An *in silico* approach for evaluation of drug conjugated gold nanoparticles against receptors associated with breast cancer

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A thesis submitted in partial fulfilment of the requirements for the degree of

BS Applied Biosciences

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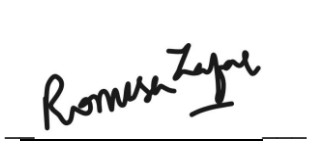
I, Romesa Zafar, Hijab Zahra, Maria Naeem declare that all work presented in this thesis is the result of our own work. Where information has been derived from other sources, we confirm that this has been mentioned in the thesis. The work here in was carried out while we were undergraduate students at Atta-ur-Rahman school of Applied Biosciences, NUST under the supervision of Dr. Rumeza Hanif.



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Dedicated to our beloved Parents and
Honorable Teachers

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful

All praises and thanks are due to the Almighty Allah, Who always guides us to the right path and gives us strength and patience to face all the challenges and difficulties. We seek refuge in Allah from the evils of our souls and the wickedness of our deeds. Whomsoever Allah guides, none can misguide, and whomsoever Allah misguides, none can guide. And may the peace and blessing be on the most noble of Prophets and Messengers, our Prophet Muhammad (SAW) and on his family and all his Companions. We offer to Him all praise and gratitude. .

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HIJAB ZAHRA

MARIA NAEEM

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List of Abbreviations

Full Name	Abbreviation	Full Name	Abbreviation
Estrogen Receptor	ER	Ductal carcinoma *in situ*	DCIS
Cyclin Dependent kinase 4	CDK4	Lobular carcinoma *in situ*	LCIS
Cyclin Dependent kinase 6	CDK6	Invasive ductal carcinoma	IDC
Cyclin Dependent kinase 4 or Cyclin Dependent kinase 6	CDK4/6	Invasive lobular carcinoma	ILC
Cytochrome P450	Cyt P450		
Gold Nanoparticle	AuNP		
Nanoparticle	NP		

ABSTRACT

Breast cancer is a leading cause of cancer related deaths in women around the globe and even in Pakistan. Treatment of breast cancer is dependent on combinatorial therapies such as chemotherapy, endocrine, and/or targeted therapy along with surgery and radiations. Systemic damage and multi drug resistance are the limitations of current therapies. To overcome these limitations, nanoparticles are conjugated with desired drugs for targeted drug delivery. The aim for this *in silico* study is to investigate the binding affinity of the nanoparticles derived from *Aloe vera*, nanoparticles derived from *Gymnema sylvestre* leaf extract and nanoparticles derived from honey conjugated with commercially available drugs such as Palbociclib, Fluversent and Letrozole against breast cancer receptor variants. The comparison was done with unconjugated drugs. The drug conjugated with gold nanoparticle (AuNP) derived from of the above natural revealed stronger hydrogen bonding with higher binding energy score using PatchDock in comparison to unconjugated drug after docking against breast cancer receptor variants. In future, we can synthesize these nanoparticles and test them on breast cancer cell lines for *in vitro* analysis and then on animal models for *in vivo* analysis. Combination of different drugs can be conjugated on AuNP to block multiple signaling pathways associated with breast cancer.

CHAPTER 1

INTRODUCTION

Breast cancer is one of the most prevalent multi-factorial heterogeneous disorder having a wide range of clinical presentations known as intratumor heterogeneity (Turashvili and Brogi 2017). Based on the site of origin, breast cancer is subdivided into ductal and lobular carcinoma. Ductal carcinoma begins in the lining of the milk ducts and can either be noninvasive or can develop into invasive ductal carcinoma. If it develops in the lobules of the breast, it is called lobular carcinoma. It is mostly invasive carcinoma (Burstein, Polyak et al. 2004). Another type is sarcoma that occurs very rarely which develops in the connective tissues (Cozen, Bernstein et al. 1999).

Classification of breast cancer also depends on whether it is hormone receptor positive or negative. This classification helps in defining treatment regime (Pusztai, Mazouni et al. 2006). Tumors that are hormone-receptor positive are treated with mostly endocrine therapy (Maughan, Lutterbie et al. 2010). It includes preventing the synthesis of estrogen or blocking its binding to receptors. These include gonadotropin releasing hormones agonists, SERMs and aromatase inhibitors. Aromatase inhibitors, in post-menopausal women, prevent the conversion of androgen to estrogen. There is some endocrine treatment most commonly used against luminal A breast cancer. It includes the use of Letrozole, Palbociclib and Fulvestrant. Hormone-receptor negative tumors are usually treated with chemotherapy drugs. Chemotherapy is also recommended for tumors of size bigger than 1 cm. Tissue-targeted therapy is for tumors over-expressing ERB2. Anti-ERBB2 monoclonal antibody is used along with chemotherapeutic drugs. Mastectomy is

the surgical removal of breast or its effected tissue which is common treatment in invasive tumors. Radiation therapy is also used for the treatment of breast cancer. In most of the cases, combinatorial treatment is suggested.

Nanoparticles (NPs) are particulate dispersion of solid particles within a size range of 1-100nm. NPs are synthesized at nanoscale and have different composition depending on their production. Different types of NPs are synthesized including nanospheres, nano shells, nanorods, nano cages and nano capsules each of which is unique in its wide range of biomedical applications. Different kinds of NP are in use such as Gold NP, Silica NP, Calcium phosphate NP, Graphene oxide NP, Magnetic NP, lipid NP, polymer base NP and the list continues. In therapeutics, we use hybrids of different NP to make them more efficient and effective carrier for drugs or genes (Estrada and Peña 2000).

Write a sentence or two why in silico approaches are need of the time and are less laborious and time efficient. Also write about the importance of binding affinity and hydrogen bonding with drugs. This project covers *in silico* studies to overcome the issues of biocompatibility and specifically focuses on binding affinity and hydrogen bonding of nanomaterials with biological systems.

1.1. Problem Statement

Breast cancer is prevalent in women worldwide and more so amongst Asian women (give a latest reference of 2018 or 2019) (Boulos, Gadallah et al. 2005). Many different therapeutic approaches have been proposed and are in practice to treat breast tumors, yet we need an efficient and more effective therapy for its treatment. One of the therapeutic approaches in

focus is the *in silico* methods for evaluation of drugs conjugated Gold nanoparticles (AuNPs) derived from three different sources against breast cancer receptors.

1.2. Hypothesis

Breast cancer is a leading cause of cancer related deaths. Its high prevalence, incidence and recurrence rate needs to explore new treatment options. One of the therapeutic options is the synthesis of drug conjugated nanoformulations for effective and targeted drug delivery to overcome the issue of recurrence.

1.3. Research Questions

The specific research questions are:

1. How many strong hydrogen bonds exist between the cancer receptors and nanoparticles-conjugated drugs?

2. Which one of the drugs, amongst those in study, binds more effectively to the receptor? 3.

3. How the binding of the drug, with the receptor, become more specific after conjugation with nanoparticles?

1.4. Objective

Following were the objectives for the study being conducted:

1. *In silico* synthesis of Drug conjugated AuNP derived from *Aloe vera*, *Gymnema sylvestre* and Honey.
2. Protein modeling and refinement of four Breast cancer inducing receptor variants.
3. *In silico* analysis of anti-breast cancer drugs conjugated AuNPs derived from three biological sources against receptor variants.
4. *In silico analysis* of anti-breast cancer drugs against their respective variant protein receptors.

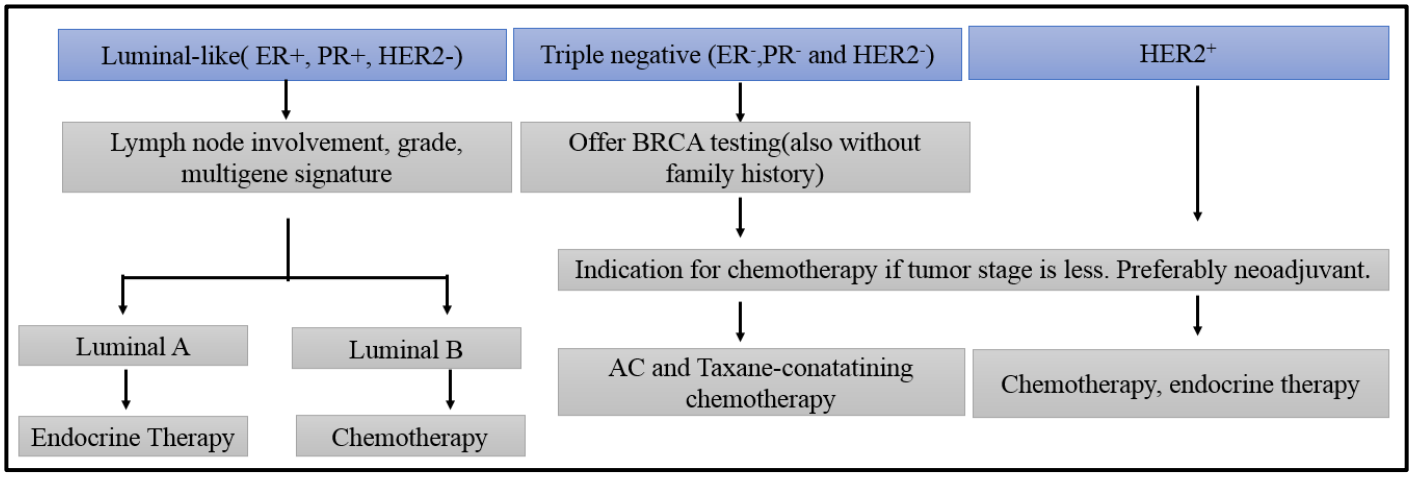

Figure 1. 1. Flowchart showing different therapeutic approaches to treat breast cancer of luminal-like, triple negative and HER2 positive breast cancers.

CHAPTER 2

LITERATURE REVIEW

2.1. Background

Breast cancer is the abnormal growth of cells that proliferates to become a lump that can either be benign or malignant (Chaurasia, Pal et al. 2018). It is a term for the combination of many subtypes of tumors in breast. These subtypes are classified based on the origin of cancerous cells, molecular status, and clinical manifestation (Weigelt and Reis-Filho 2009). Most of the breast tumors originate in the epithelium and hence are carcinomas (Keen and Davidson 2003). Looking at the anatomy of the breast, the first thing to look at is its surface anatomy. On its surface, it has two main parts, the circular part and the axillary tail. On each breast, there is a nipple which contains the smooth muscle fibers and the area around it is called areolae which is highly pigmented and contains the sebaceous glands to protect the nipples especially during pregnancy.

2.1.1. Anatomy of breast

The breast is made up of three main parts; ducts, lobules and the connective tissue (Sainsbury, Anderson et al. 2000) (Give any original reference). Lobules are the glands where milk is produced, and ducts carry this milk to the nipples. These ducts and lobules are the regions where tumors are originated. Connective tissue mainly surrounds the whole breast and is made up of fibrous and fatty tissues that supports the lobules and ducts. A pectoralis major is a layer of muscle on which a network of lobules, ducts and connective tissues are placed.

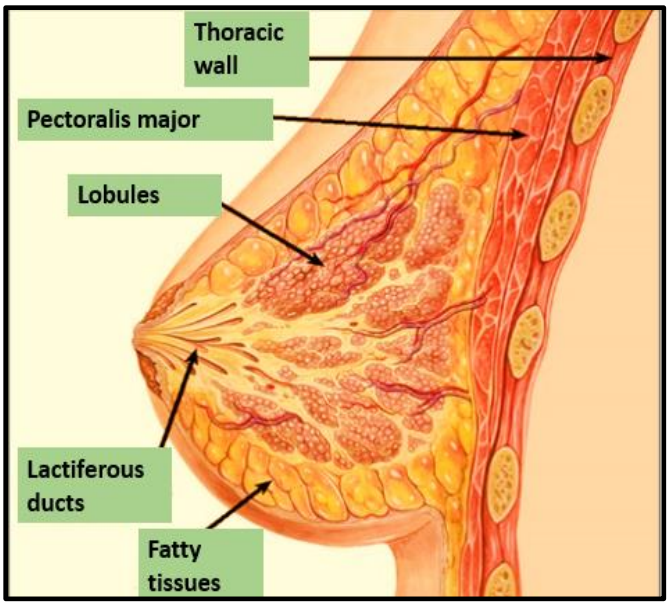

Figure 1. 2. Anatomy of the Breast showing ducts, lobules, and fatty tissues

2.2. Epidemiology of Breast cancer in Pakistan

According to the 2018 report, the leading cause of cancer-related deaths in Pakistan is breast cancer(Begum 2018). Out of a total of 6,378 malignancies caused by cancers, breast cancer related deaths were reported to be 1270 (Badar, Mahmood et al. 2015).

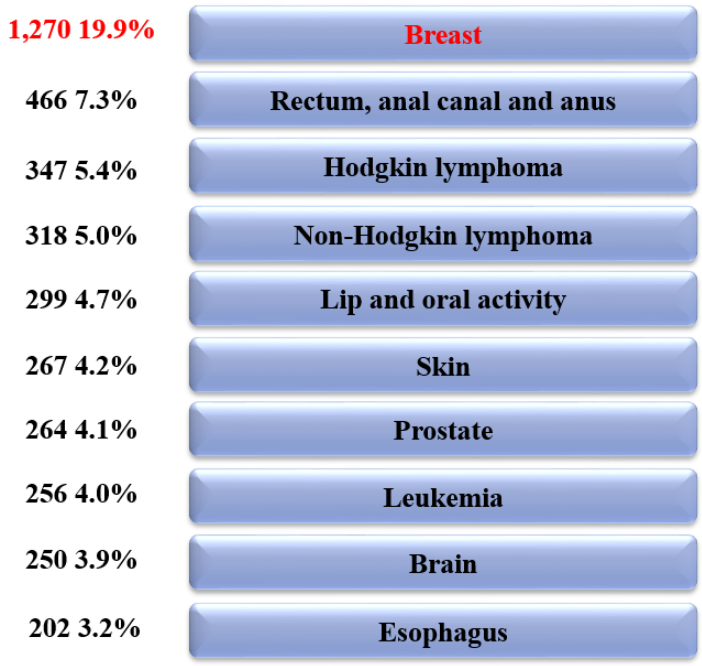

Figure 2. 1. The statistics of top 10 malignancies from June to December 2018 in Pakistan.

2.3. Types of breast cancer

Breast cancer is categorized into four different categories:

- Ductal carcinoma *in situ* (DCIS)
- Invasive ductal carcinoma
- Lobular carcinoma *in situ*
- Invasive lobular carcinoma

Invasive ductal carcinoma is most common among all types of breast cancer, with 70-80% of the cases (Borst and Ingold 1993). Although, its starts in the milk ducts but with time it spreads to other parts of the breast and hence metastasis occurs *via* blood vessels and lymphatic system (Toikkanen, Pylkkänen et al. 1997). Whereas, in case of ductal carcinoma *in situ* (DCIS), the cancerous cells stay inside the duct. The symptoms of both of these types

can vary; DCIS usually presents itself without any clear signs and symptoms but in some cases, some of the symptoms like lumps in the breast are seen (Burstein, Polyak et al. 2004). Bloody discharge from the nipple is the most common symptom in invasive ductal carcinoma.

