UNIVERSITI PUTRA MALAYSIA

PHYSIOCHEMICAL CHARACTERIZATION AND ANTICANCER EFFICACY OF CAMPTOTHECIN LOADED β-CYCLODEXTRIN-EDTA-FE3O4 NANOCARRIER

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By

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TITLE OF THESIS: PHYSIOCHEMICAL CHARACTERIZATION AND ANTICANCER EFFICACY OF CAMPTOTHECIN LOADED β-CYCLODEXTRIN-EDTA-FE_3O_4 NANOCARRIER

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Camptothecin (CPT) is a distinguished anticancer drug that effectively works on various cancers particularly colon cancer. Camptothecin is naturally occurring alkaloid that has significant amount of anti-tumor activity. However, poor solubility and instability of CPT has greatly restricted its chemotherapeutic value. Thus, various nanocarriers are continuously being formulated and designed to enhance the delivery, solubility, stability and bioavailability of CPT. As such, in the present study, we studied and determined the fabrication of novel magnetic nanocarrier by the combination of ß-Cyclodextrin-EDTA-Fe_3O_4 magnetic nanoparticle (CEF) conjugated with CPT. This nanocompound CPT-CEF were synthesized with the objective to enhance CPT's stability, bioavailability and subsequently improve its therapeutic value through the use of nanocarrier (CEF). Both β-Cyclodextrin and Fe_3O_4 magnetic nanoparticle are increasingly utilized in pharmaceutical application due to its promising therapeutic value. In this study, this hybrid nanocarrier was comprehensively characterized by NMR spectrometers and scanning electron microscopy. Particle size analysis, zeta potential measurements, drug loading efficiency and *in-vitro* drug release study was also performed to characterize CPT-CEF. The magnetic nanocarrier exhibited decent improvement in the stability of CPT encapsulated nanocarrier. Additionally *in vitro* drug release study exhibited the ability of CPT-CEF to solubilize in both acidic and neutral environment.

The ability of CPT-CEF to reflect on the anticancer activity of encapsulated CPT through the induction of apoptosis in HT29 colon cancer cells was also evaluated. MTT assay was used to show dose dependent cytotoxicity of CPT-CEF. Cell cycle analysis, Annexin V-FITC/PI staining, mitochondrial membrane depolarization (JC-1), caspase-3 activity assay were performed to detect apoptosis and cell cycle arrest. CPT-CEF evidently showed a dose-dependent cell viability reduction in HT29 cell

line. CPT-CEF also effectively induced apoptosis, which was determined by Annexin V/FITC staining. Mitochondrial membrane depolarization and activation of caspase-3 were also observed in response to CPT-CEF treatment. Interestingly cell cycle arrest was observed in the G1 phase indicating the possibility of synergistic effect contributed by the nanocarrier (CEF) on the action of CPT. These results suggests that CPT-CEF formulation has successfully maintained the anticancer potential of CPT while improving the stability and bioavailability CPT. These data suggests that CPT-CEF design has the potential to be developed as a major nanocarrier for camptothecin delivery for an effective treatment of colon cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KARAKTERISASI SISIOKIMIA DAN KECEKAPAN ANTI-KANSER CAMPTOTHECIN YANG DIMUATKAN DENGAN β-CYCLODEXTRIN-EDTA-FE_3O_4 SEBAGAI NANOCARRIER

