DERIVATIZATION AND CONJUGATION OF FOLIC ACID TO IMPROVE ITS AFFINITY TOWARDS FOLATE RECEPTOR ALPHA ON CANCEROUS MEMBRANE BILAYER: AN *IN SILICO* INSIGHT

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by

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
Å	Angstrom
h	Hour
mL	Milliliter
nm	Nanometer
μL	Microliter
μM	Micromolar
α	Alpha
β	Beta
γ	Gamma
δ	Delta
g	Gram
mol	mole
K	Kelvin
eV	Electron Volt

LIST OF ABBREVIATIONS

2D	Two-Dimensional
AMBER	Assisted Model Building with Energy Refinement
CADD	Computer-Aided Drug Design
CHARMM	Chemistry At Harvard Macromolecular Mechanics
MD	Molecular Dynamics
NAMD	Nanoscale Molecular Dynamics
NMR	Nuclear Magnetic Resonance
NPT	Constant Number of Particle (N), Pressure (P) and Temperature (T)
NVT	Constant Number of Particle (N), Volume (V) and Temperature (T)
OPLS	Optimized Potential for Liquid Simulations
PDB	Protein Data Bank
QM/MM	Quantum Mechanics/Molecular Mechanics
RMSD	Root-Mean-Square Deviation
RMSF	Root-Mean-Square Fluctuation
REUS	Replica Exchange Umbrella Sampling
HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Unoccupied Molecular Orbital

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DERIVATISASI DAN KONJUGASI ASID FOLIK UNTUK MENINGKATKAN AFINITI TERHADAP RESEPTOR FOLAT ALFA KE ATAS MEMBRAN KANSER DWI LAPISAN: PERSPEKTIF *IN SILICO*

ABSTRAK

Penyasarasn ke atas sel kanser dengan kesan sampingan yang minimal masih menjadi cabaran yang signifikan dalam pembangunan ubat kemoterapi. Kajian ini memperkenalkan pendekatan *in silico* yang baru untuk menyasar penghantaran ubat kemoterapi kepada sel kanser menggunakan asid folik (FA) yang diikattkan dengan β-siklodekstrin (βCD), bertujuan meningkatkan selektiviti melalui interaksi dengan reseptor folat alfa (FRα). Dalam kajian *in silico* sebelum ini, peningkatan sasaran telah ditunjukkan oleh deriviatif asid folik tetrazole (FOL03) dan benzothiophene (FOL08), dengan FOL03 menunjukkan interaksi yang lebih kuat terhadap FRα. Dalam kajian ini, pendokkan molekul dan simulasi dinamik molekul (MD) telah digunakan untuk menilai impak βCD terhadap kekuatan pengikatan dan kestabilan konjugat FA, FOL03, dan FOL08 dengan FRα. Tambahan pula, pensampelan payung pertukaran replika (REUS) digunakan untuk mengkaji pengekalan konjugat dan agen kemoterapi 5-fluorouracil (FLU) di dalam rongga hidrofobik βCD dalam keadaan kanser yang disimulasikan secara maya bersama persekitaran dwilapisan lipid. Dalam sistem bukan inklusi, skor penydokkan menunjukkan bahawa FRα-FOL08-βCD menunjukkan ikatan yang lebih kuat (-17.10 kcal/mol) berbanding dengan -FA-βCD (-15.20 kcal/mol) dan -FOL03-βCD (-15.50 kcal/mol). Semua ligan terikat dalam pose yang hampir sama di dalam tapak aktif FRα. Simulasi MD selama lebih 100 ns mempamerkan kompleks FRα-FOL03-βCD mengekalkan kestabilan dinamik, menunjukkan perubahan konformasi yang minimal. Analisis mekanikal kuantum,

menggunakan MOPAC-2016 dan kaedah PM7, mengukuhkan dapatan simulasi MD, mengenal pasti sifat elektronik yang memberikan kestabilan kepada kompleks FRα-FOL03-βCD. Kompleks ini juga menunjukkan interaksi yang paling kuat dan konsisten dengan FRα, seperti yang didedahkan oleh perhitungan MM-PBSA. Energi pengikatan bebas (MM-PBSA), MM-PBSA per residu, dan ikatan hidrogen menunjukkan bahawa FOL03-βCD mempunyai interaksi yang lebih konsisten dan kuat serta tenaga pengikatan residu individu yang lebih baik berbanding dengan FA-βCD. Walau bagaimanapun, FOL08-βCD tidak dapat kekal di dalam poket pengikatan dan meninggalkan tapak tersebut akibat interaksi elektrostatik yang terjejas dan pengikatan hidrogen yang lemah, terutamanya dengan ASP81. Selanjutnya, analisis isipadu dan geometri (bentuk) tapak pengikatan FRα menyarankan bahawa kelenturan poket pengikatan FRα, membolehkan ligan mengambil orientasi yang berbeza. Struktur FA yang memanjang, iaitu FA yang dikaitkan dengan cincin heterosiklik geometri kecil, muncul sebagai reka bentuk inovatif yang berpotensi untuk penghantaran ubat yang ditargetkan. Untuk menyiasat keupayaan FA-βCD dan FOL03-βCD mengekalkan FLU dalam rongga mereka, kompleks inklusi telah dikaji. Skor penyambungan mendedahkan interaksi spontan FLU dengan tapak pengikatan yang cetek dalam kedua-dua kompleks, menunjukkan geometri βCD yang sebanding. Simulasi REUS lebih daripada 350 ns menggambarkan pengikatan βCD FLU yang stabil melalui ikatan hidrogen. Simulasi REUS lanjutan dengan FRα menunjukkan βCD mengekalkan FLU dengan berkesan sepanjang simulasi 350 ns. Walaupun begitu, walaupun dengan tenaga pengikatan yang sebanding kepada FRα. Penemuan ini dapat memberikan dasar yang kuat untuk penyelidikan masa depan dalam penghantaran ubat dan terapi yang ditargetkan.

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ABSTRACT

Effective targeting of cancerous cells with minimal side effects remains a significant challenge in the development of chemotherapeutic drugs. This study presents a novel in silico approach for targeting chemotherapeutic drug delivery to cancer cells using folic acid (FA) conjugated to β-cyclodextrin (βCD), aimed at enhancing selectivity via the interaction with folate receptor alpha (FRα). In a previous *in silico* study, enhanced targeting was demonstrated by tetrazole (FOL03) and benzothiophene (FOL08) folic acid derivatives, with FOL03 exhibiting superior affinity for FRα. In this study, molecular docking and molecular dynamics (MD) simulations was employed to assess the impact of βCD on the binding affinity and stability of FA, FOL03, and FOL08 conjugates with FRα. Additionally, replica exchange umbrella sampling (REUS) was used to examine the retention of conjugates and the chemotherapeutic agent 5-fluorouracil (FLU) within βCD's hydrophobic cavity under virtual simulated cancerous conditions which also include the lipid bilayer environment. In the non-inclusion systems, the docking scores show that FRα-FOL08-βCD exhibited stronger binding (-17.10 kcal/mol) than -FA-βCD (-15.20 kcal/mol) and -FOL03-βCD (-15.50 kcal/mol). All ligands bound in a similar pose within the active site of FRα. MD simulations over 100 ns demonstrated that the FRα-FOL03-βCD complex maintained dynamic stability, showing minimal conformational changes. Quantum mechanical analysis, using MOPAC-2016 and the PM7 method, corroborated the MD simulations, identifying the electronic properties that confer