Invasive lobular carcinoma (ILC) starts in the milk glands or lobules and spreads to other parts of the breast and body through lymph nodes (Cristofanilli, Gonzalez-Angulo et al. 2005). It is less common than IDC and occurs in 5 to 10% of the patients worldwide(Borst and Ingold 1993). The symptoms are different from IDC as just a small part of the breast is thickened in ILC (Martinez and Azzopardi 1979). Lobular carcinoma in situ (LCIS) does not truly classify as a breast cancer type (Foote Jr and Stewart 1941). Since it does not have any clear signs and symptoms and does not show up on mammogram, it is commonly referred to as neoplasia rather than carcinoma (Haagensen, Lane et al. 1978). Neoplasia is a small aggregation of abnormal cancerous cells, maybe a stage prior to developing the carcinoma. The other less broad categories of breast cancer includes inflammatory breast cancer, angiosarcoma of breast, Paget 's disease of the breast and phyllodes tumors (Jaiyesimi, Buzdar et al. 1992). Paget's disease results in the formation of different tumor cells called Paget cells that occur in the skin lining of the nipple or areola. It is a very rare type of breast cancer that affects less than 4% of the patients (Ashikari, Park et al. 1970).

Angiosarcoma of the breast, as the name suggests, develops in the lymph or blood vessels surrounding the breast (Tachibana, Fukuma et al. 1996). Less than 2% of the patients develop this and it only occurs in people over the age of 70. Tumors affecting the connective tissues of the breast are called phyllodes tumor. Some of these tumors might not be cancerous and only in 25% of the cases, tumors are cancerous

(Jacklin, Ridgway et al. 2006). People who had Li-Fraumeni syndrome in their childhood are at an increased risk of developing this disease (Heymann, Delaloge et al. 2010). Some other types of breast carcinoma are medullary carcinoma, papillary carcinoma, mucinous carcinoma, adeno-squamous carcinoma, adenoid cystic carcinoma and tubular carcinoma (Ridolfi, Rosen et al. 1977).

2.4. Molecular subtypes of breast cancer

Breast cancer cells can also be classified based on genetic markers.

Based on these, breast tumors are grouped into four groups

- Group1 includes those cells that are both estrogen receptor (ER) and progesterone receptor (PR) positive but human epidermal growth factor receptor 2 (HER2) negative. These are called luminal A tumors.
- Group 2 includes tumors that are ER and PR positive and can be either HER2 positive or negative. These are called luminal B tumors.
- Group 3 includes HER2 positive tumors that are both ER and PR-negative.
- Group 4 includes called triple-negative tumors that are negative for estrogen receptors, progesterone receptors and HER2.

These subtypes are classified based on hormone receptors status and other proteins involved in the breast cancer (Ahmad and Kumar 2011). Luminal A subtype is the most common type of breast cancer that is most prevalent type in South Asian countries including Pakistan (Prat, Cheang et al. 2013). This type of breast cancer is positive for both estrogen receptor and

progesterone receptor but negative for HER2 protein. 80% of all the breast cancers are ER positive (Gao and Swain 2018).

HER2 is a human epidermal growth factor receptor-2 protein that is involved in the growth and repair of cells. Luminal B subtype cancers are less common but are faster than luminal A in spreading throughout the body (Creighton 2012). These are positive for ER and PR and also usually positive for HER2 protein (Cheang, Chia et al. 2009). HER2 positive subtypes have too many copies of the HER2 gene in the cells. Consequently, breast cancer cells express excessive HER2 receptor on their surface. It makes this type of cancer the most invasive amongst all types. HER2 binding to the cells makes the cells to rapidly grow and proliferate (Loibl and Gianni 2017).

Triple negative breast cancer (TNBC) does not have ER, PR and HER2 gene on their breast cancer cells. They are less common but most difficult to treat because of the absence of receptors (Foulkes, Smith et al. 2010). The invasive ductal carcinoma or the invasive lobular carcinoma can fall into any of the above mentioned subtypes (Rakha, El-Sayed et al. 2007).

2.5. Breast pathology

Some of the diseases of breast are benign and not necessarily cancerous. These diseases cause fibrocystic changes in the breast. These changes can cause following diseases: apocrine metaplasia, fibrosis, cysts, papillomatosis, duct ectasia, fibro adenoma, a typical ductal hyperplasia, etc. (Ali and Parwani 2007). For people with atypical ductal hyperplasia, risk for developing invasive breast cancer increases five to six times in patients. 10% of all the patients with atypical ductal hyperplasia will go on to develop invasive breast cancer (Mastitis). Among various benign tumors mentioned above, fibro adenoma is the most common. It is highly

prevalent among young women. Usually multiple fibro adenoma tumors are present among women (Dent and Cant 1989).

2.6. Current therapies for breast cancer treatment

Breast cancer can be treated by using two broadly defined approaches that includes systemic and local therapies. Treatment plan for a patient is made by keeping following factors in mind: age of the patient, the subtype of the cancer and its hormone receptor status, tumour stage and the presence of mutations in BRCA1 gene and BRCA2 gene.

2.6.1. Systemic therapies

The systemic therapies are intended to treat invasive cancers that spread throughout the body (Sachelarie, Grossbard et al. 2006). These therapies target cancerous cells over the body. For cancers that are non-invasive or smaller, surgery is used to remove the cancers but recurrence occurs in almost all the cases so additional therapies are given to remove the cancers permanently (Pondé, Zardavas et al. 2019). These therapies are called adjuvant therapies that completely remove the tumour after performing the surgery. The systemic therapies include; chemotherapy, hormonal therapy and targeted therapy (Kuroi, Toi et al. 2006).

2.6.1.1. Hormonal therapy

Hormonal therapy targets cells that are positive for hormone receptors, ER, PR and HER2. Mostly luminal A and luminal B subtypes are treated using this therapy (Locker 1998). Three categories of drugs come under hormonal therapy: hormone blocking medications (estrogen blocking medications) are aromatase inhibitors and luteinizing hormone-releasing hormone (LH-RH) analogues. Example of AH drug is letrozole, that inhibits the production of estrogen. The conversion of testosterone to estradiol is inhibited by this drug (Smith and Dowsett 2003).

Selective estrogen receptor modulators/ SERMs inhibit the action of estrogen receptor. One of the drawbacks of this types of drugs is their partial agonist action, which results in the development of resistance (Swaby, Sharma et al. 2007). An example of this type of drug is tamoxifen. The last category of this type of therapy is the selective estrogen receptor degraders/ SERDs (McDonnell, Wardell et al. 2015). An example of this type of drug is Fulvestrant.

2.6.1.2. Chemotherapy

Chemotherapy is usually recommended for those breast cancer cells that are negative for hormone receptors that is triple negative breast cancer and HER2 cancer cells (Hassan, Ansari et al. 2010). The common type of chemotherapy treatment regimen includes AC chemotherapy. AC chemotherapy is a combination of two medicines, Adriamycin(doxorubicin) and cyclophosphamide (Schlatter and Cameron 2010). Like all chemotherapeutic drugs, these drugs go inside cancerous cells, cause damage to the DNA and kills them eventually. They arrest the cell at any phase of the cell cycle. Another well-known chemotherapy treatment for breast cancers is anthracycline-based treatment. Anthracycline is extracted from bacterium *Streptomyces*. It destroys the genes within cancerous cells and inhibits their reproduction (Hatzis, Pusztai et al. 2011). The lethal side effects associated with chemotherapy are hair loss, diarrhoea, nausea, vomiting, fatigue, and heart complications is some cases.

2.6.1.3. Targeted therapy

Targeted therapy is also known as HER2 directed therapy and is most effective against breast cancer cells that are HER2 positive (Nahta, Yu et al. 2006). This type of therapy affects many different pathways, most important being cyclin dependent kinase (CDK) pathway. CDK4/6 are involved in growth and proliferation of cells and hence uncontrolled growth of cells occurs. The most important drug in this regard is palbociclib that inhibits the pathway mediated by CDK4/6.

2.6.2. Local therapies

Local therapies involve surgeries to remove the tumour. These include mastectomy, lumpectomy, breast-conserving therapy, and radiotherapy. Treatment of breast cancer using surgery not just involves removal of tumour but also surrounding healthy tissues and thorough examination of the axillary lymph nodes. Lumpectomy/breast-conserving therapy is done for non-invasive cancers in which the tumour is removed along with a small portion of healthy breast tissue. If the whole breast is not removed it is named as breast-conserving therapy. The rest of the breast tissue is then given a radiotherapy treatment for some time to avoid the growth of cancer cells anywhere else in the breast. Whereas, in mastectomy the whole breast is removed through surgery and there are several ways to do that.

2.7. Letrozole

A non-steroidal drug, third generation aromatase inhibitor which is most effective in inhibiting the higher expression of estrogen in the body (Eiermann, Paepke et al. 2001). Letrozole binds reversibly to the heme of its cytochrome p450 unit thus preventing the synthesis of estrogen *via* aromatase (Mouridsen, Gershanovich et al. 2003). It is a highly potent drug when administered orally. It is an effective drug to treat breast cancer as it acts as an estrogen receptor positive breast cancer drug.

2.7.1. Mechanism of action

Aromatase inhibitors are used to reduce estrogen production which is critical step in breast tumor regression. Letrozole is a specific aromatase enzyme antagonist. The aromatase enzyme converts androgen into estrogen in non-tumor environment and hence, this mechanism is used by letrozole to reduce the estrogen production by binding to aromatase. Thus, it inhibits the conversion of androgen into estrogen (Bhatnagar 2007).

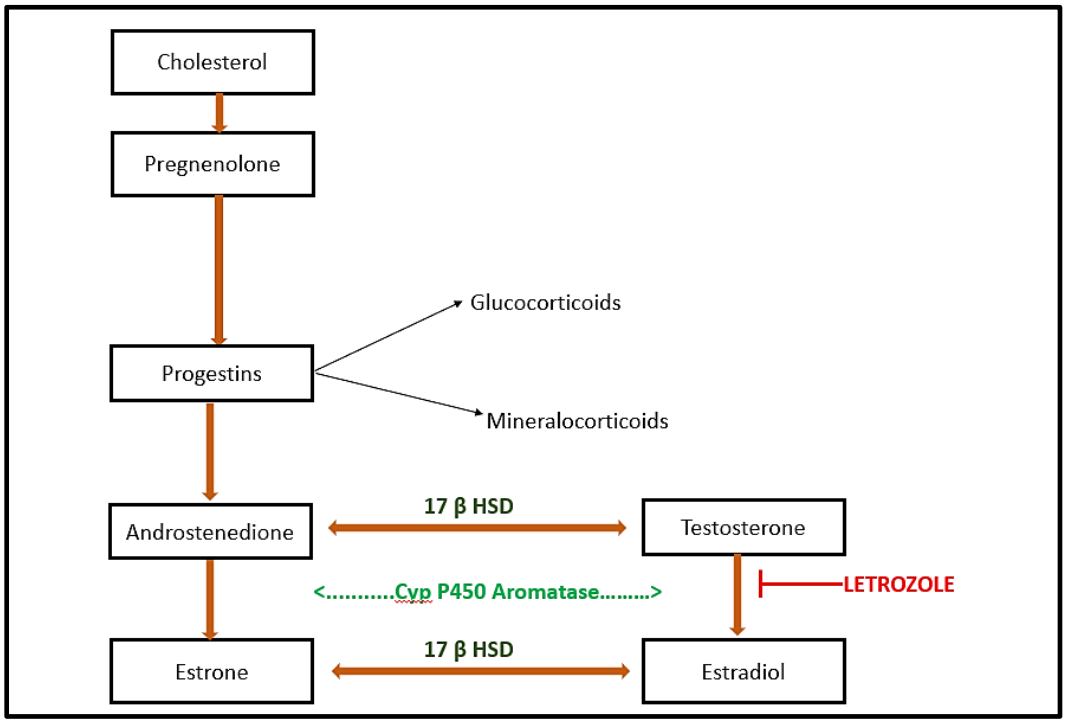

Figure 2. 2. Mechanism of action of Letrozole, (Steroid biosynthetic pathway). Androgens are converted to estrogens *via* Aromatase enzyme complex Cyp 450 (Cytochrome P450, 17 β HSD; 17 β Hydroxy Steroid dehydrogenase) that is inhibited by Letrozole.

2.7.2. Side effects of letrozole

Letrozole have not shown severe side effects in most of the studies. Though using it along with Palbociclib shows few side effects including Neutropenia, leukopenia, Fatigue, Nausea, Arthralgia, Alopecia. Letrozole itself can cause severe side effects if administered alone i.e.: hot flashes, arthralgia, bone fractures, hypercholesterolemia, and cardiac failure. Long term use of letrozole can cause hypoestrogenism which is characterized by sweating, hot flashes, arthralgia and fatigue.

2.8. Fulvestrant

Fulvestrant is used as a second-line endocrine therapy drug, after the development of resistance against some breast cancer drugs (Nathan and Schmid 2017)

2.8.1. Mechanism of action

Fulvestrant acts to disrupt the ER signaling in two different ways; firstly it binds to the ER and blocks the nuclear localization of ER by disrupting its dimerization thus inhibiting transcription of ER and estrogen response element (ERE) controlled genes (Osborne, Wakeling et al. 2004). Secondly, it disrupts ligand-independent activity of ER by disrupting the mitogen activated protein kinase (MAPK)-signaling pathway and downstream processes (Carlson 2005). In case of hormone-dependent breast cancer like luminal A subtype, estradiol binds to the estrogen receptor with high affinity, and changes the shape of the receptor. This binding results in activation of specific binding sites like activation function 1 (AF1) and activation function 2 (AF2). The dimers of the receptors are formed, resulting in activation of transcription factors sites for binding it to ERE in the nucleus that stimulate RNA polymerase enzyme to start the production of estrogen sensitive genes. When fulvestrant binds to the ER, both AF1 and AF2 sites remain inactive, dimers of receptors are formed but do not move to the nucleus. It causes the accelerated

degradation of estrogen receptors and the tumor growth is exponentially reduced (Nathan and Schmid 2017).

