Evaluation and Comparison of Two Novel Non-Platinum-Based Antitumor Agents on Pancreatic Cancer and Triple-Negative Breast Cancer

by

Xiangxuan Xu

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Cancer is one of the leading causes threatening human life. People suffering from pancreatic cancer and triple-negative breast cancer (TNBC) have particularly low survival rate among all cancer types. The novel non-platinum based antitumor agents, femtomedicine (FMD) compounds, were discovered as a new class of chemotherapeutic targeting agents to treat multiple cancers. Herein, two of the most effective FMD compounds (FMD-2Br-DAB & FMD-2I-DAB) were evaluated, based on their cytotoxicity against TNBC and pancreatic cancer cells through MTT assay, clonogenic assay, and caspase-3/7 green detection. According to the results obtained from these well-established cell-biology techniques, FMD compounds exhibit good antitumor effects in the cancer cell lines (PANC-1, BXPC-3, and MDA-MB-231), while having minimal impact on the normal cell line (GM05757), indicating FMD compounds can selectively kill TNBC and pancreatic cancer cells without being detrimental to normal cells. Furthermore, FMD-2I-DAB (compound C) shows a better efficiency than FMD-2Br-DAB (compound B), inferring that compound C could be more potent in tumor elimination. This study shows that the FMD compounds, especially for compound C, are potentially new drug candidates for effective treatment of the ‘hard-to-treat’ pancreatic cancer and TNBC.

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Chapter 1

Introduction

1.1 Cancer

Cancer has been a major health issue worldwide. According to the statistical data of human deaths around the world, Gilles et al. (2020) reported that although cardiovascular disease was the leading cause of global mortality, deaths from cancer in high-income countries and some upper-middle-income countries became more common. In Canada, it was reported that cancer already exceeded cardiovascular diseases and reached the first place leading to human death among all diseases and accidents (Canadian Cancer Society, 2022) (**Figure 1-1**). Since 2020, the pandemic (COVID-19) has brought differences to our lives including neglect of cancer. Temporary closure of some health facilities and the risk of exposure to COVID-19 significantly restrict people's actions in cancer diagnosis and treatment (Siegel, Miller, Fuchs, & Jemal, 2022). This will result in even more cases of deaths due to cancer.

Therefore, it is not surprising that research on cancer and finding more effective therapeutics are becoming more crucial and urgent.

1.2 Formation of Cancer

A healthy adult has around 30 trillion normal cells in the body. Proliferation of the great number of cells is under regulation in normal situation, therefore, the size and architecture of tissues in the body are maintained as needed (Weinberg, 1996). However, cancer cells do not obey the rule. They breach the control and reproduce unlimitedly. It is proposed that some occurrences

of cancer are due to genetic disorder which causes multiple gene mutations that overstep the boundary where cell expansion is limited (Felsher, 2004). Additionally, cancer can also be initiated by DNA damage caused by environmental factors such as ionizing radiation. Moreover, they can migrate to the sites other than where they were initiated and develop masses over time. In the late stage, they will become lethal since they interfere with the regular function of normal tissues. Fortunately, various tumor suppressors exist, which can defend growth and invasion of cancer. Cellular senescence is one of the defense lines. It can cause changes in gene expression and cell