stability to the FRα-FOL03-βCD complex. This complex also showed the strongest and most consistent interactions with FRα, as revealed by MM-PBSA calculations. The binding free energy (MM-PBSA), per residue MM-PBSA, and hydrogen bonds revealed that FOL03-βCD had stronger consistent interactions and more favourable individual residue binding energies than FA-βCD. However, FOL08-βCD was unable to remain within the binding pocket and departed from the site due to impaired electrostatic interactions and weak hydrogen bonding, particularly with ASP81. Moreover, the volume and geometry (shape) of the FRα binding site analysis suggest that the flexibility of FRα's binding pocket, enabling the ligands to adopt different orientations. The elongated FA structure i.e., FA conjugated to small geometry heterocyclic rings, emerges as a potential innovative design for targeted drug delivery. To investigate the ability of FA-βCD and FOL03-βCD to retain FLU within their cavity, inclusion complexes were studied. Docking scores revealed spontaneous interaction of FLU with shallow binding sites of both complexes, suggesting comparable βCD geometry. REUS simulation over 350 ns depicted FLU's stable βCD binding via hydrogen bonding. Further REUS simulations with FRα demonstrated βCD effectively retained FLU throughout the 350 ns simulation. However, despite comparable binding energies to FRα, FOL03 conjugation did not significantly enhance FLU retention compared to FA-βCD. These results inform the design of ligand-receptor interactions for drug delivery, providing a foundation for future research into targeted therapies.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer is one of the most dangerous and prevalent diseases that can attack any part or organ in the body (Doll *et al.*, 1981; Bray *et al.*, 2018; Yadav *et al.*, 2020). The main treatment modality of the disease is chemotherapy, which is occasionally associated with its low selectivity, leading to drug resistance and dose-limiting toxicities that could hamper the success of such treatment (Gatenby, 2009; Abdulbaqi *et al.*, 2021). These issues can be addressed by utilising a targeted drug delivery system (TDDS) which can transport drugs selectively to their site of action (Dai *et al.*, 2016). One potential receptor target for drug selective internalisation is folate receptor alpha (FRα), which is overexpressed on the surfaces of epithelial cancer cells, especially in lung, breast, kidney, and ovarian cancer (Campbell *et al.*, 1991; Chen *et al.*, 2013).

FRα is a glycosylphosphatidylinositol (GPI)-anchored protein that binds folic acid and its derivatives, specifically FOL03 and FOL08, which are reported to have a high affinity towards the FRα (Oconnor *et al.*, 2021; Al-Thiabat *et al.*, 2021). The FRα is highly expressed during the advanced stages of numerous cancers to meet the folic acid (FA) requirements of rapid cell division, as a result of low-folate concentration conditions (Jansen *et al.*, 1989; Chung-Tsen *et al.*, 1993; Brigle *et al.*, 1994). Thus, the development of a TDDS containing FA conjugated on drug carriers has been suggested as an attractive approach that can lead to a high level of cellular drug accumulation through folate-mediated endocytosis, leading to maximal chemotherapeutic efficacy with minimal side effects (Jiang *et al.*, 2008; Yin *et al.*, 2013).

One of the potential carriers that have been gaining interest to be conjugated with folic acid are cyclodextrins (CDs) (Tofzikovskaya *et al.*, 2015; Russel *et al.*, 2019; Hong *et al.*, 2021). CDs are cyclic oligosaccharides that are produced by the enzymatic degradation of starch (Caliceti *et al.*, 2003). These molecules possess an excellent ability to incorporate diverse guest molecules (Caliceti *et al.*, 2003). In this study, the guest molecule, 5-fluorouracil (FLU), interacts with the host molecule, β-Cyclodextrin (βCD), to form inclusion complexes. This interaction occurs within their hydrophobic cavities via noncovalent interactions (Caliceti *et al.*, 2003). The most common natural CDs are α-, β- and γ-CDs, consisting of six, seven, and eight glucose units, respectively (Yin *et al.*, 2013; Das *et al.*, 2020). Among the three, βCD has a moderate lumen size, high drug loading, low production cost, ability to increase lipophilic drug stability, and good toxicological properties, which makes it a suitable candidate as a drug carrier (Tofzikovskaya *et al.*, 2015). Further, it has been observed that conjugation with folic acid significantly enhances the stability of the β-cyclodextrin (βCD) unit. This stabilization is notably pronounced in the presence of the γ-carboxylic acid group located within the glutamate moiety of folic acid, demonstrating a superior stabilizing effect compared to conjugations with α- and γ-cyclodextrin (αCD and γCD) units (Yin *et al.*, 2013; Zhang *et al.*, 2013; Tofzikovskaya *et al.*, 2015).

A study by Hyun and co-workers reported that conjugation of FA to βCD has been shown to offer selective targeting of doxorubicin against breast cancer cells in a sustained manner (Hyun *et al.*, 2019). The selectivity of the delivery system helped to avoid systemic toxicity and cardiotoxicity related to the drug, when tested *in vivo* (Hyun *et al.*, 2019). The FA-appended βCD has also shown its selectivity towards

FRα-positive cell line compared to FRα-negative cell line (Okamatsu *et al.*, 2013). Various studies showed that conjugation of the two molecules can be done with linkers (Giglio *et al.*, 2015; Russel *et al.*, 2019) without any linker (Yin *et al.*, 2013; Tofzikovskaya *et al.*, 2015). These studies suggested the feasibility of utilising the system as a selective chemotherapeutic drug carrier.

Ligand-conjugated cyclodextrin (CD) development and investigation are predominantly conducted through trial-and-error approaches in experimental labs, a process that proves to be both time-consuming and costly (Wang *et al.*, 2015; Bonam *et al.*, 2017). The advent of bioinformatics offers a critical advantage, providing computational tools and models that can predict the behaviour and interactions of these complexes with high accuracy (Koukos *et al.*, 2020). This integration of computational predictions with experimental validation significantly enhances the efficiency of the research process, enabling more effective hypothesis testing and optimization of conditions before proceeding with costly and labour-intensive wet lab experiments (Wang *et al.*, 2015; Lai *et al.*, 2017). By leveraging bioinformatics, researchers can identify the most promising ligand-CD conjugates with greater precision, thereby minimizing the reliance on trial-and-error methodologies and expediting the pace of development in drug delivery systems. Yin and co-workers (Yin *et al.*, 2013), for example, investigated the potential of a novel drug delivery system, consisting of folic acid-conjugated CD carriers for the delivery of adamantane (Ada) and doxorubicin (DOX). This FACD–Ada–DOX system was predicted *in silico* by molecular docking. The prediction was validated in an *in vitro* assay where the cellular uptake of these nanoparticles was eight-fold higher in comparison to conventional systems in FR-positive tumour cells via endocytosis (Yin *et al.*, 2013). In another study, docking was

used to predict the conformation of a POH/β-CD inclusion complex. The most stable predicted structure with 1:1 molar ratio, was selected for formulation as well as in *in vitro* and *in vivo* studies (Rezende *et al.*, 2021).