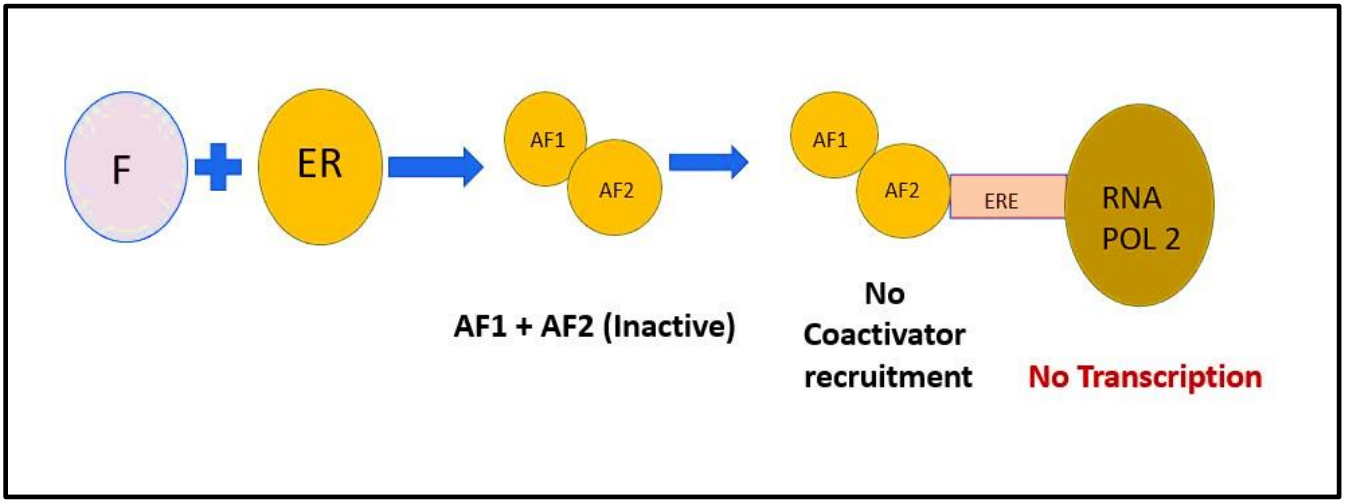

Figure 2. 3. Mechanism of action of Fulvestrant. Fulvestrant blocks transcription by binding with estrogen receptor thereby inactivating AF1 and AF2.

2.8.2. Side effects of Fulvestrant

One of the limitations of Fulvestrant drug delivery is its mode of administration. It is delivered *via* intramuscular injection because of its poor solubility (Young, Renshaw et al. 2008). But intramuscular injection has some limitations like the dose volume of drug is reduced because of intramuscular injection, 250 mg of the dose is administered every 28 days but much of the research is going on knowing the optimal dose of fulvestrant. The higher dose of fulvestrant degraded estrogen receptor more quickly than the lower dose. 5% reduction in the expression of estrogen receptor was observed with 500 mg fulvestrant dose in comparison to reduction observed after administration of 250 mg of dose. Many side effects associated with intramuscular

(IM) injection include muscle site pain, nausea, swelling at injection site, etc. (Johnston and Cheung 2010). Other side effects are associated with the systemic toxicity of healthy tissues.

2.9. Palbociclib

Palbociclib is a selective inhibitor of the CDK4 and CDK6 and is very potent in its mechanism of action. The CDK are important for the control of normal functioning of the cells as they are required for the cell cycle control and regulation (Turner, Ro et al. 2015).

2.9.1. Mechanism of action

The CDK 4 and CDK 6 are the cyclin dependent kinases. The proliferation or growth signals allows the Cyclin D to bind to CDK4 or CDK 6, then this combined cyclin and CDK complex further allows the phosphorylation of Rb protein which is the retinoblastoma tumor suppressor protein (de Dueñas, Gavila-Gregori et al. 2018). The retinoblastoma (RB1) protein is bound to E2F transcription factor as well. When the Cyclin-CDK 4 or 6 complex binds to Rb protein and phosphorylates it, the E2F transcription factor promotes gene expression that leads towards cellular proliferation upon entry into S phase of cell cycle. If this pathway expresses continuously it can lead towards tumor formation and cancer progression. Palbociclib drug is used as an inhibitor for these cyclin dependent kinases. The mechanism of action of the drug is to arrest cells in the G1 phase by blocking RB phosphorylation at CDK4/6 (Klein, Kovatcheva et al. 2018).

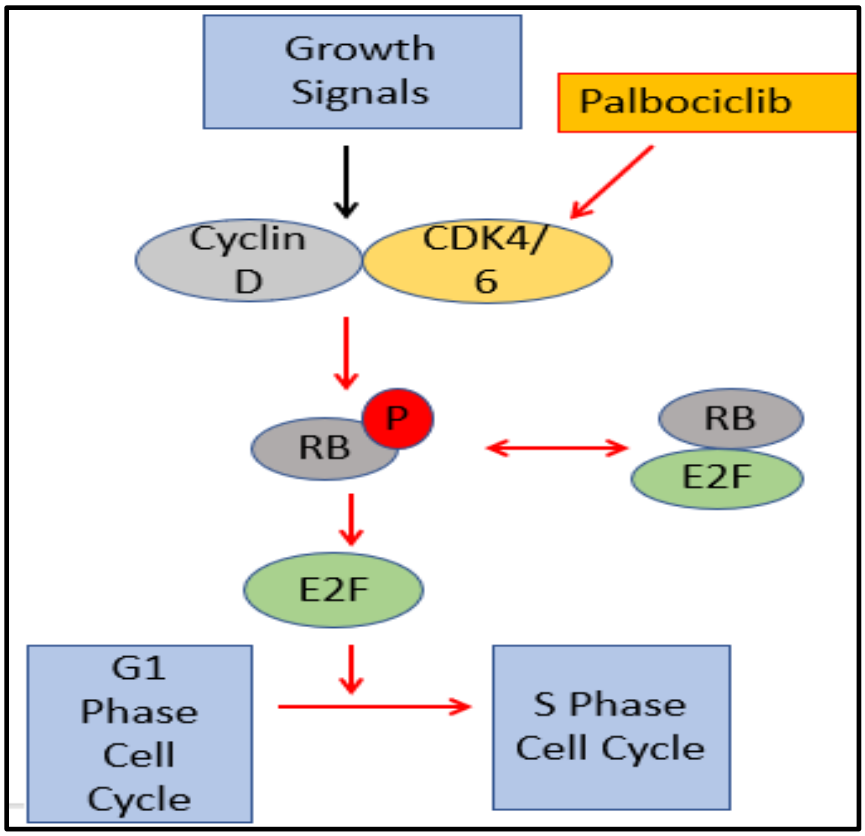

Figure 2. 4. Mechanism of action of Palbociclib. Palbociclib arrests breast cancer cells at cell cycle by inhibiting CDK4/6 to phosphorylate retinoblastoma (RB) protein.

2.9.2. Side effects of Palbociclib

The side effects of the drugs were neutropenia, anemia, fatigue, upper respiratory infections, diarrhea, decreased appetite, alopecia among others (Ahsan, Malik et al. 2017).

2.10. Clinical trials of drug

Drugs before marketing should pass clinical trials. Some of the successful clinical trials for drugs against breast cancer include combinatorial therapies. These trials include PALOMA 3 and SOLAR 1.

2.10.1. PALOMA 3 Trials

In Paloma 3 trials, Palbociclib after combining with fulvestrant were given to hormone receptor positive, HER2 negative breast cancer patients (Iwata, Im et al. 2017). This treatment was given to patients who failed in the hormone therapy previously. It was designed in 2010, with a total of 521 patients assigned to two groups. One group was fulvestrant-placebo group in which 147 patients were placed and the other group, Palbociclib-fulvestrant group, had 347 patients. A total of 201 deaths occurred in the Palbociclib-fulvestrant group compared to the 109 deaths in the placebo group. 16.1% of more people survived in the Palbociclib-fulvestrant group as compared to the placebo group. So, rate of survival was 50% in the fulvestrant-palbociclib group compared to 41% in the placebo group. The survival was prolonged by the 7 months by the addition of palbociclib to the fulvestrant. So this treatment regime was considered a big success (O'Leary, Cutts et al. 2018).

Another trail combines hydroxychloroquine, letrozole and palbociclib (Vijayaraghavan, Karakas et al. 2017) for the treatment of hormone receptor positive, HER2 negative breast cancers. This study was done to analyze the side effects caused by hydroxychloroquine when given along with letrozole and palbociclib. This study was designed in 2018 and is completed in 2020 which is comprised of 54 participants. The main objective was to check whether hydroxychloroquine augments the anti-tumor effects of letrozole and palbociclib.

2.10.2. SOLAR 1 Trials

The hormone receptor positive and HER2 negative breast cancer due to resistance against therapies results in Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) mutations. In this study, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PIK3)-Alpha inhibitor was combined with fulvestrant for the treatment of hormone receptor positive

breast cancers. A total of 572 patients were enrolled in the study with more than half of the patients with PIK3CA mutations. One group were given alpelicib-fulvestrant treatment, and the other group was given placebo fulvestrant. A progression-free survival of 11 months was seen in the first month as compared to the 5.7 months in the latter group. So it was concluded that survival was increased with the use of PI3K-Alpha inhibitor in PI3K mutated, hormone receptor positive, HER2 negative breast cancers (André, Ciruelos et al. 2018).

2.11. Limitations in current therapies

Now that we have adjuvant and neoadjuvant therapies to treat breast cancer, combinatorial use of chemotherapy and hormonal therapy, hormonal therapy and targeted therapy, surgery, and radiotherapy, still the diagnosis and prognosis of this disease is somewhat poor. There are some serious limitations of these current therapies. The recurrence of cancer occurs in 70% of the cases even after trying all the possible treatment options (Arnedos, Vicier et al. 2015). We have no way of knowing who will develop invasive metastatic disease and who will not. The drugs have high cytotoxicity in most cases. Resistance to hormone therapy is a well-known phenomenon. The activation of alternative pathways in TNBC results in the development of resistance mechanisms. The crosstalk of these pathways leads to the generation of resistance. Resistance is also developed due to the mutations in the drug target, or activation of pathways downstream or upstream of the target.

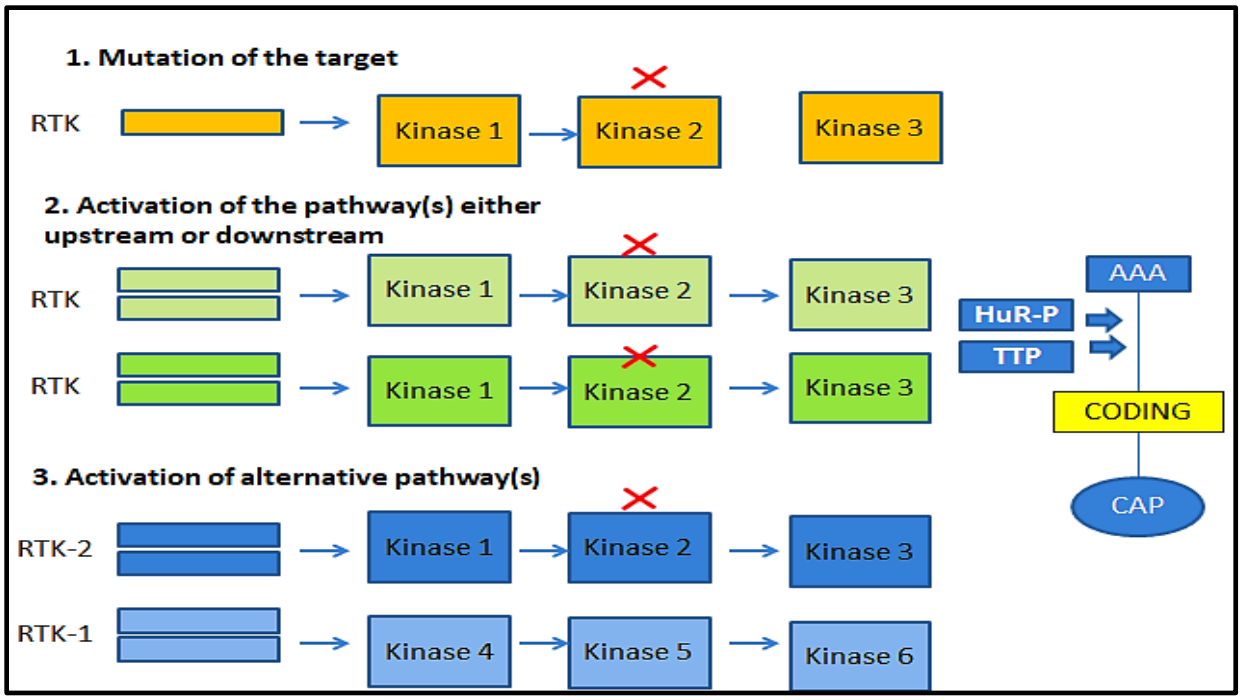

Figure 2. 5. Development of resistance mechanisms against drugs

2.12. Nanoparticles

The field of research is progressing exponentially with new therapies and new inventions being made to help solve the problems at a better and faster rate. In biomedical applications one of the new treatment options is to deliver drug by using the nanoparticles as a delivery vehicle and to check how the drug interacts with the entire body (Mohanraj & Chen, 2006).

Another great use for nanoparticle is in the treatment of cancer. There are many improvements in fighting against breast cancer with vast range of clinical presentations and multiple treatment options available but still it is a leading cause of death worldwide. It is through recent knowledge the concept of precision medicine is being described and how each individual person has a different tumor microenvironment and responds differently to the current treatment regimes. NP

offer a great help to solving this problem as being easy to produce and being loaded with various drugs catering to the specific person at hand requiring the treatment.

The NP can be manipulated easily by changing the drug to attach on it and their shape can be modified according to the desired response required, having better retention time in *in vivo* settings. The nanotechnology system for targeted drug delivery offers new and innovative solution for the treatment of the cancer (Muntimadugu et al., 2017).

2.13. Gold Nanoparticle

The AuNP utilize the gold atom that are then conjugated with various drugs and components to help increase their efficacy and targeted delivery. The AuNP has benefits of its own such as anti-bacterial property which helps suggest it to be used for drug delivery process. (Grace, Pandian, 2007). They can be loaded with different materials such as proteins, antibodies, and DNA for targeted drug delivery. The shape can also be modified to suit the need as Nano rods, Nano sphere and Nano cluster. The following diagram is taken from the article (Singh et al., 2018) and tells how the surface of the gold can be modified by drugs, genes, proteins, or likers. It states the fact that the shape and size of gold nanoparticle can be tunable according to the desired protocol.