Oleh

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Camptothecin (CPT) adalah ubat anti-kanser yang berkesan untuk pelbagai jenis kanser terutamanya kanser kolon. Camptothecin yang didapati secara semula jadi merupakan sejenis alkaloid yang mempunyai aktiviti anti-tumor yang tinggi. Walau bagaimanapun, kelarutan yang rendah dan ketidakstabilan camptothecin telah menyebabkan penggunaaan campthothecin menjadi terhad untuk rawatan kanser. Oleh itu, pelbagai jenis *nanocarrier*s dirangka dan dikaji secara berterusan untuk meningkatkan penghantaran, kelarutan, kestabilan dan bioavailabiliti CPT. Dalam pada itu, dalam kajian ini, kami mengkaji dan menentukan penggunaan *nanocarrier* magnet Fe_3O_4 besama gabungan komponen lain untuk menghasilkan ß-cyclodextrin-EDTA-Fe_3O_4 nanopartikel magnetik (CEF) yang digunakan untuk merangkumi CPT. Nanokompaun CPT-CEF ini disintesis dengan objektif untuk meningkatkan kestabilan dan bioavailabiliti CPT dan seterusnya untuk meningkatkan nilai terapeutiknya dalam rawatan kanser. ß-cyclodextrin dan nanopartikel magnetik sedang digunakan secara meluas dalam bidang farmasi kerana ianya mempunyai nilai teraputik yang tinggi. Untuk itu, dalam kajian ini *Nanocarrier* hibrid ini dikaji untuk ciri-cirinya melalui penggunaan spektrometer NMR dan mikroskop elektron imbasan. Analisis saiz zarah , ukuran potensi zeta, kecekapan pemuatan CPT dan kajian pelepasan *in-vitro* CPT juga turut dijalankan untuk mencirikan CPT-CEF. *Nanocarrier* magnet ini didapati meyumbang kepada peningkatan dalam kestabilan CPT yang terkandung dalam *nanocarrier*. Selain itu, kajian pelepasan i*n-vitro* CPT menunjukkan keupayaan CPT-CEF untuk larut dalam keadaaan yang berasid dan juga neutral.

Keupayaan CPT-CEF untuk mencungkil aktiviti anti-kanser melalui induksi *apoptosis* dalam sel kanser kolon HT29 turut dinilai. MTT telah digunakan untuk menunjukkan dos *cytotoxicity* yang bersandar kepada penggunaan CPT-CEF. Analisis kitaran sel, Annexin V / FITC, penyahkutuban membran mitokondria (JC-1) dan aktiviti caspase-3

telah dikaji untuk mengesan *apoptosis*. CPT-CEF secara jelas menunjukkan pengurangan sel HT29 selaras dengan penigkatan dos CPT-CEF. CPT-CEF juga berkesan dalam mendorong apoptosis dalam sel HT29 dan ini telah dipastikan melalui Annexin V-FITC/PI. Penyahkutuban membran mitokondria dan pengaktifan caspase-3 juga turut diperhatikan sebagai tindak balas kepada rawatan CPT-CEF. Yang menariknya, penangkapan kitaran sel diperhatikan dalam fasa G1 dan ini mungkin menunjukkan kesan sinergi *nanocarrier* (CEF) dalam aktiviti CPT. Kesimpulannya, kajian ini menunjukkan bahawa CPT-CEF telah berjaya merangkum CPT and menunjukkan aktiviti anti-kanser yang baik. Data-data ini mencadangkan bahawa reka-bentuk CPT-CEF mempunyai potensi yang tinggi untuk dimajukan sebagai *nanocarrier* utama bagi camptothecin untuk memberikan rawatan yang berkesan untuk kanser kolon.

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I remember taking up masters aiming to wrap it in a year but unfortunately I was all wrong. I have learnt that setting the goals too strongly is a terrible idea because sometimes it just not meant to be so. This is not even the project which I started with but eventually became the project I managed to complete and finish. I have also learnt other precious lessons in this period of time and I consider them more valuable than the degree itself.

I have realized there are at times certain point in life exist only for us to grow and where the end goal matters the least. The last two years means that for me. I might not the person who received what I want but now I am a person with all the skills and values I need and I am extremely grateful for it.

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I certify that a Thesis Examination Committee has met on 9 May 2017 to conduct the final examination of Poorani a/p Krishnan on her thesis entitled "Physiochemical Characterization and Anticancer Efficacy of Camptothecin Loaded β-Cyclodextrin-EDTA-FE_3O_4 Nanocarrier" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This is to confirm that:

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ACS	American Cancer Society
AO	Acridine orange
APC	Adenomatous polyposis coli
BAX	BCL2 associated X protein, apoptosis regulator
BAD	BCL2 Associated Agonist of Cell Death
BID	BH3-Interacting Domain Death Agonist
β-CD	β-Cyclodextrin
Bmpr1	Bone morphogenetic protein receptor, type IA
BSA	Bovine serum albumin
BRAF	B-Raf encoding protein
CCN	Connective tissue growth factor (CTGF), Cystein rich protein (Cyr61), and Nephroblastoma overexpressed gene
CD	Cyclodextrins
CDK	Cyclin-dependent kinases
CDP	CD polymers
CEF	β-Cyclodextrin+EDTA+Fe_3O_4
$CHCl_3$	Chloroform
CIN	Chromosomal instability pathways
CS	Chitosan
CTGF	Connective tissue growth factor
CPT	Camptothecin
CPT-CEF	CPT + β-Cyclodextrin+EDTA+Fe_3O_4
DEVD-pNA	N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide
DNA	Deoxyribonucleic acid
DPC4	Deleted in pancreatic cancer, locus 4
DTPA	Diethylenetriaminepentaacetic acid
DMSO	Dimethyl sulfoxide

