RATIONAL DRUG DESIGN OF NEW FOLIC ACID ANALOGUES WITH HIGH BINDING AFFINITY AND ANTICANCER ACTIVITY

MOHAMMAD GASEM MOHAMMAD ALTHIABAT

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by

MOHAMMAD GASEM MOHAMMAD ALTHIABAT

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LIST OF ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
5-MTHF	5 Methyl Tetra Hydro Folate
Å	Angstrom (10^{-10}m)
AMBER	Assisted Model Building and Energy
CLC-PRED	Cell Line Cytotoxicity Predictor
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
DPF	Docking Parameter File
FA	Folic Acid
FDA	Food and Drug Administration
FF	Force Field
FR	Folate Receptor
FRα	Folate Receptor Alpha
FR-β	Folate Receptor Beta
FR-γ	Folate Receptor Gamma
FR-δ	Folate Receptor Delta
GAFF	General Amber Force Field
GPF	Grid Parameter File
GPI	Glycosylphosphatidylinositol
K	Kelvin
MAXCYC	Maximum Cycle of minimization
MD	Molecular Dynamics

MFRS	Membrane Folate Receptors
MM-PBSA	Molecular Mechanics-Poisson Boltzmann Surface Area
MTX	Methotrexate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PDB	Protein Data Bank
PDBQT	Protein Data Bank, Partial Charge (Q), & Atom Type (T)
RFC	Reduced Folate Carrier
RMSD	Root Mean Square Displacement
RMSF	Root Mean Square Fluctuation
SAR	Structure Activity Relationship
SBDD	Structure Based Drug Design
THF	Tetra Hydro Folate
TS	Thymidylate Synthase

REKABENTUK SECARA RASIONAL BAGI ANALOG ASID FOLIK DENGAN AFINITI IKATAN YANG TINGGI DAN AKTIVITI ANTI KANSER

ABSTRAK

Sejak lebih daripada 50 tahun yang lalu, ubat yang mengeksploitasi laluan asid folik (FA) telah memainkan peranan yang penting dalam rawatan pelbagai penyakit seperti antikanser, antibakteria dan imunomodulator. Dalam kajian klinikal dan industri farmaseutikal, pencarian molekul antifolat sebagai agen antikanser merupakan bidang penyelidikan yang aktif. Proses merekabentuk dan mencari drug antifolat baharu dengan sifat dan aktiviti yang unggul dianggap sebagai strategi rawatan antikanser yang sangat menarik. Kebelakangan ini, beberapa ubat antifolat baharu dengan sifat yang unggul telah mula memasuki pasaran. Ubat-ubatan ini termasuk raltitrexed, pralatrexate, pemetrexed dan edatrexate telah menunjukkan keberkesanan terhadap sel kanser tertentu. Walau bagaimanapun, masalah yang melibatkan kerosakan pada sel yang sihat tetap berlaku. Sebilangan besar ubat antifolat memasuki sel melalui pembawa folat terturun (RFC), yang juga terdapat pada permukaan sel yang sihat. Dapatan kajian terkini melalui asai *in vitro* dan kajian praklinikal yang dilaporkan dalam literatur telah menunjukkan peralihan tapak sasaran daripada RFC kepada reseptor folat sebagai pendekatan yang dapat mengurangkan risiko ketoksikan ubat antifolat ke atas sel yang sihat. Reseptor folat alpha (FRα) telah dianggap sebagai sasaran tumor yang penting kerana jumlahnya yang besar pada permukaan sel tumor, iaitu sekitar 100-300 kali ganda berbanding sel normal, iaitu 1-10 juta salinan reseptor setiap sel. Peningkatan selektiviti untuk FRα dipercayai dapat membantu mengurangkan kesan sampingan yang tidak diingini terhadap sel yang sihat. Objektif

tesis ini ialah untuk merekabentuk analog FA yang baru dengan kepilihan yang lebih tinggi terhadap FRα. Lima puluh analog FA dengan pelbagai variasi cincin heterosiklik telah direkabentuk dan interaksi mereka dengan FRα telah dikaji. Hasil pendokkan molekul telah menunjukkan 20 daripada 50 analog tersebut mempunyai kekuatan ikatan yang lebih tinggi terhadap FRα berbanding FA dan metotreksat (MTX). Mekanisme pengikatan terhadap FRα bagi MTX, FA dan analog-analog dengan tenaga ikatan pendokan yang paling negatif (FOL03, FOL08, FOL21, dan FOL50) telah dikaji seterusnya melalui simulasi dinamik molekul bagi tempoh 100 ns. Analisis konformasi menunjukkan konvergensi yang jelas, dengan ligand ini memasuki poket ikatan melalui moieti pteridine dan moeiti asid glutamik terkeluar daripada mulut poket FRα. Menariknya, 3 dari empat (FOL03, FOL08, FOL50) telah membentuk interaksi yang lebih kuat terhadap FRα dan menunjukkan tenaga ikatan dan elektrostatik yang lebih rendah berbanding FA dan MTX melalui perhitungan oleh MM-PBSA. Keberkesanan analog baru FA terhadap sel kanser yang berbeza juga diperiksa secara *in silico* menggunakan pangkalan data CLC-PRED. Hampir kesemua 50 analog FA yang direka mempunyai ciri antikanser yang setanding dengan MTX terhadap sel kanser ovari, paru-paru, payudara dan pankreas. Penambahan cincin heterosiklik (contohnya cincin pteridine) dalam struktur FA menghasilkan aktiviti antikanser yang lebih baik berbanding FA. Diharapkan hasil daripada kajian ini akan dapat memberikan gambaran mengenai reka bentuk ubat antifolat yang lebih berkesan dengan ciri kepilihan yang lebih baik.

RATIONAL DRUG DESIGN OF NEW FOLIC ACID ANALOGUES WITH HIGH BINDING AFFINITY AND ANTICANCER ACTIVITY

ABSTRACT

For more than 50 years, drugs that exploit the folic acid (FA) pathway have had a major role in the treatment of diseases such as an anticancer, antibacterial and immunomodulatory. In the clinical study and pharmaceutical industry, the quest for antifolates as an anticancer remains an active area of research. Therefore, designing and discovering new antifolate drugs with superior properties and activities are considered a very attractive strategy. Recently, new novel antifolate drugs with superior properties started to enter markets. Such drugs include raltitrexed, pralatrexate, pemetrexed, edatrexate, etc are effective against certain cancers. However, the problem remains with their effect that damage healthy cells. Majority of the antifolate drugs enter the cells by the reduced folate carrier (RFC), which are also present in healthy cells. Recently, evidence from *in vitro* assays and preclinical studies has shown the shift in targeting the folate receptor instead of RFC as a promising approach to reduce the toxicity of antifolate drugs toward healthy cells. Folate receptor alpha (FRα) has been considered as important tumor target as it is overexpressed on the tumor cells surface about (100-300) folds more than the normal cells, i.e. ~1-10 million copies of receptors per cell. The increased selectivity for FRα may help to reduce the undesirable side effects towards healthy cells. Therefore, the goal of this thesis is to design new FA analogues with higher selectivity to bind with FRα. Fifty FA-analogues with variant heterocyclic rings were designed and studied for their interaction with FRα. Docking results showed 20 out of the 50 FA-analogues showed higher binding affinity than methotrexate (MTX) and the parent folic acid (FA)