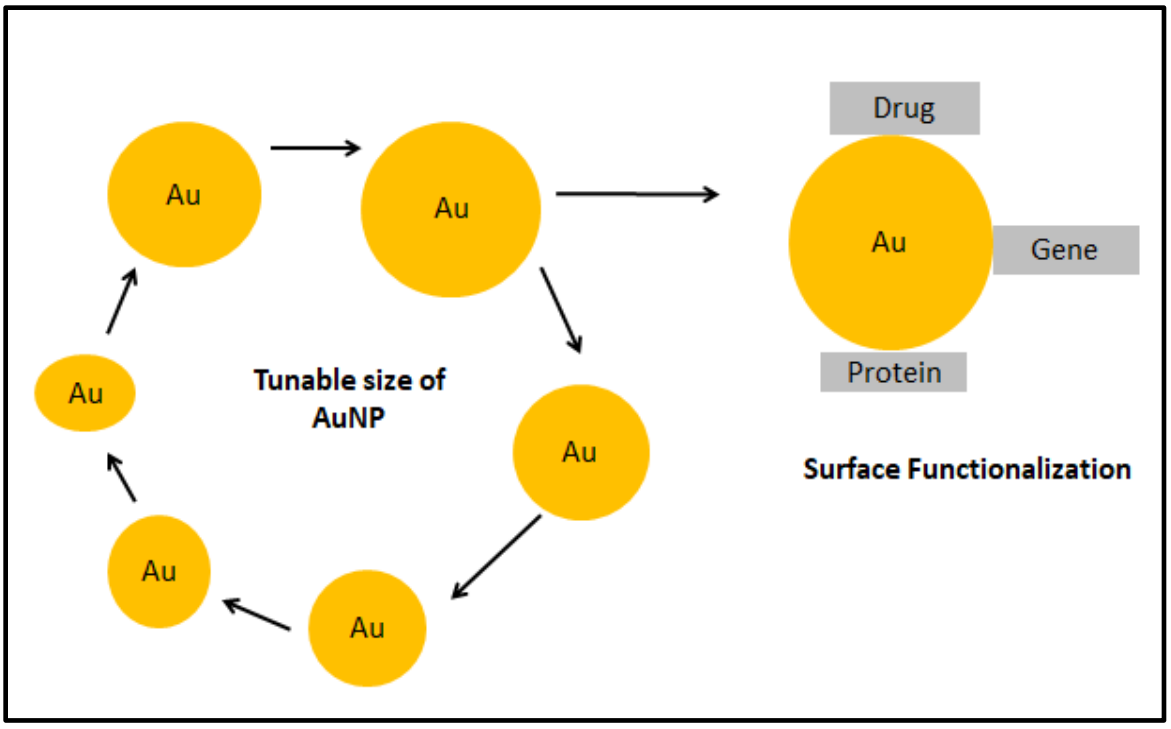

Figure 2. 6. Gold nanoparticle (AuNP) surface functionalization by adding various components along with optimizing the size and shape of the AuNP.

2.14. *In silico* approaches for combining AuNPs with drugs

In silico means the experiments that are carried out using computer simulation. ChemDraw is the name of the software that lets users draw the chemical structures of different receptors, ligands, drugs, linkers etc. by drawing the two-dimensional structures of organic molecules to predict the connectivity between molecules based on bonds between them. These *in silico* approaches are highly effective before going into *in vitro* or *in vivo* studies. For virtual screening and molecular docking AutoDock Vina, PatchDock and many other online tools are available that was used by researchers to identify the inhibitors against three most common breast cancer receptors, epidermal growth factor receptor/ EGFR, HER2 receptor and heat shock protein/ HSP90 (Sandeep, Nagasree et al. 2011). Some flavonoids were tested for their anti-tumor activity by

docking them with estrogen receptor alpha. Docking was performed by PatchDock and visualization was done using Pymol (Froufe, Abreu et al. 2011). Thiohydantoin Derivatives were tested for anti-tumor activity against tyrosine-protein kinase ERB-3 since it showed interactions when visualized through *in silico* approaches. PatchDock software was used for the docking (Suzen and Buyukbingol 2000).

CHAPTER 3

MATERIALS AND METHODS

This research was conducted *in silico* by using online available tools for collecting the data and processing it. In this study, the drug conjugated gold nanoparticles were firstly synthesized by downloading the mol. files of the drug and molecules required to make nanoparticles from the online sources available. Later, the complete drug conjugated nanoparticle structure was drawn by using ChemDraw software. The docking of drug with cancer receptor/ protein variant and also drug conjugated gold nanoparticles with cancer receptor/ protein variant were done using PatchDock, an online available tool. Then, further visualization of these docked complexes was done using discovery studio software and Pymol to check the conventional hydrogen bonds and bond length between the docked complexes. The brief overview of the steps followed in methodology of this research given in Figure 3.1.

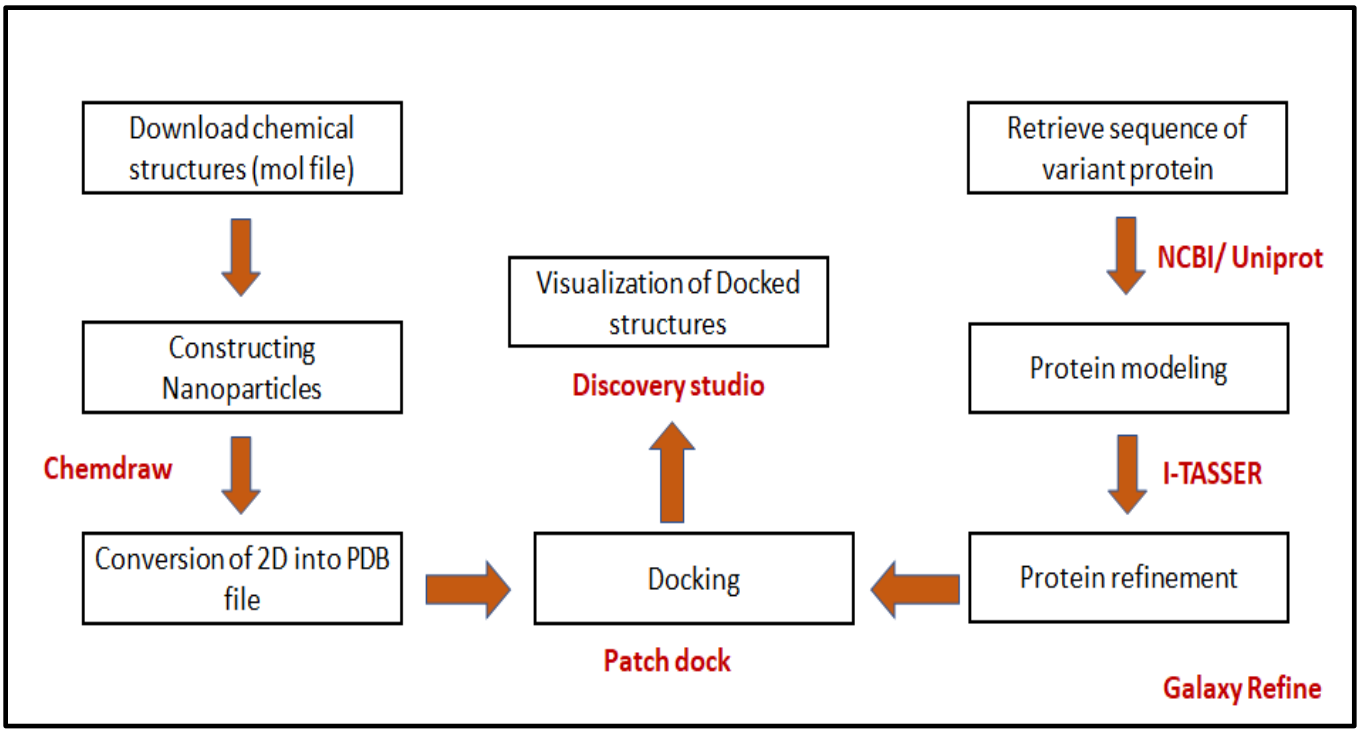

Figure 3. 1. Overview of steps involved in methodology.

3.1. Computational tools and software's under *in silico* approaches

There are some tools used for *in silico* studies. In this study, the tools used were mentioned in Table 3.1.

Table 3. 1. Online available tools with their URLs used in this study for *in silico* analysis of drug conjugated AuNPs.

Tools Used	Description	URLs
UniProt	Receptor/ protein FASTA sequences were retrieved	. https://www.uniprot.org/
I-TASSER	For modelling of variant receptor/ protein	https://zhanglab.ccmb.med.umich.edu/I-TASSER/

GalaxyRefine	For refining the modelled cancer variant receptor/ protein	http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE
ChemDraw	Free software was used for the constructing nanoparticles	
PatchDock	Online docking software for proteins and ligands.	https://bioinfo3d.cs.tau.ac.il/PatchDock
Discovery Studio	Free online software was used to view the docked structures and to see their conventional hydrogen bonds.	https://discover.3ds.com/discovery-studio-visualizer-download-thank-you
Pymol	Open-source molecular visualization tool	https://pymol.org/2/

3.2. Letrozole conjugated Au nanoparticles docked on breast cancer inducing Cytochrome P450 Aromatase variant

The .mol files of Letrozole, PEG, Aloin, Gymnemic acid and honey were taken from the online sources for the construction of drug conjugated gold nanoparticles. The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot (https://www.uniprot.org). The sequence ID for the Cyt P450 Aromatase variant involved in breast cancer is P04798 (CP1A1_HUMAN) (VAR_016941) (Gonullu et al., 2007). AuNP derived from *Aloe Vera***,** nanoparticle derived from *Gymnema sylvestre* and AuNPs derived from Honey AuNPs have gold metal atom in center that is joined to Aloin, Gymnemic acid and honey respectively and by using PEG as a linker the drug Letrozole was conjugated with AuNPs on ChemDraw (Donga et al., 2021, Boldeiu et al., 2019, Nirmala et al., 2018).

The mutant variant of breast cancer receptor/ proteins including the Cyt P450 Aromatase was converted into 3D models using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and further refined by GalaxyRefine software (Samavarchi et al., 2020). Hence the docking of Docking of Cyt P450 Aromatase variant with Letrozole conjugated gold nanoparticle was performed using the PatchDock (https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php) (Krisnamurti et al., 2019). The visualization of Letrozole conjugated AuNP derived from Honey docked Cyt P450 Aromatase variant the software DiscoveryStudio was used (Krisnamurti et al., 2019) and for the visualization of Letrozole conjugated AuNP derived from *Aloe Vera* with Cyt P450 Aromatase variant the Pymol was used. The visualization of Letrozole conjugated nanoparticle derived from *Gymnema sylvestre* docked Cyt P450 Aromatase variant the software Pymol (https://pymol.org/2/) was used.

3.3. Fulvestrant conjugated Au nanoparticles docked against Estrogen α-receptor variant

The .mol files for the drug Fulvestrant, and PEG, Aloin, Gymnemic acid and Honey were taken from the online sources for the generation of the drug conjugated gold nanoparticles. The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website. The sequence ID for the ER variant involved in breast cancer is P03372 [264 - 264] (VAR_033029) and the AuNP derived from *Aloe Vera***,** AuNP derived from *Gymnema sylvestre* and AuNP derived from Honey AuNPs have gold metal atom in center that is joined to Aloin, Gymnemic acid and honey respectively and by using PEG as a linker the drug Fulvestrant was conjugated with AuNPs (Donga et al., 2021, Boldeiu et al., 2019, Nirmala et al., 2018, Gonullu et al., 2007).

The mutant variant of breast cancer receptor/ proteins including the ER variant was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software (Samavarchi et al., 2020).

Hence the docking was done between the Cyt P450 Aromatase variant with Fulvestrant conjugated gold nanoparticle by using the PatchDock Software (Krisnamurti et al., 2019). The visualization of Fulvestrant conjugated gold nanoparticle docked ER variant the software DiscoveryStudio software (Krisnamurti et al., 2019).

3.4. Palbociclib conjugated Au nanoparticles docked on breast cancer CDK4 variant

The .mol files for the drug Palbociclib, and PEG, Aloin, Gymnemic acid and Honey were taken from the online sources for the generation of the drug conjugated gold nanoparticles.

The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website (Gonullu et al., 2007). The sequence ID for the CDK4 variant involved in breast cancer is the P11802 (CDK4_HUMAN) UBERON:0000310. The CDK4 FASTA sequence was taken form Uniprot and then manually mutated at the 43 positions, which is involved in the breast cancer variant. AuNP derived from *Aloe Vera***,** AuNP derived from *Gymnema sylvestre* and AuNP derived from Honey have gold metal atom in center that is joined to Aloin, Gymnemic acid and honey respectively and by using PEG as a linker the drug Palbociclib was conjugated with AuNPs (Donga et al., 2021, Boldeiu et al., 2019, Nirmala et al., 2018)

The mutant variant of breast cancer proteins including the CDK4 variant was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software (Samavarchi et al., 2020).

Hence the docking was done between the CDK4 variant with Palbociclib conjugated gold nanoparticle by using the PatchDock Software (Krisnamurti et al., 2019). The visualization of Palbociclib conjugated gold nanoparticle docked CDK4 variant the software Discovery Studio software (Krisnamurti et al., 2019).

3.5. Palbociclib conjugated Au nanoparticles docked on breast cancer CDK6 variant

The .mol files for the drug Palbociclib, and PEG, Aloin, Gymnemic acid and Honey were taken from the online sources for the generation of the drug conjugated AuNPs. The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website. The sequence id for the CDK6 variant involved in breast cancer is P42773 (CDN2C_HUMAN) VAR_001490. AuNP derived from *Aloe Vera***,** AuNP derived from *Gymnema sylvestre,* AuNP derived from Honey have gold metal atom in center that is joined to Aloin, Gymnemic acid and honey respectively and by using PEG as a linker the drug Palbociclib was conjugated with AuNPs (Donga et al., 2021, Boldeiu et al., 2019, Nirmala et al., 2018, Gonullu et al., 2007).

The mutant variant of breast cancer proteins including the CDK6 variant was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software. (Samavarchi et al., 2020)

Hence the docking was done between the CDK6 variant with Palbociclib conjugated gold nanoparticle by using the PatchDock Software (Krisnamurti et al., 2019). The visualization of

Palbociclib conjugated gold nanoparticle docked CDK6 variant the software Discovery Studio software (Krisnamurti et al., 2019).

3.6. Unconjugated Drugs docked against breast cancer receptor/ protein variant

3.6.1. Letrozole docked with cytochrome p450 Aromatase

The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website. The sequence id for the Cyt P450 Aromatase variant involved in breast cancer is P04798 (CP1A1_HUMAN) (VAR_016941). (Gonullu et al., 2007)

The mutant variant of breast cancer receptor/ proteins including the Cyt P450 Aromatase was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software (Samavarchi et al., 2020). The docking was done between the drug Letrozole with their respective Cytochrome P450 variant by using the PatchDock Software (Krisnamurti et al., 2019). The visualization of Letrozole docked Cytochrome P450 variant was viewed using Pymol software.

3.6.2. Fulvestrant docked with Estrogen receptor α protein breast cancer variant

The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website. The sequence id for the ER variant involved in breast cancer is P0 P03372 [264 - 264] (VAR_033029) (Gonullu et al., 2007).

The mutant variant of breast cancer receptor/ proteins including the ER variant was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software (Samavarchi et al., 2020)**.** The docking was done between the ER variant

with Fulvestrant by using the PatchDock Software (Krisnamurti et al., 2019). The visualization of Fulvestrant docked with Estrogen Receptor variant was viewed using DiscoveryStudio software (Krisnamurti et al., 2019).

3.6.3. Palbociclib docked with CDK4 breast cancer variant

The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website. The sequence id for the CDK4 variant involved in breast cancer is the P11802 (CDK4_HUMAN) UBERON:0000310. The CDK4 FASTA sequence was taken form Uniprot and then manually mutated at the 43 positions, which is involved in the breast cancer variant.

