DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF 5-FLUOROURACIL LOADED GOLD NANOPARTICLES CONJUGATED WITH CD133 ANTIBODY TARGETING POSITIVE-SURVIVIN COLORECTAL CANCER STEM CELLS

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by

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2-D	two-dimensional
3-D	three-dimensional
5-FU	5-fluorouracil
β-ME	beta-mercaptoethanol
AIF	apoptosis-inducing factor
ALDH1	aldehyde dehydrogenase-1
AJCC	American Joint Committee on Cancer
AP	ammonium persulphate
Au	gold
AuNPs	gold nanoparticles
BCA	bicinchoninic acid
BE	barium enema
bFGF	human recombinant basic fibroblast growth factor
BIRC5	baculoviral inhibitor of apoptosis repeat-containing 5
BSA	bovine serum albumin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CH2THF	5,10-methylene tetrahydrofolate
CC	conventional colonoscopy
CD	cluster of differentiation
CPC	chromosomal passenger complex
CRC	colorectal cancer
CRCSCs	colorectal cancer stem cells
CSCs	cancer stem cells
CT	computed tomography
CTAB	cetyltrimethylammonium bromide
CTC	circulating tumor cells
DAPI	4′,6-diamidino-2-phenylindole
DDS	Drug delivery system
DLS	dynamic light scattering
DMARD	disease-modifying anti-rheumatic drugs
DMEM	Dulbecco Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic Acid
DOX	doxorubicin
DPD	dihydropyrimidine dehydrogenase
dUMP	deoxyuridine monophosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
EGF	epidermal growth factor
EpCAM	epithelial cell adhesion molecules
EPR effect	enhanced permeability and retention effect
ECM	extracellular-cell matrix
FBS	fetal bovine serum

FDA	Food and Drug Administration
FdUMP	fluorodeoxyuridine monophosphate
FFPE	formalin-fixed paraffin-embedded
FIT	fecal immunochemical test
FITC	fluorescein isothiocyanate
FOBT	fecal occult blood test
FR	folate receptor
FdUTP	fluorodeoxyuridine triphosphate
FUTP	fluorouridine triphosphate
H&E	hematoxylin and eosin
HCl	Hydrochloric acid
HNSCC	head and neck squamous cell carcinoma
HS-PEG-COOH	poly(ethylene glycol) 2-mercaptoethyl ether acetic acid
IAP	inhibitor of apoptosis protein
IL4	interleukin-4
Lgr5	leucine-rich repeat-containing G-protein-coupled receptor 5
M-NBI	magnifying narrow-band imaging
mAb	monoclonal antibody
MCE	magnifying chromoendoscopy
MNS	mononuclear phagocytic system
MRI	magnetic resonance imaging
MSNs	mesoporous silica nanoparticles
NES	nuclear exportation signal
NHS	N-hydroxysuccinimide
NPs	nanoparticles
NPV	negative predictive value
OCT4	Octamer-binding transcription factor 4
OPRT	orotate phosphoribosyl transferase
PAGE	Protein separation by polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PE	phycoerythrin
PEG	poly(ethylene glycol)
PFA	parafolmaldehyde
PLGA	poly(lactic-co-glycolic acid)
PLL	poly-L-lysine
PMSF	phenylmethanesulfonyl fluoride
PPV	positive predictive value
PVDF	polyvinylidene fluoride
PVP	poly (N-vinyl-2-pyrrolidone)
RES	reticuloendothelial system
rhTNF	recombinant human tumor necrosis factor
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SFM	serum free media
SOX2	Sex-determining region Y-box2
TBS	Tris-buffered saline

TEMED	N,N,N’N’-Tetra-methylethylenediamine
TGF-β	transforming growth factor beta
TS	thymidylate synthase
UTP	uridine triphosphate
XRD	X-ray diffraction
TEM	transmission electron microscopy
MTT	thiazolyl blue tetrazolium bromide
TNM	Tumor, Node and Metastasis
UV-Vis spectroscopy	ultraviolet-visible spectroscopy
XIAP	X-linked inhibitor of apoptosis

PEMBANGUNAN, PENCIRIAN DAN PENILAIAN TINDAKAN NANOZARAH EMAS BERMUATAN 5-FLUOROURASIL YANG DIKONJUGASI DENGAN ANTIBODI CD133 UNTUK PENYUTURAN KHUSUS SEL STEM KANSER KOLOREKTAL POSITIF-SURVIVIN

ABSTRAK

Dikenali sebagai penyebab kedua tertinggi kematian berkaitan kanser, kanser kolorektal (CRC) memberikan cabaran besar dalam rawatan, seperti ketahanan terhadap kemoterapi, keberulangan laku, dan kesan toksik yang tidak diingini. Strategi kemoterapi konvensional yang menyasarkan sel yang membahagi secara aktif tidak dapat membunuh sel stem kanser (CSCs) disebabkan oleh sifat kitaran CSCs yang perlahan, lalu mengakibatkan perulangan kanser dan metastasis. Penggunaan nanoteknologi dalam penghantaran ubat untuk menyasarkan CSCs secara khusus merupakan strategi yang sangat menjanjikan untuk meningkatkan keberkesanan rawatan sambil mengurangkan kesan sampingan. Kajian ini memberi tumpuan kepada sintesis nanopartikel emas (AuNPs) menggunakan kaedah Turkevich, yang kemudiannya telah diubah suai dengan polyethylene glycol, dan dikonjugatkan dengan antibodi monoklonal anti-CD133 (mAb), serta dimuatkan dengan 5-fluorourasil (5-FU) untuk membangunkan sistem pembawa nano yang disasarkan. Nanopartikel yang disintesis telah dicirikan menggunakan difraksi sinar-X (XRD), spektroskopi UV-vis, mikroskop elektron transmisi (TEM), dan penyebaran cahaya dinamik (DLS). Pendekatan proses fungsian dengan anti-CD133 mAb memudahkan pengambilan AuNPs secara khusus ke dalam sel HCT116 CRC, seperti yang ditunjukkan oleh mikroskopi pendar fluor, yang menunjukkan lokasi mereka di dalam kawasan nuklear sel. Kajian sitotoksisiti menunjukkan bahawa 5FU-AuNPs-CD133 mengurangkan

kebolehan sel ($p < 0.0001$) dan menghalang pembentukan kolonosfera yang berasal dari HCT116 berbanding dengan formulasi AuNPs yang tidak dikonjugatkan dengan CD133 mAb, yang menunjukkan perencatan tinggi bagi aktiviti menswapulihkan CRCSCs. Pemeriksaan Western blot menunjukkan penurunan dalam survivin, iaitu protein anti-apoptotik yang dikaitkan dengan kelangsungan hidup sel kanser dalam kolonosfera yang dirawat. Penemuan ini menekankan potensi AuNPs yang disasarkan kepada CD133 sebagai sistem pembawa nano yang berkesan untuk terapi CRC, terutamanya melalui penyasaran memilih CSCs dan meningkatkan penghantaran 5-FU. Kajian masa depan haruslah memberi tumpuan kepada penjelasan mekanisme asasi yang terlibat serta meningkatkan strategi formulasi untuk penambahbaikan jendela terapeutik dan pelaksanaan klinikal yang berjaya.

