InSilico Structure Based Designing of Dihydrofolate Reductase Enzyme Antagonists and Potential Small Molecules That Target DHFR Protein to Inhibit the Folic Acid Biosynthetic Pathways

A project submitted

by

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to

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This work is dedicated to my family for their unconditional love and support

Certification Statement

This is to certify that this project titled "*Insilico* Structure Based Designing of Dihydrofolate Reductase Enzyme Antagonists and Potential Small Molecules That Target DHFR Protein to Inhibit the Folic Acid Biosynthetic Pathways" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Mohammad Kawsar Sharif Siam, Senior Lecturer. MSc in Pharmacogenetics (UCL, UK) PhD Candidate (University of Cambridge, UK) Department of Pharmacy. BRAC University, Dhaka, Bangladesh and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

Countersigned by the supervisor

Acknowledgement

Alhamdulillah, all the admiration belongs to Allah S.W.T. from whom I got the strength and health to complete this project work. I would like to show my thankfulness and gratitude to Almighty Allah for blessing me with immense patience, strength, gratefulness and assistance when necessary to complete this process of bachelors in Pharmacy.

I would like to express my earnest pleasure and honor to work with the very dedicated & supportive supervisor, **Mohammad Kawsar Sharif Siam**, Senior Lecturer. MSc in Pharmacogenetics (UCL, UK) PhD Candidate (University of Cambridge, UK) Department of Pharmacy, BRAC University, Dhaka, Bangladesh

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Abstract

Cancer has several pathways by which it is developed in our body. Among them folic acid biosynthetic pathway is one where dihydrofolate reductase (DHFR) enzyme converts dihydrofolate into tetrahydrofolate which leads to unwanted and uncontrollable growth of tissues. Our aim of this study is to design DHFR antagonistic potential small molecules that inhibits Folic Acid Biosynthetic Pathways. In this study, Human DHFR obtained from Protein Data Bank (PDB) were docked with several established anticancer drugs including Afatinib, Doxorubicin, Trimetrexate, Curcumin & Trimethoprim and several potential small molecules including Acarbose, Adenosine monophosphate, Abacavir, Aceprometazine & Isoxyl; obtained from PubChem and Drug Bank respectively. PyMOL and PyRx were used to visualize, curate and dock. For validation purpose Discovery Studio and Ramachandran Plot were run. Results after docking showed best binding affinities of established anticancer drugs with Human DHFR throughout the generations for example Methotrexate to Trimethoprim. Potential small molecules which belong from different therapeutic classes.

Contents:

List of Tables:

List of Figures:

List of Abbreviations:

- ✓ DHFR= Dihydrofolate Reductase Enzyme
- ✓ MTHFR= Methyltetrahydrofolate
- ✓ Rmsd= Root mean square deviation
- ✓ u.p.= Upper bound
- ✓ l.p.= Lower bound

Chapter One

Introduction

1 Introduction

Cancer, a disease involving uncontrollable growth of cells leading towards massive damage of human body. This unwanted event can cause tumor which is a solid mass of highly divided tissues or liquid cancer such as leukemia or cancer related to bone marrow. It is one of the main sources of death all through the world, in which the principle medicines include surgery, chemotherapy as well as radiotherapy (Gangaraju Vamsi K. Lin Haifan, 2009). Chemotherapy involves using lower molecular weight molecule to destroy or inhibit the proliferation of

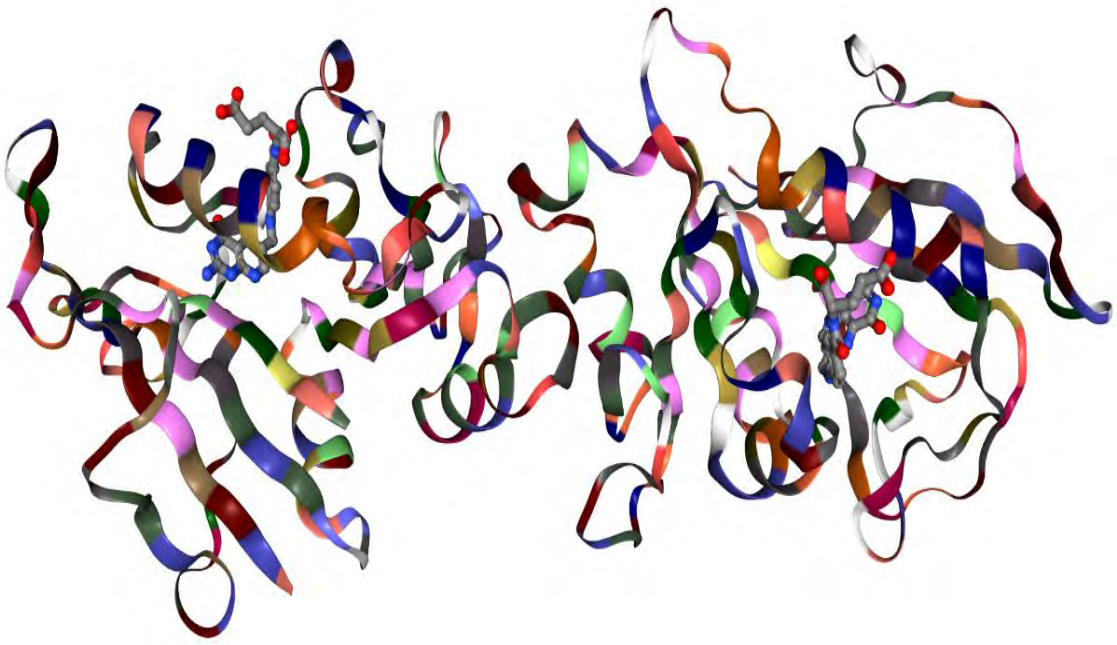


Figure 1.1: Crystal structures of Recombinant Human Dihydrofolate Reductase.

tumor cells. Hindrances of numerous cytotoxic specialists incorporate bone marrow concealment, gastrointestinal tract sores, male pattern baldness, queasiness, and the advancement of clinical protection. These symptoms happen in light of cytotoxic specialists which follow up on both tumor cells and sound cells (Thurston, 2007). Chemotherapy began

in 1940 with the use of Nitrogen mustards which is a very strong alkylating agent and antimetabolite in nature. Early success of these activities opened up the doorway to the development of new anticancer drugs (Gangaraju Vamsi K. Lin Haifan, 2009).

Anticancer drugs can be classified into several groups depending upon their component of activity, for example, DNA-intuitive specialists, anti-metabolites, hostile to tubulin operators, sub-atomic focusing on operators, hormones, monoclonal antibodies and other biological agents (Thurston, 2007). Anticancer drugs mainly work on Dihydrofolate reductase enzyme to inhibit the cell division process. Basically, the catalytic reduction of folic and dihydrofolic acid to

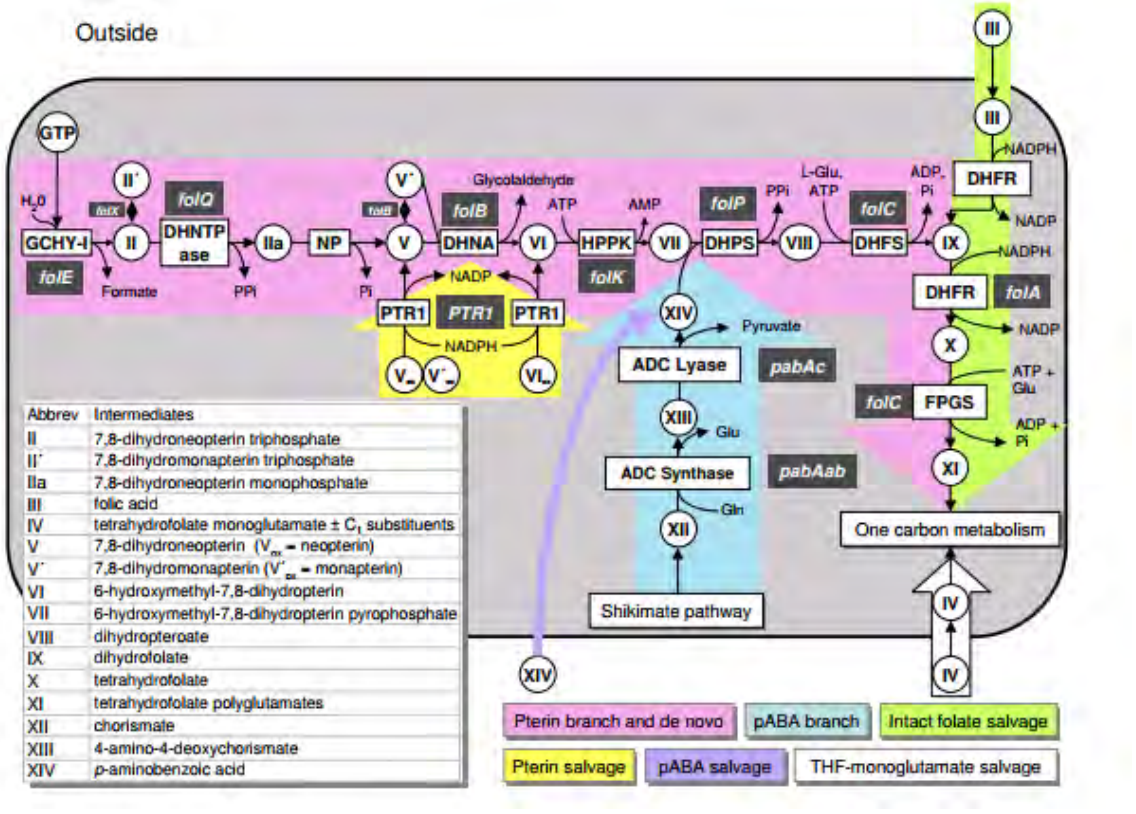


Figure 1.2: Dihydrofolate Reductase related pathways.(de Crécy-Lagard, El Yacoubi, de la Garza, Noiriel, & Hanson, 2007).

tetrahydrofolate is the main path way which proceeds under the action of dihydrofolate reductase and the coenzyme NADPH (Polshakov, 2001).