The mutant variant of breast cancer proteins including the CDK4 variant was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software (Samavarchi et al., 2020)**.** The docking was done between the CDK4 variant with Palbociclib by using the PatchDock Software (Krisnamurti et al., 2019). The visualization of Palbociclib docked with CDK4 variant was viewed using Discovery Studio software (Krisnamurti et al., 2019).

3.6.4. Palbociclib docked with CDK6 breast cancer variant

The FASTA sequence of the breast cancer receptor/ protein variants were retrieved from the Uniprot website. The sequence ID for the CDK6 variant involved in breast cancer is P42773 (CDN2C_HUMAN) VAR_001490 (Gonullu et al., 2007).

The mutant variant of breast cancer proteins including the CDK6 variant was created into 3D models using the online I-TASSER software and the results were further refined by GalaxyRefine software (Samavarchi et al., 2020). The docking was done between the CDK6 variant with Palbociclib by using the PatchDock (Krisnamurti et al., 2019). The visualization of

Palbociclib docked with CDK6 variant was viewed using DiscoveryStudio software (Krisnamurti et al., 2019).

Chapter 4

Results

4.1. *In silico* evaluation of drug conjugated gold nanoparticles

Drug delivery through drug conjugated gold nanoparticles is an efficient way to deliver drugs at target receptors and treat breast cancers. Drug formulations can be analyzed carrying out *in silico* procedures using different online softwares. These computational tools enable us to study binding efficacy and bonding interactions between ligand and receptor proteins to let us choose the best among these interactions to conclude our results. After *in silico* analysis we will proceed our study further *in vitro* and *in vivo*.

4.2. Letrozole conjugated Au nanoparticles against breast cancer inducing Cytochrome P450 Aromatase

Letrozole conjugated gold nanoparticles constructed from three sources: *Aloe vera*, *Gymnema sylvestre*, *Honey*. Letrozole was conjugated with gold nanoparticles derived from these three sources and these nanoparticles were constructed in ChemDraw as shown in Figure 4.1. These conjugated nanoparticles were used for the *in silico* evaluation against cytochrome p450 aromatase variant protein. The gold particles are positioned at center highlighted in golden, the Gymnemic acid, aloin and honey are highlighted in green, Polyethylene glycol (PEG), the linker is highlighted in blue whereas letrozole is highlighted in pink, Figure 4.1.

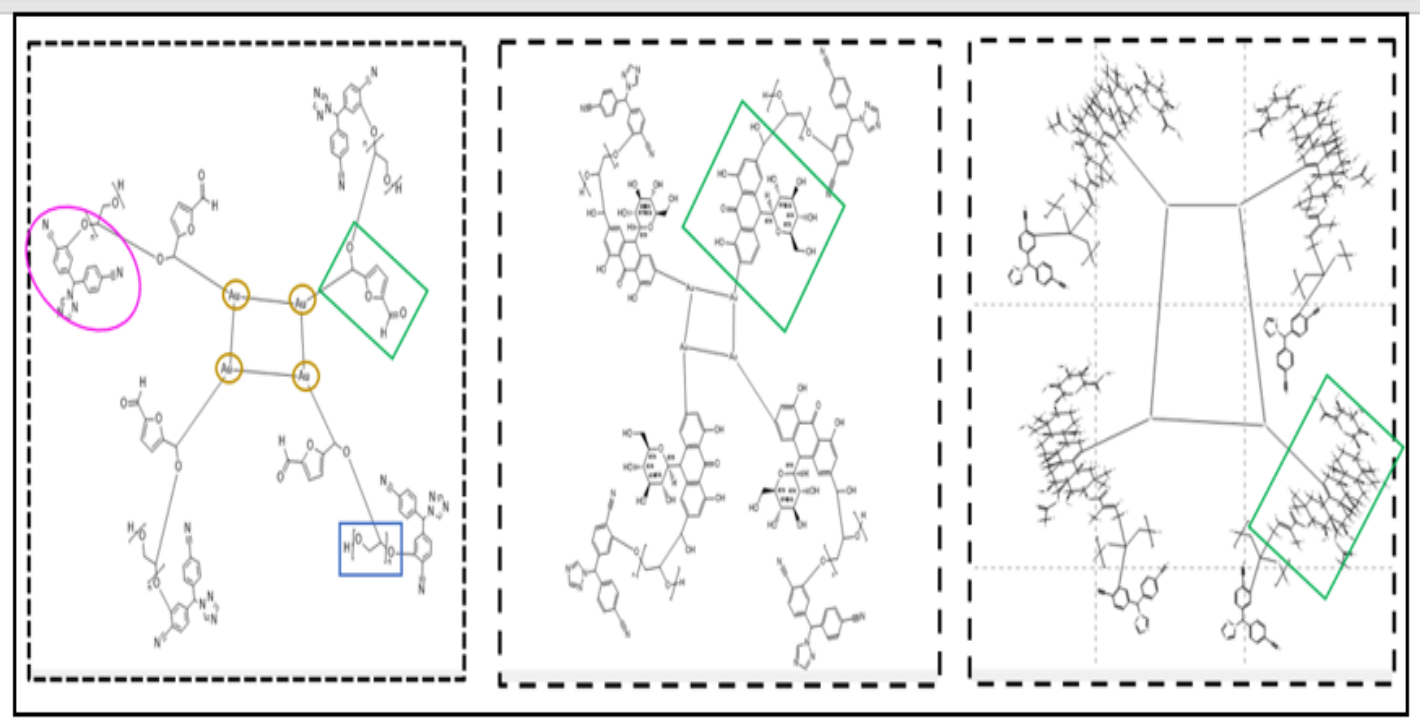

Figure 4. 1. Synthesis of drug conjugated gold nanoparticles a) letrozole conjugated gold nanoparticles derived from Honey b) letrozole conjugated gold nanoparticles derived from *Aloe vera* c) letrozole conjugated gold nanoparticles derived from *Gymnema sylvestre* in ChemDraw.

The sequence of variant protein was retrieved by Uniprot having protein ID ()P04798 [CP1A1_HUMAN] VAR_016941) and was uploaded on I-TASSER for protein modelling giving C-score (-0.12), TM score (0.70±0.12), RMSD (7.6±4.3Å), Poor Rotamer (9.2), Rama Favored (85.9), Figure 4.2. The protein model was refined through GalaxyRefine with RMSD value (0.333 Å), Poor Rotamers (0.4), Rama favored (96.5) shown in Figure 4.9 with change in values of RMSD (7.267 Å), Poor Rotamer (8.8), Rama favored (10.6).

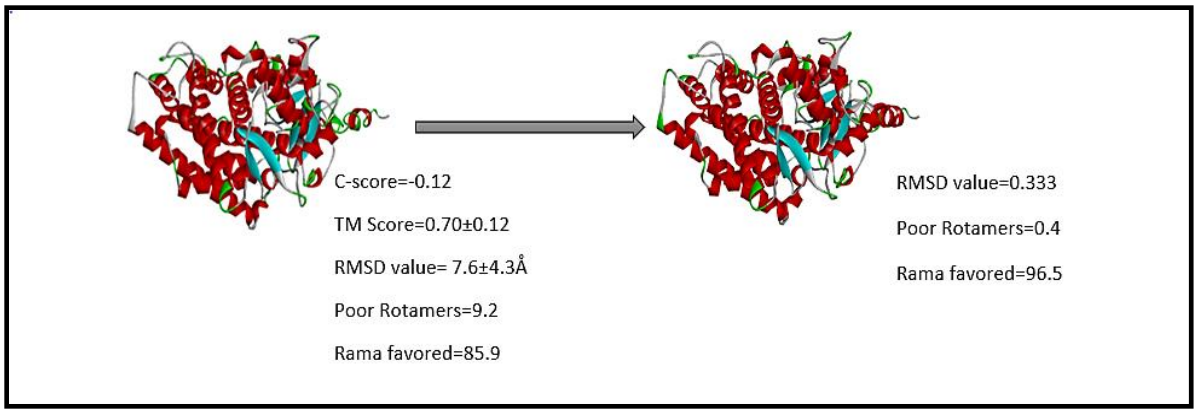

Figure 4. 2. The Cytochrome P450 aromatase variant protein modelled using I-TASSER and is refined using GalaxyRefine giving change of RMSD was 7.267 Å, Poor Rotamer was 8.8 and Rama favoured value was 10.6.

The refined protein structures were docked with letrozole conjugated gold nanoparticles derived from *honey*, *Gymnema sylvestre* and *Aloe vera*, Figure 4.3. Docking was performed using PatchDock giving different number of hydrogen bonds with each nanoparticle derived source though highest number of conventional hydrogen bonds were observed in case of *Aloe vera* derived gold nanoparticle, Table 4.1. Binding energies for *honey* derived, *Aloe vera* derived and *Gymnema sylvestre* derived nanoparticles were -451.1kcal/mol, -917.89kcal/mol, -1319.24kcal/mol, respectively.

Table 4. 1. Hydrogen bonds formed between Letrozole conjugated gold nanoparticles docked with Cytochrome P450 Aromatase with respective bond distances.

Letrozole conjugated Au NP derived from *Aloe vera* docked with Cytochrome P450		
From	**To**	**Distance**
A:SER29:HG	:UNK0:O	2.78456
A:GLN32:HE21	:UNK0:O	2.60684
A:ARG77:HE	:UNK0:O	2.96623
A:ARG77:HH21	:UNK0:O	2.22513
A:ASN245:H	:UNK0:N	2.7378
A:PRO238:CD	:UNK0:O	3.32396
Letrozole conjugated Au NP derived from *Gymnema sylvestre* docked with Cyt P450		
From	**To**	**Distance**
A:TRP44:H	:UNK0:O	2.24981
A:TRP44:H	:UNK0:O	1.86686
A:LEU48:H	:UNK0:O	1.8501
Letrozole conjugated Au NP derived From Honey docked with Cyt450		
From	**To**	**Bond Distance**
A:LEU53:CA	:UNK0:N	3.6614
A:LEU53:CA	:UNK0:N	3.6614

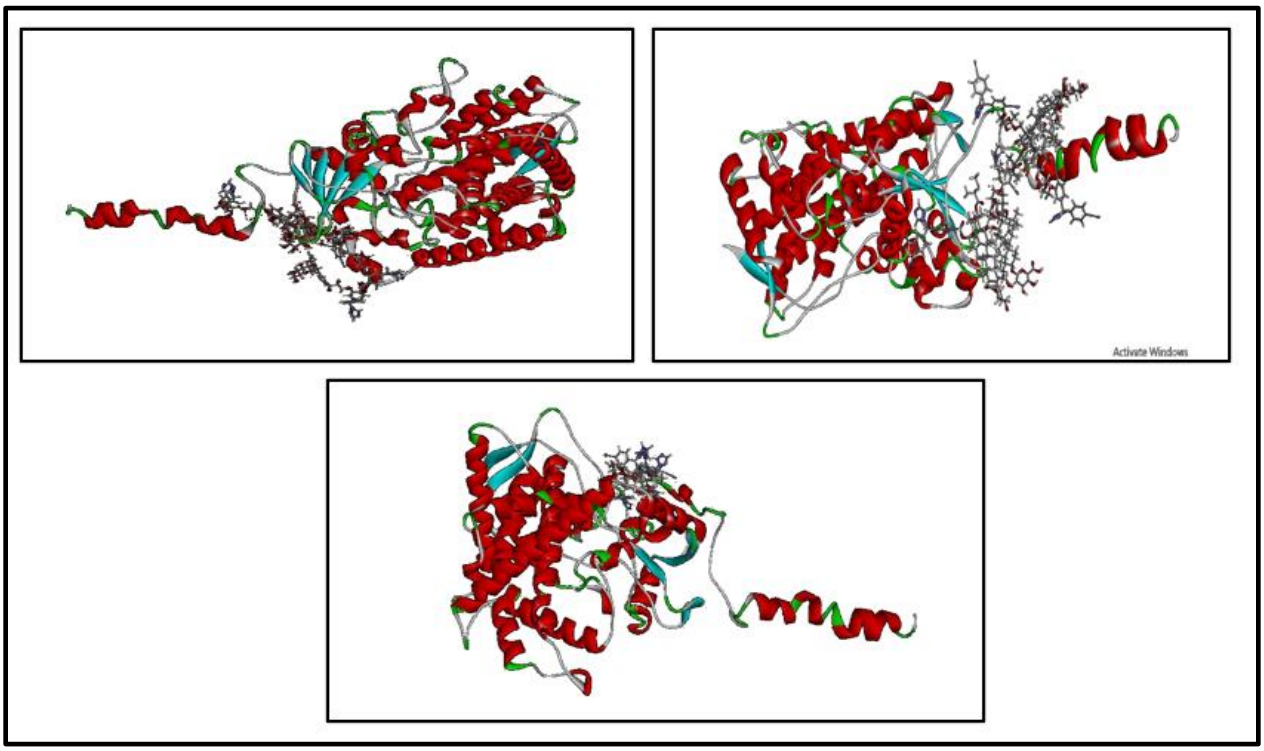

Figure 4. 3. Docking of drug conjugated gold nanoparticles with Cytochrome P450 Aromatase (A) letrozole conjugated gold nanoparticles derived from *Aloe vera* (B) letrozole conjugated gold nanoparticles derived from *Gymnema sylvestre* (C) letrozole conjugated gold nanoparticles derived from Honey.

4.3. Fulvestrant conjugated Au nanoparticles against breast cancer inducing Estrogen α- receptor

Three gold nanoparticles were created in ChemDraw each derived from *Aloe vera, Gymnema sylvestre* and *honey,* respectively. These nanoparticles were conjugated to Fulvestrant *via* linker

Polyethylene glycol (PEG) highlighted in blue where Fulvestrant is highlighted in red, Figure 4.4.