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ABSTRACT

Ranked second in cancer-related mortality, colorectal cancer (CRC) presents significant challenges in treatment, such as resistance to chemotherapy, recurrence, and adverse toxic effects. Conventional chemotherapeutic strategies that target actively dividing cells are unable to eradicate cancer stem cells (CSCs) due to the slow-cycling nature of CSCs, which results in disease recurrence and metastasis. The use of nanotechnology in drug delivery to specifically target CSCs presents a highly promising strategy to improve treatment effectiveness while reducing adverse effects. This study focused on the synthesis of gold nanoparticles (AuNPs) using the Turkevich method, subsequently modified with polyethylene glycol, conjugated with anti-CD133 monoclonal antibody (mAb), and loaded with 5-fluorouracil (5-FU) to develop a targeted nanocarrier system. The synthesized nanoparticles were characterized by X-ray diffraction (XRD), UV-vis spectroscopy, transmission electron microscopy (TEM), and dynamic light scattering (DLS). The approach of functionalization with anti-CD133 mAb facilitated the specific intracellular intake of AuNPs in HCT116 CRC cells, as demonstrated by fluorescence microscopy, which indicated their localization within the nuclear region of the cells. Cytotoxicity studies demonstrated that 5FU-AuNPs-CD133 significantly reduced cell viability ($p < 0.0001$) and hindered HCT116-derived colonospheres relative to non-targeted AuNP formulations, highlighting a strong inhibition of self-renewal activity in CRCSCs. Western blot

examination revealed a decrease in survivin, an anti-apoptotic protein associated with the survival of cancer cells, in the treated colonospheres. These findings highlight the potential of CD133-targeted AuNPs as an effective nanocarrier system for colorectal cancer therapy, particularly through the selective targeting of cancer stem cells and enhancing the delivery of 5-FU. Future studies should focus on elucidating the fundamental mechanisms involved and enhancing formulation strategies for the improvement of therapeutic window and successful clinical implementation.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer has become a remarkable public health problem worldwide. In United States (US), cancer is the second leading death after heart disease (Ahmad & Anderson, 2021; Siegel et al., 2023a). Colorectal cancer (CRC) is the third most common malignancy worldwide, represents 10.0% of all cancer cases and ranks second in terms of mortality (9.4% of the total cancer deaths) with over 1 million deaths in 2020 (Sung et al., 2021). In Malaysia, CRC was reported to be the second most diagnosed cancer in both males and females (Iyadorai et al., 2020). According to the National Cancer Registry-Colorectal Cancer 2008-2013, the overall incidence and mortality rate for CRC in Malaysia was 21.3 cases and 9.8 cases per 100,000 populations (Hassan et al., 2014). A retrospective study conducted between 2012 and 2016, reported that Malaysia's 5-year survival rate for CRC was 18.4%. This rate is significantly lower than that of other countries, including Thailand, Martinique, Singapore, the USA, and high-income countries, which had an overall survival rate of 67% (Nor Asiah Muhamad et al., 2023). The delayed detection of CRC is a major factor contributing to this low survival rate. Furthermore, the 5-year trend indicates rising incidence for both gender at the late-stage diagnosis with poor prognosis (Veettil et al., 2017; Ali & Manan, 2018). Previously, it was often believed that CRC is more prevalent among older individuals, as the risk of developing it increases with age. Nevertheless, the perspective is shifting because of a growing inclination among younger age cohorts (Syed Soffian et al., 2022). According to the age features of CRC

patients recorded in the cancer registry in Malaysia from 2003 to 2005, individuals under the age of 50 accounted for 14.6% of the cases (Veettil et al., 2017). This suggests that regular screening should be introduced and the recommended age for screening should be reduced to detect and treat CRC at an earlier stage.

The current standard treatment for CRC is by multimodal approaches including surgery, radiotherapy, and/or chemotherapy (Bhimani et al., 2022). While surgery is the most common treatment modalities in CRC management, it is noteworthy that different stages require different treatments. In fact, chemotherapy is commonly used as the first-line treatment for unresectable patients or as adjuvant therapy after surgery to prevent cancer recurrence. The most widely used chemotherapeutic agent was fluorouracil-based regimens. 5-fluorouracil (5-FU) is an anti-metabolite drug that is commonly used to treat various solid tumors particularly CRC (Xie et al., 2020). Since 5-FU is an analogue of uracil, a pyrimidine molecule of ribonucleic Acid (RNA), 5-FU binds to the thymidylate synthase and hence interfering deoxyribonucleic Acid (DNA) and RNA functions that lead to the cell death (Lind, 2020; Mafi et al., 2023).

Despite the effectiveness of 5-FU, several side effects after the treatment have been reported including diarrhea, vomiting, hair loss, peripheral neuropathy, rash, and hepatic injury (Negarandeh et al., 2020; Ozer et al., 2021). Furthermore, the development of drug resistance has limited the 5-FU effectiveness and reduced its response rate. Thus, current chemotherapy 5-FU regimens for CRC particularly in patients with metastatic CRC are usually combined with other anti-cancer drugs such as oxaliplatin (FOLFOX) and irinotecan (FOLFIRI) to improve patients' survival (Lee et al., 2020a). However, combinational treatments have been reported to introduce more toxicity rather than efficacy including hematologic toxicity (Boige et al., 2010; Lee et al., 2020a; Zhang et al., 2022). Additionally, due to non-specificity,

conventional chemotherapy regimens not only kill fast-growing tumor cells but also kill other fast-dividing healthy cells such as hair follicles and skin cells, leading to numerous side effects (Schirrmacher, 2017).

Moreover, the existence of a subpopulation of cancer stem cells (CSCs) within the bulk tumor has garnered significant interest among researchers because of their poorly differentiated characteristics and their dormancy ability to remain in the G_0 phase of the cell cycle. This unique feature allows them to escape the impacts of chemotherapy, which mainly focuses on rapidly dividing and fully developed cancer cells (Lee et al., 2020b; Xie et al., 2022). In colorectal cancer stem cells (CRCSCs), the elevated levels of CD133, a transmembrane glycoprotein, have been associated with progression of the tumor (Rezaee et al., 2021), the response to therapy (Ong et al., 2010), and the overall survival (Park et al., 2019).

