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EVALUATION OF CYTOTOXICITY AND CELL MORPHOLOGICAL CHANGES BY MODIFIED FOLFIRINOX IN PANCREATIC CANCER CELL LINE

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BY

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ABSTRACT
EVALUATION OF THE CYTOTOXICITY AND CELL MORPHOLOGICAL CHANGES BY MODIFIED FOLFIRINOX IN PANCREATIC CANCER CELL LINE

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Introduction: FOLFIRINOX (a combination of leucovorin, 5-fluorouracil, irinotecan and oxaliplatin) is the new first-line drug regimen of chemotherapy in treating pancreatic cancer disease. Patients receiving 400mg/m^2 leucovorin, 180mg/m^2 irinotecan, 85mg/m^2 oxaliplatin and bolus 400mg/m^2 5-fluorouracil followed by 2,400mg/m^2 over a 46-hours infusion has resulted 11.1 months of overall survival with common unwanted side effects encountered including neutropenia. As it is associated with high toxicity, few different patients-tailored modifications of FOLFIRINOX have been made without prior *in vitro* testing, resulting in an unknown efficacy. **Objective:** This study was aimed to determine the cytotoxicity effect and cell morphology changes by FOLFIRINOX, and to determine the best drugs concentration combination in inhibiting Capan-2 cells. **Methodology:** Identification of the half maximum inhibitory (IC_{50}) of all drugs in FOLFIRINOX at different concentrations (3.125, 6.25, 12.5, 25, 50 and 100uM) against Capan-2 cells were performed. Different concentration combinations of FOLFIRINOX were then generated by performing an orthogonal design in SPSS using a range of IC_{50} values of each drug. The cytotoxicity, cell morphology changes and drug interaction of the generated drug combinations were confirmed using CCK-8 assay, AO/PI staining and Response Surface Methodology (RSM), respectively. **Results:** The IC_{50} of 5-fluorouracil, oxaliplatin, irinotecan and leucovorin are 17.9uM, 16.61uM, 17.38uM and 9.289uM, respectively. Few morphological changes such as chromatin condensation, membrane blebbing and cell fragmentation were observed in FOLFIRINOX treated Capan-2 cells. Among the 9 combinations generated by SPSS, combination of 30uM of 5-fluorouracil, 20uM of oxaliplatin, 1uM of irinotecan and 5uM of leucovorin gave the highest percentage of cell inhibition (62.49%). However, analyses via RSM gave a combination of 1uM of 5-fluorouracil, 1uM of oxaliplatin, 15uM of irinotecan and 10uM leucovorin as the best drugs combination. **Conclusions:** The modified FOLFIRINOX concentration found in this study differs from standard combination used in pancreatic cancer treatment. Further studies needed to validate the findings by determining the maximum response achievable from the modified FOLFIRINOX in research settings, and the capacity for sufficient therapeutic effect with less occurrence of unwanted side effects in clinical settings.

Keywords: pancreatic cancer; modified FOLFIRINOX; Capan-2; CCK-8

ABSTRAK

PENILAIAN SITOTOXISITI DAN PERUBAHAN MORFOLOGI SEL OLEH FOLFIRINOX YANG DIUBAH KEATAS SEL BARAH PANKREAS

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Pengenalan: FOLFIRINOX (gabungan leucovorin, 5-fluorouracil, irinotecan dan oxaliplatin) adalah rejimen ubat kemoterapi barisan pertama yang baru dalam merawat penyakit barah pankreas. Pesakit yang menerima 400mg/m^2 leucovorin, 180mg/m^2 irinotecan, 85mg/m^2 oxaliplatin dan bolus 400mg/m^2 5-flourouracil diikuti dengan 2,400mg/m^2 selama 46 jam infusi telah menghasilkan 11.1 bulan keseluruhan kelangsungan hidup dengan kesan sampingan yang tidak diingini dan sering berlaku termasuk neutropenia. Oleh kerana ia berkaitan dengan ketoksikan yang tinggi, beberapa modifikasi FOLFIRINOX yang disesuaikan dengan pesakit telah dilakukan tanpa ujian *in vitro* sebelumnya, sehingga menghasilkan keberkesanan yang tidak diketahui. **Objektif**: Kajian ini bertujuan untuk menentukan kesan sitotoksisitas dan perubahan morfologi sel oleh FOLFIRINOX, dan untuk menentukan kombinasi kepekatan ubat terbaik dalam menghalang sel Capan-2. **Metodologi**: Pengenalpastian perencatan separuh maksimum (IC_{50}) semua ubat dalam FOLFIRINOX pada kepekatan yang berbeza (3.125, 6.25, 12.5, 25, 50 dan 100uM) terhadap sel Capan-2 dilakukan. Kombinasi kepekatan yang berbeza dari FOLFIRINOX kemudian dihasilkan dengan melakukan reka bentuk ortogonal dalam SPSS menggunakan rangkaian nilai IC_{50} setiap ubat. Sitotoksisitas, perubahan morfologi sel dan interaksi ubat didalam kombinasi ubat yang dihasilkan, disahkan menggunakan ujian CCK-8, pewarnaan AO / PI dan Metodologi Permukaan Respons (RSM). **Hasil:** IC_{50} untuk 5-fluorouracil, oxaliplatin, irinotecan dan leucovorin masing-masing adalah 17.9uM, 16.61uM, 17.38uM dan 9.289uM. Beberapa perubahan morfologi seperti pemeluwapan kromatin, peleburan membran dan pemecahan sel diperhatikan pada sel Capan-2 yang dirawat oleh FOLFIRINOX. Di antara 9 kombinasi yang dihasilkan oleh SPSS, gabungan 30uM 5-fluorouracil, 20uM oksaliplatin, 1uM irinotecan dan 5uM leucovorin memberikan peratusan perencatan sel tertinggi (62.49%). Walau bagaimanapun, analisis melalui RSM memberikan gabungan 1uM 5-fluorouracil, 1uM oxaliplatin, 15uM irinotecan dan 10uM leucovorin sebagai kombinasi ubat terbaik. **Kesimpulan**: Kepekatan FOLFIRINOX yang diubah suai yang terdapat dalam kajian ini berbeza dengan kombinasi asas yang digunakan dalam rawatan barah pankreas. Kajian lebih lanjut diperlukan untuk mengesahkan penemuan dengan menentukan tindak balas maksimum yang dapat dicapai dari FOLFIRINOX yang telah dimodifikasi dalam tetapan penyelidikan, dan kapasiti untuk kesan terapeutik yang mencukupi dengan kurang berlakunya kesan sampingan yang tidak diingini dalam keadaan klinikal.

Kata kunci: barah pankreas; FOLFIRINOX yang diubah suai; Capan-2; CCK-8

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CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

Cancer has been one of major health problems where the incidence and mortality rate are rapidly growing worldwide. Globally, about 1 in 6 deaths was due to cancer and about 70% of cancer deaths occurred in countries with low and middle incomes (WHO, 2018). Asia has been accounted to have a higher number of patients who died from cancer (57%) compared with the numbers of new cases (48.4%) reported (TATEMICHI, 2003). These both incidence and mortality rates of Asia take up more than half of the worldwide proportion. This may happen due to poorer diagnosis of disease and limited access to timely treatment along with infection-related cancer. In Malaysia, cancer is the fourth leading cause of deaths in which it accounts for 12.6% deaths in hospitals and 37,000 newly diagnosed cases of cancer every year (MOH, 2019). There are 26,395 deaths caused by cancer recorded up until May 2019 (The Global Cancer Observatory, 2019). There are one in ten and one in nine of lifetime risk estimation from 2007 to 2011 data reported in developing cancer for Malaysian male and Malaysian females respectively (MOH, 2019).

Cancer is used to define a group of disease that forms due to rapid growth of abnormal cells and this cells can invade to others areas of the body (WHO, 2018). The uncontrollably growth of the cells leading to the development of solid lumps. When the solid lumps or is called as tumors grow malignantly, it is cancerous (Murphy & Charnay-Sonnek, 2019). Cancer originates in the body's cells but both intrinsic and extrinsic factors could influence and promote cancer development. For example, some infections and environmental pollutants can act as a stimulus to induce cancer development (Murphy & Charnay-Sonnek, 2019). Cancer developments were called carcinogenesis. In carcinogenesis, the cell undergoes 3 stages which are initiation, promotion and

progression (Murphy & Charnay-Sonnek, 2019). Malignant tumors are developed in the progression stage where the tumors were spread and invaded the nearby tissue to form new tumors in other parts of the body, resulting in cancer to occur in any part of the body. There are 5 major types of cancer based on their location, which are carcinoma, sarcoma, melanoma, lymphoma and leukemia (WebMD, 2020). Carcinomas is the cancer of skin, lung, pancreas, glands and other organs. Sarcoma is a cancer of bone, muscle, fat or other connective tissue in the body. Melanoma arises in melanocyte cells and lymphoma is a cancer of lymphocytes. While, leukemia is a cancer of the white blood cells which usually does not form solid tumors.

Pancreatic cancer, a carcinoma, is an aggressive disease with poor prognosis. It is the fourth leading cause of cancer death in the United States (Malwinder et al., 2018). About 458,000 new cancer cases and more than 432,000 number of deaths are estimated worldwide in 2018 (Siegel al., 2019). In Malaysia, pancreatic cancer has increases rank for deaths (11^{th}) compared to the new cases reported (14^{th}) indicates this disease as one of most lethal-cancer type (Malaysia, 2018). There also decrease in relative survival in 5 year disease detection (14%) compared with 1 year disease detection (33.2%) (Malaysia, 2018). The low percentage of survival of pancreatic cancer was due to the late detection of the disease (Brunner et al., 2019). This may due to lack of symptoms in the early stages and eventually the symptoms was recognized only when the disease are at an advanced stage (stage 3 and 4) (Neureiter et al., 2014)(Brunner et al., 2019). In addition, pancreatic cancer has 71 years as the median age at diagnosis. It is rarely diagnosed in young people with age less than 40 years (Ryan et al., 2014). Inability to diagnose earlier usually causes cancer cells to grow uncontrolled and undergo widespread metastasization. This also could lead to higher cost of treatment and reduce chances of cure. There are several types of pancreatic cancer, depending on the types of cells the cancer began with, whether in the exocrine or endocrine cells.

In this study, we were looked at the most common type pancreatic adenocarcinoma disease where the cancerous cells were formed in exocrine cells of the pancreas, which functionally produce enzymes that help with digestion. About 85% of pancreatic cancer patients having this pancreatic adenocarcinoma cancer worldwide ranges from 1 to 10 cases per 100,000 people (Ryan et al., 2014). A very high rate of activating mutations in *KRAS* is becoming one of the defining features of pancreatic adenocarcinoma (Ryan et al., 2014). Mutation in this gene interferes with the cells normal function of proliferating, differentiating and apoptosis (Fan et al., 2018). Therefore, an integrated management of the pancreatic cancer disease is needed to ensure patients received better prognosis and live healthily.

Treatment options for pancreatic cancer are based on stages. For potentially curable pancreatic cancer which are resectable and borderline resectable (stage 1 and 2); surgery, neoadjuvant and adjuvant therapy were selected as the treatments of choices. Surgery is the removal of the tumor which helps to remove the cancerous cells. Neoadjuvant therapy is chemotherapy that is given to the patient before the surgery which functions to shrink the tumor size so that it more easily and can be removed during surgery. Meanwhile, adjuvant therapy is chemotherapy that is given to the patients after the surgery, usually starts within 8 weeks after surgery which function to increase the chance of cure. Late diagnosis in pancreatic cancer causes the tumor lesion to become unresectable and thus the surgical resection as curative treatment option is often not possible (Brunner et al., 2019). For locally advanced pancreatic cancer (stage 3 and 4), first-line chemotherapy becomes the treatment of choice. There are 2 common drugs used in chemotherapy which are monotherapy gemcitabine and FOLFIRINOX. Gemcitabine is an anti-cancer chemotherapy drug that acts as an antimetabolite and was given intravenously since no pill form of gemcitabine. Based on previous study, FOLFIRINOX has shown to be more effective in

treating patients with advanced stage pancreatic cancer compared to gemcitabine since the median overall survival of patients treated with FOLFIRINOX are 4 months more than gemcitabine (Conroy et al., 2011)

FOLFIRINOX was first reported as a therapy for pancreatic adenocarcinoma in 2010 (Quinn et al., 2015). It is a combination of 4 cancer drugs which are 5-fluorouracil, oxaliplatin, irinotecan and leucovorin (Quinn et al., 2015). 5-Fluorouracil (5-FU) is a pyrimidine analog that interferes with the DNA and RNA synthesis through inhibition of thymidylate synthase (TS) and the incorporation of its metabolites into DNA and RNA (Wang et al., 2014). Oxaliplatin acts as an alkylating agent play its cytotoxicity by forming DNA adducts through binding of alkyl group into DNA (Modi et al., 2016). This causing damage to the DNA and prevent DNA to complete its normal replication process. Irinotecan is a pro-drug which functions to inhibit topoisomerase 1, prevent relaxation of DNA supercoiled and lead to broken of DNA double stranded strands (Thomas & Pommier, 2019). Lastly, leucovorin is also known as folinic acid that enhanced the inhibition effect of 5-FU (Longley et al., 2003). As a result, by administering all of these 4 drugs, cancerous cells were stopped from growing, replicating and undergoing cell death. FOLFIRINOX was administered to the patients intravenously as a drip every 2 weeks for 6 months of treatment. Patients receiving the standard dose of FOLFIRINOX would had 11.1 months of overall survival associated with 46% of grade 3/ 4 neutropenia as one of the side effects (Marsh et al., 2015). This shows that although FOLFIRINOX is effective to treat pancreatic cancer, FOLFIRINOX also has a high toxicity on neutrophils. This leading few version of modified FOLFIRINOX to be introduced. A study by Blazer et al., 2015, they had modified the standard FOLFIRINOX by treating the patients directly with no bolus 5-FU and 165mg/m^2 irinotecan, producing 0% of incidence grade 3/ 4 neutropenia and 9 months overall survival. The modifications made had

successfully decrease the occurrence of the side effect (neutropenia) but associated with decrease survival rates.

1.2 PROBLEM STATEMENT

FOLFIRINOX is a drug regimen that was introduced into clinical practice to be used as an advanced treatment of metastatic pancreatic cancer in 2010. Despite yielding good effectiveness to treat patients, FOLFIRINOX also exert their high toxicity by causing 46% grade 3 or 4 neutropenia. Therefore, few modifications on FOLFIRINOX combinations were made to decrease its toxicity. There are many different kinds of modifications on concentrations of FOLFIRINOX have been carried out by previous researchers, where the best one could not be identified. Those modifications was successfully decrease the occurrence of neutropenia but it also associated with decrease median overall survival. Besides, the modifications on concentration of FOLFIRINOX combination were also carried out directly on the patients without prior *in vitro* testing, where the efficacy of the modifications is unknown. Also, there are lack of studies that testing the individuals drug of FOLFIRINOX or their modifications via *in vitro* study. Therefore, this study is important in determining the best modifications on concentrations of FOLFIRINOX combination via *in vitro* testing for its better therapeutic effects associated with minimal adverse side effects.

1.3 RESEARCH QUESTIONS

The present research was conducted to answer the following questions:

1. What is the half maximal inhibitory concentration (IC_{50}) for each 4 drugs in FOLFIRINOX (5-fluorouracil, oxaliplatin, leucovorin, and irinotecan) against pancreatic cancer cells?

2. What is the different drugs concentration combinations which consists of all drugs in FOLFIRINOX?

3. What is the best drugs concentration combination of FOLFIRINOX that is effective to treat pancreatic cancer cells with less toxicity effects?

1.4 RESEARCH OBJECTIVE

1.4.1 GENERAL OBJECTIVE

To determine the cytotoxicity effects and cell morphology changes by FOLFIRINOX and to determine their best drugs concentration combinations in inhibiting pancreatic cancer cells.

1.4.2 SPECIFIC OBJECTIVE

1. To determine the half maximal inhibitory concentration (IC_{50}) for each drug in FOLFIRINOX (5-flurouracil, oxaliplatin, leucovorin, irinotecan) against Capan-2 pancreatic cancer cells.

2. To determine the drugs concentration combinations consist of all drugs in FOLFIRINOX.

3. To identify the best drugs concentration combination of FOLFIRINOX by testing each of combinations on Capan-2 pancreatic cancer cells.

1.5 RESEARCH HYPOTHESIS

It is hypothesized that the best drugs concentration combination of FOLFIRINOX found from this study will be effective to treat pancreatic cancer patients by inhibiting proliferation of cancerous cells and decreasing the toxicity effects on normal cells or neutrophils.

