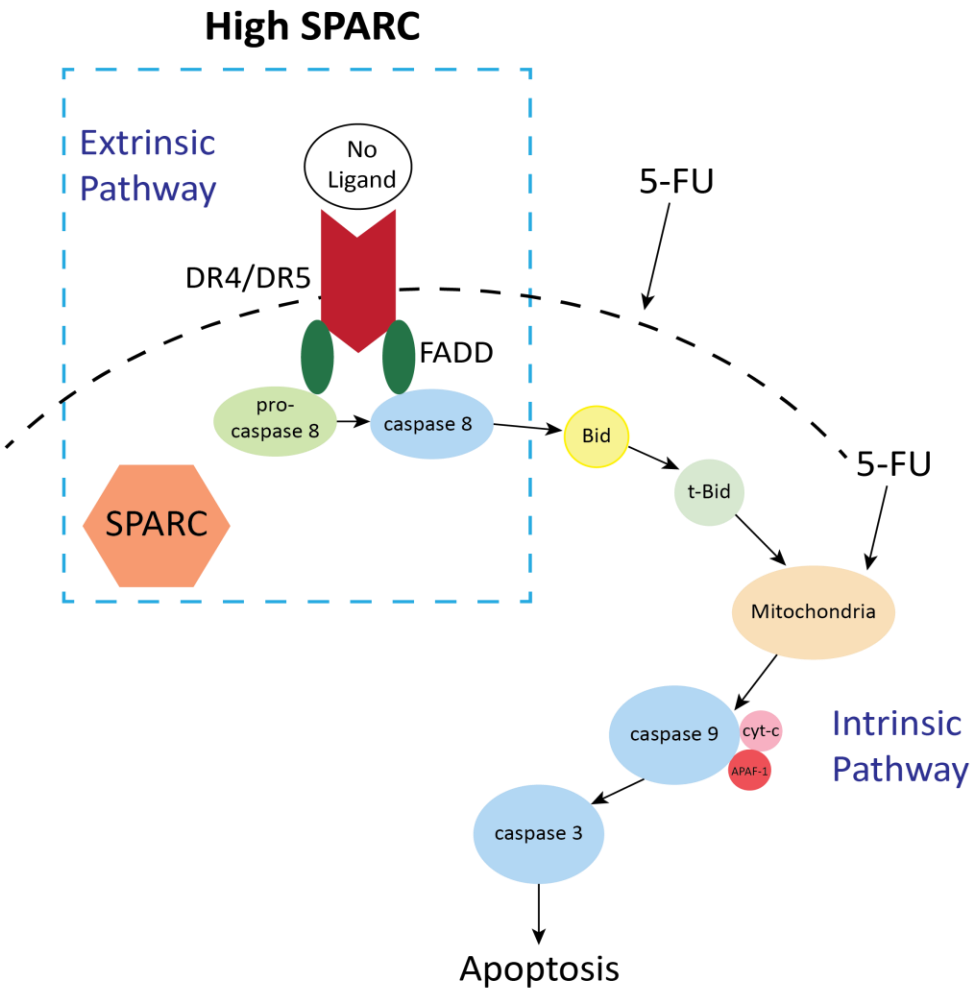


Figure 1: Structure of SPARC (obtained from PyMOL version 1.8.4.0) (Seeliger & de Groot, 2010)

SPARC also acts as tumor suppressor for some types of cancer like bladder cancer, ovarian cancer, cervical cancer and colorectal cancer. Several research have also affirmed that the transcription and expression of SPARC were substantially down regulated in bladder cancer cell line. High SPARC expression is connected to higher rate of disease free survival (Neveen Said et al., 2013). It is also seen that SPARC is amply expressed in advanced phases of ovarian cancer. Nevertheless, normalization of tumor microenvironment by SPARC has been indicated by new evidence and that it reverses tumor growth (N. Said et al., 2007). A high rate of anomalous methylation caused by SPARC has been discerned in a screening study of hyper methylated genes in invasive cervical cancer (Parwani, 2007). There is a mutual connection between overexpression of SPARC and a sharp increase of it in serum level with the buildup of cervical cancer (SHI et al., 2016). SPARC is acknowledged as a tumor suppressor in colorectal cancer in consonance with clinical studies and experimental cell models. High efficacy in tumor regression has been observed in animal xenografts when chemotherapy combined with exogenous SPARC were used (Tai, Dai, Owen, & Chen, 2005).

Besides, SPARC acts as tumor promoter in several cancer types. Fluctuating levels of SPARC has been observed in cell lines of human gastric cancer indicating its role as a tumor suppressor. Regarding diffuse type and intestinal type gastric cancer, SPARC transcript and protein level were raised and it showed a mutual connection between poor diagnosis and invasiveness of cancer (Wang et al., 2004). Inhibition of growth and expression of high expressing gastric line cells have been seen when SPARC was downregulated. Hence, SPARC can be a good target for cancer therapy and drug development. Combination drug through drug repurposing can be considered as a strategy since it can exert its effect by interacting with this protein of stomach cancer named SPARC.

The Figure 1 shows the alpha helices, beta sheets and the coils of SPARC. Red portions specifies alpha helices; yellow portions show beta sheets and green portions are coils. These are caused due to protein folding which in turn provides stability to the protein structure.



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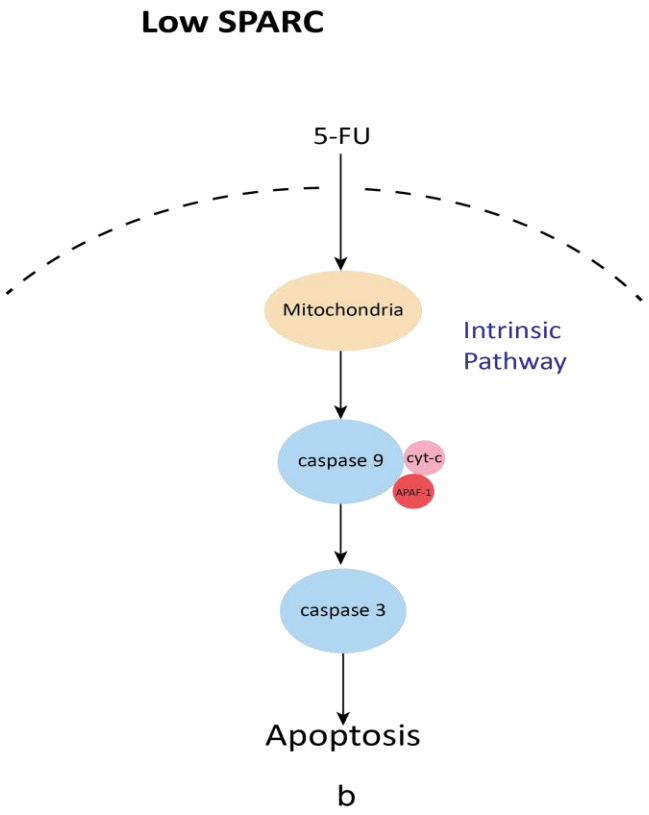


Figure 2: Occurrence of apoptosis which is SPARC induced (Rahman, Chan, & Tai, 2011)

(a)Pro-Caspase 8 is split to caspase 8 when SPARC combines with pro- caspase 8.