To date, treating cancer has been an utmost importance and urgency. Although various pharmacological agents including synthetics and natural products have been investigated to treat cancer, targeted drug delivery by nanoparticles has become a new cornerstone of cancer therapy and generated considerable research interest. Owing to the advantageous properties of gold nanoparticles (AuNPs) such as biocompatibility, small sizes and the ability for surface functionalization, AuNPs have been engineered as nanocarriers for drug delivery system in pre-clinical and clinical settings (Jahangirian et al., 2019). Previously, Phase I and pharmacokinetic studies on AuNP-based product, Aurimune (CYT-6091), revealed that pegylated AuNPs carrying recombinant human tumor necrosis factor alpha (rhTNF) were localized in tumor tissue after 24 hours post-administration of CYT-6091 but not in the healthy tissue, indicating that they had selectively targeting tumor tissue to deliver their therapeutic payloads (Libutti et al., 2010). The aggregation of nano-sized particles at tumor sites

through the increased permeability and retention (EPR) effect, also referred to as passive targeting, was proposed by Matsumura and Maeda (1986) which opens a new wave for targeted drug delivery in tumor. However, to provide more selective and enhance chemotherapeutic effect, nanoparticles can also be designed for actively targeting tumor by incorporation of various ligand-directed targeting on nanoparticles. The incorporation of targeting ligands including antibodies, peptides and folic acid on nanoparticles surface have shown promising results stemming from the fact that cancer cells can be distinguished from healthy cells by several tumor-specific receptors (Rana et al., 2023). For instance, anti-CD133 antibody has previously been used for tumor-targeting of copolymer nanoparticles consisting of poly(ethylene glycol), PEG and poly(*ε*-caprolactone) to specifically target CRCSCs cell surface receptors in which the cytotoxicity effect of targeted nanocarriers was greater than nontargeted nanocarriers in overexpressed CD133 glycoprotein HCT116 CRCSCs (Ning et al., 2016).

1.2 Research problem statement

Despite recent advances in diagnostic tools and treatment modalities, the 5-year cumulative survival rate of CRC remains around 64 - 67% (Mattiuzzi et al., 2019). Meanwhile, 20 – 30% CRC patients with stage I-III relapse or develop cancer recurrence (Qaderi et al., 2021). Accumulating evidence shows that tumor recurrence is due to the development of drug resistance associated with the presence of cancer stem cells (CSCs) (Touil et al., 2013; Martins-Neves et al., 2016). It has been proposed that CSCs forms a small subpopulation within tumor bulk and characterized by resistance to conventional therapies as well as self-renewing capabilities to maintain tumor growth (Batlle & Clevers, 2017). CSCs population can be found in CRC (*i.e.* CRCSCs) and they have been associated with CRC recurrence (Zhou et al., 2018;

Jahanafrooz et al., 2019). Owing to the slow-cycling nature of CSCs, conventional treatment modalities involving chemotherapy targeting actively dividing cells fail to kill CSCs, leading to disease recurrence and metastases (Biserova et al., 2021). In CRC patients, CRCSCs represents only 0.3 – 2.2% of the bulk cancer population (Gupta et al., 2019). They are characterized by several protein markers such as cluster of differentiation (CD)-133, CD44, CD24, epithelial cell adhesion molecules (EpCAM), aldehyde dehydrogenase-1 (ALDH1), leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) and NANOG (Pádua et al., 2020; Kalantari et al., 2022; Pashirzad et al., 2022).

CD133 is a transmembrane glycoprotein, and its overexpression is associated with anticancer drug resistance and poor prognosis (Patsalias & Kozovska, 2021). It is also known as stem cell marker and involves in Wnt/β-catenin signalling pathway (Behrooz et al., 2018). Moreover, previous study reported that there was a positive correlation between CD133 expression and survivin expression, a member of the inhibitor of apoptosis family, linked to 5-FU resistance in cancer (Lee et al., 2015). Meanwhile, study by Gu et al. (2019) reported that overexpression of biomarker survivin was significantly associated with oxaliplatin plus 5-FU resistance in patients with advanced CRC. Besides, increased expression of the survivin protein has also been reported in $CD133^{+}$ CRCSCs in comparison to $CD133^{-}$ cells (Li et al., 2017). Numerous CRCSCs studies have shown that survivin is regulated by interleukin-4 (IL4) via STAT6 signaling pathway, promoting cancer cells to escape chemotherapeutic actions (Di Stefano et al., 2010; Kim et al., 2014; Gundamaraju et al., 2018). Thus, it is noteworthy that overexpression of CD133 and survivin may be a predictive biomarker for CRCSCs that could benefit oncologists in determining chemotherapy regimens or personalized treatments for advanced CRC patients.

Currently, there is a lack of research focusing on CD133 with the use of nanoparticles, especially AuNPs, for the selective inhibition of CRCSCs.

1.3 Justification of study

To date, CRC indeed is a global burden affecting both men and women equally with their increasing prevalence among individuals younger than 50 years (Venugopal & Stoffel, 2019; Chang et al., 2022). Despite the effectiveness of current treatments, chemotherapy-induced side effects, cancer recurrence and metastasis have hindered the efficacy of treatments. It is noteworthy that, side effects after chemotherapy regimen were widely contributed by the non-specific targeting of the chemotherapeutic agents. Therefore, it is crucial to design anti-cancer drug that is specifically targeting cancer cells while sparing healthy cells. In addition, accumulating evidences associate chemotherapy resistance and cancer recurrence with the presence of CSCs (Batlle & Clevers, 2017; Xu et al., 2022). Presently, there is significant focus in cancer research in targeting CRCSCs in order to eradicate all cancer cells. Nanoparticles have emerged as a promising means of delivering chemotherapeutic drugs directly to the targeted region through passive and active targeting. A recent study demonstrated promising results with functionalized AuNPs, indicating that the combination of folate receptor-α (FR) and Tyro3, a newly discovered tyrosine kinase receptor, on AuNPs resulted in an enhanced uptake of these nanoparticles in CRC cells that overexpress FR and Tyro3 receptors (Patel et al., 2021). However, to date, there is no exploitation study on CD133 antibody as a molecular target to accumulate 5-FU loaded AuNPs on CRC model. Additionally, it is unknown if the synthesized AuNPs may inhibit the stemness characteristics of CRC cells by suppressing the expression of anti-apoptosis protein, survivin. Hence, the goal

of this study was to implement nanotechnology principles to design a targeted therapy using 5-FU (a potent anti-metabolite) loaded in functionalized AuNPs that target the bulk HCT116 CRC and HCT116 colonospheres, reducing cell viability and stemness by lowering the anti-apoptosis survivin marker. The summary of the flow chart of the study were summarized in Figure 1.1.

