UNIVERSITI PUTRA MALAYSIA

THE EFFECT OF 1-METHYL PROPYL 2-IMIDAZOLYL DISULFIDE (PX- 12) ON THE INVADOPODIA FORMATION OF HYPOXIA-INDUCED HCT116 COLORECTAL CANCERCELLS.

SHARAH BINTI MUHAMMAD SHAN

ABSTRACT

The Effect of 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) on the Invadopodia Formation of Hypoxia-Induced HCT116 Colorectal CancerCells.

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Background: Colorectal cancer is one of the most common solid tumour in both women and men reported by The American Cancer Society. It is the second most common cause of cancer deathsglobally in 2020. Cancer metastasis is a multi-step process that includes the local infiltrations ofcancer cells into the adjacent tissues, migration of cancer cells into the vessels system known asintravasation, followed by extravasation and lastly colonization of secondary sites. Extravasationprocess is the key role in cancer metastasis that allow cancer cells to migrate and invade via protrusive structures known as invadopodia. The ability of invadopodia to degrade extracellular matrix allows the migration of primary tumour to secondary sites. Several studies have evaluated therole of 1-methylpropyl 2-imidazolyl disulfide (PX-12), a thioredoxin inhibitory drug on cancer migration and invasion under normoxic condition, however, little is known on the effects of PX-12 on tumour cell invasion and invadopodia formation in colorectal cancer cells under hypoxic condition. **Objective:** This study aims to evaluate the cytotoxic effects and invadopodia formation of PX-12 in HCT116 human colorectal cancer cells in normoxic and hypoxic conditions. **Hypothesis:** It is hypothesised that PX-12 may significantly reduce invadopodia formation of HCT116 colorectal cancer cells under hypoxia condition**. Methodology:** HCT116 cells were seeded into 96-well plate prior to 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay in order to determine the inhibitory concentrations of PX-12. For gelatin degradation assay, HCT116 cells were treated with IC_{20} of PX-12 for both normoxic and hypoxic conditions and the cells were re-seeded onto gelatin-coated coverslips followed by staining with Rhodamine to observe the invadopodia formation. **Results and Discussion:** The IC_{20} values of PX-12 for HCT116 cells at 24 and 48 hours were ranging from 1.5µM to 4µM while the IC_{50} values ranging from 11µM to 14µM in normoxic and hypoxic conditions. The gelatin degradation assay showed that PX12 at IC_{20} concentration significantly reduced the number ofcells with invadopodia suggesting that low concentrations of PX-12 (1.5µM to 4µM) were sufficient to reduce colorectal cancer cells invasion**. Conclusion:** This study suggests that PX-12 can potentially be an anti-invasive drug candidate in colorectal cancer.

Keywords: Colorectal cancer, metastasis, hypoxia, invadopodia, thioredoxin

ABSTRAK

Kesan 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) ke atas Pembentukan Invadopodia Sel Kanser Kolorektal (HCT116) Terinduksi Hipoksia.

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Pengenalan: Kanser kolorektal adalah salah satu tumor pepejal yang paling terkenal di kalangan wanita dan lelaki yang dilaporkan oleh The American Cancer Society. Ia adalah punca kematian barah kedua di seluruh dunia pada tahun 2020. Metastasis kanser ialah proses complex yang merangkumi pergerakkan sel kanser primari ke dalam tisu bersebelahan, intravasasi, diikuti dengan ekstravasasi dan terakhir mendiami tisu sekunder. Proses ekstravasasi adalah proses utama dalam metastasis kanser yang membolehkan sel-sel kanser berhijrah dan menyerang melalui struktur protrusif yang dikenali sebagai invadopodia. Keupayaan invadopodia untuk menembusi matriks ekstraselular membolehkan penghijrahan tumor primer ke tapak sekunder. Beberapa kajian telah menilai peranan 1-methyl propyl 2-imidazolyl disulfide (PX-12), ubat perencatan thioredoxin pada penghijrahan dan pencerobohan kanser di bawah keadaan normoksik, bagaimanapun, sedikit yang diketahui mengenai kesan PX-12 pada pencerobohan sel tumor dan pembentukan invadopodia dalam sel kanser kolorektal di bawah keadaan hipoksik. **Objektif:** Kajian ini bertujuan untuk menilai kesan sitotoksik dan pembentukan invadopodia oleh PX-12 terhadap sel kanser kolorektal manusia (HCT116) dalam keadaan normoksik dan hipoksik. **Hipotesis:** Adalah dihipotesiskan bahawa PX-12 boleh mengurangkan dengan ketara pembentukan invadopodia sel kanser kolorektal (HCT116) di bawah keadaan hipoksia. **Metodologi:** Sel HCT116 telah disemai ke dalam plat 96-telaga sebelum ujian 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) untuk menentukan kepekatan perencatan PX-12. Bagi ujian degradasi gelatin, sel HCT116 telah dirawat dengan IC_{20} PX-12 untuk kedua-dua keadaan normoksik dan hipoksik dan kemudian sel-sel telah disemai semula pada sisip kaca bersalut gelatin diikuti dengan pewarnaan dengan Rhodamine untuk melihat pembentukan invadopodia. **Keputusan dan Perbincangan:** Nilai IC_{20} PX-12 untuk sel HCT116 pada 24 dan 48 jam adalah antara 1.5µM hingga 4µM manakala nilai IC_{50} antara 11µM hingga 14µM dalam keadaan normoksik dan hipoksik. Ujian degradasi gelatin menunjukkan bahawa PX12 pada kepekatan IC_{20} mengurangkan dengan ketara bilangan sel dengan invadopodia menunjukkan bahawa kepekatan rendah PX-12 (1.5µM hingga 4µM) adalah mencukupi untuk mengurangkan pencerobohan sel kanser kolorektal. **Kesimpulan:** Kajian ini mencadangkan bahawa PX- 12 berpotensi menjadi calon ubat anti-invasif dalam kanser kolorektal.

Kata kunci: Kanser kolorektal, metastasis, hipoksia, invadopodia, thioredoxin

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µM	Micro Molar
5-FU	5-fluorouracil
ACS	American Cancer Society
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
APC	Advanced pancreatic cancer
Arp	Actin related protein
ASCO	American Society of Clinical Oncology
ATCC	American Type Culture Collection
CDC	Centre for Disease Control and Prevention
CRC	Colorectal cancer
DMOG	Dimethyloxalylglycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene-diamine tetra-acetic acid
EMT	Epithelial-mesenchymal transition
EQI	Environmental Quality Index
FITC	Fluorescein Isothiocyanate
GI	Gastrointestinal
GLOBOCAN	Global Cancer Data
HIF-1α	Hypoxia inducible factor-1α
HNPCC	Hereditary non-polyposis colorectal cancer
IBD	Inflammatory bowel disease
IC	Inhibitory concentration
LV	Leucovorin
mCRC	Metastatic colorectal cancer
MMP	Mitochondrial membrane potential
MMPs	Metalloproteinases
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase

NCI	National Cancer Institute
NCPR	National Cancer Patient Registry
Tks5	Tyrosine kinase substrate 5
TNM	Tumour node metastasis
Trx	Thioredoxin
VEGF-A	Vascular endothelial growth factor-A

CHAPTER 1

INTRODUCTION

1.1 Background

National Cancer Institute (NCI) defined cancer as a disease in which the body's cells become abnormal and growth uncontrollably that leads to the capability of metastasizing into surrounding tissues (National Cancer Institute, 2021). Colorectal cancer (CRC) ranks second most common cause of cancer in both male and female reported in the United States (Siegel et al., 2020). Global Cancer Observatory (GLOBOCAN) reported that colorectal cancer had caused 935 173 death cases and 1 931 590 new cases in 2020 which ranks the second most common leading cause of cancer death and third most common cancer across the world in both sexes (GLOBOCAN, 2020). There is no population-based CRC screening programme implemented in Malaysia making it the second most common cancer in males and third most common cancer among females (Veettil et al., 2017).

Colorectal cancer develops in the colon or rectum which is part of the lower digestive system. It all begins with an abnormal growth known as polyps in the inner lining of the colon or rectum which will develop into tumours over time. According to American Cancer society (ACS), it is estimated that approximately 1 in every 23 men and 1 in every 25 women will develop colorectal cancer during their lifetime. Development of colorectal cancer is often associated with genetic and environmental factors leads to poorer understanding of the disease.

Tumour node metastasis (TNM) system is being used in classifying CRC stages. It is the most common system used globally (American Society of Clinical Oncology, 2013). Colorectal cancer was categorized into five stages that begins with stage 0 until stage 4. This

staging system serves as a general guideline for determining the best treatment plan for colorectal cancer patients. The earliest stage is stage 0 while the advanced stage is classified as stage 4. Stages 0 and 1 have no metastases involvement, whereas stage 2 and 3 have cancer that has spread through the walls of the colon or rectum as well as nearby tissues (Wint & Nelson, 2021). Liver being the most common metastases site among colorectal cancer patients (Qiu et al., 2015).

Early diagnosis of colorectal cancer does have the best chance of curing it. However, patients with colorectal cancer does not exhibit any signs and symptoms in their early stage and thus, often being diagnosed at later stage through routine screenings. According to the American College of Gastroenterology, they suggested that routine screening for colorectal cancer begins as early as 40 years old. Colorectal cancer may be diagnosed via genetic screening, fecal sampling, blood test and sigmoidoscopy, reported by The American Cancer Society.

Numerous colorectal cancer treatments had been implicated in the medical field as one of the interventions in curing and lowering the incidence rate of colorectal cancer. Among the most common treatment courses are surgery, chemotherapy, and radiotherapy as well as targeted therapy. Laparoscopic is one of the modern surgery intervention in CRC management. A large number of meta-analysis studies have demonstrated that laparoscopic colorectal surgery provides the same benefits as other minimally invasive treatments. (Pascual et al., 2016). Bevacizumab is the most common targeted therapy of the metastatic colorectal cancer (mCRC) (Fakih, 2015). Although some of these treatments may increase the survival rate, but some do poses adverse effects such as bleeding and prone to infections. Due to the availability of current treatment poses some toxic on CRC patient, therefore we need to find a better drug to cure CRC cases.

Metastasis was recognized as one of the leading causes of death globally (Christofori et al., 2016). It has been a major concern among patient and clinicians as it could cause significant effects in the body's system and leads to mortality. According to National Cancer Institute, approximately 50% of the colorectal cancer patients are highly treatable through the primary treatment of colorectal cancer which is surgery, yet, recurrence incidence is still there in the population. Approximately 30–40% of patients experience recurrence after surgery, with 40–50% of recurrences occurring within the first few years after initial surgical resection (Walker et al., 2014). Another study conducted by the Memorial Sloan-Kattering Cancer Center, demonstrated that 18% of individuals with metastatic colorectal cancer having recurrence. Apart from recurrence, CRC often diagnosed at advance stage making chemotherapy and targeted therapy less effective in overcoming the survival rate due to drug resistance. Therefore, developing an alternative drug with the ability to inhibit cancer metastases remains as the major attention among researchers all over the world.