This when gets exposed to chemotherapy stimulates the mitochondrial pathway of apoptosis

(b)When SPARC level is low apoptosis only happens without the presence of caspase 8

1.8 Role of Statin Drugs in Cancer

Statins are a class of drugs that originate from fungus. It was first isolated from the fungus *Penicillium citrinum.* They are lipid lowering drugs and they are used in the treatment of cardiovascular diseases (Endo, 1988).

Statins inhibit the hydroxymethylglutaryl-CoA (HMG-CoA) reductase enzyme. Structurally they are related to hydroxymethylglutaryl coenzyme A. Statins inhibit a key step of the biosynthetic pathway of sterols. This makes them a powerful medication to lower cholesterol levels, and for this reason it is also widely used for prevention of cardiovascular diseases (Sirtori, 2014).

Statins decrease cellular cholesterol content by preventing the HMG-CoA reductase enzyme to function. This restricts the cholesterol biosynthesis and decreases the concentration of cholesterol in the liver. This increases the expression of LDL-receptors in the liver cell membranes, which enhances the clearance of LDL-cholesterol from the blood circulation (Sirtori, 2014). Adverse effects linked with statins include Amnesia, Myopathy, Diabetes mellitus and other muscle related problems (Jamolowicz, Chen, & Panegyres, 2015)

Among the random combinations of Statin class of drugs, the combination that we had created of Atorvastatin and Pitavastatin that has a substantial binding affinity of -9.2 Kcal/Mol . Thus it was our drug of choice in the treatment of stomach cancer.

1.8.1 Atorvastatin and Pitavastatin

Atorvastatin is acknowledged as the first choice drug for stomach cancer treatment since it has a good therapeutic index, low reduction in LDL-C level from blood and also has little side effects (Jones et al., 2017). On the other hand, Pitavastatin can act as an anti-cancer drug as it can decline the Hydroxymethylglutarate coenzyme-A reductase (HMGCR) which is observed at a high

concentration in ovarian cancer (De Wolf et al., 2017). It shows side effects like myopathy (S., A., & S., 2016). Atorvastatin exhibits low systemic bioavailability (Hausner et al., 2017). Hence, the combination of Atorvastatin and Pitavastatin can be recommended as a combination drug therapy for treating stomach cancer. Combination form can be administered with less amount of side – effects and improved therapeutic response.

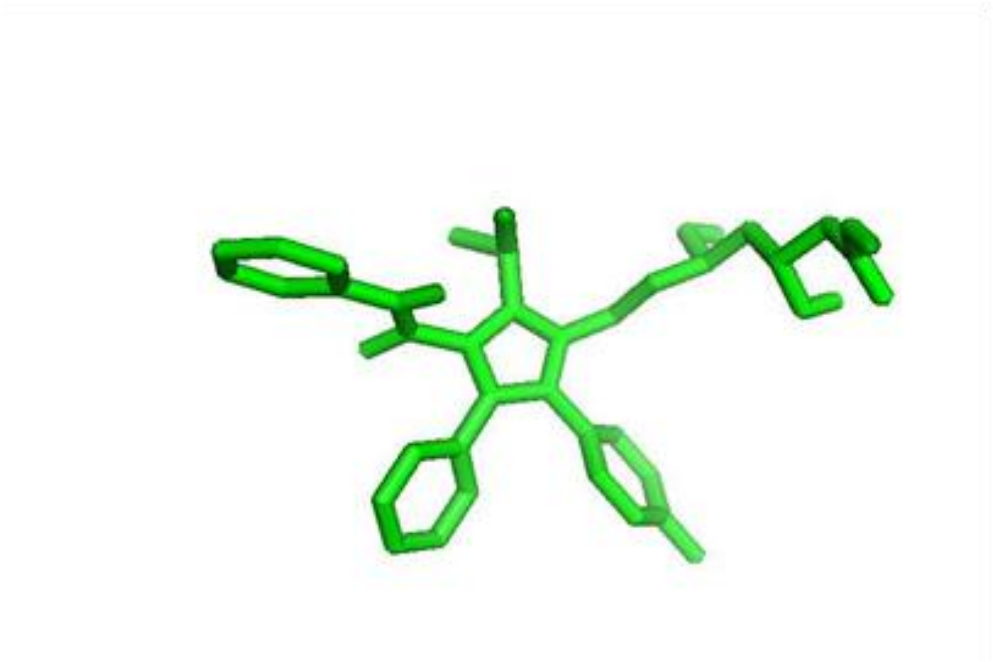


Figure 3: Combination of Atorvastain and Pitavastatin by using Avogadro and PyMOL (Hanwell et al., 2012) (Lill & Danielson, 2011)

1.9 Rationale for the study

The chances of finding newer medication with less side effects can be increased through applying drug repositioning strategy, randomized screening of drugs that have been previously approved by the FDA (Pessetto et al., 2014). The biggest obstacles in the field of drug delivery for achieving a competent solution in a short time are increased amount of money and time, lower toxicity profile, low bioavailability and extended examination process and trials. On the other hand, therapeutic switching or drug repurposing by utilizing the existing pharmacokinetic and pharmacodynamics parameters is becoming a compelling solution for this problem (Gupta, Sung, Prasad, Webb, & Aggarwal, 2013). Hence, by applying drug repurposing strategy combination drug therapy will enable the use of safer cancer medications with lower toxicity which in turn will save both money and time (Mokhtari et al., 2017).

Chapter 2

Methodology

This is basically an in *silico* based study, where by applying computational biology and molecular docking technique we have tried to identify the anti-cancer use of combination drugs in stomach/ gastric cancer . In the beginning section of the methodology, an in-depth review from the past literatures on this specific topic has been incorporated. Later on, through molecular docking, binding affinities between receptor and ligands have been identified. To perform computational docking, it was mandatory to have the three dimensional structures of ligand or small molecules and macromolecules. Articles from reliable sources like PubMed, Springer, Elsevier, Nature and so on were taken for this study purpose.

2.1 Online software, tools and databases used for molecular Docking, Visualization and Validation

RCSB-PDB (Protein Data Bank) (Berman et al., 2000), PubChem (Li, Cheng, Wang, & Bryant, 2010), DrugBank (Wishart et al., 2018), NCBI (Geer et al., 2009) are few online databases that were used to obtain the protein (SPARC) and the ligands. To validate the three dimensional structure of the protein (SPARC) ProSA Web Server (Sippl, 1993; Wiederstein & Sippl, 2007),), ERRAT (Colovos & Yeates, 1993), Ramachandran Plot (Lovell et al., 2003) were used.

Table 1: Software and other tools used in the study

Sl.	Software and tools used in the study	Version
01.	PyMOL	2.0.4
02.	Open Babel	2.4.1
03.	Avogadro	1.2
04.	AutoDock Vina	1.1.2
05.	AutoDock tools	1.5.7
06.	BIOVIA Discovery Studio Visualizer	17.2.0.16349

In Table 1, all of the software and tools are mentioned that were used throughout the study for several purposes.

2.2 Validation of Three Dimensional Protein structure

From the established source named Protein Data Bank (Berman et al., 2000), the three dimensional structure of our desired protein SPARC (PDB ID:- 1BMO) was downloaded. A visualization tool named PyMOL (DeLano, 2002) was used to curate the protein (SPARC). Since both of the Chain A & B were similar, one chain was deleted for the simplification of work. The curated protein structure was further verified in ERRAT (Colovos & Yeates, 1993), ProSA web viewer (Wiederstein & Sippl, 2007) Ramachandran Plot (Lovell et al., 2003) and Verify 3D .