1.4 Significance of study

This research may offer a unique targeted treatment for colorectal cancer patients, particularly those with $CD133^{+}$ CRCSCs that express the survivin marker. Furthermore, the results could play a significant role in enhancing nanoparticle-mediated drug delivery systems in cancer treatment, allowing for the precise targeting of tumor cells. As a result, the findings could assist oncologists in formulating enhanced treatment strategies for cases of drug-resistant colorectal cancer and possibilities of cancer recurrence.

1.5 Research questions

1. What is the procedure for synthesizing gold nanoparticles loaded with 5-FU and conjugated with CD133 monoclonal antibodies, and what are the physicochemical characteristics of the resulting gold nanoparticles?
2. Do the synthesized AuNPs exhibit cytotoxic effects on both cancer cell lines and normal cell lines, and are there any differences in localization between unconjugated AuNPs and those conjugated with CD133 mAb?
3. How to establish HCT116 colonospheres as a model for cancer-related stem cells, and does the pre-treatment of HCT116 cells with synthesized gold nanoparticles inhibit the stemness of these colonospheres?
4. What impact does the treatment with AuNPs have on the anti-apoptotic survivin marker in HCT116 colonospheres?

1.6 Hypothesis

5-fluorouracil loaded gold nanoparticles conjugated with CD133 antibody (5FU-AuNPs-CD133) markedly reduces the proliferation of human colorectal cancer HCT116-derived sphere-forming cells through the downregulation of survivin expression. 5FU-AuNPs-CD133 promotes targeted intracellular uptake and induces cellular apoptosis.

1.7 Research objective

1.7.1 General objective

To study the mechanism of gold nanoparticles loaded with 5-fluorouracil, conjugated with CD133 antibody, designed for targeting survivin-positive colorectal cancer stem cells.

1.7.2 Specific objectives

1. To synthesize and characterize 5-fluorouracil loaded gold nanoparticles conjugated with CD133 antibody using UV-vis spectrophotometry, X-ray diffraction, transmission electron microscopy, and dynamic light scattering.
2. To assess the cytotoxicity effects and intracellular localization of gold nanoparticles, 5-fluorouracil loaded gold nanoparticles, and 5-fluorouracil loaded gold nanoparticles conjugated with the CD133 antibody on HCT116 colorectal cancer cells using cell viability assay and fluorescence microscopy, respectively.
3. To generate sphere-forming cells derived from HCT116 and assess the stemness properties of cells subjected to treatment with 5-fluorouracil loaded gold nanoparticles conjugated with CD133 antibody through a sphere formation assay.
4. To assess the expression levels of survivin in HCT116 colorectal cancer stem cells subjected to 5-fluorouracil loaded gold nanoparticles conjugated with CD133 antibody using Western blotting technique.

1.8 Flow chart of study

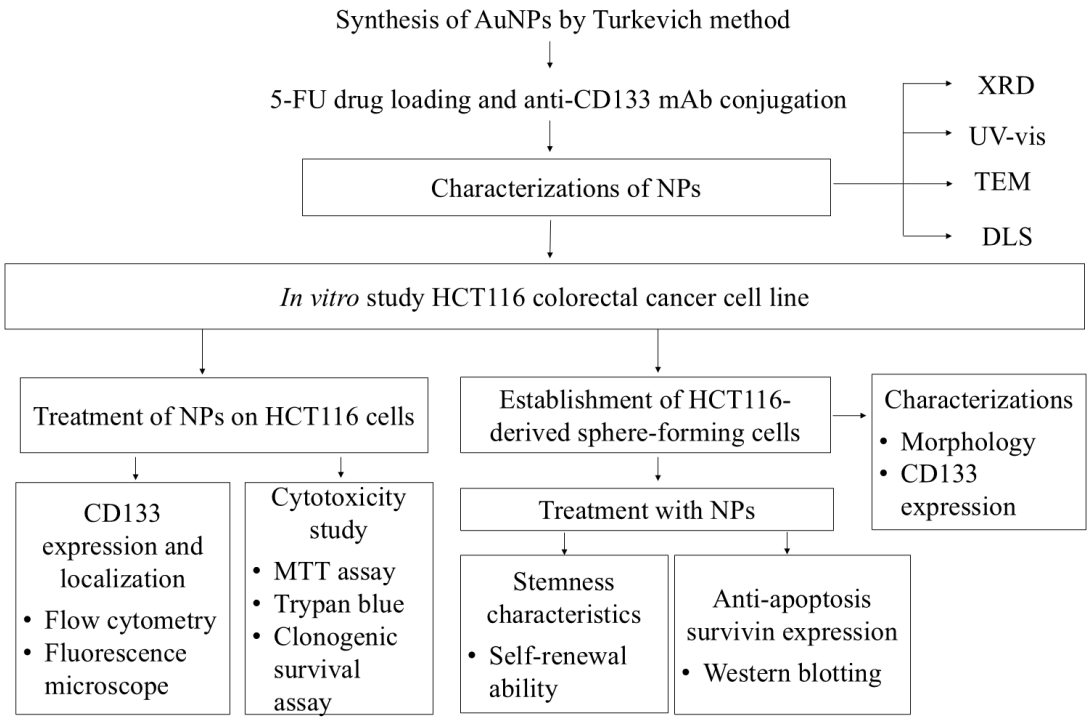

Figure 1.1 Summary of the flow chart of the study. NPs, nanoparticles

1.9 Research framework

This work examines the synthesis of gold nanoparticles (AuNPs) conjugated with anti-CD133 monoclonal antibody (mAb) and loaded with 5-fluorouracil (5-FU) for targeted drug delivery in colorectal cancer. The synthesized AuNPs undergo modifications to improve their stability, specificity, and therapeutic efficacy. The use of a CD133 mAb for functionalization allows for the precise targeting of cancer stem-like cells that express CD133, and the incorporation of drug loading ensures a regulated release of 5-FU directly at the tumor location.

The interaction of these functionalized AuNPs with cancer cells is a pivotal element of our study, especially in improving the selective uptake by CD133-expressing cancer stem-like cells while preserving normal cells that do not express CD133. Figure 1.2 illustrates a schematic representation of the study framework, emphasizing the synthesis of functionalized AuNPs, their specific interaction with CD133-expressing cancer cells, and the anticipated therapeutic effects.