1.1 Main Components of the Folic Acid Biosynthesis Cycle

DHFR = dihydrofolate reductase; MTHFR = methylenetetrahydrofolate reductase.

1.1.1 Reactions

- **Pathway 1** – Nucleotides are synthesized for the conversion of DNA to RNA.
- **Pathway 2** – Homocysteine is remethylated to form methionine (vitamin B12 which serves as a coenzyme).
- Substrates including DNA, RNA, phospholipids and proteins are being methylated.
- Formation of 5-methylfolate catalyzed by MTHFR which is needed for methylation reactions.
- DHFR enzyme.

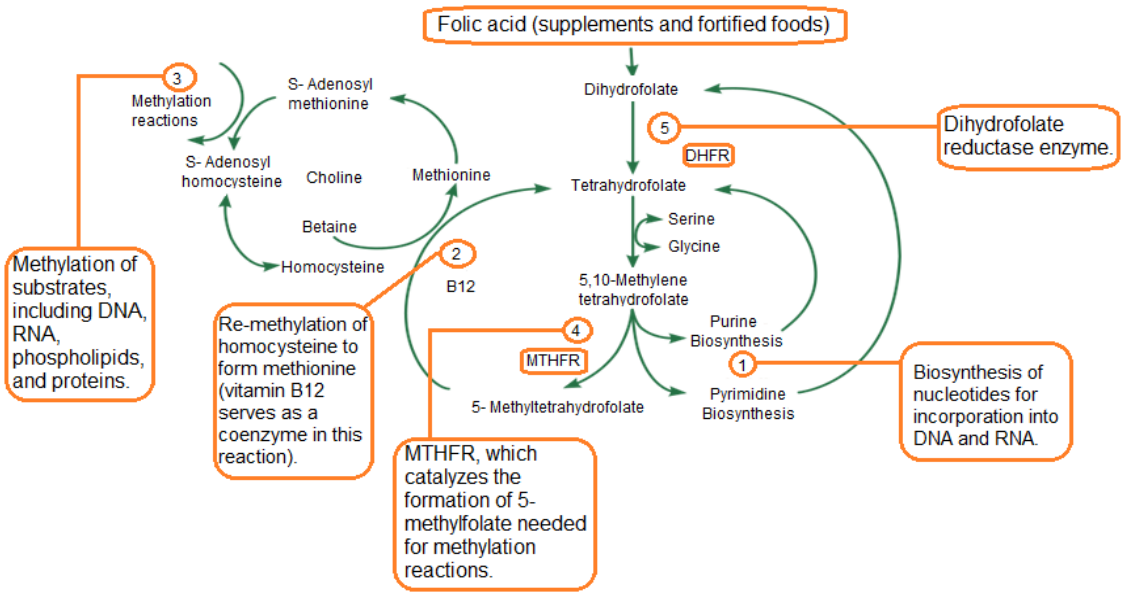


Figure 1.1.1: Dihydrofolate reductase enzyme biosynthetic pathway.

1.2 Choice of Drugs

For this computational study two types of drugs have been used; such as established anticancer drugs and different classes of potential small molecules which were selected upon screening. Five classes of small molecules such as Aceprometazine an antipsychotic agent (Overington, Al-Lazikani, & Hopkins, 2006), Adenosine monophosphate a nutritional supplement (Vourekas et al., 2016), Acarbose an antidiabetic agent (Yamagishi, Matsui, Ueda, Fukami, & Okuda, 2009), Abacavir an antiretroviral agent (Yang, Chen, & He, 2009) and Isoxyl as antibacterial agent (Overington et al., 2006) were selected after screening through more than 200 small molecules randomly (Fig.1.2.2). Among the established anticancer drugs Doxorubicin (Pommier, Leo, Zhang, & Marchand, 2010), Afatinib (D. Li et al., 2008), Trimetrexate (Wong, Woolf, Chang, & Whitfield, n.d.), Curcumin (Wishart et al., 2017) & Trimethoprim (Gordin, Simon, Wofsy, & Mills, 1984) are noteworthy (Fig.1.2.1).

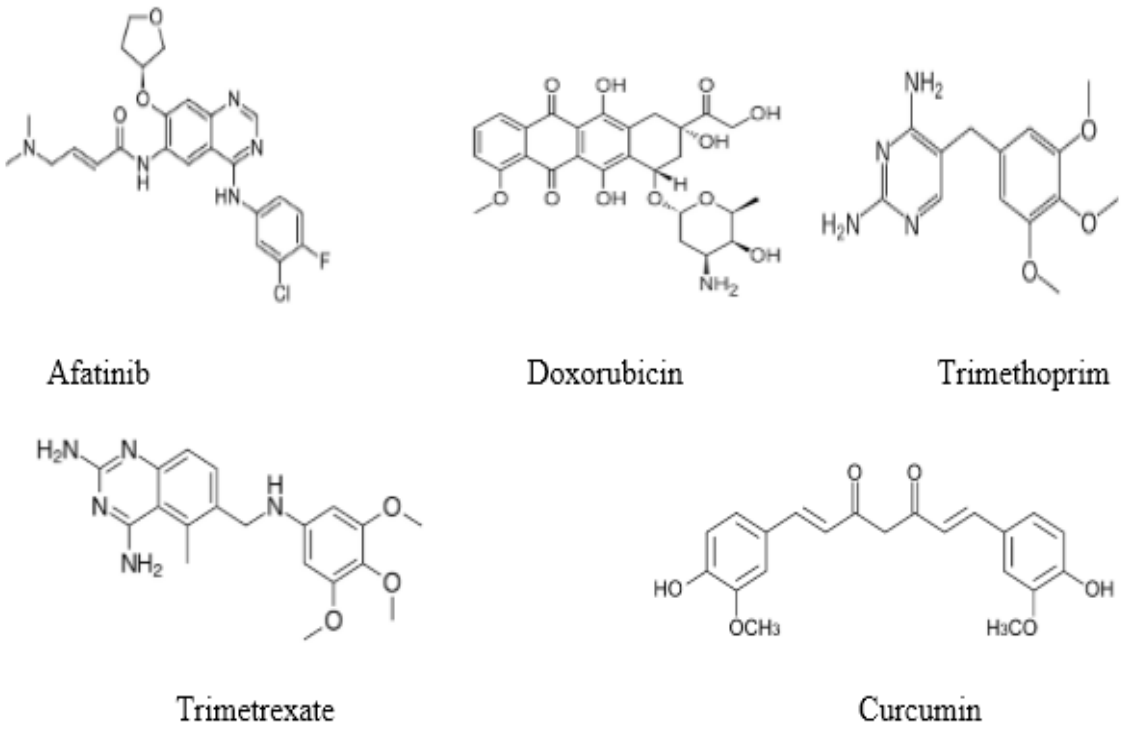


Figure 1.2.1: Established anticancer drugs. (Q. Li, Cheng, Wang, & Bryant, 2011).

Some examples of anticancer drugs include Trimethoprim, methotrexate, doxorubicin, trimetrexate, 5-fluro uracil etc. Nowadays small molecules are considered to play a significant role in the discovery of cancer therapy. Novel tubulin inhibitor STK899704, which is a small molecule has suppressed the proliferation of cancerous cells with IC_{50} values ranging from 0.2 to 1.0 μM, unlike the effects of Taxol and doxorubicin which comparatively have less activity against multidrug resistant cell line cancer (Sakchaisri et al., 2017).