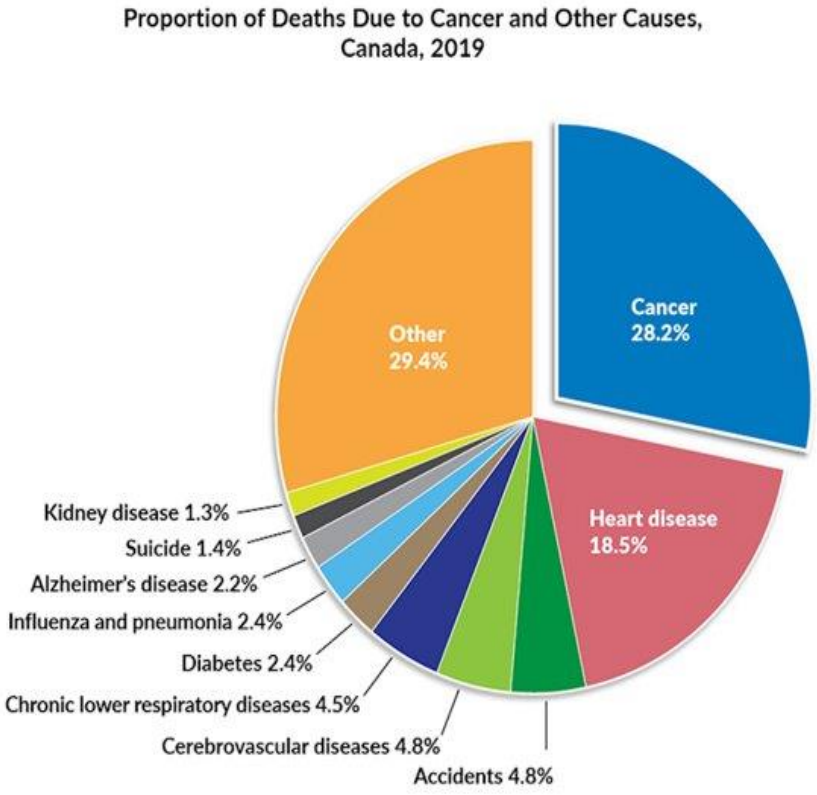

Figure 1-1: Proportion of Deaths in Canada in 2019 (Canadian Cancer Society, 2022)

morphology that result in cancer cell-cycle arrest (Vesely, Kershaw, Schreiber, & Smyth, 2011). In addition, ligation of death receptors with corresponding ligands can activate caspase in cancer cells to begin apoptosis. Furthermore, the immune system can kill cancer cells by expression of tumor-specific antigens when it discovers that the cells have become abnormal and eluded intrinsic suppressing mechanisms (Vesely et al., 2011). Although these mechanisms are strongly effective against initial occurrence of cancer, a small proportion of cancer cells may accidently escape and survive which will subsequently disseminate and invade endothelial cell layer where metastatic tumors form (Coghlin & Murray, 2010). It is commonly identified as late stage of cancer when these metastatic tumors develop. Patients detected with a late-stage cancer are extremely hard to cure, since treatment should not only target on the local site (where cancer cells were initiated) but also on distant sites. In addition, it is difficult to detect every single metastatic site because it is sometimes too small (Sajib & Taibur, 2012). Therefore, it is important for patients suffering from cancer to have early diagnosis and treatment.

1.3 Cancer Diagnosis and Current Treatments

Although overall survival rates of various types of cancer remain low, there are many factors that can potentially impact the consequence. A study by Ries et al. (2007) suggested that relative cancer survival rate is related to various factors, including sex, race, age, historic stage, grade, tumor size, histology (**Figure 1-2**). Among the listed factors, the five-year survival rate dramatically decreases when the level of historic stage becomes higher. While in early/localized

stage, most patients can survive by taking proper measures, whereas few patients can survive if cancer has been developed to an advanced stage. Hence, early diagnosis is extremely important.