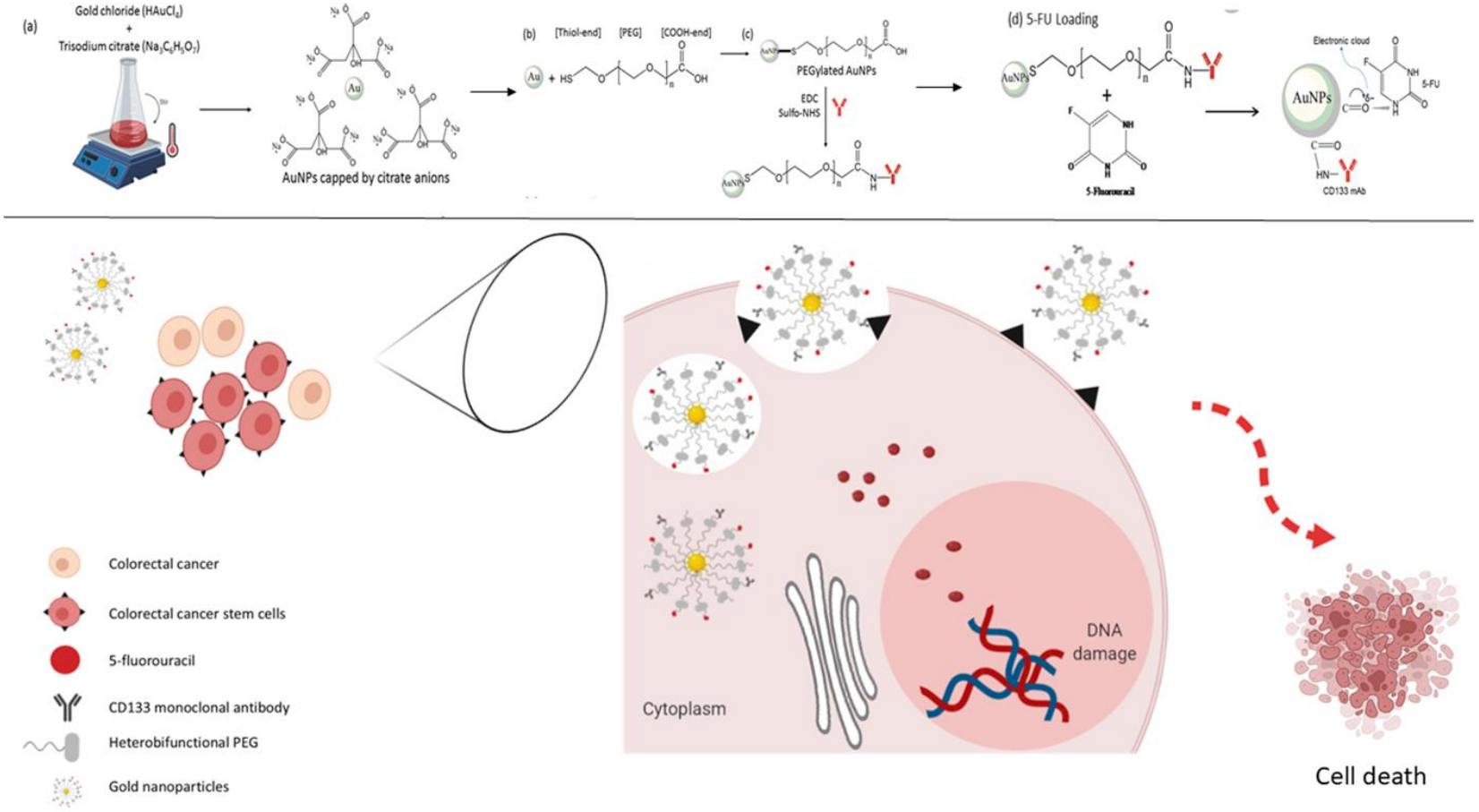

Figure 1. 2 Framework of AuNPs synthesis and functionalization for targeted drug delivery.

CHAPTER 2

LITERATURE REVIEW

2.1 Colorectal cancer

2.1.1 Overview of colorectal cancer

Colorectal cancer (CRC) is a global burden affecting both men and women equally. In clinical practice, colon cancer and rectal cancer are referred to as CRC due to their similar anatomical, histological, functional and organ of origin i.e., large intestine (Alzahrani et al., 2021). CRC arises from certain glandular epithelial cells in the large intestine due to a sequence of genetic or epigenetic abnormalities that give them a favorable edge in proliferation (Rawla et al., 2019). CRC progresses through distinct stages, i.e. initiation to invasion and metastasis. Commonly, TNM staging system by the American Joint Committee on Cancer (AJCC), which refers to Tumor, Node and Metastasis, is used by physicians to assess the extent of cancer spread and guide the treatment decisions (Li et al., 2016; Nearchou et al., 2019).

The pathogenesis of CRC is multidimensional and very complex, but certain risk factors are identified. Genetic and environmental factors such as diet and lifestyle are the most of common risk factors (Cho et al., 2019). Sporadic cases involving diets rich in red and processed meats, insufficient fiber consumption, a sedentary lifestyle, obesity, excessive alcohol intake, and tobacco use comprise 70% of CRC cases (Tuan & Chen, 2015; Malki et al., 2020; Chang et al., 2021a; Sharif et al., 2021). Meanwhile, having family history of CRC and genetic disorder with malignant polyps comprise 25% and 5-10% of the cases respectively (Malki et al., 2020).

The treatment for CRC can vary based on the stages of cancer and tumor localization. To date, surgery, chemotherapy, radiotherapy and immunotherapy have

always been standard-of-care therapy (Johdi & Sukor, 2020; Beets, 2021). A retrospective study conducted by Augestad et al. in 2015 and a randomized controlled trial conducted by Pugh et al. in 2016 both found that 15.7% and 17% of patients, respectively, experienced recurrence after undergoing surgery for CRC. Nevertheless, the response rate for patients on disease remains low and worsens by tumor relapse and potential toxicities.

2.1.2 Clinical presentation and diagnosis of colorectal cancer

CRC originates from colonic and rectal epithelium and is characterized by uncontrolled cell proliferation and division. In a recent prospective cohort study conducted by Holtedahl et al. (2021), it was shown that rectal bleeding, abdominal pain, constipation, and diarrhea were the predominant symptoms reported with high frequency. Meanwhile, 35% of patients diagnosed with colon cancer and 28% of patients diagnosed with rectal cancer exhibited nonspecific symptoms. Since most symptoms are related to changes in bowel movements and no prior symptoms suggestive to CRC, some advanced patients may experience sudden intestinal blockage or peritonitis due to a tumor perforation (Akbulut et al., 2022). It is noteworthy that, a previous retrospective study conducted in Sweden revealed that CRC was the common diagnosis associated with diagnostic errors in primary healthcare and emergency departments (Fernholm et al., 2019). This finding necessitates the implementation of preventive measures to ensure safer healthcare and prevent delayed diagnoses that result in the progression of diseases to advanced stages.

