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Mechanistic study of the anti-cancer effect of a Chinese herbal extraction in colon cancer

Chi Chiu Wong

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THESIS TITLE:  Mechanistic Study of the Anti-cancer Effect of a Chinese Herbal Extraction in Colon Cancer

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Mechanistic Study of The Anti-cancer Effect of a Chinese Herbal Extraction in Colon Cancer

WONG Chi Chiu

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

Principal Supervisor:
Prof. Wong Ka-Leung (Hong Kong Baptist University)

April 2019
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of MPhil at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

I have read the University’s current research ethics guidelines and accept responsibility for the conduct of the procedures in accordance with University’s Research Ethics Committee (REC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

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Date: April 2019
Abstract –

Mechanistic study of the anti-cancer effect of a Chinese herbal extraction in colon cancer

Gynostemma pentphyllum (Thunb.) Makino, or called Jiaogulan (絞股蓝), is a perennial creeping herb, which belongs to the Cucurbitaceae family. In China, it can be found in the South Shaanxi and the southern area of the Yangtze River. It is also widely distributed in Japan, India, Indo-China, and Indonesia. \( G. \) pentphyllum is an herbal drug of diverse therapeutic effects without obvious toxic effects in the view of traditional Chinese medicine. Saponins extracted from \( G. \) pentphyllum are most likely to be the main active ingredients for its various biological function and clinical effect. Over a hundred of saponins have been isolated and identified in \( G. \) pentphyllum Gynostemma total saponins (GpS).

Pharmacokinetics is one of the important concepts in pharmacology, which interested in relations among absorption, distribution, metabolism and excretion (ADME) of drug. The blood concentration of the drug is used to study the disposition of drugs and to monitor the progress of therapy. In this project, it aims to determine the pharmacokinetic of total saponins extraction of \( G. \) pentphyllum at a single oral dosage of 6 g/kg GpS in C57BL/6J wild type mice plasma by UPLC-MS analysis with the use of gypenoside III, IV, VIII, G, 20(S) and 20(R)-Rg3 as markers of total gypenosides. Method has been developed and validated for the quantification of GpS in plasma samples.

Previous studies show that cancer cell may perform higher glucose uptake rate while producing lactic acid as final metabolite rather than undergoes the complete oxidation of pyruvate. Gp-VIII was chosen to be further studied regarding to the role of glycolic
pathway. MTT assay were performed to determine the LD50 value of Gp-VIII in HCT116 cells. In onwards experiment, HCT116 cells were harvested in the presence of up to 250nM Gp-VIII. Reduction of glucose uptake was observed. Downregulation of different glycolic enzymes including aldolase, TPI, GAPDH, enolase, PKM2 and LDH were observed. Transcription factor HIF-1α was also found to be inhibited by Gp-VIII.

Oral administration of 10mg/kg Gp-VIII in ApcMin/+ mice model was applied to investigate the inhibitory effect of polyp formation. Briefly, about 35% suppression of total number of polyp formation was observed in Gp-VIII treatment group. Number of polyps in different size of ≤ 2mm were reduced by 70%. Reduction of the number of polyps in different intestinal regions was observed with the greatest suppression effect in medial and distal of intestine.
Acknowledgement

I would like to express my gratitude to my principal supervisor Prof. Gary Wong for the useful comments and engagement through the learning process of my MPHIL study.

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CHAPTER 1.
Pharmacokinetic study on *Gynostemma pentaphyllum*

1. Introduction

1.1 *Gynostemma pentaphyllum* (*G. pentaphyllum*)

1.1.1 History and traditional usage of *Gynostemma pentaphyllum*

*Gynostemma pentaphyllum* (Thunb.) Makino, or called *Jiaogulan*, is a perennial creeping herb, which belongs to the Cucurbitaceae family. In China, it can be found in the South Shaanxi and the southern area of the Yangtze River\(^1\). It is also widely distributed in Japan, India, Indo-China, and Indonesia. Among the 13 known species in *Gynostemma* Genus, 11 of them exist in China with abundant natural resources.\(^2\) *G. pentaphyllum* has been used as a folk medicine to relief cough or as an expectorant to treat bronchitis and asthma. Also, it is believed that supplementation before and after daily work helps to increase endurance and relieve fatigue. It is become popular and widely used over the world.

The earliest record of the plant is in the book named “Materia Medica for Famine” (<<救荒本草>>\(^3\)) in 1406 A.D., which was published in the Ming dynasty (1368-1644A.D), when Zhu Xiao first described this plant and presented a sketch of it in his book. In his book, he described *G. pentaphyllum* as ‘Grow in fields, creeping, leaves taste sweet, can use in starvation’. As a result, *G. pentaphyllum*’s was treated more likely as herbal food in dietary usage than a supplement in medicinal aspect.\(^4\)

In the 1980s, Japanese research give a better understanding of the properties of *G. pentaphyllum* with its possible anticancer effect. Later at the 1991 International Conference
on Traditional Medicine, the herb was rated as one of the ten most important tonic herbs. [5]

The extinguished herbalist Li Shi-Zhen from ancestral China described *G. pentaphyllum* with effect on the treatment such as haematuria, oedema, tumors and trauma [6, 7]. *G. pentaphyllum* is slightly bitter in taste. In view of traditional Chinese medicine, it shows neutral and warm properties. It may help to promote ‘Yin’ and supporting ’Yang’, implying that *G. pentaphyllum* ‘would be used to increase the resistance to infection and for anti-inflammation’ and a list of indications is included [8-9]. The herb has been introduced in the Pharmacopoeia of The Peoples Republic of China since 2010 version. It is prescribed in traditional Chinese medicine for different usage such as heat clearing, detoxification and antitussive [10,11].

![Figure 1.1.1 Gynostemma pentaphyllum](image-url)

*Figure 1.1.1 Gynostemma pentaphyllum*
1.1.2 Active ingredients in *Gynostemma pentaphyllum*

*G. pentaphyllum* contains variety of compounds including saponins\[^{13,14}\], flavonoids\[^{15}\], sterols\[^{16,17}\], amino acids, vitamins, trace elements and a sweet component called phyllodulcin\[^{18,19}\]. Among them, previous phytochemical studies of the *G. pentaphyllum* have shown the saponins are the major constituents and contribute to most of its pharmacological properties.\[^{20}\]

Saponins are categorized as glucosides that with foaming characteristics and bitter taste. The molecular structure consists of a polycyclic aglycones attaching to sugar side chains. Either the steroid (C27) or a triterpene (C30) part of aglycone (or called sapogenin) can be found. The foaming ability of saponins is probably combination of the hydrophobic part of sapogenin as well as the hydrophilic part of sugar side chain.

Over hundred of saponins have been isolated and identified\[^{21}\] in *G. pentaphyllum*. Gynostemma total saponins, namely gypenoside or gynsaponins exist as dammarane type-triterpene gypenoside\[^{22,23,24}\]. Several gypenosides are the same as the protopanaxadiol-type Ginsenoside Rb1 (Gypenoside III)\[^{25,26}\], Rc,\[^{27}\] Rb3 (Gypenoside IV), F2 (Gypenoside VII)\[^{27}\], Rg3\[^{28}\], malony-Rb1 and malony-Rd (6”-malonyl Gypenosides III and VIII)\[^{25}\] found in *P. ginseng*. 
Figure 1.1.2 The Carbon Skeleton of the Aglycone of Dammarane-Type Triterpenoid Saponins
1.1.3 Biological effects of *Gynostemma pentaphyllum*

### 1.1.3.1 Effects on cancer

Previous researches have revealed that some of the saponins of *G. pentaphyllum* have effect in the treating tumors [35, 36]. Dose-dependent manner of the herb shows suppressive effect in the human cervix intact cancer cells and cytosols to the NAT activity and its mRNA expression. In the use of a conventional tonic mixture including *G. pentaphyllum*, it is observed that body resistance is strengthened while tumor cell growth in human lung is inhibited [44]. Growth of mice sarcoma (S180) is found to be inhibited with administration of total gypenoside [37]. Direct cytotoxic effects on S180 cells was found with gypenoside (0.38–0.75% in vitro) [38]. Gypenosides have showed effect in prevention of cyclophosphamide induced mutagenesis and promoted DNA recovery in mice model study [39, 40]. Supplimentation of gypenoside XXVII is found to lengthen the survival days of mice having ascites tumor [41].