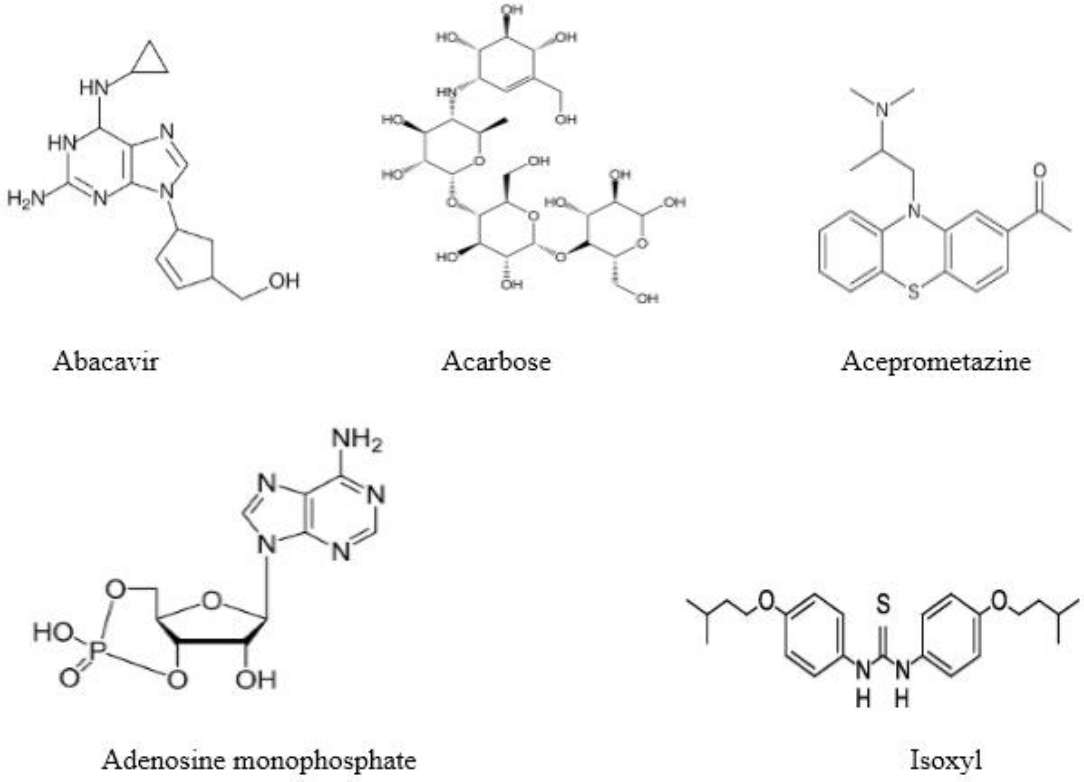


Figure 1.2.2: Potential small molecules of different classes. (Knox et al., 2011).

In the current years molecular docking has changed the idea of medication revelation. New parameters and methods have allowed us to discover many new molecules in cancer research field. DHFR (Dihydrofolate Reductase) allows the genetic evolution of carcinoma

cells which is protein in nature. In order to eliminate the effects on these cells, the DHFR pathway must be stopped. Several basic ideologies such as Structure based drug designing, Computational issues, Complementarity, docking strategies, Rigid and flexible approaches of docking are the keys to develop new drugs. In this work, we have utilized numerous drugs and updated tools to discover more effective and potent small molecules that can be considered as drugs of choice for cancer treatment.

1.3 Literature Review

Dihydrofolate reductase (DHFR) enzyme converts dihydrofolate into tetrahydrofolate requiring a help from shuttling methyl group for the De novo synthesis of thymidylic acid, purines and certain amino acids. DHFR plays a critical role in supplying tetrahydrofolates in the cells (Schnell, Dyson, & Wright, 2004). Certain mutant cells have this DHFR gene and some of them which lacks this DHFR gene require glycine which is an amino acid and thymidine (Urlaub & Chasin, 1980). The polypeptide backbone folding of DHFR is made up with a central eight stranded beta-pleated sheets (Matthews et al., 1977). Seven among these are parallel and one is antiparallel (David J. Filman, Jeffrey T. Bolin4, David A. Matthews, 1982). Successive beta strands are connected via four alpha helices (David J. Filman, Jeffrey T. Bolin4, David A. Matthews, 1982). The N-terminal is the active site for the DHFR where Tryptophan plays a role in binding with the substrate (David J. Filman, Jeffrey T. Bolin4, David A. Matthews, 1982).

General mechanism of DHFR proceeds stepwise. Specifically, the substrate that binds with the enzyme and NADPH initiates the catalytic reaction which followed by the transfer from NADPH to the substrate(Rod & Brooks, 2003) . However, two later steps are not simultaneous in action in the same transition state (Wan et al., 2014). In a computational study and experimental approaches (Liu et al., 2014) state that the protonation step is prior to the hydride transfer. The enzymatic mechanism of DHFR is pH dependent particularly the hybrid transfer step as pH change has drastic effects on the electrostatic basis of the active site and ionization of the amino acid residues (Liu et al., 2014). Acidity of the targeted Nitrogen on substrate is important as it attaches with the binding site of the enzyme which is

quite hydrophobic in nature even though it is in direct contact of water (Czekster, Vandemeulebroucke, & Blanchard, 2011; Rod & Brooks, 2003).

Dihydrofolate reductase inadequacy has been connected to megaloblastic iron deficiency & the treatment is with diminished types of folic acid. Since tetrahydrofolate, the result of this response is the dynamic type of folate in people, restraint of DHFR can cause practical folate insufficiency. DHFR is an alluring pharmaceutical focus for hindrance because of its crucial part in DNA forerunner combination. Trimethoprim, an anticancer, hinders bacterial DHFR while methotrexate, a chemotherapy specialist, represses mammalian DHFR. Be that as it may, resistance has occurred against a few medications, because of mutational changes in DHFR itself (Cowman & Lew, 1989).

DHFR transformations cause an uncommon autosomal passive characteristic blunder of folate digestion that outcomes in megaloblastic frailty, pancytopenia and extreme cerebral folate inadequacy which can be amended by folinic acid supplementation. Quickly separating cells require folate for their cell development and this can be utilized as a restorative window to treat ailments. DHFR can be the earlier focus to treat disease. As DHFR controls the level of tetrahydrofolate in human and hindrance of it can cause the stoppage of cell division and development of mutant cells. Methotrexate, a focused inhibitor of DHFR which is utilized as anticancer medication. Trimethoprim, pyrimethamine and trimetrexate are generally utilized hostile to DHFR drugs nowadays. Folic acid is the critical factor that is expected to create carcinogenic cells. The metabolic pathway of Folic acids gives us the simple focus to lessen the hazard factor of creating tumor. Classes of small-molecules employed as inhibitors of dihydrofolate reductase include diaminoquinazoline & diaminopyrroloquinazoline, diaminopyrimidine, diaminopteridine and diaminotriazines.

Pneumocystis carinii almost affects every human only upon reactivation of dormant infection by immunodeficiency which prompt a disease characterized by a crippling pneumonia (Bartlett & Smith, 1991; Murray & Mills, 1990). Very likely, this is the main agent of morbidity and mortality in persons who are HIV-infected (Mills, 1986; Murray & Mills, 1990).On a scale of 60 to 85% of AIDS patients will somehow be affected by P. carinii induced pneumonia if there is no chemoprophylaxis, and rest will be victimized by

death (Banka et al., 2011; Bartlett & Smith, 1991; Kovacs, Masur, Kovacs, & Masur, 2013; Mills, 1986). Based on published sequence alignments (Nare, Hardy, & Beverley, 1997), ***P. carinii*** DHFR displays highest similarity with that of vertebrates. Sequence identities of 3540% and homologies near 70% are observed. In high resolution, crystal structures of human (Davies et al., 1990) and ***P. carinii*** (Oefner, Arcy, & Winkler, 1988) DHFR conformation there are very few minor differences. There are six non-identical residues residing within the folate binding pocket. Aspartate a common amino acid to bacterial and protozoan DHFR is replaced with glutamate in ***P. carinii,*** as in all vertebrate DHFRs which is the active site of action (Gschwend, Good, & Kuntz, 1996).

Anticancer peptides (ACPs) are small peptides ranging from 5-30 amino acids that are mostly derived from antimicrobial peptides (AMPs) express cationic nature (Cory & Adams, 1998; Lynch & Price, 2007; Mai, Mi, Kim, Ng, & Robbins, 2001). Relating to previous studies it is proven that many of the cationic AMPs that causes toxicity to bacteria, behaves quite normally with normal cells (Benkovic, Fierke, & Naylor, 1988; Yi-bing Huang, Wang, Wang, Liu, & Chen, 2011; Yibing Huang, Feng, Yan, Hao, & Chen, 2015). In terms of cytotoxicity these AMP's show a broad spectrum against various cancer cells. As a rapidly emerging field, the mechanism of action of the ACP's remain indefinable. Studies have shown that there are minor differences between the cancerous and normal cell membranes and selective inhibition of cancer cells by certain ACPs (Hu, Chen, & Zhao, 2016).

