Drug Repurposing: Search for a Drug Having Effective Anticancer Activity

A project submitted

by

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Dedicated to my parents and siblings

Certification Statement

This is to certify that the project titled “Drug Repurposing: Search for a Drug Having Effective Anticancer Activity” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Professor Dr. Eva Rahman Kabir, Chairperson, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

_____

Countersigned by the Supervisor

_____

Acknowledgement

The blessings and mercy of the Almighty Allah who is the source of our life and strength of our knowledge and wisdom, has helped me to continue my study in full diligence which I hope will reflect in my project.

This research could not have been completed without the support of many people who are gratefully acknowledged here.

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Abbreviations

mTOR = Mammalian/mechanistic target of rapamycin

ADMET= Absorption, distribution, metabolism, excretion and toxicity

COX = Cyclooxygenase

PI3K = Phosphoinositide 3- kinase

FATC = FAT carboxy domain

mTORC = mTOR complex

RMSD = Route mean square deviation

AA= Amino acid

Abstract

In search of finding better and safer drugs and due to the high cost and decreasing productivity of novel drug discovery programs, scientists are now becoming more interested in finding new therapeutic indications for the existing drugs, popularly known as drug repurposing. In drug repurposing, a conventional drug is used to cure a condition which was not earlier known to be therapeutically effective. Many drugs which have failed clinical trials as they were not effective in their intended therapeutic indication have been repurposed. As a result they have led to huge fortune for the pharmaceutical industries. For instance, sildenafil failed its clinical trials and was repurposed and currently in use as a repurposed drug. Many methods are available for drug repurposing but computational docking is a very cheap and convenient method for drug repurposing which uses computer software to find a possible binding site of a drug within a protein. For its advantages, computational docking approach was used for the present drug repurposing study of mTOR protein, where the drugs chosen were metformin, aspirin and rosuvastatin. Autodock Vina and PyMol was used to complete the study and it was found that aspirin and metformin have poor affinity (-5.8 kcal/mol) for this protein which is upregulated in various types of cancer such as- breast cancer and ovarian cancer. On the other hand, rosuvastatin was found to have a high affinity (-7.8 kcal/mol in case of flexible docking and -10.2 kcal/mol in case of rigid docking) for mTOR and binds to the same binding pocket where the immunosuppressant and anticancer drug rapamycin binds. The study indicates that rosuvastatin might have significant immunosuppressive and anticancer activity by downregulating the activity of mTOR and needs further studies to prove it.

1.1 Introduction

As drug discovery and development program is highly expensive and it takes almost 10 years to complete, scientists are now looking forward to a new approach called drug repurposing. Drug repurposing is using an old drug for a new therapeutic purpose (Roder & Thomson, 2015). Due to the very high cost and risks associated with new molecule development, people are now becoming more interested in drug repurposing. Moreover, drug repurposing is found to be less expensive than the novel drug development as we use a drug that has been accepted in the medical community for many years. As a result, the drug is now available far more quickly than conventional drug discovery programs (Pessetto et al., 2013). In the last few years, scientists have developed various approaches for drug repurposing (Dudley, Deshpande & Butte, 2011). These approaches are-

a) using chemically identical drugs for the treatment of the same disease (Keiser, et al., 2009),
b) using drugs which have similar side effects (Campillos, 2008),
c) targeting drugs which have similarity in molecular activity (Li & Agarwal, 2009),
d) targeting drugs which have similar or the same molecular pathology (Pessetto et al., 2013).

Computational docking is one easy way to repurpose drugs in a very cost effective way. It tries to simulate the possible drug-receptor binding using *in silico* techniques. Therefore, computational docking approach was used for the present drug repurposing study of mTOR protein, where the drugs chosen were metformin, aspirin and rosuvastatin.

Drug repurposing or repositioning tries to find new therapeutic indications for an existing drug. This process is very efficient in discovering new indications because the existing drugs have-

i. well established formulations and their process of manufacture is also well known,
ii. known pharmacokinetic data (ADMET information or absorption, distribution, metabolism, excretion and toxicity data),
iii. qualified phase III or late stage clinical trials and that is why have less chances to fail due to safety issues,
iv. phase IV clinical trial data (Post-marketing surveillance) which ensures the drug's safety as well.

According to various studies 46 different drugs have been repurposed by now and many more are still being repurposed (Deotarse, Jain, Baile & Kulkarni, 2015). As the drugs have already been approved or they have already passed phase III or late stage clinical trials, this will provide these drugs leverage regarding the risk with the safety issues. Moreover, this process of finding new therapeutic indications can also be very lucrative as well as cost effective. For example, drug repositioning is a lot economical (approximately 8.4 million US dollars) than developing a new drug entity which costs billions of USD. Developing a new moiety has other backlashes such as it takes more than 10 years of time. Moreover, this process is even cheaper than launching a new formulation of a drug for its existing indication. In this way, the pharmaceutical companies can earn humongous amounts of money. Also, a company can tremendously increase its market share. For example, repurposing and redesigning thalidomide allowed Celgene to earn more than 2 billion USD (Deotarse, Jain, Baile &

Kulkarni, 2015). Furthermore, the return on investment of a repurposed drug is also very high as the repositioning cost is low. In addition to that, after repurposing a drug a company can get proprietary rights and gain lot of advantages over the competitors. Thereby, drug repurposing has a very good prospect in discovering new therapeutic indications (Deotarse, Jain, Baile & Kulkarni, 2015).

1.1.1 Metformin

Metformin is a drug of the biguanide class and still widely prescribed for type II diabetes mellitus (Figure 1.1). Recently it was found that it has significant anticancer property as well. According to Libby et al. (2009), diabetic patients taking metformin have a reduced risk of cancer by 37% which is a significant amount (Libby et al., 2009; Bridges, Jones, Pollak & Hirst, 2014). This intriguing study result piqued the interest of many researchers to repurpose this renowned drug. In various research done to shed light on the actual mechanism of metformin action, it has been found that metformin shows its anticancer activity by inactivating the mToR pathways (Zakikhani, Dowling, Fantus, Sonenberg, & Pollak, 2006; Leclerc, Leclerc, Kuznetsov, Desalvo, & Barredo, 2013; Nair, et al., 2014). Moreover, some other pathways are also involved in this anticancer activity of this drug such as the AMPK pathway and others such as RAS, HIF-1 and AKT may also play a role here (Zakikhani, Dowling, Fantus, Sonenberg, & Pollak, 2006). Some researchers have also proposed that metformin works on mitochondria and builds mechanical stress on the cancer cells (Andrzejewski, Gravel, Pollak, & St-Pierre, 2014). A very recent study by Sun et al. have tried to build a signaling pathway of genes using which metformin shows its anticancer effects and proposed seven genes and another MYC-centered pathway that might be acting in this regard (Sun et al., 2017).

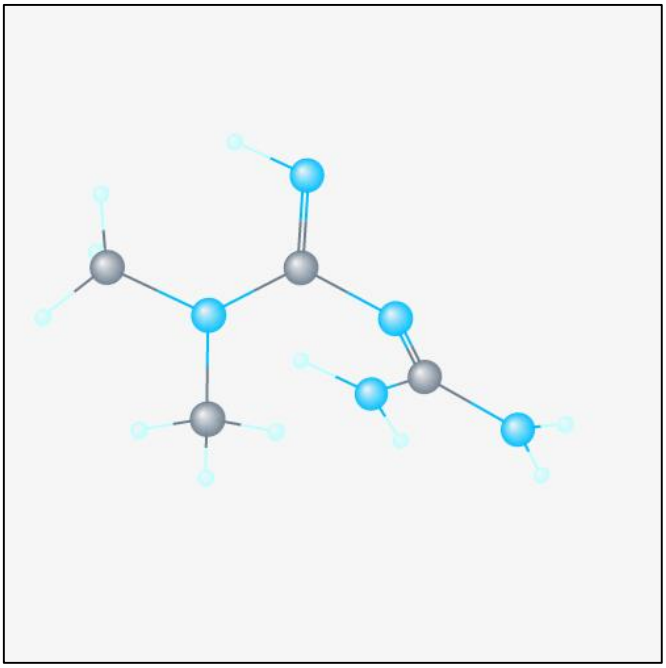

Figure 1.1: Structure of Metformin (Obtained from NCBI database) (Voter, Manthei, & Keck, 2016).

1.1.2 Aspirin

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) sold as over the counter (OTC) all over the world (Figure 1.2). It is used to treat pain, fever as well as inflammation. The mechanism of its analgesic, antipyretic and anti-inflammatory effect is nonselective inhibition of the cyclooxygenase (COX) iso-enzymes (Vane, 1971). The COX-1 and COX-2 iso-enzymes convert arachidonic acid to form prostaglandins (PG) and related compounds like prostacyclins and thromboxanes (Vane, Bakhle, & Botting, 1998). COX-1 is called the constitutive enzyme that produces various prostaglandins and controls physiological functions like production of mucus in stomach and protection from gastric acid. On the other hand, COX-2 is called the inducible enzyme that is induced by various cytokines and chemical

messengers to initiate inflammatory processes and that is why they are mostly expressed in the inflammatory cells (Masferrer et al., 1994). Recently, aspirins anticancer property was reported by researchers (Burn, et al., 2011). For this reason aspirin was chosen for this study.

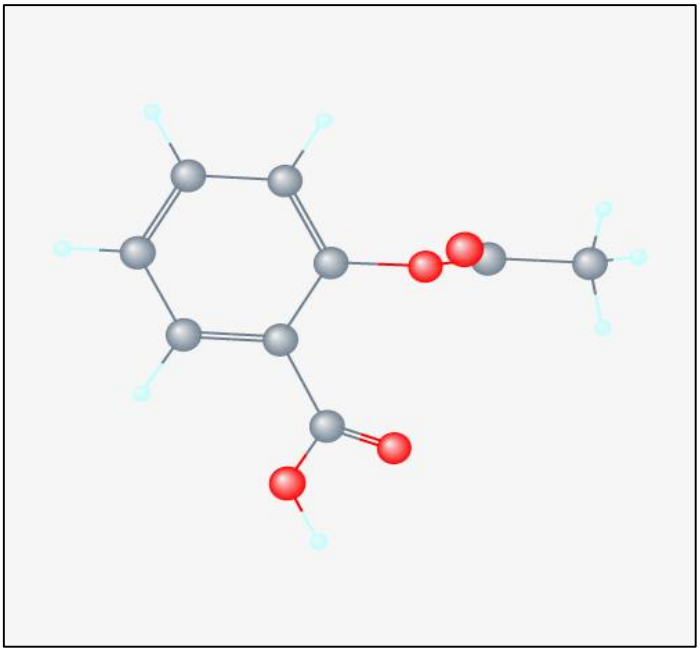

Figure 1.2: Structure of Aspirin (Obtained from NCBI database) (Saunders, et al., 2006).

1.1.3 Rosuvastatin

Rosuvastatin, a completely synthetic statin or HMG-CoA reductase inhibitor, is one of the statin molecule used for the treatment of hyperlipidemia and lowering of LDL cholesterol, as well as total cholesterol levels (Figure 1.3). Though its chemical structure has similarity with the previous statins, the chemical modifications allows it to bind with greater affinity with the enzyme HMG-CoA reductase. It is proven in many scientific studies that statin treatment reduces the risks associated with cardiovascular diseases. Statins have also shown to be effective in lowering cardiovascular risks associated with ischaemic heart diseases. Moreover, it was seen that these drugs ameliorate the condition of endothelium and also stabilize the

plaques formed by atherosclerosis. They also reduce the risk of thrombosis and inflammation in the walls of the arteries. This is why rosuvastatin is used in the primary prevention of cardiovascular diseases and it is quite effective as well. Rosuvastatin's possible anticancer activity was reported recently. It is a safe drug and what other beneficiary effect it may have was the first reason why it was chosen for this docking study (Barth, Luvai, Mbagaya & Hall, 2012).

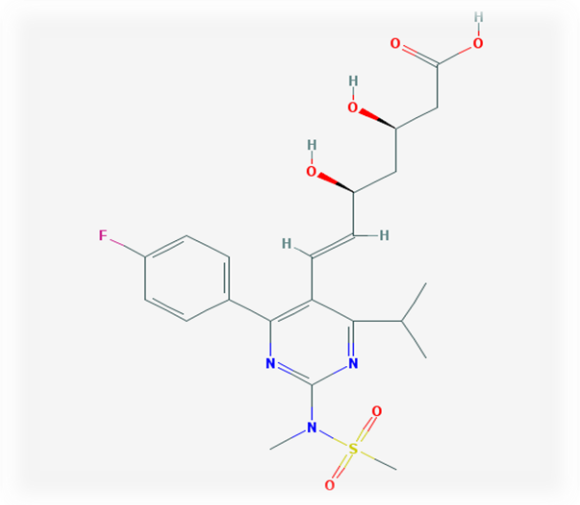

Figure 1.3: Structure of Rosuvastatin (Obtained from NCBI database) (Stein, 2001).