During the diagnostic procedure, clinicians are required to perform physical examinations and evaluate the cancer history of the patient's family before conducting laboratory analysis. The fecal occult blood test (FOBT) is a non-invasive method and is commonly used for CRC screening to detect the presence of blood in stool (Chan &

Liang, 2022; Zou et al., 2022). FOBT is effective in asymptomatic patients, aiding in early-stage cancer detection by guiding patient selection for follow-up tests like colonoscopies (Kaur & Adamski, 2020). Nevertheless, a more advanced screening technique called fecal immunochemical test (FIT) has been developed, offering improved specificity, sensitivity, and reduced costs (Kaur & Adamski, 2020). Despite the guaiac-based chemical reaction used in FOBT, FIT employs antibodies that specifically target human globin, a protein frequently linked to lower gastrointestinal bleeding (Guimarães et al., 2019). Studies recently show that, in terms of sensitivity and specificity, FIT is more effective than FOBT in detecting CRC (Clark et al., 2020; Zou et al., 2022).

Besides stool-based CRC screening, diagnosis for CRC primarily includes colonoscopy, pathology, and radiology. Colonoscopy is considered the most reliable and accurate method for the initial evaluation and tissue diagnosis of CRC due to its exceptional sensitivity, specificity, and ability to provide histological diagnoses (Akbulut et al., 2022). Moreover, if initial screening tests yield positive results, a colonoscopy is advised to exclude the possibility of cancer, identify it at an earlier stage, and excise polyps, thereby reducing the risk of subsequent colorectal neoplasia and mortality associated with CRC (Zauber et al., 2012; Forbes et al., 2020). Meanwhile, the pathology service employs digital whole-slide images (WSIs) of hematoxylin and eosin (H&E)-stained specimens obtained from formalin-fixed paraffin-embedded (FFPE) or frozen tissues to establish diagnoses (Wang et al., 2021).

Apart from CRC treatment, radiography plays a vital role in screening, diagnosing, staging, and performing imaging-guided treatments for disease surveillance. Barium enema (BE) is an X-ray examination that involves the use of a contrast agent called barium sulfate to identify abnormalities in the large intestine of

the gastrointestinal tract (Rubesin et al., 2000). Interestingly, the utilization of BE examination has had a fall in recent years, since The US Preventive Services Task Force no longer includes BE as an option for CRC screening in its guidelines (Whitlock et al., 2008; Levine & Yee, 2014). Thus, a retrospective analysis study compared the diagnostic accuracy of BE, conventional colonoscopy (CC), magnifying narrow-band imaging colonoscopy (M-NBI), and magnifying chromoendoscopy (MCE) to assess the invasion depth of colorectal epithelial neoplasms (Kawasaki et al., 2021). The study found that while BE had lower specificity, positive predictive value (PPV), and overall accuracy compared to M-NBI and MCE, it had higher sensitivity and negative predictive value (NPV) compared to CC, M-NBI, and MCE. This explains the rationale for the continued use of BE as an adjunctive diagnostic technique for early-stage CRC in certain areas, including Malaysia. In addition, computed tomography (CT) is used to assess the extent and spread of cancer, while magnetic resonance imaging (MRI) is used for rectal cancer assessment to accurately determine the disease's stage and extent (Nakayama et al., 2014; Sawicki et al., 2021).

2.1.3 Incidence of colorectal cancer

CRC is a prevalent global disease, affecting both males and females. The American Cancer Society predicts 153,020 new cases in the US in 2023, with 13% of cases in individuals under 50 (Siegel et al., 2023b). In addition, the disease is projected to cause 52,550 deaths in 2023, with 3750 deaths in those under 50 (Siegel et al., 2023b). Furthermore, the trend towards diagnosing individuals at a more advanced stage of the disease is increasing, with a higher number of people being diagnosed at an advanced stage. Malaysia's 2012-2016 cancer report revealed that 70% of CRC patients were diagnosed at stage III or IV, leading to more complex treatment and less favorable prognosis (Ministry of Health Malaysia, 2021).

2.1.4 Staging and treatment of colorectal cancer

Accurate clinical staging is essential to provide appropriate treatment for CRC. Commonly, CRC staging is determined using the TNM system, which was initially established in the 1950s (Delattre et al., 2022). This approach has been continuously improved and updated by the AJCC, with the most recent version being the 8th edition in 2017 (Delattre et al., 2022; Ueno et al., 2023). This system classifies cancer according to the dimensions and spread of the main tumor (T), the existence of cancer cells in neighboring lymph nodes (N), and the presence of metastasis, which refers to the spread of cancer to other body parts (M).

In a retrospective analysis conducted by Tong et al (2018), the 8th edition of the AJCC manual was compared to the seventh edition for CRC (Figure 2.1 and Figure 2.2). The main difference in the eighth edition is the inclusion of a new stage, known as IVC, obtained by extending the M category with the addition of M1c for peritoneal metastases (Tong et al., 2018). Furthermore, the AJCC-8th CRC staging system still advises considering the infiltration of vascular lymphatic vessels and tumor deposition as indicators of prognosis. Additionally, the microsatellite instability status and mutations in the RAS pathway, namely in genes such as KRAS, BRAF, and NRAS, are used to predict treatment effectiveness (Tong et al., 2018; Weiser, 2018).