CHAPTER 2
LITERATURE REVIEW

2.1 EPIDEMIOLOGY

One type of common cause of cancer-related death is pancreatic cancer or pancreatic adenocarcinomas. Pancreatic adenocarcinomas is a diseases with high incidences and poor prognosis. The poor prognosis accounts for a low five-year survival which ranks last amongst all other cancer (McGuigan et al., 2018). In 2018, 458,918 new cases of pancreatic cancer have been reported worldwide, representing 2.5% of all cancers and made this disease as the seventh leading cause of global cancer in industrialized countries (Rawla et al., 2019). The age-standardized rate (ASR) incidence for pancreatic cancer is the highest in Europe and North America with 7.7 and 7.6 per 100,000 people respectively compared in Malaysia with 2.2 per 100,000 people. This standardization is important in comparing several populations that differ in age as the age has a great influence on the risk of dying from cancer, although it was also influenced by the country's income all the year. In short, the age-standardised incidence rate is higher in high-income regions compared to low-income regions (Pourshams et al., 2019). Based on that, pancreatic cancer is uncommon in Malaysia where the incidence rates of pancreatic cancer are ranked at 14th (Malaysia, 2018)(Malwinder et al., 2018). A studied by National Cancer Registry (2011) has also reported only 1829 cases of pancreatic cancer in Malaysia compared to other types of cancer. However, pancreatic cancer is a disease with high mortality rates. As in 2007 until 2011 and 2016 studies, 1263 (87.8%) of total deaths from 1438 of total new cases were reported in Malaysia (Malaysia, 2018). Also, there is increases rank for deaths (11th) compared to new cases (14th) with low 5-year survival has reported (Malaysia, 2018). Aside from poor prognosis, late of disease detection influences the survival rates of patients diagnosed with pancreatic cancer. This may due to lack of

symptoms such as painless jaundice, abdominal pain and weight loss. Those symptoms were commonly appear late and mostly when patients developed to an advanced stage (Neureiter et al., 2014). A studied by National Cancer Institute (2019) has reported about 82% of pancreatic cancer patients are diagnosed at the regional or distant stage where the cancer had spread to other parts of the body and accounts for low 5-year relative survival of pancreatic cancer (15.3%). Besides, the relative survival rate for one year of disease detection shows higher percentage (33.2%) compared with five year of disease detection (14%) (Malaysia, 2018). This late of disease detection had become the reason of pancreatic cancer as the third leading cause of cancer death in the United States (National Cancer Institute, 2019).

2.2 CAUSE AND RISK FACTOR

There is no specific cause of pancreatic cancer. But, there are some common factors that can increase a person's risk to develop pancreatic cancer. An action of cells is controlled by genes and changes in those genes could lead to development of cancer. Therefore, the most significant factor is the gene mutation. There are inherited gene mutations and acquired gene mutations. The inherited gene mutation was passed by the previous generations to the newest generation. As the gene mutations were not repaired along the generation, the mutation reached to the new generation, causing them to develop the cancer. This leads to a family cancer syndrome, where an inherited family gene causes the members of one family to develop pancreatic cancer. A nine times higher risk of developing pancreatic cancer was in patients with familial risk factors compared to patients with no family history (McGuigan et al., 2018). Besides, people with hereditary pancreatitis where a mutated gene that inherited from one parent causes an inflammation of pancreas, accounts for 1% of the incidence of pancreatic cancer (Cancer Research UK, 2017). However, most pancreatic

cancers are non-hereditary, representing only 5 to 10% of pancreatic cancer patients have a family history of it (Cancer Research UK, 2017).

The acquired gene mutations mostly caused by environmental factors such as smoking, obesity, infection and increased age. Cigarette smoking has been known to increase the risk for pancreatic cancer (Pandol et al., 2012). A combined data from 12 case control studies on pancreatic cancer risk demonstrated that there is a significantly increased risk of pancreatic cancer amongst the smoker (Bosetti et al., 2012). To add on, a meta-analysis of 82 published studies found that the risk will remain for 10 years following smoking cessations (Iodice et al., 2008). Cigarettes contain 7,000 chemicals, where 250 are harmful and 60 of them are carcinogenic (Hecht, 2006). Nicotine is a major component of tobacco smoke where it was quickly absorbed into the bloodstream when it reached lungs. At lungs, nicotine was metabolized into its carcinogenic metabolites which stimulated the growth of pancreatic cancer cells through B-adrenergic receptor-mediated activation of Cox2 (Pandol et al., 2012) (Weddle, 2001).

Next, being overweight or obese are about 20% more likely to develop pancreatic cancer. There are 23 individual studies on association of high body mass index (BMI) and pancreatic cancer, where about 10% increased risk of pancreatic cancer for every 5 BMI index was reported. This findings supported that the obese people has higher risk to developed pancreatic cancer (American Institute for Cancer Research, 2018). Increased food consumption and less physical activity of obesity patients may enhance the hormonal imbalance, tumor promoting effect and carcinogens exposure promoting cancer development (Bureau, 2006).

Other than that, patients with several infections such as *Helicobacter pylori* or hepatitis C infection have increased risk of pancreatic cancer (McGuigan et al., 2018). The colonization of H.pylori on the stomach antrum could result in secretion of secretin, leading to increased

proliferation of pancreatic ductal cells. The ductal proliferation would enhance the effect of a carcinogens and cause pancreatic cancer to develop (Wang & Li, 2015). Lastly, age played a significant factor since pancreatic cancer was a common disease of the elderly. 90% of diagnosed patients are aged over 55 years (Midha et al., 2016). The late diagnosis of pancreatic cancer has made pancreatic cancer as a deadly disease that mainly affects the elderly. In addition, the elder typically associated with immunosuppressive conditions cause them to be more prone in developing these cancers (Higuera et al., 2016).

2.3 CARCINOGENESIS AND CHEMOPREVENTIVE AGENTS

Generally, pancreatic cancer was initiated by gene mutations resulting in local proliferation of abnormal cells and migration to the other parts of the human body. This cancer development is carcinogenesis, a multistep process where it was first proposed by Berenblum and Schubik in 1948 and later supported by other studies (Murphy & Charnay-Sonnek, 2019). There are initiation, promotion and progression phases in cancer development **(Figure 2.1).** Better understanding of carcinogenesis helps to identify the type of pancreatic lesion and method of disease management. During initiation, the genes of the cells undergo mutations leading to gene alterations and cellular changes. This could occur spontaneously or induced by exposure to a carcinogenic agent such as radiation. When the cellular genome undergoes mutations, an initiated cell or potential cell for subsequent neoplastic transformation was created. For example, a single point mutation of proto-oncogene into a potent oncogene. Such gene mutation will result in problems on proto-oncogene to control the cell proliferation, communication and differentiation.

During promotion phases, the initiated cells were actively proliferating and accumulating to forms preneoplastic cells. This due to the repeated and prolonged exposures of the initiated cells to stimuli (carcinogenic agent). During progression, preneoplastic cells were transformed to

neoplastic cells where they are more committed to malignant development. It was the final stage of neoplastic transformation and involved a rapid increase in the tumor size. The cells undergo further mutation causing them to have an invasive and metastatic potential. Metastasis causes the neoplastic cells to spread to other parts of the body from the primary site through the bloodstream or lymph system, forming malignant tumors (Siddiqui et al., 2015). The malignant tumor could produce its own blood vessel in the process of angiogenesis to help their invasion to other parts of the body. The normal functions of the body could interfere with the metastasized cancer cells and lead to death (Friedman, 2012).

On the contrary, there are chemicals or substances which have anticancer properties by inhibiting, delaying and reversing the carcinogenesis before invasion. This substance was called chemopreventive agents. There are two main types which are blocking and suppressing agents **(Figure 2.1)** (Siddiqui et al., 2015). During initiation, blocking agents altered the process and prevent the mutation of the genes. Meanwhile, suppressing agents altered both promotion and progression process and prevent the neoplastic transformation of the cells. These agents are also able to inhibit angiogenesis and therefore prevent the metastasis of the cancer cells. In this study, we study a combination of drugs which played a role as the anticancer against pancreatic cancer cells

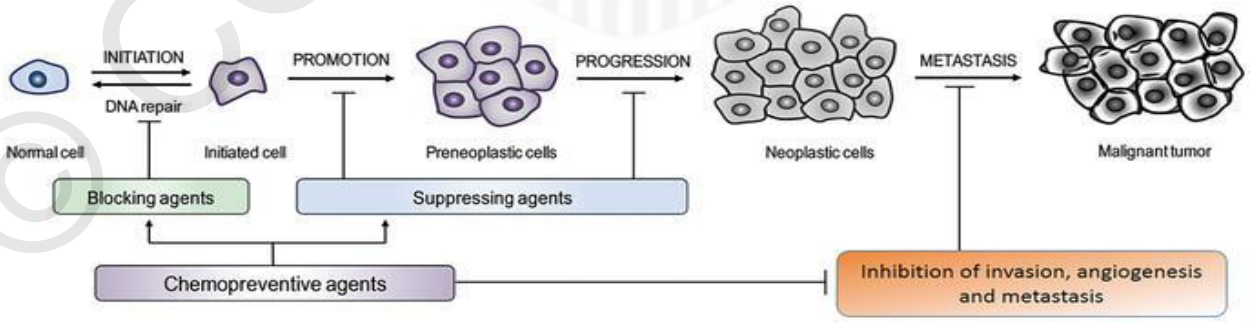


Figure 2. 1: Steps in carcinogenesis and type of chemopreventive agent (Adapted from Siddiqui et al., 2015)

2.4 TYPE AND STAGES

Pancreatic cancer is classified based on their cell type of origin, structure and behaviour. Pancreas is the organ where it has two main types of cells: exocrine and endocrine cells **(Figure 2.2).** The exocrine cells which consist of duct cells and acinar cells were functioned to produce digestion enzymes such as protease, lipase and amylase, and secreted into pancreatic duct (Mitra, 2019). In contrast, the endocrine cells which contain h cells and other types of endocrine cells were functioned to produce hormones into bloodstream, including insulin that is required for glucose metabolism (Mitra, 2019).

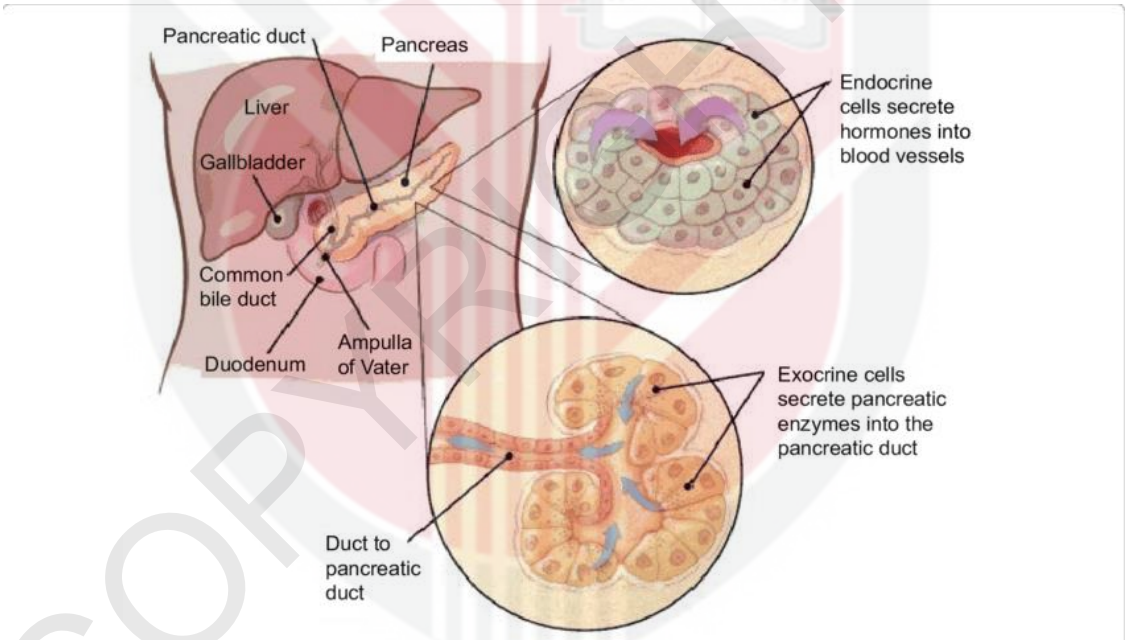


Figure 2. 2: The type of pancreas cells (Adapted from Mitra, 2019)

Pancreatic cancers that originated from the exocrine cells are the most common. Adenocarcinomas that account for 90-95% of all pancreatic cancer including the malignant type originated in ductal and acinar cells of exocrine (Vareedayah et al., 2018). Meanwhile, cancers of the endocrine pancreas are less common. They account for less than 5% of all pancreatic cancers,

where the type of tumor were insulinomas and gastrinomas which were detected due to the symptoms caused by excessive hormone levels (Mullan et al., 2001). This leads to higher survival rates among endocrine pancreatic cancer cases compared to exocrine cases. There is a study reported that, about 51.6% of endocrine cases were alive compared with 4.6% of adenocarcinoma cases (Fesinmeyer et al., 2005). In addition, the median survival of endocrine pancreatic cancer cases is 2 years longer than for adenocarcinoma cases (Fesinmeyer et al., 2005).

Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) were reported to be the precursor for invasive ductal adenocarcinoma of the pancreas (Hruban et al., 2020). PanIN is a non-invasive lesion that occurs in the small pancreatic duct (McGuigan et al., 2018). The resected main duct IPMN accounts for 70% of containing malignant cells, including carcinoma in situ (Tanaka et al., 2006). MCN is a premalignant lesion of the pancreas where it accounts for 25% of pancreatic cysts undergoing resection (Mohammed et al., 2014). Lower grade PanIN lesions have mutations in the *KRAS* oncogene and exhibit telomere shortening leading to a pathway for invasive malignancy (Hruban et al., 2020). Meanwhile, the higher grade PanIN lesions have mutation mutations in p16, CDNK27, p53 and SMAD4, promoting pancreatic adenocarcinomas (McGuigan et al., 2018).

Other than types of cells of origin, the clinical stage of tumor has also been used to classify pancreatic cancer. There are 4 stages which are stage I (no spread or resectable), stage II (local spread or borderline), stage III (wider spread or unresectable) and stage IV (metastatic). Stage I have IA where the cancer was limited to the pancreas and has grown 2 cm, while IB where the cancer has grown greater than 2 cm but less than 4 cm. In stage II, the cancer was over 4 cm and was limited to the pancreas, or the cancer may spread locally to the nearby lymph nodes. In stage III, the cancer may have expanded to the nearby blood vessels or nerves, but has not metastasized

to distant sites. While in stage IV, the cancer has spread to distant organs. At early stages, pancreatic cancer usually has a lack of symptoms causing late time of disease detection. But there are gradual onset of non-specific symptoms including weight loss, abdominal pain, nausea and fatigue upon progression of the cancer.

2.5 PANCREATIC CANCER CELL LINE

Cancer cell line are important for cancer research since they provide an accessible and cost effective model to study the biological behavior and response of a cell. A thorough understanding on its characteristics also helps in choosing the cell lines used for *in vitro* experimentation. Characteristics such as expression of a growth receptor had influenced the proliferating of pancreatic cancer cells. Capan-2 pancreatic cancer cell line expressed an elevated levels of the epidermal growth factor receptor (EGFR) (Fogh et al., 2020). This correspond with the defining features of pancreatic adenocarcinoma where an abnormal rate of cells proliferating and differentiating occurred due to mutations in KRAS genes, a gene involved in the EGFR pathway (Ryan et al., 2014) (Fan et al., 2018).

Besides, biological characteristics such as adhesion potential would also influenced the growth and proliferation of the cancer cells via mediating the interaction of extracellular matrix components (for example; collagen, fibronectin) with cell-surface molecules (Deer et al., 2010). A studied by (Sawai et al., 2005) shown a reduction of adhesiveness of Capan-2 compared with other cell lines such Capan-1, MIA PaCa-2 and PANC-1. This allowed Capan-2 to not follow the normal cell communication, resulting destruction to the cells structure which as one of the hallmarks of malignant cancer (pancreatic adenocarcinoma) (Hirohashi & Kanai, 2003)

In addition, a studied by (Kada et al., 1999) demonstrated that Capan-2 cells had 2-fold greater invasive abilities compared to other cell line such as AsPC-1. This allowed Capan-2 to spread and migrate aggressively to other part of the body which eventually made the another defining phenotype of pancreatic adenocarcinoma where almost all patients presenting metastasis at the time of disease detection (Deer et al., 2010).

2.6 DIAGNOSIS

The poor outcome of pancreatic cancer is mainly because of the late diagnosis of these diseases which at the time of diagnosis, the cancer cells already spread to nearby tissue. The main reason for the late detection is because of non-specific symptoms of the disease and the close proximity of major blood vessels which can be readily invaded by the tumor cells (Canto et al., 2013). Therefore, by determining the most appropriate protocols for earlier disease detection, the prognosis could be improved. There are few tests that a person needs to undergo when they have suspected with this disease. Firstly, imaging tests such as computerized tomography (CT) scans, magnetic resonance imaging (MRI) and positron emission tomography (PET) scans are used to diagnose pancreatic cancer (MayoClinic, nd). CT is the imaging examination that is most widely used for the detection and staging of pancreatic carcinoma (Miura et al., 2006). There are 3 types of CT that have been used which are contrast-enhanced CT, helical CT and multi-detector-row CT. In contrast-enhanced CT, pancreatic adenocarcinoma looked like a hypoattenuating area (Prokesch et al., 2002). Helical CT accounts for 89% and 97% of sensitivity in detection pancreatic carcinoma (Miura et al., 2006). While, multi-detector-row (MD) CT was precise in early detection and staging of pancreatic carcinoma by allowing uses of thin collimation for the acquisition of high-resolution scans during multiple phases of contrast enhancement (Miura et al., 2006). Using MRI, the pancreatobiliary system could be examined non-invasively and produced better soft

tissue contrast compared with CT (Miura et al., 2006). Local tumor extension and vascular involvement in pancreatic cancer is enhanced using MRI which have gadolinium and mangafodipir trisodium (Miura et al., 2006). PET scanning uses a radiolabeled glucose analog ^{18}F-fluorodeoxyglucose (FDG) which functions to differentiate between benign and malignant lesions (Miura et al., 2006). There are studies that reported the sensitivity and specificity of FDG-PET for detecting malignant pancreatic tumors to be 71–100% and 64–90%, respectively (Sendler et al., 2000).