1.1.4 Rationale

Drug repurposing (or repositioning) tries to find new therapeutic indications for an existing drug. This process is very efficient in discovering new indications because the existing drugs have-

- **i.** well established formulations and their process of manufacture is also well known,
- **ii.** known pharmacokinetic data (ADMET information or absorption, distribution, metabolism, excretion and toxicity data),

iii. qualified phase III or late stage clinical trials and that is why have less chances to fail due to safety issues,

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As the prevalence of cancer is increasing day by day, safe chemotherapeutic and chemopreventive drugs are sought by the scientists as well as by the patients. Anticancer properties of numerous existing drugs are being studied worldwide. According to Libby et al. (2009), diabetic patients taking metformin have a reduced risk of cancer by 37%. Moreover, aspirin was reported to have some significant anticancer effect in colorectal cancer (Burn et al., 2011). According to Sharkawi, Shemy & Khaled (2014) rosuvastatin may have anticancer activity as well (Sharkawi, Shemy, & Khaled, 2014). Unfortunately, for these drugs the underlying mechanism behind their anticancer effect is not well established. The present study considered metformin, aspirin and rosuvastatin as candidates for repurposing as they generally do not harm the neoplastic cells. These drugs were chosen since they are being worked on for their anticancer properties. As these drugs are already in use it will save a lot of development cost and no clinical trials would be needed as these drugs have already passed them. For these reasons, the current study was undertaken in search for safe anticancer drugs.

1.2 mTOR

mToR (Mammalian/Mechanistic Target of Rapamycin) is a kinase enzyme belonging to the PI3K (Phosphoinositide 3- kinase) family that controls various intracellular processes (Figure 1.4). It is a serine-threonine kinase enzyme present in mammals which is coded by a single gene and forms various complexes with proteins which are mToRC1 (mToR complex 1) and mToRC2 (mToR complex 2) (Wullschleger, Loewith & Hall, 2006; Hay, 2004). The key functions of this enzyme is to control cellular growth, cellular proliferation, autophagy as well as protein synthesis and survival (Yuan, Kay, Berg & Lebwohl, 2009; Shaw & Cantley, 2006). Where mToRC1 regulates different types of cap-dependent translation, mToRC2 controls actin which is used to make cytoskeleton and also regulates spine structure and memory (Huang and Manning, 2009; Darnell & Klann, 2013). Moreover, it senses and then takes action based on various signals generated from actions like nutrient intake and growth factors or other stimuli to control downstream processes such as protein synthesis. Using this regulation mToR prevents cells from growth and replication when there is a lack of nutrients and when it is abundant mToR allow cells to grow and divide (Shaw & Cantley, 2006). Furthermore, it has been seen that in various types of cancer there is a loss of this function and for this reason it should be a good target for drug repurposing (Crespo & Hall, 2002; Bjornsti & Houghton, 2004). For example, in breast cancer mTOR is dysregulated and rapamycin is used to treat alongside other anticancer drugs (Zagouri, Sergentanis, Chrysikos, Filipits, & Bartsch, 2012; Wheler et al., 2016). It is also dysregulated in ovarian cancer and that is why it should be researched (Campos, 2011).

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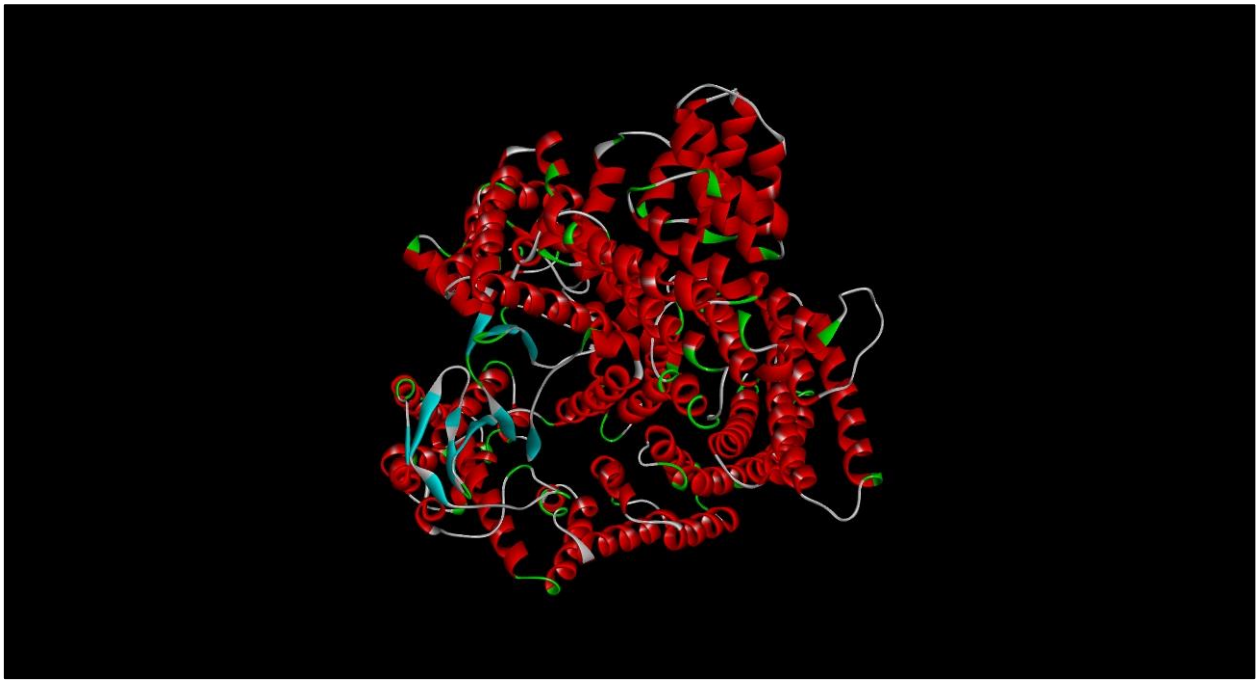


Figure 1.4: Structure of mTOR (Obtained from NCBI Database and Visualized in Biovia Discovery Studio v4.5) (Pavletich & Yang, 2013).

As mTOR controls cellular growth, proliferation as well as survival, its signaling is often enhanced in cancer and for this reason it is of great interest to scientists to develop drugs targeting this enzyme. Rapamycin, an mTOR inhibitor and its other analogs are used in the treatment of certain cancers and they bind to a domain which is separate from the catalytic site of the enzyme. Thus mTOR inhibitors control some functions of this enzyme and show their anticancer effect. This group of drugs are very selective for this enzyme and they are being used clinically for the treatment of many cancer types, but the problem is they can turn on the mTOR dependent survival function which might lead to the failure of the treatment. For this reason, scientists are trying to find mTOR inhibitors who do not activate this survival pathway.

1.2.1 Phosphatidylinositol 3-kinase (PI3K) Enzyme and Related Kinase Enzymes

mTOR is a serine-threonine protein kinase enzyme whose catalytic domain has great resemblance with the catalytic domains of lipid kinases of PI3K family. According to their structure and substrate specificity the enzymes can be subdivided into 3 groups. PI3k enzymes phosphorylate the inositol lipids and give a cascade of reactions. Class I enzymes of PI3k enzyme family have four catalytic segments which are different to each other and they form heterodimers with different regulatory subunits. The catalytic subunits are p110α (p110-alpha), p110β (p110-beta), p110γ (p110-gamma) and p110δ (p110-delta). These iso-enzymes do the task of phosphorylating phosphatidylinositol 4, 5-diphosphate in the body. The structure activity relationship (SAR) of various PI3K inhibitors were discovered using the various crystal structures of p110γ attached to ATP and other inhibitors. The alpha subunit, in other words p110α binds to a specific regulatory subunit. Class II (C2) of PI3K enzymes have 3 subtypes- C2α, β and γ and they phosphorylate PI as well as PI(4)P outside the body and they have a unique C2 homology that is not present in other PI3Ks. The class III of PI3K enzymes may have a role to control functions of mTOR and they phosphorylate PI (Marone, Cmiljanovic, Giese, & Wymann, 2008). On the other hand, the type IV PI4K (PI4-Kinase enzymes) phosphorylate the inositol rings' 4′-hydroxyl group and they closely resemble PI3Ks.

mTOR is a member of the PI3K-related protein kinase enzyme family also known as the PIKKs. This group also includes ATM/ataxia-telangiectasia mutated, DNA dependent PK (Protein kinase), Rad3-related called ATR and SMG-2/suppressor of morphogenesis present in genitalia-1 (Marone, Cmiljanovic, Giese, & Wymann, 2008; Wullschleger, Loewith & Hall,

2006; Showkat, Beigh, & Andrabi, 2014). These proteins are quite large, weighing almost 300 to 500 kDa (kilo Daltons). In addition, they have different regions present such as a conserved kinase catalytic domain (KD) and other domains like HEAT repeats, FATC (FAT carboxy terminal) and FAT domains, repressor domain or RD domain which is an autoinhibitory domain. The HEAT repeats stay in tandem to each other and form a superhelical structure with other large interfaces and it facilitates interaction between proteins. After the HEAT segments, lies the FAT domain which is followed by FRB domain (FBBP12-rapamycin binding site), where rapamycin binds to the protein FK506-12. After this domain the KD/kinase domain, Repressor domain and FATC domain lie after one another in a line. Moreover, the FATC domain is a must for the kinase activity of this enzyme (mTOR). Even upon the deletion of one amino acid molecule it loses its catalytic activity. The FATC and FAT domains interact with each other and this interaction most possibly leads to expose the catalytic domain, controlling the catalytic kinase activity of mTOR (Showkat, Beigh, & Andrabi, 2014).

The protein mTOR has also a binding site for the anticancer drug rapamycin called FRB shown in Figure 1.5. This is sometimes referred to as FKBP12-rapamycin-binding domain. This site allows for the rapamycin-FKBP12 complex to bind with mTOR. The other proteins like SMG-1, ATM, DNA-PK and ATR control surveillance of DNA and mRNA and also the repair pathways. Thus, mTOR actually combines signals generated by growth factors in presence of adequate nutrients and energy with permissive factors to control growth, survival and proliferation. Since, signaling of mTOR is upregulated or enhanced in cancer cells, proliferative disorders and some growth disorders, targeting the activity of this enzyme is thought to stunt the progress of these disorders (Showkat, Beigh, & Andrabi, 2014).

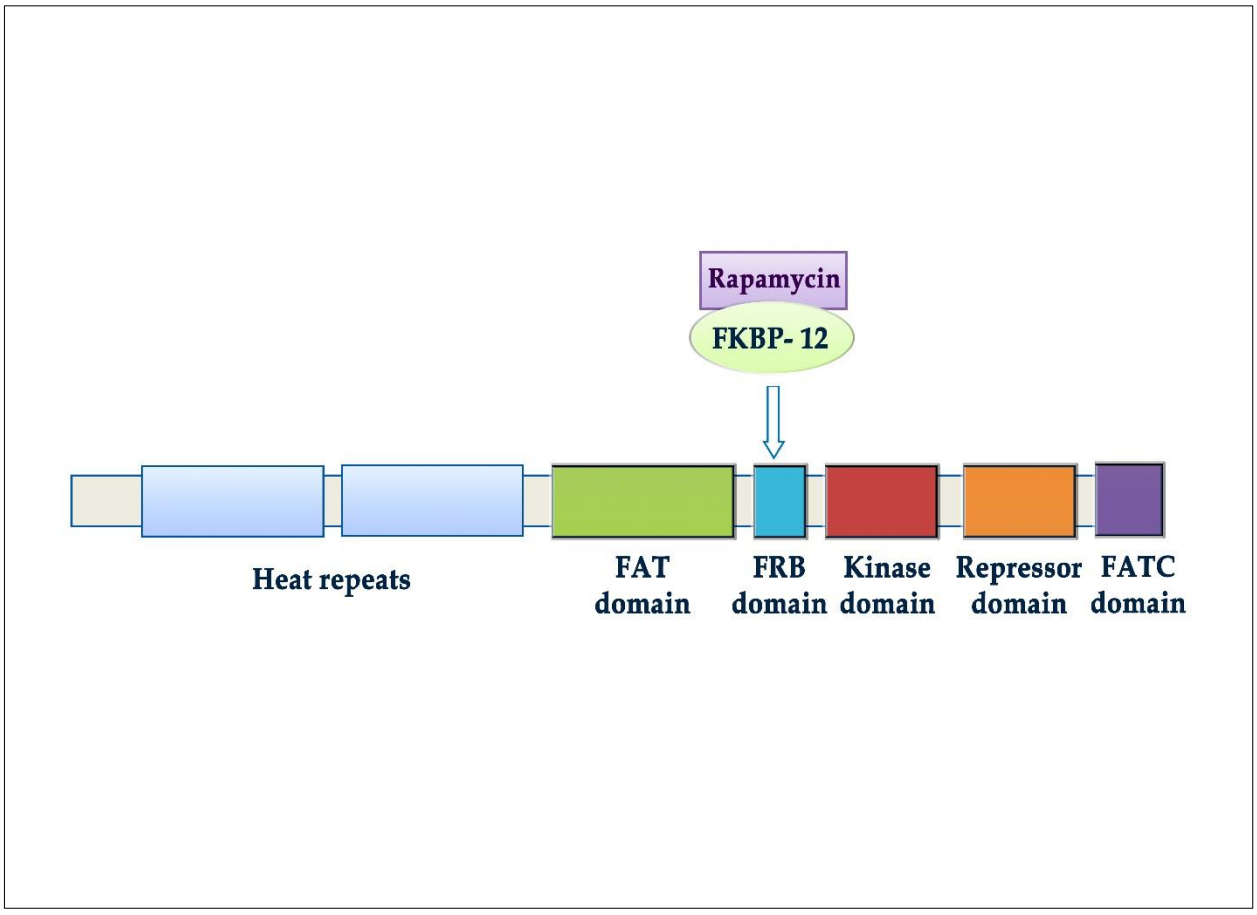


Figure 1.5: Schematic Representation of mTOR Domain Structure (Showkat, Beigh, & Andrabi, 2014).