towards FRα. The binding mechanisms of FA, MTX, and the most negative docked binding energy analogues (FOL03, FOL08, FOL21, and FOL50) with the FRα were studied by 100 ns molecular dynamics simulations. The conformational analysis showed an apparent convergence, with the ligands entered the binding pocket from pteridine part, while the glutamic part stuck at the mouth of FRα pocket. Strikingly, 3 out of the four (FOL03, FOL08, FOL50) have formed stronger interactions with the most negative free binding and electrostatic energies more favourable than FA and MTX as calculated by MM-PBSA. The efficacy of new FA-analogues towards different cancer cells were also examined *in silico* utilizing the CLC-PRED database (http://www.way2drug.com/Cell-line/). Nearly all the 50 FA analogues designed have anticancer properties comparable to MTX against the ovary, lung, breast, and pancreatic cancer cells. The addition of heterocyclic rings (e.g. pteridine ring) in the FA structure, results in improved anticancer activity of the FA. Thus, it is hoped the results from this study might give an insight into the design of promising, more efficacious and selective anti-folate drugs.

CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Cancer is the second-largest recognized cause of death for human making it one of the most serious diseases known to humanity (WHO, 2018). The symptoms of cancer vary with the type of organ affected, and chemotherapy, radiation and/or surgery are part of the treatment modality (Gomis *et al.*, 2016).

Antifolates are the earliest of the antimetabolite class of anticancer agents but are also one of the first modern anticancer drugs. The drugs consist of folic acid (FA) scaffold (Gonen *et al.*, 2012; Scaglione *et al.*, 2014). They possess a strong binding affinity to glycoprotein receptor available on the tumor cell surface known as folate receptors, and enter the cells through endocytosis process (McGuire, 2003; Salazar *et al.*, 2007).

Folate receptors are available on the surface of both cancerous and normal cells, but they are overexpressed in the epithelial cancerous cells about (100-300) folds more than the normal cells. With an estimate of 1 to 10 million copies of receptors per cell, the receptors are considered as important target in the cancer treatment (Gibbs *et al.*, 2005; Salazar *et al.*, 2007; Kalli *et al.*, 2008; Vlahov *et al.*, 2012; Chen *et al.*, 2013; Patel *et al.*, 2016; Siwowska *et al.*, 2017; Frigerio *et al.*, 2019). Cells need FA to mediate one-carbon metabolism inside the cell, which is essential for cell growth and DNA synthesis. Clinical studies have showed that the cell uptake for FA is much higher in the tumor cells than in the normal cells as meet the rapid growth demands of the cancer cells (Anand *et al.*, 2008; Du *et al.*, 2015; Shuvalov *et al.*, 2017).

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FA competes with antifolate drugs in the binding with FRα. This leads to a decrease in the efficacy of the antifolates drugs, and raise the concentration of unbonded drugs in the blood circulating. As a consequence, the unbounded anti-folates will interact with the healthy cells and cause damage to them (Westerhof *et al.*, 1995; Miotti *et al.*, 1997; Corona *et al.*, 1998; Nakashima-Matsushita *et al.*, 1999; Matherly *et al.*, 2003; Visentin *et al.*, 2012). Despite their proven clinical efficacy towards different types of cancer, none of the existing antifolate drugs, have shown enough selectivity towards FRα compared to FA (Chattopadhyay *et al.*, 2006; Kelemen, 2006; Walling, 2006; Chen *et al.*, 2013).

The major challenge in cancer therapy is to find a selective and potent anticancer drug in low dose with a potentially lower risk of effects towards healthy cells (McGuire, 2003; Asif, 2014; Du *et al.*, 2015; Seitz *et al.*, 2015; Dos Santos *et al.*, 2018).

1.2   Overview of Cancer

Cancer is a general term for a broad range of diseases characterised by an unregulated proliferation of abnormal cells where the propagation of these irregular cells outside the boundaries may invade and distort the healthy neighbouring cells and thus kill them (WHO, 2018). Other known names that are used to describe cancer disease are malignant tumors and neoplasms. The World Health Organisation (WHO) reported that cancer is the second leading cause of death in 2018 and considered as one the most serious diseases in history. The symptoms of cancer vary with the type of organ affected, and chemotherapy, radiation and/or surgery are part of the treatment modality

(Gomis *et al.,* 2016). The mortality rates are reported to be around 9.6 million worldwide. The most prevalent cancers in men are the prostate, lung and colorectal cancer, while lung, cervix and breast cancers are highly prevalent among women (WHO, 2018).

Environmental factors such as malnutrition, overweight, physical inactivity, oxidative stress, pollution and radiation are also considered to be the main causes for cancers (Thun *et al.*, 2006; Anand *et al.*, 2008). In addition, genetic (defected gene inheritance) has also played a minor role in the incidence of tumor (Roukos, 2009). At the genetic level, the mechanistic development of cancer creates an immortal cell by altering a nucleotide sequence for both cancer genes suppressor and oncogenes (Knudson, 2001). Furthermore, the alteration of epigenetic proteins that regulate chromosomal accessibility also plays a significant role in cancer pathophysiology (Baylin *et al.*, 2006).

1.3   Drug Targeting Cancer's Receptors

Many criteria should be considered when selecting suitable drug target. The most important is to select receptors that significantly appears in the tumor cells compared to the normal cells. This feature gives the opportunity for the anticancer drug to target these receptors thus halting its activity and spread.

Recently, on a group of glycoproteins with molecular weights in the range of 35–40 kDa identified as folate receptors (FR), was identified in increased concentration in tumor cells as compared to the normal cells. Thus, they have been considered to be among receptors that meet these requirements (Antony, 1996; Assaraf *et al.*, 2014;

Quici *et al.*, 2015). In general, there are four FR isoforms (FRα, FR-β, FR-γ, and FR-δ) (Clifton *et al.*, 2011; Frigerio *et al.*, 2019). Isoforms FRα, FR-β, and FR-δ are linked to tumor surface cells by a glycosylphosphatidylinositol anchor (GPI); whilst, FR-γ which lack of GPI, thus rendering it to be a freely soluble secreted protein (Shen *et al.*, 1994; Shen *et al.*, 1995; Assaraf *et al.*, 2014). Physiologically, the FR possess a strong affinity for FA and vitamin N5-methyltetrahydrofolate (Antony, 1996; Kelemen, 2006; Assaraf *et al.*, 2014).

FR-β isoform is preferentially activated in myeloid cells that are associated with autoimmune and inflammatory diseases (Puig-Kröger *et al.*, 2009; Xia *et al.*, 2009). The FR-β isoform has an active appearance with related tumors-associated macrophages (TAMs) of several tumors, including those in the lung, kidney, blood, soft tissue, skin and liver (Kurahara *et al.*, 2012; Shen *et al.*, 2015). The TAMs have the ability to permeate solid tumors, develop their metastasis and expansion by secretion of proangiogenic factors and suppression of CD8+ T cells (Feng *et al.*, 2011). Consequently, the FR-β isoform can act as a prospective target for selective cytotoxic agents in cancer treatment (Antony, 1996; Feng *et al.*, 2011). However, despite FR-β expression on many types of cancers, the FRα is the most abundance isoform presence in cancer cells, compared to all other isoforms of FR (Kalli *et al.*, 2008; Clifton *et al.*, 2011; Feng *et al.*, 2011). In fact, FRα is overexpressed in various types of epithelial tumors including ovarian, lung, kidney, and breast cancers, therefore, considered as a strong target for cancer treatment (Kalli *et al.*, 2008; Clifton *et al.*, 2011; Assaraf *et al.*, 2014; Frigerio *et al.*, 2019).