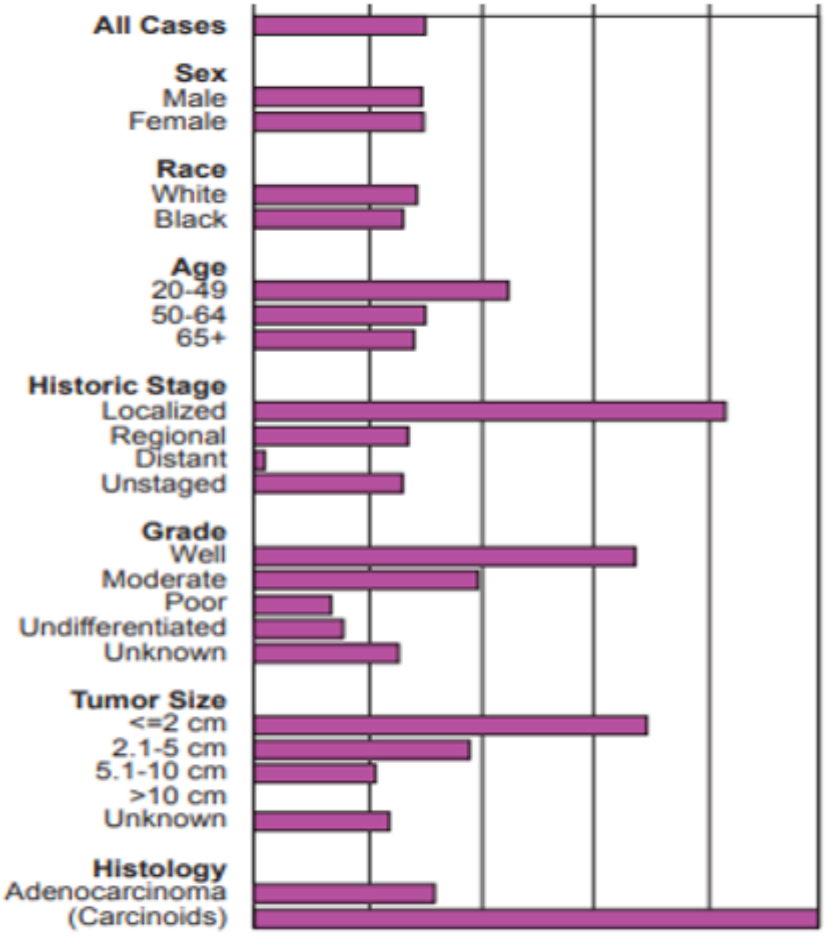

Figure 1-2: : Five-year relative survival rates by sex, race, age, historic stage, grade, tumor size, and histology (Ries et al., 2007). Note: the data depend on evaluation of 12 different sites in human body, so survival rate within each cancer site may vary substantially

Cancer diagnosis has shown strong dependence on imaging techniques in recent decades. Medical imaging started in 1895 with the great discovery of X-ray. In the 1950s, another big progress was made by invention of nuclear medicine. A widely used modality today in terms of

nuclear medicine is positron emission tomography (PET), which can offer better understanding in cancer's biologic functions and its microenvironment by use of radioactive compounds (Bradley, 2008). The first use of ultrasound in clinic was in 1970s, which can safely produce tomographic images by reflection of sound waves. Magnetic resonance imaging (MRI) and computed tomography (CT) which were also invented in 1970s are the most commonly used modalities nowadays. They provide insightful information on morphology, size, and structural shift. They are often used in combination with PET to maximize the strengths, since MRI or CT can provide a better spatial resolution that is lacked if using PET solely (Histed, 2012). Recently, with the rapid development of computer science, diagnosis relying on computer is progressing. Deep learning with many algorithms established can produce high-level representation of cancerous features directly from raw images (Munir, Elahi, Ayub, Frezza, & Rizzi, 2019). This technique may become the dominant cancer screening modality in the future.

Although screening can be effective for people to know whether they are suffering from cancer, most people are reluctant to do the screening until they feel something wrong. However, some types of cancer, including ovarian and pancreatic cancer, do not reveal noticeable symptoms until metastatic stage is developed. By then, they can hardly be cured. Therefore, development of cancer treatments is important.

A typical cancer treatment is surgery if the cancer is still localized. Surgery is the major curative treatment against localized cancer by manually eliminating tumors together with surrounding tissues. It is effective at an early stage for most cancers, whereas less useful if tumors have already metastasized since it is impossible to surgically remove tumors in every single site.

Therefore, palliative surgery, instead of curative surgery, may be performed against late-stage cancer.

Radiation therapy is a modern technique against cancer through ionizing radiation. When radiation passes through biomolecules (mainly DNA), chemical modifications will occur, which produce various lesion sites (**Figure 1-3)**. Ionizing radiation can result in cancer cell death but may also cause death or genetic changes to normal cells (Lomax, Folkes, & O'Neill, 2013).