There is a need to investigate the anti-metastatic effects of the PX-12 against HCT116 colorectal cancer cells by observing the ability of the compound to inhibit invadopodia formation through inhibition of the thioredoxin (Trx) system. A study with breast cancer cells had been done on the effect of thioredoxin upon cancer migration and invasion (Bhatia et al., 2016) in metastatic cancers, however, the effects of thioredoxin inhibitors, PX-12 on the invadopodia formation in hypoxia-induced human colorectal cancer cells have yet to be explored.

1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) is known as an irreversible inhibitor of thioredoxin (Trx) (Baker et al., 2006). PX-12 were selected as the candidate drug in this study as it targets the Trx system which is often highly expressed in colorectal cancer tissues that contribute to poor prognosis. Low dose of PX-12 inhibited colorectal cancer cell migration and invasion shown in a study conducted by Wang et al (2014). Thus, we aimed to identify the

cytotoxic effects of PX-12 against the hypoxia induced HCT116 human colorectal cancer cells by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as well as to observe the invadopodia formation via gelatin degradation assay.

1.2 Objectives

1.2.1 General Objective

This study aims to determine the effect of 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) on the invadopodia formation on hypoxia induced HCT116 human colorectal cancer cells.

1.2.2 Specific Objective

This study specifically aims to determine the cytotoxic effects and to observe the effect of PX-12 on the invadopodia formation under normoxic and hypoxic conditions by using MTT assay and gelatin degradation assay.

1.3 Hypothesis

It is hypothesized that 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) exhibits cytotoxic and anti-invasive effects on HCT116 human colorectal cancer cells under normoxia and hypoxia condition.

CHAPTER 2

LITERATURE REVIEW

2.1 Colorectal Cancer

2.1.1 Introduction

Colorectal cancer was uncommon several decades ago. However, it has become one of the most predominant cancer around Western regions and accounting for about 1 out of 10 cancer-related mortality (Kuipers et al., 2015). Colorectal cancer (CRC) attributes for about 1.9 million cases and 0.9 million deaths worldwide described in 2020 thus makes it the third most common malignant disease and the second most lethal cancer (Xi & Xu, 2021).

According to what has been defined by the Centers for Disease Control and Prevention (CDC), colorectal cancer begins when colon or rectum grow abnormally. These small cluster growths are called polyps. Polyps are benign mass that may somehow turn into tumour over time. The likelihood of these polyps to turn into cancer depends on the type of the polyps as well as other factors including the size and the number of polyps formed. According to studies, polyps ≤ 5 mm in size are less likely to be cancerous and are therefore easier to remove endoscopically using the usual methods (Aarons et al., 2014).

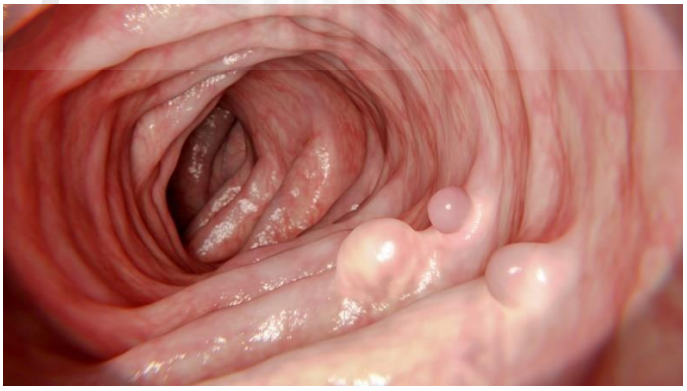


Figure 2.1.1: Colonic polyps that appear on the surface of colon (Adapted from Healthline, 2019).

2.1.2 Epidemiology

According to Kuipers et al. (2015), colorectal cancer is the second and third most prevalent cancers in both men and women, respectively. GLOBOCAN 2020 reported that colorectal cancer is responsible for 1 931 590 new cases worldwide in 2020, accounting for 10% of all new cancer cases. Meanwhile, colorectal cancer ranks second after lung cancer in cancer mortality statistics with 935 173 (9.4%) number of death cases reported in all sexes in 2020. Based on cancer incidence statistic by GLOBOCAN 2020, males have higher cumulative risk (2.71%) compared to females with only 1.83% worldwide and when combined, the cumulative risk is 2.25%.

According to Malaysia National Cancer Registry (2019), between 2007 and 2017, a total of 6172 colorectal cancer cases recorded to the registry in Northern Malaysia and among all the ethnic groups, Chinese recorded the highest number of CRC cases (57.5%), followed by Malay (35.3%) and Indian (5.9%). Over 80% of colorectal cancer cases in Malaysia are detected in individuals over the age of 50, this aging trend may raise the prevalence even more (Veettil et al., 2016)

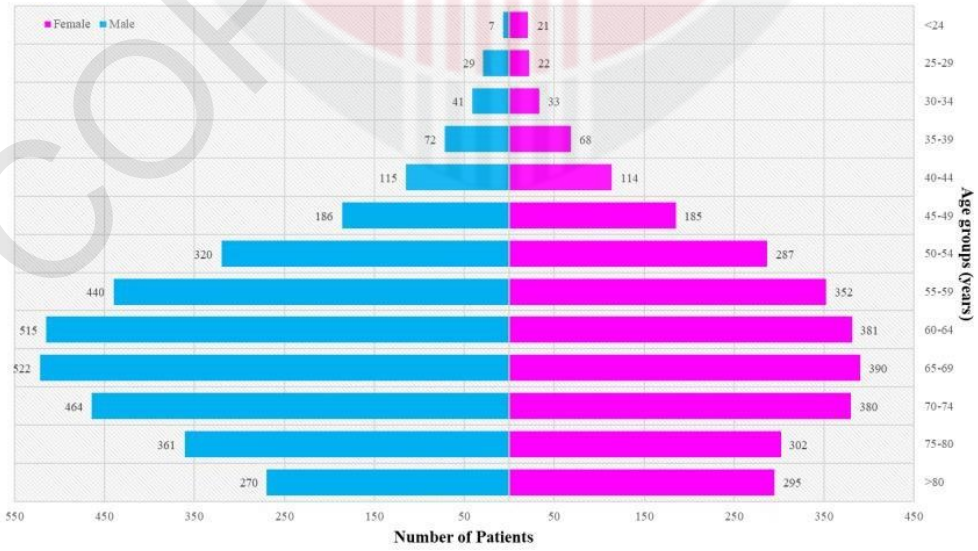


Figure 2.1.2: Colorectal cancer patient age distribution by gender in Northern Malaysia (National Cancer Patient Registry -Colorectal Cancer, 2007-2017).

2.1.3 Pathogenesis

Pathogenesis of colorectal cancer is still unclear, however it is often associated between the individual's genetic make-up and the environment. Mutation often begins in our deoxyribonucleic acid (DNA) sequence that alter the body's system which will then be pass down through the germline onto next generation. According to Kuipers et al. (2015), tumour development is promoted by the gradual accumulation of genetic and epigenetic changes that leads to the activation of oncogenes and deactivation of tumour suppressor genes. When the tumour suppressor genes are no longer functioning, cells that grow abnormally will keep on proliferate which leads to tumour development. About 90% of colorectal cancer cases are due to chromosomal instability initiated by mutation that occurs at the adenomatous polyposis coli (APC) gene, which is a key gene in the development of colorectal cancer (Dekker et al., 2019). APC gene is the key tumour suppressor gene which prevent cells from growing and dividing uncontrollably. Mutation of the APC gene mostly found in colon cancers and is associated with the initiation and expansion of the colon cancers (Aoki & Taketo, 2007).

Apart from that, environmental factors do play role in the development of colorectal cancer as well. A study published in The American Cancer Society (2017) proposed that exposure to low environmental quality may increase the risk of having cancer in a population. Based on the data obtained from Environmental Quality Index (EQI), Jagai et al. (2019) reported that poor environmental quality counties had nearly 40 more cancer cases per 100,000 people than high environmental quality counties. Although this study does not include other comorbidity factors, however, Kuipers et al. (2015) stated that some modifiable lifestyle factors such as smoking, excess alcohol intake and increase body weight are strongly associated with the development of CRC.

2.1.4 Stages

In CRC, tumour node metastasis (TNM) is the most common staging system being used globally (American Society of Clinical Oncology, 2013). This system provides information on the size of the tumour, parts of the colon or rectum that have cancer as well as to determine the spread of the cancer in patients. TNM system classified colorectal cancer into five stages starting from stage 0 to stage 4 as shown in figure 2.1.2 below. According to the American Joint Committee on Cancer (AJCC), TNM system allows prediction of the prognosis of colorectal cancer patients and to guide adjuvant therapy like chemotherapy and radiotherapy following potentially curative surgery.

Table. Colon and Rectum Cancer Staging[6]

AJCC Stage	TNM Stage	Description
0	Tis N0 M0	Tumor is confined to mucosa
I	T1 N0 M0	Tumor invades submucosa
I	T2 N0 M0	Tumor invades muscularis propria
IIA	T3 N0 M0	Tumor invades subserosa or beyond, no other organs involved
IIB	T4 N0 M0	Tumor invades adjacent organs or perforates visceral peritoneum
IIIA	T1-2 N1 M0	Metastasis to 1-3 regional lymph nodes with tumor invasion of submucosa and/or muscularis
IIIB	T3-4 N1 M0	Metastasis to 1-3 regional lymph nodes with tumor invasion of subserosa or adjacent organs
IIIC	Any T, N2 M0	Metastasis to 4 or more lymph nodes
IV	Any T, any N, M1	Metastasis to distant organs

Abbreviations: AJCC, American Joint Committee on Cancer; Tis, tumor (carcinoma) in situ.

Figure 2.1.3: Colon and rectum cancer staging (AJCC Cancer Staging Manual, 2010)

2.1.5 Risk factors

CRC incidence can be influenced by a number of risk factors including age and hereditary factors both of which an individual has no control over (Haggar & Boushey, 2009). Moreover, Kuipers et al. (2015) also mentioned that both genetic hereditary and other

environmental factors have an impact on the aetiology of the CRC incidence. Several risk factors are discussed below.

Family history. About 75% of patients with colorectal cancer have zero link to family history, which suggest that most of the CRC cases are sporadic (Kuipers et al., 2015). However, they mentioned that the risk increases in individuals with first-degree family member having colorectal cancer over the age of 50. In addition, about 7-10% of all colorectal cancer cases are due to hereditary CRCs including non-polyposis colorectal cancer (HNPCC) (Rawla et al., 2019).

Age. In Malaysia, highest proportion of cases are contributed by patients over the age of 60, with nearly 90% of the CRC cases diagnosed in individuals above 40 years old (Veettil et al., 2016). A study conducted by Myers et al. (2013) shows an increase in CRC incidences among young patients below the age of 50 with poor prognosis. In another study, they mentioned that the incidence rate among those over 50 has decreased in recent decades, whereas the incidence rate among those under 50 has actually increased (Rawla et al., 2019).