1.3.1 Folate Alpha Receptor

FRα is also known as folate binding protein. Due to its high concentration found on the tumor cell surface compared to that of healthy cell, many efforts focus on understanding the properties of FRα which is the prerequisite in developing anti-cancer treatment, including anti-FRα antibodies, high-affinity folate antagonists, folate-based imaging agents and conjugated folate drugs (McGuire, 2003; Deng *et al.*, 2009).

FRα is globular-like shaped protein highly stabilized by disulphide bonds made up of cysteine amino acid residues. It has four long α-helices (α-1, α-2, α-3, and α-6), two short α-helices (α-4, α-5), four short β-strands (β1-β4), and several loop areas (Monaco, 1997), as illustrated in Figure 1.1.
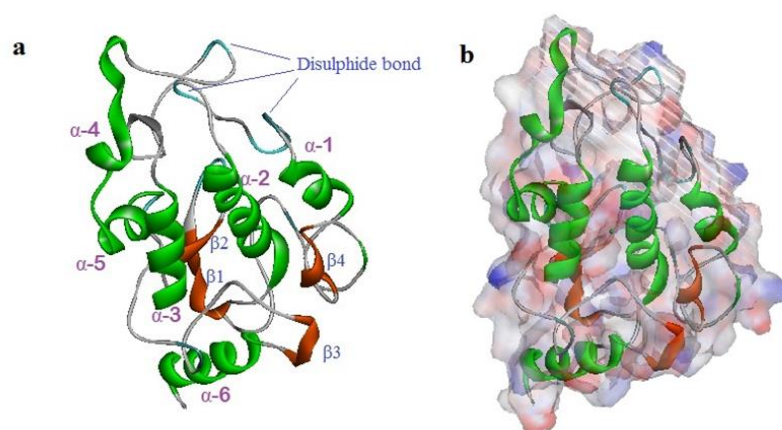
Figure 1.1: a. FRα alpha helices (green), beta helices (orange), and disulphide bonds (blue), b. FRα in a solvent surface of hydrophobicity with semi-transparency.

The structure of FRα includes an open and lengthy binding pocket as shown in Figure 1.2. The binding pocket of folate is formed behind of α-1, α-2, α-3, at the bottom of β-1 and β-2 terminal strands; on the left and top of α-1, α-2, α-3, and α-4 helices and on the right of α-4, α-5, β-3 and β-4 regions (Chen *et al.*, 2013). FRα binding pocket consists of a high number of tryptophan residues (Figure 1.3) that can create a large hydrophobic environment to fit the aromatic folate component (Maziarz *et al.*, 1999).

In addition, it also has many cysteine residues which can bind with high affinity with

FA to facilitate its cellular uptake (Kelemen, 2006).
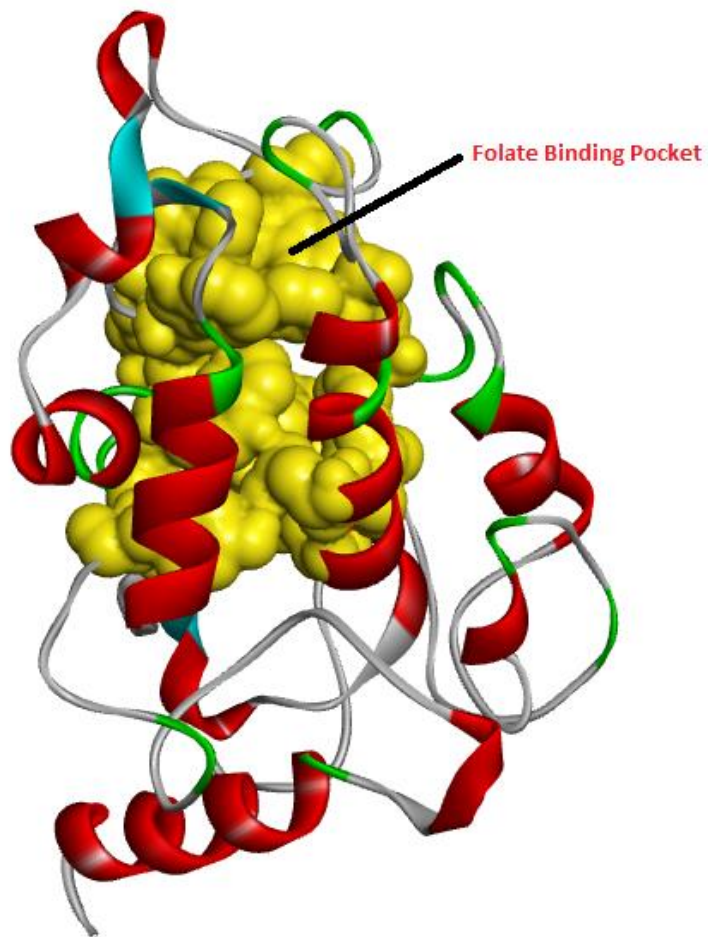
Figure 1.2: FRα binding pocket in yellow colour
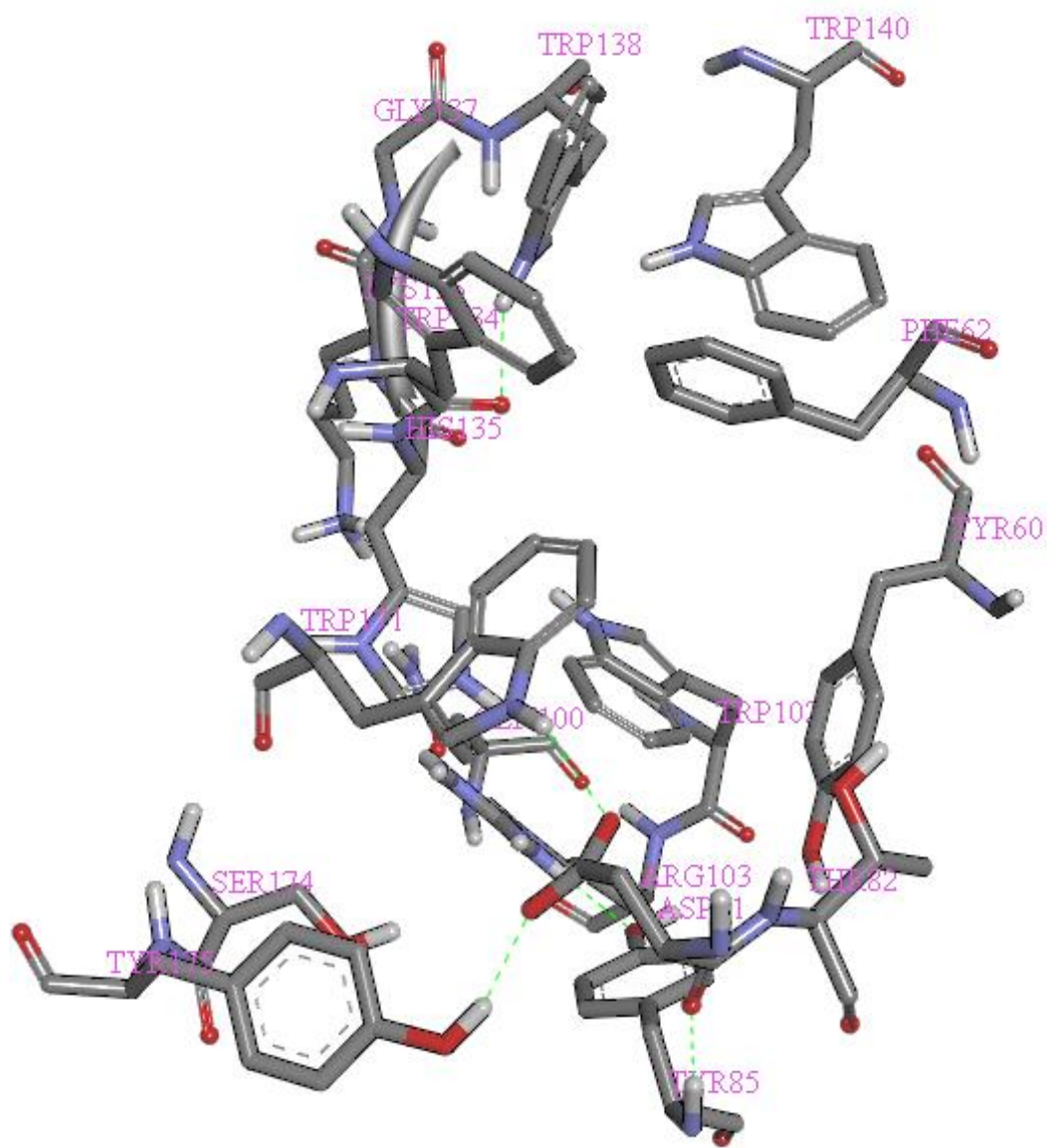
Figure 1.3: FRα binding pocket with labeled amino acids.