Secondly, a scope which is called endoscopic ultrasound (EUS) was used to create ultrasound pictures of the pancreas when a thin and flexible tube was down the esophagus and into the pancreas (MayoClinic, nd). EUS allowed an inhomogeneous solid mass with irregular borders to appear in pancreatic cancer patients (Miura et al., 2006). These techniques are also very usable to identify small tumor cells that are less than 2cm and invasion of these cells into vascular when compared to CT and MRI (Miura et al., 2006). Biopsy using EUS-guided fine needle is a safe and highly accurate method for cytology diagnosis of suspected pancreatic cancer patients where some abnormal features in pancreatic cysts was determined (Vander Noot et al., 2004). Other than that, blood tests which are radioimmunoassay (RIA) used to test specific tumor markers or protein released by cancer cells was also useful in pancreatic cancer detection. Serum cancer antigen 19-9 (CA 19-9) has been the most commonly used marker and the only marker approved by the United States Food and Drug Administration for treatment management of pancreatic cancer patients (Kim et al., 2004). People with pancreatic cancer patients has an elevated level of CA 19-9, but decreasing CA 19-9 indicates the cancer treatment is working in order to stop the cancer growing (Kim et al., 2004).

2.7 TREATMENT

Treatment is the major factor that determines the outcome of a disease. Treatment for pancreatic cancer patients is based on the tumor stages. Surgery remains as the fundamental of treatment for patients with resectable or borderline pancreatic cancer since it is the most efficient technique in order to remove the cancer mass. Pancreatectomy is one of the minimally invasive surgical methods where it involves vascular reconstruction and allows safety with no significant oncologic outcomes (Mohammed et al., 2014) (Strobel et al., 2019). Resection of the body or tail of the distal portion of pancreas and spleen were called as distal pancreatectomy, where it is the standard procedure to cure pancreatic cancer. However, there are major complications from this procedure which are delayed gastric emptying and hemorrhage (Sun & Zhu, 2014). Besides, performing the traditional pancreaticoduodenectomy (PD) by resect the pancreatic head, duodenum, distal common bile duct, gallbladder, and gastric antrum has decrease the perioperative mortality rate to less than 5%, while the morbidity rate remains high at 40% (Cameron et al., 2006). Laparoscopically is one of invasive approaches to treat pancreatic patients where when it was performed safely, it is able to facilitate prognosis (Kooby et al., 2008).

Borderline tumors are those tumors that are neither clearly resectable nor clearly unresectable. The tumor may be located in an unresectable region because it is difficult to do resection on it, leading a neoadjuvant therapy was used prior the surgeries. Neoadjuvant are efficient in downsizing the size of the tumor which could result in good prognostic (Assifi et al., 2011). This therapy also has high sensitivity on curing micrometasases of pancreatic tumors by understanding the biology of each tumor, leading to early relapse post-surgery prevention (Assifi et al., 2011). However, neoadjuvant requires histological and cytological analysis of the tumor before start the chemotherapy, but there are no techniques that are sufficient enough to obtain such

diagnosis (Seufferlein & Ettrich, 2019). Delayed on the diagnosis could result in delay to the patient to obtain the neoadjuvant treatment, causing the tumor to become unresectable. Even repeated EUS-guided FNA accounts for 25% of insufficient (Hewitt et al., 2012).

Throughout a few years, surgical resection alone or surgical resection with neoadjuvant were developed into advanced surgical techniques. Surgical resection with adjuvant systemic chemotherapy made up the surgeries improvement and provides the only chance of long-term survival (Strobel et al., 2019) (Seufferlein & Ettrich, 2019). Gemcitabine or a combination chemotherapy which is FOLFIRINOX play a role as adjuvant treatment (Seufferlein & Ettrich, 2019). About 15-20% of patients initially diagnosed with pancreatic cancer are ready to have a resection with those advances (Zuckerman & Ryan, 2008). Advances in surgical techniques also allowed patients to undergo resection with decreasing perioperative morbidity and mortality into 3% of mortality and 5-year survival (30%) (Mohammed et al., 2014) (Strobel et al., 2019). In addition, although the majority of pancreatic cancer patients present with metastatic disease and only 10–20% was diagnosed with localized and surgically resectable, surgery remains underused with present adjuvant therapy (Strobel et al., 2019).

For patients diagnosed with locally or metastatic advanced pancreatic cancer, chemotherapy or combination chemotherapy has become treatment of choice to manage the patients. Gemcitabine has been established as a standard agent of choice that is approved to treat advanced pancreatic disease since 1997 (Conroy et al., 2011). However, in order to improve gemcitabine performances, gemcitabine was used in combination with other chemotherapy agents of choice. For instance, Erlotinib which a HER 1/EFGR-targeted agent was used with gemcitabine in study of pancreatic cancer since an overexpression of human epidermal growth factor receptor type 1 (HER 1/ EFGR) was observed in pancreatic tumor (Moore et al., 2007). These regimens

have achieved a significant increase in one-year survival (23% *vs* 17%) and median overall survival (6.7 *vs* 5.7 months) compared to gemcitabine monotherapy (Moore et al., 2007). Nab-paclitaxel is a target therapy with nanotechnology where the drug was transported in albumin, a human protein that is present in nanometric size directly to the tumor lesion (R. et al., 2018) (Cohen et al., 2016). Nab-paclitaxel plus gemcitabine has demonstrated an improvement of one-year survival (35% *vs* 22%) and median overall survival (8.7 *vs* 5.7 months) when compared with gemcitabine alone (Von Hoff et al., 2013). This regimen was well-tolerated and reported to have a safer toxicity profile which became an option for patients who cannot tolerate FOLFIRINOX because of poor performance status (Mohammed et al., 2014). Besides that, there are studies that show some agents that demonstrated a distinct decrease of median survival when compared with gemcitabine alone. Gemcitabine in association with oxaliplatin (GEMOX) achieved median (4.5 *vs* 5.7 months) as response with a significant neurological toxicity due to oxaliplatin (Louvet et al., 2002).

Identification of effective combinations of existing chemotherapeutic agents, such as the FOLFIRINOX has improved survival for pancreatic cancer patients especially those at metastatic stage, although the toxicity profiles of these combinations still remain. (Mohammed et al., 2014). FOLFIRINOX is a drug combination of 4 drug which are 5-fluorouracil, oxaliplatin, irinotecan and leucovorin. The first drug, 5-fluorouracil is an antimetabolite drug that exert its anticancer effects through the inhibition of thymidylate synthase (TS) activity by incorporation of its metabolites (5-fluorodeoxyuridine monophosphate) into TS (Phua et al., 2013). These binding catalyzed the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using 5,10-methylenetetrahydrofolate (CH2THF) leading to inhibition of DNA synthesis of the cancer cell (Longley et al., 2003). The co-treatment of 5-FU with leucovorin

and methotrexate demonstrated an improvement in 5-FU anticancer activity (Longley et al., 2003). Oxaliplatin is an alkylating agent that acts as an anticancer chemotherapy drug when given by infusion into the vein. It is often given in combination with other anticancer drugs. There is a phase II trial of oxaliplatin plus gemcitabine that has shown clinical benefit in 40% of patients, with acceptable toxicity (Louvet et al., 2002). However in Phase III CONKO-003 trial, oxaliplatin showed better therapeutic response when acted with 5-fluorouracil. This trial involving the combination of oxaliplatin/5-fluorouracil/folinic acid (OFF) was tested as second-line therapy for advanced pancreatic cancer, OFF differs from folinic acid/ 5-fluorouracil (FF) by an improved in median overall survival with 5.9 *vs* 3.3 months. The time to progression also improved with OFF (2.9 months) compared with FF (2.0 months). However, due to insufficient accrual, the trial was terminated and was reopened with a change of comparison arm to FF (Pelzer et al., 2011).

Irinotecan or a trade name of Camptosar® is a topoisomerase 1 inhibitor where it prevents the separation of the two strands of double-stranded DNA. These inhibitions would prevent synthesis of DNA and therefore inhibit proliferation of cancer cells. Irinotecan also showed a better response when used in combination. The regimen of 5-FU/liposomal irinotecan as a standard-of-care second-line therapy has shown an improved of median overall survival with the nanoliposomal irinotecan/5-FU/leucovorin combination to 6.1 months (95% CI, 4.8–8.9) compared with nanoliposomal irinotecan (4.9 months) and 5-FU/leucovorin (4.2 months) (Wang-Gillam et al., 2016). This study was also supported by Level I evidence from the NAPOLI-1 trial (Wang-Gillam et al., 2016). Leucovorin is the active metabolite of folic acid where it was used as an inhibitor to block the conversion of folic acid to folinic acid. There is a long time study that reported the progression-free time in pancreatic cancer was substantially better with response to combination of leucovorin and 5-fluorouracil compared to other regimens (Bruckner et al., 1988).

These 4 drugs of FOLFIRINOX were chosen based on preclinical and clinical studies reporting there are synergy and non-overlapping toxic effects between these different drugs (Saung & Zheng, 2017). FOLFIRINOX as the most effective reference regimens in order to treat locally advanced and metastatic pancreatic cancer, has demonstrated a higher response rate than gemcitabine alone (Mohammed et al., 2014) (Ulusakarya et al., 2019). A significant improved of median overall survival of FOLFIRINOX (11.1 *vs* 6.8 months) compared to gemcitabine alone was observed in the Actions Concertées dans les Cancer Colo-Rectaux et Digestifs (ACCORD) 11 trial where 342 patients with metastatic pancreatic cancer were randomly assigned to receive either of both treatment. In this trial, they received FOLFIRINOX with leucovorin; 400mg/m^2, oxaliplatin; 85mg/m^2, irinotecan; 180mg/m^2 and 5-fluorouracil; 400mg/m^2 given as a bolus followed by 2400mg/m^2 given as 46 hour continuous infusion for every 2 weeks or received gemcitabine at 1000mg/m^2 for every week. Both treatment in this study was carried out for 6 months of therapy. With the improved survival, FOLFIRINOX demonstrated better efficacy compared to gemcitabine (Conroy T et al, 2011). Other than that, a study with 21 patients with pancreatic cancer who received FOLFIRINOX as their neoadjuvant treatment, shows a 33% R0 resection rate was achieved and 24% of patients demonstrated a significant pathologic response (Boone et al., 2013).

FOLFIRINOX shows a promising effectiveness towards advanced pancreatic cancer disease but associated with significant toxicity. In the ACCORD 11 trial, an adverse side effect of FOLFIRINOX was noted where there are 54% in the group having febrile neutropenia (Conroy et al., 2011). There were significantly grade 3 or 4 toxicities, such as cytopenias, diarrhea, and neutropenic fever in the treatment group. Besides, in regimen of irinotecan/5-fluorouracil/ leucovorin has shown more common of grade 3 or 4 as the adverse events, where 27% neutropenia

vs 15% with nanoliposomal irinotecan monotherapy (Wang-Gillam et al., 2016). About 61/380 (16%) of treatment groups received 5-fluorouracil, leucovorin and oxaliplatin has shown grade 3/4 neutropenia (Bruckner et al., 1988). A subsequent study which was conducted to confirm the efficacy of the FOLFIRINOX regimen has demonstrated few concerns on the applicability and tolerability to patients of older age, poor performance status, co-morbid conditions (Mohammed et al., 2014). There are studies that reported manageable side effects as well as significant toxicity resulting in treatment discontinuation of FOLFIRINOX (Faris et al., 2013). Despite concerns of toxicity and tolerability, FOLFIRINOX demonstrates a good potential for improved oncologic outcomes (Mohammed et al., 2014).

Therefore, a modified version of FOLFIRINOX was introduced through the PRODIGE 24-ACCORD study to reduce its toxicity effects. In this study, 493 patients were randomly assigned to receive a modified FOLFIRINOX regimen (without bolus fluorouracil) or gemcitabine for 24 weeks. The median overall survival in the modified FOLFIRINOX group was 54.4 months and 35 months in gemcitabine, associated with manageable side effects of FOLFIRINOX (Conroy et al., 2011). Besides that, a modified FOLFIRINOX protocol has demonstrated a better efficacy in the adjuvant setting compared to gemcitabine alone with a median disease-free survival (mDFS) of 21.6 months compared to 12.8 months and a 3-year DFS rate of 39.7% compared to 21.4% with gemcitabine alone (Seufferlein & Ettrich, 2019). In addition, there is also a study where the pancreatic ductal adenocarcinoma patients were treated with dose-modified FOLFIRINOX in 2016 at Paul Brousse University Hospital. The dose-reductions were made based on patient profile and toxicity which irinotecan was administered whenever initial serum bilirubin was less than 1.5 (upper limit of normal) and oxaliplatin was stopped for severe sensory. As a result, a significant improvement in median overall survival (14.6 months) was observed. (Ulusakarya et al., 2019).

Other than increased the FOLFIRINOX efficiency, the modifications also decreased its efficiency but associate with manageable side effect. A study which drop bolus fluorouracil and add 6mg pegfilgrastim to 60 advanced pancreatic cancer patients produce a lesser overall survival (9 months) and incidence of neutropenia (3%) (Mahaseth et al., 2013). Another study treating 43 patients without bolus 5-FU and decreasing the doses of irinotecan from 180mg/m2 to 165mg/m2, have made patients to survive about 9 months with 0% neutropenia (Blazer et al., 2015). In addition, a study which treated 44 patients with modified FOLFIRINOX (100mg.m2 5-FU and 45mg/m2 irinotecan) produced lesser median overall survival and incidence of grade 3 or 4 neutropenia which 10.2 months and 12.2% respectively (Stein et al., 2016). Another study also showed lesser overall survival with 10.3 months compared to 11.1 months of the standard but associated with 29% incidence of neutropenia (Li et al., 2017).

To conclude, those studies had evaluated the efficacy and the safety profile of modified FOLFIRINOX in order to determine the modification that helps to reduce the significant toxicity of FOLFIRINOX. However, since the components of the FOLFIRINOX regimen have been dose-attenuated or patients-tailored modified, these raise the concern of physicians where the modification of the regimen may affect patient outcomes (Ulusakarya et al., 2019). The modification should be individualized with an emphasis on minimizing side effects and maximizing quality of life.

CHAPTER 3
METHODOLOGY

3.1 STUDY DESIGN

This study is a quantitative study where an experimental *in vitro* testing has been carried out. The cytotoxicity effects and cell morphological changes by the individuals FOLFIRINOX and their best drugs concentrations combination were assessed using Capan-2 pancreatic cancer cell line.

3.2 CELLS AND CULTURE

3.2.1 Capan-2 Pancreatic Cancer Cell Line

Capan-2 is the human pancreatic cancer cell line that was obtained in kind from Professor Dr Johnson Stanslas from Universiti Putra Malaysia (UPM). This cell line was from a 56 year old Caucasian male with pancreatic adenocarcinoma disease. It is an adherent cell with a median doubling time of 46 to 60 hours and seeding density of $1x10^4$ cells per cm^2. The cells received is at number of passage of 19. The cells required 7 days to be a confluent monolayer in the culture flask. The subcultivation ratio recommended for these cells is 1:2 to 1:4. This cells was cryopreserved in 90% media and 10% DMSO solution and stored in -80° freezer (Characteristics & Conditions, n.d.)

In order to stimulate the growth of Capan-2 *in vitro*, Capan-2 was cultured and maintained in T-25 tissue-culture flasks with 25 cm^2 surface area containing complete culture media. The complete culture media was prepared by added 40% of RPMI media with 10% fetal bovine serum and 1% penicillin. In a culture flask, 5 ml of complete media and desired volume of cell suspension (contained of $25x10^4$ cells) were added. Then, the cells were incubated in a 5% CO_2 incubator at

37°C for 1-7 days. The media renewal was done 2 to 3 times per week when needed, which is when there is change of original color of the media. The cells was examined within 6 day of culture under inverted microscope and once the cells had reached 70-80% confluence, the cells was subculture into another new culture flasks.