### 1.1.3.2 Effects on cardiovascular system

The herb was found to have effect on cardiovascular activities. Coronary artery flow of anaesthetized dogs was found to be improved with decreasing in blood pressure, blood flow resistance, cardiac tension-time index with administration of total gypenoside [38]. Gypenosides also showed effects on protecting myocardial cells in neonatal rat myocardium, resulting in down regulation of the release of creatine phosphokinase and lactate dehydrogenase. Also, treatment of gypenoside also inhibited the damage caused by glucose starvation and oxygen insufficiency [38].
1.1.3.3 Effects on central nervous system

DNA and RNA damages in cerebral cortex and hippocampus was significantly improved with treatment of gypenosides in vascular dementia rat \[^{42}\]. Protective effect of gypenosides (40 mg kg\(^{-1}\) i.g) against ischemia reperfusion injury of hippocampus structure in rats was observed \[^{43}\]. Gypenosides molecules were also found to have inhibitory effect on ATP in uncompetitive way \[^{44}\].

1.1.3.4 Effects on immune functions

Immunopotentiating properties of gypenosides was found. Cyclophosphamide induced-reduction in serum haemolysin antibody level is observed with 5 days administration of total gypenosides to mice.

1.1.3.5 Other protective effects

\textit{G. pentaphyllum} also display anti-aging effect and anti-oxidant effect. \textit{G. pentaphyllum} also shows enhancing effect on lymphocyte transformation to reduce immunosuppression effect in chemotherapy \[^{45}\]. Toxicity of lidocaine was reduced, resulting in lower lethal rate of the mice upon gypenoside administration \[^{38}\].
1.2 Pharmacokinetic Studies of gypenoside in GpS

Pharmacokinetics is one of the important concepts in pharmacology, which interested in relations among Absorption, Distribution, Metabolism and Excretion (ADME) of drug. The study of pharmacokinetics is aimed to reach the prescription of each drug at the dosage which ensures the best efficiency and the minimized side effects. The blood concentration of the drug is used to study the disposition of drugs and to monitor the progress of therapy.

The fate of drug composed of following procedure.

**Administration** which means the drug is taken intravenously, intramuscularly, subcutaneously, intraperitoneally or orally etc.

**Absorption** is the movement of a drug into the bloodstream, it is affected by the routes of administration (such as orally, topically, intravenously) and the dosage form (such as capsule, tablet). For drug administered intravenously, it can be directly absorbed with near 100% bioavailability. Absorption efficiency is always one of the main focus during drug development. Sight adjustment in the factors that affect absorption may significantly alter the pharmacokinetic profile.

**Distribution** refers to the diffusion or dissemination of the stuff between the fluid parts and tissues parts of the body. Many factors such as solubility, cardiac flow, permeability and nature of surrounding compartment may affect the distribution of a drug through tissues. Distribution to interstitial and intracellular fluids happen upon the entrance of drug into
systemic circulation. Drug is easily distributed in high perfused organ including liver while it is less distributed in low perfused organs such as muscle. Movement of the drug is permitted until equilibrium is established between plasma and tissue.

**Metabolism** describes their biochemical modification or degradation through specialized enzymatic systems. Conversation of lipophilic compounds into polar products for excretion can often been seen in metabolism. Metabolic rate of a drug raises an important factor determining the pharmacological action.

**Excretion** is the elimination of waste products of metabolism and other useless materials from the body. Kidneys are the main excretory organ.

**The pharmacokinetics parameters:**

(1) **half-life (t₁/₂)**

It describes the time it takes for the blood plasma concentration of a substance/drug to halve ("plasma half-life") of its steady-state.

(2) **Maximum concentrations under the curve (Cₘₐₓ)**

It refers to the maximum concentration that a drug achieves in tested area after the drug has been administrated and prior to the administration of a second dose.

(3) **The time to reach the maximum concentrations (tₘₐₓ)**

It is the time at which the maximum concentration (Cₘₐₓ) of drug in the plasma occurs.

(4) **The area under plasma concentration-time curve (AUC)**

It corresponds to the integral of the plasma concentration versus an interval of definite time; it allows the measurement of the bioavailability of a drug.
(5) The volume of distribution \( (V_d) \)

It is an assumed volume, in which the drug would have been distributed in the assumption of homogeneous concentration. It allows evaluation of the dose to administer.

(6) Clearance (Cl)

It is the fraction of a theoretical volume completely purified per unit of time. Plasma clearance is the apparent volume of plasma purified per unit of time. Total clearance refers to which is completely purified per unit of time.

(7) Mean Retention Time (MRT)

Residence time often refers to the amount of time that a pharmaceutical spends in the body. MRT is the average drug dose clearance time.
1.3 Rationale

Colorectal cancer is one of the most commonly diagnosed cancers worldwide with relatively high death rate. Patient diagnosed colorectal cancer in early stage shows significant higher survival rate compared to that detected at late stage. Method developments of an effective early diagnosis and staging precisely are thus important.

By targeting on the steps of carcinogenesis, chemical agents are used to suppress or prevent the carcinogenic progression in chemoprevention. Although some drugs and natural agents have been in practice such as NSAIDs, aspirin, etc. However, the associated toxicity and side effects of those drugs are inevitable.

*G. pentaphyllum* is an herbal drug of diverse therapeutic effects without obvious toxic effects in the view of traditional Chinese medicine. Saponins extracted from *G. pentaphyllum* are most likely to be the main active ingredients for its various biological function and clinical effect. However, the questions about the pharmacokinetic is still a riddle. In this chapter, the following objectives will be focused on:

(1) To determinate the pharmacokinetic of total saponins extraction of *G. pentaphyllum* at a single oral dosage of 6 g/kg GpS in C57BL/6J wild type mice plasma by UPLC-MS analysis. This study will use on gypenoside III, IV, VIII, 20(S) and 20(R)-Rg3 as a marker of total gypenosides.
2. Materials and Methods

2.1 Animals

Wild type mice in the C57BL/6J background were used in the plasma concentration-time course study. The mean age was 5 weeks and the body weight was between 21-27 kg.

2.2 Chemical

Gypenoside IV (Ginsenoside Rb3) and Gypenoside VIII (Ginsenoside Rd) (purity ≥ 98 %) were purchased from Aktin chemical Company (China). Gypenoside III (Ginsenoside Rb1), and 20(S) and 20(S)-Rg3 were provided by Prof. Zhi-Hong Jiang. Gypenoside G (Gp-G) and G. pentaphyllum powder were extracted and purified in our lab. Diethyl ether was used for anesthesia. The saline (0.9 % sodium chloride) was purchased from Baxter. Methanol was of HPLC grade from Lab-Scan. Acetonitrile was of HPLC grade from Tedia.

2.3 Drug preparation

The GpS solution was prepared by dissolving the GpS powder in MilliQ H2O at a concentration of 200 mg/mL and then filtered by 0.2 μm syringe filter. The drug is newly prepared at the experiment day.

2.4 Drug Administration and Sample Collection

Total of 41 mice were used in the pharmacokinetic study of gypenoside in GpS by parallel sampling technique. After fasted overnight, each mouse administrated 6 g/kg GpS
solution by oral administration at the 0 min. Mice were sacrificed by overdose of diethyl ether inhalation and blood sample was collected from the posterior vena cava by 1 mL heparinzed syringe at 0, 0.5, 0.75, 1, 2, 3, 6, 9, 12 and 24 hours. Plasma samples taken from 3-4 individual mice were collected at each time point for an independent experiment with total 10 time points. Plasma samples are prepared by centrifuge at 1500 x g for 15mins at 4 °C. The plasma samples were frozen at −80 °C for future use.

2.5 Preparation of Plasma Samples

Aliquot 50 μL plasma samples into clean Eppendorfs. Same volume ratio (1:1) of ACN (acetonitrile) was added into the plasma to precipitate the proteins. The supernatants were filtered through 0.22μm syringe filter and then were 10 fold diluted by Mini Q water. The plasma samples were then submitted for quantitative bioanalysis HPLC-MS (Triple-Q). PK parameters were calculated using non-compartmental analysis (NCA).

2.6 UPLC-MS Analysis

Analysis was performed on a 1290 Infinity LC system (Aglient), equipped with a binary solvent manager, an auto-sampler and a degasser. The UPLC separation was performed on an ACQUITY UPLC HSS C18 1.8μM (2.1 x 100mm column) connect with an Aglient ZORBAX Eclipse plus C18, Rapid Resolution HD (2.1 x 50mm) 1.8μm. The mobile phases consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The gradient elution was 10 % B at 0-2 min; 26 % B at 2-16 min; 40 % B at 16-25 min; 100% B at 25-29 min; 10 % B at 29.1-33 min. The flow-rate was 0.35 mL/min, the column temperature was kept at 40 °C, and the injection volume was 4 μL.

Detection was performed on an Agilent 6460 LC Triple Quadrupole Mass
Spectrometer (Agilent) with an ESI source. The ionization of analytes was carried out in negative mode. Mass parameters were as follow: drying gas (N₂) temperature, 350 °C, drying gas flow-rate, 8 L/min; Nebulizer 8psi; Sheath gas temperature, 400 °C and flow-rate at 12 L/min; Capillary, 3500 V and Nozzle Voltage, 1500 V.