1.4   Folic Acid

Folic acid (FA) is consists of three central units: pteridine ring, PABA and glutamic acid residues (Figure 1.4). It is an oxidized synthetic form of folate structure which is essential in several human metabolic pathways. Folate functions as a coenzyme or cosubstrate in single-carbon transfer reactions that are important in nucleic acid synthesis, amino acid metabolism, cell division, growth and survival, particularly for those rapidly dividing cells (Morris *et al.*, 2007). Folate occurs naturally in foods such as green leafy vegetables while FA is the synthetic form of folate and given as food supplements or added to fortified food (Morris *et al.*, 2007). FA is an inactive form where once inside the cell, it is transformed into vitamin B9 active form which is also known as folate or 5-methyltetrahydrofolate (5-MTHF) via several enzymes as shown in Figure 1.5 (Paniz *et al.*, 2017). FA can be actively transported into cells by the RFC or via the membrane folate receptors (MFRs) either by potocytosis or endocytosis (Anderson *et al.*, 1992; Matherly *et al.*, 2007; Gonen *et al.*, 2012).
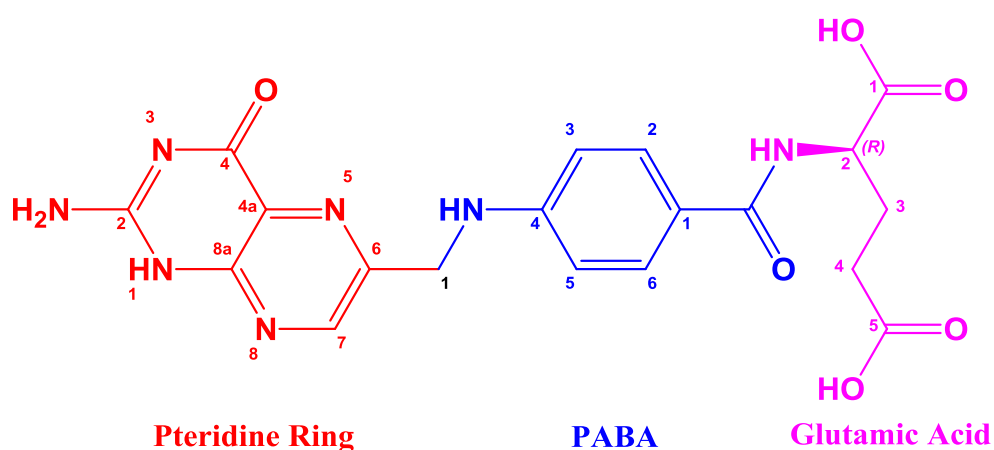
Figure 1.4: 2D-Chemical Structure of FA.
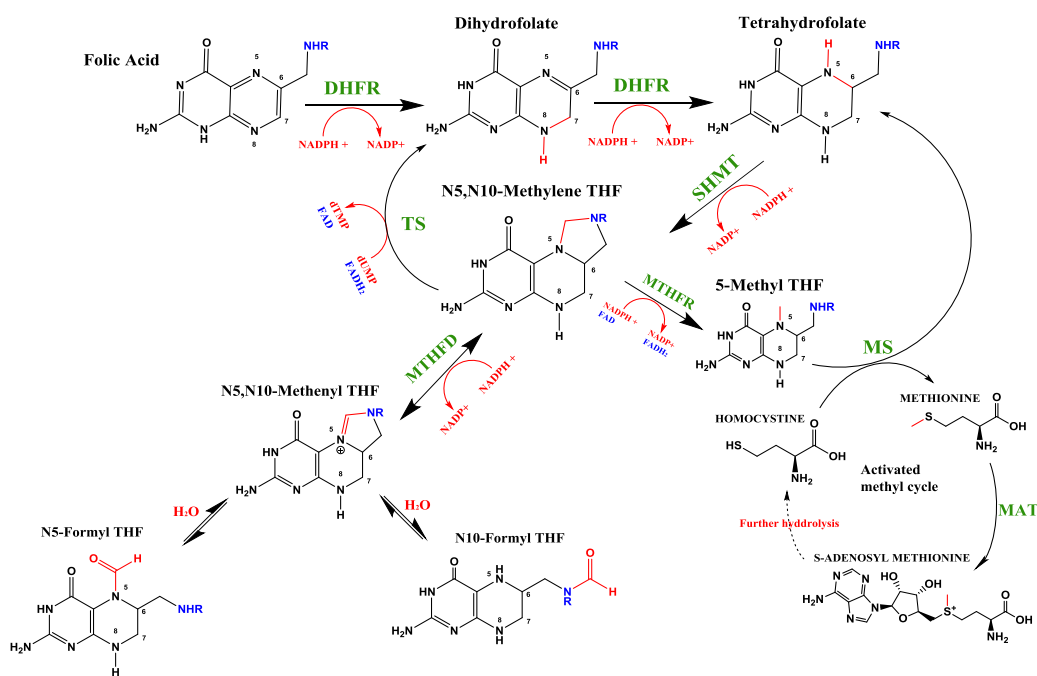
Figure 1.5: FA pathway inside the cell.