2.3 Protein and Drug list

The curated protein (SPARC) structure was obtained after performing the validation process. The protein structure was ready for docking by using AutoDock Vina. AutoDock Tools (Morris et al., 2009) played a huge role in changing the polarity of the desired protein SPARC (by adding polar hydrogens to it) and it was further saved in a format which is suitable for AutoDock Vina to

perform its operations. Before that the removal of all other groups from the protein was necessary. From the Grid menu of ADT, 'Gridbox' was used to specify the area of coordinates inside the protein SPARC. A docking folder was created and the protein after perfect positioning of the 'Grid Box' was saved as 'Protein.pdbqt' format to perform docking.

Randomly, structures of more than 400 drugs of different classes were obtained by using both PubChem (Li et al., 2010) and DrugBank (Wishart et al., 2018). Structures obtained from PubChem were in SDF format which is not suitable to perform docking by using AutoDock or PyRx .Open babel was used to convert the SDF structures to the desired PDB format (O'Boyle et al., 2011). However, drugs structures which were obtained from DrugBank (Wishart et al., 2018) were already in PDB format and ready to use .Furthermore, Avogadro software was used to make the combinations of several group of drugs. In some cases, combination drug structures were drawn by using Avogardo software and the structures were derived from PubChem (Li et al., 2010) and DrugBank (Wishart et al., 2018) were used as a template to draw those structures. For further procedure, all of the structures were saved in PDB format into the particular folder of the computer. From the tool menu of AutoDock toolbox, 'Torsion tree' option was used to fix the rotatable bonds of the specified ligands. Finally, the ligands were ready as rigid and flexible to perform docking. This is considered as a manual process to prepare ligand by using Autodock. In case of PyRx, the process is automated starting from the very beginning till end and quite easy to use. Moreover, side chain flexibility is a prime feature in the most recent version of AutoDock.

2.4 Molecular Docking and Screening

In the study, both rigid and flexible docking were performed by using AutoDock Vina and AutoDock tools. For this manual docking using AutoDock Vina and AutoDock Tools, both the protein and ligand named ‘Protein.pdbqt’ and ‘Ligand.pdbqt’ were saved in the Vina folder. Afterwards, various coding operations were performed by using cmd.exe for docking. All of the output files containing the results were saved in the classified folders. To perform operations, changes were made in the flexibility of ligands according to the manual to get desired results.

For further study, output file containing the best result was considered. Since the operation represents an exothermic reaction, it provides nine best binding affinity of negative values. More negative value is considered as the strongest binding affinity.

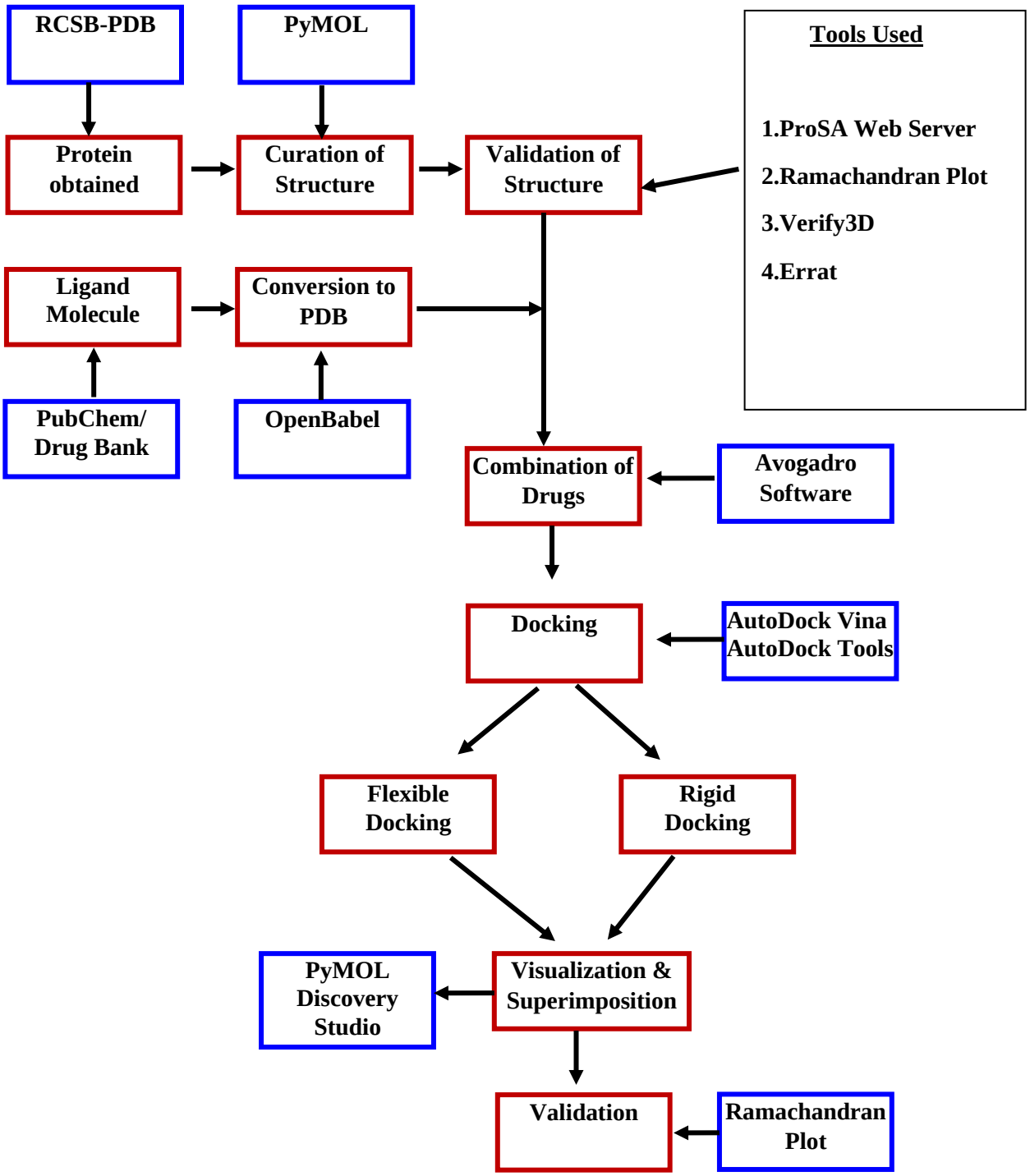


Figure 4: Flowchart showing the steps of molecular docking

2.5 Visualization and Validation Process

After the docking process, the ‘Output.pdbqt’ file of ligands were saved and visualized along with the protein (SPARC) in PyMOL to assess all of the binding sites present in between the ligands and the protein. The Discovery Studio Visualizer (Dassault Systèmes BIOVIA, 2010) was used for visualizing purpose by which assessment of the protein-ligand interaction was seen that involves amino acids, types of bonds, subsequent distance between bonds etc. Ramachandran plot, ProSA Web Server, ERRAT, Verify3D were used for validation purpose.