Inflammatory bowel disease (IBD). According to CDC, inflammatory bowel disease is a chronic inflammation of the gastrointestinal (GI) tract due to ulcerative colitis and Crohn's disease. A recent Swedish cohort study of 9405 patients discovered that patients with childhood-onset inflammatory bowel disease had an increased risk of any cancer, particularly digestive system related malignancies in childhood and later in life (Rawla et al., 2019). Furthermore, a paper by Dekker et al. (2019) described that individuals with prolong inflammatory bowel disease, as well as those with a history of colorectal cancer or adenomas, are at an increased risk of CRC and require close monitoring.

Diabetes mellitus. Kuipers et al. (2015) stated that individuals with type 2 diabetes mellitus is at a higher risk of having colorectal cancer. Based on National Cancer Patient

Registry (2007-2017), in a total of 6,172 colorectal cancer cases, 16.9% patients were diagnosed with diabetes mellitus, thus, being the underlying risk factor.

Lifestyle habits. Certain lifestyle habits may also contribute to the incidence of CRC. Examples of habits that may increase risk of colorectal cancer include smoking, excess alcohol intake, obesity and physical inactivity. Individuals who are physically active have a 25% lower risk of acquiring CRC, whereas those who are inactive have a 50% higher risk of developing CRC (Rawla et al., 2019). Meanwhile in Malaysia, smoking has contributed about 13.4% in overall CRC cases which is the second most common risk factor among Malaysian (NCPR, 2007-2017)

Colorectal cancer incidences could be influenced by any of these factors, thus any possibilities shall not be underestimated and neglected.

2.1.6 Treatment Management

Therapeutics for metastatic colorectal cancer (mCRC) have advanced substantially, shifting from monotherapy to combination therapy and even recently it involved sequential combination therapy (Venook, 2005). Over the last two decades, significant advances have been made in the treatment of (mCRC) (Fakih, 2015) and the advancements in treatment programmes did enhanced survival rate in colorectal cancer patient but the outcomes best seen in non-metastasized CRC while in advanced stage CRC, the prognosis is still poor which leads to high morbidity and mortality rate (Dekker et al., 2019). The current treatment interventions for colorectal cancer include surgery, chemotherapy and targeted therapy.

Surgery. Surgery is the most common treatments which involves the removal of the tumour and some surrounding healthy tissues to be sure all tumour has been remove. Surgery is more likely to be beneficial in patients who are in the early stages of cancer and do not have

metastatic disease. According to American Cancer Society (2021), early stage of CRC can be easily removed through colonoscopy including removal of the polyps. Laparoscopic surgery, sometimes known as keyhole surgery, is a revolutionary surgical intervention that allows physicians to reach the abdomen without the need for major incisions. A large number of meta-analysis studies have demonstrated that laparoscopic colorectal surgery provides the same benefits as other minimally invasive treatments. (Pascual et al., 2016). A study conducted in 2005 shows that, laparoscopic colectomy was related with earlier bowel function recovery, the need for fewer analgesics, and a shorter hospital stay (Veldkamp et al., 2005).

Chemotherapy. Chemotherapy is a treatment that uses medications to halt the proliferation of cancer cells, either by killing them or preventing them from dividing which reduces the extent of the colorectal cancer. Fluorouracil (FU) was the standard of therapy for people with mCRC for more than 40 years as it has a time-dependent method of action (Venook, 2005). Fluorouracil (FU) often used in combination with other biochemical agents such as folinic acid, Leucovorin (LV) to enhance the effectiveness of the chemotherapy. A study by Venook (2005) shows that patients getting FU/LV had a greater response than those receiving FU alone. However, the possible risk of adverse effects from chemotherapy often worrying in colorectal cancer patients as it cause excessive bleeding, nausea, neuropathy as well as prone to infections.

Targeted therapy. In order to specifically target the cancer cells, targeted therapy employs medications to target certain genes and proteins that aid in the survival and growth of cancer cells (American Society of Clinical Oncology, 2022). Targeted therapy can alter the tissue environment of the affected area and even targeting cancer-related cells. A vascular endothelial growth factor A (VEGF-A) targeting monoclonal antibody, bevacizumab, is the only antiangiogenic medication licenced by the US Food and Drug Administration for first-line targeted treatment of mCRC (Fakih, 2015). According to Hurwitz et al (2004), the median

period of survival in the group given irinotecan, bolus fluorouracil, and leucovorin (IFL) with bevacizumab was 20.3 months, compared to 15.6 months in the group given IFL with placebo alone.

2.1.7 Metastasis

Metastasis occurs when the primary tumour migrates from its primary site into a secondary site or different body tissues (ASCO, 2019). Metastasis is recognized as one of the leading causes of death globally (Christofori et al., 2016). Metastasis has been a source of concern for both patients and clinicians because it can be fatal, causing a massive effect and altering with the body's homeostasis (Riihimaki et al., 2016). Van Der Geest et al. (2015) reported that approximately 20% of CRC patients have metastases at the time of diagnosis, and this figure has remained stable over the last two decades. According to Qiu et al. (2015), the most common metastatic site in colorectal cancer patient is liver. In a finding reported by Rhiihimaki et al. (2016), liver metastases is greatly significant in higher stage of colorectal cancer compared to thoracic metastases regardless of the gender, anatomical location, or the number of metastases.

Metastatic cascade of solid tumour progression is divided into five basic steps which are invasion and cell migration, intravasation, survival in the circulation, extravasation and lastly colonization in the secondary site. In order for metastasis to occur, tumour cells undergo epithelial-mesenchymal transition (EMT) which lowered the E-cadherin expression and increased N-cadherin expression that leads to tumour cells dissociation from the primary tumour site. Tumour cells then invade through the basement membrane via secretion of proteolytic enzymes, metalloproteinases (MMPs) which allows tumour cells to invade and migrate. Then, tumour cells intravasate into the vascular system by disrupting the endothelial

cells' junctions which create pores that promote the entry of tumour cells into the circulation. Tumour cells adhere to the endothelium in the circulation, allowing extravasation, where tumour cells exit the circulation and colonise the secondary site (Figure 2.1.3) (Hapach et al, 2019).

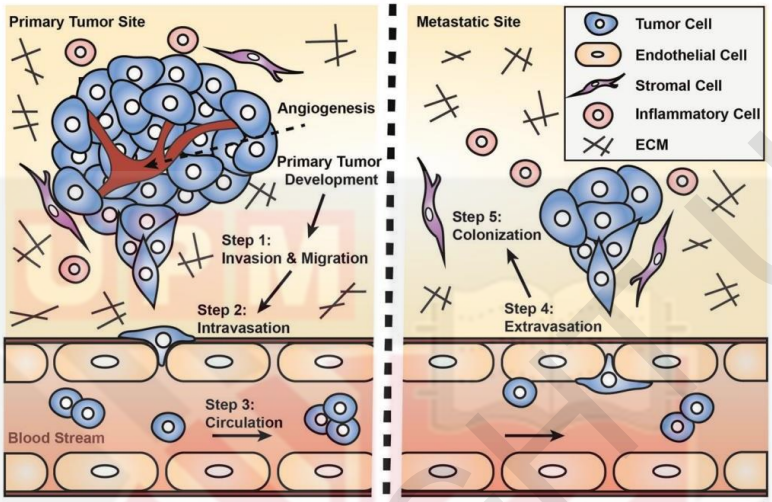


Figure 2.1.4: Illustration of the metastatic cascade (Hapach et al., 2019).

2.2 Invadopodia

Invadopodia are a form of invadosomes that are essential in the integration of tumour signalling to support tumour cell invasion and distribution (Eddy et al., 2017). According to Weaver (2006), invadopodia, are F-actin rich protrusive structures that enhance matrix-degradation activity. Invadopodia are able to degrade the extracellular matrix (ECM) as it contain proteolytic functions which allows the secretion and accumulation of the proteolytic enzymes, metalloproteinases (MMPs) (Eddy et al., 2017; Jacob and Prekeris, 2015). These proteolytic enzymes allow cancer cells to invade and migrate into secondary sites.

According to Augoff et al. (2020), the process of invadopodia formation are highly complex which involved three phases including initiation, stabilization and maturation. In initiation stage, it begins with the assembly of actin-based precursor complexes, which drives

the elongation of cellular protrusions. The activation of the protein (Arp) 2/3 complex during this phase is critical for actin filament branching (Augoff et al., 2020). Actin filaments starts to group together into tight packages and secured themselves to the plasma membrane to form three-dimensional (3D) structure in the stabilization stage. Finally, as the invadopodia begins to mature, the proteolytic enzymes are recruited and ready to degrade the extracellular matrix (ECM).

Invadopodia formation has been recognized as the key driver in the invasiveness of cancer metastasis in human. Several studies in human tumours have found that an enrichment of the invadopodia markers, such as cortactin and Tks5/FISH, at the invading human tumours are consistent with invadopodia-mediated invasion (Seals et al., 2005; Zhang et al., 2006).

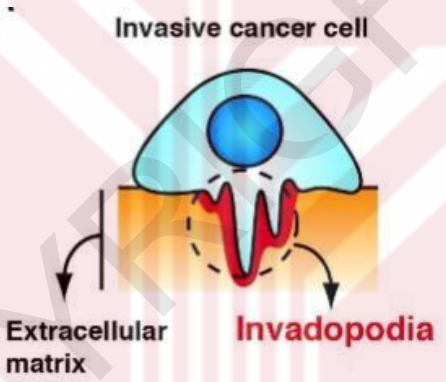


Figure 2.2.1: An illustration of invadopodia. Reproduced by Sasaki Institute with permission from Yamaguchi et al., Oncotarget (2010), and Yamaguchi et al., Eur J Cell Biol (2012).

2.3 Hypoxia

Hypoxia is a common key feature presents in most solid tumours including mCRC, however, the severity depends on the types of the tumour (Muz et al., 2015). Hypoxia is a condition in which the body's tissues lack oxygen availability, and when this occurs in tumour cells, it increases the risk of resistance to treatment and promotes tumour progression (Brahimi-Horn et al., 2007). Moreover, hypoxia occurs due to an increase in oxygen demand outweigh

the oxygen supply when tumour cells undergo uncontrolled proliferation activity. According to Welsh et al (2003), the activation of hypoxia inducible factors-1 (HIF-1) in the tumour microenvironment promotes tumour metastatic potential, such as development of angiogenesis that results from adaptation to hypoxic condition. Apart from that, HIF-1 signalling also promotes tumour cell migration and invasion by regulating the epithelial-mesenchymal transition (EMT). It is known that cancer cells resist the negative impacts of hypoxia by inducing angiogenesis, which is primarily mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1) (Md Hashim NF et al., 2013). A study conducted by Gould et al (2014) described that the expression level of HIF will be high in hypoxia condition hence, influenced the invadopodia formation in tumour cells under HIF-dependent manner.