The utilization of computational methods has been proposed as an effective strategy to expedite the development of folic acid-conjugated drug carriers and enhance the efficiency of loaded chemotherapy (Tagde *et al.*, 2020; Marverti *et al.*, 2021). However, the impact of βCD on the affinity and stability of FA conjugated within the active binding site of FRα, assessed through molecular modeling approaches, remains unexplored. This applies both in its guest-free state (where βCD does not contain any guest molecules) and its loaded state (characterized by βCD's inclusion of guest molecules). This knowledge gap has motivated the undertaking of this investigation in the current study.

1.2 Problem Statement

Chemotherapy is pivotal in treating, but its toxicity and the side effects of many anticancer drugs pose significant challenges. Since selectivity is the key to destroying cancerous cells and reducing the undesirable effects of chemotherapeutic drugs, innovative approaches are needed to improve targeted drug delivery. Folic acid (FA) conjugated to β-cyclodextrin (βCD) has emerged as a novel strategy to enhance selectivity by targeting drug delivery towards folate receptor alpha (FRα), thereby improving the bioavailability, biosafety, and loading capacity of the chemotherapeutic agent.

However, despite their potential advantages in delivering anticancer agents and reducing harm to healthy cells, the synthesis and deployment of folic acid-conjugated delivery systems are complex, challenging, and costly. The development and incorporation of folic acid-conjugated agents require laborious chemical modifications, which are time-consuming and require expertise in synthetic chemistry. Furthermore, the availability and cost of folic acid-conjugated agents pose additional barriers to their practical implementation in cancer therapy.

Computational methods offer a cost-effective solution by exploring virtual folic acid-conjugated agents. These computational approaches allow for the screening and evaluation of a diverse range of virtual folic acid-conjugated agents, expanding the potential pool of candidates for targeted drug delivery. By leveraging molecular docking simulations and molecular dynamics simulations, the binding affinity and stability of these conjugated systems can be assessed, providing valuable insights into their potential efficacy in targeting FRα and delivering anticancer drugs selectively.

Previously, two folate derivatives, FOL03 (an FA analog designed by substituting the primary amine in the pteridine moiety with a tetrazole ring) and FOL08 (an FA analog designed by substituting the primary amine in the pteridine moiety with a benzothiophene ring), exhibited superior targeting towards FRα compared to FA as predicted *in silico* (Al-Thiabat, 2020; Al-Thiabat *et al.*, 2021), making them potential candidates for further exploration. This study, thus, aspires to delve deeper into the potential of folic acid conjugation and its alternative, providing more insight into the development of more selective and targeted drug delivery systems.

1.3 Objectives of Study

The primary aim of this research project is to investigate the influence of βCD on the affinity and stability of FA-conjugated and the folate derivatives (FOL03 and FOL08) conjugated βCD within the binding site of FRα. In addition, this study also aims to explore the retention ability of conjugated βCD, when loaded with 5-fluorouracil (FLU). These will be accomplished through the following objectives:

1. To evaluate the effect of β-cyclodextrin conjugation on the affinity of folic acid (FA) and its derivatives (FOL03 and FOL08) towards FRα in the guest-free state using classical molecular dynamics simulation (MD).

2. To analyze the stability, compactness, and dynamic behavior of FRα - (FA-, FOL03-, FOL08-βCD) MD systems and assess the conformational changes.

3. To delineate the average pocket shapes, volume, and flexibility of FRα systems when bound to the conjugated ligands (FA-, FOL03-, FOL08-βCD).

4. To apply advanced sampling (replica exchange umbrella sampling (REUS)) simulation technique in order to determine the binding affinity and explore the stability of ligand-βCD with guest molecules (FLU) in the absence of the protein (FRα).

5. To investigate the interaction of the ligand-βCD complex with guest molecules (FLU) in the presence of the FRα linked to the GPI-linker on cancerous cell surfaces, in order to gain insights into the conformational changes of the

protein-ligand complex and their influence on guest molecule retention using the REUS technique.

1.4 The Scope of Study

This study investigated the impact of βCD on the affinity and stability of FA-conjugated compounds and their derivatives (FOL03 and FOL08) (Figure 1.1) within the active binding site of FRα. In addition, it explored the ability of conjugated βCD, when loaded with 5-fluorouracil (FLU) (Figure 1.1), to effectively maintain its guest molecule. The study was divided into two major parts, as presented in Figure 1.2.

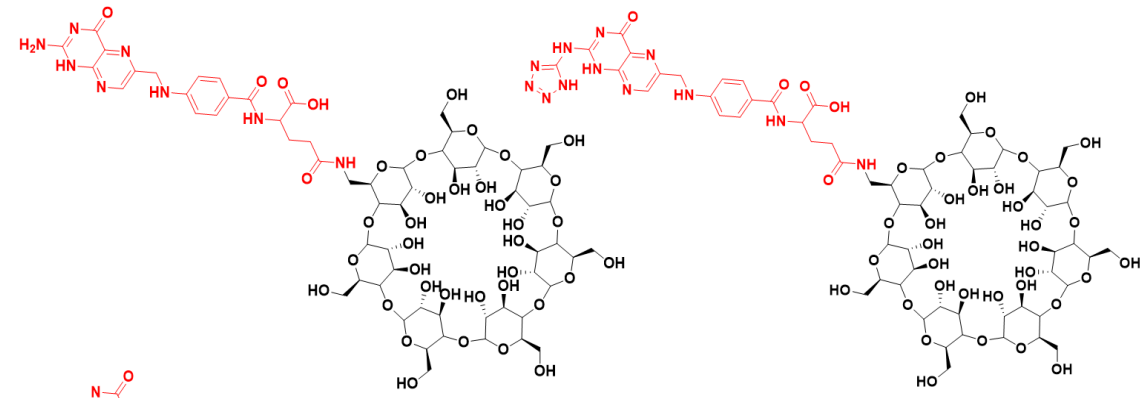
Part one focused on investigating the impact of βCD on the affinity and stability of FA-conjugated compounds and their derivatives (FOL03 and FOL08) in the guest-free state (non-inclusion complexes) using classical molecular dynamics simulations. This part aimed to provide comprehensive insights into the molecular interactions, stability, shape, and geometry of the binding site after complexes with the conjugated βCD.

Part two explored the ability of FA-conjugated compounds and their potential derivatives (identified as promising in part one) in the host-guest state to effectively retain their guest molecules. It took into consideration complex parameters such as the protein GPI-linker and temperature changes, especially within a cancerous cell environment (Knapp *et al.*, 2022). These factors could induce conformational changes in both the FRα protein and the conjugated ligands.