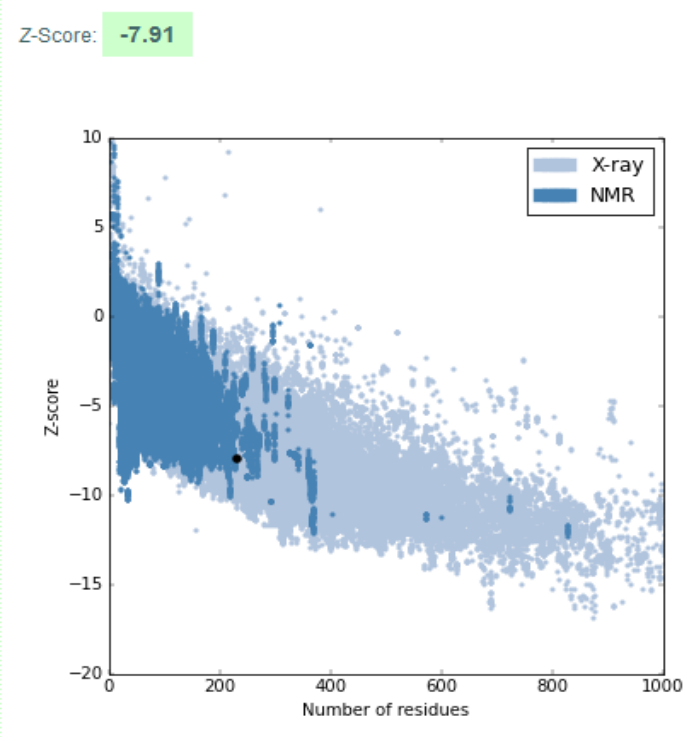
Chapter 3

Results and Validation

In this section, binding affinities after docking, visualization and validation of the three dimensional structure of protein SPARC and the interacting ligands are discussed.

3.1 Validation of the Structure of Protein (SPARC)

The quality of the three-dimensional structure of the SPARC was analyzed by using ProSA (Protein Structure Analysis). It is a widely used online tool to determine the validity of protein structure by generating Z score value. From the study, SPARC (PDB Code-1BMO) achieved a Z-score value of -7.91.



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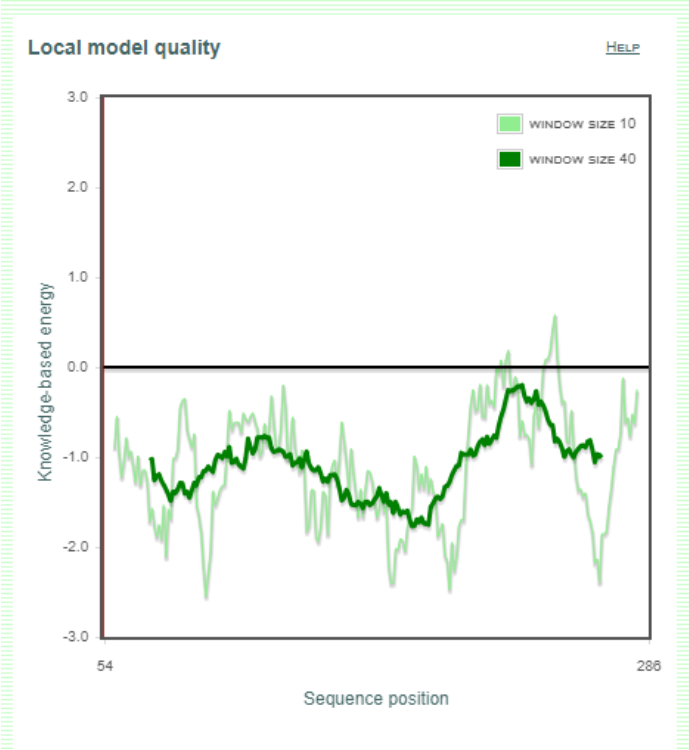


b

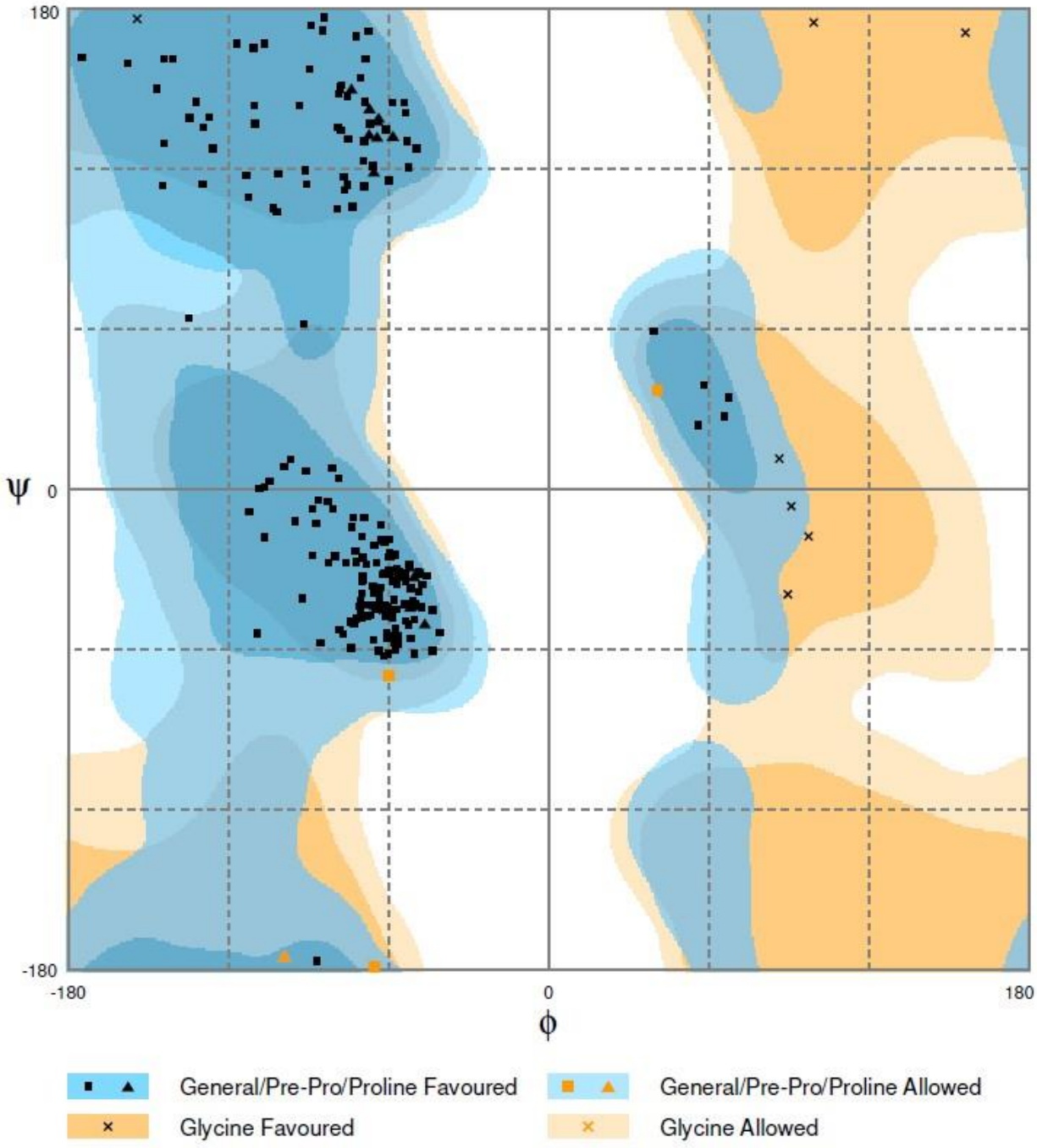
Figure 5: (a) The z-score (-7.91) of SPARC (PDB ID-1BMO) obtained from ProSA Web Server

(b) Local model quality of SPARC

From figure 5(a), the z-score value -7.91 of SPARC is in the range since values away from zero towards negative is considered as best result .The result is seen in both X ray and NMR region. Also, in Figure 5 (b) the local model quality of SPARC , it is below positive value which so it can be said that the 3D structure of the protein has no error (Wiederstein & Sippl, 2007). Hence, the structure of protein SPARC can be considered as valid.