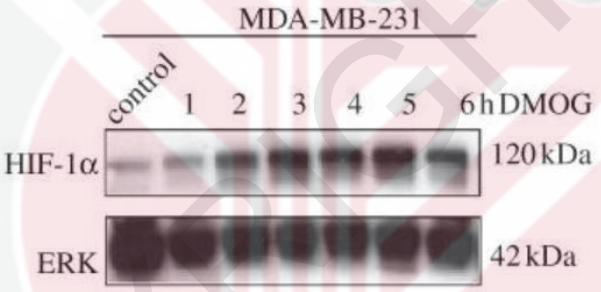


Figure 2.3.1: MDA-MB-231 cell stimulated with DMOG and total ERK used as a control (Md Hashim NF et al., 2013)

It is well established in a paper by Md Hashim et al. (2013) that dimethyloxalylglycine (DMOG) is able to mimic hypoxia condition within 6 hours of incubation as shown in Figure 2.3.1 above. They also reported that the HIF-1 expression in MDA-MB-231 is significantly increased after DMOG stimulation compared to the basal level. DMOG is commercially available and well-characterized as hypoxia mimetic which mimics the hypoxia response (Md Hashim et al., 2013). It is a prolyl hydroxylase inhibitor that inhibit the hydroxylation process in hypoxia condition which leads to an increase in the HIF-1 protein expression.

2.4 Thioredoxin system

The Thioredoxin system (Trx) functions as a cellular antioxidant as well as the transcription factor activity regulator (Mukherjee & Martin, 2008). In Trx system, Trx reductase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) work together to convert Trx from its inactive oxidized form to an active reduced form. Then, the active form of Trx is re-oxidized, supplying reducing equivalents to target molecules such as peroxiredoxins (cellular antioxidant) and numerous transcription factors (Mukherjee & Martin, 2008). Trx protein levels are inversely correlated with the development of aggressive malignancies, which affect patient prognosis and are resistant to cancer treatments, and other diseases developed by an imbalance in the antioxidant system and oxidative stress (Tonissen, 2007).

Trx proteins are highly expressed in breast, lung, liver as well as colorectal cancer and was reported in a paper by Lin et al. in 2017, where there was overexpression of Trx in 70% of primary colorectal cancer samples, hence promotes cancer invasion and migration. Trx system can act as anticancer by protecting cells from reactive oxygen species (ROS) and it can also enhance cancer formation by promoting angiogenesis through increase expression of HIF-1α (Figure 2.4.1) (Mahmood et al., 2013).

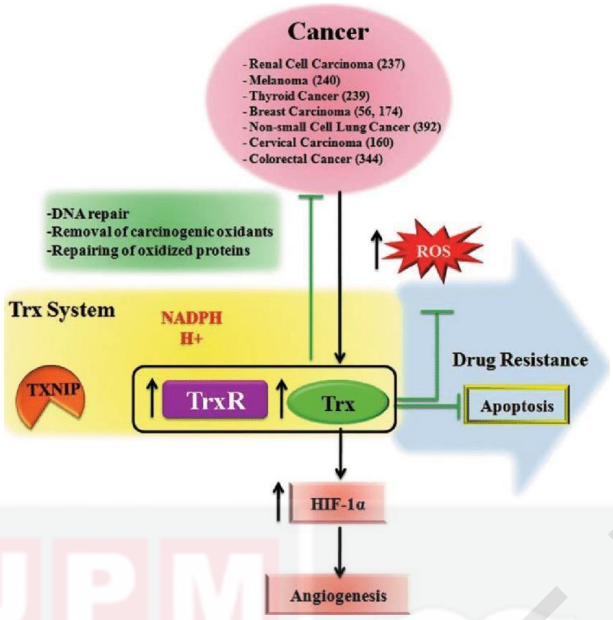


Figure 2.4.1: The role of Trx system in cancer (Mahmood et al., 2013).

Trx system is reported to be associated with HIF-1 in the paper by Welsh (2003). They stated that Trx-1 is highly expressed in the majority of human cancers, which increases the HIF-1 protein in the cells, resulting in increased expression of downstream genes. PX-12 has the ability to reduce HIF-1 transactivation in a dose-dependent manner under hypoxic conditions. (Mukherjee & Martin, 2008).

2.5 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12)

According to Welsh et al. (2003), 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) is being developed as an effective Thioredoxin-1 (Trx-1) inhibitor that irreversibly thioalkylating the Trx-1 cysteine (Cys73). They further characterized that PX-12 is effective for both anti-tumour and indirect anti-angiogenic effects (Welsh et al., 2003). The effects of PX-12 were reported in A549 lung cancer cells where it inhibits the growth of the lung cancer cells via induction of cell cycle arrest as well as apoptosis (You et al., 2014). A study conducted by Wang et al. (2014) reported that their study demonstrated anti-tumour effects and clinical

application of PX-12 in colorectal cancer is in dose-dependent manner. PX-12 once entered clinical trials and was terminated at Phase II due to insignificant anti-tumour activity due to low baseline of Trx-1 levels in treated advanced pancreatic cancer (APC) (Ramanathan et al., 2011). However, this study emphasis on the understanding of the effect of PX-12 in the invadopodia formation, known as one of the key step in cancer cell invasion.

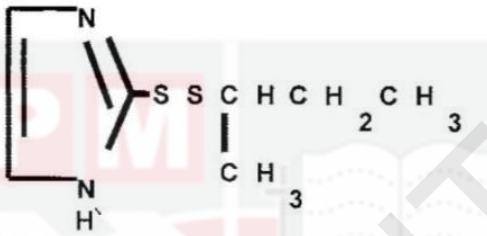


Figure 2.4.1: Structure of PX-12 (Welsh et al., 2003)

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Reagents

McCoy's 5A Medium, 0.25% Trypsin-EDTA and Fetal Bovine Serum (FBS) were purchased from Cytiva HyClone™ (Utah, United State). Phosphate-buffered saline (PBS) tablet was purchased from VWR® Life Science AMRESCO (Ohio, USA). Penicillin-Streptomycin (Pen/Strep) solution (10x) was purchased from BBI Life Sciences (Shanghai, China). Dimethyloxalylglycine (DMOG) was purchased from MedChemExpress LLC (Princeton, United States). Dimethyl sulfoxide (DMSO) was purchased from Fisher BioReagents™ (Massachusetts, USA). Trypan Blue Stain solution (0.4%) was purchased from Solarbio Life Sciences (Beijing, China). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich® (St. Louise, USA). 95% ethanol was purchased from Alcheny Supplies (Selangor, Malaysia). Oregon Green™ 488 conjugate was purchased from Invitrogen™ (California, United States). Triton X-100, bovine serum albumin (BSA) and sucrose were purchased from Sigma-Aldrich® (St. Louise, USA). Sodium borohydride was purchased from R&M Chemical (USA). Paraformaldehyde (PFA) and glutaraldehyde were purchased from Sigma Aldrich® (St. Louise, USA). Phalloidin-Tetramethyl-rhodamine B isothiocyanate was purchase from Sigma Aldrich® (Germany). Hoechst 33342 was purchased from Invitrogen (USA). Anti-Fluorescence Quenching Agent was purchased from Elabscience (United State).

3.1.2 Cell lines

The HCT116 cells (human colorectal cancer cells) were purchased from an international biological resource which is the American Type Culture Collection (ATCC), USA with the ATCC accession number of CCL-247™. HCT116 cells were cultured in McCoy's 5A Medium along with 10% FBS and 1% Penicillin-Streptomycin solution as per recommended by the ATCC. Cells were cultured in cell culture flasks and incubated in 5% CO_2 incubator at 37℃.

3.1.3 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) compounds

The treatment compound used in this study, 1-Methyl Propyl 2-Imidazolyl Disulfide (PX-12) was purchased from MedChemExpress LLC (Princeton, United States). 0.9 mg of PX-12 compound was then diluted with 478 µL of DMSO as the compound was purchased in powder form. The stock solution with a concentration of 10 mM was stored in -20℃ freezer. Different concentrations were produced from this stock solution were 0, 1.25 µM, 2.5 µM, 5.0 µM, 10.0 µM and 20.0 µM respectively.

3.2 Methodology

3.2.1 Cell culture

Strict aseptic techniques were applied throughout the cell culture procedures including the usage of Biosafety Cabinet (BSC) class II in order to prevent unnecessary contaminations being introduced to the cells. HCT116 cells were grown in a complete growth medium which was the McCoy's 5A Medium containing 10% FBS and 1% Penicillin-Streptomycin solution

within the cell culture flasks. The cells were then incubated in the 37℃ incubator with 5% CO_2.

Cell were monitored daily and medium renewal was conducted every 2 to 3 days depending on the appearance of the growth medium.

3.2.2 Subculture of cells

Subculture cells were procedures involved in transferring cells into a new culture flask with a new culture medium upon reaching 80% of confluency. This permits further propagation of the cells as well as maintaining the cell growth. Failure in doing so may inhibit cells proliferation which will results in cell death once they reach contact inhibition. Growth medium was discarded from the T25 culture flask and added with 2 mL of Phosphate Buffered Saline (PBS) to wash off excess growth medium as well as death cells from the culture flask. In order to dislodge the cells from the culture flask's surface, 1 mL of trypsin-EDTA was added into the flask and then incubated in 37℃ incubator with 5% CO_2 for 3 minutes. After 3 minutes of incubation with trypsin-EDTA, the side of tissue culture flask were tapped several times to ensure most of the cells detached from the flask's surface. For further confirmation, the cells were then observed under the inverted microscope to make sure that all the cells had fully dislodged from the flask. 5 mL of complete growth medium with 10% fetal bovine serum (FBS) was added into the flask to stop the trypsin activity and transferred all the cell suspension into a 15 mL falcon tube for centrifugation. The cell suspension was then centrifuged for 5 minutes at 1500 rpm in 24℃. After centrifugation, the supernatant was removed and the cell pellet were gently flicked before 5 mL of complete growth medium with 10% FBS were added into the falcon tube. The cells were sub-cultured into two T25 flask with 2.5 mL of cell suspension in each of the cell culture flaks. After that, another 2.5 mL of complete growth medium with 10% FBS was added onto each flask and incubated overnight.

3.2.3 Cryopreservation of cells

Cryopreservation of cells enable cells to be preserved at a low temperature for long term storage as well as reducing the risk of morphological and genetic changes towards the cell line. Cryovials were labelled with the name of the cell line, the number of cells, the date as well as the passage number of the cells. Freezing medium or freeze mix was prepared by adding 1 mL of DMSO into 9 mL of FBS. Cells were trypsinized as mentioned in section 3.2.2 and then centrifuged for 5 minutes at 1500 rpm in 24℃. Supernatant was removed and the cell pellet were gently flicked before 2 mL freeze mix were added into the 15 mL falcon tube. The cell pellet was resuspended with the freezing medium and then transferred 1 mL of cell suspension into each labelled cryovials. The cryovials were sealed with parafilm and were placed into the ice box containing ice packs. It is then transferred into Mr Frosty and were stored in the freezer at -80℃.