1.2.2 mTOR Signal Transduction and Cellular Functions

mTOR pathway controls cell growth and proliferation using the signals it receives from mitogen, nutrients as well as from energy status within the cell. It is found to be dysregulated in cancer, diabetes and other disorders. In recent studies, it has been seen that mTOR signaling plays a vital role in tumorigenesis and it is reported to be activated in many cancers in humans such as breast cancer, ovarian cancer etc (Zagouri, Sergentanis, Chrysikos, Filipits, & Bartsch, 2012; Wheler et al., 2016; Campos, 2011). Moreover, rapamycin, a partial inhibitor of mTOR kinase enzyme activity, has played a huge role in unveiling mTOR, to understand its mechanism of action within the body. The major criterion which was used to determine mTOR

controlled events is rapamycin sensitivity, but now newer means are available as well. At present we know that mTOR binds to various regulatory subunits to form complexes that have distinct signaling functions as well as distinct rapamycin sensitivity.

The mTORC1/mTOR complex I is composed of mTOR, mLST8 (also known as GβL/G-protein-β-subunit-like protein), deptor (death domain that contains the proteins interacting with mTOR), PRAS40 (proline rich 40 kDa component called AKT/PKB substrate) and raptor (mTORs regulatory associated protein) which is shown in Figure 1.6 (Beauchamp & Platanias, 2012). Rapamycin inhibits mTORC1 functions by binding with FKBP12 which interact with mTOR alone or mTORC1 in bringing conformational changes. This is how rapamycin shows its mTORC1 effects. mTORC1 controls translation by phosphorylating S6K1 (S6-kinase-1) at Thr389. 4EBP1, the translational suppressor is also phosphorylated by it and is rapamycin sensitive as well.

Raptor (regulatory associated protein) is a protein associated with mTORC1 that weighs 150 kDa and has one N-terminal which is highly conserved. After that lies the HEAT repeats (in tandem) and then the 7 WD40 repeats present in the half of C-terminal. Raptor binds strongly using its N-terminal (which contain the HEAT repeats) with mTOR. This complex functions as a major scaffold protein by forming a link between mTOR and its substrates S6K1 and 4E-BP1, thus controlling mTORC1 activity mediated by the mitogenic signals. Raptor proteins phosphorylation state controls the activities of mTORC1 and when it is phosphorylated at S722 and S792 by 5 prime AMPK/AMP-activated protein kinase it is inactivated. On the other hand, when raptor is phosphorylated at position S863 by mTOR, mTORC1 becomes activated as a result of mitogenic stimulation. In addition, PRAS40, which is another subunit of mTORC1 is a down-regulator of this complex. Initially it was thought that PRAS40 was an

AKT substrate as it was directly phosphorylated by AKT at T246 position by insulin. In later studies, it has been found that PRAS40 is an inactivator of mTORC1 and is phosphorylated on S183 by mTORC1 when it is associated with mTORC1 through raptor. However, this binding is abolished if a mutation occurs on S183 by an aspartate molecule. Moreover, the function of mLST8 is not well understood and its removal is found to also not hamper the function of mTORC1 within the body (Ballou & Lin, 2008; Showkat, Beigh, & Andrabi, 2014).

Different growth factors like nutrients regulate the activity of mTORC1. The complex of tuberous sclerosis (TSC1-TSC2) inhibits mTORC1. On the other hand, mTOR complex I is activated by the PI3K/AKT pathway. PI3K has other downstream targets such as proteins and also enzymes like S6K1 and AKT (a serine-threonine kinase enzyme). When PI3K is activated by the growth factors, it phosphorylates phosphatidylinositols (at D3 position) and produces PtdIns-3, 4, 5-triphosphate which is a second messenger system. This second messenger binds to pleckstrin homology (PH), a domain of AKT and this binding moves the kinase enzyme PI3K to plasma membrane and upstream kinase enzymes activate it there. PDK1 enzyme phosphorylates T308 molecule of AKT and mTORC2 does that at position S473. These 2 phosphorylation are a must for complete activation of kinase activity. PTEN (Phosphatase and tensin homologs) has a negative effect on AKT activation and it does that by dephosphorylating PtdIns-3, 4, 5-P3 to its diphosphate form and leads to decreased transport of AKT to cell membrane. Two activated AKT substrates which are its downstream substrates, are involved in down-regulating the activities of mTORC1, PRAS40 and TSC2 and they are phosphorylated and thus inhibited by AKT. Rheb GTPase (Ras homolog which is abundant in brain) is a positive regulator of mTORC1 and it shows this activity by binding

directly to the catalytic domain of mTOR. TSC1 and TSC2 form a complex between themselves and they down-regulate mTORC1 activity. This is done by performing as a Rheb GAP which is a GTPase activation protein and it is involved in the conversion of Rheb into its inactive form, its GDP bound form. When growth factors stimulate AKT, it can directly phosphorylate TSC2 at multiple locations and prevents it to form complex with TSC1, thus allowing Rheb to turn back to its active form/GTP-bound state and eventually leads to mTOR activation. Morover, PI3K/AKT pathway acts as activator of mTORC1 and Rheb by phosphorylating and inhibiting TSC2 and thus impairing its function to inhibit Rheb (Ballou & Lin, 2008; Showkat, Beigh, & Andrabi, 2014)

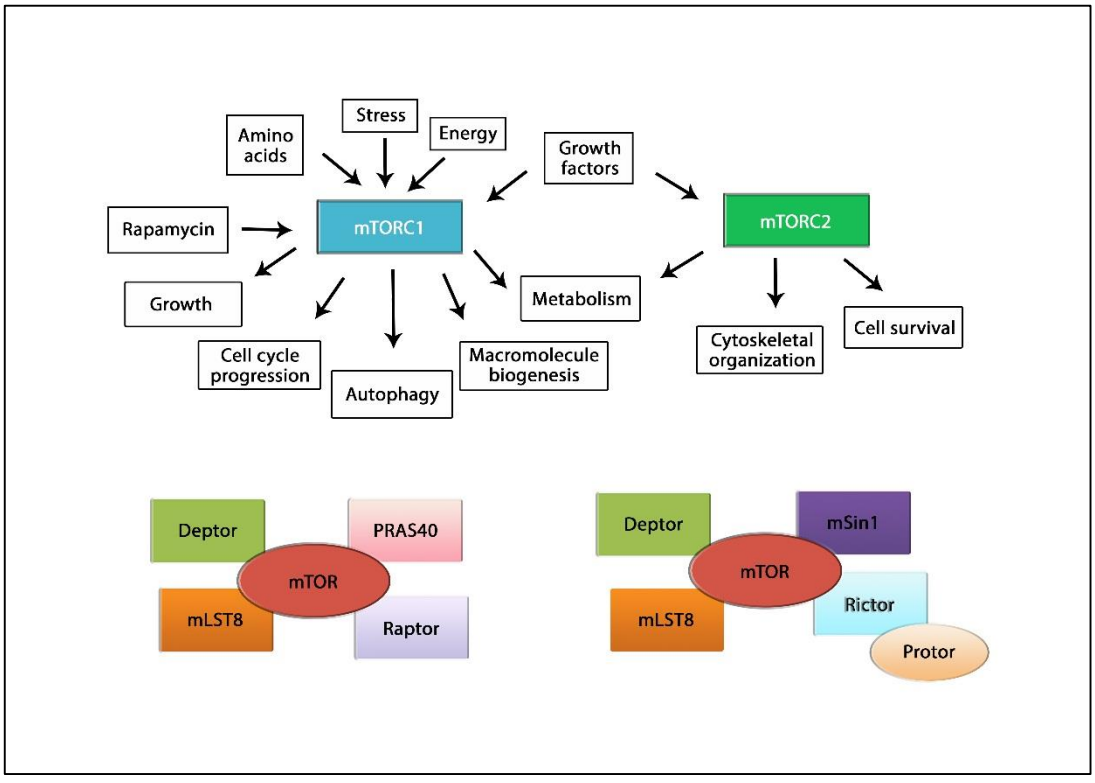


Figure 1.6: mTORC1 and mTORC2 Complexes and their Function (Showkat, Beigh, & Andrabi, 2014).

mTORC2 is composed of the following subunits- RICTOR (mTOR's rapamycin insensitive domain), mLST8, PRAS4, Protor (protein which is observed with RICTOR), deptor and mSin1 (a mammalian SAPK enzyme/stress activated protein interacting protein 1) (Zoncu, Efeyan, & Sabatini, 2010). Initially it was thought that this complex is not sensitive to high dose rapamycin treatment. RICTOR has mTOR complex and it is not bound to FRB domain (FKBP12-rapamycin binding protein) and that is why it was thought to be insensitive. Further research showed that prolonged treatment blocks the mTORC2 assembly and function as well. mLST8 and deptor, both are present in mTORC1 and mTORC2 where deptor down-regulates both mTOR complexes. mLST8 only affects the function of mTORC2. Another important subunit of mTORC2 complex is mSin1 and it is an essential component of this complex. Absence of it abolishes activity as its absence hampers the interaction of mTOR and RICTOR. Protor has interaction with RICTOR as well, but it is not a must for the assembly of the various subunits of mTORC2 complex (Pearce, et al., 2007).

mTORC2 controls many functions like apoptosis (controlled by activated-AKT), glucose metabolism, cell growth and proliferation. mTORC2 regulates these activities by phosphorylating AKT at S473 position and thus activating it to control the above mentioned cellular processes. mTORC2 also mediates the phosphorylation of a conserved domain of AKT and thus mTOR controls both upstream (controlled by mTORC2) and downstream (regulated by mTORC1) processes of AKT. Protein kinase Cα (PKCα) and SGK which means serum and glucocorticoid-inducible kinase enzymes are also essential substrates of mTORC2. mTORC2 further regulates protein kinase C maturation and its stability and actin cytoskeleton's organization (Showkat, Beigh, & Andrabi, 2014).

1.2.3 mTOR inhibitors

Rapamycin also known as Sirolimus is an antibiotic of macrolide class and it is a natural product made by the *Streptomyces hygroscopicus* bacterium. This bacterium can be found in the Easter Island soil. It was discovered as a powerful antifungal agent. However, after its discovery, scientists had observed another troublesome side effect which was immunosuppression and this side effect eventually allowed the scientists to develop a very useful immunosuppressant drug. Later their anticancer activities were detected and they are now used as anticancer drugs (Zagouri, Sergentanis, Chrysikos, Filipits, & Bartsch, 2012; Wheler et al., 2016). Rapamycin and tacrolimus (FK506) are structural analogs and these two drugs bind to the same protein receptor inside the cell which is FKBP12. These two drugs still differ in their mechanism of action though they bind to the same intracellular receptor. Cytokine signaling is affected by rapamycin and this is how rapamycin shows its activity. On the other hand, tacrolimus (FK506) shows its activity by inhibiting T cell proliferation. It blocks Ca (II) ion and calcineurin dependent transcriptional activation of genes which is essential for growth and thus it blocks T cell proliferation. S6K activation induced by interleukin-2 (IL-2) within a T cell has been found to be highly sensitive to inhibition by rapamycin. In comparison, mTORC1 kinases were found to be much less sensitive to FKBP12/rapamycin in vitro. The reason behind this sensitivity difference is not well understood at all.

Alongside rapamycin, other analogs of rapamycin known as rapalogs are now clinically used in human beings. Temsirolimus (CCI779) is a rapamycin's prodrug. It is in dihydroxymethyl propionic acid ester form of rapamycin and this allows the drug to be water soluble and thus it can be used intravenously. When injected temsirolimus quickly converts to rapamycin and

this is how all or most of its actions in the body is generated. Everolimus (RAD001), at its C40 position contains an ortho-2-hydroxyethyl substitution that is a chain substitution. Deforolimus on the other hand has a substituted phosphine oxide and it is at the same position on the rapamycin's lactone ring. All the rapamycin analogs (rapalogs) work in the same way like rapamycin *in vivo*. They interfere with mTOR's FRB domain by binding to FKBP12 and it's the same for all rapalogs.

Kinase inhibitors are drugs that target the ATP-binding domain which is the catalytic site basically. Unlike these drugs, rapalogs are much more selective for mTORC1. Unfortunately, the underlying mechanism of action on FRB interaction which inactivates mTORC1 is still not clearly understood. 4EBP1 is stopped from getting phosphorylated in vitro and also mTOR's autophosphorylation is inhibited by FKBP12/rapamycin and it suggests that the changes that occur in the FRB site may have an allosteric action on the catalytic site. Initially it was thought that rapamycin only interacts with FRB site, just after binding with FKBP12. FKBP12-rapamycin-FRB complex crystal structure obtained via X-ray crystallography shows us that rapamycin sits simultaneously in two hydrophobic binding sites within FKBP12 and FRB domain and thus brings the two protein parts closer to each other. However, the two proteins do not interact with them much. Eventually, rapamycin was found to be binding to FRB domain in absence of FKBP12, but with a low affinity. By the help of this information and combining it with the structure of FRB domain obtained by a solution NMR, Leone et al. have made a chemical library and they have also discovered some small molecules capable of binding to FRB domain when FKBP12 is absent.