Molecular docking is a tremendous way to understand bio-molecular interactions of drug to establish the rational drug design and discovery. In the mechanistic study a molecule (ligand) is placed into the specific binding site of the DNA/protein (receptor) at a target specific region that's mainly in a non-covalent fashion which allows to form a stable complex having a potential efficacy and specificity. With optimized conformation the principle objective of this is to attain ligand-receptor complex with the intention of less binding free energy possession. To find the target with proper PDB format which is a practical approach of molecular docking a data bank is required and a methodology to prepare preferred ligand as a PDB file. There are various software's (Discovery studio, etc.,) available from where the PDB format of the ligand can be produced. Depending upon the ability to interact with given target proteins/DNA these tools provide the organization to the

ligands. Molecular docking of small molecules to a target includes a preferred sampling of possible conformation of ligand in the particular groove of target in an order to establish the optimized conformation of the complex. This can be made possible by using scoring function of software. Magnetic Resonance (NMR) spectroscopy, infrared spectroscopy, X-ray crystallography and Nuclear are the techniques for the investigation and establishment of three dimensional structures of any organic molecule targets which provides a proxy approach for establishing the target structure that forms starting point for *in silico* drug discovery (Meng, Zhang, Mezei, & Cui, 2011).

1.3.1 Approaches of Molecular Docking

Primarily two types of approaches are used for performing molecular docking (Meng et al., 2011).

1.3.2 Approach for simulation

The ligand and target are being separated here by physical distance which allows the ligand to bind into target groove after "definite times of moves" in its conformational space (Meng et al., 2011).

1.3.3 Optimization of the lead

On its target optimized orientation of a ligand can be predicted by molecular docking. In the groove of target molecule binding modes of the ligand can also be predicted. This helps to produce and develop more potent, selective and efficient drug molecules (Meng et al., 2011).

1.3.4 Hit identifications

To find out potent drug candidates *insilico*, combination of docking with scoring function can be used to evaluate large databases by which the molecule of interest can be targeted (Meng et al., 2011).

1.3.5 Interaction of Drug-DNA

In the initial prediction of molecular recognition between ligand and target, molecular docking plays a vital role. However, this method takes longer duration of time to sum up optimal docked conformer because of the large energy vanishing for each conformation (Meng et al., 2011).

1.3.6 Types of Docking

Docking tools maneuvers search algorithms for instance Monte Carlo algorithms, genetic algorithm, molecular dynamics algorithms and fragment-based algorithms which are comprehensively utilized. Besides there are some tools such as DOCK, Auto DOCK Vina, PyRx and ICM which are mostly applied for high output docking simulations. There are numerous types of docking strategies with either ligand flexible or rigid depending on the docking simulations, like flexible ligand docking (target as rigid molecule), rigid docking (both the protein and ligands as rigid molecules) and flexible docking (interacting molecules as flexible) (Meng et al., 2011).

Molecular docking is a computational mockup of the complexes formed upon the interaction of two or more molecules. It forecasts the 3D structure of adducts established upon binding properties of interacting molecules. Different candidate structures are graded and grouped together utilizing scoring function generated by molecular docking. (Meng et al., 2011).

Chapter Two

Methodology

2 Methodology

The study is comprised of extensive literature review on the specific topic which is followed by molecular docking to comprehend the binding affinities between the ligands and receptors.

2.1 Software for Docking

The computational methods during this *insilico* study used exclusive software such as AutoDock Vina, PyMOL, PyRx, Swiss PDB Viewer, Discovery Studio, MEGA 6, Clustal Omega, MGL tools, Open Babel, POCASA.

Table 2.1: Software used in this Computational Study.

Sl.	Software Used	Version
01.	AutoDock Vina	1.1.2
02.	AutoDock Tools	1.5.7
03.	PyRx	0.8
04.	PyMOL	2.0.4
05.	Swiss PDB Viewer	4.1
06.	MEGA 6	6.06
07.	Open Babel	2.4.1
08.	Discovery Studio	4.5
09.	Clustal Omega	1.2.4
10.	POCASA	1.2

Concomitantly, few online databases such as RCSB-PDB (Protein Data Bank), PubChem Project, Swiss Model, EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute), Drug Bank, CASTp (Computed atlas of surface topography of proteins) have been used to collect data for the purpose of this study.

The objective of molecular docking is to produce a predictive modeling of the ligand-receptor complex structure through computational techniques (Rarely, Kramer & Klebe, 1996). There are two interrelated steps by which docking can be done (Meng, Zhang, Mezei

& Cui, 2011). The first step involves conformations sampling of the ligand of interest in the active site of the protein molecule, followed by the second step that involves conformation ranking of ligands and proteins via a scoring function (Halperin, Ma, Wolfson, & Nussinov, 2002). Sampling algorithms should be able to regenerate the experimental binding mode. Among all generated conformations the scoring function should also rank it highest. With these two perspectives a brief concept of basic ideology of docking theory can be achieved. There are six degrees of translational and rotational freedom available. And the conformational degrees of freedom for both the ligand and protein are used to find out the best binding mode between these two molecules. Unfortunately, it has become too expensive to computationally generate all the possible conformations. Because of this various sampling algorithms have been produced and have been widely used in molecular docking software such as DOCK, FLOG, SLIDE, DOCK 4.0, LibDock, and SANDOCK programs, Matching algorithms (MA), FlexX, Hammerhead, SLIDE and eHiTS, Multiple Copy Simultaneous Search (MCSS) and LUDI. Scoring function is to define the correct poses from incorrect poses and that is the importance of it. Scoring functions involve estimating rather than calculating the binding affinities between the protein and ligand. It takes in various assumptions and simplifications as well.

In the protein or receptor AutoDock, PyMOL, PyRx and Vina utilize rectangular boxes for the coupling site recognizable proof. Amid module, by giving unequivocal facilitates in AutoDock Tools or PyMOL the container focus can be characterized (e.g. a reference ligand). For this investigation, AutoDock Tools and PyRx have been utilized to characterize the container for recognizing the coupling site. In AutoDock Tools the container focus was figured from the mean directions of the iotas which are in the AutoDock Tools chosen lattice box segment. The docking confine was shown in the AutoDock Tools window. To isolate the entire protein in little parts, the size and the correct position of the crate was likewise balanced all together. This approach was extremely useful to discover the correct position of the coupling site in protein of intrigue. Besides for representation purposes the module was taken in two distinctive perception programming to distinguish if any distinctions occurred or not.

AutoDock Tools gives an intuitive technique to characterizing the torsional tree, generally for a given ligand and once in a while for the receptor. Each progression in this procedure has been robotized to permit programmed relegation, for example, virtual screening. Ligand adaptability is appointed in a few stages. Initially, a specific ligand or medication atom is picked, which will be unbending amid change in the docking reenactment. To locate the ideal ligand, the quantity of iotas in each branch is assessed and the base of the ligand that limits the extent of the biggest branch is additionally picked. Now and again, adaptability of the ligand is limited by inactivating every one of the torsions in a ligand. AutoDock Tools gives two decisions to do this work naturally. Initially, it is conceivable to choose the arrangement of torsions of opportunity that will move the biggest number of iotas and it is done close to the root. The other decision is to include torsions dynamically from the branches, moving the least number of particles and leaving the center of the atom unbending.

Secondly, PyRx was also used to determine the binding affinity of the drug towards the protein and its binding site. PyRx is an automated software in computational approach where all the parameters and algorithms are prefixed or default in nature. The protein and the ligands were selected and docked with each other for several times to see and compare the affinity values among them. The protein of interest is termed here as the macro molecule and the drug is termed as ligand and after defining these two they are being run through AutoDock and Vina in a default manner to get the results which come in an excel form that can be saved for further information and research purpose.

2.2 Steps Involved in Molecular Docking

Protein and drugs of interest are downloaded from databases such as RCSB PDB (Protein Data Bank) and PubChem. PDB provides the pdb files of protein and PubChem gives the sdf files of the drugs which later is converted with OpenBable that converts almost every format of protein and drugs into format of interest. For more information and research purpose if we need to see the sequence similarities MEGA6 comes in handy to use and see the conserved region of that protein sequence with reference sequence by using CLUSTAL Omega which is an EMI-EMBL database software from European region. To find out the

binding site within the protein for the drug POCASA 1.2 and CASTp are used. CASTp deals with more detailed information regarding binding pocket where POCASA gives the specific and exact position of amino acids that deal with binding of drug within the protein of interest.

During the *insilico* study, the ligand chosen for this experiment was prepared for subsequent docking runs through PyMOL selections. It was done to specify a directory containing a library of ligands to be docked. After defining the binding site with the help of the grid box, receptor and ligand were selected (De Lando, 2002). Input materials were entered to the specific software to run the docking from the command line. Both Auto Dock and Vina were employed for predefining flexible side chains during docking. Here, the plugin facilitated the selection of flexible side chains. Side chains within the docking box could be visualized conspicuously and PyMOL selections were translated into a flexible receptor definition.