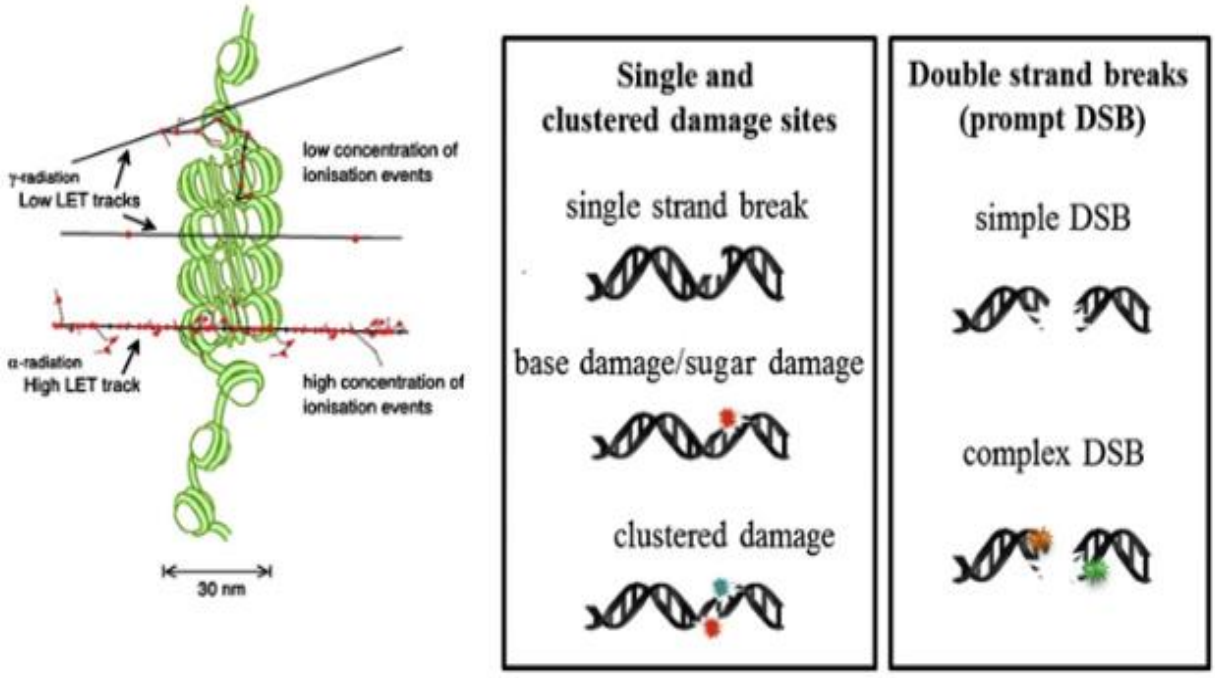

Figure 1-3: Schematic of the types of DNA damage (Lomax, Folkes, & O'Neill, 2013)

Targeted therapy can be applied, relying on the difference between normal cells and tumors. Proteins produced in cancer cells, for instance, are mostly different due to DNA modification. Therefore, treatments can be applied targeting on the specific proteins that are especially rich in cancer cells so that cancer will be selectively eliminated whereas most normal

cells will remain their activities. P53 is known as a tumor suppressor gene. It was found that there was around 50% occurrence of mutated p53 in various cancer cells (Hollstein et al., 1991). It was also found that expression of mdm-2 protein, which could inhibit transcription of p53, was highly elevated in many cancer cells (Momand et al., 1992). The evidence shows that theses cancer cells grow by producing mdm-2 to block the activity of p53. This brings an insight that targeted therapy can be utilized by restricting production or activity of mdm-2, which consequently liberates the function of p53. In 2004, Vassilev discovered that Nutlins could inhibit interaction between p53 and mdm-2 and reveal the normal function of p53. This discovery shows that targeted therapy is a specific and useful tool to kill tumors.