The inclusion of the membrane bilayer is pivotal for accurately simulating the cellular environment pertinent to FRα functionality (Gocheva *et al.*, 2019). The

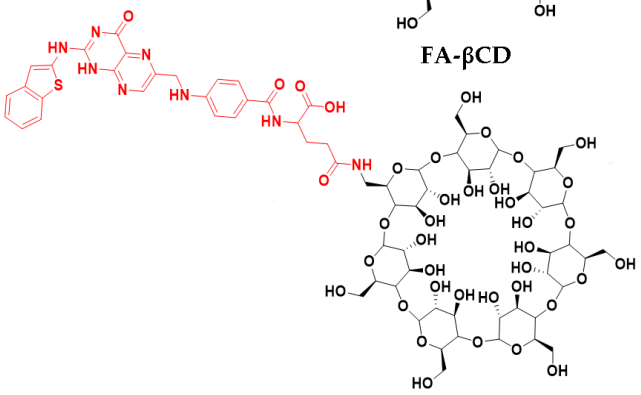
membrane bilayer mediates the accessibility and dynamics of ligand-receptor interactions, a critical aspect for receptors like FRα that are anchored through GPI-linkers (Gocheva *et al.*, 2019). This aspect of the study may enhance the understanding of the interactions between βCD-conjugated compounds and the cell membrane, offering insights into their navigational mechanisms towards the target receptor and providing a more comprehensive evaluation of their potential efficacy *in silico*.

Most importantly in this study, an attempt was made to apply Replica Exchange Umbrella Sampling (REUS), a sampling advanced simulation technique to gain insights into the ability of conjugated βCDs to retain their guest molecule in the absence and presence of FRα. The application of REUS has been applied for the first time to study protein-ligand interactions in a complex environment by our research group.

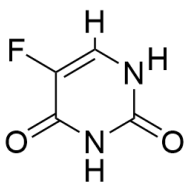


FA-βCD

FOL03-βCD



FOL08-βCD



5-fluorouracil

Figure 1.1 Illustration of the structures for FA-βCD, FOL03-Bcd, FOL08-βCD, and 5-fluorouracil (FLU). The folic acid (FA), FOL03, and FOL08 structures are in red, and the beta-cyclodextrin (βCD) chemical structure is in black colour.

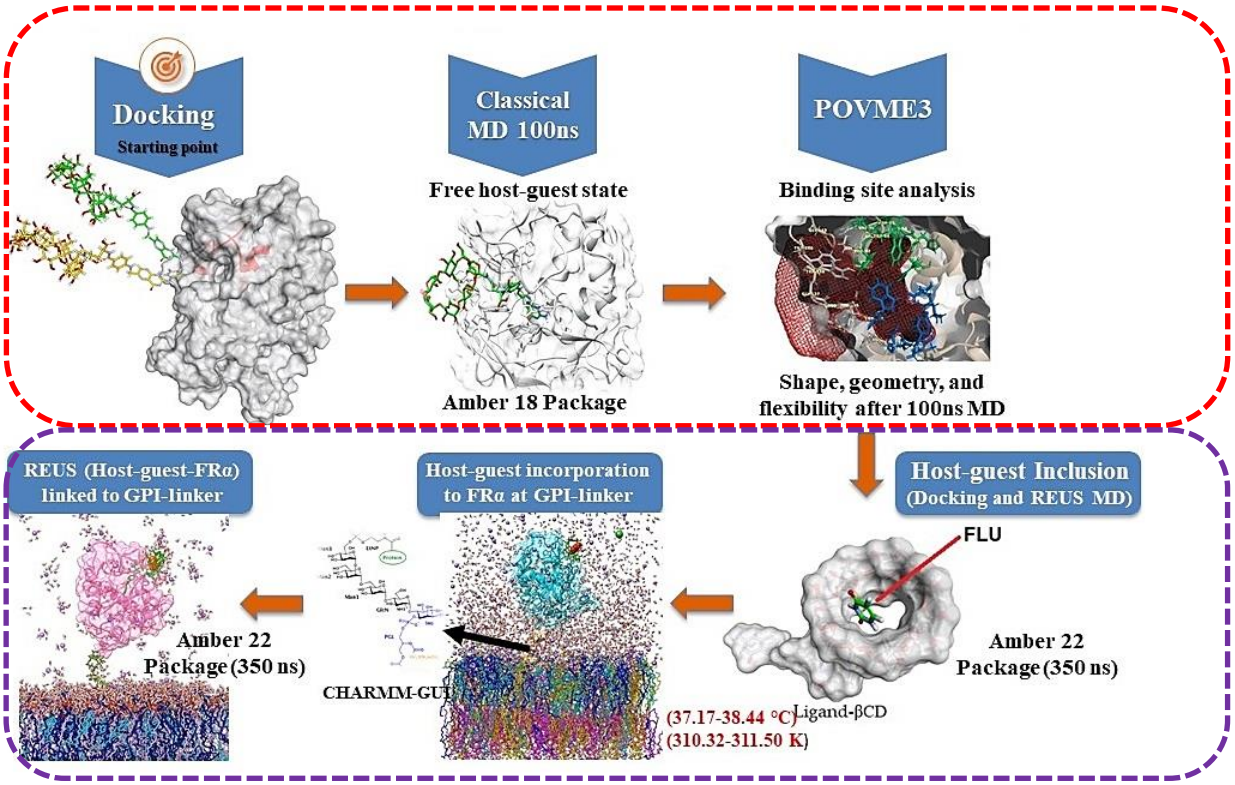


Figure 1.2 The general scheme for the flow of the research study. The red and purple dotted boxes indicate Part 1 and Part 2 of the study, respectively.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer Overview

Cancer is a disease characterized by the uncontrolled growth and spread of abnormal cells in the body (Doll *et al.*, 1981; Bray *et al.*, 2018; Yadav *et al.*, 2020). Its progression mechanism involves a multistep process that includes mutation and selection for cells with progressively increasing capacity for proliferation, survival, invasion, and metastasis (Valastyan *et al.*, 2011). The development of cancer is often explained using the three-stage theory of carcinogenesis, which divides cancer development into initiation, promotion, and progression stages (Barrett, 1993; Abel *et al.*, 2009). Initiation involves the mutation of a single cell, which then begins to proliferate abnormally (Vineis *et al.*, 2010). During the promotion stage, additional mutations occur, followed by selection for more rapidly growing cells (Balmain, 2020). In the progression stage, cancer cells invade normal tissues and may spread to other parts of the body (Arneth, 2019; Entenberg *et al.*, 2023).

Cancer progression is attributed to the accumulation of genetic alterations in cells that lead to the transformation of normal cells into cancerous cells (Preston-Martin *et al.*, 1990; Yokota, 2000; Takeshima *et al.*, 2019). Followed by tumor formation, growth, and metastasis (the spread of cancer to other parts of the body) (Preston-Martin *et al.*, 1990; Yokota, 2000; Takeshima *et al.*, 2019). This process is driven by a combination of genetic, epigenetic, and environmental factors that contribute to the acquisition of hallmark cancer traits, such as sustained proliferative signalling, resistance to cell death, angiogenesis, and tissue invasion (Preston-Martin *et al.*, 1990; Yokota, 2000; Takeshima *et al.*, 2019; Fane *et al.*, 2020; Lian *et al.*, 2020). Understanding the molecular mechanisms of cancer metastasis and the genes involved

in this process is crucial for developing effective treatments and prevention strategies (Yokota, 2000; Modugno *et al.*, 2012).

Despite advances in cancer detection, diagnosis, and treatment, the disease continues to progress, often due to the heterogeneous nature of tumours and the development of resistance to therapy (Dagogo-Jack *et al.*, 2018). One key aspect of cancer research is the identification and targeting of cancer cells, which has the potential to improve therapeutic outcomes and minimize side effects in patients (Kennedy *et al.*, 2011; Rahim *et al.*, 2021; Yang *et al.*, 2021).