In order to subdivide the proliferating cells and allow propagating of a cell line, subculture was performed. To subculture the pancreatic cancer cell line, firstly, the media was discarded and the cells were washed for 2 times using 2 ml of phosphate buffer saline solution. Then, this wash solution was discarded and 1 ml of pre-warmed dissociation reagent (trypsin) was added. The cells were incubated in 5% CO2 at 37°C for 5 minutes. After 5 min, the cells were observed under an inverted microscope for the detachment of the cells from the bottom of the flask which characterized by cells floating over the flask. When more than 70% of the cells have detached, 2 ml of complete culture media was added to the culture flask. The media together with the cells then was transferred to a 15 ml centrifuge tube which is then centrifuged at 1000 rpm for 5 minutes. After centrifuge, the supernatant was discarded and 1 ml of complete media was added into the tube and the cell pellet was rinsed with the media. To count the cells, 100 µl of cell suspension were mixed with 100 µl trypan blue solution and 100 µl from the mixture were loaded into a haemocytometer. The live cells which appear in 4 large corners in the haemocytometer grid were calculated and a total of cells per ml was obtained using a formula. After calculating, 5 ml of culture media and desired volume of cells was added in a new culture flasks. Refer Appendix 1.1 for calculation of total cells per ml.

3.3 CYTOTOXICITY TEST AND CELL MORPHOLOGY ANALYSES

3.3.1 CCK-8 assay for IC_{50} values determination

Cell Counting Kit-8 (CCK-8) was also used to check the cell viability of the treated cells and obtained the half maximal inhibitory concentration (IC_{50}) of each FOLFIRINOX drug. CCK-8 utilizes Dojindo's highly water soluble tetrazolium salt (WST-8) for the determination of the number of viable cells. WST-8(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-sulfophenyl)-2H-tetrazolium,monosodium salt) is reduced by dehydrogenase in live cells producing an orange colored formazan, where then it absorbance was read through ELISA multiplate reader (Dojindo, 2009).

After the cell number was determined by the trypan blue dye exclusion test, 3×10^3 cells per well were seeded in a 96-well flat-bottom plate by adding 200 µl from cell suspension prepared into each well of the plate. Refer Appendix 1.3 for cell seeding calculation in a 96-well plate. Then, the cells were allowed to adhere to the plate by incubating in 5% CO_2 incubator at 37°C for 24 hours. After that, each drug was diluted in 1:1000 dilution factor with the complete media. From this, different drugs solutions with their respective concentration was prepared. Refer Appendix 2.3.1 for drug solutions calculation. Then, 200 µl from each drug (5-fluorouracil, oxaliplatin, irinotecan, leucovorin) solutions with concentration of 3.125, 6.25, 12.5, 25, 50 and 100 µM were added to the cells in their respective well and incubated for 46 hours. The control cells which is the drugs untreated cells and the culture media as the blank was also added to their respective well on the plate. After incubate, the media were discarded and 20 µl of CCK-8 dye was added into each well and incubated in 5% CO_2 incubator at 37°C for 4 hours. Then, the absorbance value produced was read at 570 nm using ELISA multiplate reader. From the absorbance value,

percentage of cell viability and cell inhibition was calculated and plotted against drug concentration via Graphad prism. From that, IC_{50} values of the drugs were obtained by extrapolating the linear regression as the concentration of drug producing a 50% reduction in corrected absorbance. Each drug was tested in duplicate and the data represents mean of two.

3.3.2 AO/PI staining for cells morphological changes analysis

AO/PI staining was used to study the cytotoxic effects of drugs on treated cells by assessing the morphological of cells. Acridine orange (AO) was a cationic dye that is permeable to the cell membrane allowing it to stain both live and dead cells, producing a green fluorescence. Meanwhile, propidium iodide (PI) was a non-membrane permeable dye but it could penetrate the membranes of dead cells and stain dead cells red. A fluorescence resonance energy transfer (FRET) will occur when the dead cells contained both AO and PI, where AO emission will be absorbed by PI molecules, causing dead cells to fluoresce red and only live cells fluoresce green (Chan et al., 2016)(Gothai S et al, 2018).

After the cell number was determined by the trypan blue dye exclusion test, 3 $x10^4$ cells per well were seeded in a 24-well flat-bottom plate by adding 400 µl from cell suspension prepared into each well of the plate. Refer Appendix 1.2 for cell seeding calculation in a 24-well plate. Then, the cells were allowed to adhere by incubating in 5% CO2 incubator at 37°C for 24 hours. After that, each drug was diluted in 1:1000 dilution factor with the complete media. From this, different drugs solutions with their respective concentration was prepared. Refer Appendix 2.3.2 for drug solutions calculation. Then, 400 µl of each drug (5-Fluorouracil, Oxaliplatin, Irinotecan, Leucovorin) solutions with concentration of 3.125, 6.25, 12.5, 25, 50 and 100 µM were added to the cells in their respective well and incubated for 46 hours. The control cells which is the drugs

untreated cells and the culture media as the blank was also added to their respective well on the plate. After incubation, the cells were trypsinized for staining purposes. The media was discarded from each wells and the cells were washed for 2 times using 600 µl of phosphate buffer saline solution. Then, this wash solution was discarded and 300 µl of pre-warmed dissociation reagent (trypsin) was added to each well. The cells were incubated in 5% CO2 at 37°C for 5 to 10 minutes.

Once the cells were detached, 600 µl of media was added to each well and the media together with the cells then was transferred to a 1.5 ml centrifuge tube which is then centrifuged at 4000 rpm for 5 minutes. After centrifuge, the supernatant was discarded and 100 µl of complete media was added into the tube and the cell pellet was rinsed with the media. To stain the cells, AO/PI dye solution was prepared by mixing 100 µl of AO dye and 100 µl of PI dye together. Then, 10 µl from the prepared dye solution was added into 10 µl of cell suspension on a glass slide. The different color and morphological changes of the stained cells treated with different drugs at different concentration was determined using fluorescent microscopes based on respective wavelengths. The images produced were snapped and recorded using computer software. Each drug was tested in duplicate and the data represent the clearest images.

3.4 BEST DRUG COMBINATIONS DETERMINATION

3.4.1 Orthogonal design in SPSS for designing different combinations of FOLFIRINOX concentration

Orthogonal experiment design is an efficient and flexible design method with orthogonal table to arrange multi-factor experiment in which optimal combination scheme was created (Zhou et al., 2012). The process of creating FOLFIRINOX combinations via experimental orthogonal design with SPSS software is shown in **Figure 3.1**.

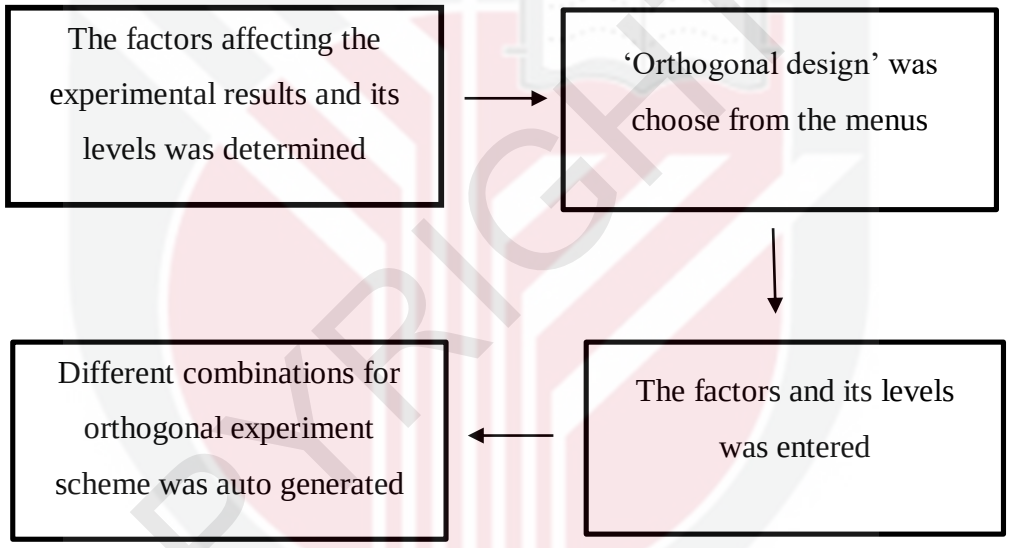


Figure 3. 1: Orthogonal experiment design flow with SPSS software

The orthogonal experiment design was carried out with percentage of cell inhibition as an evaluation index. Four factors (drugs in FOLFIRINOX) with three levels (range of IC_{50} values) were used in the design. The factors and levels were shown in **Table 3.1**.

Table 3. 1: Factors-levels of orthogonal experiment

Factor	Level		
Range of IC_{50} of 5-Fluorouracil	1 µM	15 µM	30 µM
Range of IC_{50} of Oxaliplatin	1 µM	10 µM	20 µM
Range of IC_{50} of Irinotecan	1 µM	15 µM	30 µM
Range of IC_{50} of Leucovorin	1 µM	5 µM	10 µM

3.4.2 CCK-8 assay and AO/PI staining for cytotoxicity test and cell morphology analyses of different FOLFIRINOX combinations

Based on different combinations of FOLFIRINOX concentration generated above, CCK-8 assay was used to obtain the percentage of cell inhibition of each different combination. After the cell number was determined by the trypan blue dye exclusion test, 3 x 10^3 cells per well were seeded in a 96-well flat-bottom plate by adding 200 µl from cell suspension prepared into each well of the plate. Refer Appendix 1.3 for cell seeding calculation in a 96-well plate. Then, the cells were allowed to adhere to the plate by incubating in 5% CO2 incubator at 37°C for 24 hours. After that, each drug was diluted in 1:1000 dilution factor with the complete media. From this, different drugs solutions with their respective concentration was prepared. Refer Appendix 2.4 for drug solutions calculation. Then, 200 µl from each drug (5-fluorouracil, oxaliplatin, irinotecan, leucovorin) solutions with their respective concentration of 1 µM to 30 µM were added to the cells in their respective combinations well and incubated for 46 hours. The control cells which is the drugs untreated cells and the culture media as the blank was also added to their respective well on the plate. After incubate, the media were discarded and 20 µl of CCK-8 dye was added into each well and incubated in 5% CO2 incubator at 37°C for 4 hours. Then, the absorbance value produced

was read at 570 nm using ELISA multiplate reader. From the absorbance value, percentage of cell inhibition was calculated. Each combinations was tested in duplicate and the data represents mean of two.

AO/PI staining was used to access the morphological changes on cells treated with different FOLFIRINOX combinations. After the cell number was determined by the trypan blue dye exclusion test, 3 $x10^4$ cells per well were seeded in a 24-well flat-bottom plate by adding 400 ul from cell suspension prepared into each well of the plate. Refer Appendix 1.2 for cell seeding calculation in a 24-well plate. Then, the cells were allowed to adhere by incubating in 5% CO2 incubator at 37°C for 24 hours. After that, each drug was diluted in 1:1000 dilution factor with the complete media. From this, different drugs solutions with their respective concentration was prepared. Refer Appendix 2.4 for drug solutions calculation. Then, 400 µl from each drug (5-fluorouracil, oxaliplatin, irinotecan, leucovorin) solutions with their respective concentration of 1µM to 30 µM were added to the cells in their respective combinations well and incubated for 46 hours. The control cells which is the drugs untreated cells and the culture media as the blank was also added to their respective well on the plate. After incubation, the cells were trypsinized for staining purposes. The media was discarded from each wells and the cells were washed for 2 times using 600 µl of phosphate buffer saline solution. Then, this wash solution was discarded and 300 µl of pre-warmed dissociation reagent (trypsin) was added to each well. The cells were incubated in 5% CO2 at 37°C for 5 to 10 minutes.

Once the cells were detached, 600 µl of media was added to each well and the media together with the cells then was transferred to a 1.5 ml centrifuge tube which is then centrifuged at 4000 rpm for 5 minutes. After centrifuge, the supernatant was discarded and 100 µl of complete media was added into the tube and the cell pellet was rinsed with the media. To stain the cells,

AO/PI dye solution was prepared by mixing 100 µl of AO dye and 100 µl of PI dye together. Then, 10 µl from the prepared dye solution was added into 10 µl of cell suspension on a glass slide. The different color and morphological changes of the stained cells treated with different drugs combinations was determined using fluorescent microscopes based on respective wavelengths. The images produced were snapped and recorded using computer software. Each combinations was tested in duplicate and the data represents the clearest images.

3.4.3 Response Surface Methodology (RSM) analyses

Based on above results, further studies on different combination of FOLFIRINOX concentration were performed. A face centered central composite design (FCCD) was employed to study the interaction of 4 factors (5-fluorouracil, oxaliplatin, irinotecan, and leucovorin) and determine the best combination among different combinations generated in 3.4.1. Each factors was studied at 3 different levels encoded with (-1, 0, +1) and a model was generated using Design-Expert 6.0 software. This model was analyzed using ANOVA, 3D response curve plots and one contour plot. All test have performed duplicate and the data represent a mean of two. The experimental plan with respect to actual and coded form of factors are listed as in **Table 3.2**. Percentage of cytotoxicity were considered as the responses.

Table 3. 2: Experimental range and levels of the four independent factors used in RSM in term of actual and coded form.

Factor	Level					
	Actual	Coded	Actual	Coded	Actual	Coded
Range of IC_{50} of 5-Fluorouracil	1 µM	-1	15 µM	0	30 µM	+1
Range of IC_{50} of Oxaliplatin	1 µM	-1	10 µM	0	20 µM	+1
Range of IC_{50} of Irinotecan	1 µM	-1	15 µM	0	30 µM	+1
Range of IC_{50} of Leucovorin	1 µM	-1	5 µM	0	10 µM	+1

CHAPTER 4
RESULTS

4.1 Growth of Capan-2 cells

The culture of Capan-2 cells in RPMI 1640 complete media was composed of cells exhibiting a round shape with a transparent cytoplasm a day after sub culturing into a new 25cm^2 culture flask. Majority of the cells were single celled and located apart from each other. However, as the day goes by, the cells exhibited proliferation where at day 7 of culture, the size of cells were increased and epithelial-like morphological characteristics were shown. The cells also located closely to each other **(Figure 4.1).** This indicates that the cells were well-growing under the culture conditions.

a) b)

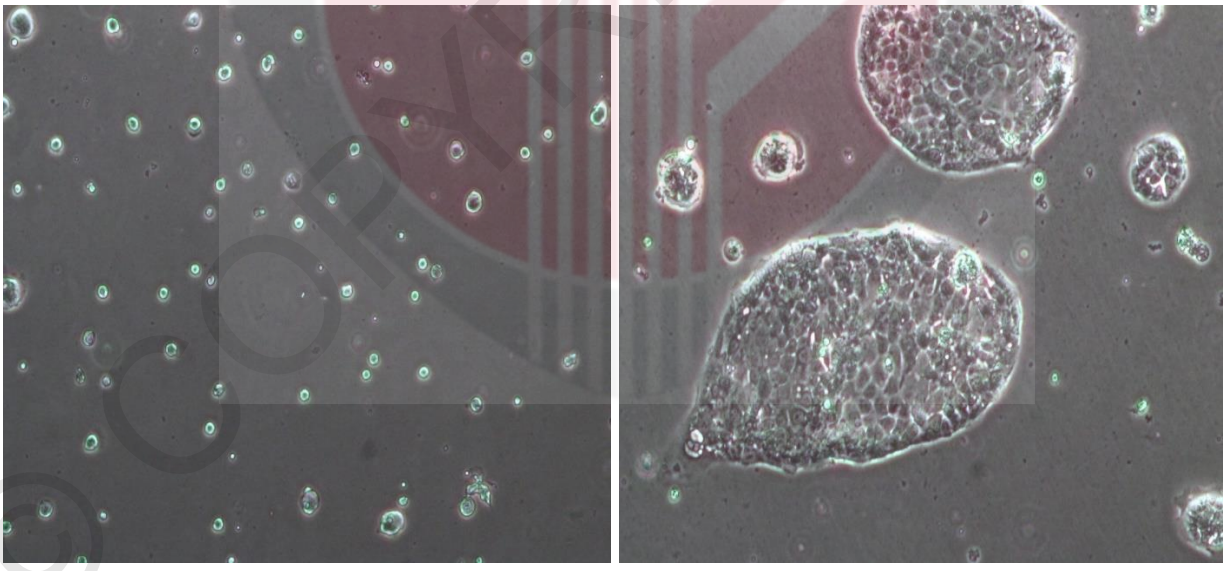


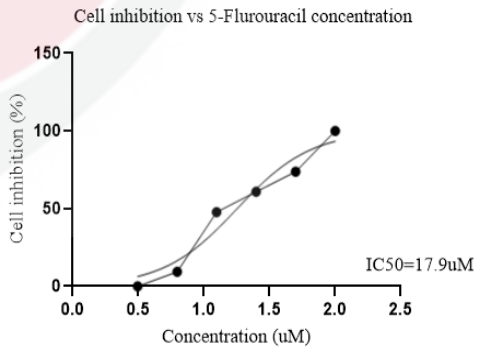
Figure 4. 1: Capan-2 cells under inverted microscope after (a) 1 day of culture and (b) 7 day of culture at 40x magnification.