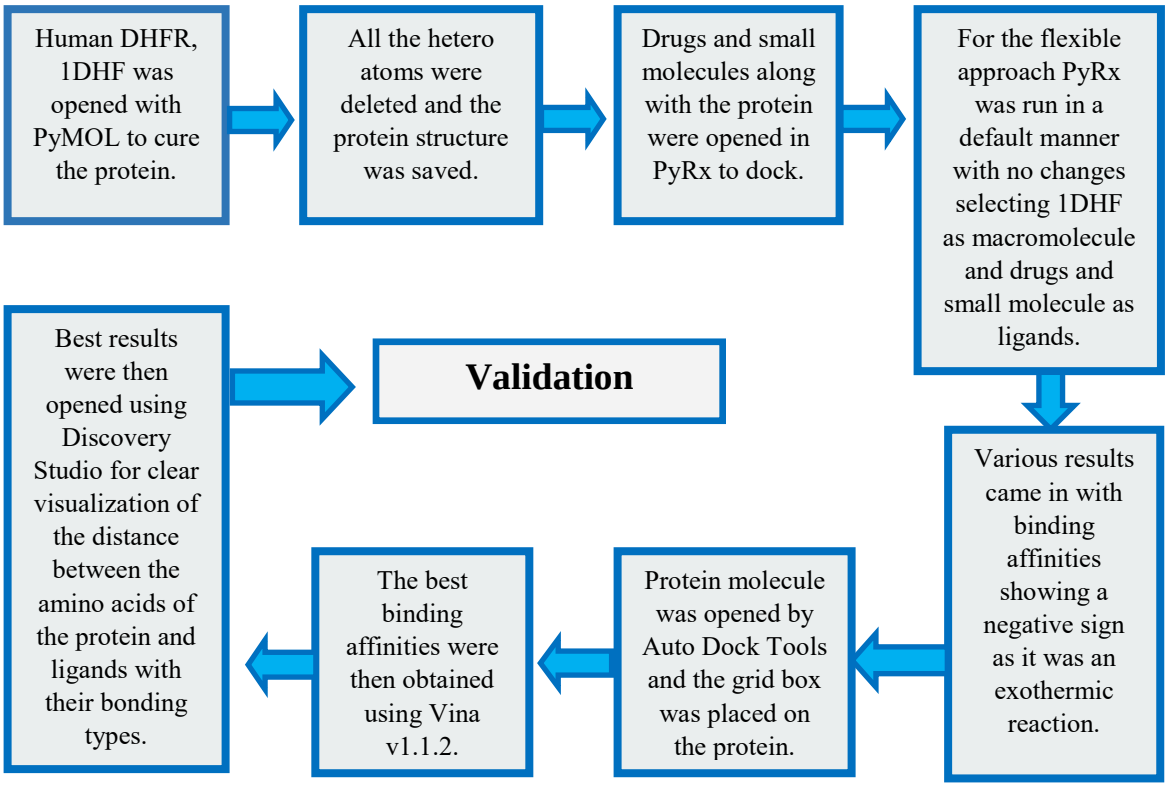


Figure 2.2.1: Flow diagram of steps involved in Molecular docking.

Human DHFR 1DHF was opened with PyMOL to cure the protein. Because protein molecule contained different hetero atoms and ligand within them. All the hetero atoms were deleted and the protein structure was saved. Two classes of drugs were selected, one is established anticancer drugs and other one is small molecules that belongs to five different classes. 1DHF, the Human DHFR protein downloaded from PDB and at the same time small molecules and drugs were downloaded from Drug Bank and PubChem Project respectively. Drugs and small molecules along with the protein were opened in PyRx to dock. For the flexible approach PyRx was run in a default manner with no changes selecting 1DHF as macromolecule and drugs and small molecule as ligands. Various results came in with binding affinities showing a negative sign as it was an exothermic reaction. The more the negative value greater the binding affinity. Again, the drugs and small molecules were run through PyRx in a rigid approach. This time there was a slight change in the preference and torsion was activated so that the joints within the structure of the drugs and small molecules could twist on its own order to have the best fit with the binding pocket of the protein. This time the binding affinities were better than the flexible approach.

Results from rigid docking of established drugs and small molecules gave upon one best drug and one small molecule which were again docked in AutoDock Tools. Protein molecule was opened by AutoDock Tools and the grid box was placed on the protein in different sections of it. This helped to find the best possible binding site for the 1DHF, the human DHFR. The best binding affinities were then obtained using Vina v1.1.2. Best results contained nine best binding affinities which were then opened using Discovery Studio for clear visualization of the distance between the amino acids of the protein and ligands with their bonding types. Some parameters were defined according to the ligand and protein before receiving the interaction details of the drug and ligands (Fig: 2.2.1).

Chapter Three

Results & Validation

3 Results

Molecular docking can be done in two ways, flexible docking and rigid docking. Flexible docking allows the movement of the molecules where the torsion of the drug is not fixed. On the other hand, rigid docking does not allow the movement of the molecules and torsions are fixed. Flexible approach more often done by default manners where in terms of rigid approach torsions which are fixed in a certain amount. This makes the drug molecules less shaky while being docked with the receptor. For rigid docking approach torsions were fixed and further steps were operated.

Table 3.1: Rigid docking of established anticancer drugs with Human DHFR where both the protein and drugs are rigid in nature.

Pose	Affinity (kcal/mol)	Distance from best mode (rmsd l.b.)	Distance from best mode (rmsd u.b.)
Doxorubicin	-12.9	0.000	0.000
Afatinib	-11.6	0.000	0.000
Trimetrexate	-10.5	0.000	0.000
Curcumin	-10.4	0.000	0.000
Trimethoprim	-8.7	0.000	0.000

Table 3.1 contains the results of rigid docking of different anticancer drugs with DHFR protein with their binding affinity. These are the best possible values to get and other values were removed to show the best results only.

Table 3.2: Flexible docking of established anticancer drugs with Human DHFR where Protein is rigid and drugs are flexible in nature.

Pose	Affinity (kcal/mol)	Distance from best mode (rmsd l.b.)	Distance from best mode (rmsd u.b.)
Doxorubicin	-9.6	0.000	0.000
Afatinib	-8.8	0.000	0.000
Trimetrexate	-8.6	0.000	0.000
Curcumin	-8.4	0.000	0.000
Trimethoprim	-7.9	0.000	0.000

Table 3.2 contains the results of Flexible docking of some established anticancer drugs with Human DHFR protein along with their binding affinities.

Table 3.3: Rigid docking of different classes of small molecules with Human DHFR where both the protein and small molecules are rigid in nature.

Pose	Affinity (kcal/mol)	Distance from best mode (rmsd l.b.)	Distance from best mode (rmsd u.b.)
Acarbose	-13.0	0.000	0.000
Adenosine Monophosphate	-10.9	0.000	0.000
Abacavir	-10.0	0.000	0.000
Aceprometazine	-9.3	0.000	0.000
Isoxyl	-9.0	0.000	0.000

Table 3.3 contains different classes of small molecules that are subjected to rigid docking with Human DHFR. Results show that they have higher amount of binding affinity towards Human DHFR and they can be the target drug choice for further research purpose.

Table 3.4: Flexible docking of different classes of small molecules with Human DHFR where the protein is rigid and small molecules are flexible in nature.

Pose	Affinity (kcal/mol)	Distance from best mode (rmsd l.b.)	Distance from best mode (rmsd u.b.)
Acarbose	-9.6	0.000	0.000
Adenosine Monophosphate	-8.8	0.000	0.000
Abacavir	-8.6	0.000	0.000
Aceprometazine	-8.4	0.000	0.000
Isoxyl	-7.9	0.000	0.000

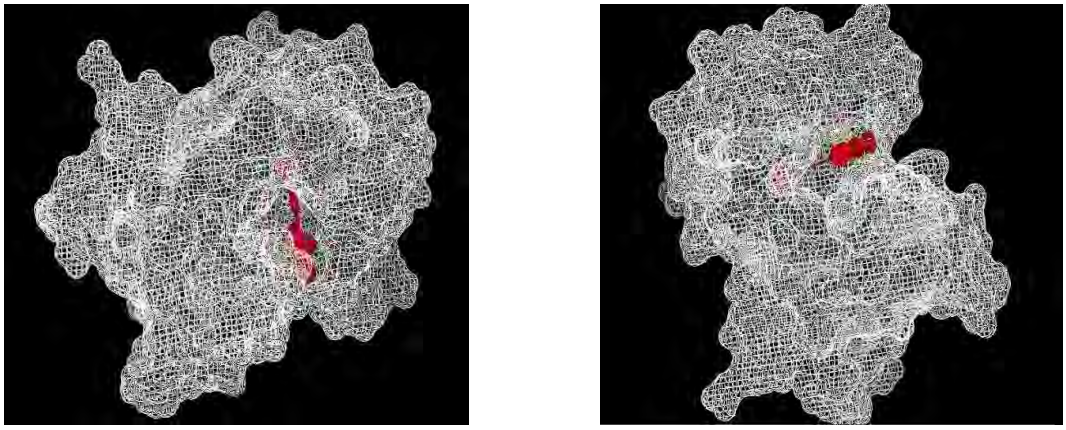
Table 3.4 contains small molecules that were docked against Human DHFR in flexible mode. Results show that they have higher amount of binding affinity towards Human DHFR and they can be the target drug choice for further research purpose.