2.2 Cancer Targeting and Folates

Cancer targeting is a rapidly expanding area of research that seeks to develop innovative strategies to deliver therapeutic agents selectively to cancer cells, thus avoiding the harmful side effects of systemic treatment on healthy tissues (Sharma *et al.*, 2010; Sherman *et al.*, 2010; Falese *et al.*, 2021). Various approaches have been explored, including monoclonal antibodies, small molecule inhibitors, RNA interference, and targeted drug delivery systems, such as liposomes and nanoparticles (Mehta *et al.*, 2019; Alzhrani *et al.*, 2020; Falese *et al.*, 2021). These strategies rely on the identification and exploitation of specific molecular markers or signalling pathways that are overexpressed or dysregulated in cancer cells, allowing for the selective targeting of malignant cells (Mehta *et al.*, 2019; Alzhrani *et al.*, 2020; Falese *et al.*, 2021).

Folate deficiency has been implicated in cancer progression by contributing to DNA damage, impaired DNA repair, and altered methylation patterns (Wang *et al.*,

2023). Folate, which is a type of B vitamin known as vitamin B9, is a general term encompassing folic acid (FA) and various oxidized forms of it such as tetrahydro folic acid (THF), dihydro folic acid (DHF), 5-formimino THF, 5,10-formyl THF, 5,10-methylene THF, and 5-methyl THF, that have metabolic activity (as shown in Figure 2.1) (Kim, 2004; Shulpekova *et al.*, 2021).

Folates could not be synthesized in mammalian cells and are delivered from exogenous sources are richest in folates such as mushrooms, spinach, yeast, green leaves, animal liver and kidney, and could be from supplementary vitamins (Strobbe *et al.*, 2018; Shulpekova *et al.*, 2021). These folates play a crucial role in one-carbon metabolism, which is necessary for DNA synthesis, repair, and methylation (Yang *et al.*, 2021). All tissues require one-carbon metabolism for growth and survival (Yang *et al.*, 2021). And its deficiency has been linked to an increased risk of developing different types of cancers, including breast, lung, and colorectal cancer (Duthie, 2011; Keshteli *et al.*, 2015).

The importance of folates in cancer progression has been widely studied (Van-Guelpen *et al.*, 2006; Yang *et al.*, 2021; Zarou *et al.*, 2021; Fatima *et al.*, 2022). Cancer cells present an increased demand for folates to support their rapid proliferation, leading to overexpression of folate receptors on their cell surface (Toffoli *et al.*, 1997; Fernández *et al.*, 2018; Scaranti *et al.*, 2020). This characteristic has been exploited in the development of targeted therapies, such as the use of folic acid-functionalized nanoparticles for drug delivery (Khattabi *et al.*, 2017; Mauro *et al.*, 2020; Rostami *et al.*, 2022). Moreover, modulation of folate metabolism has been employed as a

therapeutic strategy in cancer, with drugs such as methotrexate and antifolates gaining prominence (Taddia *et al.*, 2015).

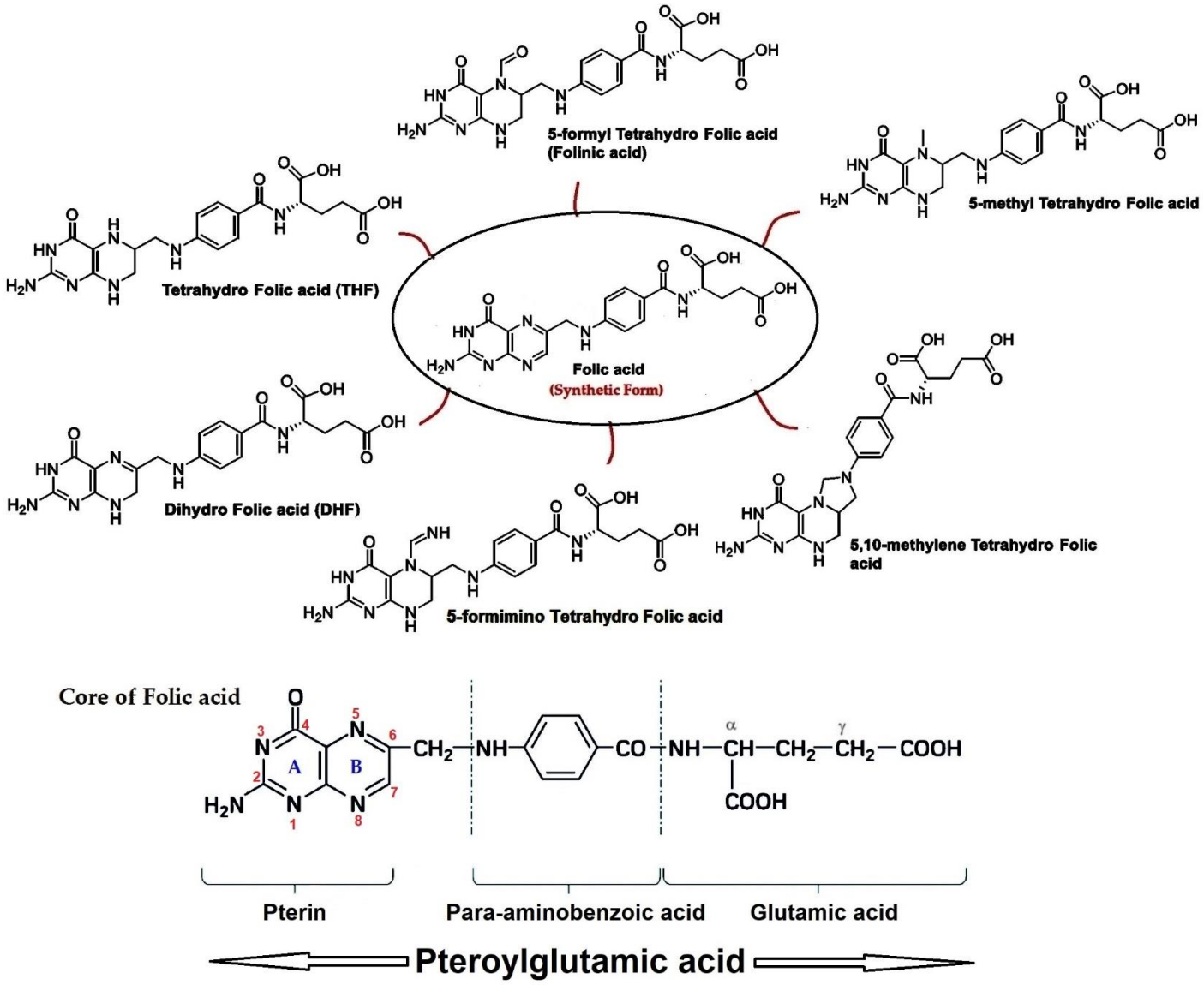


Figure 2.1 Physiologically important folates.