Secondly, ERRAT was used to identify the overall quality factor of the 3D structure. The overall quality factor of the protein was 95.067 which is considered as a very good results for proteins with higher resolutions (Colovos & Yeates, 1993).

Furthermore, Ramachandran plot was used to validate the three dimensional structure of the protein SPARC.



Number of residues in favoured region (~98.0% expected)	: 218 (98.2%)
Number of residues in allowed region (~2.0% expected)	: 4 (1.8%)
Number of residues in outlier region	: 0 (0.0%)

Figure 6: Ramachandran Plot for Protein SPARC (PDB ID: 1BMO)

In figure 6, the detail Ramachandran plot for the protein SPARC (PDB ID:1BMO) is represented. The outlier region was devoid of any amino acid residue. The favorable region contains 98.2% of all the residue. Only four amino acids are in the allowed that allows for a percentage of (2.1%). Since, all of the mentioned results are considered as ideal the protein SPARC can be termed as validated.

Lastly, Verify3D was used where it showed that 89.70% of the residue have averaged 3D-1D >= 0.2 and it passed. So, it depicts the validity of the structure.

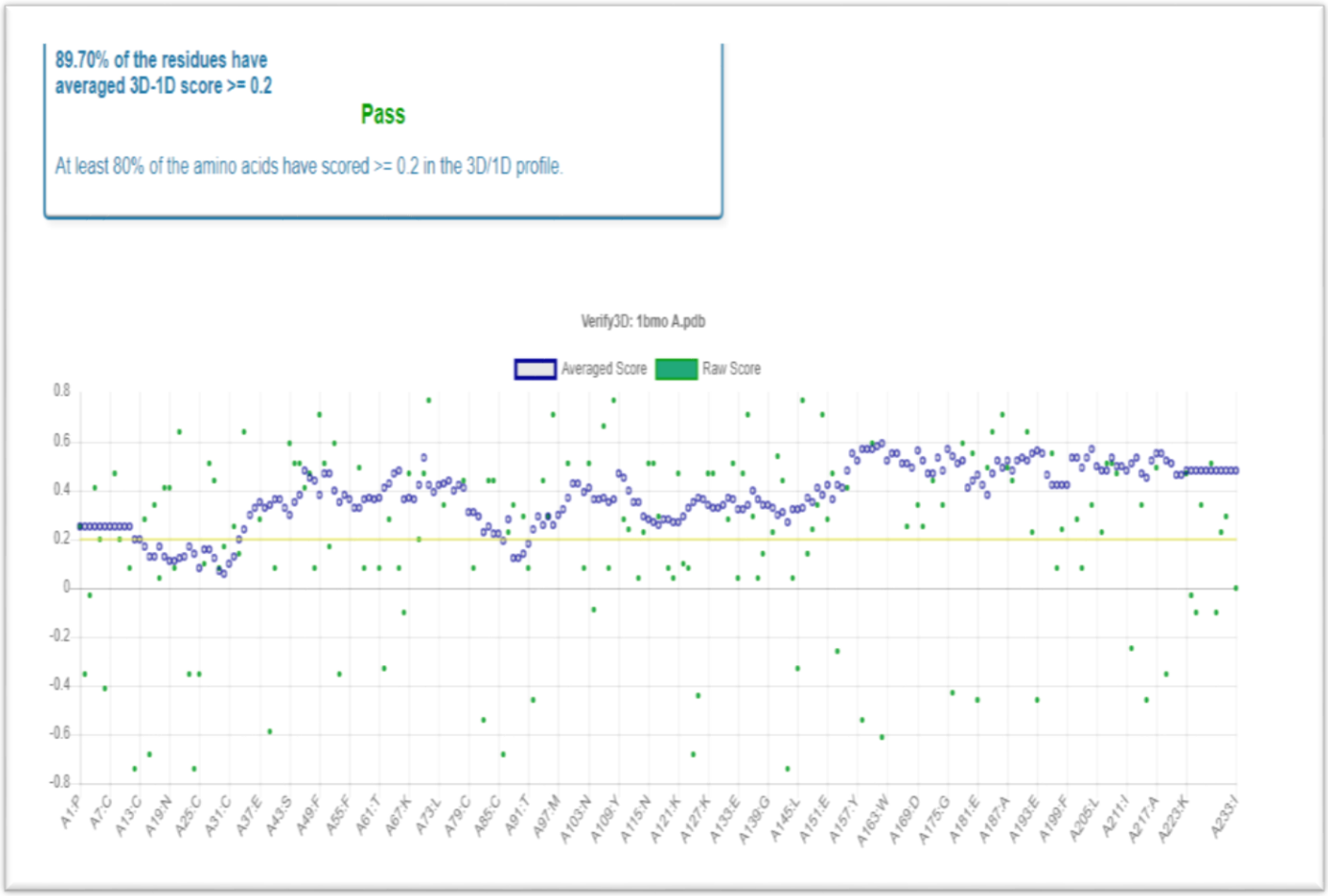


Figure 7: Verify3D score for the Protein SPARC (PDB ID: 1BMO)

3.2 *In silico* binding results of SPARC after docking

A various classes and numbers of drugs were screened among which statin class of drugs became ideal in making combinations. Avogadro software was used to make the combinations. SPARC was considered as the macromolecule and different combinations of statins were considered as ligands. Both rigid and flexible docking were performed by using AutoDock Vina. In case of rigid docking, molecules needed to be in a non-rotatable form so the torsions were fixed. But in flexible docking the torsions were not fixed and a various torsions and torsion routes were used. Flexible docking is set as the by default system in most of the molecular docking software. Rigid docking is performed by fixing the torsions in a non-rotatable manner using AutoDock Tools. Furthermore, the docking through manual input of the codes in cmd.exe was performed. For this study, the results are summarized in the table below:

Table 2: Rigid and Flexible docking results of Combinations of Statins with SPARC (PDB ID-1BMO) by using AutoDock Vina (Trott & Olson, 2010)

Different Combinations of Statins	**Flexible Docking Affinity (kcal/mol)**	**Rigid Docking Affinity (kcal/mol)**
Atorvastatin + Pitavastatin	-8.4	-9.2
Simvastatin + Rosuvastatin	-7.5	-8.5
Atorvastain + Rosuvastatin	-8.7	-10.9
Atorvastatin + Pravastatin	-8.0	-9.1
Atorvastatin + Lovastatin	-8.1	-9.0

Different combinations of statins along with their rigid and flexible binding results are mentioned in table 2. Where we can see that combination of Atorvastatin and Pitavastatin have shown rigid binding affinity of -9.2 kcal/ mol. On the other hand, combination of Atorvastatin and Rosuvastatin has a binding affinity of -10.9 kcal/ mol but it does not superimpose with marketed combination drugs that are available to treat stomach/ gastric cancer. Furthermore, In case of the combination (Atorvastatin + Rosuvastatin), there is only one amino acid which is common with the marketed standard drug .So, for our further study, we have considered the combination of (Atorvastatin + Pitavastatin) as a drug of choice.