All the instrument control, data acquisition, qualitative and quantitative data analysis, and reporting were controlled by MassHunter Workstation Software (Agilent).

2.7 Method Validation

2.7.1 Calibration Curve

The stock solution of Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(R) and 20(S)-Rg3 and were prepared by dissolving the accurately weight reference compounds powder in 100% MeOH at a concentration of 10 mg/mL and stored at -20 °C. The stock solution was then dilution to yield median standard working solutions at concentration of 4000, 2000, 1000, 500, 100, 50, 10, 5 and 1 ng/mL. The median working solutions were stored at 4 °C before use.

Calibration curve samples were prepared by spiking aliquots of the median working solutions of Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(R) and 20(S)-Rg3 into drug-free (blank) plasma samples. The final concentrations of calibration curve samples were 2000, 1000, 500, 100, 50, 10, 5, 1 and 0.5 ng/mL of Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(R) and 20(S)-Rg3 respectively. The calibration curve was constructed by plotting peak area ratio of Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(R) and 20(S)-Rg3 versus their corresponding concentrations. The regression parameters of slope, intercept and correlation coefficient were calculated by weighted (1 / X²) linear regression.
2.7.2 Gypenoside Recovery

The extraction recoveries were determined by five replicates of mice plasma spiked with standard Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(R) and 20(S)-Rg3 compound at the concentrations of 125 ng/mL. The recovery rate is calculated by comparing the peak area of each analyte in plasma samples which process in two different ways. The analyte pre-spiked plasma samples were prepared by spiking the working standard solutions into drug-free plasma which then were together processed by ACN precipitation method. The post-spiked samples were prepared by spiking the analytes in the blank plasma which has been treated by ACN precipitation process.
3. Results

3.1 Method development and validation

3.1.1 Chromatogram and Specificity

This study is focus on investigating the pharmacokinetic of GpS after single dosage of 6 g/kg GpS by oral feeding to mice. An identification and quantitative analytical procedure for the determination of gypenosides in plasma has been developed and validated. The analyte were quantified using Triple Quadrupole Mass spectrometer in negative electrospray ionization mode (ELS). The specificity of the method was demonstrated by comparing MS chromatograms for reference standard (Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3) for a drug-free plasma, a spiked plasma sample and a plasma sample from mice different time point after oral administrated of drug.

For confirmation, two transitions were monitored and one ion ratio was determined which was within 50% of that of the known calibration standards. The range of concentration analyzed for each compound was 0.5 to 500 ng/mL. Under the ionization of ESI, the mass mainly produced from Gp-III, Gp-IV, Gp-VIII, Gp-G and 20(S) and 20(R)-Rg3 were chosen as Quantifier (Ai), Table 1, while one more fragmented ion of each gypenoside was also determined as Qualifier (Qi). Areas of the above masses were determined by Triple-Q MS, the corresponding concentration was found by using calibration curve. The validity of the method was ensuring by the concentration ratio of Qi/Ai. The new EU guidelines for identification and quantification were fulfilled.\textsuperscript{[57]} The following figures show the ion chromatograms of the above referred mass and the tolerance of the analytes.
<table>
<thead>
<tr>
<th>Gypenoside</th>
<th>Gp-III</th>
<th>Gp-IV</th>
<th>Gp-VIII</th>
<th>Gp-G</th>
<th>20(S)-Rg3</th>
<th>20(R)-Rg3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantifier (m/z)</td>
<td>1107.50</td>
<td>1077.56</td>
<td>945.50</td>
<td>769.40</td>
<td>783.50</td>
<td>783.50</td>
</tr>
<tr>
<td>Qualifier (m/z)</td>
<td>945.60</td>
<td>783.60</td>
<td>783.50</td>
<td>637.50</td>
<td>621.50</td>
<td>621.50</td>
</tr>
</tbody>
</table>

*Table 1.3.1 The Quantifiers and Qualifiers mass of gypenosides in GpS*
(A) 

(B) 

(C) 

(D) 

C53H92O23 Gypenoside III 

C48H82O18 Gypenoside VIII 

C53H90O22 Gypenoside IV
Figure 1.3.1 Mass Spectra of (A) Gypenoside III, (B) Gypenoside IV, (C) Gypenoside VIII, (D) Gypenoside G, (E) Gypenoside 20(S)-Rg3 and 20(R)-Rg3.
Figure 1.3.2 The tolerance of the analytes used in this study. Two transitions were monitored and one ion ratio was determined, all results were within 50% of that of the known calibration standards. The monitored ions are shown in (A) Gp-III, (B) Gp-IV, Gp-VIII, (D) Gp-G and (E) 20(S) and 20(R)-Rg3.
The mass spectra of Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3 are shown below. Under the developed chromatographic conditions, the retention times for Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3 were 12.03, 14.09, 15.84, 20.14, 20.25 and 20.40 min respectively. Under the LC-MS running condition, the peaks of the analytical mass of the gypenosides were well separated to each other in both G. pentaphyllum total extraction and plasma without significant interfering peaks. The results indicated the specificity of the method was acceptable.

Firstly, the percentage amount of Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3 in GpS was found using the above method. It is found that there is 1.10% of Gp-III, 3.21% of Gp-IV, 1.69% of Gp-VIII, 1.98% of Gp-G, 1.52% and 1.38% of 20(S)-Rg3 and 10(R)-Rg3 in GpS respectively.
Figure 1.3.3 Mass Spectra of (A) 100%MeOH (B) Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3 in GpS dissolved in 100% MeOH at the 1ppm
For the mice plasma sample analysis, mice drug-free plasma was used as matrix of the calibration curve to eliminate the irreverence interference peaks. The following figures show the ion chromatograms of blank plasma, blank plasma spiked with Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3 and the plasma sample after oral administration of the GpS.

(A) 

(B) 

(C) 

Figure 1.3.4 Extracted ion chromatogram of standard (A) Gp-III, IV, VIII, G and 20(S) and 20(R)-Rg3 in 100%MeOH, (B)Blank plasma, and (C)standard mix spiked in blank plasma(C) at 1 ppm concentration.
3.1.2 Standard curve for GpS time course

Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(S) and 20(R)-Rg3 standard were spiked in blank plasma and prepared for standard Linear calibration curves. Results were shown that the linear calibration curves with linear coefficients $R^2$ greater than 0.98 were obtained in the concentration range 0.5-2000 ng/mL in plasma for the standards.
Figure 1.3.5 Linear standard calibration curves of (A) Gp-III, (B) Gp-IV, (C) Gp-VIII, (D) Gp-G, (E) 20(S)-Rg3 and (F) 20(R)-Rg3 spiked with plasma in the concentration range 0.5-2000 ng/mL.
<table>
<thead>
<tr>
<th>Concentration(ng/mL)</th>
<th>Gp-III</th>
<th>Gp-IV</th>
<th>Gp-VIII</th>
<th>Gp-G</th>
<th>20(S)-Rg3</th>
<th>20(R)-Rg3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>125.41</td>
<td>254.07</td>
<td>405.28</td>
<td>145.44</td>
<td>411.25</td>
<td>382.02</td>
</tr>
<tr>
<td>1</td>
<td>219.14</td>
<td>498.20</td>
<td>795.30</td>
<td>199.58</td>
<td>809.24</td>
<td>639.56</td>
</tr>
<tr>
<td>5</td>
<td>1074.93</td>
<td>2426.87</td>
<td>3216.23</td>
<td>249.99</td>
<td>3631.21</td>
<td>2784.37</td>
</tr>
<tr>
<td>10</td>
<td>2253.28</td>
<td>5423.80</td>
<td>8089.19</td>
<td>660.80</td>
<td>7856.51</td>
<td>6399.18</td>
</tr>
<tr>
<td>50</td>
<td>9561.55</td>
<td>24534.61</td>
<td>36660.05</td>
<td>2546.72</td>
<td>39268.99</td>
<td>30928.62</td>
</tr>
<tr>
<td>100</td>
<td>25376.30</td>
<td>64829.34</td>
<td>95666.72</td>
<td>7430.49</td>
<td>93707.26</td>
<td>77484.93</td>
</tr>
<tr>
<td>500</td>
<td>181502.50</td>
<td>471174.64</td>
<td>661842.90</td>
<td>70170.20</td>
<td>857262.74</td>
<td>725470.50</td>
</tr>
<tr>
<td>1000</td>
<td>404354.74</td>
<td>995812.76</td>
<td>1324081.94</td>
<td>153470.31</td>
<td>1780709.60</td>
<td>1499998.69</td>
</tr>
<tr>
<td>2000</td>
<td>782530.83</td>
<td>2092135.87</td>
<td>2965076.33</td>
<td>327374.05</td>
<td>3484036.05</td>
<td>2969952.98</td>
</tr>
</tbody>
</table>

Table 1.3.2 The area reading of linear calibration curves of (A)Gp-III, (B)Gp-IV, (C)Gp-VIII, (D)Gp-G, (E)20(S)-Rg3 and (F)20(R)-Rg3 spiked with plasma in the concentration range 0.5-2000 ng/mL.
3.1.3 Recovery of the Standard gypenosides after the ACN treatment in plasma

Extraction recovery experiment has been performed in order to determinate the effect of adding 1:1 ACN on the concentration of gypenoside in mice blank plasma samples during the protein precipitation process. Two sets of the plasma samples each with five replicates have been prepared, which means the standard solution of Gp-III, IV, VIII, Gp-G, 20(s) and 20(R)-Rg3 were spiked either into pre or post ACN-protein precipitation treated plasma samples. The concentration of gypenoside in all sample were 125 μg/mL. The results of Extraction recoveries of plasma samples are shown below.