However, despite the advancements made in cancer targeting and folate deficiency research, significant challenges remain (Miele *et al.*, 2012; Rahim *et al.*, 2021). Resistance to targeted therapies is one of the significant issues faced in cancer

treatment (Holohan *et al.*, 2013; Aldea *et al.*, 2021). It mostly results from the emergence of compensatory pathways or the heterogeneity of cancer cells (Holohan *et al.*, 2013; Aldea *et al.*, 2021). To achieve more efficacious therapies, a deeper understanding of the interactions between cancer cells, the tumour microenvironment, and the host immune system is necessary (Mbeunkui *et al.*, 2009; Chew *et al.*, 2012; Mittal *et al.*, 2014).

2.2.1 Folic Acid (FA)

FA is a synthetic form of folate, commonly found in supplements and fortified foods (Fernández-Villa *et al.*, 2019). Its core structure consists of a heterocyclic pterin ring with a methyl group in the sixth position bound to para-aminobenzoic and glutamic acid (Figure 2.1) (Shulpekova *et al.*, 2021). This gives rise to the chemical name pteroylglutamic acid (Miller *et al.*, 2013; Mahendran *et al.*, 2018; Shulpekova *et al.*, 2021). The pterin ring is made up of pyrimidine and pyrazine rings with amino and keto groups in the second and fourth positions, respectively, providing aromatic heterocyclic structures that allow for reversible electron-accepting (Feirer *et al.*, 2017; Mahendran *et al.*, 2018; Shulpekova *et al.*, 2021).

FA is considered the parent structure of folates because it is fully oxidized and highly stable in the bloodstream (Shulpekova *et al.*, 2021). Inside cells, FA is converted to its active form, 5-methyltetrahydrofolate (5-MTHF), through a series of enzymatic reactions that occur primarily in the liver and other tissues (Verhaar *et al.*, 1998; Scaglione *et al.*, 2014). The conversion process involves the reduction of FA to dihydrofolate (DHF) by the dihydrofolate reductase (DHFR) enzyme, followed by the reduction of DHF to tetrahydrofolate (THF) by dihydrofolate reductase/thymidylate

synthase (DHFR-TS) enzymes, and finally, the methylation of THF to produce 5-MTHF by methylenetetrahydrofolate reductase (MTHFR) (Blancquaert *et al.*, 2010; Fernández-Villa *et al.*, 2019). Once converted to 5-MTHF, this active form of folate can be utilized in various metabolic pathways within the cell, such as DNA synthesis, repair, and methylation, as well protein synthesis and homocysteine metabolism (Verhaar *et al.*, 1998; Coşar *et al.*, 2014; Mahmood, 2014). In recent years, FA has gained increasing interest as a potential therapeutic agent in the treatment of cancer due to its unique cellular uptake in mammalian cells (Ebbing *et al.*, 2009; Cheung *et al.*, 2016; Newstead, 2022).

Cellular uptake is a crucial process whereby nutrients, ions, or signalling molecules are absorbed into cells, which is indispensable (absolutely necessary) for the proper functioning of mammalian cells (Zhao *et al.*, 2011; Augustine *et al.*, 2020). Intriguingly, research has shown that the uptake of FA in cancerous cells is significantly elevated compared to normal cells (Miller *et al.*, 2013; Bhunia *et al.*, 2016; Tagde *et al.*, 2020). This finding is particularly noteworthy, given that a multitude of potent anticancer drugs, referred to as antifolates, are structurally derived from FA (Hagner *et al.*, 2010; Gonen *et al.*, 2012). These antifolates such as methotrexate, edatrexate pemetrexed, raltitrexed, and pralatrexate accomplish their therapeutic objectives by inhibiting enzymes that play central roles in DNA synthesis, repair, and cellular replication – all of which are indispensable for cancer progression (Gonen *et al.*, 2012; Visentin *et al.*, 2012; Wróbel *et al.*, 2021).

In biological membranes, folates cannot be passively dragged across by passive diffusion due to their hydrophilicity and anionic structure at physiological pH (Dong

et al., 2014; Behzadi *et al.*, 2017; Rahim *et al.*, 2021). Consequently, mammalian cells have developed several sophisticated transporter systems for absorbing folic acid (Desmoulin *et al.*, 2012; Newstead, 2022). The uptake of folic acid into mammalian cells is mediated by three major distinct pathways: the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT), and folate receptors (FRs) (Elnakat *et al.*, 2004; Matherly *et al.*, 2007; Zhao *et al.*, 2007; Oconnor *et al.*, 2021). These systems are genetically distinct and functionally diverse, each playing a unique role in mediating folic acid uptake across epithelia and into systemic tissues (Oconnor *et al.*, 2021).

2.3 Folic Acid Cellular Uptake

Folate transporters, such as RFC, PCFT, and FR, play important roles in the cellular uptake and distribution of folate (Desmoulin *et al.*, 2012; Z. Hou *et al.*, 2014). These transporters have been studied for their potential as therapeutic targets in cancer, as their expression levels and activity can be altered in tumour cells (Elnakat *et al.*, 2004; Matherly *et al.*, 2007; Zhao *et al.*, 2007; Oconnor *et al.*, 2021). For example, FRα is overexpressed in various malignancies, including ovarian, lung, and breast cancers, making it an attractive target for cancer therapy (Fernández *et al.*, 2018; Scaranti *et al.*, 2020). Targeting folate transporters may not only enhance the delivery of anticancer agents to tumour cells but also provide insights into the role of folate metabolism in cancer progression and response to therapy (Zhao *et al.*, 2007; Cheung *et al.*, 2016; Wright *et al.*, 2022).

2.3.1 Reduced folate carrier (RFC)

Reduced folate carrier (RFC) is a protein that transports reduced folates, a type of vitamin B9, across cell membranes (Matherly *et al.*, 2007; Desmoulin *et al.*, 2012). It belongs to the major facilitator superfamily (MFS) of transporters (Hou *et al.*, 2014). MFS proteins transport an assortment of substrates including amino acids, neurotransmitters, sugars, vitamins, nucleosides, and organic phosphate in a uniport, symport, or antiport fashion (Chang *et al.*, 2004). RFC is expressed in a variety of tissues, including the liver, kidney, intestine, and brain (Au *et al.*, 1999; Shulpekova *et al.*, 2021). Also, it is expressed in cancer cells, where it plays a role in the uptake of folate and antifolate drugs (Au *et al.*, 1999; Shulpekova *et al.*, 2021). RFC is a target for cancer therapy, and inhibitors of RFC have been developed as potential new anti-cancer drugs (Au *et al.*, 1999; Shulpekova *et al.*, 2021). RFC is the major transport system for reduced folates in mammalian cells and tissues (Matherly *et al.*, 2007; Desmoulin *et al.*, 2012). Its physiologic substrate is 5-methyltetrahydrofolate (5-MTHF), the major circulating folate form (Matherly *et al.*, 2007; Desmoulin *et al.*, 2012). RFC has a much lower (~50- to 100-fold) affinity for FA than for reduced folates (Desmoulin *et al.*, 2012).