Besides, docking was also performed for all other statin drugs as a single molecule with SPARC. It was done to assess the individual binding affinity of these particular groups of drugs. The results are stated below:

Table 3: Rigid and Flexible docking results of Statin drugs with SPARC (PDB ID-1BMO) by using AutoDock Vina (Trott & Olson, 2010)

Individual Drug	**Flexible Docking Affinity (kcal/mol)**	**Rigid Docking Affinity (kcal/mol)**
Atorvastatin	-9.5	-9.6
Pitavastatin	-8.1	-10
Lovastatin	-8.6	-9.2
Pravastatin	-8.2	-9.3
Simvastatin	-8.7	-8.9
Rosuvastatin	-7.8	-8.6

From Table 3, Atorvastatin has a binding affinity of -9.6 kcal/ mol. Pitavastatin has the highest binding affinity of -10 kcal/mol. To assess the binding affinity of combinations of statins, a lot of combinations were made. However, other combinations were not proof to be effective enough in further *in silico* studies. Combination of (Atorvastatin + Pitavastatin) showed effective results and individually also they have shown best binding affinities compared to other drugs of statin group.

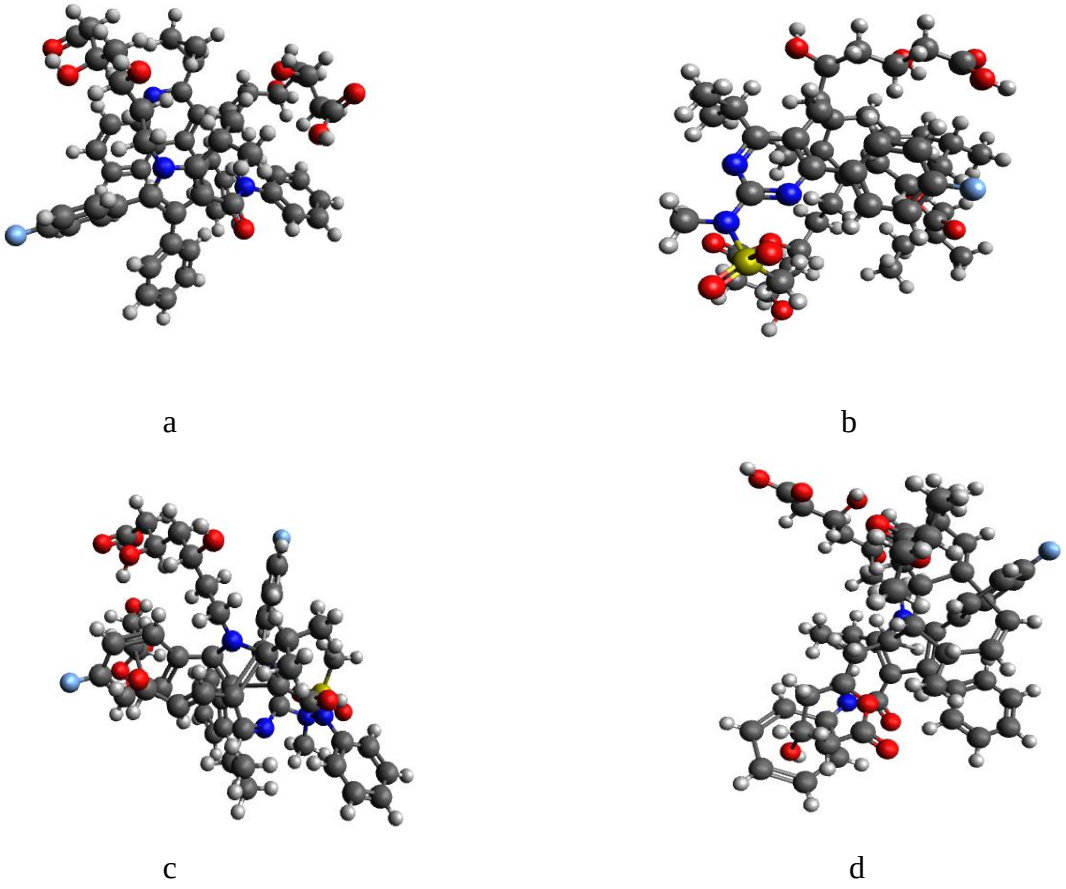


Figure 8: Structures of different combinations of Statin Drugs after energy optimization through Avogadro (Hanwell et al., 2012)

(a) Combination of (Atorvastatin +Pitavastatin)

(b) Combination of (Simvastatin + Rosuvastatin)

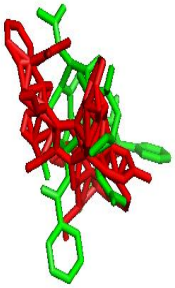
(c) Combination of (Atorvastatin+ Rosuvastatin)

(d) Combination of (Atorvastatin+Lovastatin)

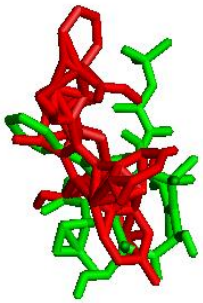
3.3 Visualization and Validation by using PyMOL

PyMOL was used for visualization purpose, then ligand-protein interactions were visualized and validated through Discovery Studio and Ramachandran Plot.

Visualization by PyMOL means, to visualize the protein (SPARC) which is bound with the desired combinations of statins along with a reference drug. It is considered as a standard drug and currently available in market. After performing the rigid docking method the "pdbqt" file was visualized when it was loaded with the SPARC protein molecule (PDB ID: 1BMO).Ligands were bound in the nine binding sites of the protein. To validate with a reference any of the existing nine binding sites can be used. Combination of Statin (Atorvastatin + Pitavastatin) was superimposed with two different standards that are available in the market.



a



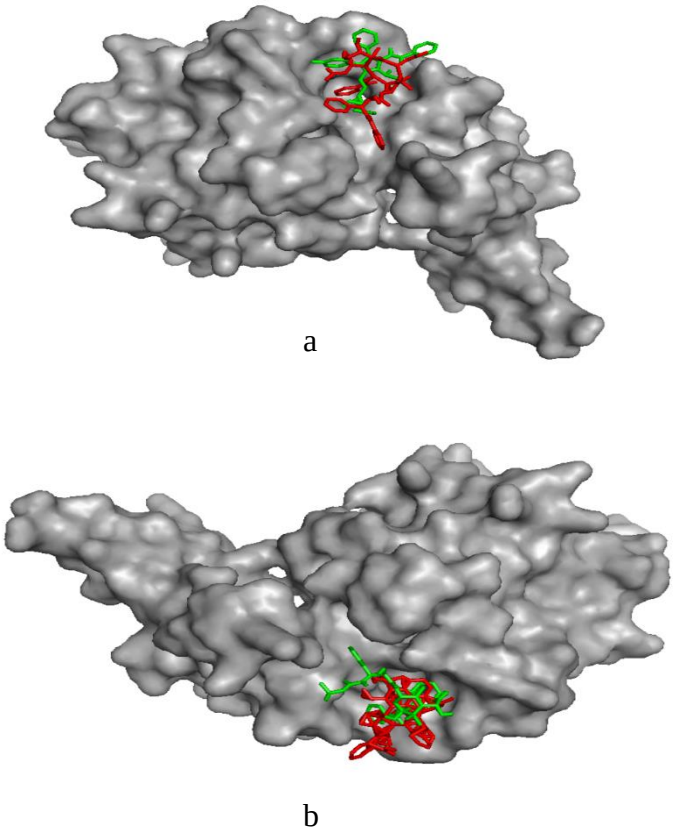
b

Figure 9: (a) Superimposition of combination of (Atorvastain + Pitavastatin) with (Paclitaxel + Capecitabine)