1.4.1   Entry of FA to the FRα binding pocket

The mechanism by which FA is incorporated into the folate receptor is a prerequisite in understanding the binding process and to improve FA-competitor drugs at the binding site (Gabizon *et al.*, 1999; Chen *et al.*, 2013; Jones *et al.*, 2017). FA enters FRα from pteroate part of FA and seated deeply inside the receptor, where the glutamate moiety can be found superficially stuck at the mouth of pocket as shown in Figure 1.6. The prevalent binding and interactions of those formed between the FRα and FA demonstrate the high affinity of binding for this FR isoform, and act as a prototype for modeling in order to synthesize drugs that target the folate receptor more efficiently (Chen *et al.*, 2013).
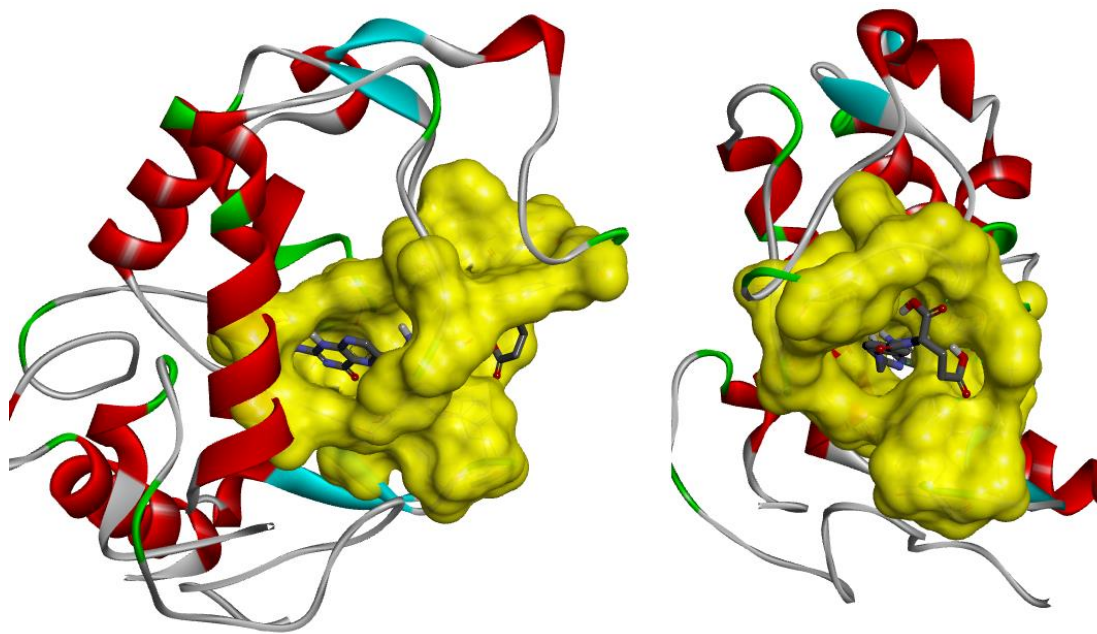
(i)
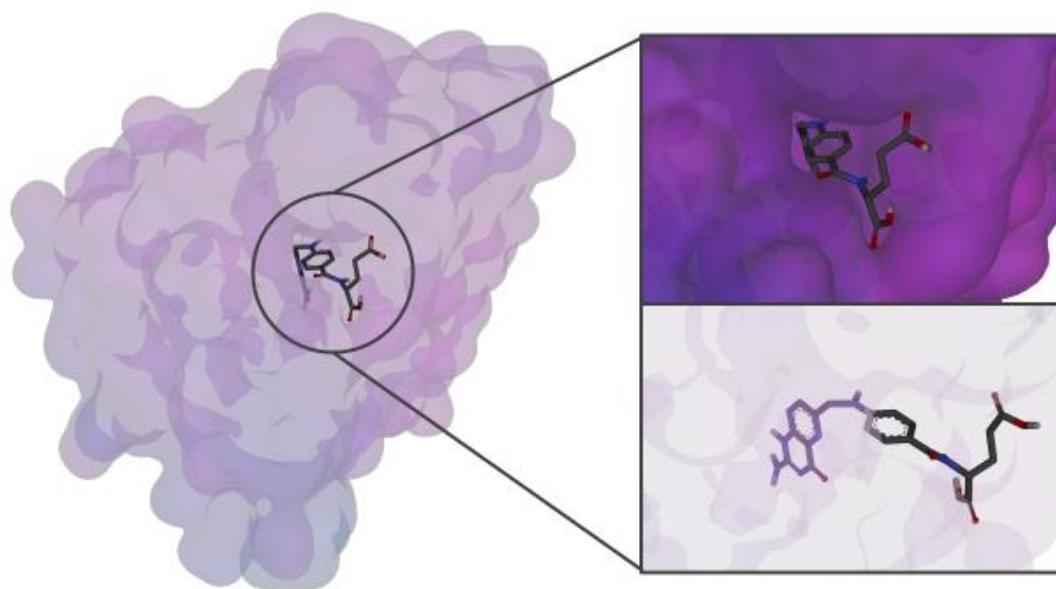
(ii)

Figure 1.6: 3D crystal structure (4LRH.PDB) interaction of FA with FRα (i).3D solvent accessibility interaction of FA with FRα (ii).

Many current cancer treatments are FA drug-conjugates or antifolate moieties that play a key role in the binding with FRα in the cancer cells (Theti *et al.*, 2004). Studies showed that many antifolates have better binding affinity for FRα than FA. This indicates that the types of bonds (ionic bond, H-bond, polar covalent bond, and hydrophobic interaction) and the diverse of functional groups are critical to increase the binding affinity between folate protein and antifolate structure (Patil *et al.*, 2010; Assaraf *et al.*, 2014; Lopez *et al.*, 2014).

However, each functional group of FA gives different effect as they bind to the protein. This was due to the difference of the functional groups used, the stereochemistry of structure, the distance of each interaction, and the type of amino acids inside the receptor pocket that participate in the interactions. All these factors play crucial roles in bond formation like irreversible covalent bonds and reversible non-covalent bonds (ionic bond, ion-dipole, H-bond, hydrophobic bond) (Kastritis *et al.*, 2013).

1.5   Antifolate drugs

Antifolate is a group of drugs that block the action of FA inside the cell by inhibiting several enzymes such as dihydrofolate reductase (DHFR) and/or thymidylate synthase (TS) (Visentin *et al.*, 2012). Aminopterin is the first clinical approved antifolate. This drug was first shown to be appropriate to induce remission in children suffering from acute lymphoblastic leukaemia and brought hope for the treatment of other types of cancer (Farber *et al.*, 1948). In the early 1950s, Aminopterin was substituted by methotrexate (MTX) due to unexpected toxicity of aminopterin such as crystalluria and acute renal failure (Toth *et al.*, 1964). MTX was considerably less potent than

aminopterin but has a more favourable therapeutic index (Goldin *et al.*, 1955; Kremer, 2004). Notwithstanding its early therapeutic effectiveness, an understanding of the mechanism of action of MTX has slowly developed over the decades. Even until today MTX is used at a minimum dose in clinical treatments to avoid any unexpected side effects (Visentin *et al.*, 2012). Subsequently, in 2004, pemetrexed the second antifolate was approved after more than 50 years since the release of MTX for treating lung and mesothelial tissue cancers (Rollins *et al.*, 2005), and in 2009 pralatrexate follows suit for the treatment of non-Hodgkin lymphomas cancer (Casanova *et al.*, 2011).