The recovery of Gp-III, IV, VIII, Gp-G, 20(s) and 20(R)-Rg3 at concentration were more than 95% with the RSD less than 15%. These data indicated that the ACN precipitation method, used to remove protein in plasma sample, almost no effect on the actual gypenoside concentration in plasma. This extraction method is valid for the sample preparation for the downward experiment.

<table>
<thead>
<tr>
<th>Recovery (%) of gypenosides after or before ACN treatment in plasma samples</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Gp-III</td>
</tr>
<tr>
<td>Recovery</td>
<td>98.74%</td>
</tr>
<tr>
<td>RSD</td>
<td>4.77%</td>
</tr>
</tbody>
</table>

*Table 1.3.3 Extraction recovery (%) of Gp-III, IV, VIII, Gp-G, 20(S) and 20(R)-Rg3 in blank mice plasma*
3.2 Pharmacokinetic Analysis

3.2.1 Plasma concentration-time curve of glyenoside after a single dosage oral administration of 6 g/kg GpS.

The plasma concentrations of the six glyenoside of the 31 mice (randomly divided into 9 time point) after oral administration of the GpS at the dosage of 6 g/kg were determined using the established UPLC-MS method. The average plasma concentration-time profiles of the six glyenoside standards after oral administration of the GpS to mice were shown in the figures below. The graphs were overall sharing three pattern, the concentration of Gp-I, III and VIII increasing steadily after oral administration and peak at 9 hours, it then decreased gradually, the compound still could be observed after 24 hours. The plasma concentration-time graph of Gp-G, 20(S)-Rg3 increased steadily, from time 0 and seems peak at 3 hours but it is then reached another much greater peak at 9 hours rapidly.
Figure 1.3.6 (A) The plasma concentration-time curve of the six marker gypenosides after oral administration of the GpS to mice. The results were shown in one graph together. The error bar was calculated in term of SEM. (B) Amplify the bottom part of (A).
Figure 1.3.7 The average plasma concentration-time profile (A) Gp-III, (B) Gp-IV, (C) Gp-VIII, (D) Gp-G, (E) 20(S)-Rg3 and (F) 20(R)-Rg3 after oral administration of GpS to C57BL/6J mice. The error bar was calculated in term of SEM.
3.2.2 Pharmacokinetic Parameters of Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(S) and 20(R)-Rg3

Pharmacokinetic parameters of the six gypenoside after oral administration of the GpS were calculated by using the Kinetica 5.0 analytical software (Thermo Fisher Scientific Inc). The results show there is an obvious variation in the elimination of gypenosides in GpS after oral administrated. The details of PK parameter of each gypenoside were shown in the table below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gp-III</th>
<th>Gp-IV</th>
<th>Gp-VIII</th>
<th>Gp-G</th>
<th>20(S)-Rg3</th>
<th>20(R)-Rg3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2 E \text{ phase}}$ (hr)</td>
<td>13.5828</td>
<td>13.4190</td>
<td>11.7177</td>
<td>5.5832</td>
<td>5.9446</td>
<td>8.0272</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>2004.94</td>
<td>46088.20</td>
<td>16938.70</td>
<td>411.80</td>
<td>1706.56</td>
<td>1459.69</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>3.0</td>
</tr>
<tr>
<td>$\text{AUC}_{0-t}$ (ng hr/mL)</td>
<td>27695.20</td>
<td>641432.00</td>
<td>219332.00</td>
<td>2165.93</td>
<td>13308.70</td>
<td>11702.40</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (ng hr/mL)</td>
<td>45411.3</td>
<td>1040000.0</td>
<td>330145.0</td>
<td>2428.2</td>
<td>14810.1</td>
<td>14061.3</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>24.8718</td>
<td>24.6109</td>
<td>22.0027</td>
<td>11.6111</td>
<td>10.7827</td>
<td>12.9419</td>
</tr>
<tr>
<td>$\text{Vd}$ (mL/kg)</td>
<td>0.0822</td>
<td>0.0035</td>
<td>0.0100</td>
<td>0.7173</td>
<td>0.1092</td>
<td>0.1381</td>
</tr>
<tr>
<td>CL (mL hr/kg)</td>
<td>0.0033</td>
<td>0.0001</td>
<td>0.0005</td>
<td>0.0618</td>
<td>0.0101</td>
<td>0.0107</td>
</tr>
</tbody>
</table>

Table 1.3.4 Pharmacokinetic parameters of the six targeted gypenosides after oral administration of the 6 g/kg GpS in mice plasma sample.
4. Discussion and Conclusion

4.1 Discussion

4.1.1 Preparation of plasma sample

The aim of sample preparation was to remove proteins and interference substances from plasma samples to prevent interference of the UPLC-MS analysis. We have used acetonitrile (ACN) precipitation to remove proteins which has been already extensity used in protein precipitation. Our results have shown that ACN precipitation can effectively remove proteins with high recovery rate of gypenosides in plasma samples which facilitated the downstream UPLC-MS analysis.

4.1.2 Pharmacokinetic study of mice plasma

In our pharmacokinetic study, mice were used because we extensively used mice as an animal model in studying pharmacologic effects of GpS. In this study parallel blood sampling technique was employed, i.e. each mouse is subject to only one blood draw. Previous researches showed that parallel blood sampling technique is a valid study method\cite{61,62}. This method can ensure enough plasma samples can be harvested at each time point without causing potential stress on animals which might affect the pharmacokinetic results.

4.1.3 Pharmacokinetic parameters of Gypenosides

For Gp-III, IV and VIII compounds, the drug concentration in plasma steadily increase over time after oral administration and peak at around 9 hours; it then decreases gradually, gypenosides could still be detected at 24 hours treatment. The increasing trend of the curve represents the absorption phase of the drug, and decreasing trend is due to the distribution and elimination of drug. As the above gypenosides could
still be observed at 24 hours, in order to further understand the retention time of gypenosides in mice, a longer plasma time course could be done.

The plasma concentration-time curves of Gp-G, 20(S)-Rg3, 20(R)-Rg3 exhibited distinct double peaks after oral administration. There are two possible reasons: (1) involvement of enterohepatic recirculation\cite{63,64}, (2) fluctuation cause by fasted body states. The double-peak phenomenon might be due to the absorption of these gypenosides in enterohepatic recirculation, i.e. gypenoside absorbed from the intestine (the first peak) and transported to the liver and gall bladder, where gypenosides secreted into the bile and again re-enter the intestine by bile secretion (the second peak). The identification of enterohepatic recirculation could be determined by a comparison of AUCs obtained after oral administration of Gp-G, 20(S)-Rg3, 20(R)-Rg3 in normal and bile-duct cannulated animals.

Another reason of the double-peak phenomenon might due to the fluctuation cause by fasted body states. The bioavailability of a substance is better in fasted animals and with great amount of water supply. Alteration in the release rate and absorption rate of certain drug may be affected by intake of different food due to the change of integrity of the dosage form. Therefore, pharmacokinetic studies, including our study, were done under fasted states of animals. Extensive literatures have shown that administration of a single dose to mice in fasted states may generally result in double-peak phenomenon\cite{65}. This phenomenon may be happened due to changes in stomach emptying, changes in intestinal motility or alteration of the dosage form.