4.2 IC_{50} of the FOLFIRINOX drugs on Capan-2 cells

The cytotoxicity testing of 4 drugs in FOLFIRINOX (5-fluorouracil, oxaliplatin, irinotecan and leucovorin) via CCK-8 viability assay had showed a prominent cells inhibitory as the drugs concentration increased. As in **Table 4.1**, the percentage of normalized cell inhibition for all 4 drugs was increase when the concentration of drugs increase. This findings indicates that all drugs were played a great cytotoxicity effects in inhibiting the growth of Capan-2 cells. From this, the concentration which induced 50% cell growth inhibition (IC_{50}) on Capan-2 cells were calculated. The IC_{50} values obtained from this study were 17.9 µM for 5-fluorouracil, 16.61 µM for oxaliplatin, 17.38 µM for irinotecan and 9.289 µM for leucovorin.

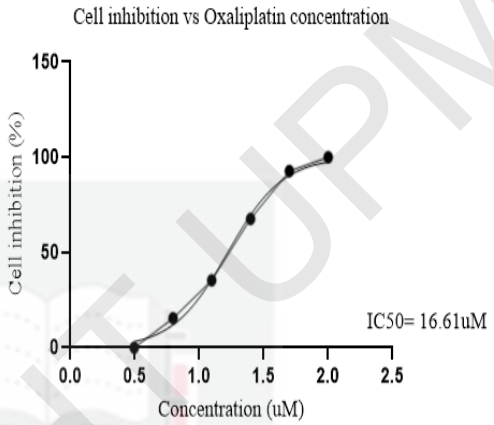
Table 4. 1: The average of absorbance of duplicate experiment, percentage of normalized cell inhibition and IC_{50} graph and value for all drugs a) 5-fluorouracil b) oxaliplatin c) irinotecan d) leucovorin

a) 5-Fluorouracil

Concentration (µM)	Average of absorbance (nm)	Normalized cell inhibition (%)	IC_{50} graph and value
3.125	0.883	0	 Range IC_{50}: 11.64 to 28.06
6.25	0.858	9.38	
12.5	0.728	47.87	
25	0.698	60.95	
50	0.671	73.80	
100	0.586	100	

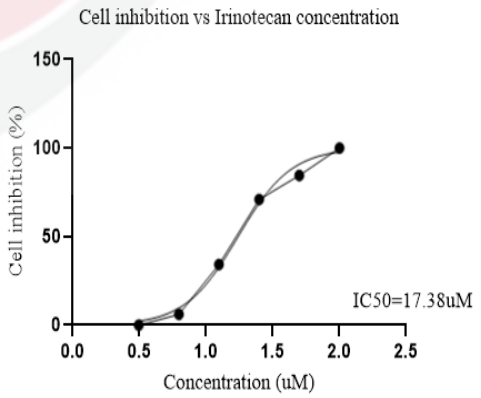
Mean ± SD for 5-flurouracil = 48.7± 38.28, with R^2 = 0.9522

b) Oxaliplatin

Concentration (µM)	Average of absorbance (nm)	Normalized cell inhibition (%)	IC_{50} graph and value
3.125	0.834	0	 Range IC_{50}= 14.64 to 18.83
6.25	0.787	15.61	
12.5	0.731	35.40	
25	0.626	67.68	
50	0.538	92.81	
100	0.517	100	

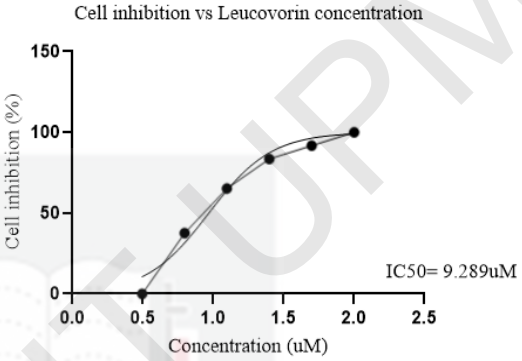
Mean ± SD for oxaliplatin = 51.92± 41.3, with R^2 = 0.9963

c) Irinotecan

Concentration (µM)	Average of absorbance (nm)	Normalized cell inhibition (%)	IC_{50} graph and value
3.125	1.227	0	 Range IC_{50}= 14.81 to 20.48
6.25	1.142	6.23	
12.5	1.037	34.32	
25	0.888	71.12	
50	0.836	84.60	
100	0.767	100	

Mean ± SD for irinotecan = 53.2± 38.8, with R^2 = 0.9921

d) Leucovorin

Concentration (µM)	Average of absorbance (nm)	Normalized cell inhibition (%)	IC_{50} graph and value
3.125	1.54	0	 Range IC_{50}= 7.027 to 12.44
6.25	1.483	37.65	
12.5	1.440	65.29	
25	1.413	83.53	
50	1.400	91.76	
100	1.387	100	

Mean ± SD for leucovorin = 64± 38, with R^2 = 0.9739

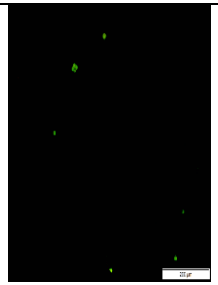
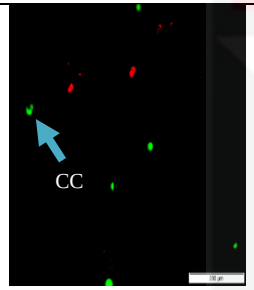
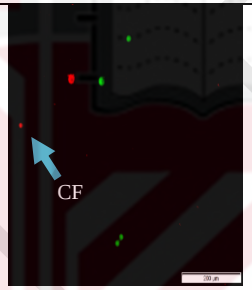
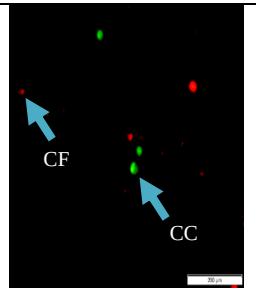
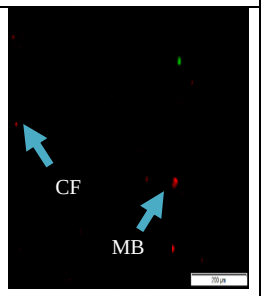
4.3 Cells morphological changes analyses on Capan-2 cells treated with FOLFIRINOX

The cells morphological analyses via AO/PI staining generally had showed induction of apoptosis in the Capan-2 cells treated with all 4 drugs in FOLFIRINOX. The cells displayed the apoptotic change in treated cells in terms of colour and morphology. The control cells displayed a bright green colouration and intact membrane structure indicate a healthy live cells, where the treated cells displayed red colouration and some apoptotic features signifying the presence of death cells. As the drugs concentration increased, the number of green cells gradually decreased while the number of red cells increased. In addition, the treated cells had displayed a change in cell morphology where the apoptotic features such as cells fragmentation, chromatin condensation and membrane blebbing had become more apparent as the drugs concentrations increased **(Table 4.2).**

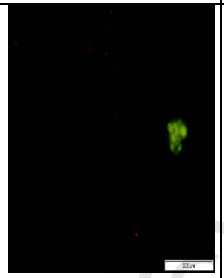
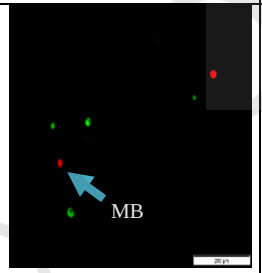
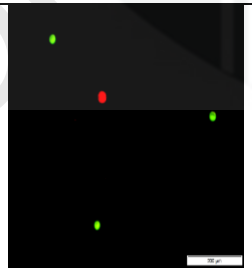
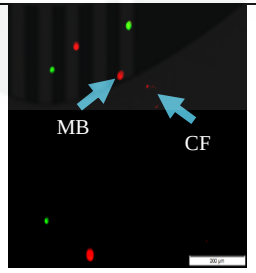
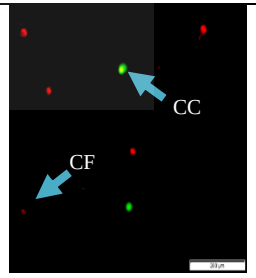
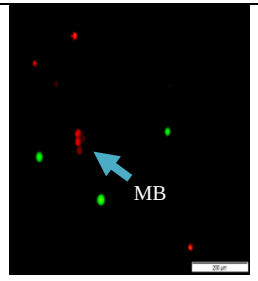
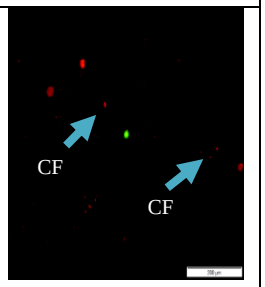
At 3.125 µM of 5-fluorouracil, there were clear indication of chromatin condensation while at 50 to 100 µM of 5-fluorouracil, more chromatin condensation, cells fragmentations and blebbing of Capan-2 membrane were observed. Similarly, at 3.125 µM of oxaliplatin, chromatin condensation were observed while there were more fragment and membrane blebbing cells at 50 to 100 µM of oxaliplatin. At 100 µM of irinotecan, more cells undergo fragmentation and membrane blebbing compared to low concentrations (3.125 to 12.5 µM). Lastly, at 50 to 100 µM of leucovorin, the cells showed a prominent cells fragmentation and membrane blebbing compared to 3.125 µM of leucovorin. The morphological changes as well as the changes in number of live and death cells that were observed from AO/PI staining indicating gradual cell death via apoptosis with increasing concentration of the drugs. This showed the drugs had worked greatly in exerting their cytotoxicity effects to induced cell death of Capan-2 as concentration increased.

Table 4. 2: AO/PI fluorescence images of Capan-2 cells treated with all 4 drugs: a) 5-fluorouracil, b) oxaliplatin, c) irinotecan d) leucovorin with arrows indicating chromatin condensation (CC), cells fragmentation (CF) and membrane blebbing (MB) at 200x magnification

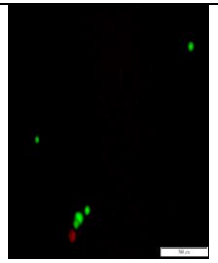
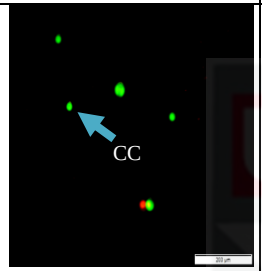
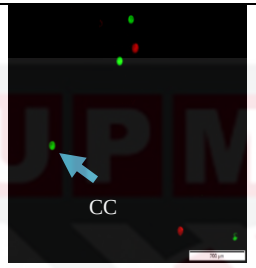
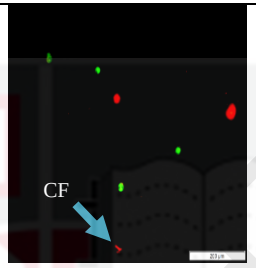
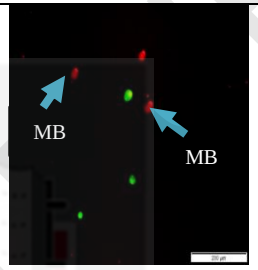
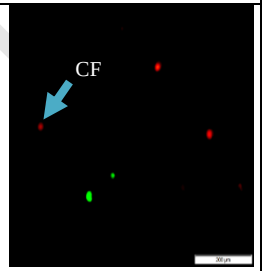
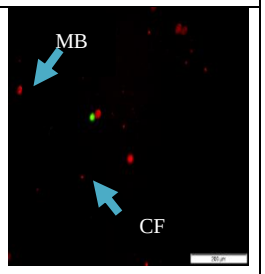
a) 5-Fluorouracil

Concentration (µM)	Control	3.125	6.25	12.5	25	50	100
AO/PI							

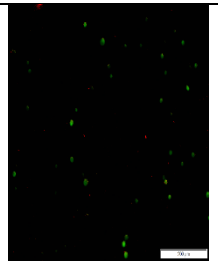
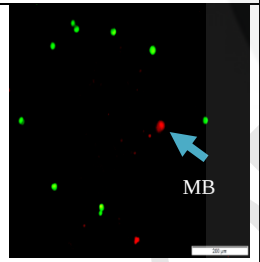
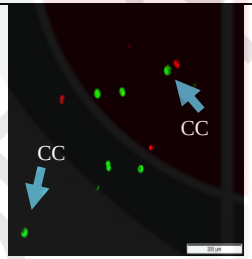
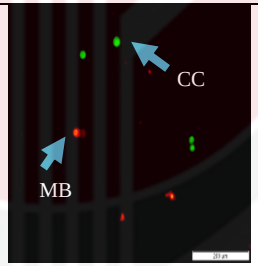
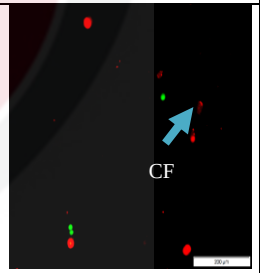
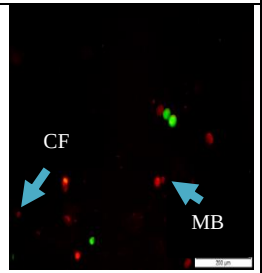
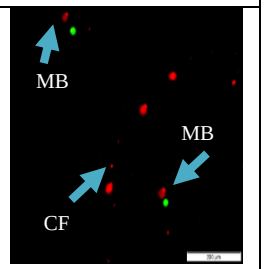
b) Oxaliplatin

Concentration (µM)	Control	3.125	6.25	12.5	25	50	100
AO/PI							

c) Oxaliplatin

Concentration (µM)	Control	3.125	6.25	12.5	25	50	100
AO/PI							

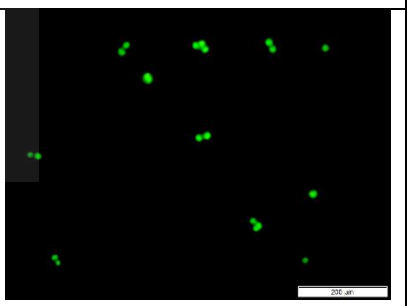
d) Leucovorin

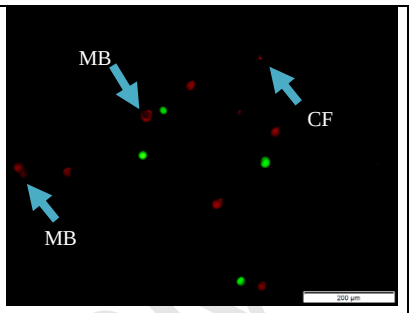
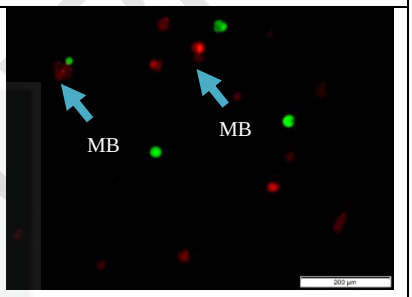
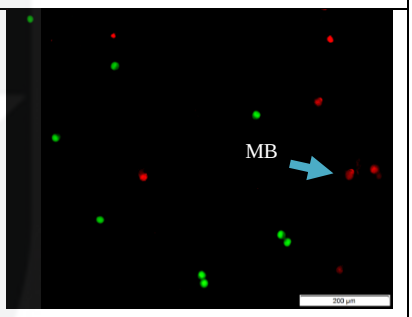
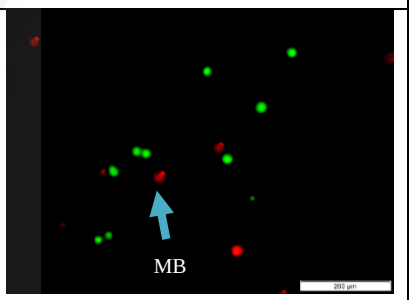
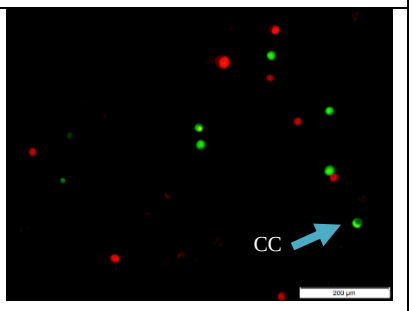
Concentration (µM)	Control	3.125	6.25	12.5	25	50	100
AO/PI							

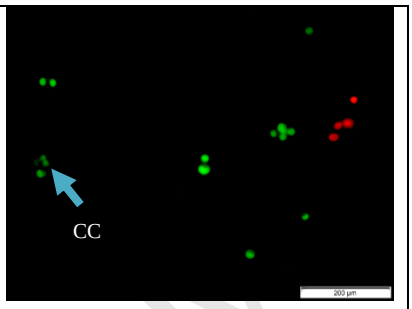
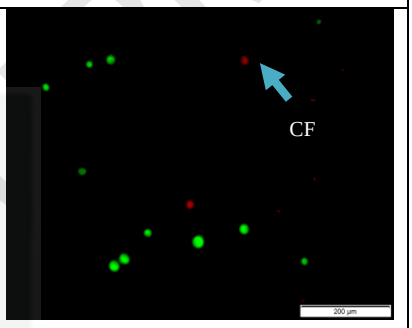
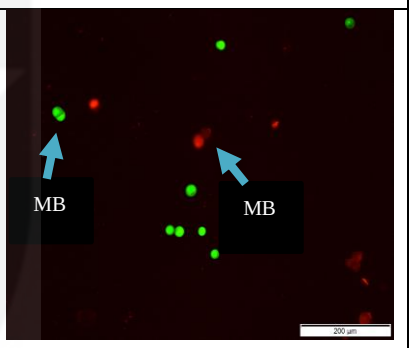
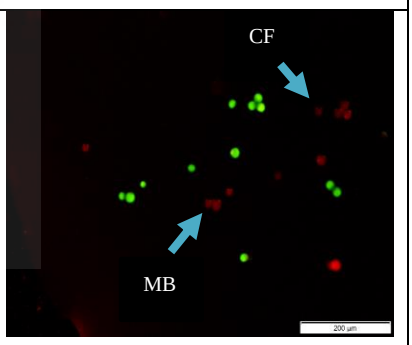
4.4 Cytotoxicity and morphological changes by different combinations of FOLFIRINOX

The orthogonal design via SPSS had generated 9 different combinations of FOLFIRINOX concentration. The cytotoxicity testing of this different combinations via CCK-8 viability assay had showed a promising cells inhibitory on Capan-2 as all the combinations showed 40 to 60% of cell inhibition. Besides, there were also a clear apoptotic features which are chromatin condensation, cell fragmentation and membrane blebbing had shown on Capan-2 treated with different combinations FOLFIRINOX compared to the untreated cells (control) **(Table 4.3).** Among the 9 different combinations, combinations 2 which consists of 30 µM of 5-flurouracil, 20 µM of oxaliplatin, 1 µM of irinotecan and 5 µM of leucovorin had showed the highest percentage of cell inhibition with 62.4% and blebbing on the cells membrane.