In general, antifolates are classified into two major categories: "classical" and "nonclassical" antifolate. The classical has a general FA-like structure and enters via polyglutamylation intracellularly. While the non-classical antifolate, due to lack of glutamic acid site and being more lipophilic, cannot enter via polyglutamylation process, thus enters the cells by passive diffusion instead (Walling, 2006; Gonen *et al.*, 2012).

Antifolates can be actively transported into cells by reduced folate carrier (RFC) or via the membrane folate receptors (MFRs) either by potocytosis or endocytosis (Anderson *et al.*, 1992; Matherly *et al.*, 2007; Gonen *et al.*, 2012). The majority of antifolate drugs enter cells effectively through RFC, nonetheless, there are also some that bind with MFRs in high affinity (Jackman *et al.*, 2004). There is evidence that the way of transportation process might affect the activity of antifolates, especially, when the protein is extremely over-expressed or when the extracellular of folate concentration is very low (Jackman *et al.*, 2004).

Antifolates such as MTX, edatrexate, pemetrexed, lometrexol, etc, are effective in cancer treatment (Figure 1.7). However, despite their proven clinical efficacy towards different types of cancer, none of these compounds binds better to FRα than FA (Chattopadhyay *et al.*, 2006; Kelemen, 2006; Walling, 2006; Chen *et al.*, 2013), thus the possibility of producing many side effects.
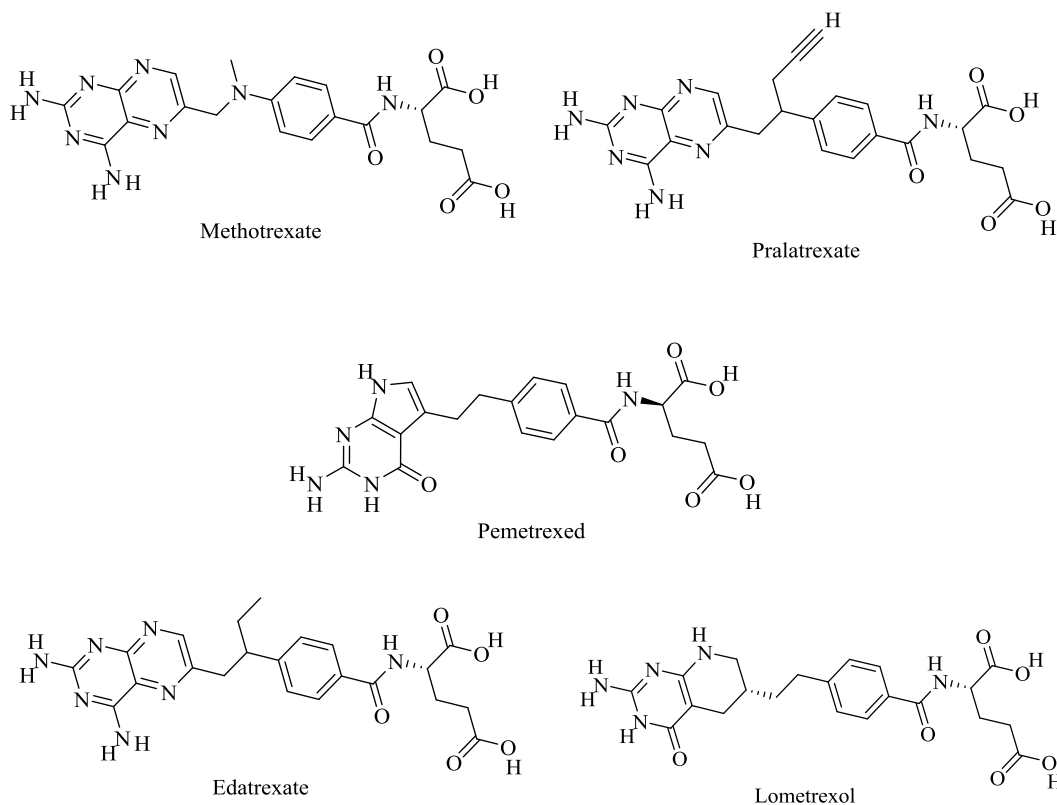
Figure 1.7: Classical antifolates available in the markets.

Methotrexate (MTX) is a well-known drug for the management of several kinds of tumors such as (neck, breast, bladder cancers, osteosarcoma, and non-Hodgkin's lymphoma). It is classified as a polyglutamate antifolate because it involves fragments

of glutamic acid, thus undergoes intracellular polyglutamylation. Generally, MTX is designed to attack and suppress nucleotide biosynthesis in folate-dependent enzymes, and to destroy cancer cells sequentially (Walling, 2006). Many studies demonstrated that MTX has strong activity in inhibiting DHFR enzyme (Figure 1.8). DHFR converts dihydrofolic acid to tetrahydrofolic acid; thus, inhibiting this pathway causes aggregation of dihydrofolic acid substrate and purine biosynthesis (Stone *et al.*, 1986; Assaraf, 2007).

Figure 1.8: Inhibition activity/mechanism of MTX against dihydrofolate reductase (DHFR) enzyme in the folate cycle inside the cell.

Scientific literature analysis in the last 10 years showed that all new DHFR inhibitors from classical antifolate drugs are distinguished by a heterocyclic rings in the structure, which play key role in increasing the affinity for the folate receptor and enzymes

inhibition inside the cancer cell (Gonen *et al.*, 2012; Sharma *et al.*, 2012; Anderson *et al.*, 2014; Jendele *et al.*, 2019; Raimondi *et al.*, 2019; Wróbel *et al.*, 2020) . Multi-targets drug in this scenario is a promising approach for new antifolate drugs.

1.6   Rational drug design

Recently, pharmaceutical studies have become highly dependent on modern medicinal chemistry and molecular modeling. This field of research allows us to study complex biological and chemical systems by understanding the structure-activity relationship (SAR) (Hughes *et al.*, 2011). The integration of the software and mathematical tools reflected an important and effective outcome of revising the basic structure of the ligand, its stable conformation, and how binding mechanism occur with the receptor. This contribution reduced the costs of research and experiments (Ferreira *et al.*, 2015).

Rational drug design (RDD) is used to explore novel compounds and enhancement of the selectivity based on the knowledge of the characteristic features of the ligands and the receptors. Different computational programs with smart algorithms are currently available for the screening and matching the modeling design with the previous experimental assay (Mavromoustakos *et al.*, 2011). One of the methods used in RDD is structure-based drug design (SBDD).

SBDD is the development and optimization of a chemical model to identify a novel drug candidate appropriate for clinical assay. Its strategy is based on the knowledge of the 3D structure of the ligand and how its configuration causes it to interact with its

biological target, eventually producing a therapeutic effect (Mavromoustakos *et al.*, 2011).