Compare to Gp-III, IV and VIII, after oral administration, Gp-G, 20(S)-Rg3 and 20(R)-Rg3 has a relatively low plasma concentration. Commonly, the low plasma concentration is caused by the following reasons: (1) drug entered into gastrointestinal tract has been decomposed by enzyme in gastrointestinal tract or bacteria in intestinal
tract. Gypenosides decomposed into metabolites with different analytical mass required in UPLC-MS analysis. (2) involvement of first-pass effect. After oral administration, drugs are absorbed by the digestive system and enter the hepatic portal system. The drugs are carried into the liver before they can arrive to the rest of the body. Although a large amount of drugs are metabolized in the liver, there may only be small amount of active drug that can be delivered to the rest of the body via circulatory system. As a result, a great reduction of drug bioavailability thus occurs.

To sum up, the two possible reasons of the double-peak phenomenon might due to the enterohepatic recirculation of gypenosides and fasted states of animals. The relatively low plasma concentration of Gp-G, 20(S)-Rg3 and 20(R)-Rg3 might be due to bioactivity of enzyme and gastrointestinal bacterial and involvement of first pass effect. The suggestions need further studies.
4.2 Conclusions

A sensitive and specific UPLC-MS analysis method has been developed and validated for the quantification of GpS in plasma samples by using Gp-III, Gp-IV, Gp-VIII, Gp-G, 20(S) and 20(R)-Rg3 as its markers. The same method also valid in studying of single gypenoside compound (Gp-VIII). Calibration curves displayed good linearity over the range of 0.5-2000 ng/mL with \( r^2 > 0.98 \). The plasma samples were prepared by ACN precipitation with recovery more than 95 %. Moreover, a valid plasma time course of GpS in mice has been developed by using parallel blood samples sampling technique.

The two developed methods were subsequently applied to the pharmacokinetic studies of the gypenosides above in mice successfully. The elimination half-life, area under the plasma concentration-time curve (AUC), mean-residence time (MRT), volume of distribution and clearance (Cl) were calculated by using non-compartmental analysis. The pharmacokinetic parameters of the marker gypenosides have shown that GpS and Gp-VIII alone were absorbed in mouse blood after oral administration.
CHAPTER 2.
Study of the effect of Gp-VIII on glycolytic pathway

1. Introduction

1.1 Glucose as energy source

In general, glucose consumption is required by all living systemic cell. To maximum energy production in form of ATP, glucose is broken down into pyruvate, followed by series of oxidation via tricarboxylic acid (TCA) cycle. Glucose would be converted into CO₂ and H₂O through TCA cycle plays key role in energy production. Apart from ATP biosynthesis, citric acid cycle also contributes in constitution of DNA, RNA and phospholipids.

1.2 Energy production in normal cells

Glycolytic pathway is a universal in all living cell for glucose metabolism [77]. Briefly, it happens in cytoplasm. Energy is released in form of two ATPs and two NADH molecules during glycolysis process. A molecule of glucose produce undergoes glycolytic pathway to give two molecules of pyruvate.

In the presence of oxygen, the two molecules of pyruvates are converted into two acetyl-CoA molecules which then enters mitochondrion followed by the metabolism of citric acid cycle. Each glucose yields six NADH and two FADH. These molecules then enter electron transport system for completely utilization, producing ATPs efficiently. Theoretically, ideal yield of numbers of ATPs are 40 per glucose molecule. However, final yield may vary depending on enzymes encoded in different individual.

1.3 Glycolysis in cancer cells

Although glycolysis is a relatively inefficient way in generating energy when
comparing to complete oxidation in term of net yield of ATP per unit glucose, the rate of glucose metabolism through glycolysis is up to 100 times faster than complete mitochondrial respiration\[^{74}\].

### 1.3.1 The Warburg Effect

Cancer cells is highly proliferative without normal programmed cell growth due to DNA damage or abnormal expression. It may be highly invasive and may spread to other part of body. Unlike normal cells which utilizing complete oxidation of glucose, cancer cells shift glucose metabolism into less efficient aerobic glycolysis. The phenomenon was first described by Otto Warburg in 1924 \[^{73,77}\].

It is observed that in the presence of normal oxygen tension, mitochondrial respiration is responded in the generation of energy. However, in cancer cells, glycolytic pathway in the cytosol region contributes over 50% of energy produced. The shift of utilization of glycolytic pathway in cancer cell is not due to lack of oxygen. As mitochondrial respiratory system also works normally contributing the remainder energy requirement.

To meet extra energy demand required for cancer cell growth, cancer cells may adopt increased glucose import via different mechanism \[^{76,77}\]. For example, it is observed that glucose transporters such as Glut1 and Glut3 in plasma membrane were overexpressed. Moreover, regulation of efficiency of glycolysis also contributes the increase of glucose consumption. For example, change of activity of glycolytic enzymes helps regulate glycolytic flux and favors the accumulation of metabolite for cancerous biosynthetic needs.

### 1.3.2 Adaptation to hypoxic condition

HIF-1 is key transcription factor that regulate genes involved in hypoxia induced
metabolic switch and regulation of pH\textsuperscript{[77,78]}. Hypoxia-inducible factor-1 (HIF-1) plays role for the adaptation of cells from normoxia condition to hypoxia condition\textsuperscript{[79]}. Under oxygen-deprived hypoxic conditions, depletion of oxygen directly decreases the prolyl hydroxylation activity of HIF-1α. Stabilized HIF-1α together with HIF-1β forming a heterodimer of HIF-1. Activated heterodimer binds to hypoxicresponsive element in its cognate DNA sequence. Expression of various genes related to angiogenesis, metastasis and glycolysis are regulated by HIF-1. Glycolysis is hence indirectly regulated by expression of HIF-1α.

1.4 Rationale

Previous studies show that cancer may perform higher uptake rate of glucose while producing lactic acid as final metabolite rather than undergoes the TCA cycle for complete oxidation\textsuperscript{[73]}. The increasing bioenergetics via metabolic shift observed in cancer cell is known as the Warburg Effect. As metabolic precursors, intermediate metabolites during glycolysis may also involve in other biosynthesis for biosynthesis\textsuperscript{[75]}. These key benefits in aerobic glycolysis result in the shift of cancer cells to favor glycolysis over complete mitochondrial oxidation. To study the effect of Gp-VIII in glycolytic pathway, inhibitory studies of different glycolytic enzymes were carried out in human colon cancer cell line, HCT116\textsuperscript{[80]}.

1.4.1 MTT assay

MTT assay is an assay for assessing cell metabolic activities in colorimetric approach. It is commonly used as cell viability to reflect the number of viable cells upon drug treatment. In this project, to adjust the maximal concentration of Gp-VIII administration, MTT assay is used to determine the lethal dosage of Gp-VIII to HCT116 cells. Optimization of drug concentration is done without greatly affecting the cell viability upon Gp-VIII administration.
1.4.2 Inhibitory study

Inhibitory studies of Gp-VIII on glycolytic enzymes were carried out. Upon treatment of Gp-VIII, relative fold change of different glycolytic enzymes is measured. Base on the known information of glycolytic pathway, proposed mechanism behind the effect of Gp-VIII is drawn.

1.4.3 Apoptotic analysis

Apoptotic analysis of cells in the presence of Gp-VIII would also be investigated. Inhibition in glycolytic pathway may leads to insufficient energy required in cell proliferation, resulting in cell cycle arrest. Abnormally arrested cell maybe detected via checkpoint and projected into apoptosis.
2. Materials and Methods

2.1 Cell culture and drug treatment

HCT116 cells were kept in M5A with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37°C and 5% CO2 in a humidified atmosphere. The cells were grown in monolayer and passaged routinely twice a week.

2.2 MTT assay

4000 cells were seeded in each well of 96-well plates. After 24 hours, the medium was replaced by Gp-VIII containing medium. The treatments were 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 nM for 24, 48 and 72 hours, respectively. Cells were observed under microscope after incubation at harvest time. MTT solution (20μL) was added to each well, and the plates were incubated in the dark for 4 hours at 37°C. Then, the media was removed. 100 μL dimethyl sulfoxide (DMSO) was added to each well and shook the plate for 10 minutes. Afterwards, the mixture was read at absorbance 570nm.

2.3 Gp-VIII treatment, cells collection, cell lysate preparation and protein estimation

For Gp-VIII treatment, cells were seeded in 60mm plate for 24 hours. They were then treated with 0, 100, 150, 200 and 250nM Gp-VIII media for 72 hours. At the time of harvest, the cells were scrapped in ice-cold PBS and pelleted by centrifugation at 800xg at 4°C for 5 minutes. The supernatant was discarded. Then, the cells were washed in 1 mL ice-cold PBS and centrifuged at 800xg at 4°C for 5 minutes. The supernatant was discarded, and the cell pellets were stored at -80°C until use.

For cell lysis, cells were lysed in RIPA buffer with protease inhibitors for 30 minutes on ice. The lysate was then centrifuged at 25,000xg in 4°C for 10 minutes. The
supernatant was collected and used for Western Blot analysis. To determine the protein concentration of the cell lysate, DC protein assay kit (Bio-Rad) was used as per manufacturer’s instructions.