1.3 Molecular Docking

Molecular docking is an approach that predicts the interaction between a protein molecule (macromolecule) and a small molecule (ligand) at atomic level. It allows us to obtain the binding behavior of the small molecules and also to attain information on the basic biochemical processes. This method involves two basic steps: the first step is predicting how the small molecule's conformation would be within the binding pocket along with its orientation and position in the binding pocket (known as the pose) and the second step involves the calculation of the binding affinity. These steps are associated with sampling and scoring functions of the docking tool (Meng, Zhang, Mezei, & Cui, 2011).

If we know the location of the binding sites within the protein or macromolecule before performing the docking process, it significantly enhances the docking efficiency. Most of the time, the researchers know the locations of the binding domain before running the docking function. Moreover, the information on the probable binding site can be obtained by comparing the protein with another known protein that has a high similarity with it or with proteins that are co-crystallized with different ligands. If knowledge of the binding sites is not known, online servers or binding pocket detecting programs like GRID, Surfnet, POCKET, MMC and PASS are available that can give information on the possible binding sites within the proteins. If we dock without any prior information on the binding sites then it is termed blind docking.

Lock and key is a theory proposed by Fischer and was used for the early elucidation of ligand-receptor binding mechanism. According to this theory, the ligand is like a key that fits into the binding site of the receptor, like a key is fitting inside a lock. The earliest attempts of

docking used this theory and considered that the ligand molecule and the protein/receptor were both rigid. Then came the "induced fit" theory given by Koshland that says that the binding site of the receptor protein is continuously modified due to the interactions with the various ligand molecules. This theory considers the receptor and ligand molecules flexible and reshape themselves during the docking procedure (or ligand-receptor binding). This resulted in the increased accuracy of the docking procedure than the previous rigid docking mechanism.

Due to limitations in computer resources, the conventional method uses a ligand that is flexible and a receptor that is rigid in nature. This method is still found to be the most popular way of docking. At present, scientists are trying to run docking that considers a receptor flexible in nature. However, flexibility in receptors is still posing a major problem for the docking methods that are currently available.

1.3.1 Theory of Molecular Docking

The first and foremost aim of molecular docking is to obtain a prediction of the complex of ligand and the receptor and we do that by computational methods. It can be achieved by two interconnected steps. Firstly, the different conformations of the ligand that are possible in the binding pocket of the protein are sampled and then these conformations are ranked by using a scoring function. Sampling algorithms are ideally supposed to reproduce the experimental binding mode and then, the scoring function is supposed to rank them from highest to lowest based on all of the generated conformations (Meng, Zhang, Mezei, & Cui, 2011).

1.3.2 Sampling Algorithms

The different binding modes that are possible between the ligand and the receptor molecule is a large number as there is six degrees of both rotational and translational freedom and freedom of conformational degrees between the drug and ligand molecules. However, it would be very expensive to generate every possible conformation. Different sampling algorithms are designed and developed for this purpose and they are widely used by various docking software. The various sampling algorithms are matching algorithms, incremental contruction or IC method, MCSS or multiple copy simultaneous search and LUDI, genetic algorithms (GA), monte carlo (MC) and stochastic methods and molecular dynamics (MD) method (Halperin, Ma, Wolfson & Nussinov, 2002; Meng, Zhang, Mezei, & Cui, 2011). The different algorithms and their method of operation is shown below in Table 1.1.

Table 1.1- Different Sampling Algorithms and their Method of Operation.

Algorithms	**Characteristic**
Matching algorithms	Geometry-based approach
Molecular dynamics	Can allow further refinement docking
Monte carlo	Utilizes stochastic search
LUDI	De novo drug design (fragment-based)
Incremental construction	Fragment based approach
MCSS	De novo drug design (fragment based)
Genetic algorithm	Utilizes stochastic search

1.3.3 Scoring Functions for Molecular Docking

Using the scoring function we try to determine the correct poses of the ligands in the binding pocket and eliminate the incorrect poses. In other words, elimination of the inactive compounds from the binders need to be done as quickly as possible. Scoring functions estimate the binding affinity between the protein and receptor molecule rather than calculating it. Using the various functions different simplifications and assumptions were adopted. The different scoring functions are force field based scoring function, empirical and knowledge based (Halperin, Ma, Wolfson & Nussinov, 2002; Meng, Zhang, Mezei, & Cui, 2011).

1.3.4 Docking Methodologies

There are three different ways by which docking can be done, as mentioned below:

1. **Rigid ligand, rigid receptor docking**

In this method, the docking software considers both the ligand and receptor as rigid components. The search space is very small and allows only three rotational and translational degrees of freedom. For example- DOCK, FLOG and FTDOCK used this method (Meng, Zhang, Mezei, & Cui, 2011).

2. **Flexible ligand, rigid receptor docking**

This method considers only the ligand as flexible and the receptor a rigid body. In other words, the ligand changes its conformation during docking. Almost all the docking software use this method as it is cheap and also saves a lot of computational time. E. g- AutoDock (Meng, Zhang, Mezei, & Cui, 2011).

3. Flexible ligand, flexible receptor docking

This method considers change in shape of both the ligand and receptor during docking. The problem with this approach is that generation of flexibility in the receptor is a very challenging task. This method involves a longer computational time as well as increases the computational expense to a great extent. E. g- AutoDock 4 has flexibility in side chain (Meng, Zhang, Mezei, & Cui, 2011)

2. Methodology

The study started with extensive literature review on the topic followed by molecular docking to ascertain the binding affinities between the ligand and receptors.

Computational docking requires 3D structures of the ligand and macromolecule or protein molecule. Different databanks were used for this purpose where the structure of the macromolecules or receptor protein molecules are available. From different data banks like Protein Data Bank (PDB), Pubchem (for ligand structures) the different structures were obtained.

2.1 Software for Docking and Visualization

Various software can be used for docking and visualization purposes, such as - PyMOL, AutoDock Vina, BIOVIA Discovery Studio, AutoDockTools (also known as MGLTools). Open Babel was another software which helps in converting file formats. PyMOL was used for modifying and visualizing the 3D structure of proteins and ligands. Several structures of the macromolecule was found to contain unnecessary water and other compounds being attached to the protein molecule that needed to be purified and modified before starting computational docking. This modification of the macromolecules (proteins) was also facilitated by PyMOL. Discovery studio was another software that was used for the visualization of the ligand and receptor molecule and validation of the results of docking. In addition, AutoDock Vina was used to run the docking procedure. AutoDockTools (ADT) is an extension of AutoDock Vina that allowed the modification of the polarity of the

macromolecule. Polar hydrogen was added to the protein making the protein more reactive so that it could easily bind to ligand molecules. Furthermore, ADT also allowed to fix the rotatable bonds of a ligand molecule. Thus, the ligand was made flexible and a flexible ligand docking was performed which mimics the binding behavior of drugs in the body. In addition, a specific portion of the protein molecule was selected to perform site specific docking. Open Babel was used whenever the files required to be converted to a particular format. The software used are given below with their versions in Table 2.1.

Table 2.1 – Software used in this Drug Repurposing Study.

Serial	Software used	Version
01	AutoDock Vina	1.1.2
02	AutoDock Tools	1.5.4
03	Discovery Studio	4.5
04	PyMol	1.8.4.0
05	OpenBabel	2.4.0

2.2 Visualization and Subsequent Docking

Firstly, PyMOL was used to purify the protein structure, mTOR (mammalian target of rapamycin) that had multiple ligand and protein segments attached to it. For example, mTOR had mLST8 protein subunit attached to it that was removed using Discovery Studio. It also had Mg ions and AGS (ATP gamma S) molecules attached that were removed as well. After the removal of all the groups, AutoDockTools was used to change the polarity of the protein mTOR (by adding polar hydrogens to it) and saved in the format suitable for AutoDock Vina.

The ligand molecules were then opened in AutoDockTools and the maximum number of torsions possible were allowed from the torsion tree menu and the files were saved for subsequent docking. These torsions made flexible ligand docking possible. Moreover, the most recent version of AutoDock Vina generated side chain flexibility as well as made the procedure for receptor docking flexible giving the most accurate prediction of the binding between the ligand and drug molecule. The coordinates for specifying the area within the protein was also needed and the area was specified using the Grid box from the Grid menu of ADT. The results of docking was obtained after sampling and scoring functions calculated the binding affinities.

3. Result and Discussion

3.1 Result and Discussion of Aspirin's Flexible Docking

The binding affinities for aspirin's flexible docking with mTOR are given below in Table 3.1-

Table 3.1: Results of Docking of Aspirin and mTOR.

Mode or Binding pose	Affinity (kcal/mol)	Distance from best mode RMSD lower bound (l. b.) (in Angstrom)	Distance from best mode RMSD lower bound (l. b.) (in Angstrom)
1	-5.8	0.000	0.000

Several docking simulation was performed but from the binding affinities it was seen that aspirin has a poor affinity (Trott & Olson, 2010) for mTOR receptor. Visualization was carried out as a confirmation that aspirin and mTOR did not have a strong binding affinity with mTOR.

After analyzing the binding affinities and RMSD distance values, the binding pocket of aspirin was visualized and it is shown in Figure 3.1.

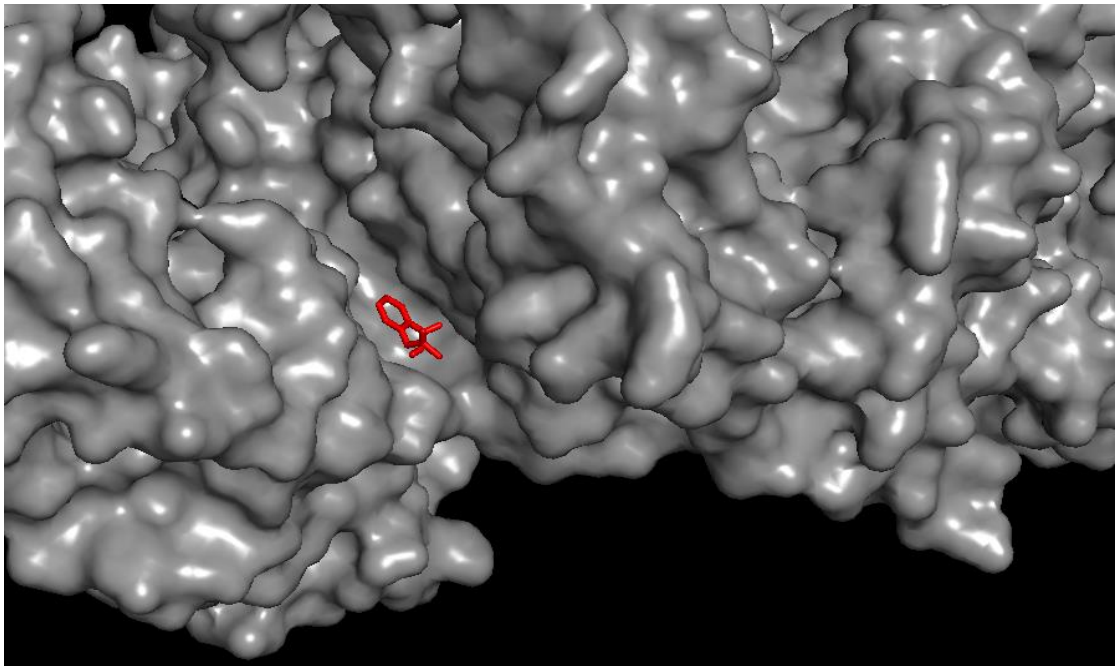


Figure 3.1: Binding Pocket of Aspirin within mTOR (Visualized in PyMOL v1.8.4.0).