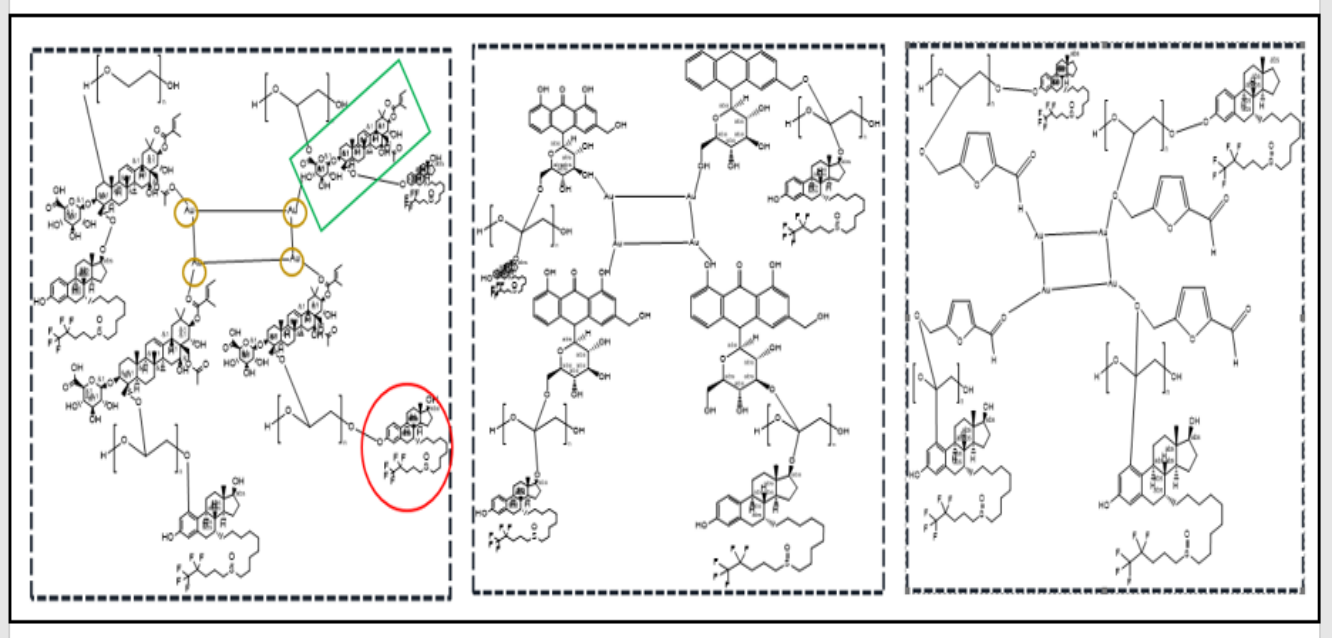

Figure 4. 4. Synthesis of drug conjugated gold nanoparticles a) Fulvestrant conjugated gold nanoparticles derived from *Gymnema sylvestre* b) Fulvestrant conjugated gold nanoparticles derived from *Aloe vera* c) Fulvestrant conjugated gold nanoparticles derived from Honey in ChemDraw.

The sequence of variant protein was retrieved by Uniprot searching for the protein variants of Estrogen receptor which causes breast cancer induction in humans. The protein ID (P03372[262-262] VAR_033029) was retrieved and was uploaded on I-TASSER giving C-score (2.71), TM score (0.40), RMSD (14.5Å), Poor Rotamers (9.5), Rama Favored (86.2). The refinement of modeled protein was done on GalaxyRefine giving values of RMSD (0.541Å), Poor rotamers (1.0), Rama Favoured (92.2) with the change of RMSD, Poor rotamer and Rama favoured was 13.959 Å, 8.5, 6 respectively as shown in Figure 4.5.

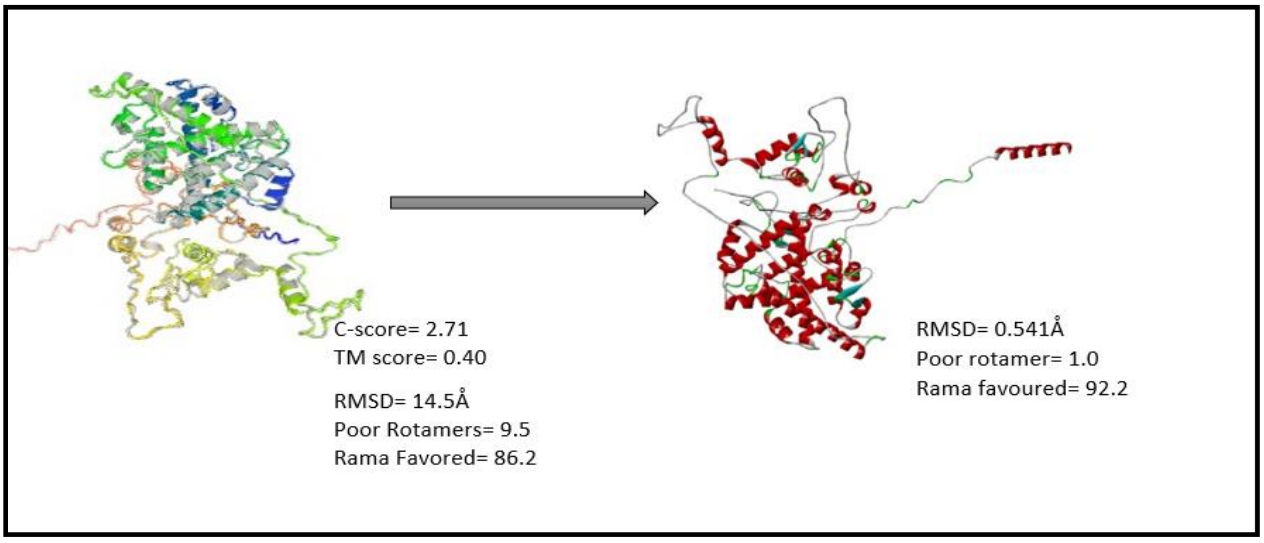

Figure 4. 5. The Estrogen receptor variant protein modelled using I-TASSER and is refined using GalaxyRefine giving change of RMSD was 13.959 Å, Poor Rotamer was 8.5 and Rama favoured value was 6.

The refined protein was docked with fulvestrant conjugated gold nanoparticles from three sources as mentioned above, Figure 4.6. *Aloe vera* derived AuNPs, *Gymnema sylvestre* AuNPs and *Honey* derived AuNPs docked with Fulvestrant was done using PatchDock giving binding energies -1516.52 kcal/mol, -1017.64 kcal/mol and -970.86 kcal/mol, respectively. Whereas hydrogen bonds in each docked complex was found to be only one in the case of Fulvestrant as shown in Figure 4.6. Honey conjugated AuNP docked with ER formed one conventional hydrogen bond: UNK0:N :A:LEU53:CA having bond distance 3.66A° unlike Letrozole and Palbociclib which showed more than one hydrogen bonds, Table 4.2.

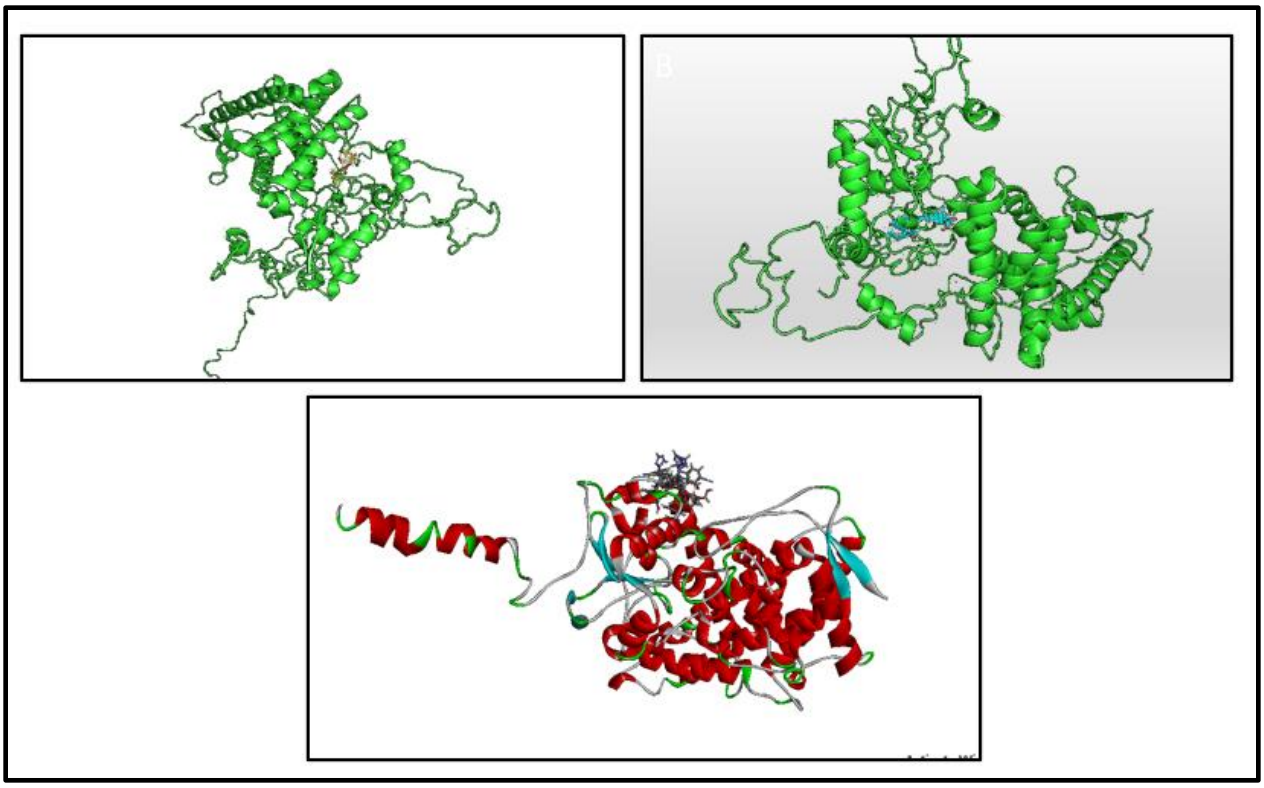

Figure 4. 6. Docking of drug conjugated gold nanoparticles with Estrogen receptor (A) Fulvestrant conjugated gold nanoparticles derived from *Aloe vera* (B) Fulvestrant conjugated gold nanoparticles derived from *Gymnema sylvestre* (C) Fulvestrant conjugated gold nanoparticles derived from Honey.

4.4. Palbociclib conjugated Au nanoparticles against breast cancer inducing CDK4 protein

Palbociclib was conjugated to gold nanoparticles derived from the same three sources as mentioned above. The gold nanoparticle structures were drawn in ChemDraw using *Aloe vera, Gymnema sylvestre* and *honey* and Palbociclib highlighted in violet. Figure 4.7.

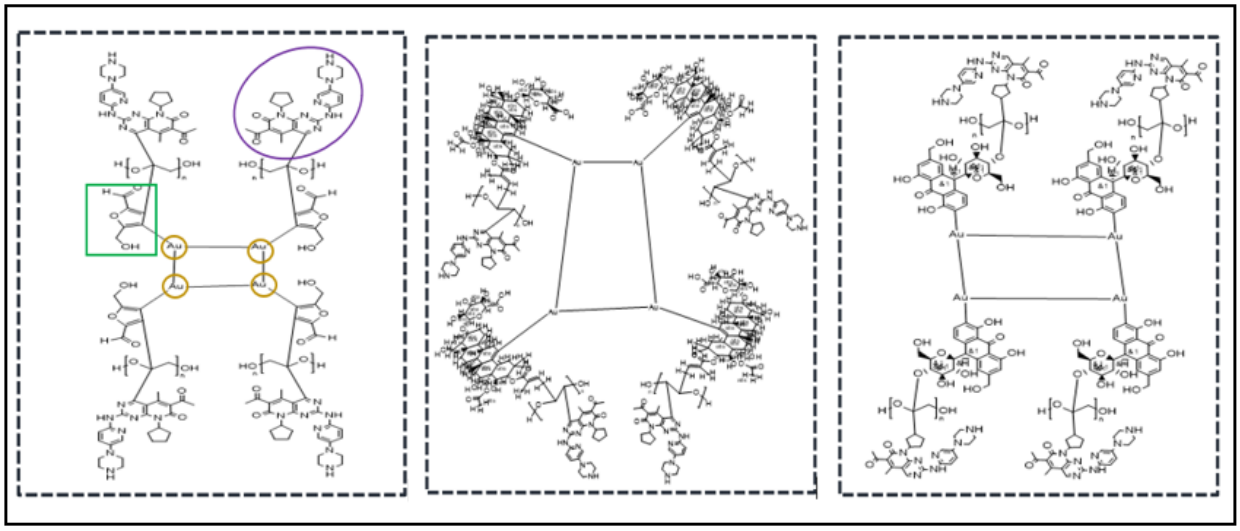

Figure 4. 7. Synthesis of drug conjugated gold nanoparticles a) Palbociclib conjugated gold nanoparticles derived from honey b) Palbociclib conjugated gold nanoparticles derived from *Gymnema sylvestre* c) Palbociclib conjugated gold nanoparticles derived from *Aloe vera* in ChemDraw.

The normal protein sequence of CDK4 was retrieved from Uniprot and the mutated sequence in the CDK4 protein sequence was acquired from Biochem Hive and the normal protein sequence was then edited to insert mutated sequence having protein ID (P11802 [CDK4_HUMAN] UBERON:0000310). It was then uploaded on I-TASSER providing C-score (0.32), TM score (0.76±0.10), RMSD (5.5±3.5Å), Poor Rotamers (12.4), Rama Favored (82.4). Refining modelled protein using GalaxyRefine give RMSD, Poor rotamer and Rama favoured values were 0.338 Å, 0.0, 96.7 respectively, Figure 4.8.

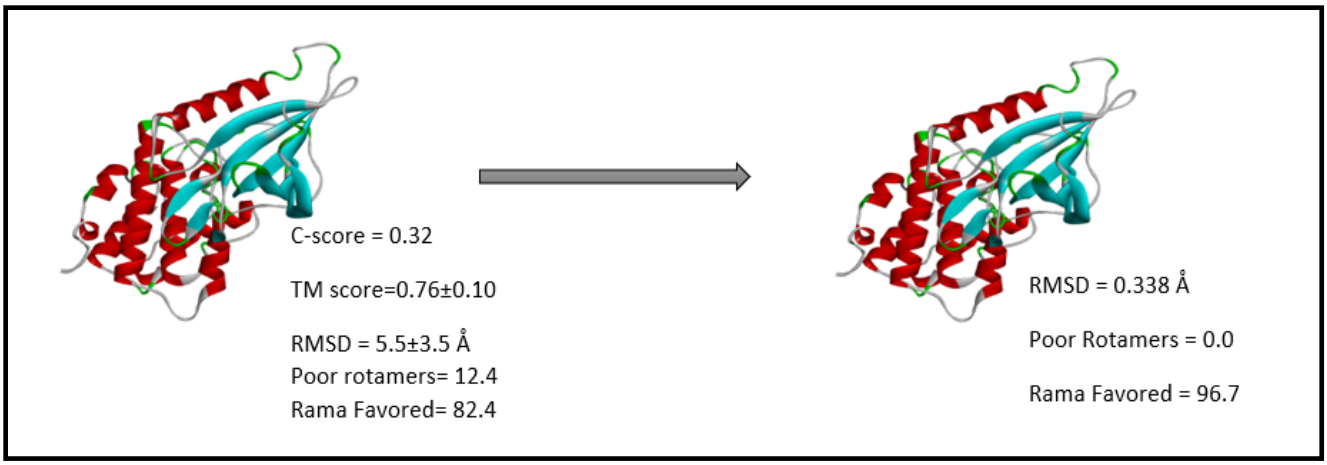

Figure 4. 8. The CDK 4 variant protein modelled using I-TASSER and is refined using GalaxyRefine giving change of RMSD was 5.162 Å, Poor Rotamer was 12.4 and Rama favoured value was 14.3.