2.4 Glucose uptake assay

Medium was collected from plate and glucose uptake was analyzed using glucose assay kit as per the manufacturer’s instructions.

2.5 Western Blotting

Sample was prepared by mixing cell lysate with sample buffer. Then, the protein was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane using semi-dry transfer system. The membrane was then blotted in 5% non-fat skim milk for 1 hour at room temperature. After that, the membrane was probed with primary antibody in 5% BSA overnight at 4°C. After that, the membrane was washed with 1XTBST (mixture of Tris-Buffered Saline and Tween 20) for 12 minutes at 200rpm at room temperature. Then, corresponding secondary antibody was added to the membrane for 45 minutes at room temperature. The membrane was washed in 1XTBST for 30 minutes. Protein bands were visualized by Amersham ECL Western Blotting Analysis System (GE Healthcare).

PKM1, PKM2, triosephosphate isomerase, pyruvate carboxylase, enolase, aldolase A, GAPDH, LDH, β-actin antibodies were purchased from Santa Cruz Biotechnology (USA). Protein levels were quantified by scanning autoradiogram and densitometry.
3. Results

3.1 MTT assay

Median Lethal Dose (LD50) refers to the concentration of drug administration which results in 50% of cell death.

Result shows that from 0 to 250nM of Gp-VIII administration, there were not great drop in cell viability. Upon 500nM and 1000nM Gp-VIII treatment, most of the cells were dead. By calculation, LD50 of Gp-VIII for 24, 48 and 72 hours cell incubation were 474.3nM, 453.4nM and 446.3nM respectively.

Based on the MTT result, 250nM Gp-VIII was used as the highest dosage in the following experiments.

![Figure 2.3.1 Effect of the increasing concentration of Gp-VIII on cell viability by MTT assay](image)
3.2 Decrease of glucose consumption

Medium after incubation was collected and glucose uptake assay were done. 11.2% decrease of relative glucose consumption was observed after addition of 200nM Gp-VIII. Up to 21.3% decrease of relative glucose consumption from media after incubation were observed with 250nM Gp-VIII administration. It shows that glucose uptake rate may probably be down-regulated by Gp-VIII treatment.

*Figure 2.3.2 Result of glucose uptake assay after cell culture with Gp-VIII treatment*
3.3 Effect of Gp-VIII on regulation of glycolytic enzyme

Protein were extracted by cell lysis, followed by western blot analysis. Corresponding primary anti-bodies was used to measure the expression of target glycolytic enzyme upon Gp-VIII treatment. Housekeeping gene of β-actin was also used as reference to normalize protein concentration for fair comparison.

3.3.1 Aldolase A

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that concentration of aldolase decrease with addition of Gp-VIII. A decrease of 37% of relative fold change of aldolase was observed with 200nM Gp-VIII while 27% decrease can be seen upon 250nM treatment of Gp-VIII.

![Western blot result and relative fold change of Aldolase A with Gp-VIII treatment](image)

*Figure 2.3.3.1 Western blot result and relative fold change of Aldolase A with Gp-VIII treatment*
### 3.3.2 Triosephosphate isomerase

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that concentration of Triosephosphate isomerase (TPI) decrease with addition of Gp-VIII. A decrease of 22% of relative fold change of TPI was observed with 200nM Gp-VIII while 55% decrease can be seen upon 250nM treatment of Gp-VIII.

![Western blot result and relative fold change of Triosephosphate isomerase with Gp-VIII treatment](image)

*Figure 2.3.3.2 Western blot result and relative fold change of Triosephosphate isomerase with Gp-VIII treatment*
3.3.3 GAPDH

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that there were a mild decrease in concentration of GAPDH with addition of Gp-VIII. A decrease of 15% of relative fold change of GAPDH was observed with 200nM Gp-VIII while 13% decrease can be seen upon 250nM treatment of Gp-VIII.

Figure 2.3.3.3 Western blot result and relative fold change of GAPDH with Gp-VIII treatment
3.3.4 Enolase

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that concentration of enolase decrease with addition of Gp-VIII. A great decrease of 37% of relative fold change of enolase was observed with 250nM Gp-VIII while a decrease of 26% can be seen in 200nM Gp-VIII group.

![Western blot result and relative fold change of Enolase with Gp-VIII treatment](image)

*Figure 2.3.3.4 Western blot result and relative fold change of Enolase with Gp-VIII treatment*
3.3.5 PKM2

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that concentration of PKM2 decrease with addition of Gp-VIII. A decrease of 14% of relative fold change of PKM2 was observed with 200nM Gp-VIII while 20% decrease can be seen upon 250nM treatment of Gp-VIII.

Figure 2.3.3.5 Western blot result and relative fold change of PKM2 with Gp-VIII treatment
3.3.6 LDH

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that concentration of LDH decrease with addition of Gp-VIII. A mild decrease of 14% upon 150nM GP-VIII administration can be seen. A decrease of 27% of relative fold change of LDH was observed with 200nM Gp-VIII while 33% decrease can be seen upon 250nM treatment of Gp-VIII.

Figure 2.3.3.6 Western blot result and relative fold change of LDH with Gp-VIII treatment
3.3.7 PKM1

Concentration of 100nM, 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that upon treatment of Gp-VIII, an increase of Gp-VIII concentration had no obvious effect on relative fold change of PKM1.

Figure 2.3.3.7 Western blot result and relative fold change of PKM1 with Gp-VIII treatment
3.3.8 HIF-1α

Concentration of 150nM, 200nM and 250nM of Gp-VIII administration were used as treatment group. Results show that concentration of HIF-1α decrease with addition of Gp-VIII. A decrease of 21% of relative fold change of HIF-1α was observed with 200nM Gp-VIII while 36% decrease can be seen upon 250nM treatment of Gp-VIII.

*Figure 2.3.3.8 Western blot result and relative fold change of HIF-1α with Gp-VIII treatment*
3.4 Effect of Gp-VIII on cell growth

3.4.1 Induction of cell cycle arrest

150nM, 200nM and 250nM of Gp-VIII were used as treatment group. Results show that increase concentration of Gp-VIII administration results in more cells getting arrested in G2 phase. In the treatment of 250nM Gp-VIII, 39.39% cells were arrested at G2 phase while there were only 7.29% of cells in G2 phase in control group. It reveals that cell proliferation maybe inhibited by Gp-VIII.

![Figure 2.3.4.1 Analysis for cell cycle distribution of HCT116 cells with different concentration of Gp-VIII treatment](image)

*Figure 2.3.4.1 Analysis for cell cycle distribution of HCT116 cells with different concentration of Gp-VIII treatment*
### 3.4.2 Induction of cell apoptosis

Increase concentration of Gp-VIII administration results in more cells were categorized in Q3-UR window. In the presence of 250nM Gp-VIII, 64.3% cells were distributed in Q3-UR. It indicates more cells were available for the binding of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) dye in the presence of Gp-VIII probably due to cell apoptosis induced by abnormal cell cycle arrest.

![Figure 2.3.4.2 Flow cytometry analysis for cell apoptosis](image)

**Figure 2.3.4.2 Flow cytometry analysis for cell apoptosis**
4. Discussion and Conclusions

4.1 Discussion

4.1.1 Importance of MTT assay

MTT assay is a traditional practice in confirming the viability of mammalian cell after drug treatment. In the project, to investigate the inhibitory effect of Gp-VIII in glycolytic pathway, MTT assay was used to determine the LD50 value of Gp-VIII on Homo sapiens colorectal cancer cell line HCT116. Maximum concentration of Gp-VIII was thus chosen to minimize the lethal effect. Based on the MTT result, 250nM Gp-VIII was used as the highest dosage in the onward experiments.

4.1.2 Glycolytic pathway in cancer development

In general, glucose consumption is required by all living systemic cell. To maximum energy production in form of ATP, glucose is broken down into pyruvate, followed by series of oxidation via tricarboxylic acid (TCA) cycle. Previous studies show that cancer may perform higher uptake rate of glucose while producing lactic acid as final metabolite rather than undergoes the TCA cycle with pyruvate \(^1\). The increasing bioenergetics via metabolic shift observed in cancer cell is known as the Warburg Effect. Although glycolysis is a relatively inefficient way in generating energy when comparing to complete oxidation in term of net yield of ATP per unit glucose, the rate of glucose metabolism through glycolysis is up to 100 times faster than complete mitochondrial respiration \(^2\). As metabolic precursors, intermediate metabolites during glycolysis may also involve in other biosynthesis for biosynthesis \(^3\). These key benefits in aerobic glycolysis result in the shift of cancer cells to favor glycolysis over complete mitochondrial oxidation.
4.1.3 Potential of Gp-VIII in reducing aerobic glycolysis

Targeting glycolysis in cancer cell remains one of the attractive therapeutic approaches in cancer treatment. Recently, different investigations have demonstrated the effectiveness of this therapeutic intervention\(^4\).