The following Figure 3.2 shows the different bonds formed between aspirin and mTOR:

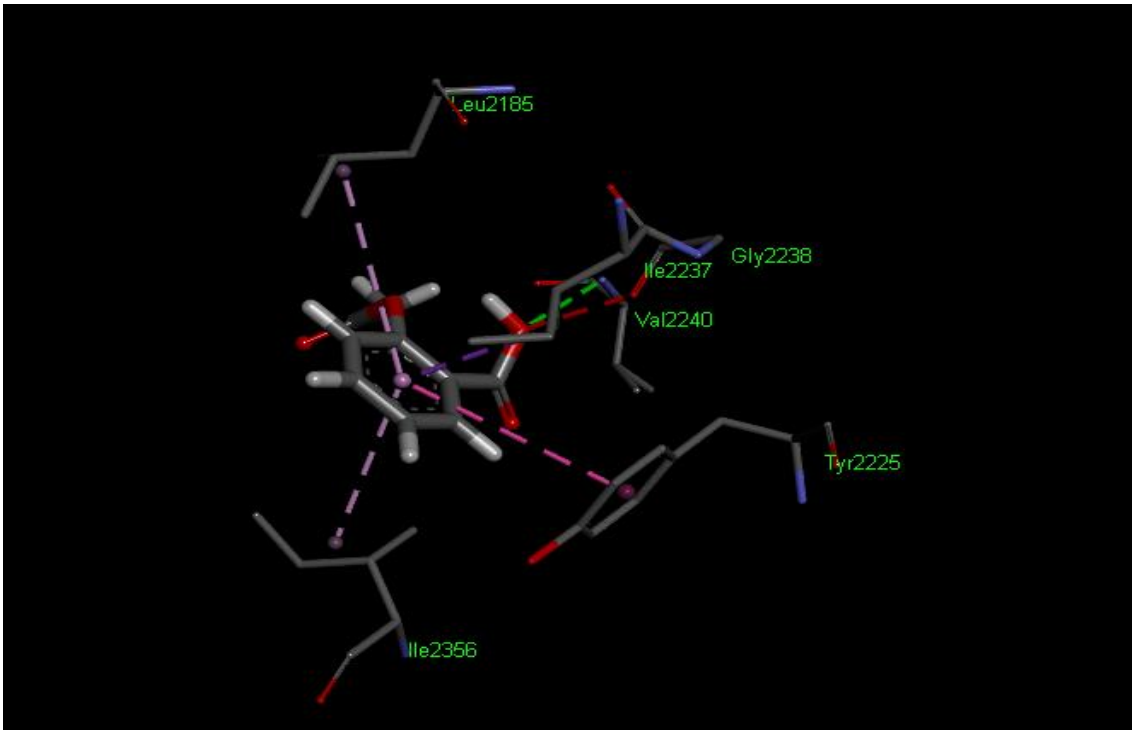


Figure 3.2: Aspirin's Binding Interactions with mTOR's Amino Acid Molecules (Visualized in Discovery Studio v4.5).

Multiple bonds were formed between mTOR and aspirin and they are showed in the following table (Table 3.2).

Table 3.2: Non-bond Interactions of Aspirin and mTOR.

Ligand	Binding Affinity (kcal/mol)	Category of Bond	Type	Amino acid…Ligand atom Interaction	Distance in angstroms (Amino acid…Ligand)
Aspirin	-5.8	Hydrogen Bond	Conventional Hydrogen Bond	VAL2240 (N…O)	3.21648
		Hydrophobic	Pi-Sigma	ILE2237	3.64421
		Hydrophobic	Pi-Pi T-shaped	TYR2225	5.13153
		Hydrophobic	Pi-Alkyl	LEU2185	5.0062
		Hydrophobic	Pi-Alkyl	ILE2356	4.23379

3.2 Result and Discussion of Metformin's Flexible Docking

The result of metformin's flexible docking with mTOR obtained from AutoDock Vina are given in the following table (Table 3.3).

Table 3.3: Results of Docking of Metformin and mTOR.

Mode or Binding pose	Affinity (kcal/mol)	Distance from best mode RMSD lower bound (l. b.) (in Angstrom)	Distance from best mode RMSD upper bound (u. b.) (in Angstrom)
1	-5.8	0.000	0.000

Several docking simulation was performed but from the binding affinities it was seen that metformin has a poor affinity (-5.8 kcal/mol) (Trott & Olson, 2010) for mTOR receptor. Visualization was carried out as a confirmation that metformin and mTOR did not have a strong binding affinity with mTOR.

Then the binding pocket for metformin within mTOR was visualized in Figure 3.3-

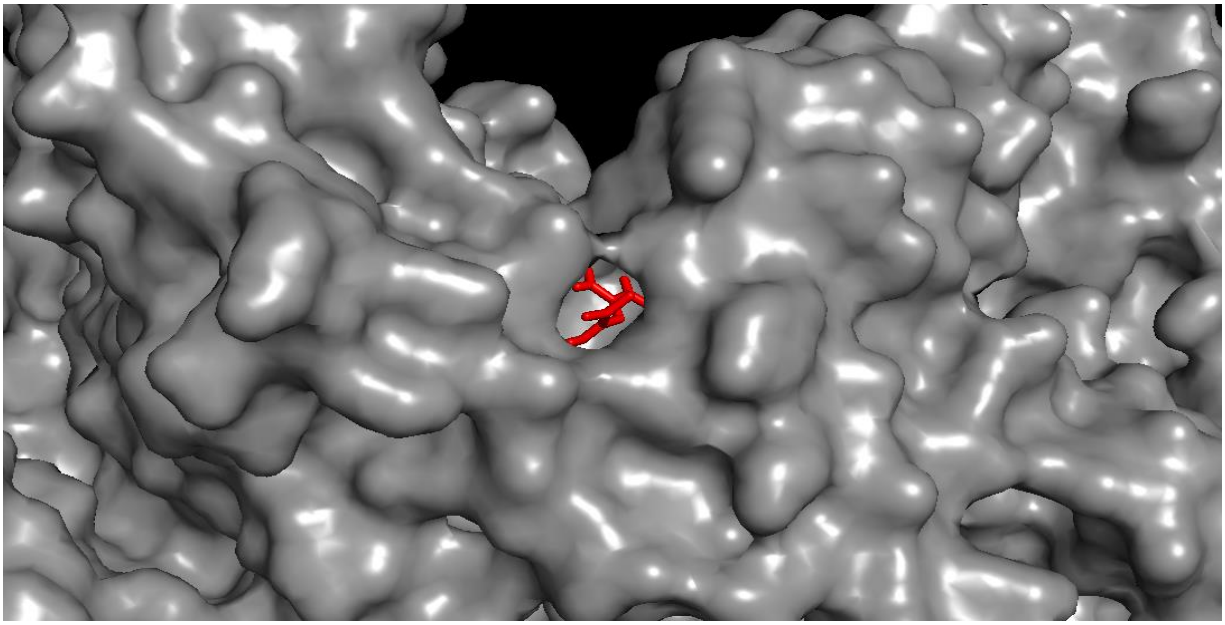


Figure 3.3: Metformin's Binding Pocket within mTOR (Visualized in PyMOL v1.8.4.0). The red sticks represent metformin molecule and the gray surface represents the receptor molecule mTOR in Figure 3.3. Non-bond interactions are shown below in Figure 3.4-

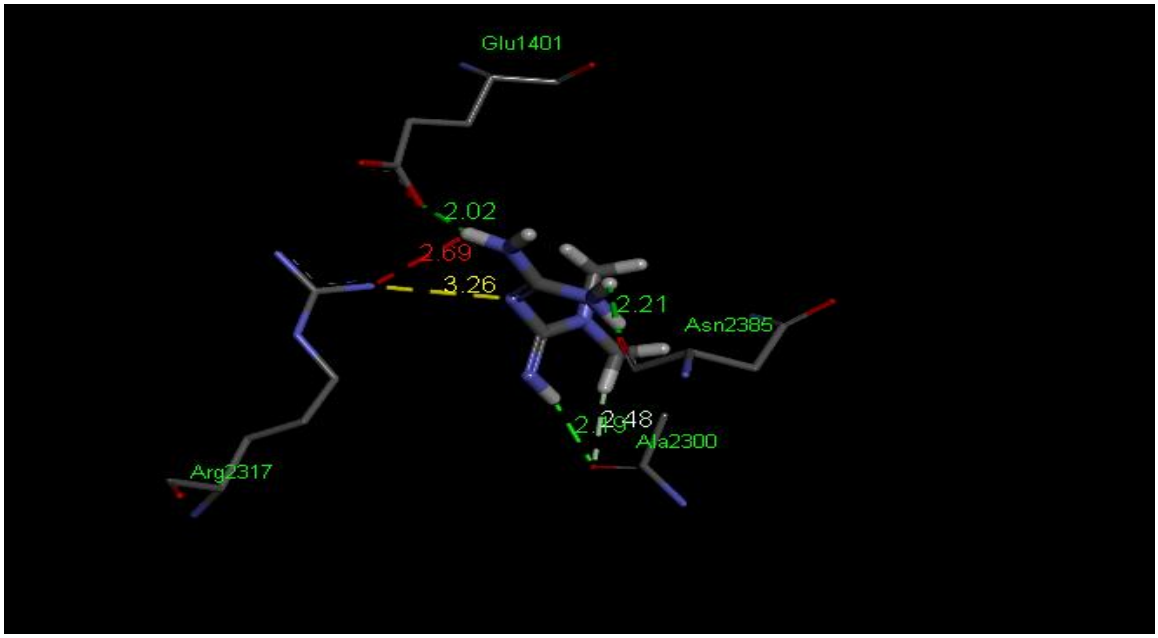


Figure 3.4: Non-bond Interactions of Metformin and mTOR (Visualized in Discovery Studio v4.5).

The different bonds formed between metformin and mTOR are shown in the following table (Table 3.4).

Table 3.4: Non-bond Interactions of Metformin and mTOR.

Ligand	Binding Affinity (kcal/mol)	Category of Bond	Type	Amino acid…Ligand atom Interaction	Distance in angstroms (Amino acid…Ligand)
Metformin	-5.8	Hydrogen Bond	Conventional Hydrogen Bond	ARG2317 (N…N)	3.26462
		Hydrogen Bond	Conventional Hydrogen Bond	GLU1401 (O…H)	2.02353
		Hydrogen Bond	Conventional Hydrogen Bond	ASN2385 (O…H)	2.21359
		Hydrogen Bond	Conventional Hydrogen Bond	ALA2300 (O…H)	2.19009
		Hydrogen Bond	Carbon Hydrogen Bond	ALA2300 (O…H)	2.47589

3.3 Result and Discussion of Rosuvastatins's Flexible Docking

The binding affinities for rovastatin's flexible docking with mTOR are given below in Table 3.5.

Table 3.5: Binding Affinity of Rosuvastatin and mTOR's Docking.

Mode or Binding pose	Affinity (kcal/mol)	Distance from best mode RMSD lower bound (l. b.) (in Angstrom)	Distance from best mode RMSD upper bound (u. b.) (in Angstrom)
1	-7.8	0.000	0.000
2	-7.0	5.142	7.575
3	-6.8	5.531	7.853
4	-6.6	4.740	8.149
5	-6.4	5.155	7.400
6	-6.3	4.995	8.345
7	-6.2	4.972	7.697
8	-6.0	38.029	39.965
9	-6.0	4.872	7.533

From the binding affinities it was found that rosuvastatin binds with mTOR with an affinity of -7.8 kcal/mol which was the highest among the different binding poses. The higher the binding affinity between the drug and protein, the greater is the bond strength is. Moreover, the binding affinity was negative which meant that it was an exothermic reaction. In other words, the drug protein interaction released energy due to the binding. Thus ligand and protein molecules formed a complex that was more stable than their previous entities. As the affinity was exothermic, this meant that no energy would have to be provided and the binding would be autonomous. This is the reason why negative binding affinities are the ones that can be exploited within the human body for pharmacologic effects as they do not need to provide extra energy. Furthermore, the first binding pose with the highest affinity was docking very close to the protein. The rest of the docking poses were not as close as the first pose and this can be deduced by analyzing their RMSD upper and lower bound values. According to Cole et al. when the value of RMSD is found to be less than 2 angstroms, it is deemed an effective docking prediction (Cole, Murray, Nissink, Taylor, & Taylor, 2005). The first docking distance was far smaller than 2 angstrom and this meant that the result of this prediction was a fruitful one. Unfortunately, the rest of the docking poses had RMSD values far above 2 angstrom which made them unacceptable.

After analyzing the affinity and RMSD values for the different poses, the binding pocket was visualized using PyMOL. Output file generated by AutoDock was used to visualize the binding of drug within the binding pocket as given in Figure 3.5.

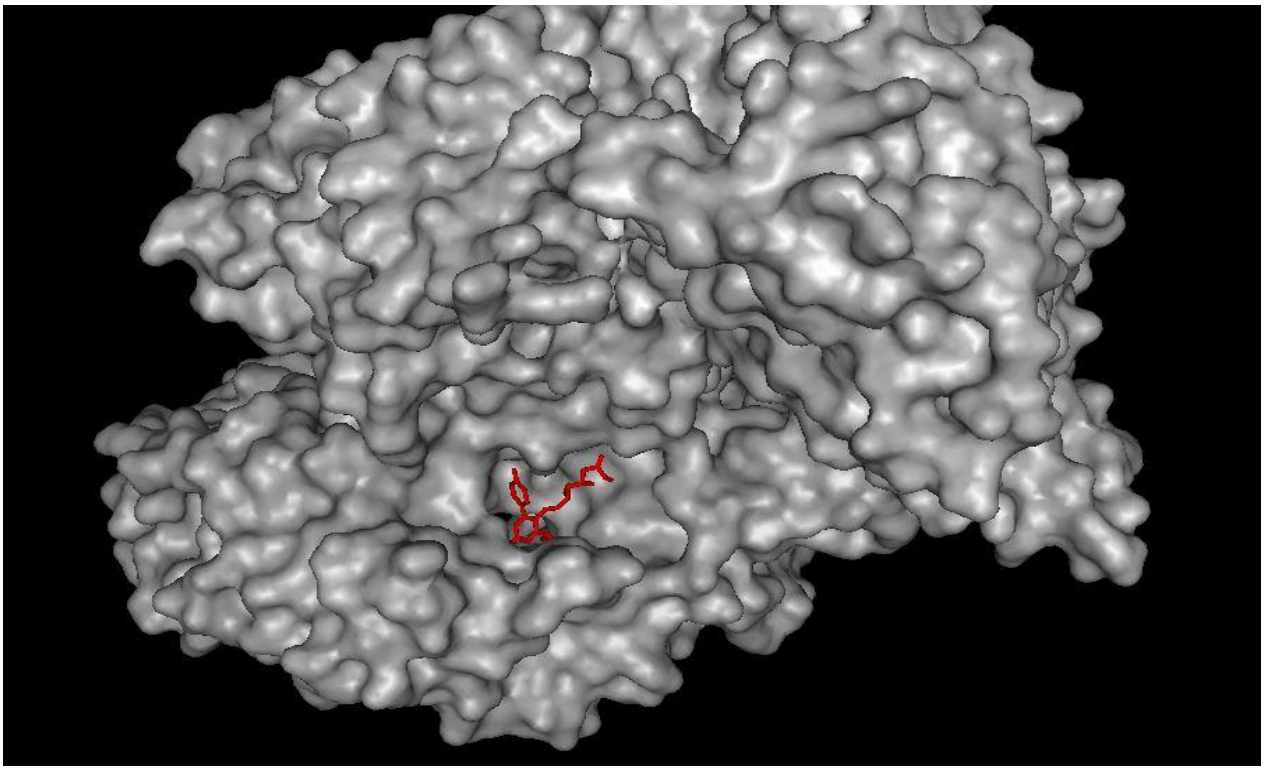


Figure 3.5: Binding site of Rosuvastatin (red) within mTOR (dark gray) (Visualized in PyMOL v1.8.4.0).