Transport by RFC is characterized by a neutral pH optimum and markedly decreased transport activity below pH 7 (Matherly *et al.*, 2007; Zhao *et al.*, 2007; Zhao *et al.*, 2009; Desmoulin *et al.*, 2012). RFC can transport classic antifolates, which are drugs that inhibit the function of folate, with high (micromolar) affinities (Desmoulin *et al.*, 2012). These antifolates include methotrexate (MTX), aminopterin (AMT), pemetrexed (PDX), raltitrexed (RTX), and pralatrexate (PMX) (Matherly *et al.*, 2007; Desmoulin *et al.*, 2012). While these analogues are also substrates for other folate

transporters like PCFT and FRα, the antifolate PT523 and the benzoquinazoline antifolate GW1843U89 are selective RFC substrates with no apparent transport activity for PCFT (Matherly *et al.*, 2007; Desmoulin *et al.*, 2010; Desmoulin *et al.*, 2012; Wang *et al.*, 2012).

2.3.2 Proton-coupled folate transporter (PCFT)

The proton-coupled folate transporter (PCFT), also known as solute carrier family 46 member 1 (SLC46A1), was first identified in 2006 (Qiu *et al.*, 2006). PCFT functions as a unidirectional symporter that transports folates with protons into the cells (Qiu *et al.*, 2006; Alam, 2020). For instance, in the upper small intestine, the concentration of protons is high due to the activity of the Na+/H+ exchanger (Zhao *et al.*, 2009). Thus, the high extracellular H+ concentration acts as the driving force for the PCFT symport into the cell (Qiu *et al.*, 2006). It functions optimally at pH 5.5 (Qiu *et al.*, 2006). The transporter activity decreases as the pH increases (Qiu *et al.*, 2006). Unlike RFC, PCFT has an equal affinity for both folic acid and reduced folates (Zhao *et al.*, 2009).

Similar to RFC, PCFT is a MFS with 459 amino acids transmembrane protein with a molecular mass of 55 kDa (Qiu *et al.*, 2006; Unal *et al.*, 2008). It has 12 transmembrane domains (TMDs) and both its N- and C-termini are in the cytosol (Qiu *et al.*, 2006). PCFT displays a 14% amino acid identity with RFC (Desmoulin *et al.*, 2012; Zhanjun *et al.*, 2012). To date, there is no available human X-ray crystal structure of PCFT in the protein data bank database (Westbrook *et al.*, 2003), and low-resolution (≥ 3.30 Å) crystal structures of RFC (PDB ID: 8DEP, 7TX6, and 7TX7) are available (Wright *et al.*, 2022), which impedes a structure-based drug design approach.

2.3.3 Folate Receptors (FRs)

Folate receptors (FRs) are a type of receptor known for its high availability in epithelial malignancy cells (Salazar *et al.*, 2007; Shulpekova *et al.*, 2021). It is a glycoprotein located on the surface of cells with a molecular weight ranging from 38–45 kDa and it is attached to membranes by a glycosylphosphatidylinositol (GPI) anchor (Yi, 2016; Oconnor *et al.*, 2021). FRs are characterized by high affinity for FA and 5-MTHF (Kd 1–10 nM), and lower to other folate derivates such as 5-formyl-THF (Kd 10–300 nM) (Mahmood, 2014; Yi, 2016).

Membrane-bound FRs are responsible for transporting ligands, such as FA and 5-MTHF, into cells via a process called receptor-mediated endocytosis (Hamid *et al.*, 2009; Desmoulin *et al.*, 2012). During this process, the ligands bind to the FRs located at the cell membrane, which then leads to invagination and the formation of cytoplasmic vesicles, also known as endosomes (Hamid *et al.*, 2009; Desmoulin *et al.*, 2012). The release of bound ligands occurs when the endosome's acidity increases, which promotes the dissociation of the ligand-FR complex (Kamen *et al.*, 1988; Hamid *et al.*, 2009; Desmoulin *et al.*, 2012). The ligand is then released from the endosome to the cytoplasm by either diffusion or a transport-mediated process that operates at acidic pH (Kamen *et al.*, 1988; Desmoulin *et al.*, 2012).

There are four human FR isoforms (FRα, FRβ, FRγ, and FRδ) (Spiegelstein *et al.*, 2000; Vergote *et al.*, 2015). FR isoforms are homologous, with 68–79% identical amino acid sequences and have N-glycosylation sites critical for their proper folding (Desmoulin *et al.*, 2012; Yi, 2016; Shulpekova *et al.*, 2021). FRα, FRβ, and FRδ are cell surface GPI-anchored glycoproteins (Desmoulin *et al.*, 2012; Yi, 2016; Fernández

et al., 2018), whereas FRγ is found only in hematopoietic cells (Mironava *et al.*, 2013) and lacks the GPI component, making it freely soluble (Ledermann *et al.*, 2015; Quici *et al.*, 2015; Fernández *et al.*, 2018).

Notwithstanding FRβ, FRγ, and FRδ's expression in some cancers, the FRα isoform is the most common isoform on the cancer cell surface (Kelley *et al.*, 2003; Cheung *et al.*, 2016; Yi, 2016; Mcord *et al.*, 2021). This isoform is widely expressed in cancers of epithelial tissues, such as lung, breast, kidney, and ovarian cancers (Yi, 2016; Scaranti *et al.*, 2020). The expression in these carcinomas is 100–300 times higher than in healthy cells, with 1–10 million receptor copies per cell (Vlahov *et al.*, 2012; Sun *et al.*, 2015; Fernández *et al.*, 2018). The increased expression of FRα during advanced stages of various cancers is needed to meet the folate requirements of rapid cell division under the effect of low-folate concentration conditions (Rizzo *et al.*, 2018).

FA possesses unique properties that distinguish it from other molecules. For example, it has a high affinity for FRα compared to RFC and PCFT, which has been confirmed in studies conducted by (Bailey *et al.*, 2009; Tagde *et al.*, 2020; Oconnor *et al.*, 2021). This property is retained even when a drug payload is attached to it to form a FA-conjugated system, as demonstrated by (Leamon *et al.*, 2009; Vlahov *et al.*, 2012; Yin *et al.*, 2013). In addition, FA is highly soluble in both organic and aqueous solvents, and it has a low molecular weight (441.4 g/mol), low immunogenicity, and low cost, which makes it a viable candidate for exploitation in cancer diagnostics and targeted drug delivery systems (TDDS) (Vlahov *et al.*, 2012; Liu *et al.*, 2018; Soe *et al.*, 2018; Zhang *et al.*, 2018; Tagde *et al.*, 2020; Fatima *et al.*, 2022).