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EPI	Epichlorohydrin
Fas-L	Fas ligand
Fe_3O_4	Iron magnetic nanoparticles
FT-NMR	Fourier Transform - Nuclear Magnetic Resonance
FITC	Fluorescein isothiocyanate
FT-IR	Fourier transform infrared spectroscopy
GI	Gastrointestinal
GLA	Glutaraldehyde
GO	Graphene oxide
HCl	Hydrochloric acid
hCPT	HomoCamptothecins
HT29	Human colorectal cancer cells
JC-1	5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide
MLH1	MutL Homolog 1
MMR	Mismatch repair genes
MNP	Magnetite Nanoparticle
MPT	Mitochondrial permeability transition
MSI	Microsatellite instability
MSH2/6	MutS Homolog 2/6
MTT	3-(4, 5-methylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide
MYH	Myosin heavy chains
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
NP	Nanoparticles
PBS	Phosphate buffer solution
PI	Propidium iodide

PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PMS2	PMS1 homolog 2, mismatch repair system component
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SEM	Scanning Electron Microscope
STK-11	Serine Threonine Kinase 11
SPION	Superparamagnetic iron oxide nanoparticles
TGF-β	Transforming growth factor beta
TGFBR2	Transforming growth factor beta receptor 2
Topo 1	Topoisomerase 1
ZnO	Zinc oxide
$\Delta\Psi_M$	Mitochondrial membrane potential

CHAPTER 1

INTRODUCTION

1.1 Outline and framework of study

Cancer is among the primary cause of death globally. Multiple factors are attributed to the occurrence of cancer in humans. Internal factors such as genetic mutations and hormones and external factors such as lifestyle, diet and infectious agents (Stratton et al., 2009; Willett, 2000) are among the contributing factors of cancer.

As such, colon/colorectal cancer are one among the major cancers that are affecting people globally. Colorectal cancer contributes a high percentage of cancer related death worldwide in which almost 500 000 related deaths are reported yearly thus making it as the third leading cause of cancer death world-wide and the second most prevalent cancer globally (Gill et al., 2003; Parkin, 2001). According to (Greenlee et al., 2001), colon cancers are the second leading cause of cancer incidence and cancer death among adult Americans, with 135,000 new cases and 57,000 deaths in 2001, and with a 6% lifetime risk of developing the disease. In Malaysia about 4,501 cases of colorectal cancer was reported in the NCPR-CC National Cancer Patient Registry-Colorectal Cancer from 2008 to 2013 and stands as the second most common cancer in Malaysia (Abu Hassan et al., 2016).

Colorectal cancers identified at early stages are treatable with standard therapies such as surgical resection however when it reaches a stage where surgery is not feasible, chemotherapy will be implemented. Chemotherapy has become one of the major treatment options for several cancers. In the case of colorectal cancer numerous chemotherapies have been shown to provide an average improved survival. In the light of this, topoisomerase I inhibitor Camptothecin has shown immense potential in being an anticancer drug for colorectal cancer (Ulukan & Swaan, 2002).

Camptothecin (CPT), a naturally occurring plant alkaloid is a potent anticancer agent that inhibits topoisomerase I during the S-phase of the cell cycle (Hertzberg et al.1989). Following its isolation from *Camptotheca acuminata* and characterization in 1966 by Wall (Wall et al., 1966) the use of CPT as potential anti-tumor agents was comprehensively studied and tested (Swaminathan et al., 2010). As a reputed chemotherapeutic agent Camptothecin is known as a prominent apoptosis inducer in cancer cells (Shao et al., 2001; Sharma, Lansdell, Peddibhotla, & Tepe, 2004). Camptothecin is broadly studied due to its immense potential as anticancer drug however it is subjected to various limitations especially in terms of stability and solubility. Due to this, studies are endlessly being conducted to enhance the anticancer effect of CPT by subduing its limitation. To achieve this, nanotechnology is among the distinct technology that is being adapted to enhance the affectivity of chemotherapeutic drugs such as CPT. Particularly nanomaterial-based drug carriers

are broadly being applied and studied due to their efficient drug loading capasity, targeted delivery, and controlled release of drugs ability (Burger et al., 2002; Santra et al., 2009; Zhang et al., 2010)

CPT has been encapsulated in various formulation of nanoparticles, microspheres, microemulsions, polymeric micelles, and polymeric implants (Hatefi & Amsden, 2002; Shenderova et al., 1999) in attempt to efficiently deliver and achieve an optimum anticancer effect (Tyner et al., 2004).