The red sticks represented the drug rosuvastatin and the dark gray surface represented the receptor molecule mTOR in Figure 3.5. mTOR was a huge protein molecule and it had multiple possible binding pockets. Then the output file was saved and visualized with Discovery Studio to obtain information on interactions which is shown below in Figure 3.6.

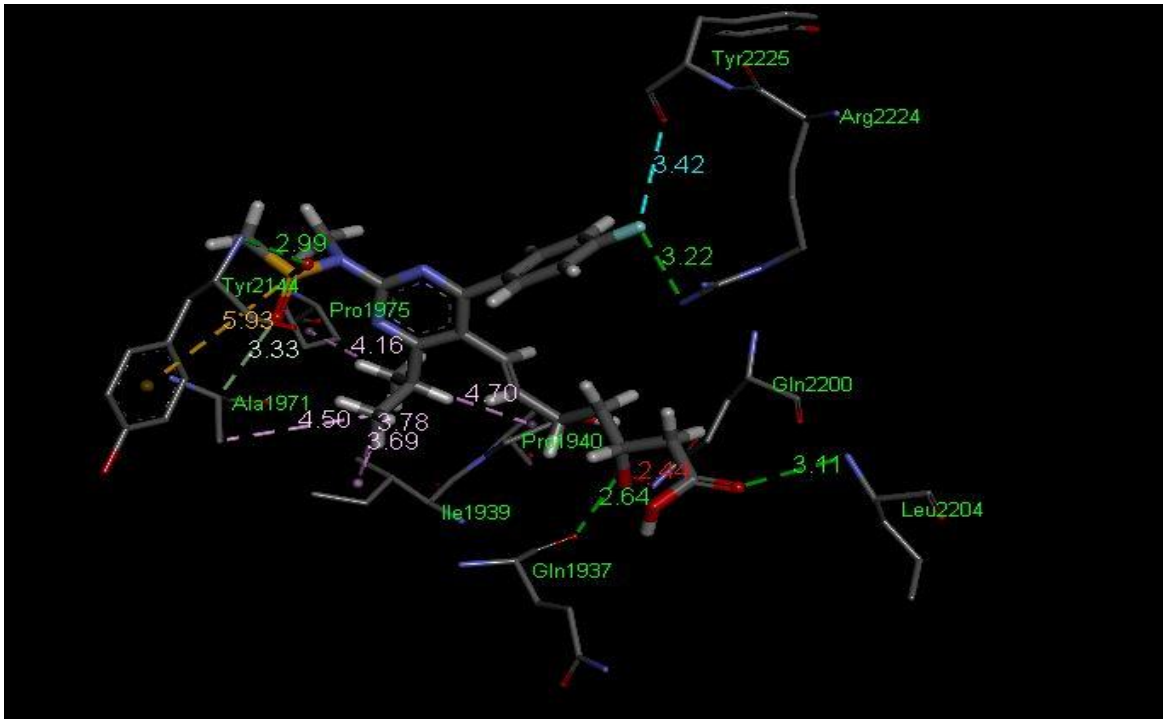


Figure 3.6: Different Nonbonding Interactions between mTOR and Rosuvastatin (Visualized in Discovery Studio v4.5).

Multiple bonds were formed between the drug and receptor molecule mTOR. Among the twelve different non-conventional bonds, five were hydrogen bonds shown in the Figure above (Figure 3.6) and also in Table 3.6. The first hydrogen bond was formed between tyrosine's (2144 no. aa) nitrogen atom and oxygen atom of the ligand rosuvastatin and the distance was 2.98 angstroms.

Table 3.6: Non-bond Interactions of Rosuvastatin and mTOR's Docking (Hydrogen bonds).

Ligand	Binding Affinity (kcal/mol)	Category of Bond	Type	Amino acid…Ligand atom Interaction	Distance in angstroms (Amino acid…Ligand)
Rosuvasta-tin	-7.8	Hydrogen Bond	Conventional Hydrogen Bond	TYR2144 (N...O)	2.98
		Hydrogen Bond	Conventional Hydrogen Bond	LEU2204 (N...O)	3.11
		Hydrogen Bond: Halogen	Conventional Hydrogen Bond: Halogen (Fluorine)	ARG2224 (NH_2…F)	3.21
		Hydrogen Bond	Conventional Hydrogen Bond	GLN1937 (O…H)	2.64
		Hydrogen Bond	Carbon Hydrogen Bond	ALA1971 (CA...O)	3.32

The second hydrogen bond was formed between the leucine's (2204 no. aa) nitrogen atom and oxygen atom of rosuvastatin and the distance was 3.11 angstroms. The third hydrogen bond was formed with a halogen atom which was between arginine's (2224 no. aa) nitrogen atom and fluorine atom of rosuvastatin and the distance was 3.21 angstroms. The fourth hydrogen bond was formed between glutamine's (1937 no. aa) oxygen atom with hydrogen atom of rosuvastatin and the distance was 2.64 angstroms. The last conventional hydrogen bond was a carbon hydrogen bond. In other words, the bond formed between alanine's (1971 no. aa) carbon atom with oxygen atom of rosuvastatin.

It can be seen from the values that all the hydrogen bonds were very close to each other and they all had values less than 4 angstroms. According to Wade and Goodford a hydrogen bond formed between two components that has a distance less than 2.3 angstroms can increase the binding affinity to a great extent (Wade & Goodford, 1993). Though these hydrogen bonds were not below 2.3 angstroms, they were quite close to 2.3 angstroms. Glutamine's hydrogen bond value was 2.64 and it was very close to 2.3 angstroms. Moreover, there were multiple hydrogen bonds formed. Thus these hydrogen bonds allowed rosuvastatin to bind closely with mTOR.

Among the rest of the seven bonds five are hydrophobic bonds, one is halogen bond and another one is interaction between sulfur atom with pi-electrons. They are shown below in Table 3.7. There is presence of one halogen bond between the rosuvastatin's fluorine atom and receptor moiety. According to Lu et al., halogen bonds play a key role to stabilize the drug-protein complex and also results in the enhancement of selectivity and binding affinity. There was presence of another uncommon type of bond present as well, which is the pi-sulfur interaction.

Table 3.7: Non-bond Interactions of Rosuvastatin and mTOR's Docking.

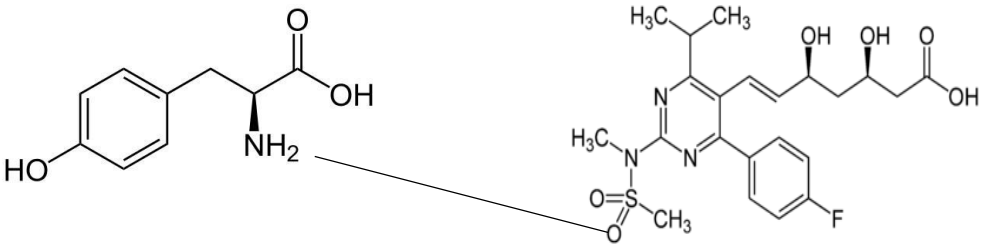
Ligand	Binding Affinity (kcal/mol)	Category of Bond	Type	Amino acid…Ligand atom Interaction	Distance in angstroms (Amino acid...Ligand)
Rosuvastatin	-7.8	Halogen	Halogen (Fluorine)	TYR2225 (O…F)	3.42
		Hydrophobic	Alkyl	ALA1971	4.49
		Hydrophobic	Alkyl	ILE1939	3.78
		Hydrophobic	Alkyl	PRO1940	4.70
		Hydrophobic	Alkyl	PRO1975	4.15
		Hydrophobic	Alkyl	ILE1939	3.68
		Other	Pi-sulfur	TYR2144 (Pi…S)	5.92

The other five bonds were hydrophobic bonds which are shown in Table 3.7. The amino acids (aa) involved in the five hydrophobic interactions are Ala1971 (aa Alanine), Ile1939 (aa Isoleucine), Pro1940 (aa Proline), Pro1975 (aa Proline) and Ile1939 (aa Isoleucine). Hydrophobic interactions play a very important role in nonpolar molecules. According to Davis and Teague hydrophobic interactions influence the binding affinity of a drug for its receptor molecule to a great extent. They also stated that hydrophobic bonds can enhance binding per methyl group by almost 3.2 times. Thus all these hydrophobic bonds present here were very important in the binding affinity of rosuvastatin for mTOR.

3.3.1 Visualization of Bonds

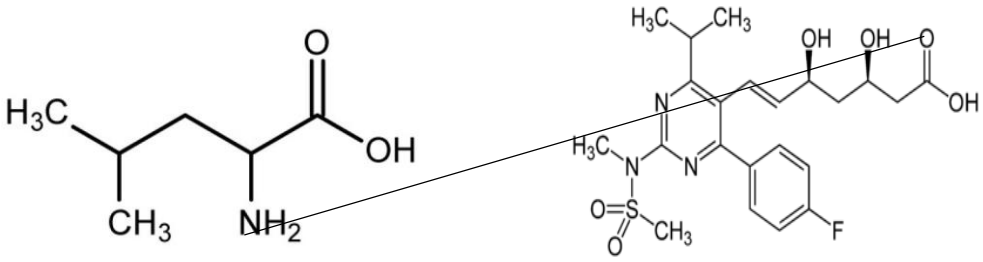
All the twelve bonds formed were visualized in the following manner generated using ChemDraw free 8 Pro (Mendelsohn, 2004):-