As written previously, the immune system plays a crucial role in killing cancer cells. However, some cancer cells possess the ability to hide from the arrest. One way they can achieve this is to create specific proteins shown on cellular surfaces that alleviate the immune function on cancer cells (Saha et al., 2021). In this case, immunotherapy could be an effective treatment to strengthen the immune function by stimulating the immune system. The enhanced ability of tumor identification and elimination can strongly limit the growth and existence of cancers. However, the drawback of immunotherapy is that the treatment may lead to immune selection for cancer cells so that they become more adaptable and harder to kill by the immune system. Therefore, caution needs to be made before immunotherapy.

Hormonal therapy, as indicated by its name, specifically focuses on hormones produced in human body. Some cancer cells (for example, most breast cancer cells, which will be discussed in later sections) require hormones to survive and grow. Therefore, removing or blocking specific

hormones will result in death of tumor cells. Hormonal therapy is frequently used together with other treatments including radiation therapy, surgery, etc.

Chemotherapy is used by treating cancer cells with cytotoxic chemicals. The main target for chemotherapy is DNA in tumors. Typically, agents used in chemotherapy possess reactive groups that can react with DNA and form a solid bond, which consequently leads to cross linking of DNA. It is extremely difficult for cross-linking DNA to successfully replicate or transcript. Therefore, cancer cells will die due to the dysfunction of DNA. A commonly used example of chemotherapy is the platinum agent: Cisplatin, which will be specifically discussed in later sections.

1.4 Relevant Features in Cancer Cells

Apart from DNA mutations, there are multiple features mainly existing in cancer cells and tumor microenvironment. These features can be critical to differentiate from normal cells when a treatment is applied.

1.4.1 Redox Adaption

There are various oxidants in cytoplasm, so called reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radical, etc. On the other hand, reductive stress is controlled by reductive equivalents, such as reduced glutathione, nicotinamide adenine dinucleotide (NADH), etc. (Chun, Kim & Surh, 2021). The redox balance is important for cells to

be alive. However, because of the rapid proliferation of cancer cells, cancerous metabolism is reprogrammed and highly elevated, which leads to increased yield of ROS as the byproducts of metabolic activities (K. Wang et al., 2019). To deal with the overwhelming ROS, cancer cells tend to produce abundant antioxidants constantly, which dramatically affect the overall redox environment (Bae et al., 2004). Although the exact mechanism is still puzzled today, many researchers proposed the intracellular environment in many cancer cells is reductive. DeNicola et al. (2011) suggested that Nrf2, a transcription factor that plays a key role in antioxidant program, was found to be elevated following by the expression of endogenous oncogenes. The ROS is therefore lower creating a reductive intracellular environment in cancer cells.

1.4.2 Hypoxia

One of the most hallmarked features of tumor microenvironment (TME) is hypoxia, which represents a condition that the level of oxygen is extremely low in cancerous tissues. Hypoxia mainly exists in TME due to the fact that their vasculature is abnormal compared with normal vasculature (Siemann, 2011). It is known that different kinds of nutrients including oxygen are necessary for cell viability and growth and that they are carried by the flow of blood in blood vessels. To unlimitedly grow, cancer cells overexpress the pro-angiogenic factors that cause the formation and development of blood vessel to be immature and tortuous (**Figure 1-4**). As a result, vessels for cancer cells lose their conventional hierarchy, where arterioles, capillaries and venules cannot be clearly distinguished (Siemann, 2011). The poorly developed vessels possess abnormal shape and diameter which largely limit the blood flow. Therefore, oxygen transportation is usually

restricted leading to hypoxia in cancer cells (Tong et al., 2004). Some traditional treatments reveal low efficiency under the TME, so appropriate strategies are needed against cancers.