7th edition	8th edition
Tx: Primary tumor cannot be assessed	Tx: Primary tumor cannot be assessed
T0: No evidence of primary tumor	T0: No evidence of primary tumor
Tis: Carcinoma *in situ*, limited to intraepithelial or invasive lamina propria	Tis: Carcinoma *in situ*, limited to intraepithelial or invasive lamina propria
T1: Tumor invading submucosa	T1: Tumor invading submucosa
T2: Tumor invading the muscularis propria	T2: Tumor invading the muscularis propria
T3: Tumor penetrating the muscularis propria and arriving at colorectal fat tissue	T3: Tumor penetrating the muscularis propria and arriving at colorectal fat tissue
T4: Tumor directly invading other organs or structures	T4: Tumor directly invading other organs or structures
T4a: Tumor penetrating visceral peritoneum	T4a: Tumor penetrating visceral peritoneum
T4b: Tumor directly invading or adhering to other organs or structures	T4b: Tumor directly invading or adhering to other organs or structures
Nx: Regional lymph nodes cannot be assessed	Nx: Regional lymph nodes cannot be assessed
N0: No lymph node metastasis and no tumor deposits (TD)	N0: No lymph node metastasis and no TD
N1: 1-3 lymph nodes metastases	N1: 1-3 lymph nodes metastases
N1a: 1 lymph node metastases	N1a: 1 lymph node metastases
N1b: 2-3 lymph nodes metastases	N1b: 2-3 lymph nodes metastases
N1c: Although there was no regional lymph node metastasis, TDs were submucosal, mesangial, or peritoneum-covered para-colorectal tissue.	N1c: Although there was no regional lymph node metastasis, TDs were submucosal, mesangial or peritoneum-covered para-colorectal tissue.
N2: More than or equal to 4 lymph node metastases	N2: More than or equal to 4 lymph node metastases
N2a: 4-6 regional lymph node metastases	N2a: 4-6 regional lymph node metastases
N2b: More than or equal to 7 lymph node metastases	N2b: More than or equal to 7 lymph node metastases
M1: There is distant lymph node metastasis	M1: There is distant lymph node metastasis
M1a: Metastasis is limited to one organ or site (*e.g.*, liver, lung, ovary, and extra-regional lymph node metastases)	M1a: Metastasis is limited to one organ or site (*e.g.*, liver, lung, ovary, and extra-regional lymph node metastases)
M1b: Transfer more than one organ or site, or to the peritoneum[1]	M1b: Transfer more than one organ or site[1]
	M1c: Peritoneal metastases with or without metastasis of other organs[1]

[1]Differences between the two versions.

Figure 2.1 Comparison of the tumor-node-metastasis stage between the 7th edition and the 8th edition [Extracted from Tong et al. (2018)]

7th edition				8th edition			
Stage	**T**	**N**	**M**	**Stage**	**T**	**N**	**M**
0	Tis	N0	M0	0	Tis	N0	M0
I	T1-2	N0	M0	I	T1-2	N0	M0
ⅡA	T3	N0	M0	ⅡA	T3	N0	M0
ⅡB	T4a	N0	M0	ⅡB	T4a	N0	M0
ⅡC	T4b	N0	M0	ⅡC	T4b	N0	M0
ⅢA	T1-2	N1/N1c	M0	ⅢA	T1-2	N1/N1c	M0
	T1	N2a	M0		T1	N2a	M0
ⅢB	T3-4a	N1/N1c	M0	ⅢB	T3-4a	N1/N1c	M0
	T2-3	N2a	M0		T2-3	N2a	M0
	T1-2	N2b	M0		T1-2	N2b	M0
ⅢC	T4a	N2a	M0	ⅢC	T4a	N2a	M0
	T3-4a	N2b	M0		T3-4a	N2b	M0
	T4b	N1-2	M0		T4b	N1-2	M0
ⅣA	Any T	Any N	M1a	ⅣA	Any T	Any N	M1a
ⅣB	Any T	Any N	M1b	ⅣB	Any T	Any N	M1b
				ⅣC	Any T	Any N	M1c

Figure 2.2 Colorectal cancer tumor-node-metastasis staging American Joint Committee on Cancer 7th and 8th editions [Extracted from Tong et al. (2018)]

The management of CRC is tailored based on the stage, site, and patient's health. Surgery serves as the principal intervention to resect the tumor and adjacent lymphatic nodes. The traditional open incision method and the minimally invasive laparoscopic procedure are two surgical approaches employed to remove CRC (Dekker et al., 2019). Additional modalities for the treatment of colorectal cancer including chemotherapy, radiation, and immunotherapy. In Malaysia, the national guideline Ministry of Health Malaysia (MOH) recommends standard cytotoxic chemotherapy (often based on 5-fluorouracil or oral Capecitabine), alone or combined with Oxaliplatin, for adjuvant therapy, especially in stage II (high-risk) and stage III CRC after surgery.

2.2 5-fluorouracil

2.2.1 Overview of 5-fluorouracil

5-FU is an antimetabolite that was identified in 1957 by Heiderberger et al. (1957) for its tumor inhibitory properties. This compound is a natural pyrimidine uracil analog where a fluorine atom replaces hydrogen at the C-5 position (Figure 2.3) (Handali et al., 2018). In addition to colorectal cancer, 5-FU is frequently utilized in the treatment of various solid tumors, including those originating from the breast, stomach, pancreas, and head and neck regions (Zhang et al., 2008; Bracht et al., 2010).

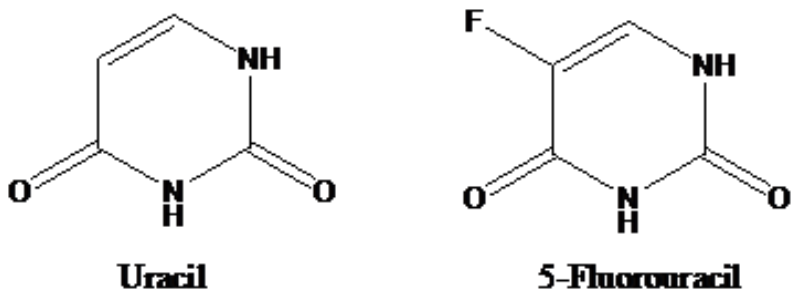

Figure 2.3 Chemical structure of uracil and 5-fluorouracil

Due to its structural similarity to uracil, 5-FU can be integrated into RNA and DNA, disrupting nucleoside metabolism. Nonetheless, chemotherapy that relies on 5-FU also disrupts the healthy cells that are rapidly dividing because of its non-specific targeting, leading to prevalent side effects like hair loss, nausea, and vomiting (Tummala et al., 2015; Cisterna et al., 2016). Moreover, a rapid clearance from the bloodstream and inadequate distribution hinder the therapeutic efficacy of 5-FU (Liu et al., 2015).

2.2.2 Mechanism of action

The regulation of 5-FU metabolism encompasses the enzymes thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and orotate phosphoribosyl transferase (OPRT) (Ishikawa et al., 2008). 5-FU functions as a thymidylate synthase inhibitor, disrupting DNA and RNA synthesis, which subsequently affects their processing and functionality, ultimately leading to cell death (Longley et al., 2003; Saif et al., 2009; Baudino, 2015).

5-FU, an analog of uracil, easily crossed the cell membrane through the same facilitated transport mechanism as uracil and was incorporated into nucleic acids, ultimately contributing to its cytotoxic effects (Wohlhueter et al., 1980; Longley et al., 2003). 5-FU and uracil are in constant competition with one another because they share the same metabolic pathway (Figure 2.4). Fluorouridine triphosphate (FUTP), rather than uridine triphosphate (UTP), is the active metabolite of 5-FU that gets incorporated into ribonucleic acid (RNA), disrupting its processing and functioning (Saif et al., 2009). Fluorodeoxyuridine triphosphate (FdUTP) serves as an additional metabolite of 5-FU that can be integrated into DNA in place of deoxythymidine triphosphate (dTTP). Furthermore, rather than typical pyrimidines, deoxyuridine monophosphate (dUMP), a metabolite of 5-FU, interacts to form a covalent ternary

complex with TS and 5,10-methylene tetrahydrofolate (CH2THF), leading to the inhibition of deoxythymidine monophosphate (dTMP) production, which ultimately disrupts DNA synthesis (Saif et al., 2009; Focaccetti et al., 2015). Typically, 5-FU induces cancer cell death by disrupting DNA synthesis as well as RNA processing and function.