3.2.4 Thawing of cells

Thawing cells involved the procedure of unfreezing the cryopreserved cells. A 50 mL falcon tube containing complete growth medium with 10% FBS were prewarmed in the water bath at 37℃, meanwhile a T25 flask was labelled with the name of the cell, the passage number, date as well as name. The cryovials were taken from -80℃ freezer were thawed in the water bath at 37℃ for about 2 to 3 minutes. The T25 flask was filled with 2 mL of complete growth medium and the newly thawed cells were slowly introduced into the culture flask containing the growth medium. The flask was swirled for a minute to evenly spread the cells on the flask's surface. Then the cells were observed under the inverted microscope followed by overnight incubation at 37℃ incubator with 5% CO_2.

3.2.5 Cell count

Cell counting is a method of counting the viable cells in a cell suspension by using trypan blue solution. Cells in the flask were trypsinized as per mentioned in section 3.2.2. After centrifugation, the supernatant was removed and the cell pellet were resuspended in 15 mL of complete growth medium. 50 µL of cell suspension were transferred into an Eppendorf tube and added with 50 µL of trypan blue solution (Sigma-Aldrich) with ratio of 1:1. 10 µL of the solution were loaded onto the hemocytometer and the viable cells were counted manually under the inverted microscope (Olympus Research Microscope, CKX41) using the following formula:

$$\text{Total viable cells per mL (stock cells)} = \frac{A + B + C + D}{4} \times 2 \times 10^4$$

3.2.6 Cytotoxicity assay

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is classified as a colorimetric assay that measures the metabolic activity of the cells through cell viability (Bahuguna et al., 2017). MTT assay is routinely used in the laboratory to assess cell viability and cytotoxic screening of novel drugs in order to determine its safety. This assay enables the quantification of the cells' metabolic activity by observing the ability of the NADPH-dependent cellular oxidoreductase enzymes to reduce the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (yellow colour) into an insoluble formazan crystal (purple colour). Formazan crystals that formed from this assay will reflect the cell viability where it is found to be precipitated only in the viable cells (Riss et al., 2013). The purple-coloured formazan crystals were dissolved in DMSO and the absorbance is quantified using microplate reader at 570 nm and 630 nm as a reference wavelength. (Bahuguna et al., 2017).

Higher intensity indicates higher cell viability while low intensity indicates reduce number of viable cells which reflects the cytotoxicity of the substance. This MTT assay was conducted in three independent experiments in triplicates for each experiment.

3.2.6.1 Cell seeding

The HCT116 cells were seeded at a density of 1 x 10^4 cells/well by using the $M_1V_1 = M_2V_2$ formula. The cell suspension prepared using the formula were pipetted into two different 96-well plates (24 and 48 hours). Each plate contained cells for both conditions which were normoxia and hypoxia. The cells were then incubated overnight in 37℃ incubator with 5% CO_2.

3.2.6.2 Preparation and application of treatment

After overnight incubation of the HCT116 cells, cells were treated for 24 and 48 hours. PX-12 stock compound containing 0.9 mg of PX-12 was diluted in 478 µL of DMSO with a concentration of 10 mM. Six 15 mL falcon tubes were labelled with different working concentrations of PX-12 (1.25, 2.5, 5, 10, 20, and 40 µM). Tube with the highest concentration of PX-12 was prepared by adding 12 µL of the stock compound into 2988 µL of complete growth medium with a final concentration of 40 µM. Then, 1500 µL of complete growth medium were added into the remaining five falcon tubes. Two-fold serial dilution were performed to obtain the desired PX-12 working concentrations by transferring 1500 µL of solution from the 40 µM tube into 20 µM tube and these steps were repeated for the subsequent tubes. In order to chemically induced hypoxia towards the cells, dimethyloxalylglycine (DMOG) was introduced into the well plates that were designed for hypoxia conditions. The working concentration of DMOG was 2 mM and was prepared from 500 mM stock compound.

After 24 hours incubation, growth medium were discarded and replaced with 100 µL of PX-12 (1.25, 2.5, 5, 10, 20, and 40 µM). These steps were repeated for the 48 hours incubation plate. 100 µL of DMOG were added into the hypoxia well to induce hypoxic condition. The first well contained only cells and growth medium which act as a negative control. Fluorouracil (5-FU) with two different concentrations (10 and 20 µM) were used as the positive control for normoxia and hypoxia conditions.

3.2.6.3 MTT solution preparation

5 mg of MTT powder were dissolved in 1 mL of PBS with a final concentration of 5 mg/mL. The freshly prepared MTT solution were mixed using vortex. The stock solutions were stored in -20℃ freezer (stable for 6 months).

3.2.6.4 MTT application and absorbance reading

After 24 hours incubation period, 20 µL of MTT solution with a working concentration of 5 mg/mL were added into each well and incubated for 3 hours in the CO_2 incubator at 37℃. After 3 hours of incubation with the MTT solution, media was discarded from wells and 100 µL of DMSO were added. The absorbance was read using a microplate reader (Infinite® F50, Tecan) at 570 nm and 630 nm as a reference wavelength. These steps were repeated for 48 hours incubation plate. Data obtained were tabulated and the percentage of cell viability was computed using the following formula below.

$$\text{Cell viability (\%)} = \frac{\text{Average of sample absorbance}}{\text{Average of control absorbance}} \times 100\%$$

3.2.7 Incubation time optimization for invadopodia assay

The incubation time post reseeding was optimize to find the optimal incubation period for gelatin degradation activity in the invadopodia assay. It was well established that with 3 hours of incubation after reseeding of MDA-MB-231 breast cancer cells there are formation of invadopodia (Harun et al., 2018), however, no gelatin degradation activity was observed in HCT116 colorectal cancer cells after three hours. In optimization assay, cells were seeded at densities of 1 x 10^4 and 2 x 10^4 cells/mL and incubated for 3, 24 and 48 hours. The invadopodia formation were observed under fluorescent microscope and images were captured. The range of the incubation periods was determined based on the previous studies using HCT116 cells, with incubation times ranging from 20 to 72 hours (Mansour et al., 2014; Liu et al., 2020; Jang et al., 2022). Based on Figure 3.2.7.1, invadopodia formed by HCT116 cells were observed at 48 hours with a cell density of 1 x 10^4 cells/mL. There is absent of invadopodia formation at 3 hour and 24 hours of incubation after reseeding of HCT116 cells onto gelatin-coated coverslips. Hence, 48 hours of incubation time were selected for this invadopodia assay.

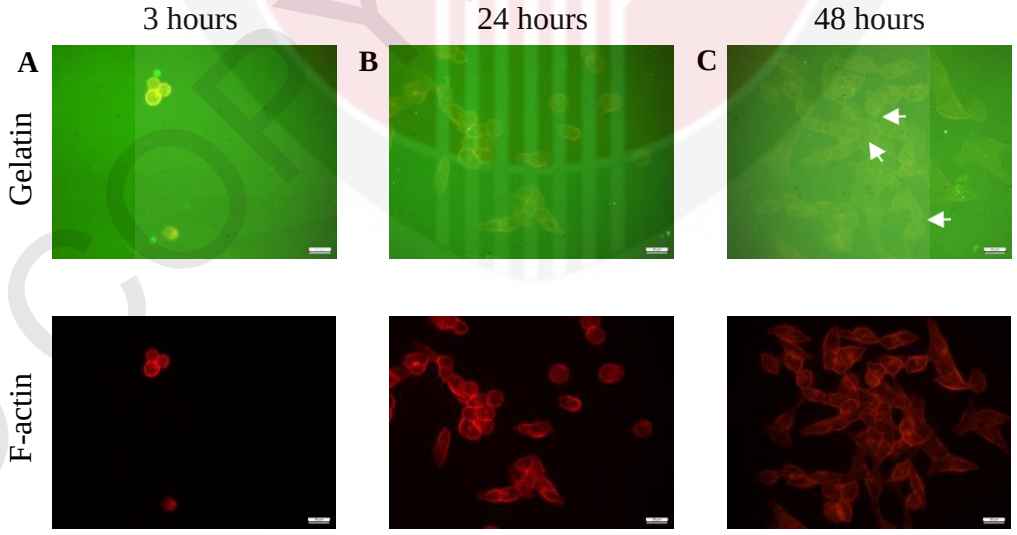


Figure 3.2.7.1 Optimization in the incubation time after reseeding of HCT116 cells. A. HCT116 cells after 3 hours of incubation with seeding density of 2 x 104 cells/mL. B. HCT116 cells after 24 hours of incubation with seeding density of 1 x 104 cells/mL. C. HCT116 cells

after 48 hours of incubation with seeding density of 1 x 104 cells/mL. White arrows indicate gelatin degradation. (Scale: 25 µm)

3.2.8 Invadopodia assay

Invadopodia assay allows the assessment and quantification of invadopodia activity through gelatin degradation. According to Weaver (2008), invadopodia assay involved plating cells overnight on a thin layer of fluorescent ECM, such as crosslinked FITC–gelatin where any invadopodia-associated ECM digestion will results in the removal of fluorescent ECM, revealing degraded areas as dark spots in the fluorescent background. Invadopodia are quantified when there's co-localization with the dark spots observed on the fluorescent gelatin (Md Hashim NF et al., 2013).

3.2.8.1 Treatment of glass coverslips

Coverslips treatment involved series of washing and cleaning the coverslips in the Biosafety Cabinet (BSC) class II. The coverslips with a diameter of 13 mm were gently placed in a petri dish containing 70% ethanol for 30 minutes with rocking motion at room temperature. After 30 minutes, the 70% ethanol were discarded followed by the addition of absolute ethanol into the petri dish containing coverslips for 30 minutes. The coverslips were left to air- dried on a paper towel before being placed in a cleaned petri dish. All the coverslips were kept sterile in the petri dish and stored at room temperature for long term storage.

3.2.8.2 Preparation of fluorophore-conjugated gelatin coated coverslips

Gelatin-coated coverslips were prepared fresh each time before seeding cell for invadopodia assay. Fluorophore-conjugated gelatin that was used to coat the 13 mm coverslips was Oregon Green® 488 Conjugate obtained from Porcine Skin and manufactured by Invitrogen, CA, USA. Fluorophore-conjugated gelatin-coated coverslips were prepared according to method adapted from (Harun et al., 2018). The working concentration of the gelatin was 0.2 mg/mL and was prepared from a stock of 1 mg/mL. In order to obtain the working concentration, gelatin was diluted in PBS containing 2% sucrose. 30 µL of 0.2 mg/mL gelatin were pipetted onto a piece of parafilm where the coverslips were then transferred onto the gelatin using forceps and were incubated for 10 minutes in the dark. After that, the coverslips were transferred to a new parafilm containing 100 µL of 0.5% glutaraldehyde prepared from 25% stock solution. The coverslips were then incubated for 15 minutes in the dark with humidified environment. After 15 minutes of incubation, the coverslips were transferred into labelled 24-well plate with the gelatin-coated side facing up and followed by washing with PBS three times. 1 mL of 5 mg/mL sodium borohydride was added into each well containing coverslips for 3 minutes with shaking the plate for every minute to avoid air bubbles. The sodium borohydride was discarded after 3 minutes followed by washing the coverslips gently with PBS three times and another three times with 70% ethanol. Lastly, the 70% ethanol were aspirated off and the coverslips were left to air-dry in the BSC for about 2 to 3 minutes in the dark. The 24-well plate containing gelatin-coated coverslips were wrapped in an aluminium foil and stored in the 4℃ refrigerator for maximum of one week.