2.4 Targeted Drug Delivery System (TDDS)

Various chemotherapy agents, such as paclitaxel (PTX), 5-fluorouracil (FLU), doxorubicin (DOX), idarubicin, 5-docetaxel (DTX), are commonly used to treat cancer patients (Spei *et al.*, 2019; Tagde *et al.*, 2020; Wright *et al.*, 2022). Although these agents can save lives, they may also cause severe side effects and systemic toxicity (Spei *et al.*, 2019; Tagde *et al.*, 2020; Mazayen *et al.*, 2022; Wright *et al.*, 2022). Because of their narrow margin of safety, high doses are often necessary to achieve maximum efficacy, and they cannot distinguish (poor targeting) between healthy and cancerous cells (Spei *et al.*, 2019; Tagde *et al.*, 2020; Mazayen *et al.*, 2022; Wright *et al.*, 2022). To address the limitations of conventional drug delivery methods, targeted drug delivery systems (TDDS) have emerged as a promising approach. These systems aim to deliver drugs directly to cancer cells, minimizing exposure to healthy cells and enhancing therapeutic outcomes (Greenwald *et al.*, 1999; Yin *et al.*, 2013; Li *et al.*, 2019; Tewabe *et al.*, 2021). One effective strategy involves conjugating folate (FA) to drug carriers, which has shown potential for achieving high levels of drug accumulation within cancer cells (Yin *et al.*, 2013; Dong *et al.*, 2014; Quici *et al.*, 2015; Tofzikovskaya *et al.*, 2015; Marverti *et al.*, 2021). By selectively targeting cancer cells, this approach maximizes the efficacy of chemotherapy while minimizing side effects (Yin *et al.*, 2013; Dong *et al.*, 2014; Quici *et al.*, 2015; Tofzikovskaya *et al.*, 2015; Marverti *et al.*, 2021).

2.4.1 FA-Conjugated Drug Carriers

FA-conjugated drug carriers have been suggested as an attractive approach in TDDSs due to their ability to enhance the delivery of therapeutic agents specifically to cancer cells that overexpress FRα (Yin *et al.*, 2013; Yi, 2016; Narmani *et al.*, 2019).

FRα is the only receptor that mediates the transport of FA conjugates designed for diagnostics and therapeutics via the receptor-mediated endocytosis process (Yin *et al.*, 2013; Yi, 2016; Narmani *et al.*, 2019). The high binding affinity of FRα for oxidized folates, such as FA, has led to the development of drug conjugates with FA as the targeting entity, offering several benefits: it is non-immunogenic, inexpensive, and readily available (Fernández *et al.*, 2018; Narmani *et al.*, 2019). FA-conjugated drug carriers, including liposomes, polymeric nanoparticles, protein toxins, and dendrimers, have demonstrated improved drug uptake in FR-expressing cancer cells, both *in vitro* and *in vivo* (Vasir *et al.*, 2005; Avval *et al.*, 2020; Jurczyk *et al.*, 2021). This targeted approach not only increases the therapeutic efficacy of anti-cancer agents but also reduces their toxicity to normal cells, making FA-conjugated drug carriers a promising strategy for advancing cancer treatment (Mcord *et al.*, 2021).

FA-conjugated drug carriers have demonstrated success in various cancer treatment studies. For instance, Dua et al. (2021) demonstrated the potential of folate receptor-targeted drug delivery systems (Dua *et al.*, 2021). Their study utilized FA-conjugated liposomes loaded with celastrol and irinotecan, showing greater drug uptake in folate receptor cells and targeted drug delivery in a mouse tumor model (Dua *et al.*, 2021). In another study, Patra et al. (2022) developed folate receptor-targeted and PEGylated poly(lactide-co-glycolide) nanoparticles containing genistein (GEN) for targeted delivery to ovarian cancer cells (Patra *et al.*, 2022). The NPs showed sustained release of GEN and increased cellular uptake in folate receptor-overexpressing ovarian cancer cells (Patra *et al.*, 2022). The GEN-containing PLGA-PEG-FA NPs showed superior anticancer activity than non-targeted PLGA and PLGA-PEG NPs, with an IC50 of 11.98 μg/ml (Patra *et al.*, 2022). The study suggested

that folate-targeted PLGA nanoparticles could be developed for potential target-specific delivery of GEN in the treatment of ovarian cancer (Patra *et al.*, 2022).

In addition to the promising results of Patra's et al. (2022) study, recent research by Parvathaneni et al. (2023) also aimed to develop a scalable drug delivery system for cancer therapy using FA-conjugated polymeric nanoparticles loaded with amodiaquine (FA-AQ NPs) (Parvathaneni *et al.*, 2023). In their study, FA was successfully conjugated with a poly(lactic-co-glycolic acid) (PLGA) polymer, and the resulting nanoparticles demonstrated uniform size distribution and spherical shapes under transmission electron microscopy (Parvathaneni *et al.*, 2023). Cellular uptake studies showed enhanced internalization of the nanoparticles in non-small cell lung, cervical, and breast cancer cells (Parvathaneni *et al.*, 2023). Cytotoxicity studies revealed the superior efficacy of FA-AQ NPs against various cancer cells, including MDAMB-231 and HeLA (Parvathaneni *et al.*, 2023). These findings suggested that FA-AQ NPs could serve as a promising drug delivery system for cancer therapy (Parvathaneni *et al.*, 2023).

Furthermore, previous research by Fan et al. (2019) aimed to develop a safe and effective therapy for liver cancer using multifunctional nanoparticulate systems (Fan *et al.*, 2019). They investigated the potential of doxorubicin (DOX)-loaded FA–polyethene glycol–β-cyclodextrin (FA–PEG–β-CD) nanoparticles (NPs) as a drug-delivery system for liver cancer therapy (Fan *et al.*, 2019). The researchers synthesized the FA–PEG–β-CD nanoparticles and loaded them with DOX (Fan *et al.*, 2019). They investigated the physicochemical characteristics of the nanoparticles, including size and drug-loading content, and tested their drug release and blood compatibility (Fan

et al., 2019). *In vitro* antitumor activity was evaluated using HepG2 cells (Fan *et al.*, 2019). The researchers found that the FA–PEG–β-CD NPs were effective in carrying the drug to the tumour tissues and releasing it effectively (Fan *et al.*, 2019). The DOX-loaded NPs did not induce blood haemolysis and had high drug encapsulation efficiency (95.2%) and drug-loading efficiency (11.9%) (Fan *et al.*, 2019). The researchers concluded that FA–PEG–β-CD/DOX NPs could be a potential platform for improving the treatment of liver cancer (Fan *et al.*, 2019). These stories highlight the potential of FA-conjugated drug carriers in targeted cancer therapy, paving the way for more effective and safer treatments. Several studies have highlighted the potential use of cyclodextrin as a drug carrier when conjugated with FA (Yin *et al.*, 2013; Tofzikovskaya *et al.*, 2015; Hong *et al.*, 2021). This is attributed to its excellent ability to form stable host-guest inclusion complexes with various chemotherapy drugs through noncovalent interactions (Zhang *et al.*, 2013; Giglio *et al.*, 2015; Ceborska *et al.*, 2018; Bakshi *et al.*, 2020).

2.4.2 Folic Acid conjugated cyclodextrins (FA-CD)

2.4.2(a) Cyclodextrins (CDs) Inclusion Complexes

One of the potential carriers that has been gaining interest in being conjugated with FA is cyclodextrins (CDs) (Yin *et al.*, 2013; Russel *et al.*, 2019; Hong *et al.*, 2021). CDs are cyclic oligomers of glucopyranosyl units linked by α-1,4 glycosidic bonds (Giglio *et al.*, 2015). α, β, and γ-CD are the three most common types of CDs, each with 6, 7, and 8 glucopyranose units, respectively, to create different-sized hydrophobic cavities (Figure 2.2) (Samuelsen *et al.*, 2020). CDs have truncated cone-like shapes, with the larger and smaller openings of the cone exposed to the solvent through secondary and primary hydroxyl groups respectively (Figure 2.2) (Giglio *et*