3.1 Validation

Validation was done by two different ways. First one is by visualizing using PyMOL and other one by ligand protein interactions using Discovery Studio.

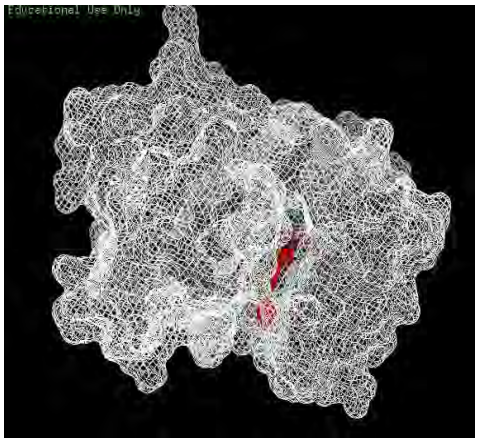
3.1.1 Validation using PyMOL

PyMOL validation mainly related to visualization of the protein with the attached ligand and other small molecules & established drugs as ligands. The out pdbqt file from the PyRx are being visualized after loading the protein molecule. This process shows the exact position of the binding pocket with drugs or ligands residing inside. To be specific there are nine positions in which molecules are to be found and any of them can be used to validate the binding site and binding pocket with reference to the given ligand with the protein molecule.

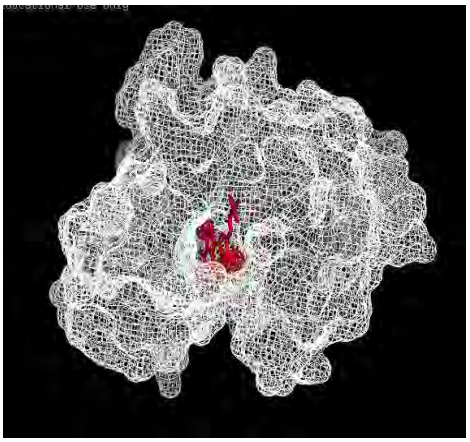


A **B**

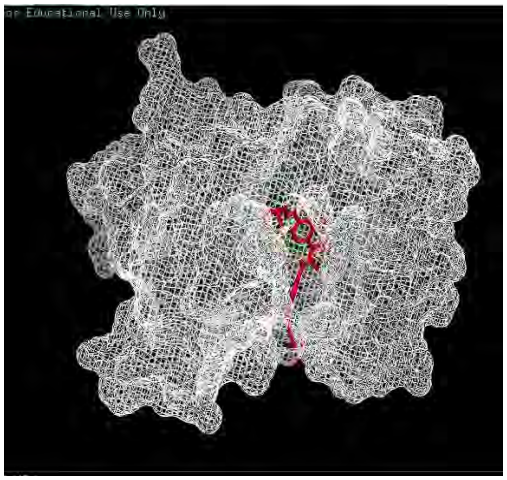
Figure 3.1.1: Visualization of Human DHFR with potential Small molecules Acarbose (A), Aceprometazine (B), Adenosine Monophosphate (C), Abacavir (D) & Isoxyl (E) using PyMOL (DeLano, 2002; Wishart et al., 2017).



C



D



E

Figure 3.1.2: Visualization of Human DHFR with potential Small molecules Acarbose (A), Aceprometazine (B), Adenosine Monophosphate (C), Abacavir (D) & Isoxyl (E) using PyMOL (DeLano, 2002; Wishart et al., 2017).

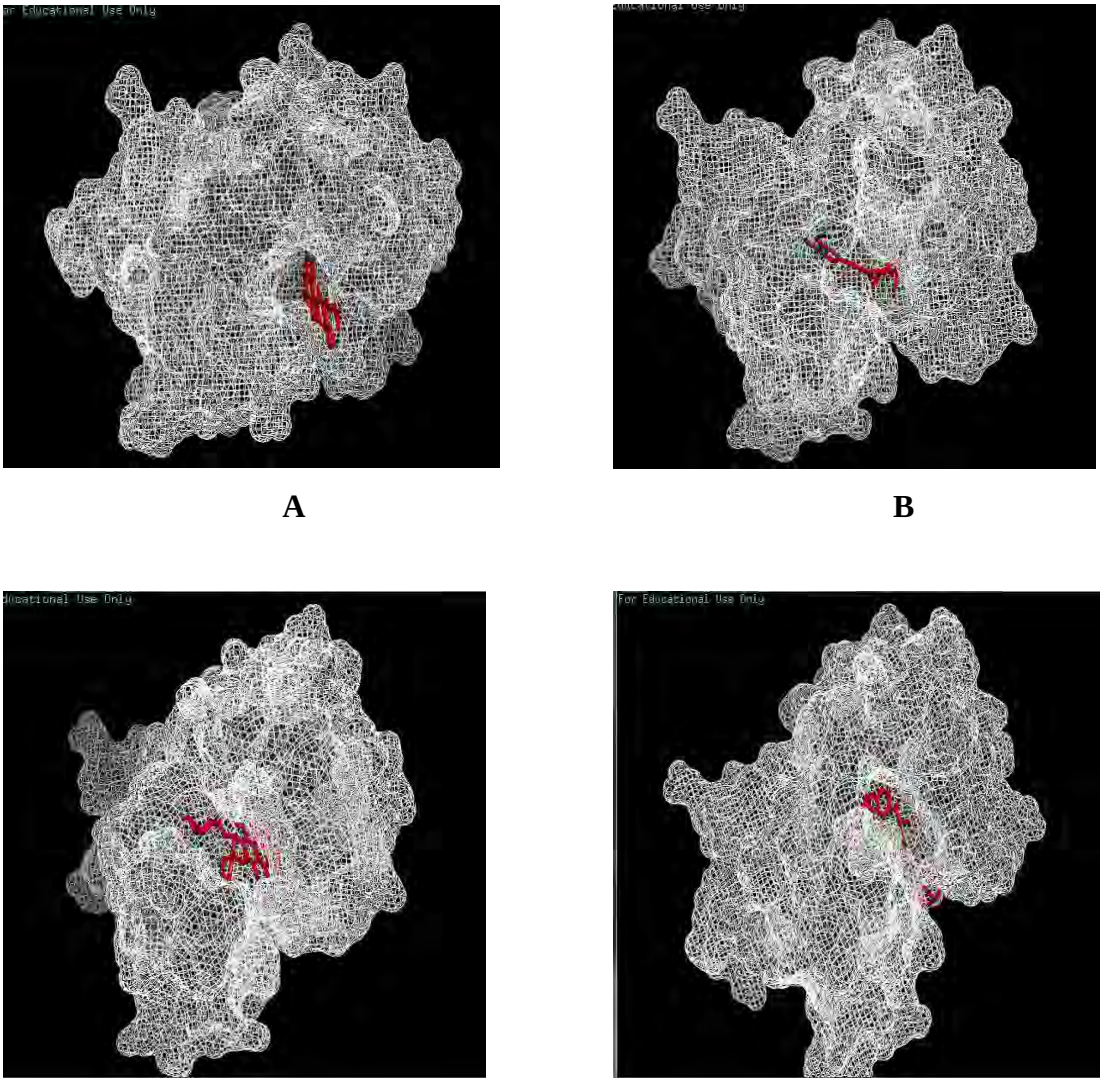
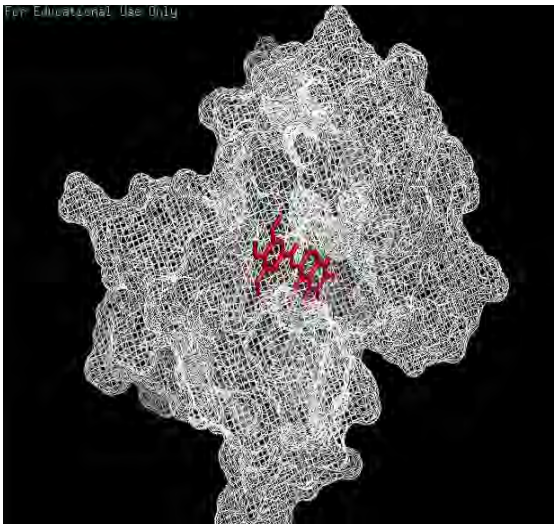


Figure 3.1.3: Visualization of Human DHFR with potential Small molecules Acarbose (A), Aceprometazine (B), Adenosine Monophosphate (C), Abacavir (D) & Isoxyl (E) using PyMOL (DeLano, 2002; Q. Li et al., 2011).



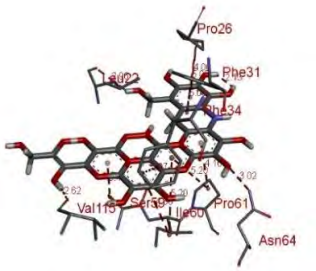
E

Figure 3.1.4: Visualization of Human DHFR with potential Small molecules Acarbose (A), Aceprometazine (B), Adenosine Monophosphate (C), Abacavir (D) & Isoxyl (E) using PyMOL (DeLano, 2002; Q. Li et al., 2011).