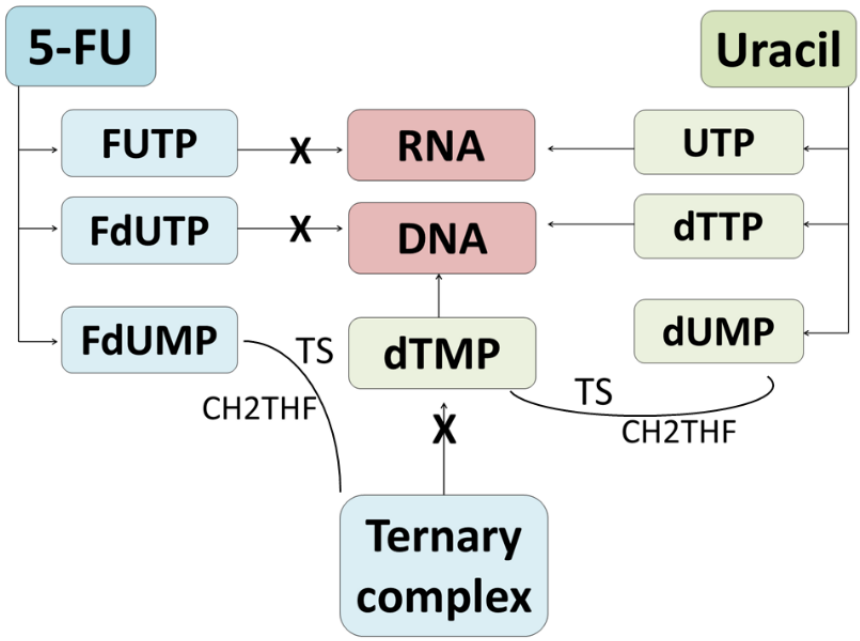

Figure 2.4 Metabolism routes of 5-fluorouracil and uracil. 5-FU metabolites inhibit DNA synthesis and RNA processing and function which eventually attributes to the cell death

2.2.3 Side effects

While 5-FU demonstrates significant efficacy as a chemotherapeutic agent, it is constrained by a short plasma half-life of 10-20 minutes due to rapid degradation (Handali et al., 2018). Indeed, the rate at which substances degrade and are cleared from the body plays a crucial role in determining the duration of exposure to active drug forms, thereby impacting their therapeutic effectiveness.

Furthermore, because of the non-selective nature and limited bioavailability of 5-FU at the tumor site, a higher dosage is necessary to achieve an effective concentration of the drug. However, the administration of high doses of chemotherapeutic agents results in elevated systemic side effects and a greater prevalence of drug resistance, thereby constraining the efficacy of 5-FU in the chemotherapy treatment of colorectal cancer.

2.3 Colorectal cancer stem cells

2.3.1 Cancer stem cells theory

The cancer stem cells (CSCs) theory proposes that a minor subpopulation of cells exhibits characteristics akin to embryonic stem cells (Munro et al., 2018), leading to the emergence of metastatic cancer cells and contributing to the elevated recurrence rates (Zhang et al., 2017). Indeed, CSCs exhibit the same key signaling pathways as embryonic stem cells, including Wnt, Notch, transforming growth factor beta (TGF-β), and the Hedgehog signaling pathway (Abetov et al., 2015). Nonetheless, dormant cancer stem cells exhibit the expression of stemness-related genes, possess the ability to self-renew and differentiate into various non-stem cancer cell types, and demonstrate resistance to conventional chemotherapy and radiotherapy (Xiao et al., 2017). A key characteristic of these cells is their tumorigenic potential, which is

assessed by their capacity to initiate xenograft tumors following transplantation (Abetov et al., 2015).

2.3.2 CD133 as a biomarker in colorectal cancer stem cells

The process of isolating and identifying CSCs from CRC provides deeper insights into various potential stem cell markers. The identification of CRCSCs markers will assist in tracking disease progression and assessing the risk of recurrence in patients (Langan et al., 2013). Table 2.1 presents various candidate biomarkers associated with stem cells in colorectal cancer and outlines their prognostic significance as documented in prior studies.

Table 2.1 Biomarkers of colorectal cancer stem cells with the prognostic values

CRCSCs Markers	Molecular weight	Prognostic Significance	Tumorigenicity
CD133	120 kDa	• Highly correlated with low survival in CRC patients (Horst et al., 2009a). • Higher expression is associated with resistance of CRC to 5-FU based chemotherapy (Ong et al., 2010; Paschall et al., 2016).	Subcutenous injection of $CD133^+$, but not $CD133^-$ subpopulation of CRC into immunodeficient mice established tumor (O'Brien et al., 2007; Ricci-Vitiani et al., 2007).
CD44	81 kDa	• Associated with lower patient survival (Ropponen et al., 1998). • Not associated with prognosis (Horst et al., 2009a).	$CD44^+$ cells from a patients' tumor initiated a xenograft tumor in vivo and form a sphere in vitro (Du et al., 2008).
CD166	100-105 kDa	• The expression correlated with a shortened period of survival (Weichert et al., 2004). • No significant correlation with poor survival (Horst et al., 2009a).	$CD166^+/CD44^+$ or $CD166^+/ESA^+$ from human CRC cells can induce tumorigenesis in immunodeficient mice (Dalerba et al., 2007).
CD24	35-45 kDa	• No significant correlation with patient low survival (Papailiou et al., 2011).	Tumor spheroid cells capable to induce tumors upon xenotransplantation (Fanali et al., 2014).
CD26	110 kDa	• Associated with enhanced invasiveness and chemoresistance (Pang et al., 2010).	Isolated $CD26^+$, but not $CD26^-$ cells of CRC, developed distant metastasis when injected into the mouse cecal wall (Pang et al., 2010).
Epithelial cell adhesion molecule (EpCAM)	30-40 kDa	• Inversely correlated between EpCAM glycoprotein expression and tumor staging (Mokhtari & Zakerzade, 2017).	Injection of 200 to 500 EpCAMhigh/ $CD44^+$ cells in immunodeficient mice were sufficient to give rise to a tumour (Fanali et al., 2014).