In SBDD there are many of softwares helpful in drug discovery strategy, the most important of these is the software that enables us to understand and measure the Gibbs free energy for the interaction between the ligand and receptor, such as AutoDock and AMBER (Jamkhande *et al.*, 2017). Recently, understanding the size of the binding site, orientations and distances within the protein have become a major concern in enhancement affinity of binding (Zhang *et al.*, 2008). A programme call Depth, has been applied to measure depth, groove sizes and searching potential amino acids which have a probability of binding inside the protein pocket (Tan *et al.*, 2013).

1.6.1 Molecular Docking

Recently, the development of diseases and their drug resistance became more complicated, and the need has become more significant for the development of a modified drug to eliminate various diseases and increase the response of the human body to treatment. One of the more complex problems is the understanding of the binding affinity and interaction mechanism that occurs between the ligand and the receptor in the *in vitro* and *in vivo* clinical studies. One of the tools that enable us to measure and understand such interaction is molecular docking.

Molecular docking software typically measures the binding energy and prediction of the binding affinity between the receptor and ligand, that allow us to clarify the basic requirements for biochemical processes (McConkey *et al.*, 2002; Ferreira *et al.*, 2015).

Whereas the receptor is often a sequence of amino acid or enzymes, the ligand is usually a small structure or peptide (Brooijmans *et al.*, 2003). Molecular docking is the most extensively used process in SBDD since the early 1980s (Kuntz *et al.*, 1982; Meng *et al.*, 2011).

In this study, the analysis of the binding affinity was done using the molecular docking simulation approach using AutoDock 4.2 software as well as also using MM-PB/GBSA from AMBER 18 (O Tanin *et al.*, 2015). AutoDock 4.2 uses the semi-empirical force field to estimate the binding affinity through two significant actions. Initially, it determines the intramolecular energy for the protein-ligand complex from the free state to the bound state. Then, it evaluates the intermolecular energy for the protein-ligand bound complex (Huey *et al.*, 2007; Morris *et al.*, 2009).

The free binding energy of protein–ligand interactions is estimated by ΔG_{Bind} as the following Equation 1:

$$\Delta G_{Bind} = (V_{bound}^{L-L} - V_{unbound}^{L-L}) + (V_{bound}^{P-P} - V_{unbound}^{P-P}) + (V_{bound}^{P-L} - V_{unbound}^{P-L} + \Delta S_{conf}) \qquad \text{Eq. 1}$$

In the ΔG_{Bind} equation a symbol (V) is referring to a pair-wise atomic for bound and unbound of ligand (L) and protein (p) and complex of protein-ligand, and in the equation, there is also an estimation of the torsional entropy loss after the binding (Morris *et al.*).

The pair-wise energetic was estimated as the equation model (2).

$$V = W_{vdw} * \sum_{ij} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} \right) + W_{Hb} * \sum_{ij} E(t) \left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}} \right) + W_{elec} *$$

$$\sum_{ij} \frac{q_i * q_j}{\varepsilon(r_{ij}) * r_{ij}} + W_{solv} * \sum_{ij} (S_i V_j + S_j V_i) e^{-r_{ij}^2 / 2\sigma^2} \hspace{2cm} \text{Eq. 2}$$

In Equation 2, the pair-wise energy is a summation of four estimation models. Starting from Lennard-Jones 12–6 potential for dispersal/repulsion interaction with hydrogen bond interaction based on Lennard-Jones 10/12 potential equation, followed by the electrostatic interaction equation that evaluates based on coulomb potential, and finally potential solvation equation (Bitencourt-Ferreira *et al.*, 2018).

The torsional entropy loss after the binding occurred between ligand and the protein is estimated from Equation 3 (Bitencourt-Ferreira *et al.*, 2018).

$$\Delta S_{conf} = W_{conf} * N_{tors} \hspace{3cm} \text{Eq. 3}$$

Where, N_{tors} is the number of rotatable bonds in the ligand.

1.6.2 Molecular Dynamics (MD) Simulations

Molecular dynamics (MD) simulation is a computational tool to simulate and estimate the motions with the orientation of the ligand inside the receptor pocket, protein stability, molecular recognition, protein folding, and structural determination. In the design of pharmaceutical drugs, MD is becoming an essential part of a routine in studying the kinetics and thermodynamic stability of the newly designed ligand with the receptor pocket (De Vivo *et al.*, 2016).

The basic concept behind MD simulations is to investigate the time-dependent performance of microscopic systems. This is achieved by determining the second-order equations described by Newton's second law, Equation 4.

$$f_i(t) = m_i * a_i(t) = -\frac{\partial * V(x(t))}{\partial * x_i(t)} \qquad \text{Eq. 4}$$

Where $f_i(t)$ is the force on the atom at a time t, m_i is the mass, and $a_i(t)$ is the acceleration of each atom in the system at given time t (Cavalli *et al.*, 2006).

MD utilizes molecular mechanics to illustrate the geometry model for the molecules with motions by classical or Newtonian mechanics. Generally, molecular mechanics is also known as a force field (FF). FF determines the strength of the potential energy based on calculating the intramolecular interactions, van der Waals and electrostatic interactions of the atoms using the general FF functional equation 5 (De Vivo *et al.*, 2016):

$$
\begin{aligned}
FF = \;& \sum_i^{Bonds} \frac{K_{l,i}}{2}(l_i - l_{0,i})^2 + \sum_i^{Angles} \frac{K_{a,i}}{2}(a_i - a_{0,i})^2 \\
& + \sum_i^{Torsions} \left\{ \sum_K^M \frac{V_{iK}}{2} \left[1 + \cos(n_{iK} * \theta_{iK} - \theta_{0,iK}) \right] \right\} \\
& + \sum_{i,j}^{Pairs} \varepsilon_{ij} \left[\left(\frac{r_{0,ij}}{r_{ij}} \right)^{12} - 2 * \left(\frac{r_{0,ij}}{r_{ij}} \right)^6 \right] \\
& + \sum_{ij} \frac{q_i * q_j}{4 * \pi * r_{ij} * \varepsilon_0 * \varepsilon_r} \qquad \text{Eq. 5}
\end{aligned}
$$

Where the first four terms refer to total energy intramolecular or local contributions (bond stretching, angle bending, and dihedral and unsuitable torsions), while the last two terms define the interactions between the repulsive and Van der Waals (in this situation, 12-6 Lennard-Jones potential) and the Coulombic interactions (González, 2011).

1.6.3 Free Binding Energy Calculation by (MM-PBSA)

The MM-PBSA approach has become a widely adopted method in estimating the binding affinity of complexes due to its efficiency and high convergence criteria with experimental results (Wang *et al.*, 2018). In this study, Amber MM-PBSA was used to calculate the average free binding energies between FRα and ligands, with the expression of free binding energy as follows in equation 6 (Miller III *et al.*, 2012):

$$\Delta G^{MM-PBSA} = \Delta G^{vacu} + \left(\Delta G^{solv}_{PL} - \Delta G^{solv}_{P} - \Delta G^{solv}_{L} \right) \qquad \textbf{Eq.6}$$

Where, ΔG^{vacu} is the vacuum binding energy,

ΔG^{solv}_{PL} is the solvation of protein-ligand complex free energies,

ΔG^{solv}_{P} and ΔG^{solv}_{L} are the solvation of free energy for free protein and free ligand, respectively.