Using PatchDock, the Palbociclib conjugated gold nanoparticles were docked with the CDK4 protein variant as shown in Figure 4.9, giving binding energies -1095.67kcal/mol, -940.51kcal/mol and -1095.97kcal/mol for *Aloe vera* derived AuNP, *Gymnema sylvestre* derived AuNP and *Honey* derived AuNP, respectively. The protein docked complex with *Aloe vera* and *honey* derived AuNP formed six hydrogen bonds while *Gymnema sylvestre* derived AuNP nanoparticle formed four hydrogen bonds as shown in Table 4.2.

Table 4. 2. Hydrogen bonds formed between Palbociclib conjugated gold nanoparticles docked with CDK 4 with respective bond distances.

Palbociclib conjugated AuNP derived from *Aloe vera* docked with CDK4		
From	**To**	**Distance**
A:THR53:H	:UNK0:O	3.00956
A:SER166:H	:UNK0:O	3.07209

A:SER166:HG	:UNK0:O	1.88317
A:VAL174:H	:UNK0:O	2.81191
A:VAL176:H	:UNK0:O	2.96994
A:THR177:H	:UNK0:O	2.68107
Palbociclib conjugated AuNP derived from *Gymnema sylvestre* docked with CDK4		
From	**To**	**Distance**
A:VAL14:H	:UNK0:O	2.46439
A:VAL174:H	:UNK0:O	1.78916
A:LYS211:HZ2	:UNK0:O	2.75834
A:GLU219:H	:UNK0:O	3.03634
Palbociclib conjugated AuNP derived from Honey docked with CDK4		
From	**To**	**Distance**
A:TYR21:HH	:UNK0:N	1.86513
A:THR172:HG1	:UNK0:O	2.85399
A:VAL174:H	:UNK0:O	2.11757
A:VAL175:H	:UNK0:O	2.5731
A:ARG38:NH2	:UNK0	4.08407
A:THR19:HG1	:UNK0	3.23732

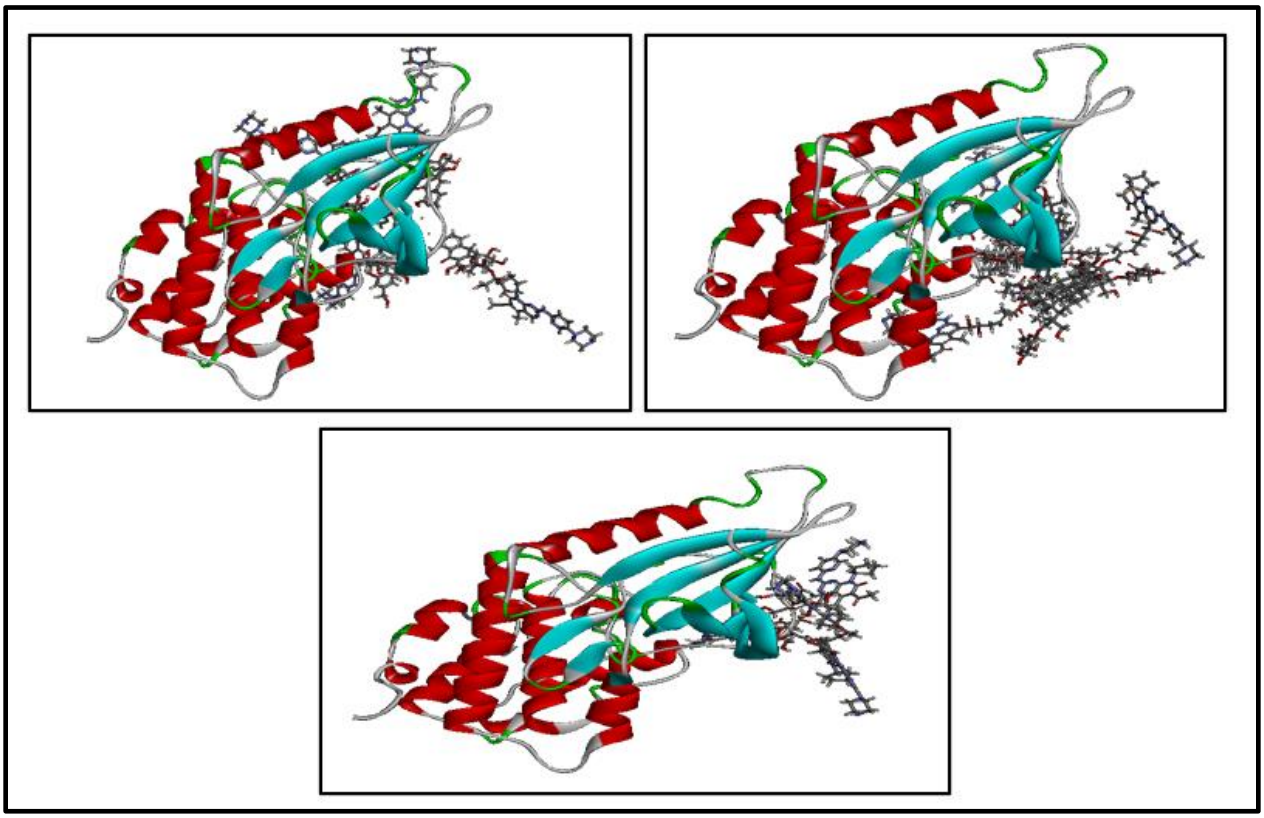

Figure 4. 9. Docking of drug conjugated gold nanoparticles with CDK 4 variant protein (A) Palbociclib conjugated gold nanoparticles derived from *Aloe vera* (B) Palbociclib conjugated gold nanoparticles derived from *Gymnema sylvestre* (C) Palbociclib conjugated gold nanoparticles derived from Honey.

4.5. Palbociclib conjugated Au nanoparticles against breast cancer inducing CDK6 receptor

Three Gold nanoparticles which were constructed in ChemDraw for docking with CDK4 were used for docking with CDK6. Palbociclib was conjugated to these nanoparticles *via* PEG against CDK4.

The protein ID (P42773 [CDN2C_HUMAN] VAR_001490) was retrieved from Uniprot and was uploaded on I-TASSER providing C-score (0.52), TM score (0.78±0.09), RMSD (3.9±2.7 Å), Poor Rotamers (22.3), Rama Favored (82.5). Refining protein model on GalaxyRefine we get values 0.361 Å, 1.5, 96.4 for RMSD, Poor Rotamers and Rama Favored, respectively. The difference in the values were 3.539 for RMSD, 20.8 for Poor Rotamers, 13.9 for Rama Favored, Figure 4.10.

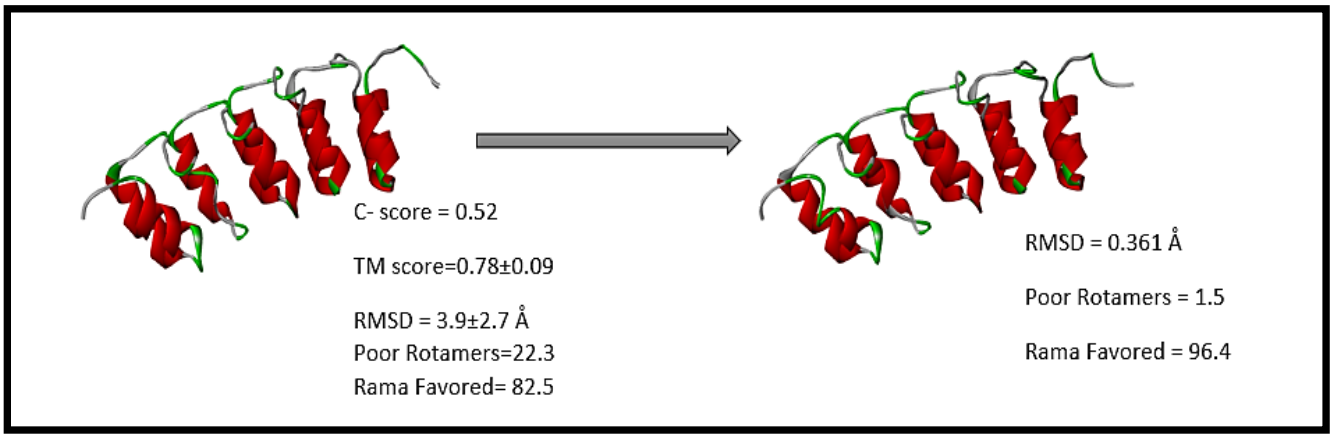

Figure 4. 10. The CDK 6 variant protein modelled using I-TASSER and is refined using GalaxyRefine giving change of RMSD was 3.539 Å, Poor Rotamer was 20.8 and Rama favoured value was 13.9.

Using PatchDock, the Palbociclib conjugated gold nanoparticles were docked with the CDK6 protein variant as shown in Figure 4.7, giving binding energies -755.49kcal/mol, -578.46kcal/mol and -656.97kcal/mol for *Aloe vera* derived AuNP, *Gymnema sylvestre* derived AuNP and *Honey* derived AuNP, respectively. The protein docked complex with *Gymnema sylvestre* derived AuNP formed six hydrogen bonds while with *honey* derived AuNP it formed three conventional

hydrogen bonds. With *Aloe vera* derived AuNP formed two hydrogen bonds as shown in Table 4.3.

Table 4. 3. Hydrogen bonds formed between Palbociclib conjugated gold nanoparticles docked with CDK 6 with respective bond distances.

Palbociclib conjugated AuNP derived from *Aloe vera* docked with CDK6		
From	**To**	**Distance**
A:ASN32:HD22	:UNK0:O	2.95655
A:ASN101:H	:UNK0:O	1.50148
Palbociclib conjugated AuNP derived from *Gymnema sylvestre* docked with CDK6		
From	**To**	**Distance**
A:LYS124:HZ1	:UNK0:O	2.86326
A:LYS124:HZ3	:UNK0:O	2.69042
A:ARG117:CD	:UNK0:O	2.7536
A:ARG117:CD	:UNK0:O	3.16254
A:SER154:CB	:UNK0:O	3.26548
A:ASP84:H	:UNK0	3.09
Palbociclib conjugated AuNP derived from Honey docked with CDK6		
From	**To**	**Distance**
A:ASN97:HD22	:UNK0:O	2.79583
A:GLY160:CA	:UNK0:O	3.58141
A:ALA164:H	:UNK0	2.81018

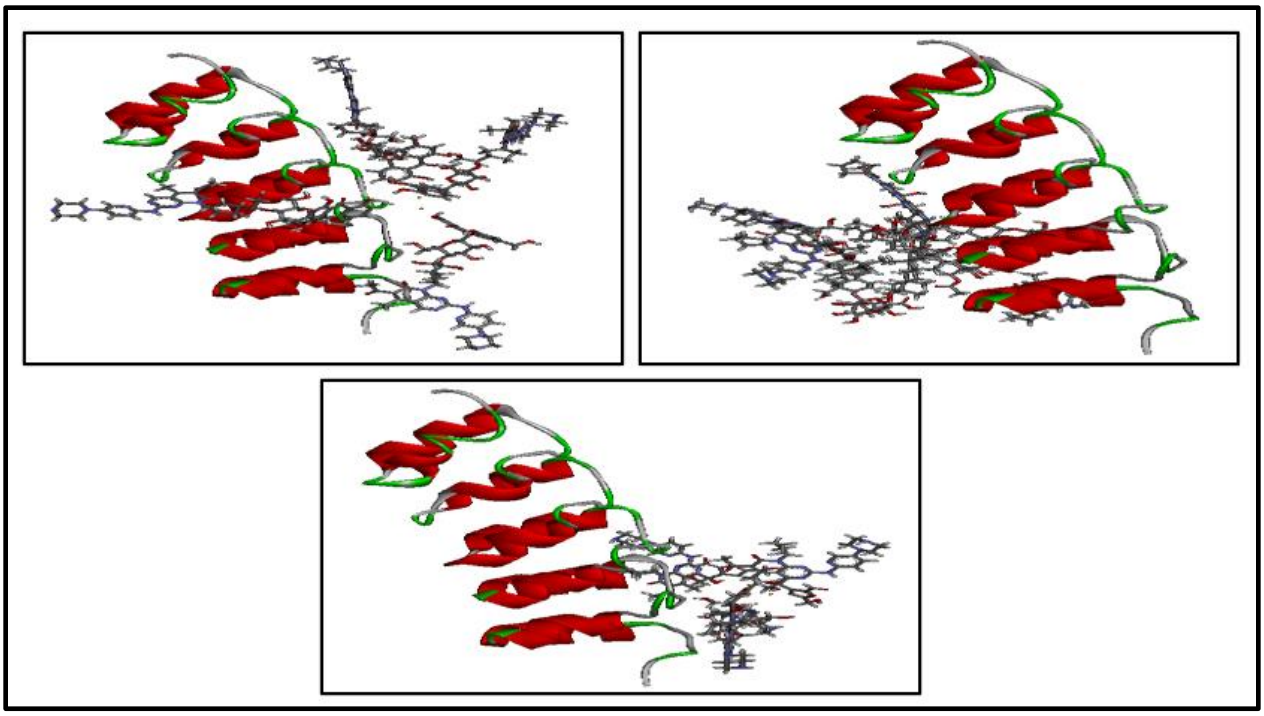

Figure 4. 11. Docking of drug conjugated gold nanoparticles with CDK 6 variant protein (A) Palbociclib conjugated gold nanoparticles derived from *Aloe vera* (B) Palbociclib conjugated gold nanoparticles derived from *Gymnema sylvestre* (C) Palbociclib conjugated gold nanoparticles derived from Honey.

4.6. Drugs docked with the associated binding protein

Unconjugated drugs were docked with their respective protein receptors. Their results were analyzed by their binding energies and conventional hydrogen bonds formed between drugs and the proteins. The results were compared to the drug conjugated gold nanoparticles docked with the associated proteins.