1. Hydrogen bond formed between TYR2144 and Rosuvastatin (N…O)-



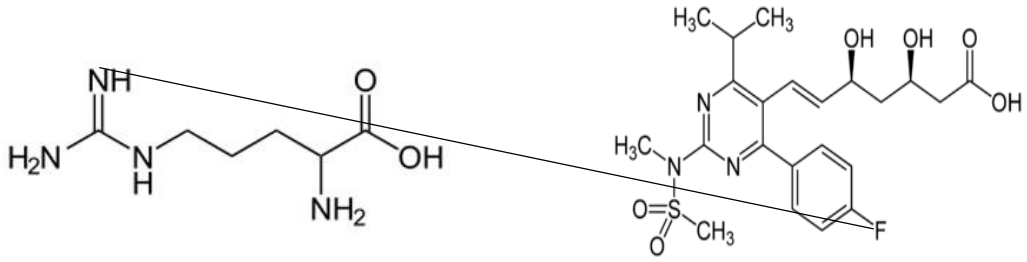
Tyrosine 2144 Rosuvastatin

2. Hydrogen bond formed between LEU2204 and Rosuvastatin (N…O)-



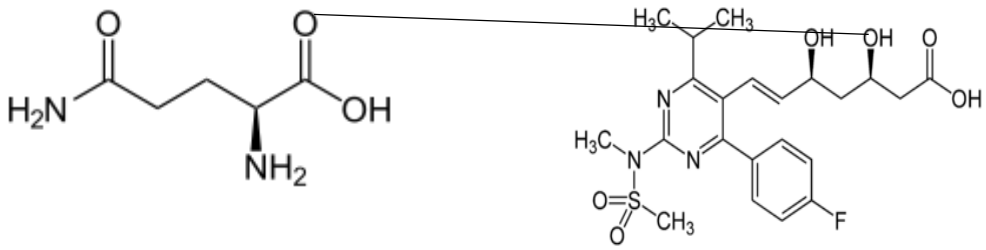
LEU2204 Rosuvastatin

3. Hydrogen bond between ARG2224 and Rosuvastatin (N…F)-



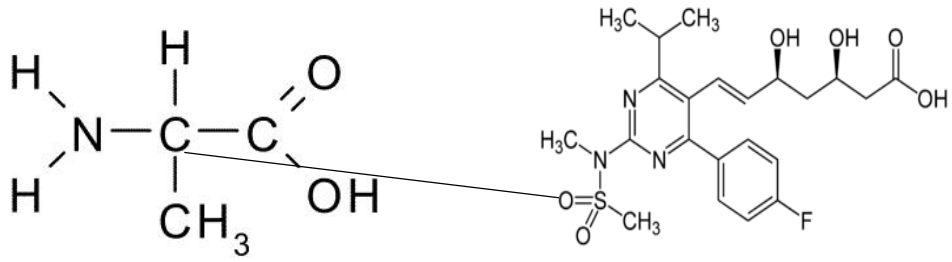
ARG2224 Rosuvastatin

4. Hydrogen bond formed between GLN1937 and Rosuvastatin (O…O)-



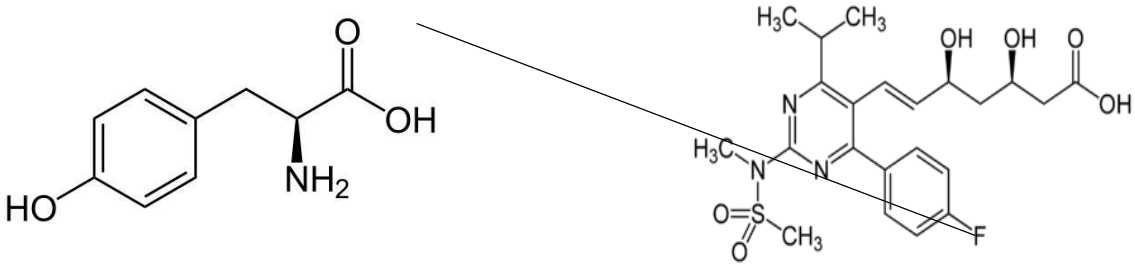
GLN1937 Rosuvastatin

5. Hydrogen bond between ALA1971 and Rosuvastatin (C…O)-



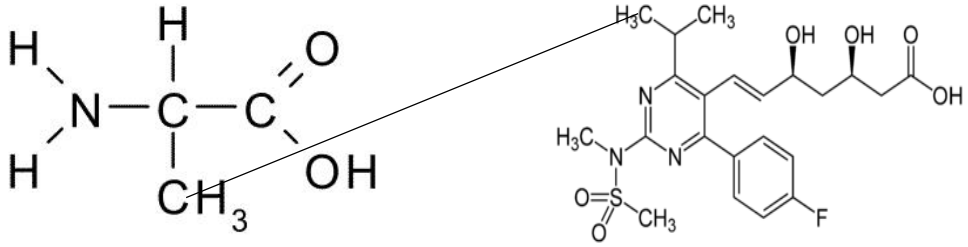
ALA1971 Rosuvastatin

6. Halogen bond formed between TYR2225 and Rosuvastatin (O…F)-



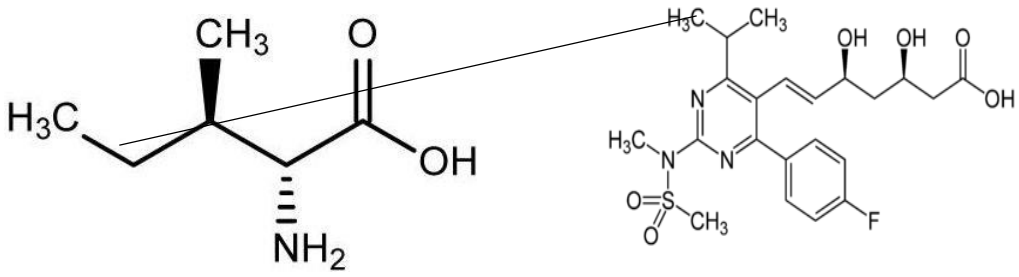
TYR2225 Rosuvastatin

7. Hydrophobic bond formed between ALA1971 and Rosuvastatin (Alkyl)-



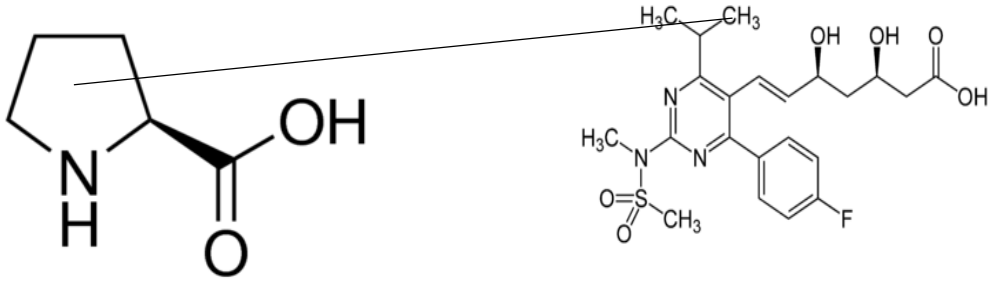
ALA1971 Rosuvastatin

8. Hydrophobic bond formed between ILE1939 and Rosuvastatin (Alkyl)-



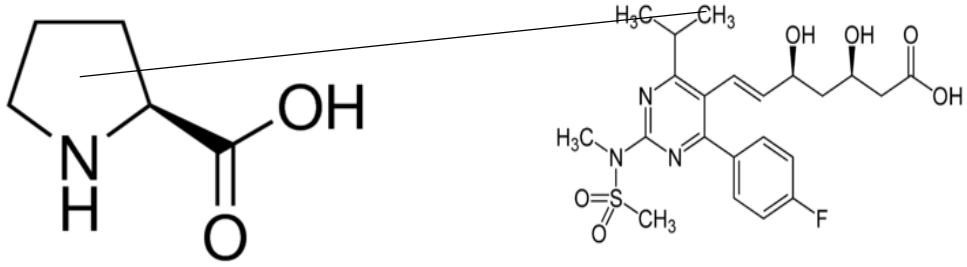
ILE1939 Rosuvastatin

9. Hydrophobic bond formed between PRO1940 and Rosuvastatin (Alkyl)-



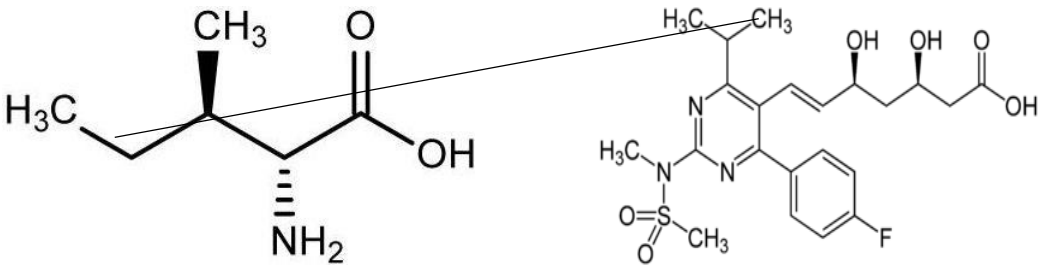
PRO1940 Rosuvastatin

10. Hydrophobic bond formed between PRO1975 and Rosuvastatin (Alkyl)-



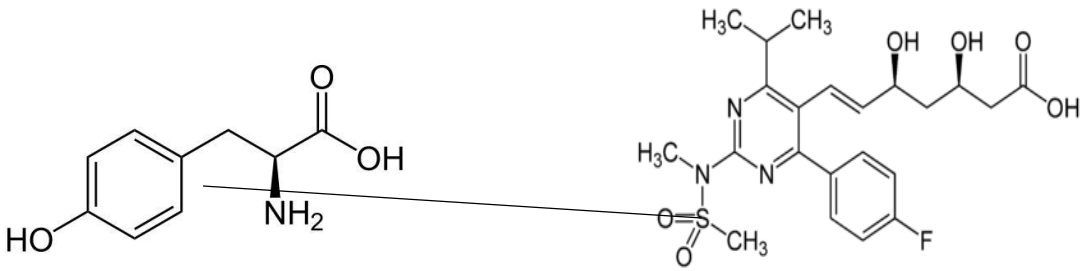
PRO1975 Rosuvastatin

11. Hydrophobic bond formed between ILE1939 and Rosuvastatin (Alkyl)-



ILE1939 Rosuvastatin

12. Bond formed between TYR2144 and Rosuvastatin (Pi-Sulfur interaction)-



TYR2144 Rosuvastatin

3.3.2 Validation of Rosuvastatin's Flexible Docking

1. Rapamycins Flexible Docking with mTOR

After flexible docking using AutoDock Vina it was seen that, rapamycin binds with mTOR with an affinity of -11.8 kcal/mol, (Trott & Olson, 2010) shown in Table 3.8. It formed ten separate bonds where six were hydrogen and the rest of the four were hydrophobic bonds. Some of the bonds were quite close having bond lengths upto 2.42 angstroms and resulting in the very high affinity of this flexible docking.

Table 3.8: Non-bond Interactions of Rapamycin and mTOR's Docking.

Ligand	Binding Affinity (kcal/mol)	Category of Bond	Type	Amino acid…Ligand atom Interaction	Distance in angstroms (Amino acid…Ligand)
Rapamycin	-11.8	Hydrogen Bond	Conventional Hydrogen Bond	ARG2224	2.90
		Hydrogen Bond	Conventional Hydrogen Bond	ARG2224	2.86
		Hydrogen Bond	Carbon Hydrogen Bond	Unknown	2.42
		Hydrogen Bond	Carbon Hydrogen Bond	GLN1970	2.74
		Hydrogen Bond	Carbon Hydrogen Bond	Unknown	2.81
		Hydrogen Bond	Carbon Hydrogen Bond	ASP1933	3.03
		Hydrophobic	Alkyl	ALA1971	4.38
		Hydrophobic	Alkyl	ALA2226	3.91
		Hydrophobic	Alkyl	LEU1936	4.32
		Hydrophobic	Pi-Alkyl	TYR2144	5.38

2. Similarity between Rapamycin and Rosuvastatin's Physicochemical Properties

Despite having a lot of differences such as molecular weight and site of action, these two drugs have many underlying similarities that gives rise to the similar interactions with mTOR. Both of them have hydrogen bond donor count of three (Stein, 2001; Ohia, Mancino, & Kulkarni, 1992). Rosuvastatin and rapamycin also have similar number of hydrogen bond acceptor count which are ten and thirteen respectively. Moreover, both of these drugs have poor water solubility (Stein, 2001; Ohia et al., 1992). For these reasons they gave similar interactions with mTOR.

3. Validation using Visualization

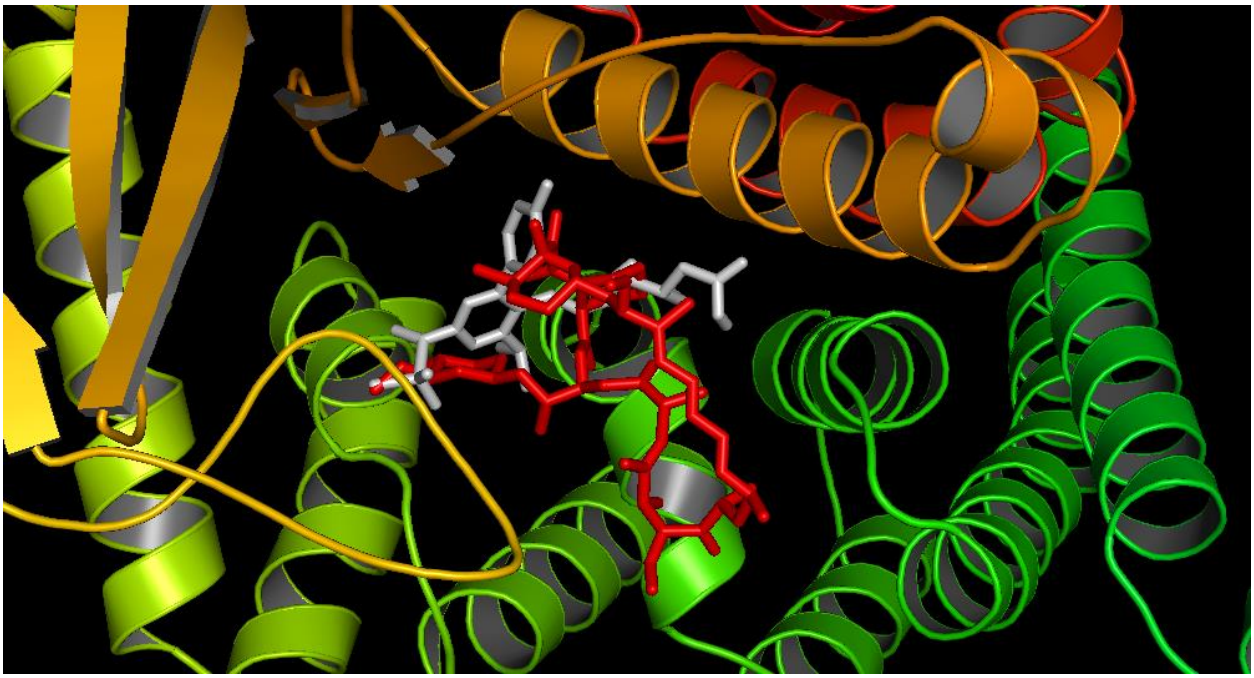


Figure 3.7: Rosuvastatin and Rapamycin Binding to the same Binding Pocket within mTOR (Visualized in PyMOL v1.8.4.0).