3.2.8.3 Gelatin degradation assay

The HCT116 cells were seeded into 6-well plate at a density of 3 x 10^5 cells/mL and were incubated overnight. After overnight incubation, cells were treated using non-cytotoxic concentration (IC_{20}) of PX-12 along with treatment with DMOG to induce hypoxic condition and then incubated for 24 hour (Figure 3.2.8.1). Prior to seeding cells, the coverslips in the 24-well plate were quenched with complete growth medium and incubated for 1 hour in the 37°C incubator with 5% CO_2. After 1 hour of quenching, the HCT116 cells in the 6-well plate were trypsinized following the procedure on section 3.2.2 and were re-seeded onto gelatin-coated coverslips in the 24-well plate with a density of 1 x 10^4 cells/well followed by 48 hours of incubation. The cells were then fixed with 4% paraformaldehyde (PFA) for about 20 minutes followed by three times washing with PBS. After that, 500 µL of 0.2% Triton X-100 were added into the 24-well plate to permeabilized the cells for 5 minutes. The cells were stained with 50 µL of 50 µg/mL Rhodamine phalloidin (Sigma, Germany) which was diluted in PBS containing 3% bovine serum albumin (BSA) and were incubated in humidified dark environment for 1 hour. The cells were then counterstained with 50 µL of 2 µg/mL Hoechst 33342 (Invitrogen, USA) prepared from the stock of 1 mg/mL to stain the nucleus. The gelatin-coated coverslips were then mounted with Anti-Fluorescence Quenching Agent (Elabscience, USA) to observe the invadopodia formation under the fluorescence microscope. GM6001 is a wide-spectrum of matrix metalloproteinase (MMP) inhibitor that was used in this assay to act as a positive control. Wang et al., 2013 demonstrated that GM6001 is capable of inhibiting invadopodia formation in tumour invasion. In this study, the analysis method involved the percentage quantification of cells that formed invadopodia where it is said to be co-localized with dark dots on the fluorescent gelatin (Md Hashim et al., 2013). This experiment where repeated for at least three independent experiments.

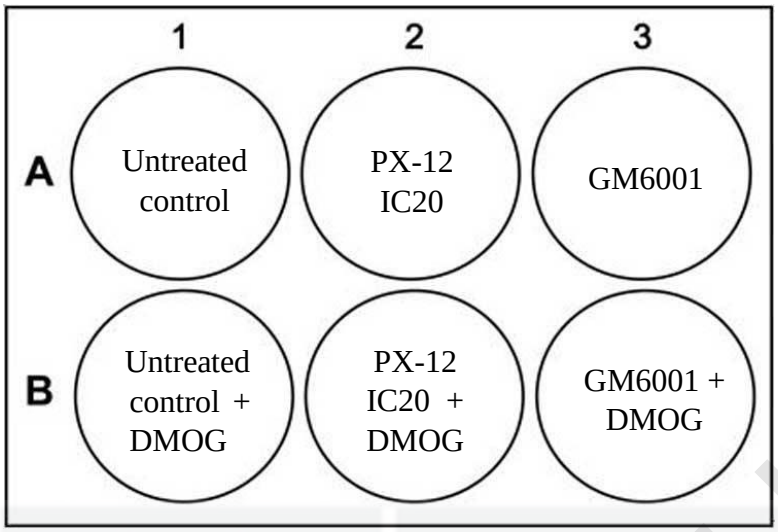


Figure 3.2.8.1: 6-well plate designed for treatment in invadopodia assay.

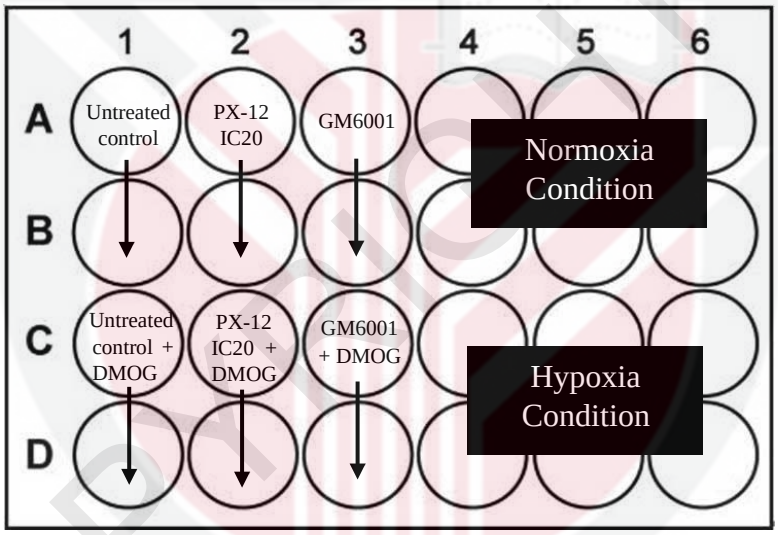


Figure 3.2.8.2: 24-well plate designed for invadopodia assay.

3.2.9 Statistical analysis

The IC_{20} and IC_{50} values of PX-12 were extrapolated from the graph made in Microsoft Excel 2013. Statistical analysis was performed using GraphPad Prism 8 where *p-value* less than 0.05 was considered significant. Gelatin degradation analysis was done using ImageJ software to merge the captured images and determine the invadopodia formation.

CHAPTER 4

RESULTS

4.1 Cell viability of HCT116 Human Colorectal Cancer Cells treated with PX-12

The cytotoxicity effects of PX-12 on the hypoxia induced HCT116 human colorectal cancer cells were evaluated using MTT assay in three independent experiments. The cells were treated with five different concentrations (1.25, 2.5, 5, 10 and 20 µM) of PX-12 in two different incubation periods which were 24 and 48 hours. Following the MTT assay, invadopodia assay was performed to evaluate the anti-invasive effects of PX-12 on the invadopodia formation of HCT116 cells under normoxia and hypoxia conditions.

Figure 4.1.1. showed the cell viability of HCT116 cells treated with 5-Fluorouracil (5-FU). 10 µM of 5-FU was used as the positive control in this assay. Results showed that 5-FU significantly reduced the cell viability of HCT116 cells in both normoxia and hypoxia conditions at 24 and 48 hours ($P < 0.005$). From these results, it showed that 5-FU is a good internal control for this MTT assay.

Figure 4.1.2, showed the cell viability of HCT116 cells treated with different concentrations of PX-12 at 24 hours under normoxia and hypoxia conditions. The HCT116 cells viability was significantly reduced after 24 hours of treatment with PX-12 starting from 5 µM to 20 µM under normoxia while in hypoxia condition, the cell viability of HCT116 cells were significantly reduced at 20 µM after 24 hours of treatment.

Figure 4.1.3 showed the cell viability of HCT116 cells treated with different concentrations of PX-12 at 48 hours under normoxia and hypoxia conditions. PX-12 significantly reduced HCT116 cells viability at 20 µM after 48 hours of treatment under normoxia and hypoxia conditions ($P < 0.005$).

Table 4.1.1 summarizes the inhibitory concentration (IC_{20} and IC_{50}) values obtained after 24 and 48 hours treatment with PX-12 under normoxia and hypoxia conditions. The IC_{20} values for 24 hours normoxia is 2 µM ± 0.667 and 24 hours hypoxia is 3 µM ± 1.000 while the IC_{50} normoxia and hypoxia are 12 µM ± 4.842 and > 20 µM ± 5.667 respectively. The IC_{20} values for 48 hours normoxia is 4 µM ± 0.882 and 48 hours hypoxia is 1 µM ± 0.333 while the IC_{50} normoxia and hypoxia are 14 µM ± 4.372 and 12 µM ± 5.608 respectively.

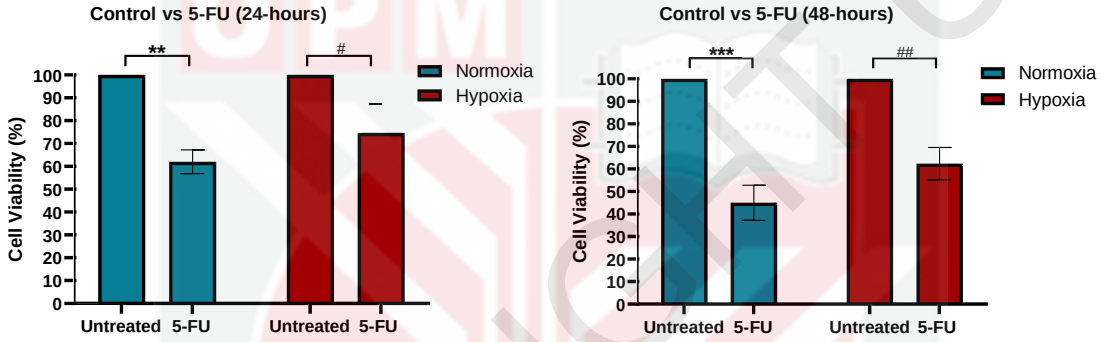


Figure 4.1.1 Cytotoxic effects of 5-FU on untreated HCT116 cells via MTT assay at 24 and 48-hours time-point. Mean data were expressed as ± SEM based on three replicated experiments. Results were analyzed by using Two-Way ANOVA followed by Tukey's post-hoc test. The *P-value indicates statistical difference (*P < 0.05, **P < 0.01 and ***P < 0.001) in 5-FU vs. control under normoxia condition while #P-value indicates statistically significant difference (#P < 0.05) in 5-FU vs. control under hypoxia condition.