In this project, Gp-VIII was chosen to further investigate the effect on the development of colon cancer development. In this part, Gp-VIII was administrated with *Homo sapiens* colorectal cancer cell line HCT116. Acidity test on the culture medium after 72 hours incubation with Gp-VIII administration shows that Gp-VIII might reduce acidic glycolytic by-products production in cancer cells. Glucose uptake assay also reveals that there is up to 16% decrease in glucose consumption upon 250nM Gp-VIII administration. It might probably due to the decrease of efficiency of glycolytic pathway.

4.1.4 Inhibitory effect of Gp-VIII to glycolytic enzyme

Expression of different glycolytic enzyme in the presence of Gp-VIII administration were investigated by western blot analysis. Housekeeping gene of beta-actin was used as reference to normalize protein concentration for comparison. Glycolytic enzyme including aldolase, TPI, GAPDH, enolase, PKM2 and LDH were found to be inhibited by Gp-VIII. The higher concentration of Gp-VIII, the lower the protein concentration of these particular glycolytic enzymes. It shows that Gp-VIII may decrease the expression of the enzymes, leading to the decrease of glycolytic efficiency.

Glycolysis is made more efficient in the presence of transcription factor HIF-1\(\alpha\)\(^5\). It up regulate the expression of glycolytic enzymes and glucose transporters in the absence of oxygen. Quantitative analysis of western blot result also reveals that Gp-VIII may have inhibitory effect toward HIF-1\(\alpha\). Up to 32% decrease of HIF-1\(\alpha\) was observed in the presence of 250nM Gp-VIII.
4.1.5 Proposed mechanism of Gp-VIII on glycolytic pathway

To sum up, GP-VIII shows effect in inhibiting several enzymes directly or indirectly involved in aerobic glycolysis. Directly inhibition refers to the direct interaction of different glycolytic enzymes such as Aldolase, TPI, GAPDH, enolase, PKM2 and LDH by Gp-VIII. In the presence of Gp-VIII, down regulation of the above glycolytic enzyme is observed. Indirectly inhibition refers to the inhibition of HIF-1α by Gp-VIII. As a transcription factor supporting cell growth during hypoxia condition, HIF-1α plays role in up-regulation of glycolytic pathway. Inhibition of HIF-1α by Gp-VIII may results in down-regulation of glycolytic pathway.

With the known glycolytic pathway and the above results, a proposed mechanism on the effect of Gp-VIII on glycolytic pathway was hypothesized as shown in the figure below. Rate of glycolysis decrease due to the inhibitory effect of Gp-VIII. With less energy generated, it is proposed that rate of cancer growth is lower. Chapter 3 in this project will discuss the actual effect of Gp-VIII on cancer development in form of polyp formation.
4.1.6 Cell cycle arrest and apoptosis induced by Gp-VIII

In control group, 51.0%, 41.7% and 7.3% cells were distributed at G1, S and G2 phase respectively. However, cells getting arrested in G2 phase increase with the concentration of Gp-VIII. In the treatment of 250 nM Gp-VIII, 39.39% cells were arrested at G2 phase. It is proposed that, inhibition in glycolytic pathway may lead to insufficient energy required in cell proliferation, resulting in cell cycle arrest. Abnormally arrested cell is thus detected via checkpoint and projected into apoptosis.

This hypothesis is supported by the results from flow cytometry analysis for cell apoptosis. In the treatment group of 250nM Gp-VIII, 64.3% of cells were sorted into the window categorized with high concentration of fluorescein isothiocyanate (FITC) and
phycoerythrin (PE). It implies that more cells were subjected into apoptosis, increasing the availability of the dyes getting into cell. It shows that high concentration of Gp-VIII may indirectly induce apoptosis, favoring in cancer cell treatment.
4.2 Conclusion

_Homo sapiens_ colorectal cancer cell line HCT116 was grown in the presence of different concentration of Gp-VIII. MTT assay was carried out and LD50 of Gp-VIII were measured as for 24, 48 and 72 hours cell incubation were 474.3nM, 453.4nM and 446.3nM respectively.

Highest dosage of 250nM Gp-VIII in onwards experiment was used. With the application of flowcytometry technique, it is found that Gp-VIII may induces cell cycle arrest and cell apoptosis.

Glucose uptake test shows that Gp-VIII might reduce acidic glycolytic by-products production in cancer cells. Decrease of glucose consumption was up to 21.3% in the presence of 250nM Gp-VIII.

Protein expression profile of different glycolytic enzyme was measured by western blotting. Upon drug administration, glycolytic enzyme including aldolase, TPI, GAPDH, enolase, PKM2 and LDH were found to be inhibited by Gp-VIII. The inhibitory effect increases with concentration of Gp-VIII. Transcription factor HIF-1α was also found to be inhibited by Gp-VIII. With the known glycolytic pathway and the above results, a mechanism on the effect of Gp-VIII on glycolytic pathway was proposed. Gp-VIII may also induce cell cycle arrest and apoptosis, favoring in cancer cell treatment.
CHAPTER 3.

Study of the anti-cancer effect of Gp-VIII in Apc^{min/+} mice

1. Introduction

1.1 Importance of animal study

Drug screening may show effect in mammalian cell line such as HCT116 used in this project. To further confirm the properties of a potential drug in treating cancer, animal model is always a good choice in the next step. C57BL/6J is a common inbred mouse strain. This genetically modified animal model is widely used for human disease.

1.2 Properties of Mice Apc^{min/+}

Genetic and epigenetic alteration are common cause contributing the arise of colon cancer. Mice with Apc^{Min/+} background is usually use as a model for adenomatous polyposis [83]. Min (multiple intestinal neoplasia) is a mutant allele of the murine Apc (adenomatous polyposis coli) locus [84]. Apc^{Min/+} refers to the existence of point mutation at the Apc gene. The animal model is predisposed to adenoma formation in intestinal region. Losing of the tumor suppressor function of Apc gene results in polyp formation and colorectal carcinogenesis.

1.3 Rationale

A polyp is a mass protruding into lumen regions that originate from epithelial cells [81]. Genetic alteration contributes one of the main causes in cancer development from polyps [82]. Although most of the neoplastic polyps would not grow into cancer, the
carcinoma progression sequence was proposed by scientist and this transformation is known as the adenoma-to-carcinoma sequence.

As refer to chapter two, it is proposed that Gp-VIII may inhibit glycolytic pathway which contributes the energy production in cancer cell. In this part, mice Apc\textsuperscript{Min/+} are used in the study of the effect of Gp-VIII on polyp formation in GI tract. If the proposed mechanism is correct, inhibition in polyp formation should be observed.
2. Materials and Methods

2.1 Preparation of animal model

Apc\(^{Min+/+}\) and wild type mice in the C57BL/6J background, purchase from Jackson Laboratory, Bar Harbor, USA, were used in the study. The mean age of the experiment mice was 5 weeks. During the study period, all mice were housed in a room with a 12 hours light-dark cycle, controlled room temperature at 21±2 °C and a relative humidity at 55%±10%, in cages with absorbent corncob bedding. Mice were given ad libitum access sterilized drinking water and 9% pelleted Fat Diet. Both diet and water were replaced weekly. Starts from week 5, mice were either treated with Gp-VIII or 0.5% CMC three times a week for 8 weeks. Drugs were given by oral administration using syringe oral gavages.

2.2 Drug preparation

Gp-VIII powder was weighted and mixed with 0.5% sodium carboxymethyl cellulose (CMC) by sonication. 0.5% CMC solution without Gp-VIII was used as control. The drugs were newly prepared weekly and stored at 4 °C in the refrigerator before used.
2.3 Drug treatment and schedule

At the age of five weeks, all the mice were randomly divided into four groups with every three to five in a cage to start the experiment for eight weeks.