(b) Superimposition of combination of (Atorvastain + Pitavastatin) with (Docetaxel + 5 Fluorouracil)

Figure 9, shows the superimposition of the combination (Atorvastatin + Pitavastatin) with both of the available market preparations of combination drug like (Paclitaxel + Capecitabine) that is

administered as a single combination drug dose and (Docetaxel + 5 FU) that is regarded as a combination therapy.



a

b

Figure 10: (a) Superimposition of combination of (Atorvastatin + Pitavastatin) with (Paclitaxel + Capecitabine) in the same binding pocket of protein SPARC (b) Superimposition of combination of (Atorvastatin + Pitavastatin) with (Docetaxel + 5 Fluorouracil) in the same binding pocket of protein SPARC

(Paclitaxel + Capecitabine) has a binding affinity of -10.4 Kcal/mol (Rigid Docking Affinity) with protein SPARC. (Docetaxel + 5FU) also has a good binding affinity of -9.7 Kcal/mol (Rigid docking Affinity

3.4 Validation by using Discovery Studio

Discovery Studio Visualizer (Dassault Systèmes BIOVIA, 2010) is used for the visualization purpose. To identify the involved amino acid, determining the bonds between amino acids and ligands, different type and category of the bonds and lastly distances between the bonds were visualized by using Discovery Studio.

3.4.1 Protein-ligand interaction of SPARC (PDB ID: 1BMO) with different combinations of Statins

Firstly, Protein-ligand interaction of SPARC was observed and the similarities of the amino acids, category of bond, types and the distance of amino acid-ligand between the established combination drug (Paclitaxel + Capecitabine) and our combination drug of choice (Atorvastatin + Pitavastatin) is shown in the table below:

Table 4: Protein-ligand interaction of SPARC (PDB ID-1BMO)-(Paclitaxel + Capecitabine) in Discovery Studio Visualizer

Amino acid..ligand and atom interaction	Category of bond	Type of bond	Distance (Å) (Amino acid-ligand)
B:ALA240	Hydrophobic	Pi-Alkyl	3.88565
B:VAL157	Hydrophobic	Pi-Alkyl	4.68175
B:LEU242	Hydrophobic	Pi-Alkyl	5.22042
B:ILE129	Hydrophobic	Pi-Alkyl	4.48254

Table 4 shows, that four amino acids are common in case of our combination drug of choice with established drug (Paclitaxel + Capecitabine). ALA240 (aa Alanine), VAL157 (aa Valine), LEU242

(aa Leucine) and ILE129 are the amino acids that mostly contains hydrophobic bonds. All of them contain Pi-Alkyl type of bonds and distance of amino acid-ligand is from 3.8-5.2 angstroms. So, it will exhibit very good protein-ligand interactions.

In this section, the protein-ligand interaction between established combination drug (Docetaxel + 5fluorouracil) and our combination drug of choice was observed. Similarities of the amino acids, category of bond, types and the distance of amino acid-ligand were shown in the table below:

Table 5: Protein-ligand interaction of SPARC (PDB ID-1BMO)-

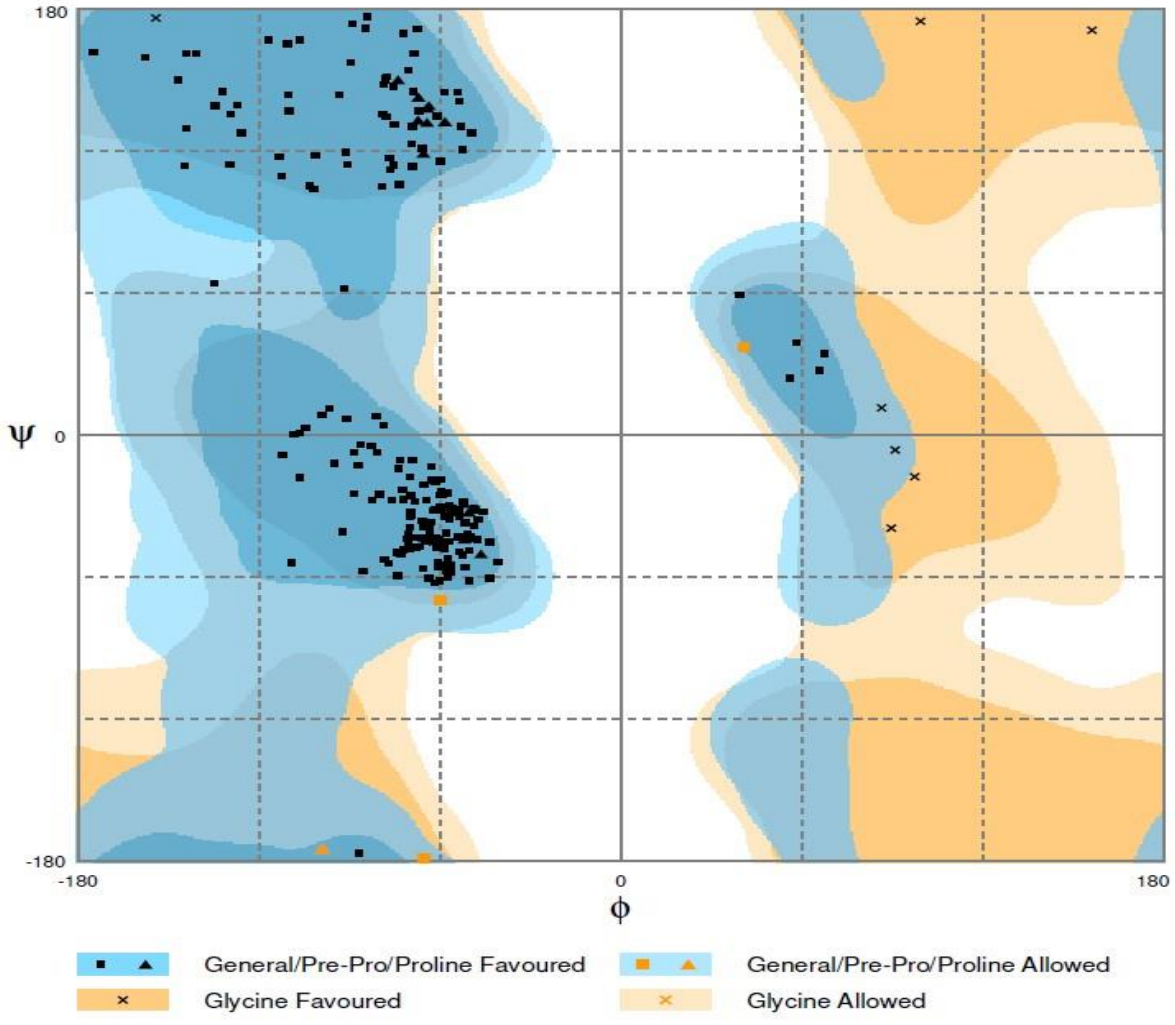
(Docetaxel + 5 fluorouracil) in Discovery Studio Visualizer