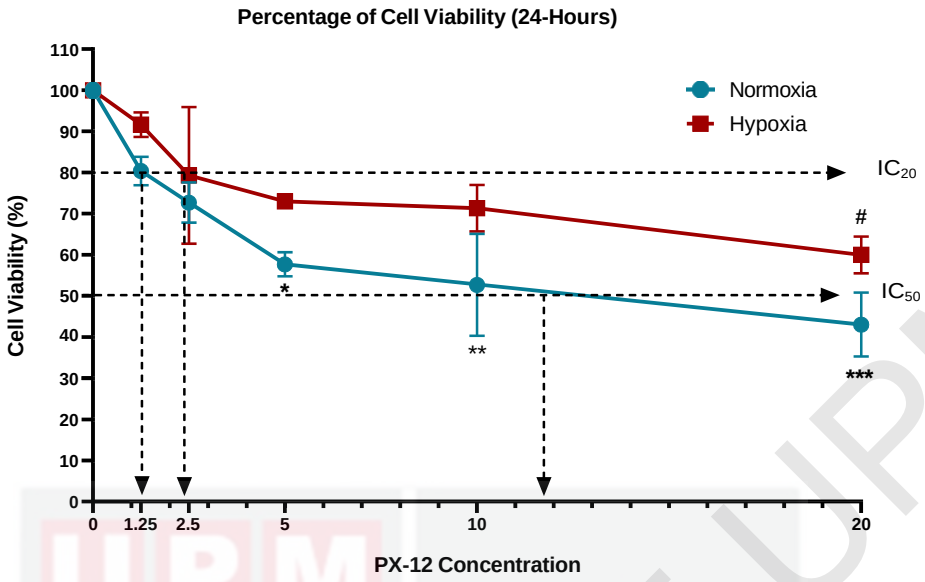


Figure 4.1.2 Cytotoxic effects of PX-12 on HCT116 cells via MTT assay at 24-hours time-point under normoxia and hypoxia conditions. Mean data were expressed as ± SEM based on three replicated experiments. Results were analyzed by using Two-Way ANOVA followed by Tukey's post-hoc test. The *P-value indicates statistical difference (*P < 0.05, **P < 0.01 and ***P < 0.001) in normoxia vs. control while #P-value indicates statistically significant difference (#P < 0.05) in hypoxia vs. control.

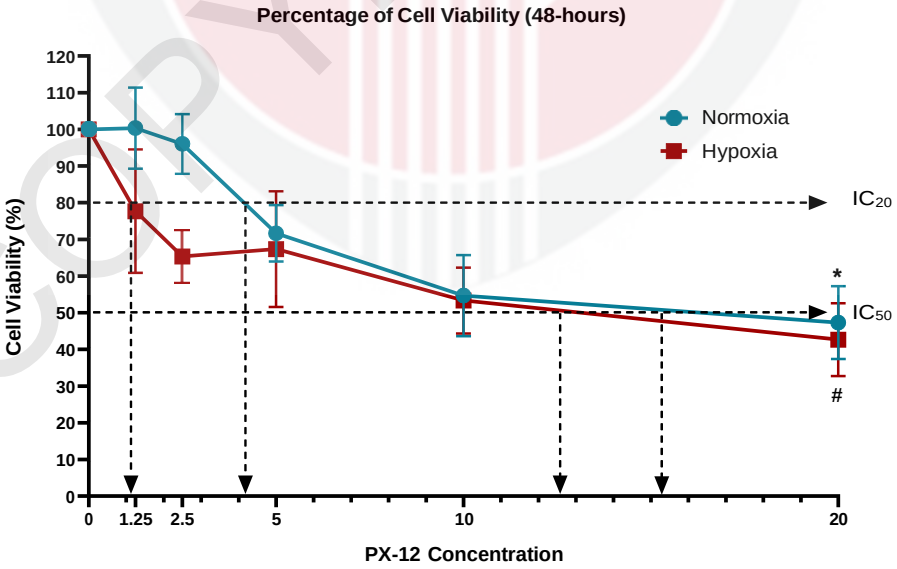


Figure 4.1.3 Cytotoxic effects of PX-12 on HCT116 cells via MTT assay at 48-hours time-point under normoxia and hypoxia conditions. Mean data were expressed as ± SEM based on three replicated experiments. Results were analyzed by using Two-Way ANOVA followed

by Tukey's post-hoc test. The *P-value indicates statistical difference (*P < 0.05) in normoxia vs. control while #P-value indicates statistically significant difference (#P < 0.05) in hypoxia vs. control.

Table 4.1.1 IC_{20} and IC_{50} values of PX-12 on HCT116 cells. IC_{20} and IC_{50} values were determined at 24 and 48-hours, respectively.

		IC_{20} HCT116 cells (µM)		**IC_{50} HCT116 cells (µM)**	
	Time	**24 hours**	**48 hours**	**24 hours**	**48 hours**
PX-12	Normoxia	2 ± 0.667	4 ± 0.882	12 ± 4.842	14 ± 4.372
PX-12	Hypoxia	3 ± 1.000	1 ± 0.333	± 20	12 ± 5.608

4.2 The effects of PX-12 on the invadopodia formation of HCT116 cells.

Invadopodia assay was conducted to observe the effects of PX-12 on the invadopodia formation of HCT116 cells. In this study, the gelatin degradation activity were quantified as the percentage of cell forming invadopodia and is defined as F-actin puncta that co-localized with black holes on the fluorescent gelatin (Md Hashim et al., 2013

In Figure 4.2.1, after 24 hours of treatment with IC_{20} PX-12, the number of cells forming invadopodia was significantly reduced under normoxia condition at a concentration of 2 µM (P < 0.005). Although, there is no statistical difference (P > 0.005) in the HCT116 cells forming invadopodia when treated with PX-12 under hypoxia condition, there is a trend of reduction in cells forming invadopodia when observed under the fluorescent microscope. GM6001 is a broad spectrum MMP inhibitor that was used as the positive control. GM6001 was shown to reduce 23% and 6% of invadopodia formation in HCT116 cells under normoxia and hypoxia conditions respectively. Figure 4.2.2 demonstrates the presence of invadopodia in

both conditions, normoxia and hypoxia for untreated, IC_{20} PX-12 and GM6001. The invadopodia formation observed as a black dot under the gelatin channel which co-localized with the presence of F-actin in the red channel.

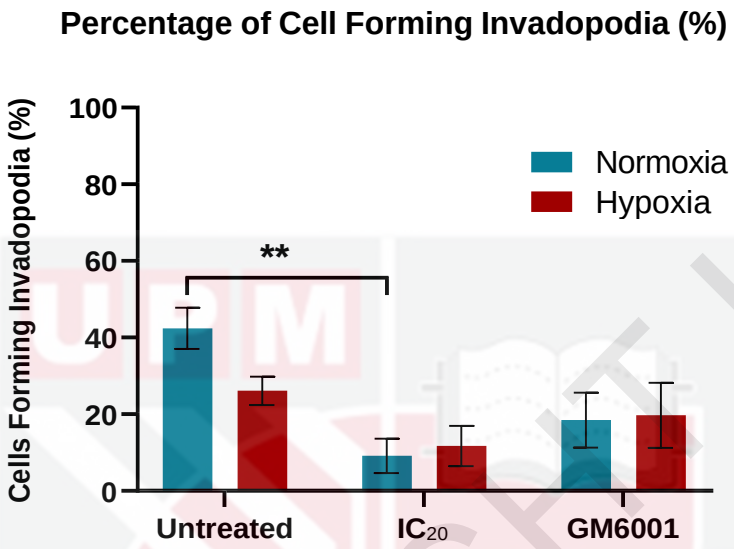
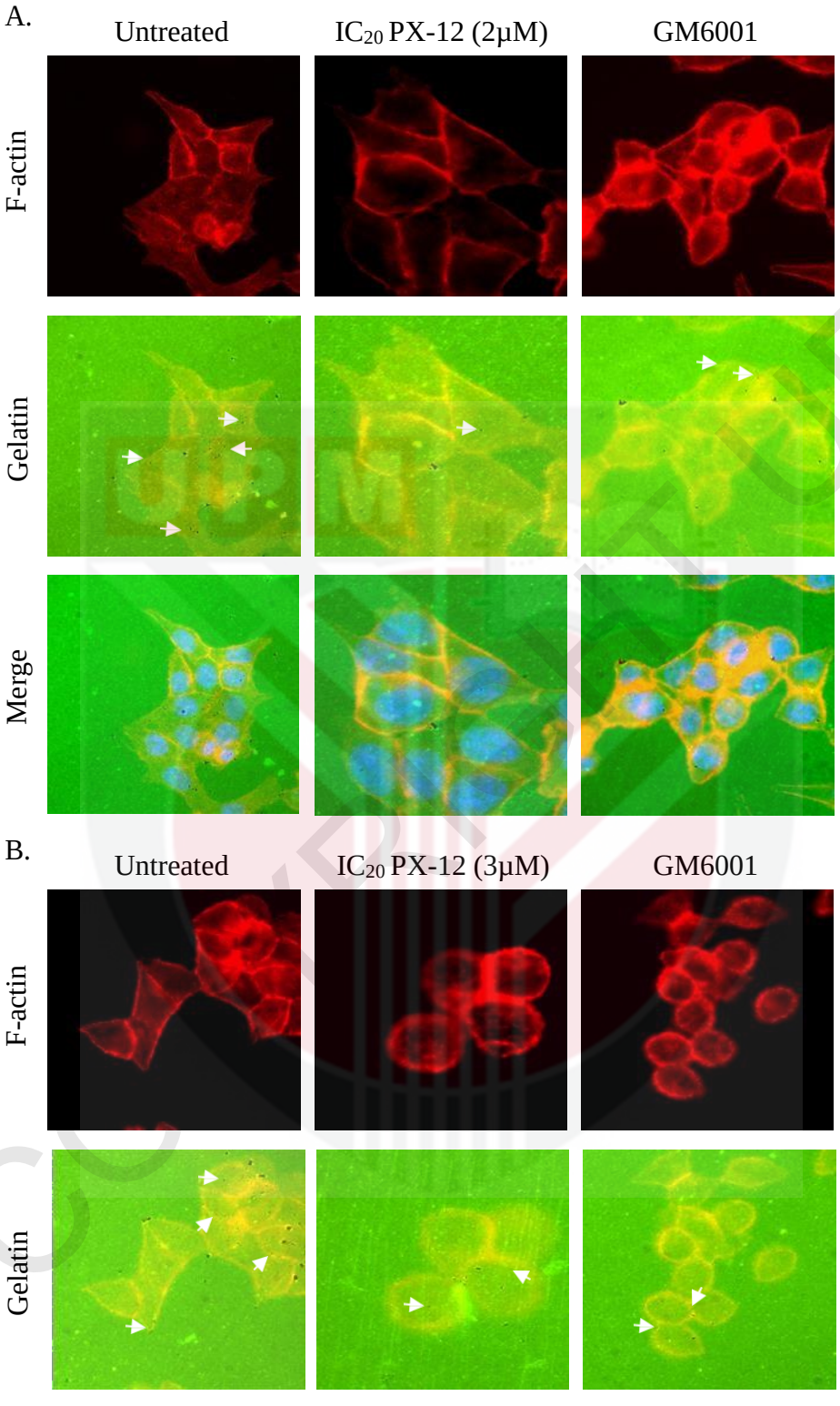


Figure 4.2.1. Percentage of HCT116 cells forming invadopodia after treatment with IC_{20} PX-12. Cells were scored for the presence of actin spots with underlying gelatin degradation for about 100 cells for each condition. Results are mean ± S.E.M of two independent experiments and were analyzed using Two-way ANOVA followed by Tukey's post-hoc test. The *P-value indicate statistical difference (**P < 0.01) compared to the untreated group of normoxia.