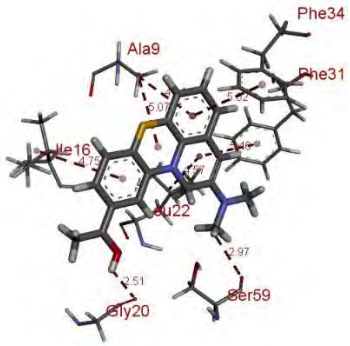
3.1.2 Validation using Discovery Studio

Discovery studio provides a clear and distinct concept regarding the ligand and protein with their interactions. It allows to observe the bonds between the amino acids along with the types. Distances between the ligands and protein molecule were also being evaluated with the help of Discovery studio. Furthermore, expansions of both distances and types of the bonds can also be observed and compared with the standard one.

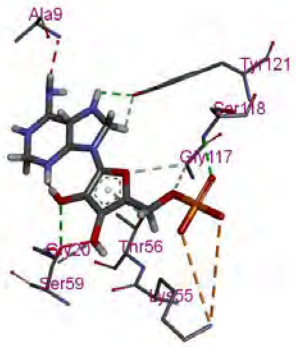
A



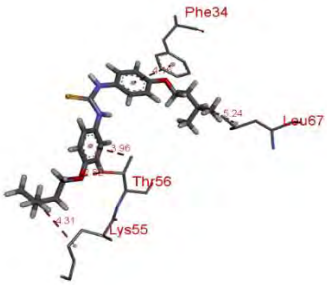
B



C

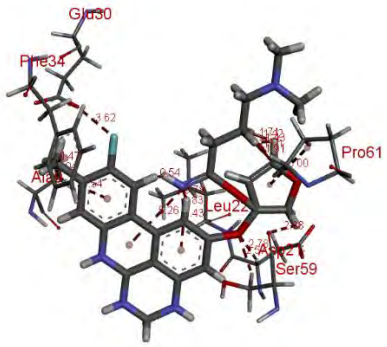


D

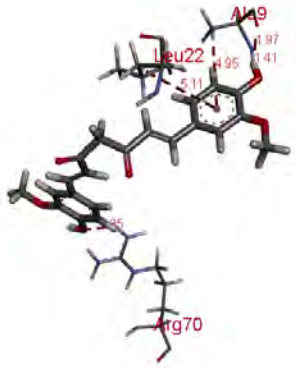


E

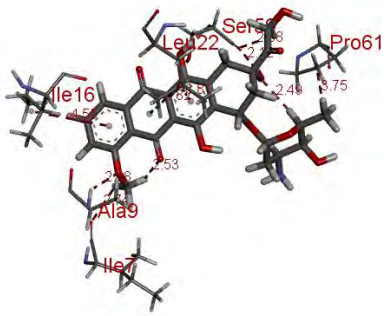
Figure 3.1.5: Observation of ligand interactions of Human DHFR with established anticancer drugs Afatinib (A), Curcumin (B), Doxorubicin (C), Trimethoprim (D) & Trimetrexate (E) using Discovery Studio (Q. Li et al., 2011; Systems, 2017).



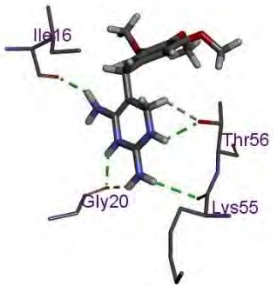
A



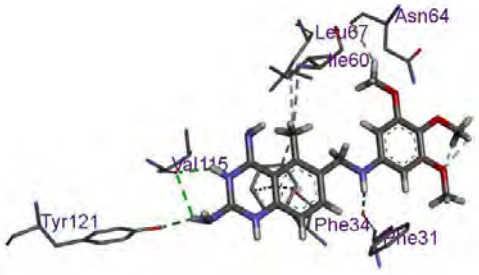
B



C



D

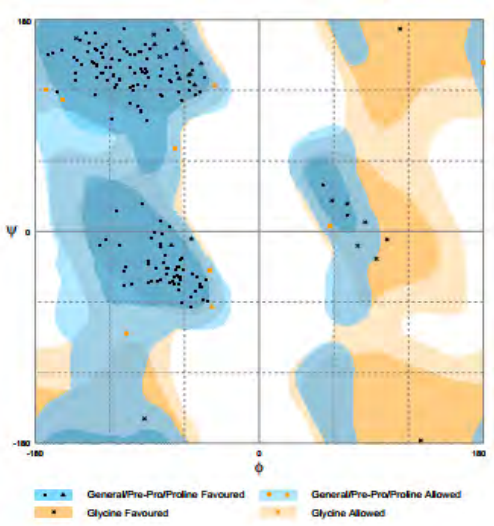


E

Figure 3.1.6: Observation of ligand interactions of Human DHFR with potential Small molecules Acarbose (A), Aceprometazine (B), Adenosine Monophosphate (C), Abacavir (D) & Isoxyl (E) using Discovery Studio (Systems, 2017; Wishart et al., 2017).

3.1.3 RAMACHANDRAN Plot

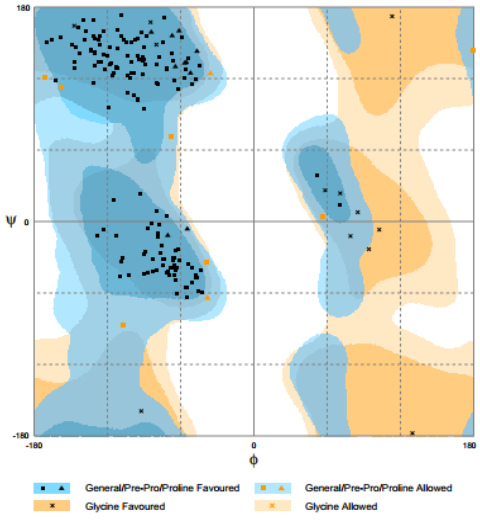
Ramachandran Plot was also done for potential small molecules & established anticancer drugs with Human DHFR and it significantly shows the energy minimization of the whole protein and other amino acids which are not quite minimized in terms of energy are shown as outliers with in the plot.



Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)



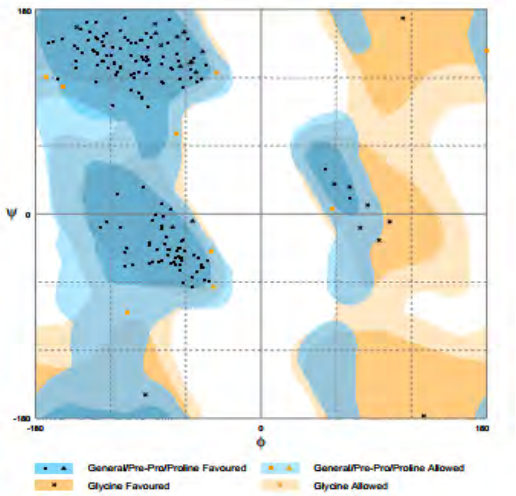
Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Acarbose **Aceprometazine**

Figure 3.1.3.1: Ramachandran Plot of potential small molecule & established anticancer drugs with Human DHFR where number of residues in favored region (~98.0% expected): 171(95.0%).Number of residues in allowed region (~2.0% expected): 9(5.0%).Number of residues in outlier region 0(0.00%) (Lovell et al., 2003).