Amino acid..ligand and atom interaction	Category of bond	Type of bond	Distance (Å) (Amino acid-ligand)
UNK1:C44-B:LEU221	Hydrophobic	Alkyl	5.40071
UNK1:C44-B:PRO237	Hydrophobic	Alkyl	4.72024
UNK1:C8-B:VAL157	Hydrophobic	Alkyl	4.68175
UNK1:C58-B:ILE129	Hydrophobic	Alkyl	4.48254
B:ALA240	Hydrophobic	Pi-Alkyl	5.21509
B:LEU242	Hydrophobic	Pi-Alkyl	5.22042

Table 5, shows that six amino acids are common in case of our combination drug of choice with established drug (Docetaxel + 5 fluorouracil). ALA240 (aa Alanine), VAL157 (aa Valine), LEU242 (aa Leucine), ILE129, PRO237 (aa Proline) and LEU 221 (aa Leucine) are the amino acids that mostly contains hydrophobic bonds. Most of them contains Pi-Alkyl type of bonds and distance of amino acid-ligand is from 4.6-5.4 angstroms. So, it will exhibit very good protein-ligand interactions.

3.5 Validation by using Ramachandran Plot

For this study, SPARC was used along with the combinations of (Atorvastatin + Pitavastatin), (Paclitaxel + Capecitabine), (Docetaxel + 5 Fluorouracil). The following graph is showing the result after the protein-ligand interaction takes place.



Number of residues in favoured region (~98.0% expected) : 218 (98.2%)
Number of residues in allowed region (~2.0% expected) : 4 (1.8%)
Number of residues in outlier region : 0 (0.0%)

Figure 11: Ramachandran Plot graph for established combinations of anti-cancer drug and drugs of different class with SPARC

Figure 11, shows that in Ramachandran Plot, the favoured region contains same amount of residue as per before which is 98.2 %. No residue is present in the outlier region and the allowed region also has only 4 residue. So, it can be said that the protein-ligand complex are validated.

3.6 Drug of choice

From the above mentioned processes and studies, the combination of (Atorvastatin + Pitavastatin) showed better efficacy with SPARC compared to all other drugs. While visualization by Discovery Studio, it was observed that the choice of combination drugs along with the established standards were placed in the same binding pocket. The bond angles of psi and phi bonds were not changed. However, the combination of (Atorvastatin + Pitavastatin) has a binding affinity of – 9.2 Kcal/mol. This combination was superimposed with our established market preparations. The Protein-Ligand interactions showed a wide range of similarities in amino acids, bonds, types and distance. So, the combination of (Atorvastatin + Pitavastatin) was considered as an anti-cancer drug of choice in the treatment of Stomach/ Gastric Cancer.

Chapter 4

Discussion

In case of tissue remodeling, secretion of SPARC as a glycoprotein is often seen. Inconsistent level of SPARC is seen in gastric tumor cell line which leads to tumor progression (Hohenester et al., 1997). Higher level of SPARC is often seen in gastric cell line due to poor diagnosis of the disease (Wang et al., 2004). Although, in various studies it was noticed that a lower level of expression can inhibit the growth of cancerous cells and reduces the chances of cancer in stomach/ gastric cell line (Yin et al., 2010). An increase in the signaling cascade is responsible to induce apoptosis when the level rise in a caspase-8 dependent manner (Tang & Tai, 2007). For that reason, a better understanding of the mechanism is a prime concern in designing anti-cancer combination drugs.

SPARC was selected as a protein of choice due to its variable expression in gastric cell line. Since, both the chain A and B were similar, one chain (chain A) was deleted for the ease of work. Heteroatoms like water molecule and associated ligand N-Acetyl-D-Glucosamine were also deleted prior to docking. Validation of the protein was carried out by using ERRAT, Verify3D, Ramachandran Plot and ProSA Web Server. An overall quality factor of 95.067 is considered as a very good result for a protein structure that has a resolution over 2.8 angstrom. A Z score value of -7.91 where it falls both in between the X-RAY and NMR region also plays an important role in validating the protein.

A number of drugs from the drug library were screened to make the possible combinations. A group of different combinations made by statin class of drugs which showed better binding affinity compared to others. The preparation of the combinations by using Avogadro software was a

challenging part since it involves the energy optimization process that leads to ensure the stability of the required structures. Greater binding affinity while performing both rigid and flexible docking suggested links between SPARC and the combination of Statins. Through, Discovery Studio the presence of amino acids, its types, distance and non-bonded interactions were measured. A more detailed date was useful in establishing the outcome of this study. Four amino acids were common in case of our combination drug (Atorvastatin+Pitavastatin) of choice with established drug (Paclitaxel + Capecitabine). ALA240 (aa Alanine), VAL157 (aa Valine), LEU242 (aa Leucine) and ILE129 are the amino acids that have hydrophobic bonds. All of them contains Pi-Alkyl type of bonds .The distance of amino acid-ligand stated in between 3.8-5 .2 angstroms. (Docetaxel + 5 fluorouracil) combination has ALA240 (aa Alanine), VAL157 (aa Valine), LEU242 (aa Leucine), ILE129 , PRO237 (aa Proline) and LEU 221 (aa Leucine) amino acids that mostly contains hydrophobic bonds and are found to be similar with the proposed drug (Atorvastatin + Pitavastatin) . Pi-Alkyl type of bonds and distance were seen in greater number and the distance between amino acid-ligand is from 4.6-5.4 angstroms. This data works in validating the proposed structure of combination drug (Atorvastatin + Pitavastatin) as a treatment of choice in stomach/ gastric cancer. Ramachandran Plot was used to further validate the SPARC-Drug complex and no significant changes were observed with the previous results. Previous studies have shown that Atorvastatin and Pitavastatin have anticancer properties. Atorvastatin with protein SPARC had a binding affinity of -9.6 kcal/mol. On the other hand, Pitavastatin showed a binding affinity of -10 kcal/mol. However, at higher dose, Pitavastatin showed side effects like rhabdomyolysis (De Wolf, De Wolf, & Richardson, 2017). Thus, a combination of (Atorvastatin+Pitavastatin) can be suggested for a better therapeutic response.

Chapter 5

Conclusion

Statin group of drugs were basically used to reduce the level of cholesterol in patient. Drug repurposing strategy was useful to understand their activity in disease like cancer. From the study, it was observed that a combination of (Atorvastatin + Pitavastatin) may significantly help in treating stomach/ gastric cancer by controlling the regulation of protein secretion. Application of several *in-silico* approaches suggested that a combination of (Atorvastatin + Pitavastatin) can exert its activity in reducing the over-expression of protein SPARC in gastric cell line. Combination of (Atorvastatin + Pitavastatin) showed promising binding result and strong protein-ligand interaction with SPARC. In conclusion, by evaluating the results of in-silico approaches it can be suggested that the combination of (Atorvastatin + Pitavastatin) has a potential future in becoming a counterpart of the already established combination drugs that are available in market to treat stomach/ gastric cancer.

Chapter 6

Future work

To evaluate the chemical stability and toxicity profile, further *in vivo* and *in vitro* studies should be carried out. Widespread acceptance of this combination drug can be achieved through concrete evidence from further studies. Thus, it can be established as a chemotherapeutic agent.

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