This study also embarks such objective where Iron oxide (Fe_3O_4) magnetic nanoparticles conjugated with β-Cyclodextrin cross linked with EDTA are formulated as a nanocarrier (CEF) for CPT. Iron oxide (Fe_3O_4) magnetic nanoparticles has rising potential in biomedical application as it aids targeted drug release, enables MRI detection and biocompatible. Magnetic properties of Iron oxide (Fe_3O_4) magnetic nanoparticles enable the use of external magnetic force for chemotherapy treatment (Jeong et al., 2007; Jun et al., 2008). Hyper-cross-linked structure of β-Cyclodextrin that consists of cyclodextrin matrix constitutes a protected environment for transport of different molecules. Besides, it also provides solubility capacities and protection for easily degradable compounds from the external environment thus allowing the delivery of insoluble compounds (Torne et al., 2013). β–Cyclodextrin are also known to be able to mediate controlled release of encapsulated compounds over time leading to a prolonged exposure, which in turn may reduce the dosage and the frequency of drug administrations (Minelli et al., 2012). Taken together, this combination of β-Cyclodextrin conjugated with Iron oxide (Fe_3O_4) magnetic nanoparticles encapsulating CPT might be an efficient nanocarrier for the effective delivery of the CPT to combat cancer cells.

This formulation is characterized by FT-NMR spectrophotometer and scanning electron Microscope (SEM) and studied for encapsulation efficiency *and in-vitro* drug release study. To further ensure the ability and effectiveness of Camptothecin+β-Cyclodextrin+EDTA+Fe_3O_4 (CPT-CEF) formulation to retain the anticancer property of the natural CPT, cytotoxicity studies, cell cycle analysis and apoptosis determination assays are also employed and studied on HT29 colorectal cancer cell lines.

1.2 Problem Statement

CPT is subjected to few drawbacks thus hindering its maximum application in cancer chemotherapy. CPT exhibits poor aqueous solubility, stability and severe side effects. Also, at physiological pH the lactone ring of CPT is opened thus yielding the inactive carboxylate form (Chourpa et al., 1998; Fassberg & Stella, 1992). This ring opening reduces the permeability of CPT to pass through the lipid bilayer of a low dielectric constant, thus altering the molecular diffusivity (Burke et al., 1993; Swaminathan et al., 2010).

Although extensive researches have been carried out to develop delivery systems for the insoluble CPT and its derivatives through entrapment into liposomes, microspheres and nanoparticles, the quest to design an ultimate nanocarrier to unleash the full potential of CPT is still being earnestly pursued. As such, this study adopts nanotechnology in which a nanocarrier formulation utilizing magnetic nanoparticle that enhances the stability, solubility, delivery and bioavailability of CPT in aqueous solution is developed and studied.

1.3 Hypothesis

1.3.1 Null Hypothesis

The formulated drug CPT-CEF is insoluble in aqueous solution and it is unable to retain the original anticancer effect of Camptothecin in HT29 colorectal cancer cell line.

1.3.2 Alternative Hypothesis

I. The CPT-CEF formulation is soluble in aqueous solution and stable at different pH.
II. The CPT-CEF formulation has cancer cell cytotoxicity inducing potential.
III. The CPT-CEF formulation has apoptosis inducing potential in HT29 colorectal cancer cell line.

1.4 Objectives

1.4.1 General objectives

To characterize the novel β-Cyclodextrin-EDTA-Fe_3O_4 magnetic nanoparticles nanocarrier for Camptothecin (CPT) delivery in colon cancer treatment.

1.4.2 Specific objectives

This study was conducted with the following objectives;

I. To characterize β-Cyclodextrin + EDTA + Fe_3O_4 +CPT as a nanocarrier for camptothecin (CPT).
II. To investigate the cytotoxicity inducing potential of CPT-CEF nanoparticle in HT29 colorectal carcinoma cells.
III. To determine the apoptosis inducing potential of CPT-CEF in HT29 colorectal carcinoma cells.

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