Table 4. 3: The percentage of cell inhibition and changes in cells morphology by 9 different combinations of FOLFIRINOX concentration

Combination	5-fluorouracil (µM)	Oxaliplatin (µM)	Irinotecan (µM)	Leucovorin (µM)	Cell inhibition (%)	AO/PI
Control	0	0	0	0	0	

1	30	10	30	1	60.3	
2	**30**	**20**	**1**	**5**	**62.4**	
3	15	1	30	5	49.5	
4	15	20	15	1	46.2	
5	15	10	1	10	60.6	

6	1	20	30	10	41.6	
7	1	1	1	1	40.6	
8	30	1	15	10	40.3	
9	1	10	15	5	48.4	

4.5 Determination on best concentration combinations of FOLFIRINOX via Response surface methodology (RSM)

The further analyses on different combinations of FOLFIRINOX concentration using RSM had showed a correspond findings between observed values and predicted values of percentage of cell inhibition **(Table 4.4).** The standard analysis of variance (ANOVA) for percentage of cytotoxicity had shown that the model term is significant as the p-value is less than 0.05 and R^2 values of 0.9983. The second order regression equation for percentage of cytotoxicity as a function of 5-fluorouracil (A), oxaliplatin (B), irinotecan (C) and leucovorin (D), is given below.

Cytotoxicity (%) = +50.90 + 2.03 × A + 12.54 × B + 5.84 × C + 16.33 × D + 15.48 × A × B + 18.75 × A × C - 7.67 × B × C

Table 4. 4: Experimental design used in RSM by using 4 drugs showing observed and predicted values of percentage of cytotoxicity.

Combination	5-fluorouracil (µM)	Oxaliplatin (µM)	Irinotecan (µM)	Leucovorin (µM)	Cytotoxicity (%)	
					Observed	Predicted
1	+1	0	+1	-1	60.3	60.1
2	+1	+1	-1	0	62.4	61.6
3	0	-1	+1	0	49.5	49.8
4	0	+1	0	-1	46.2	46.6
5	0	0	-1	+1	60.6	60.9
6	-1	+1	+1	+1	41.6	41.7
7	-1	-1	-1	-1	40.6	40.7
8	+1	-1	0	+1	40.3	40.1
9	-1	0	0	0	48.4	47.6

Mean ± SD= 49.99 ± 1.05, R^2 =0.9983, P>F-value (0.001) is being less than 0.05

The plotted three dimensional response curve were used to understand the interaction of 5-flurouracil, oxaliplatin, irinotecan and leucovorin in the combinations. Further analysis predicted the maximum cytotoxicity (114.2%) of drugs combination to occur when all 4 drugs in FOLFIRINOX are at their highest concentration **(Figure 4.2 (a)).** This findings correspond to analysis with contour plot **(Figure 4.2 (b)).** The analysis also revealed that 5-flurouracil was the most significant parameter and its cytotoxicity effects was influenced by other drugs. From **Figure 4.3 (a)**, it can be observed that the percentage of cytotoxicity was decrease to 82.84% from the maximum when irinotecan are at its lowest concentration (1 µM) correspond with the decrease in 5-fluorouracil response curve. This indicates influence of irinotecan to the cytotoxicity of 5-fluorouracil. In addition, from **Figure 4.3 (b),** a more clear indications were observed where only 50.17% of cytotoxicity were occurred when leucovorin at its lowest concentration in presence of high concentrations of 5-fluorouracil (30 µM). This indicates the great influence of leucovorin to the cytotoxicity effect of 5-fluorouracil and the needed of leucovorin at high concentrations to enhanced 5-fluorouracil activity so that the percentage of cytotoxicity of whole combination would increase. Besides, a high cytotoxicity (82.84%) was occurred when oxaliplatin at its highest concentration (20 µM), in presence of 1 µM 5-fluorouracil, 1 µM irinotecan and 10 µM leucovorin **(Figure 4.3 (c)).** This indicates that oxaliplatin also shows great cytotoxicity effects on Capan-2 cells despite lowest concentrations of 5-fluorouracil. However, since both 5-fluorouracil and oxaliplatin had showed a significant higher incidence of side effects according to literature review, further analysis were performed to obtain a combination of FOLFIRINOX with less concentrations of 5-fluorouracil and oxaliplatin. This analysis revealed a combinations consists of 1 µM 5-fluorouracil, 1 µM oxaliplatin, 15 µM irinotecan and 10 µM leucovorin produced a good and promising cytotoxicity percentage which is 73.38% **(Figure 4.3 (d)).**

a) b)

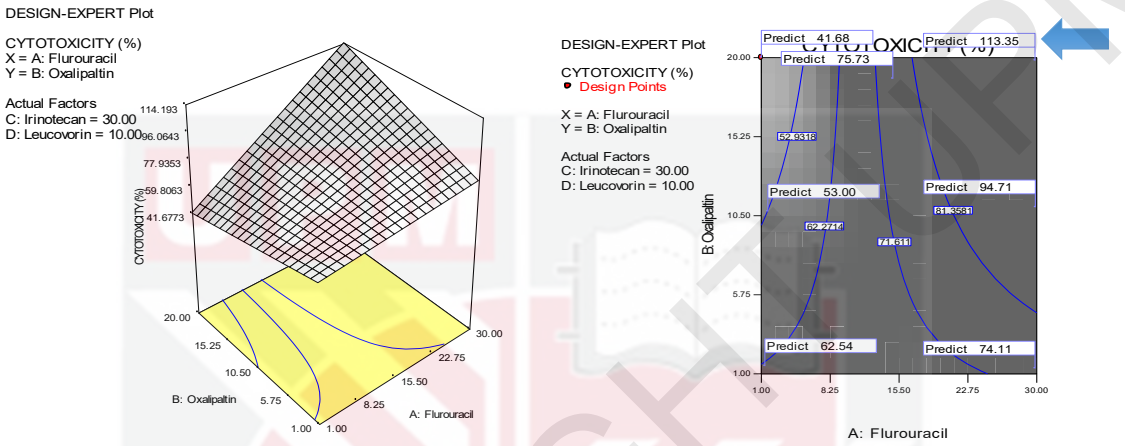
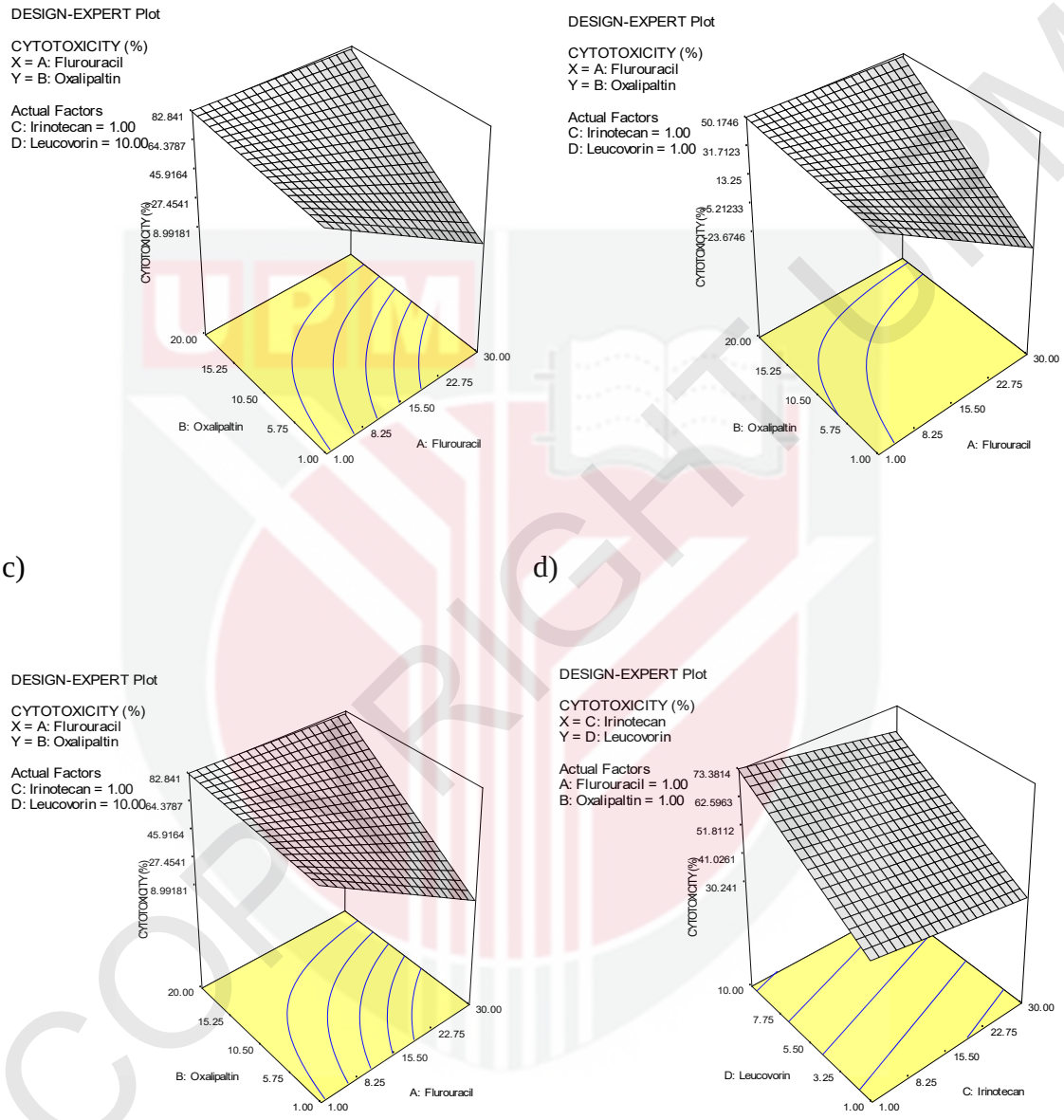


Figure 4. 2: a) 3D response surface curve and b) contour plot of percentage of cytotoxicity which showing maximum cytotoxicity of the combination

a) b)



c) d)

Figure 4. 3: Response surface curve of percentage of cytotoxicity which showing interaction between 5-fluorouracil, oxaliplatin, irinotecan and leucovorin in combinations. Figure (a) and (b) show interaction of 5-fluorouracil with irinotecan and leucovorin respectively, (c) show interaction of oxaliplatin with another 3 drugs (5-flurouracil, irinotecan, leucovorin) and (d) show interactions of all 4 drugs (5-fluorouracil, oxaliplatin, irinotecan, leucovorin) in the best combination

CHAPTER 5

DISCUSSION

Chemotherapy with potent and effective cytotoxic drugs were the main treatment modality for cancer. It is crucial to access and monitor the cytotoxicity activity of the drugs prior to patient administration as to prevent any unwanted events such as death to occur. Thus, cell-based assays which use of cell culture studies are useful in identifying the drugs cytotoxicity activity as this studies offers a controlled conditions and easy procedures (Florento et al., 2012). As in this study, Capan-2 pancreatic cancer cell line was cultured and maintained in RPMI 1640 complete media, a suitable environments to enhance their growth for cytotoxicity testing purposes. The presences of fetal bovine serum (FBS) and penicillin in the complete media provide growth supplement for cells in culture, due to high content of embryonic growth promoting factors in FBS and contamination prevention by penicillin.

The determinations of half-maximal inhibitory concentration (IC_{50}) is the most widely used to measure the cytotoxicity effects of a drug. IC_{50} is the concentrations of drugs which able to inhibit the growth of 50% of the cells. These determinants provide understanding on the pharmacological and biological properties of the drugs, whereby it give the prediction on the degree of drug's cytotoxic effect (Florento et al., 2012) (Neubig et al., 2003). A high IC_{50} value indicates the low cytotoxic effects of a drugs, while a low IC_{50} value indicates the high cytotoxic effects of the drugs (Florento et al., 2012). In this study, IC_{50} values of four drugs in FOLFIRINOX on Capan-2 cells were evaluated using a cell viability assay, CCK-8 assay. This assay measures the amount of formazan (in terms of absorbance values) produced which proportional to the number of living cells present after the drugs treatment. The production of formazan was relies on

the reduction of water-soluble tetrazolium salts to yellow colored formazans by live cells. The increase or decrease in amount of formazan formed indicates the degree of cytotoxicity effects of the drugs.

Based on the result for all 4 drugs in FOLFIRINOX, as the drugs concentration increase, the average absorbance produced was decrease and percentage of cell inhibition was increase. This indicates that all drugs had shown increases cytotoxicity activity when the concentrations of the drugs also increase. The low IC_{50} values obtained from this study indicates a high cytotoxic effects against Capan-2 cells as its growth could be inhibited effectively even with a small amount of drugs being used. For 5-fluorouracil, the IC_{50} value obtained is 17.9 µM with range of 11.64 to 28.06 µM. This findings is correlates with a studies by Elsold et al (2004) in which the IC_{50} values obtained is 19.13 µM where still in range with our findings. For oxaliplatin, the IC_{50} values obtained in this study is 16.61 µM with range of 14.64 to 18.83 µM. This findings is supported with a studies by Kornmann et al (2000) stated that IC_{50} values for oxaliplatin is less than 25.2 µM. However, the IC_{50} values for irinotecan found in this study was different from previous studies where the IC_{50} values is 17.38 µM with range of 14.81 to 20.48 µM, compared with 830 µM reported by Cancergene (nd). This may due to different materials and methodology used when performing the research. As stated by Brody et al (2018) comparing IC_{50} values between different research laboratories can be problematic since the result varies according to different type of materials and procedure applied. Thus our findings still accepted. Lastly, for leucovorin, the IC_{50} values found in this study is 9.289 µM with range of 7.027 to 12.44 µM. This findings was correlates with a studies by Beck et al (1994) stated that the IC_{50} of leucovorin for human tumour cells was ranging from 0.4 µM to 38.9 µM. The correlation of findings from this study with

previous indicates a promising findings has obtained for better understanding the cytotoxicity effects of the drugs.

Besides IC_{50} determinations, identification of the cells morphological changes due to physical destruction involved in apoptosis is used to confirm the cytotoxicity activity of the drugs. In this study, the cell morphological changes was evaluated using AO/PI staining, a dual-fluorescence staining for live or dead cells. As the acridine orange (AO) is permeable to both live and dead cells, its stains all nucleated cells with green fluorescence, while propidium iodide (PI) which membrane impermeable but can enters cells with compromised membranes would stain the dying and dead nucleated cells with red fluorescence. The apoptotic properties of the treated cancer cells were better illustrated from the morphological observations via this staining. Based on results, this study had revealed an increases of dead cells as the drugs concentration increases as well as a clear apoptotic morphological features in Capan-2 cells treated with all 4 drugs in FOLFIRINOX, including chromatin condensation, membrane blebbing and cells fragmentation. This indicates a promising cytotoxicity activity of FOLFIRINOX to induce apoptosis to Capan-2 cells. Chromatin condensation characterized by a dense green color on part of the cells indicates chromatin condensed and compacted in a random which often around the nuclear membrane (Yang et al., 2006). Membrane blebbing characterized by the alteration on red stained cells plasma membrane where the membrane is swell and form blebs on their surface (Shiratsuchi et al., 2002). While cells fragmentation showed a small pieced red stained cells indicates the cells was fragmented from the original cells when undergo cell death. All of this apoptotic features was more absorbed when the cells treated with increase concentration of 5-fluorouracil. This findings was supported with a studies by Focacceti et al (2015) stated that there are loss of membrane in cells treated with 5-fluorouracil greater than 1 µM. Similarly, cells treated with high concentration of oxaliplatin

showed a clear indications on cells fragmentation which correspond with finding by Amardori et al (2005) stated that PI staining revealed cells fragmentation after oxaliplatin treatments. Besides, a prominent membrane blebbing and cells fragmentation were also observed in this study after irinotecan treatment correlates with findings from Casado et al (2007) studies revealed that cells became rounded and fragmented after treat with irinotecan. Lastly, a more membrane blebbing and cells fragmentation obtained at high concentration of leucovorin indicates the ability of the drug to cause cell to death.