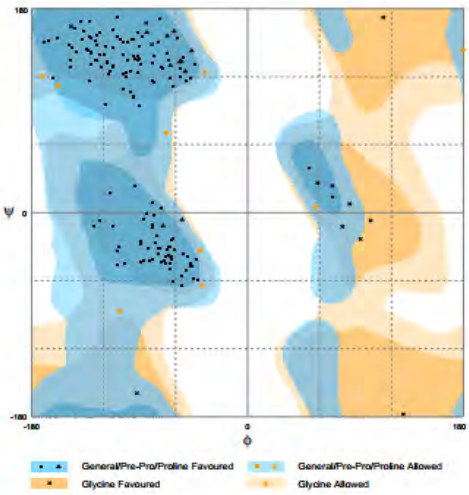


Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Adenosine Monophosphtate

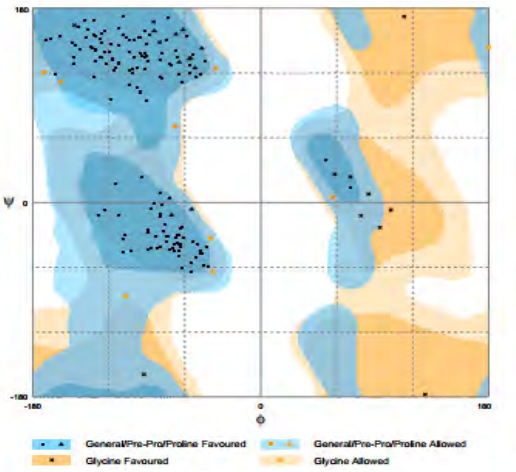


Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Isoxyl

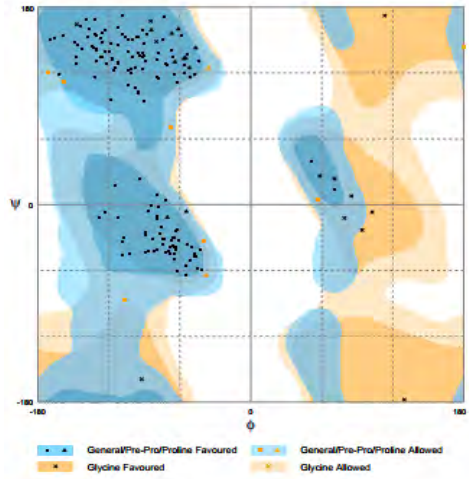


Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Abacavir



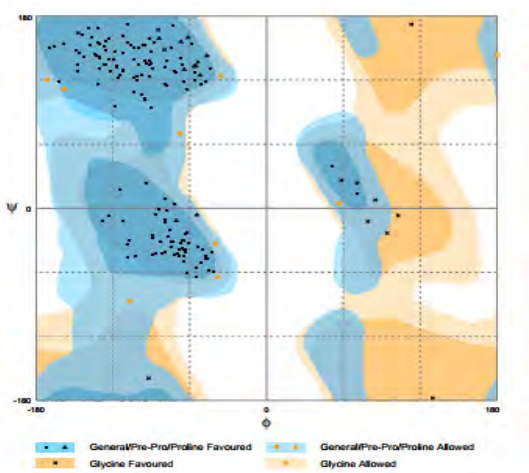
Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Afatinib

Figure 3.1.3.2: Ramachandran Plot of potential small molecule & established anticancer drugs with Human DHFR where number of residues in favored region (~98.0% expected): 171(95.0%).Number of residues in allowed region (~2.0% expected): 9(5.0%).Number of residues in outlier region 0(0.00%)(Lovell et al., 2003).

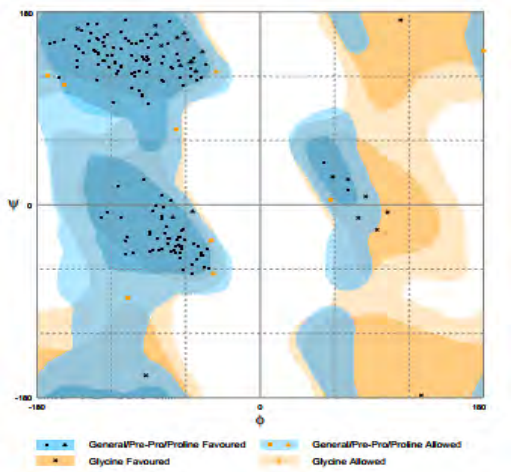


Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Curcumin

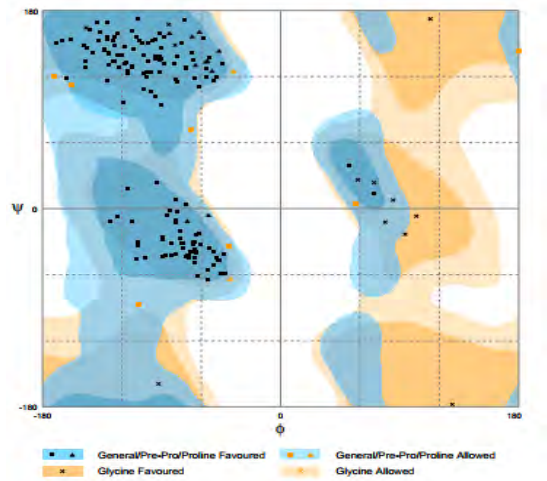


Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Doxorubicin

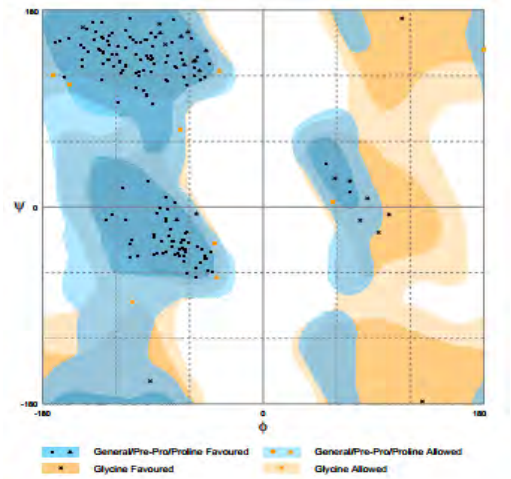


Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Trimethoprim



Number of residues in favored region (~98.0% expected): 171(95.0%).

Number of residues in allowed region (~2.0% expected): 9(5.0%).

Number of residues in outlier region 0(0.00%)

Trimetrexate

Figure 3.1.3.3: Ramachandran Plot of potential small molecule & established anticancer drugs with Human DHFR where number of residues in favored region (~98.0% expected): 171(95.0%).Number of residues in allowed region (~2.0% expected): 9(5.0%).Number of residues in outlier region 0(0.00%)(Lovell et al., 2003)**.**

Chapter Four

Discussion & conclusion

4 Discussion

Throughout this docking procedure there were a number of screening of drugs as well as docking. Different approaches of docking were also taken to validate the result by comparing binding affinities with each other. Among 200 small molecules, few of them were selected on the basis of drug classes randomly. Besides their usual therapeutic indications, they showed good amount of anticancer activity which is very valuable. Different approaches of docking were applied while completing this computational study. Rigid docking applied to reduce the atomic level shaking and rotation which leads to more of a fixed positioning of drugs within the protein molecule Human DHFR. Whereas, flexible approach of docking relates to very natural process of drug protein binding and torsions were also not definitely fixed. Both of the approaches were applied to evaluate the results and compare them depending on the situational factors such as ligand rigidity and torsion number. Table 3.1 contains results where the protein and drugs are rigid. Because of rigidity Table 3.1 contains best affinity values for established anticancer drugs compared to Table 3.2. Whereas, in Table 3.2 protein is rigid in nature but the drugs are flexible and binding affinity decreased compared to Table 3.1. On the other hand, Table 3.3 & 3.4 contains binding affinity values where human DHFR is docked with small molecules by rigid and flexible docking respectively. Results of affinities are always come with negative sign as these are exothermic reactions. The more the negative affinity values greater the binding affinities. All torsions were inactivated and torsion number 1 was set to dock in rigid approach while using PyRx. PyMOL showed the specific binding site of the human DHFR comparing the bound ligand with established anticancer drugs and small molecules at the same position (Fig: 3.1.1). Discovery Studio gave a detailed interaction of ligand with the amino acids of protein human DHFR which is almost same for established anticancer drugs (Fig: 3.1.2) and potential small molecule (Fig: 3.1.2). It also showed the distance and type of the bonds interacting with ligands and protein molecule. Discovery Studio results showed amino acids with which both of them were attached to including their bond distance and bonding types (Fig 3.1.3 & 3.1.4). Amino acids are PRO26 PRO61 LEU22 PHE31 PHE34 VAL115 GLY17 ILE60 and PRO61 PHE34 LEU22 ASP21 SER59 ALA9 GLU30 (Systems, 2017). Most of the bonds are Pi-Alkyl, Pi-Pi, Conventional, Carbon bonds etc. (Systems, 2017). Ramachandran plot showed the energy minimization for the protein

molecule (Fig 3.1.3.1). Number of residues in favored region (~98.0% expected): 171(95.0%). Number of residues in allowed region (~2.0% expected): 9(5.0%). Number of residues in outlier region:0(0.0%) (Lovell et al., 2003). Among the established anticancer drugs that have been used for this study, Curcumin, Afatinib and Doxorubicin are not directly involved with DHFR pathway rather they have different pathways to reduce cancer progression. Curcumin precedes in peroxisome proliferator-activated receptor gamma, vitamin D3 receptor, multidrug resistance-associated protein-5, carbonyl reductase (NADPH) 1, glutathione S-transferase P pathways (Wishart et al., 2017). Afatinib works on Human epidermal growth factor receptor (HER2) 2 and epidermal growth factor receptor kinases to inhibit breast cancer (D. Li et al., 2008). Doxorubicin by intercalation interacts with DNA and inhibit the progression of Topoisomerase II (Pommier et al., 2010). Trimethoprim & trimetrexate have shown their efficacy towards interacting with Human DHFR and suppress cancer mechanism.

Conclusion

The finding provides a clear and distinct concept about the efficiency of the established anticancer drugs working by interacting with DHFR pathway throughout their therapeutic generations. On the other hand, small molecules from different classes also show good binding affinities towards this DHFR protein which gave the direction of similar work in future to find out anticancer activity of very specific class of the small molecules. The results show a promising future for these molecules as potent counterparts of the already established cancer drugs. Furthermore, the three drugs other than trimetrexate and trimethoprim were previously known to follow different pathways but showed remarkable results in our experiment regarding DHFR pathway. Therefore, we can easily conclude that a doorway has opened for the repurposing of these three drugs in this particular pathway as well.

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