ΔG^{vacu} can be calculated after stripping out explicit solvent molecules from the group(s), as the following equation 7 (Amzel, 1997; Hou *et al.*, 2010):

$$\Delta G^{vacu} = V_{PL} - V_P - V_L - T\Delta S_{solute} \qquad \textbf{Eq.7}$$

Where, V_{PL}, V_P, V_L are the average Boltzmann potential energy for the complex, protein, and ligand, respectively. ΔS_{solute} is the change in solute entropy upon binding and can be estimated from standard statistical thermodynamics models or alternative models.

In equation 8, the solvation free energy (ΔG_{solv}) is computed by the Poisson-Boltzmann Surface Area tacit solvent model, which is divided into two components (Genheden *et al.*, 2015):

$$\Delta G_{solv} = \left(\Delta G_{PB}^{elec} + \Delta G_{SASA}^{hydr} \right) \qquad \textbf{Eq.8}$$

Where, ΔG_{PB}^{elec} is the contribution of electrostatic to the solvation-free energy which is calculated by Poisson-Boltzmann method and consider polar electrostatic energy. ΔG_{SASA}^{hydr} is a theoretical term of the hydrophobic contribution that is linearly dependent on used non-polar electrostatic solvent accessible surface area (SASA) model.

The ΔG_{SASA}^{hydr} term can be written as in the following equation 9:

$$\Delta G_{SASA}^{hydr} = \gamma \times SASA + b \qquad \textbf{Eq.9}$$

where, γ is a coefficient related to surface tension of the solvent (Hou *et al.*, 2010), b is fitting parameter.

1.7 Cancer Cell Line Cytotoxicity Prediction

In-silico cytotoxicity prediction is now trending in the world of biological and pharmaceutical researches. It has many advantages through saving efforts of money and time. This trend leads to the establishment of many servers and databases that are specialized in cytotoxicity research. Using these servers, one is able now to test the

effectiveness of any novel compound on a large set of cancer cell lines. One of the recently reported web-services is Cell Line Cytotoxicity Predictor (CLC-Pred) (http://www.way2drug.com/Cell-line/) (Lagunin *et al.*, 2018). This service is based on PASS (Prediction of Activity Spectra for Substances) technology and the training set created based on data on cytotoxicity retrieved from ChEMBL database. In this study, MTX and all the fifty designed FA derivatives were tested their cytotoxicity through CLC-Pred against several cancer cell lines as D54 (brain), YAPC (pancreas), MANCA (lymphoid tissue), MDA-MB-453 (breast), MOLT-4 (blood), 786-0 (kidney), DMS-114 (lung), SJSA-1 (bone), IGROV-1 (ovarian), and SK-MEL-1 (skin) cell lines.

1.8 Aims and Objectives

The major aim of this study is to design new FA-derived analogues with higher binding affinity and stronger interactions towards FRα than FA through the following objectives:

Objective 1: To design new FA-derived analogues and measure the binding affinity of the sketched FA-analogues to FRα.

Objective 2: To predict the cytotoxic activity of the new fifty FA-analogues towards several cancer cell lines against MTX as control.

Objective 3: To calculate the time-dependent behaviour of the molecular systems of the complexes of FA, MTX, and the FA- analogue with the most negative free energy of binding.

CHAPTER 2

MATERIALS AND METHODS

2.1   Overview

There are many FA analogues reported in the literature. Each of them has different binding affinity toward folate alpha receptors possibly due to the difference of the attached functional group and the stereochemistry. These factors play a major role in the bond's formation, such as irreversible covalent bonds and reversible non-covalent bonds (ionic bond, ion-dipole bond, H-bond, hydrophobic interaction).

In structure based drug design (SBDD) where molecular modeling is heavily employed, one must know more about the binding site of the protein and the characteristic of the amino acids that make up the pocket, to be able to make the most reliable prediction of the interactions and fitting inside the pocket. Once pocket is identified, virtual screening can be carried out using molecular docking techniques to filter compounds with the best binding affinity to the receptor. The filtered compounds that have the best affinity can be selected for molecular dynamics study where more detailed binding interactions between the ligand and the receptor can be investigated. Finally, compounds that meet the selection criteria can be tested experimentally using *in vitro* and *in vivo* assays.

In this study, process flow is demonstrated in Figure 2.1 and the detailed methodology is described below.

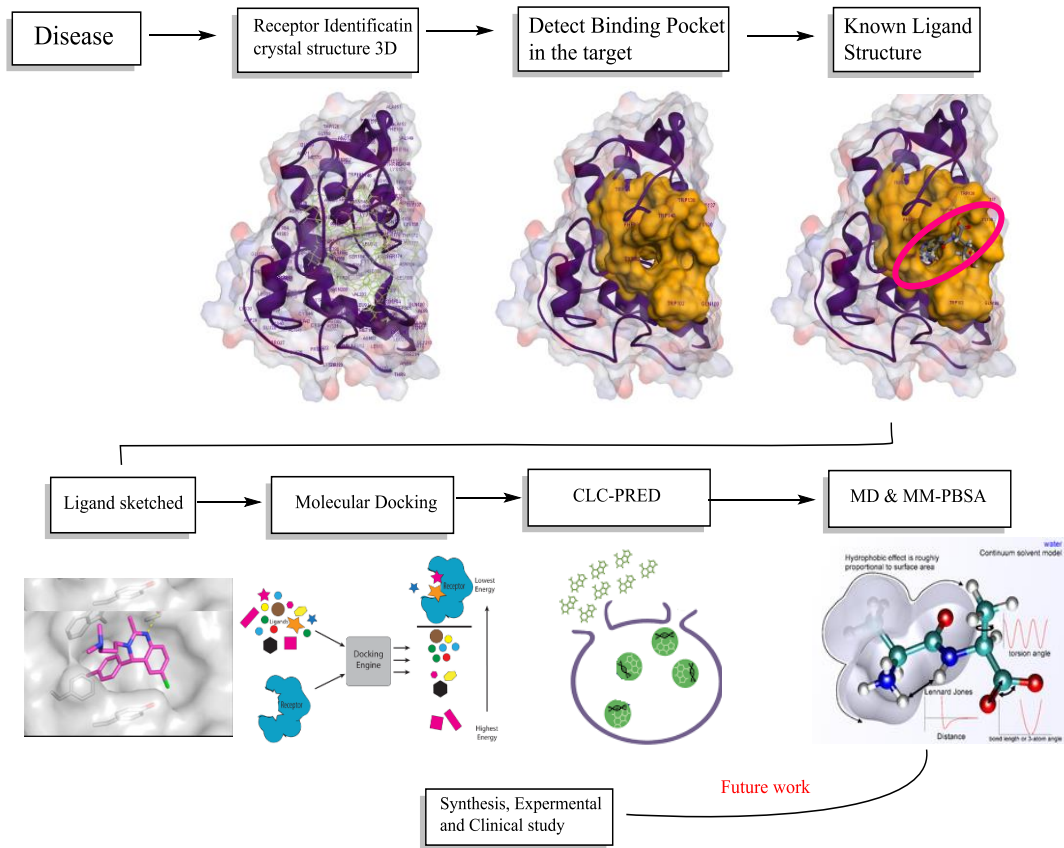**Flow process of Methodology in this study**

Figure 2.1: Summary of the study methodology flow *in silico* to reach for the promising lead compound.