A modified combination drug therapy was useful to reduce the intolerable side effects and prolong life. Therefore, the cytotoxicity of generated FOLFIRINOX combinations in this study were also evaluated. The Capan-2 cells was treated with 9 different FOLFIRINOX combinations and their cytotoxicity were assessed by CCK-8 viability assay and AO/PI staining. From the result, each drugs combinations showed a promising cytotoxicity as there are high percentage of cell inhibitory (40-60%) was observed. Among that, combinations 2 (30 µM 5-fluorouracil, 20 µM oxaliplatin, 1 µM irinotecan, 5 µM leucovorin) with 62.4% and membrane blebbing represent the best combinations as its highest percentage of cell inhibition produced. However, this findings was contradict with previous studies stated on the standard dose used in the FOLFIRINOX combinations on patients, where approximation of drugs dosages equivalent to *in vitro* are 492 µM 5-fluorouracil, 161 µM oxaliplatin, 109 µM irinotecan and 125 µM leucovorin. This may cause by the different systems used to study the drugs. It is difficult to extrapolated findings found from *in vitro* to *in vivo* due to the absence of drugs pharmacokinetic, a metabolism, absorption and transportation process occurred on the drugs *in vitro* studies (Yoon et al., 2012).

A further analysis on different combinations of FOLFIRINOX concentration was carried out by Response Surface Methodology (RSM). RSM is a uses of mathematical and statistical

techniques in development or formulation of new products (Şenaras, 2019). It study the interaction of factors which influenced the outcome of a products. In this study, the interactions of the 4 drugs in FOLFIRINOX were studied and the best combination of FOLFIRINOX concentration which provide high cytotoxicity with low side effects were determined. Based on the result, the analysis revealed that 5-fluorouracil was the most significant parameter where its cytotoxicity effects was influenced by other drugs. The decrease in percentage of cytotoxicity as well as 5-fluorouracil cytotoxic response when concentration of irinotecan is lowest indicates that irinotecan had exert synergistic influence on 5-fluorouracil. Besides, the much more clear indications of decrease in percentage of cytotoxicity and 5-fluorouracil cytotoxic response were occurred when concentration of leucovorin at its lowest, indicates the requires of leucovorin at high concentration in order to increase the cytotoxicity of whole combination and 5-fluorouracil. This findings goes well with the ultimate function of leucovorin where its use to stabilizing the binding of 5-fluorouracil's metabolites to its target enzyme (thymidylate synthase) for cellular DNA synthesis inhibition. In addition, leucovorin which as derivative of folic acid would act as chemoprotectant to lesser the side effects caused by high concentrations of 5-fluorouracil (T-johari et al., 2019) (Pubmed.,nd).

Due to this, we evaluated the percentage of cytotoxicity produced with the lowest concentrations of 5-fluorouracil in the drugs combinations. The high cytotoxicity responses exert by a combinations with highest concentrations of oxaliplatin, in presences of low 5-fluorouracil, low irinotecan and high leucovorin indicates that oxaliplatin played an efficient cytotoxic effects in inhibiting growth of capan-2 cells. However, since oxaliplatin was also exerting a significant higher incidence of side effect (grade 3 or 4 neutropenia), use of lowest concentration of oxaliplatin in the drugs combination was needed. Therefore, as we analyzed the combinations with lowest 5-

fluorouracil (1 µM), lowest oxaliplatin (1 µM), median irinotecan (15 µM) and highest leucovorin (10 µM), a high (78.33%) percentage of cytotoxicity was produced. This indicates a promising and the best drugs combinations which provide a high cytotoxic effects associated with low side effects has found. This findings was correspond with previous studies by Blazer et al (2015) and Mahaseth et al (2013) where the occurrence of neutropenia after FOLFIRINOX treatment was decreased to 0% and 3% respectively when they dropping the 5-fluorouracil as the modifications in the drug combination. However, the validation of this findings with normal cells or neutrophils was needed to determine the accuracy of the model generated.

CHAPTER 6
CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

As the conclusion, the findings in this study showed that there was cytotoxicity effects exerting by all drugs in FOLFIRINOX as the percentage of cell inhibition increased when the drug concentration increased. The baseline data of the half-maximal inhibitory concentration (IC_{50}) of all drugs in FOLFIRINOX has found for future use in research. Besides, there are apoptotic features observed in Capan-2 cells treated with FOLFIRINOX. The combination of FOLFIRINOX concentrations which produce highest percentage of cell inhibition found in this study is differs from the standard combination used in pancreatic cancer treatment. However, the drug response curve analysis via Response Surface Methodology (RSM) has revealed the best drug combination which consists of 1 µM 5-fluorouracil, 1 µM oxaliplatin, 15 µM irinotecan and 10 µM leucovorin. In short, this study provide the knowledge on cytotoxicity effects of FOLFIRINOX in *in vitro* study and the importance in determining the best drug combination with high therapeutic and low toxicity effect as a treatment therapies for a disease.

As the limitations of the study, there are time restriction caused by pandemic COVID-19 which hits our country starting from March 2020 until now. Due to that, the validation test of the best drugs combination found via RSM analyses on normal cells and neutrophils was not conducted. Therefore, further studies are needed as the recommendations to validate this findings in order to determine a modified FOLFIRINOX which give maximum response achievable of in research setting and sufficient therapeutic effect with less occurrence of side effects in clinical settings.

APPENDIX 1

CELLS SEEDING CALCULATION

1.1 Formula of Total number of cells per ml

Total number of cells/ml= (Total number of cells counted/4) x Dilution factor x 10,000 cells/ml

1.2 Calculation of cells seeding in 24-well plate

a) Calculate total number of cells from culture flask in 1 ml of media

Eg: 1. Total number of cells counted = 193

2. Dilution factor =2

3. Total cells/ml= (Total number of cells counted/4) x Dilution factor x 10,000 cells/ml

= (193/4) x 2 x 10,000 = 965,000cells/ml

b) Calculate total number of cells needed in 1 plate.

Eg: 1. Number cells needed per well= 3 x 10^4 cells

2. Number of wells used = 21

3. Total number of cells/plate= 3 x 10^4 x 21 = 630,000 cells.

c) Calculate volume of cell suspension that needed to seed cells in a plate (x).

Formula: m1 (total cells/ml) = v1 (cells suspension volume) m2 (total cells needed/ml) = v2 (x)

Eg: (965,000 cell) = (1 ml)

(630,000 cell) = (x ml)

x ml = 0.65 ml

d) Calculate total volume of media that needed to seed the cell in a plate.

Eg: 1. Volume of culture medium/well= 0.4 ml

2. Number of wells used = 21

3. Total volume of culture medium/plate= 0.4 ml x 21= 8.4 ml

1.3 Calculation of cells seeding in 96-well plate

a) Calculate total number of cells from culture flask in 1 ml of media

Eg: 1. Total number of cells counted= 193

1. Dilution factor:=2
2. Total cells/ml= (Total number of cells counted/4) x Dilution factor x 10,000 cells/ml

= (193/4) x 2 x 10,000 = 965,000cells/ml

b) Calculate total number of cells needed in 1 plate.

Eg: 1. Number cells needed per well= 3 x 10^3 cells

2. Number of wells used = 54

3. Total number of cells/plate= 3 x10^3 x 54 = 162,000 cells.

c) Calculate volume of cell suspension that needed to seed cells in a plate (x).

Formula: m1 (total cells/ml) = v1 (cells suspension volume)
m2 (total cells needed/ml) = v2 (x)

Eg: (965,000 cell) = (1 ml)

(162,000 cell) = (x ml)

x ml= 0.17 ml

d) Calculate total volume of media that needed to seed the cell in a plate.

Eg: 1. Volume of culture medium/well= 0.2 ml

2. Number of wells used = 54

3. Total volume of culture medium/plate= 0.2 ml x 54= 10.8 ml

APPENDIX 2

DRUG SOLUTIONS CALCULATION

2.1 Formula for calculation of drug based on concentration

1. Mass of drug (g) = Concentration (µM/ul) x Volume of culture per well (ul) x Molecular weight of drug (g/M)
2. Convert mass of drug (g) to mass of drug (ug) by divide 10^6
3. Multiply mass of drug (ug) with number of wells for each concentration to get total mass of drug (ug) for all well in each concentration.
4. Convert total mass of drug (ug) to total volume of drug (ul) by multiply 10

2.2 Formula for calculation of media based on concentration

1. Total volume of culture media for all well (ul) = volume of culture media in 1 well (ul) x number of well
2. Volume of media to dilute drug (ul)= total volume of culture media for all well-total volume of drug (ul)

2.3 Individual Drug Calculation

2.3.1 Table 3 Calculation for preparation of drugs solution used in CCK-8 assay

a) 5-Fluorouracil

Concentration (µM)	Volume of drug/4 well (ul)	Volume of media/4 well (ul)
3.125	1) 3.125 µM/ul x 200 ul x 130.08 g/M= 81300 g 2) 81300 g /10^6=0.0813 ug 3) 0.0813 ug x 4 well= 0.3252 ug 4) 0.3252 ug x 10= 3.25 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 3.25 ul= 796.8 ul
6.25	1) 6.25 µM/ul x 200 ul x 130.08 g/M= 162600 g 2) 162600 g /10^6=0.1626 ug 3) 0.1626 ug x 4 well= 0.6504 ug 4) 0.6504 ug x 10= 6.5 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 6.5 ul= 793.5 ul
12.5	1) 12.5 µM/ul x 200 ul x 130.08 g/M= 325200 g 2) 325200 g /10^6=0.3252 ug 3) 0.3252 ug x 4 well= 1.3008 ug 4) 1.3008 ug x 10= 13 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 13 ul= 787 ul
25	1) 25 µM/ul x 200 ul x 130.08 g/M= 650400 g 2) 650400 g /10^6=0.6504 ug 3) 0.6504 ug x 4 well= 2.6016 ug 4) 2.6016 ug x 10= 26 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 26 ul= 774 ul
50	1) 50 µM/ul x 200 ul x 130.08 g/M= 1,300,800 g 2) 1,300,800 g /10^6=1.3008 ug	1) 200 ul x 4 well= 800 ul 2) 800 ul- 52 ul= 748 ul

	3) 1.3008 ug x 4 well= 5.2032 ug 4) 5.2032 ug x 10= 52 ul	
100	1) 100 µM/ul x 200 ul x 130.08 g/M= 2,601,600 g 2) 2,601,600 g /10^6= 2.6016 ug 3) 2.6016 ug x 4 well= 10.4064 ug 4) 10.4064 ug x 10= 104 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 104 ul= 696 ul

a) Oxaliplatin

Concentration (µM)	Volume of drug/4 well (ul)	Volume of media/4 well (ul)
3.125	1) 3.125 µM/ul x 200 ul x 397.29 g/M= 248,306 g 2) 248,306 g /10^6=0.2483 ug 3) 0.2483 ug x 4 well= 0.9932 ug 4) 0.9932 ug x 10= 9.92 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 9.92 ul= 790.1 ul
6.25	1) 6.25 µM/ul x 200 ul x 397.29 g/M= 496,612 g 2) 496,612 g /10^6=0.4966 ug 3) 0.4966 ug x 4 well= 1.9864 ug 4) 1.9864 ug x 10= 19.9 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 19.9 ul= 780.1 ul
12.5	1) 12.5 µM/ul x 200 ul x 397.29 g/M= 993,225 g 2) 993,225 g /10^6=0.9932 ug 3) 0.9932 ug x 4 well= 3.9728 ug 4) 3.9728 ug x 10= 39.7 ul	1) 200 ul x 4 well= 800ul 2) 800 ul- 39.7 ul= 760.3 ul
25	1) 25 µM/ul x 200 ul x 397.29 g/M= 1,986,450 g	1) 200 ul x 4 well= 800 ul

	2) 1,986,450 g /10^6=1.9865 ug 3) 1.9865 ug x 4 well= 7.9456 ug 4) 7.9456 ug x 10= 79.5 ul	2) 800 ul- 79.5 ul= 720.5 ul
50	1) 50 µM/ul x 200 ul x 397.29 g/M= 3,972,900 g 2) 3,972,900 g/10^6=3.9729 ug 3) 3.9729 ug x 4 well= 15.8916 ug 4) 15.8916 ug x 10= 158.9 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 158.9 ul= 641.1 ul
100	1) 100 µM/ul x 200 ul x 397.29 g/M= 7,945,800 g 2) 7,945,800 g/10^6= 7.9458 ug 3) 7.9458 ug x 4 well= 31.7832 ug 4) 31.7832 ug x 10= 317.8 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 317.8 ul= 482.2 ul

b) Irinotecan

Concentration (µM)	Volume of drug/4 well (ul)	Volume of media/4 well (ul)
3.125	1) 3.125 µM/ul x 200 ul x 586.67 g/M=366,668 g 2) 366,668 g/10^6= 0.3667 ug 3) 0.3667 ug x 4 well= 1.4668 ug 4) 1.4668 ug x 10=14.7 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 14.7 ul= 785.3 ul
6.25	1) 6.25 µM/ul x 200 ul x 586.67 g/M=733,337 g 2) 733,337 g/10^6= 0.7333 ug 3) 0.7333 ug x 4 well= 2.9332 ug 4) 2.9332 ug x 10=29.3 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 29.3 ul= 770.7 ul

12.5	1) 12.5 µM/ul x 200 ul x 586.67 g/M=1,466,675 g 2) 1,466,67 g/10^6= 1.4667 ug 3) 1.4667 ug x 4 well= 5.8668 ug 4) 5.8668 ug x 10=<u>58.7 ul</u>	1) 200 ul x 4 well= 800 ul 2) 800 ul- 58.7 ul= <u>741.3 ul</u>
25	1) 25 µM/ul x 200 ul x 586.67 g/M=2,933,350 g 2) 2,933,350 g/10^6= 2.9334 ug 3) 2.9334 ug x 4 well= 11.7336 ug 4) 11.7336 ug x 10=<u>117.3 ul</u>	1) 200 ul x 4 well= 800 ul 2) 800 ul- 117.3 ul= <u>682.7 ul</u>
50	1) 50 µM/ul x 200 ul x 586.67 g=5,866,700 g 2) 5,866,700 g/10^6= 5.8667 ug 3) 5.8667 ug x 4 well= 23.4668 ug 4) 23.4668 ug x 10=<u>234.7 ul</u>	1) 200 ul x 4 well= 800 ul 2) 800 ul- 234.7 ul= <u>565.3 ul</u>
100	1) 100 µM/ul x 200 ul x 586.67 g/M=11,733,40 g 2) 11,733,40 g/10^6= 11.7334 ug 3) 11.7334 ug x 4 well= 46.9396 ug 4) 46.9396 ug x 10=<u>469.4 ul</u>	1) 200 ul x 4 well= 800 ul 2) 800 ul- 469.4 ul= <u>330.6 ul</u>

c) Leucovorin

Concentration (µM)	Volume of drug/4 well (ul)	Volume of media/4 well (ul)
3.125	1) 3.125 µM/ul x 200 ul x 512 g/M= 320,000 g 2) 320,000 g/10^6= 0.32 ug 3) 0.32 ug x 4 well= 1.28 ug	1) 200 ul x 4 well= 800 ul 2) 800 ul- 12.8 ul= <u>787.2 ul</u>

	4) 1.28 ug x 10=12.8 ul	
6.25	1) 6.25 µM/ul x 200 ul x 512 g/M= 640,000 g 2) 640,000 g/10^6= 0.64 ug 3) 0.64 ug x 4 well= 2.56 ug 4) 2.56 ug x 10=25.6 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 25.6 ul= 774.4 ul
12.5	1) 12.5 µM/ul x 200 ul x 512 g/M= 1,280,000 g 2) 1,280,000 g/10^6= 1.28 ug 3) 1.28 ug x 4 well= 5.12 ug 4) 5.12 ug x 10=51.2 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 51.2 ul= 748.8 ul
25	1) 25 µM/ul x 200 ul x 512 g= 2,560,000 g 2) 320,000 g/10^6= 2.56 ug 3) 2.56 ug x 4 well= 10.24 ug 4) 10.24 ug x 10=102.4 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 102.4 ul= 697.6 ul
50	1) 50 µM/ul x 200 ul x 512 g= 5,120,000 g 2) 5,120,000 g/10^6= 5.12 ug 3) 5.12 ug x 4 well= 20.48 ug 4) 20.48 ug x 10=204.8 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 204.8 ul= 595.2 ul
100	1) 100 µM/ul x 200ul x 512g/M= 10,240,000 g 2) 10,240,000 g/10^6= 10.24 ug 3) 10.24 ug x 4 well= 40.96 ug 4) 40.96 ug x 10=409.6 ul	1) 200 ul x 4 well= 800 ul 2) 800 ul- 409.6 ul= 390.4 ul