![Figure 3.2.1 Grouping of mice and Gp-VIII administration schedule](image)

Group A and B were Apc\textsuperscript{Min/+} mice while Group C and D were wild-type mice which serve as control. Group A and C treated with Gp-VIII (10mg/kg) while Group B and D treated with 0.5% CMC by oral feeding three times a week. The mice were fed with 9% pelleted Fat Diet and given filtered drinking water; the amount of food and water consumption was measured weekly and mice were monitored daily and weighed weekly. All mice were sacrificed at the age of 13 weeks by overdose of diethyl ether inhalation. After dissection of the mice, blood and tissues (Liver, Spleen, adipose tissue and the whole intestine) were removed. Blood was centrifuged at 4 °C 1500xg for 15minutes to obtained plasma which was store in -80 °C refrigerator for later analysis. Liver and Spleen were weighted and store at -80 °C refrigerator without dissection.
2.4 Assessment of number and sizes of intestinal polyps

The whole gastrointestinal tracts (GI) were removed and the associated mesenteric fat and blood vessels on the surface of it were removed firstly. Then the whole intestine was opened longitudinally with scissors and rinsed with 0.9% saline to remove the intestinal contents. It is fixed in 10% phosphate-buffered formalin for 16-24 hours. Following the fixation, the intestine was dehydrated by washing thoroughly with 70% ethanol and then stored at 4°C refrigerators.

In the experiment, the gastrointestinal tracts (GI) were divided into 4 sections: the duodenum (~4cm in length of the proximal part of the intestine), medial and distal (equally divided the remained proximal part into two halves), and the entire colon. Cecum was omitted because of its rare incidence of polyps.

The length of the whole GI tract and each segment was measured. Polyp and tumor numbers and size in each segment were determined by manual count using a dissecting microscope (×25 magnifications). To facilitate counting of polyps, each fixed GI tract was stained with 0.01% methylene blue solution. The diameter of each polyp was measured by ruler under dissecting microscope. The polyps were categorized according to their relative diameters, which was calculated by the mean of the largest and the smallest diameters of the polyp. There are four categories of polyps, ≤1mm, 1.1mm - 2mm, 2.1mm - 3mm, 3.1mm - 4mm.
3. Results

3.1 Change of mean body weight

Group A and B were Apc\textsuperscript{Min/+} mice while Group C and D were wild-type mice which serve as control. Group A and C treated with Gp-VIII (10 mg/kg) while Group B and D treated with 0.5% CMC by oral feeding three times a week. Mean body weight of each mice were measured and percentage change of mean body weight throughout the experiment were calculated. No obvious change was observed between treatment groups and control group.

Figure 3.3.1 Change of mean body weight of different groups of mice throughout the experiment
3.2 Change of ratio of mean vital organ weight to body weight

Weight of mean vital organs from control groups and Gp-VIII were measured. No significant difference was observed in liver and kidney samples. However, spleen to body weight was decreased by 14% while adipose tissue to body weight was increased by 37% after Gp-VIII treatment.

Figure 3.2.1 Change of liver/body weight (left) and change of kidney/body weight (right) with and without Gp-VIII treatment

Figure 3.2.2 Change of spleen/body weight (left) and change of adipose tissue/body weight (right) with and without Gp-VIII treatment
3.3 Effect of Gp-VIII on formation of polyps

Formation of polyps were analyzed in form of total number, sizes and locating region. Suppression can be observed with Gp-VIII treatment.

3.3.1 Suppression of total number of polyps

![Graph showing suppression of total number of polyps](image)

*Figure 3.3.3.1 Suppression of total number of polyps*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SEM</th>
<th>Suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td>70.23 ± 5.75</td>
<td>5.75</td>
</tr>
<tr>
<td>Treatment (n=8)</td>
<td>45.32 ± 7.32</td>
<td>35.46%</td>
</tr>
</tbody>
</table>

*Table 3.3.3.1 Suppression of total number of polyps*

A obvious decrease in the total number of polyps was observed. About 35.46% suppression of total number of polyps formation was measured upon Gp-VIII treatment. Number of polyps in different size of ≤ 2mm were reduced up to 70%. Number of polyps in different intestinal regions with the greatest suppression effect in medial and distal of intestine up to 60%.
3.3.2 Suppression of different sizes intestinal polyps

![Figure 3.3.3.2 Suppression of different sizes intestinal polyps](image)

<table>
<thead>
<tr>
<th>Treatment / Control (No. of mice)</th>
<th>Suppression (%) of Polyp Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 1mm</td>
</tr>
<tr>
<td>Control (n=5)</td>
<td>30.13 ± 8.71</td>
</tr>
<tr>
<td>Treatment (n=8)</td>
<td>19.90 ± 5.67</td>
</tr>
<tr>
<td></td>
<td>(33.95%)</td>
</tr>
</tbody>
</table>

*Table 3.3.3.2 Suppression of different sizes intestinal polyps*

Polyps were categorized according to its size. Results show that the formation of polyps with relative diameter of ≤1mm and 1.1-2 mm were obviously decreased. As summarized in the above figures, 33.95% of ≤1mm polyp formation were suppressed while 37.83% of polyps with relative diameter of 1.1-2 mm were suppressed. Since the sample size of 2.1-3mm and 3.1-4mm polyps were small, no conclusion can be drawn.
### 3.3.3 Suppression of polyp formation in different regions

#### Table 3.3.3.3 Suppression of polyp formation in different regions

<table>
<thead>
<tr>
<th>Treatment (No. of mice)</th>
<th>Suppression (%) of Polyp Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
</tr>
<tr>
<td>Control (n=5)</td>
<td>2.64 ± 0.12</td>
</tr>
<tr>
<td>Treatment (n=8)</td>
<td>1.88 ± 0.08</td>
</tr>
</tbody>
</table>

(7.69%)  (32.41%)  (28.23%)  (43.51%)

By compared with Gp-VIII treated group and the control group, obvious decrease of polyps number in medial and distal regions were observed. As summarized in the above figures, 32.41% of polyps formed in medial region were suppressed while 28.23% of polyps formed in distal regions were suppressed. Since the sample size of polyps formed in duodenum and colon regions were small, no conclusion can be drawn.
4. Discussion and Conclusions

4.1 Discussion

4.1.1 Relationship of polyp formation and colorectal cancer

A polyp is a mass protruding into lumen regions. Neoplastic colorectal polyps refer to the benign tumor that originate from epithelial cells which in response for mucus secretion\(^\text{[81]}\). Although most of the neoplastic polyps would not grow into cancer, the carcinoma progression sequence was proposed by scientist and this transformation is known as the adenoma-to-carcinoma sequence.

Genetic alteration contributes one of the main causes in cancer development from polyps\(^\text{[82]}\). For examples, induction of active oncogenes activating abnormal protein expression, mutation of tumor suppressor gene leading to reduction its activities and mutation of gene relating to regulation of DNA repair mechanism. Under this transformation stage, uncontrolled cell proliferation and autonomous growth are commonly observed.

4.1.2 Effect of Gp-VIII on polyp formation

Genetic and epigenetic alteration are common cause contributing the arise of colon cancer. Mice with Apc\(^{\text{Min/}+}\) background is usually use as a model for adenomatous polyposis\(^\text{[83]}\). Apc\(^{\text{Min/}+}\) refers to the existence of point mutation at the Apc gene. Losing of the tumor suppressor function of Apc gene play role in polyp formation and colorectal carcinogenesis.

Apc\(^{\text{Min/}+}\) mice model and wild type mice in the C57BL/6J background was used in this project. Upon 10mg/kg Gp-VIII administration, treatment group shows reduction in mean spleen/body weight. Gp-VIII might help to alleviate the inflammatory condition.
However, there was an increase in mean adipose tissue/body weight after treatment. Reason is still unknown and may need further study.

Treatment group with Gp-VIII administration shows an obvious decrease of polyp formation. Polyps with different size of \( \leq 2\text{mm} \) were reduced up to 50% while up to 64% suppression was observed in medial and distal regions.

As suggested in chapter 2 of this thesis, Gp-VIII play role in the reduction of rate of aerobic glycolysis pathway by inhibiting several vital glycolytic enzymes. To support autonomous growth in during polyp formation, large amount of energy is consumed. Administration of Gp-VIII shows the inhibitory effect of glucose uptake and energy generation. Comparing with control group, treatment group results in the reduction of polyp formation along intestinal tract due to lack of energy support.

### 4.2 Conclusion

Gp-VIII shows suppression effect in polyp formation in ApoMin/+ mice model. About 35.46% suppression of total number of polyps formation was observed in Gp-VIII treatment group. Number of polyps in different size of \( \leq 2\text{mm} \) were reduced by up to 70%. Reduction of the number of polyps in different intestinal regions was observed with the greatest suppression effect in medial and distal of intestine.
4.3 Future perspective

In this project, Gp-VIII shows suppression effect in aerobic glycolysis and polyp formation in ApcMin/+ mice model. Also, pharmacokinetic study shows that Gp-VIII can be naturally eliminate after administration. It reveals that Gp-VIII may be a suitable therapeutic agent for further study such as pre-clinal trial.

Also, to increase the effectiveness of Gp-VIII administration, molecular modification of Gp-VIII may be further investigated to improve the administration approaches and increase the retention time in target cancer cell or pre-cancer polyp for better efficacy.
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