Figure 3.7 shows the red sticks, that represent rapamycin and gray sticks represent rosuvastatin molecule. Both of the molecules are present within the same binding pocket of

mTOR. Moreover, both of the molecules form similar bond with mTOR protein and they have similar bond distance and both molecules have significant affinity for this receptor (Trott & Olson, 2010). This further clarifies that rosuvastatin has some significant interaction with mTOR. However, for confirmation this needs to be further validated using both in vitro and *in vivo* studies.

3.4 Result and Discussion of Rosuvastatins's Rigid Docking

The binding affinities for rovastatin's rigid docking with mTOR using AutoDock Vina are shown below in Table 3.9.

Table 3.9: Binding Affinity of Rosuvastatin and mTOR's Docking.

Mode or Binding pose	Affinity (kcal/mol)	Distance from best mode RMSD lower bound (l. b.) (in Angstrom)	Distance from best mode RMSD upper bound (u. b.) (in Angstrom)
1	-10.2	0.000	0.000

Several rigid docking simulation were performed but from the binding affinities it was seen that rosuvastatin has a very high affinity for mTOR receptor which is -10.2 kcal/mol (Trott & Olson, 2010). The binding affinity in case of rigid docking was much higher than that of flexible docking. The high negative affinity also indicates that this binding of drug will occur spontaneously within the body as it is exothermic in nature. Moreover, the distance from best mode rmsd values were zero angstrom which indicates the firmest binding pose possible.

The result of rosuvastatin's rigid docking is visualized below in Figure 3.8.

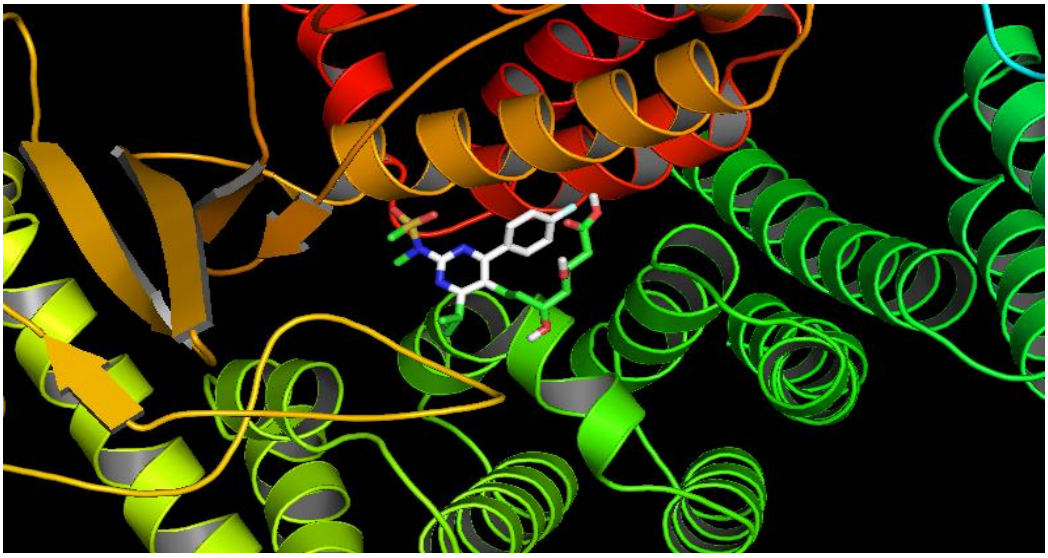


Figure 3.8: Binding site of Rosuvastatin (sticks) within mTOR (ribbons) (Visualized in PyMOL v1.8.4.0).

The non-bond interactions were then visualized and using Discovery Studio and it is shown below in Figure 3.9.

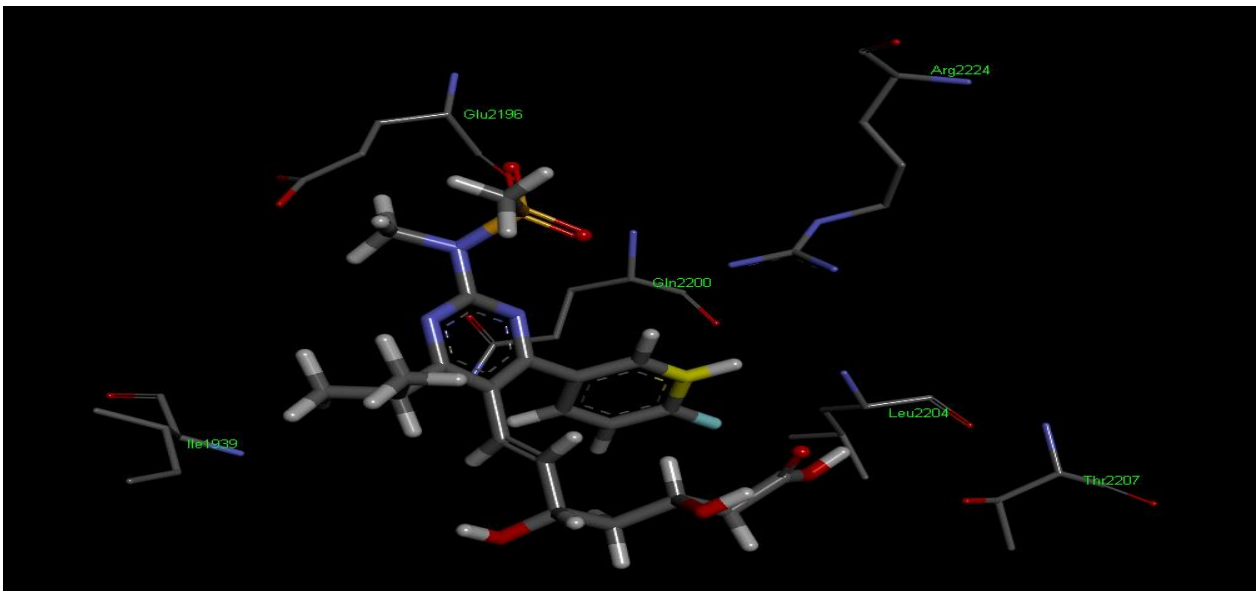


Figure 3.9: Different Nonbonding Interactions between mTOR and Rosuvastatin (Visualized in Discovery Studio v4.5).

Multiple bonds were formed between rosuvastatin and mTOR in case of their rigid docking. Among the eight different bonds six were hydrogen bonds and the rest of the two were halogen and hydrophobic bonds respectively. They are given below in the following Table 3.10.

Table 3.10: Non-bond Interactions of Rosuvastatin and mTOR's Docking (Obtained using Discovery Studio v4.5).

Ligand	Binding Affinity (kcal/mol)	Category of Bond	Type	Amino acid…Ligand atom Interaction	Distance in angstroms (Amino acid…Ligand)
Rosuvastatin	-10.2	Hydrogen Bond; Halogen	Conventional Hydrogen Bond; Halogen (Fluorine)	LEU2204 (N…F)	3.33
		Hydrogen Bond	Conventional Hydrogen Bond	ARG2224 (N…O)	3.17
		Hydrogen Bond	Conventional Hydrogen Bond	THR2207 (O…H)	2.98
		Hydrogen Bond	Conventional Hydrogen Bond	Unknown (H…H)	2.33
		Hydrogen Bond	Carbon Hydrogen Bond	GLU2196 (O…H)	3.03
		Hydrogen Bond	Carbon Hydrogen Bond	GLU2196 (O…H)	2.76
		Halogen	Halogen (Fluorine)	GLN2200 (O…F)	3.33
		Hydrophobic	Alkyl	ILE1939 (Alkyl…Alk-yl)	4.69

The first hydrogen bond formed between LEU2204 (aa Leucine) and fluorine atom of rosuvastatin having bond length of 3.33 angstroms. The second hydrogen bond formed between nitrogen atom of ARG2224 (aa Arginine) and oxygen atom of rosuvastatin having

2.98 angstroms bond length. The four other hydrogen bonds were formed by THR2207 (aa Threonine), unknown aa's hydrogen atom, GLU2196, GLU2196 with four hydrogen atom respectively. Their bond length were 2.98, 2.33, 3.03, 2.76 angstroms respectively. There was a halogen bond formed between GLN2200 (aa Glutamine) with fluorine atom of rosuvastatin. Another hydrophobic bond formed between ILE1939 (aa isoleucine) between the alkyl groups of ILE1939 and rosuvastatin.

A conventional hydrogen bond formed between mTOR and rosuvastatin had a bond length of 2.33 angstroms which was very close. According to Wade and Goodford a hydrogen bond having the bond length below 2.3 angstroms can increase the binding affinity tremendously between two components (Wade & Goodford, 1993). As a result, this bond was very significant for this rigid docking and its huge binding affinity value.

3.4.1 Validation of Rosuvastatin's Rigid Docking by Visualization

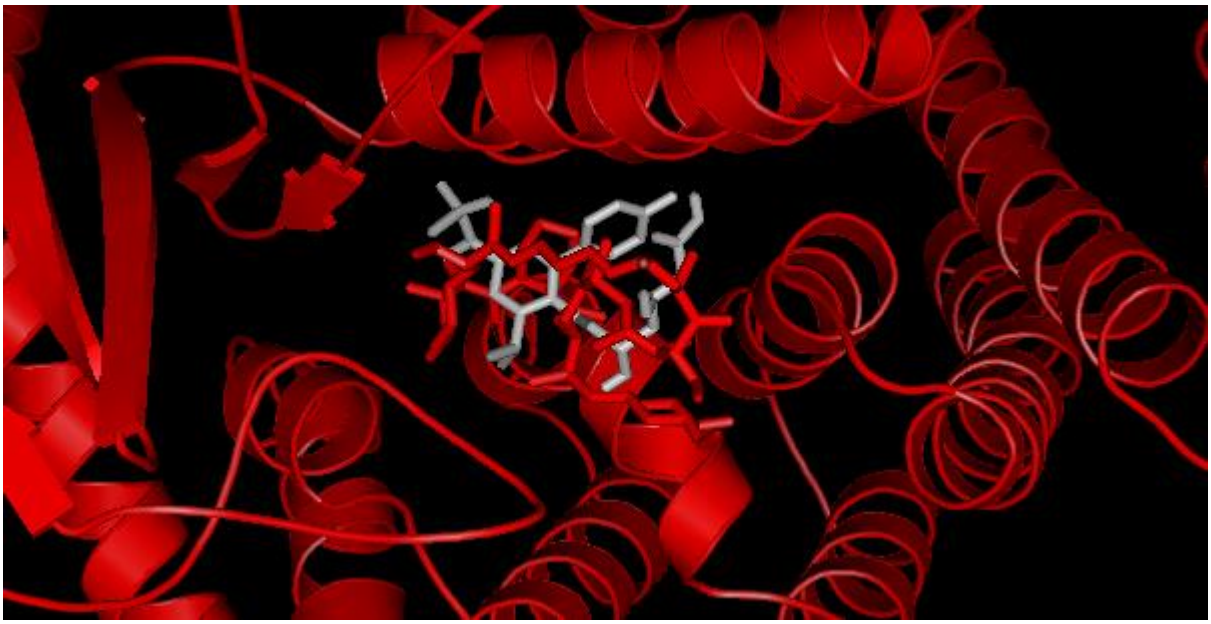


Figure 3.10: Rosuvastatin and Rapamycin Binding to the same Binding Pocket within mTOR(Visualized using PyMOL v1.8.4.0).

After completing rigid docking of rapamycin and mTOR it was found that rapamycin binds with very high affinity which is 12 kcal/mol. In Figure 5.10, the red sticks represent rapamycin and gray sticks represent rosuvastatin molecule. Both of the molecules are present within the same binding pocket of mTOR (red ribbons) which can be seen in Figure 5.10. Moreover, both of the molecules form similar bond with mTOR protein and they have similar bond distance and both molecules have significant affinity for this receptor. This further clarifies that rosuvastatin has some significant interaction with mTOR. However, for confirmation this needs to be further validated using both in vitro and *in vivo* studies.

4. Conclusion

From the result of our docking study it was seen that aspirin and metformin had a poor affinity for mTOR and their highest binding affinity were -5.8 kcal/mol for both of them. In recent studies, it was seen that both aspirin and metformin have some anticancer properties but the exact mechanism behind this property is still not well understood. That was the very reason why these drugs were chosen for this docking study and shed some light on their anticancer activity. Unfortunately, it was seen that their affinity for mTOR protein is poor and they mediate their anticancer properties in a different way which needs further studies to decipher. Besides aspirin and metformin another drug rosuvastatin was selected for this docking study. Rosuvastatin is an INN drug which is used basically to control hyperlipidemia. In case of rosuvastatin it was seen that it has significant affinity for mTOR protein which is upregulated in various types of cancer. Rosuvastatin's affinity for mTOR was -7.8 kcal/mol in case of flexible docking and -10.2 kcal/mol in case of rigid docking which suggest that it might have significant interaction with this receptor. Further testing of this interaction needs to be done *in vitro* to prove its potential interaction with the receptor molecule. If it is proven to be effective in *in vitro* test as a potent mTOR inhibitor then a safe anticancer as well as immunosuppressant medication can be obtained which would be very useful in clinical practice.

4.1 Future work

Further study of these drugs and protein should be carried out to evaluate the docking predictions and obtain concrete evidence to gain widespread acceptance.

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