A.
Untreated
IC20 PX-12 (2µM)
GM6001
F-actin
Gelatin
Merge
B.
Untreated
IC20 PX-12 (3µM)
GM6001
F-actin
Gelatin

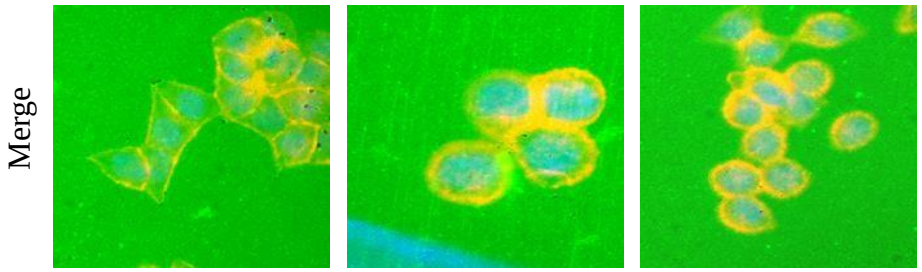


Figure 4.2.2. Representative images of invadopodia formation in HCT116 cells. Cells seeded in 6-well plate (3 x 10^5 cells/mL) were treated with IC_{20} PX-12 and then reseeded onto Oregon Green 488 gelatin-coated coverslips (1 x 10^4 cells/mL) for 48 hours, fixed and stained with Phalloidin for F-actin. Image captured at 400x magnification and merged using *ImageJ* software. White arrows indicate gelatin degradation. (A) Cells under normoxia condition. (B) Cells were treated with DMOG to induce hypoxia condition. (Scale: 25µm)

CHAPTER 5

DISCUSSION

Major prerequisite for cancer metastasis is the ability of the cancer cells to break tissue barriers and invade into surrounding tissues. Cancer metastasis involved multi-complex processes which begins with the detachment of tumour cells from the primary tumour site followed by migration and invasion through the basement membranes. The tumour cells will then intravasate into small blood vessels or lymphatic channels and therefore, colonize in a distant target organ (Alsarraj and Hunter, 2011). Chemotherapy is one of the treatment approach in colorectal cancer, however, this therapy often leads to tumour hypoxia in nearly all solid tumours which associated with poor prognosis in several tumours including colorectal cancer (Yao et al., 2017).

1-methyl propyl 2-imidazolyl disulfide (PX-12) had been characterized as an effective compound for anti-tumour in other type of colorectal cancer such as DLD-1 and SW620 (Wang et al., 2015) and in A549 lung cancer cells (You et al., 2014). According to Wang et al. (2015), they claimed to have demonstrated that PX-12 reduced the growth ability of the colorectal cancer cells (DLD-1 and SW620) by arresting the G2/M phase and inducing apoptosis. PX-12 is the inhibitor of the thioredoxin proteins and these thioredoxin proteins were often upregulated in 70% of most human cancers involving breast, lung, liver and colorectal cancer (Lin et al., 2017). Trx system is said to be crucial for cancer cell survival and proliferation through enhancement to numerous growth factors, inhibition of spontaneous apoptosis, and resistance to drug-induced apoptosis. However, the involvement of the Trx system in cancer remains uncertain (Mahmood et al., 2013). A dose dependent manner of PX-12 for the anti-tumour effects were observed on colorectal cancer in a study conducted by Wang et al, in 2014. PX-12 was the substance used in this study to examine its cytotoxicity and anti-invasive

properties on the HCT116 human colorectal cancer cells. The MTT assay was used to assess the cytotoxic effects of PX-12 on HCT116 cells since it provides rapid test execution and reproducibility of the test results. The cytotoxic effects of PX-12 were evaluated by measuring the percentage of cell viability. In this assay, viable cells reduced MTT into purple formazan crystals and DMSO were used to dilute the formazan crystals which allows the absorbance to be read at 570 nm (Riss et al., 2016). The higher the intensity of the purple colour, the more the viable cells there are. In this study, HCT116 human colorectal cancer cells were used as they were highly aggressive with little to no ability to differentiate compared to other colorectal cancer cell lines (Yeung et al., 2010).

Based on Figure 4.1.2 and 4.1.3, the cell viability of HCT116 cells treated with PX-12 were shown to reduce in a dependent manner after 24 and 48-hours of incubation in normoxia and hypoxia conditions. PX-12 significantly reduced the cell viability of HCT116 cells after 24 hours with IC_{50} between 5 µM to 20 µM under normoxia condition. In hypoxia condition, PX-12 significantly reduced the cell viability of HCT116 cells at 20 µM after 24 and 48 hours. These results indicate that the cytotoxic effects of PX-12 on HCT116 cells greatly depending on the concentration of the PX-12, as the concentration of the drug compound increases, the higher the cytotoxic effects. This was also proven in a study by Wang et al, where the cell viability of other colorectal cancer cell lines such as SW620 and DLD-1 was reduced in a dose dependent manner upon treatment with PX-12 (Wang et al., 2015). The IC_{50} value of PX-12 obtained by Wang et al. (2015) ranging between 5 µM to 8 µM under normoxia condition at 48 hours which is comparable with present study where the IC_{50} value of PX-12 obtained was 14 µM and it is not far from the range reported by Wang et al. (2015). According to You et al. (2014), PX-12 induced apoptosis by arresting the cell cycle progression that leads to cell death and it is associated with the loss of mitochondrial membrane potential (MMP).

The IC_{20} and IC_{50} values of PX-12 in HCT116 cells were extrapolated from the graph at each incubation time-point. The IC_{20} values of PX-12 ranging from 1 µM to 4 µM under normoxia and hypoxia conditions at 24 and 48-hours. IC_{20} value is the inhibitory concentration of a substance that is able to inhibit 20% of the cell population. These IC_{20} values will be used in the invadopodia assay to observe the cells that form invadopodia upon treatment with IC_{20} PX-12. Treatment with 12 µM to 14 µM of PX-12 inhibits 50% of the HCT116 cell viability after 24 hour incubation under normoxia condition and in both conditions at 48 hours of incubation. In comparison with other study, the IC_{50} value of PX-12 in SW620 and DLD-1 under normoxia condition at 48-hours of incubation were ranging from 12µM to 14µM (Wang et al., 2014). Px-12 at 20 µM under hypoxia condition in 24 hours decreased only about 60% cell viability of HCT-116 cells which was the highest concentration used, therefore, the IC_{50}'s could not be determined. In future experiments, it is suggested that we need to increase the concentration of the PX-12 in order to obtain the IC_{50} value.

Invadopodia is known as an intrusive foot processes that are rich in F-actin (Weaver, 2006) which is essential in tumour signalling to support tumour cell invasion and migration (Eddy et al., 2017). The ability of the invadopodia to degrade extracellular matrix (ECM) contribute to the metastasis of colorectal cancer. In a study by Mansour et al, 2014, it was demonstrated that invadopodia were observed in HCT116 human colorectal cancer cells where they were present as discrete puncta under the cell nucleus or located at the leading edge of the cells (Mansour et al., 2014). The HCT116 cells in this present study had successfully formed invadopodia, which is consistent with the previous research (Mansour et al., 2014) that established the association between colorectal cancer cells' ability to produce invadopodia and their metastatic potential.

In the invadopodia assay, PX-12 at 2 uM (IC_{20} concentration) significantly reduces the invadopodia formation of HCT116 cells under normoxia condition at 24 hours treatment where

33% of reduction in the cells forming invadopodia were observed. This data suggests that low concentration of PX-12 is sufficient to reduce HCT116 cells forming invadopodia which would contribute to the anti-invasive effects of PX-12 in colorectal cancer. In contrast, there are about 14% reduction in cells with invadopodia following PX-12 treatment under hypoxia condition suggesting that PX-12 can reduce invadopodia formation under low oxygen tension. Oxygen tension surrounding tumour cells were reduced and result in hypoxia condition that leads to invadopodia formation (Md Hashim et al., 2013). Reduction in invadopodia formation does reduces metastatic potential. Evidence shown in a study by Eckert and Yang (2011), it was demonstrated that knockdowns of the crucial invadopodia protein, Tks5 greatly reduced local invasion while maintaining the integrity of the primary tumours. This is the first study reported that low concentration of PX-12 can reduces invadopodia formation in colorectal cancer cell lines. Further study is needed to further evaluate the mechanism of action of PX-12 as anti-invasive agent.

Results obtained demonstrated that PX-12 does have significant cytotoxic activities towards HCT116 human colorectal cancer cells and should be further tested for its anti-migratory effects on HCT116 human colorectal cancer cells. Furthermore, from the invadopodia assay, we determined that in normoxia condition, at low concentration of PX-12, it is able to reduce gelatin degradation activity. This showed that PX-12 can be a good anti-invasion drug candidate in colorectal cancer. This study may provide further guidance in future testing on the PX-12 towards anti-invasion of the HCT116 cells especially in hypoxia condition since there is no evidence in the effects of PX-12 towards HCT116 cells in previous study.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Various factors could have influenced the results of the experiment including contaminations, pipetting errors as well as the ability to recognize invadopodia. Poor aseptic techniques could lead to the development of contamination. Aseptic technique is the most crucial step in cell culture work as it could give false positive result especially in the MTT assay. Contamination may affect the cell viability which directly affects the absorbance reading that leads to inconsistent of the results, thus, the experiment may not be able to reproduce. Pipetting technique is also an important issue in cell culture. Once the number of cells needed for seeding is inconsistent, automatically the absorbance reading will be affected. In addition, it is vital to get familiar with the structure of invadopodia so that the quantification of invadopodia would be easy and able to eliminate any possibilities of false positive result.

In conclusion, this study found that 1-methyl propyl 2-imidazolyl disulfide (PX-12) has cytotoxicity effects on HCT116 human colorectal cancer cells after 24- and 48-hours treatment under normoxia and hypoxia conditions. PX-12 significantly reduced invadopodia formation of HCT116 cells under normoxia condition upon 24 hours of treatment with PX-12. Interestingly, there is no significant difference in invadopodia formation under hypoxia condition, however, trend of reduction in cells forming invadopodia treated with PX-12 under hypoxia condition were observed in this study. Further experiments and validation work need to be done to further confirm this.

6.2 Future recommendations

Some recommendations need to take into account to further improve and have a deep understanding on the effects of PX-12 on HCT116 human colorectal cancer cells. The recommendations may include:

a) Checking the expression of the thioredoxin (Trx) protein and hypoxia-inducible factor expression level via Western blot after treatment of PX-12 to further validate the functionality of the PX-12.
b) Suggest doing 3D degradation assay since it mimic the tumour microenvironment much better than 2D assay.
c) Further validate the invadopodia assay using other invadopodia marker such as cortactin to evaluate the invadopodia formation.

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