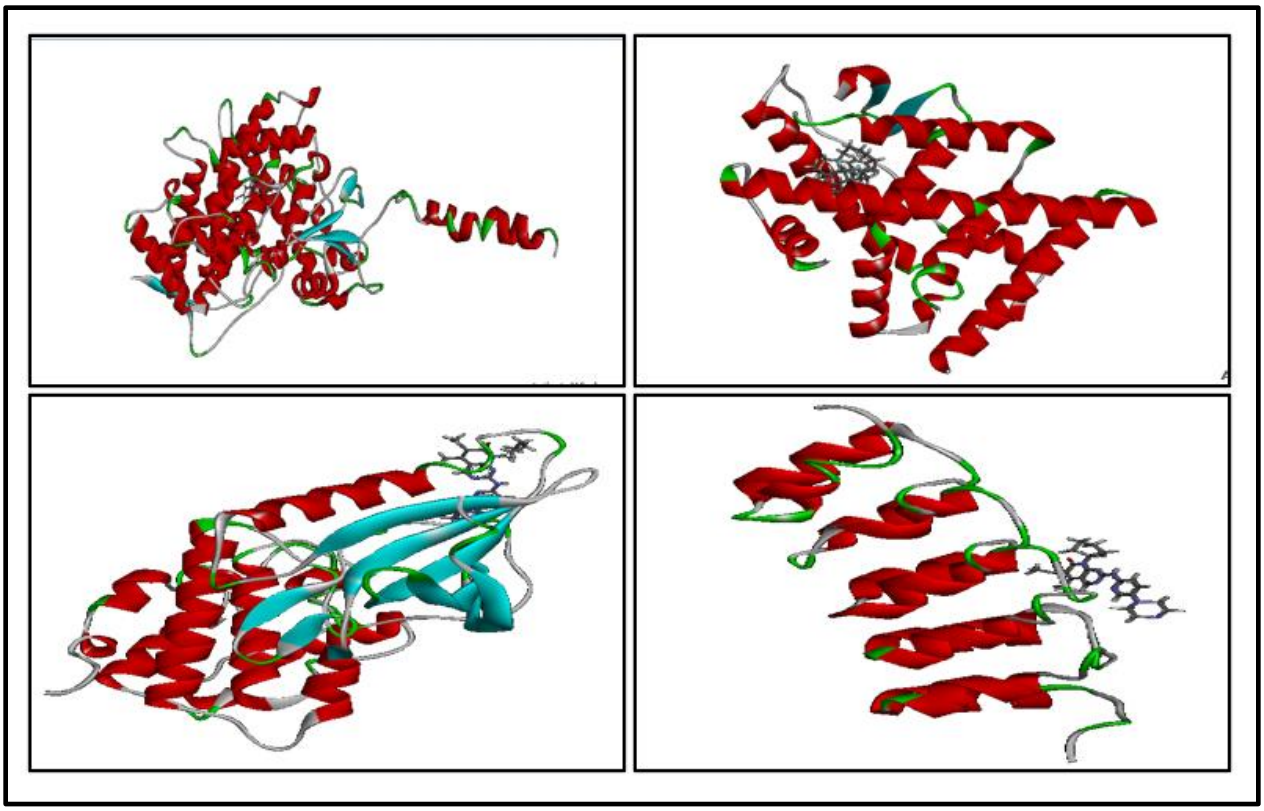

Figure 4. 12. Drugs docking with their respective protein variants (A) Letrozole docked with Cytochrome P450 Aromatase (B) Fulvestrant docked with Estrogen receptor (C) Palbociclib docked with CDK 4 (D) Palbociclib docked with CDK6.

4.6.1. Letrozole docked with cytochrome p450 Aromatase

The mol file for letrozole was downloaded and converted to pdb file and was docked with cytochrome p450 variant protein on PatchDock as shown in Figure 4.12 (A). The binding energy observed was (-386.53 kcal/mol) with one conventional hydrogen bond: UNK0:N-A:SER122:CB having bond distance (2.52 Å,), Table 4.4.

Table 4. 4. Hydrogen bonds formed between docked structure of Letrozole and cytochrome P450 along with the bond distance.

Unconjugated Letrozole Docked with Cyt P450		
From	**To**	**Distance**
A:SER122:CB	:UNK0:O	2.52

4.6.2. Fulvestrant docked with Estrogen receptor α protein variant

The .mol file of fulvestrant molecule was downloaded, converted to pdb file, and was docked with Estrogen receptor on PatchDock as shown in Figure 4.12 (B). The binding energy provided was -149.11 kcal/mol and this docked complex form only one hydrogen bond with bond distance of 4.8829 Å.

4.6.3. Palbociclib docked with CDK 4 and CDK6

The .mol file for Palbociclib was downloaded, converted to pdb file, and was docked with CDK 4 and CDK6 individually. Both docked results did not show any conventional hydrogen bond while visualizing in Discovery studio, Figure 4.12 C &D.

4.7. Analysis of Protein-ligand complexes

All docked complexes including the nine drug conjugated nanoparticle structures docked with their respective protein variants and the individual drugs docked with the respective protein variants were visualized and analyzed in discovery studio.

In case of letrozole, the letrozole conjugated gold nanoparticle derived from *Aloe vera* when docked against Cyp450 showed the best results forming six conventional hydrogen bonds with higher binding energies. In case of Palbociclib, the palbociclib conjugated gold nanoparticle derived from *Aloe vera* and *Honey* respectively, showed strongest binding interactions with CDK4, each forming six conventional hydrogen bonds. On the other hand, the Palbociclib conjugated gold nanoparticle derived from *Gymnema sylvestre* docked with CDK6 showed the strongest interaction forming maximum hydrogen bonds compared to other two docked complexes.

4.8. Comparison of drug protein complexes with drug conjugated gold nanoparticle protein complexes

Concluding from the above results, Letrozole conjugated gold nanoparticles and Palbociclib conjugated gold nanoparticles both showed an efficient drug delivery system effectively targeting the receptor proteins in breast cancer. Whereas unconjugated drugs did not show the effective binding interactions with protein through which we concluded that drug must be conjugated to nanoparticle instead of only delivering drug to the target. Moreover, Fulvestrant conjugated gold nanoparticles and Fulvestrant interaction with the receptor protein did not show a prominent difference in binding interactions as in both cases same conventional hydrogen bonds were observed. Though binding energy observed in former docked complexes were observed to be low as compared to the later one showing good interaction of Fulvestrant conjugated gold nanoparticles instead of unconjugated Fulvestrant.

CHAPTER 5

DISCUSSION

Hormonal therapy is an effective therapy to treat ER+ breast cancer patients but multi drug resistance is its biggest limitation. There are high chances of relapse and cytotoxic side effects can occur in normal cells, symptoms show joint pain, hair loss, neutropenia etc. (Normanno et al, 2009). Therefore, we need a highly targeted approach which is safe and effective. Nanotechnology due to its targeted delivery, efficacy, controlled drug release to tumor cells and reduced toxicity have gained much importance to treat metastatic breast cancer. This approach can overcome resistance mechanisms against endocrine resistance when combined with nanomaterials (Amjad et al., 2018). In a previous study, the cytotoxic potential of biosynthesized AuNP using combinatorial approach *via* four naturally derived phytochemicals such as Curcumin, Turmeric, Quercetin and Paclitaxel against breast cancer receptors. It showed maximum therapeutic activity having no or less side-effects (Gomathi, Sudha et al. 2017). In another study, resveratrol conjugated gold nanoparticles was for the evaluation of an anti-tumor activity which shows no significant drug cytotoxicity and an effective way to treat tumors (Dong Gun Lee et al., 2019). Research on different drugs conjugated AuNP to treat cancers and specifically breast cancers let us know about the effective use of AuNP instead of other metallic nanoparticles used in previous studies. In post-menopausal women most of the breast cancers are due to the 10-fold increased concentration of 17-b estradiol (E2) in breast tumor than in the plasma. Letrozole has been used to treat such breast cancers being formulated to Chitosan-NP with the linker Tripolyphosphate (Vemuri, Banala et al. 2019). Letrozole clinical trials conducted

in 2002, the letrozole toxicities reported in more than 5% patients including deep vein thrombosis (Martin H. Cohen et al., 2002). Despite some of the challenges of using gold nanoparticles, they have been used for the systemic delivery of tamoxifen drug which is of the same class of drugs as fulvestrant (Dreaden, E. C et al.,2012).

The *in silico* analysis in our study proved that Letrozole, Fulvestrant and Palbociclib conjugated gold nanoparticles is an efficient way to deliver drugs to the target protein receptors i.e: CYP450, ER-α and CDK4/CDK6, respectively. The effective binding and controlled release of the drugs can be done through conjugation of drug with NP. Moreover, the drug unconjugated with NP was observed to be less effective and having low efficacy as compared to the drug conjugated with NP. In case of AuNP conjugated with letrozole, 6 bonds were formed with CYP450 receptor protein compared to just one hydrogen bond in case of unconjugated drug interaction with Cyp450. Although AuNP bound with fulvestrant drug were able to make only one hydrogen bond but it was stronger and showed less bond distance than using the drug alone. Palbociclib conjugated with AuNPs showed a minimum of 6 hydrogen bonds with CDK4 receptor and no conventional hydrogen bond was formed in case of interacting unconjugated drug against CDK4. Similarly, palbociclib conjugated AuNPs showed more hydrogen bonds and higher binding energies while no hydrogen bond was formed between CDK6 receptor upon interaction with unconjugated Palbociclib. All these results indicate that by using AuNPs derived from biological sources, the bond between drug and its receptor can be made stronger and this could potentially help in the targeted drug delivery and reduced cytotoxicity.

There are many previous studies that uses same tools for checking binding energy scores, bonding interactions, protein modelling and refinement based on RMSD, Poor Rotamers, C-score and TM- score that uses PatchDock (Hemalatha et al., 2015; Mathew, 2009). In a study, I-

TASSER was used for modelling protein (Alsharif et al., 2020). In another study, I-TASSER was used for docking and GalaxyRefine for refinement of docked protein to repack interface (Kathwate, 2020; Rezaie et al., 2019). In a study, the *in silico* analysis of gold nanoparticles before *in vitro* cytotoxicity evaluation was carried out (Alsharif et al., 2020). In accordance with our study, the previous study has modelled the human blood serum proteins using I-TASSER and then docked with gold nanoparticles and titanium oxide particles for *in silico* analysis of protein nanoparticles interactions (Alsharif et al., 2020). Another previous study has evaluated silver nanoparticles in their research by sketching the chemical structure of *Citrus macroptera* fruit extract that has been used for synthesizing silver nanoparticles using ChemDraw. These nanoparticles have been evaluated *in silico* for interaction studies against Biofilm forming proteins of *Bacillus subtilis* and *Pseudomonas aeruginosa*. The anti-biofilm activity of these nanoformulations was evaluated using AutoDock 4.2 tool and visualized using Maestro (Schrodinger) software (Majumdar et al., 2020).

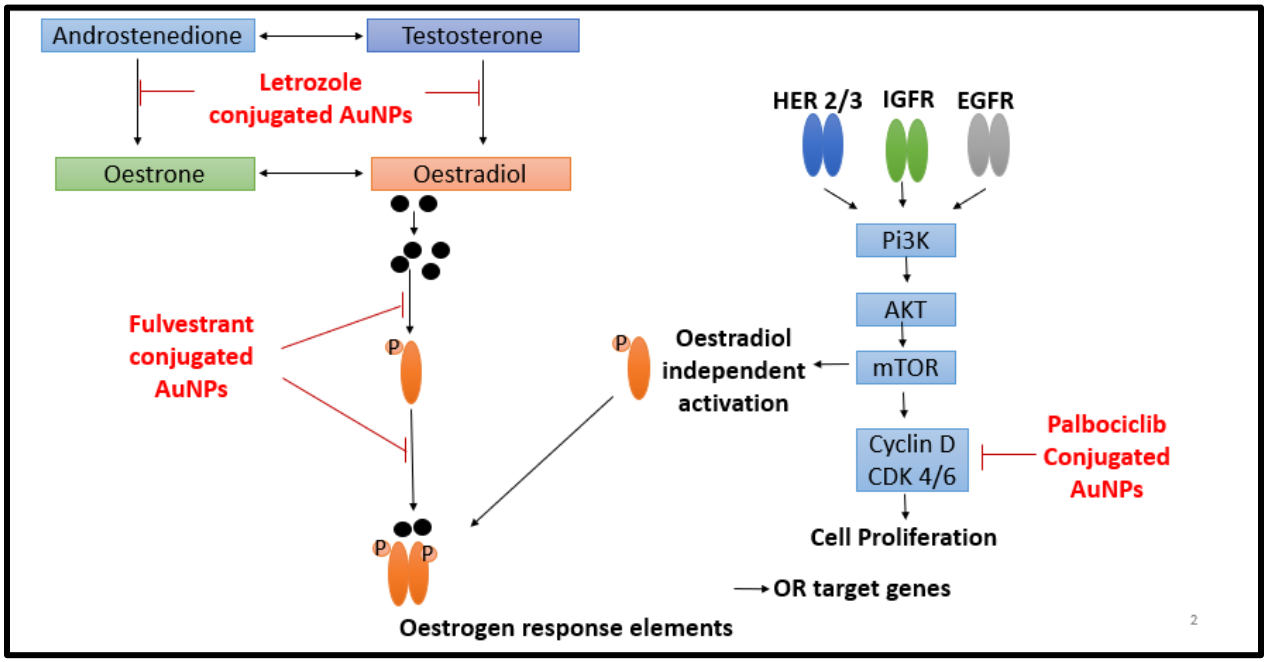

Figure 5. 1. Inhibition of breast cancer progression pathway at specific sites through Letrozole conjugated AuNP, Fulvestrant conjugated AuNP and Palbociclib conjugated AuNP.

Concluding our result discussion, different pathways in tumor environment leads to the breast cancer progression in the ER+ breast cancer, the overall pathway involved in the breast cancer progression is illustrated in the Figure 5.1. On different steps in the pathway all three drug conjugated nanoparticles inhibits the breast cancer progression by inhibiting the production of estradiol in case of letrozole conjugated AuNP and inhibition of cell proliferation in case of Fulvestrant and Palbociclib conjugated AuNP. The inhibition at these pathways provides an effective way for the treatment of ER+ breast cancers with low cytotoxicity along with high binding efficiency and effective release of drugs at target sites with increased pharmacokinetics and pharmacodynamics.

Our good *in silico* results can be effectively used *in vitro* and *in vivo* testing after choosing the best results with these drug conjugated Au-NP showing high binding energies and hydrogen bonds with the receptor proteins with higher binding efficacy. Our study is a potential target approach to use different drug combinations formulated to gold nanoparticles for the effective binding of drugs in treatment of breast tumors. This research can be proceeded further to test the synthesized nanoparticles on breast cancer cell lines *in vitro.*

Honey has also been used in a study named comparative analysis of honey and citrate stabilized gold nanoparticles: *In vitro* interaction with proteins and toxicity studies and the results proved to have reduced toxicity trends depending on the cell type, concentration of nanoparticles and exposure time toward various biomedical applications.

CHAPTER 6

SUMMARY & FUTURE PROSPECTS

In general, the *in silico* studies of the drug conjugated nanoparticles with their appropriate receptor and protein breast cancer variant showed significant improved hydrogen bonding as compared to simple drugs with the cancer protein variant receptor/ proteins. The binding energy from PatchDock of drugs Palbociclib, Fulvestrant, Letrozole conjugated on to AuNP derived from *Aloe Vera*, gold AuNP derived from *Gymnema sylvestre* and AuNP derived from Honey showed better results when docked on to the receptors CDK4/6, ER and Cyt P450 aromatase breast cancer receptor variants, respectively. The result for this study indicates further use of nanoparticles which can be conjugated with multiple drugs to help check their efficacy *in vitro* and then with satisfactory results can be taken *in vivo* and further to preclinical and clinical testing.

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