2.3.2 Table 4 Calculation for preparation of drugs solution used in AO/PI staining

a) 5-Fluorouracil

Concentration (µM)	Volume of drug/3 well (ul)	Volume of media/3 well (ul)
3.125	1) 3.125 µM/ul x 400 ul x 130.08 g/M= 162600 g 2) 162600 g/10^6=0.1626 ug 3) 0.1626 ug x 3 well= 0.4878 ug 4) 0.4878 ug x 10= 4.9 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 4.9 ul= 1195.1 ul
6.25	1) 6.25 µM/ul x 400ul x 130.08 g/M= 325200 g 2) 325200 g/10^6=0.3252 ug 3) 0.3252 ug x 3 well= 0.9756 ug 4) 0.9756 ug x 10= 9.8 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 9.8 ul= 1190.2 ul
12.5	1) 12.5 µM/ul x 400 ul x 130.08 g/M= 650400 g 2) 650400 g/10^6=0.6504 ug 3) 0.6504 ug x 3 well= 1.9512 ug 4) 1.9512 ug x 10= 19.5 ul	1)400 ul x 3 well= 1200 ul 2) 1200 ul- 19.5 ul=1180.5 ul
25	1) 25 µM/ul x 400 ul x 130.08 g/M= 1,300,800 g 2) 1300800 g/10^6= 1.3008 ug 3) 1.3008 ug x 3well= 3.902 ug 4) 3.902 ug x 10= 39 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 39 ul=1161 ul
50	1) 50 µM/ul x 400 ul x 130.08 g= 2,601,600 g 2) 2,601,600/10^6=2.6016 ug 3) 2.6016 ug x 3 well= 7.805 ug	1) 400 ul x 3 well= 1200 ul 2) 1200ul- 78 ul= 1122 ul

	4) 7.805 ug x 10= 78 ul	
100	1) 100 µM/ul x 400 ul x 130.08 g= 5,203,200 g 2) 5,203,200 g/10^6= 5.2032 ug 3) 5.2032 ug x 3 well= 15.609 ug 4) 15.609 ug x 10= 156.1 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 156.1 ul= 1043.9 ul

b) Oxaliplatin

Concentration (µM)	Volume of drug/3 well (ul)	Volume of media/3 well (ul)
3.125	1) 3.125 µM/ul x 400 ul x 397.29 g/M= 496,613 g 2) 496,613 g/10^6=0.4966 ug 3) 0.4966 ug x 3 well= 1.4898 ug 4) 1.4898 ug x 10= 14.9 ul	1) 400 ul x 2 well= 1200 ul 2) 1200 ul- 14.9 ul= 1185.1 ul
6.25	1) 6.25 µM/ul x 400 ul x 397.29 g/M= 993,225 g 2) 993,225 g /10^6=0.9932 ug 3) 0.9932 ug x 3 well= 2.9796 ug 4) 2.9796 ug x 10= 29.8 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 29.8 ul=1170.2 ul
12.5	1) 12.5 µM/ul x 400 ul x 397.29 g/M=1,986,450 g 2) 1,986,450 g/10^6=1.9865 ug 3) 1.9865 ug x 3 well= 5.9592 ug 4) 5.9592 ug x 10= 59.6 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 59.6 ul=1140.4 ul
25	1) 25 µM/ul x 400 ul x 397.29 g/M= 3,972,900 g 2) 3,972,900 g /10^6=3.9729 ug	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 119.2 ul=1080.8 ul

	3) 3.9729 ug x 3 well= 11.919 ug 4) 11.919 ug x 10= <u>119.2 ul</u>	
50	1) 50 µM/ul x 400 ul x 397.29 g/M= 7,945,800 g 2) 7,945,800 g /10^6=7.9458 ug 3) 7.9458 ug x 3 well= 23.8374 ug 4) 23.8374 ug x 10= <u>238.4 ul</u>	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 238.4 ul= <u>961.6 ul</u>
100	1)100 µM/ul x 400 ul x 397.29 g=15,891,600 g 2) 15,891,600 g/10^6= 15.892 ug 3) 15.892 ug x 3 well= 47.6748 ug 4) 47.6748 ug x 10= <u>476.8 ul</u>	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 476.8 ul=<u>723.2 ul</u>

c) Irinotecan

Concentration (µM)	Volume of drug/3 well (ul)	Volume of media/3 well (ul)
3.125	1) 3.125 µM/ul x 400 ul x 586.67 g/M=733,337 g 2) 733,337 g /10^6= 0.7333 ug 3) 0.7333 ug x 3 well= 2.2002 ug 4) 2.2002 ug x 10=<u>22 ul</u>	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 22 ul=<u>1178 ul</u>
6.25	1) 6.25 µM/ul x 400 ul x 586.67 g/M=1,466,675 g 2) 1,466,67 g/10^6= 1.4667 ug 3) 1.4667 ug x 3 well= 4.3998 ug 4) 4.3998 ug x 10=<u>44 ul</u>	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 44 ul=<u>1156 ul</u>
12.5	1) 12.5 µM/ul x 400 ul x 586.67 g/M=2,933,350 g 2) 2,933,350 g/10^6= 2.9334 ug	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 88 ul=<u>1112 ul</u>

	3) 2.9334 ug x 3 well= 8.8002 ug 4) 8.8002 ug x 10=88 ul	
25	1) 25 µM/ul x 400 ul x 586.67 g/M=5,866,700 g 2) 5,866,700 g /10^6= 5.8667 ug 3) 5.8667 ug x 3 well= 17.6004 ug 4) 17.6004 ug x 10=176 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 176 ul=1024 ul
50	1) 50 µM/ul x 400 ul x 586.67 g/M=11,733,40 g 2) 11,733,40 g /10^6= 11.7334 ug 3) 11.7334 ug x 3 well= 35.2002 ug 4) 35.2002 ug x 10=352 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 352 ul=848 ul
100	1)100 µM/ul x 400 ul x 586.67 g=23,466,800 g 2) 23,466,800 g/10^6= 23.467 ug 3) 23.467 ug x 3 well= 70.4004 ug 4) 70.4004 ug x 10=704 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 704 ul=496 ul

d) Leucovorin

Concentration (µM)	Volume of drug/3 well (ul)	Volume of media/3 well (ul)
3.125	1) 3.125 µM/ul x 400 ul x 512 g/M= 640,000 g 2) 640,000 g/10^6= 0.64 ug 3) 0.64 ug x 3 well= 1.92 ug 4) 1.92 ug x 10=19.2 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 19.2 ul=1180.8 ul
6.25	1) 6.25 µM/ul x 400 ul x 512 g/M= 1,280,000 g	1) 400 ul x 3 well= 1200 ul

	2) 1,280,000 g /10^6= 1.28 ug 3) 1.28 ug x 3 well= 3.84 ug 4) 3.84 ug x 10=38.4 ul	2) 1200 ul- 38.4 ul= 1161.6 ul
12.5	1) 12.5 µM/ul x 400 ul x 512 g/M= 2,560,000 g 2) 320,000 g /10^6= 2.56 ug 3) 2.56 ug x 3 well= 7.68 ug 4) 7.68 ug x 10=76.8 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 76.8 ul=1123.3 ul
25	1) 25 µM/ul x 400 ul x 512 g/M= 5,120,000 g 2) 5,120,000 g /10^6= 5.12 ug 3) 5.12 ug x 3 well= 15.36 ug 4) 15.36 ug x 10=153.6 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 153.6 ul=1046.4 ul
50	1) 50 µM/ul x 400 ul x 512 g/M= 10,240,000 g 2) 10,240,000 g /10^6= 10.24 ug 3) 10.24 ug x 3 well= 30.72 ug 4) 30.72 ug x 10=307.2 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 307.2 ul=892.8 ul
100	1) 100 µM/ul x 200 ul x 512 g/M=20,480,000 g 2) 20,480,000 g/10^6= 20.48 ug 3) 20.48 ug x 3 well= 61.44 ug 4) 61.44 ug x 10=614.4 ul	1) 400 ul x 3 well= 1200 ul 2) 1200 ul- 614.4 ul=585.6 ul

2.4 Combination Drug Calculation

2.4.1 Table 5 Calculation for preparation of drugs solution used in combinations

a) 5-Fluorouracil

Concentration (µM)		Volume of drug (ul)	Volume of media (ul)
CCK-8 assay	1	1) 1 µM/ul x 200 ul x 130.08 g/M= 26016 g 2) 26016 g /10^6=0.026 ug 3) 0.026 ug x 12well= 0.3122 ug 4) 0.3122 ug x 10= 3.122 ul	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 3.122 ul= 2396.9 ul
	15	1) 15 µM/ul x 200 ul x 130.08 g/M= 390240 g 2) 390240 g /10^6= 0.39024 ug 3) 0.39024 ug x 12 well= 4.683 ug 4) 4.683 ug x 10= 46.83 ul	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 46.83 ul= 2353.2 ul
	30	1)30 µM/ul x 200 ul x 130.08 g/M=780480 g 2) 780480 g/10^6= 0.7805 ug 3) 0.7805 ug x 12 well= 9.366 ug 4) 9.366 ug x 10= 93.66 ul	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 93.66 ul= 2306.3 ul
AO/PI staining	1	1) 1 µM/ul x 400 ul x 130.08 g/M= 52032 g 2) 52032 g/10^6=0.0520 ug 3) 0.0520 ug x 6 well= 0.312 ug 4) 0.312 ug x 10= 3.12 ul	1) 400 ul x 6 well= 2400 ul 2) 2400 ul- 3.12 ul= 2396.9 ul
	15	1) 15 µM/ul x 400 ul x 130.08 g/M= 780,480 g 2) 780,480 g /10^6=0.78048 ug	1) 400ul x 6well= 2400ul 2) 2400ul- 46.83ul= 2353.2ul

		3) 0.78048 ug x 6 well= 4.683 ug 4) 4.683 ug x 10= <u>46.83 ul</u>	
	30	1) 30 µM/ul x 400 ul x 130.08 g/M=1,560,960 g 2) 1,560,960 g/10^6=1.56096 ug 3) 1.56096 ug x 6 well= 9.366 ug 4) 9.366 ug x 10= <u>93.66 ul</u>	1) 400 ul x 6 well= 2400 ul 2)2400 ul- 93.66 ul=<u>2306.34 ul</u>

b) Oxaliplatin

Concentration (µM)		Volume of drug (ul)	Volume of media (ul)
CCK-8 assay	1	1) 1 µM/ul x 200 ul x 397.29 g/M = 79458 g 2) 79458 g /10^6=0.07946 ug 3) 0.07946 ug x 12 well= 0.9535 ug 4) 0.9535 ug x 10= <u>9.535 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 9.535 ul= <u>2390.5 ul</u>
	10	1) 10 µM/ul x 200 ul x 397.29 g/M = 794580 g 2) 794580 g /10^6= 0.79458 ug 3) 0.79458 ug x 12 well= 9.535 ug 4) 9.535 ug x 10= <u>95.35 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 95.35 ul= <u>2304.7 ul</u>
	20	1)20 µM/ul x 200 ul x 397.29 g/M =1589160 g 2) 1589160 g/10^6= 1.5892 ug 3) 1.5892 ug x 12 well= 19.07 ug 4) 19.07 ug x 10= <u>190.7 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 190.7 ul= <u>2209.3 ul</u>
AO/PI staining	1	1) 1 µM/ul x 400 ul x 397.29 g/M = 158916 g	1) 400 ul x 6 well= 2400 ul

		2) 158916 g/10^6=0.1589 ug 3) 0.1589 ug x 6 well= 0.9535 ug 4) 0.9535 ug x 10= <u>9.535 ul</u>	2) 2400 ul- 9.535 ul= <u>2390.5 ul</u>
	10	1) 10 µM/ul x 400 ul x 397.29 g/M = 1589160 g 2) 1589106 g /10^6= 1.5892 ug 3) 1.5892 ug x 6 well= 9.535 ug 4) 9.535 ug x 10= <u>95.35 ul</u>	1) 400 ul x 6 well= 2400 ul 2) 2400 ul- 95.35 ul= <u>2304.7 ul</u>
	20	1) 20 µM/ul x 400 ul x 397.29 g/M =3178320 g 2) 3178320 g/10^6= 3.17832 ug 3) 3.17832 ug x 6 well= 19.07 ug 4) 19.07 ug x 10= <u>190.7 ul</u>	1) 400 ul x 6 well= 2400 ul 2)2400 ul- 190.7 ul=<u>2209.3 ul</u>

c) Irinotecan

Concentration (µM)		Volume of drug (ul)	Volume of media (ul)
CCK-8 assay	1	1) 1 µM/ul x 200 ul x 586.67 g/M = 117334 g 2) 117334 g/10^6=0.1173 ug 3) 0.1173 ug x 12 well= 1.408 ug 4) 1.408 ug x 10= <u>14.08 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 14.08 ul= <u>2385.2 ul</u>
	15	1) 15 µM/ul x 200 ul x 586.67 g/M = 1,760,010 g 2) 1,760,010 g /10^6= 1.760 ug 3) 1.760 ug x 12 well= 21.12 ug 4) 21.12 ug x 10= <u>211.2 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 211.2 ul= <u>2188.8 ul</u>

	30	1)30 µM/ul x 200 ul x 586.67 g/M =3,520,020 g 2) 3,520,020 g/10^6= 3.52 ug 3) 3.52 ug x 12 well= 42.24 ug 4) 42.24 ug x 10= <u>422.4 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 422.4 ul= <u>1977.6 ul</u>
AO/PI staining	1	1) 1 µM/ul x 400 ul x 586.67 g/M = 234668 g 2) 234668 g/10^6=0.2347 ug 3) 0.2347 ug x 6 well= 1.408 ug 4) 1.408 ug x 10= <u>14.08 ul</u>	1) 400 ul x 6 well= 2400 ul 2) 2400 ul- 14.08 ul= <u>2385.9 ul</u>
	15	1) 15 µM/ul x 400 ul x 586.67 g/M = 3,520,020 g 2) 3,520,020 g/10^6= 3.52 ug 3) 3.52 ug x 6 well= 21.12 ug 4) 21.12 ug x 10= <u>211.2 ul</u>	1) 400 ul x 6 well= 2400 ul 2) 2400 ul- 211.2 ul= <u>2188.8 ul</u>
	30	1) 30 µM/ul x 400 ul x 586.67 g/M =7,040,040 g 2) 7,040,040 g/10^6= 7.04 ug 3) 7.04 ug x 6 well= 42.24 ug 4) 42.24 ug x 10= <u>422.4 ul</u>	1) 400 ul x 6 well= 2400 ul 2)2400 ul- 422.4 ul=<u>1977.6 ul</u>

d) Leucovorin

Concentration (µM)		Volume of drug (ul)	Volume of media (ul)
CCK-8 assay	1	1) 1 µM/ul x 200 ul x 512 g/M = 102400 g 2) 102400 g /10^6=0.1024 ug 3) 0.1024 ug x 12 well= 1.2288 ug	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 12.29 ul= <u>2387.7 ul</u>

		4) 1.2288 ug x 10= <u>12.29 ul</u>	
	5	1) 5 µM/ul x 200 ul x 512 g/M = 512000 g 2) 512000 g /10^6= 0.512 ug 3) 0.512 ug x 12 well= 6.144 ug 4) 6.144 ug x 10= <u>61.44 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 61.44 ul= <u>2338.6 ul</u>
	10	1)10 µM/ul x 200 ul x 512 g/M =1,024,000 g 2) 1,024,000 g/10^6= 1.024 ug 3) 1.024 ug x 12 well= 12.29 ug 4) 12.29 ug x 10= <u>122.9 ul</u>	1) 200 ul x 12 well= 2400 ul 2) 2400 ul- 122.9 ul= <u>2277.1 ul</u>
AO/PI staining	1	1) 1 µM/ul x 400 ul x 512 g/M = 204800 g 2) 204800 g/10^6=0.2048 ug 3) 0.2048 ug x 6 well= 1.229 ug 4) 1.229 ug x 10= <u>12.29 ul</u>	1) 400 ul x 6 well= 2400 ul 2) 2400 ul- 12.29 ul= <u>2387.7 ul</u>
	5	1) 5 µM/ul x 400 ul x 512 g = 1,024,000 g 2) 1,024,000 g /10^6= 1.024 ug 3) 1.024 ug x 6 well= 6.144 ug 4) 6.144 ug x 10= <u>61.44 ul</u>	1) 400 ul x 6 well= 2400 ul 2) 2400 ul- 61.44 ul= <u>2338.6 ul</u>
	10	1) 10 µM/ul x 400 ul x 512 g =2,048,000 g 2) 2,048,000 g/10^6= 2.048 ug 3) 2.048 ug x 6 well= 12.29 ug 4) 12.29 ug x 10= <u>122.9 ul</u>	1) 400 ul x 6 well= 2400 ul 2)2400 ul- 122.9 ul=<u>2277.6 ul</u>

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