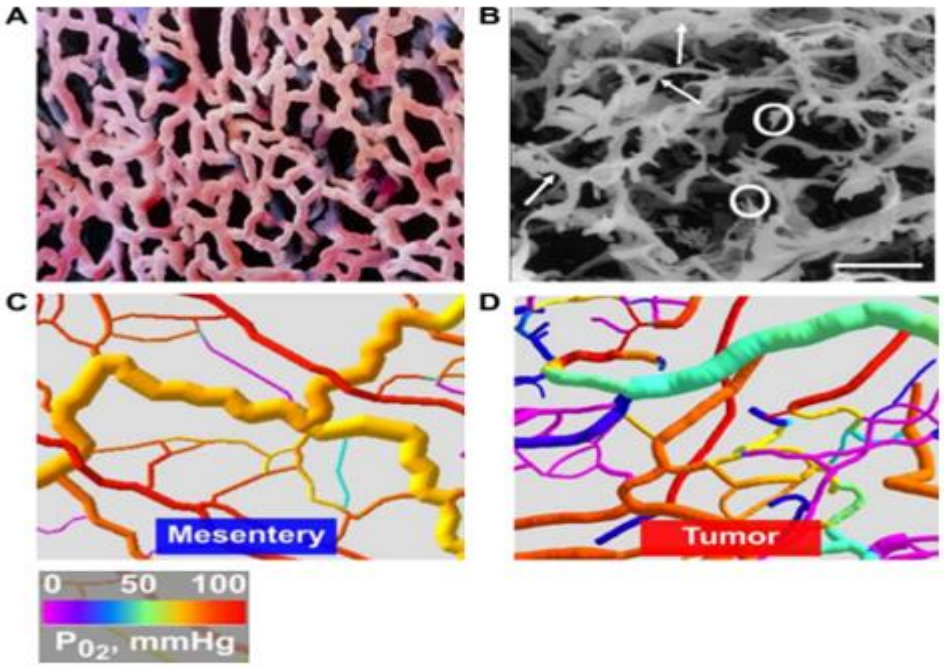

Figure 1-4: Comparison of vasculature between tumor and normal tissues. A: SEM image of normal lung tissue (Prof. P. Motta, G. Macchiarelli University, La Sapienza, Rome. Science Photo Library); B: An SEM image of a human adenocarcinoma with blind ends (circled) and abnormal bulges (arrowed) (Br J Cancer 2001); C and D: Mesentery and tumor vascular networks colored by pO2 level (Pries et al., 2009)

1.5 Study on Specific Types of Cancers

There have been various types of cancers discovered and recorded so far. Formation of cancer may appear in different organs, and they reveal disparate characters and detriments. Although all cancers severely endanger human life, some types of cancers particularly lead to

serious syndromes or even death. Herein, two of them are mainly studied and will be discussed: breast cancer and pancreatic cancer.

1.5.1 Breast Cancer

Breast cancer is reported to be the most common cancer among Canadian women. It is also the second leading cause of Canadian women death from cancer (Canadian Cancer Statistics Advisory Committee, 2022). Worldwide, more than 1.3 million cases of invasive breast cancer are diagnosed, while over 450000 women die annually due to breast cancer (Chavez, Garimella, & Lipkowitz, 2010). Although it is not common for men to have breast cancer, sometimes it happens.

For most types of breast cancer, the survival rate is not extremely terrible. According to the 5-year relative survival rate statistics (**Figure 1-5 (left)**), there are 99% patients with localized breast cancer can survive in 5 years. When it comes to regional stage, 86% percent survival rate is remained, while 29% can survive in distant stage. On the other hand, there exists a special type of breast cancer called triple-negative breast cancer (TNBC). It accounts for around 10% to 20% of all breast cancer cases. The special type of breast cancer often exhibits the same symptoms as other types including a lump or mass in the breast, breast redness and pain, a nipple turning inward and having discharge (WebMD, 2022). However, it is harder to cure, and hence yields a much lower survival rate. **Figure 1-5 (right)** illustrates the survival rate for TNBC. Patients suffering from TNBC have 91%, 65%, and 12% survival rate for localized, regional, and metastatic stage, respectively. The data clearly indicates that TNBC substantially reduce the survival rate compared with commonly appeared breast cancer, which shows TNBC have become a major challenge

among all types of breast cancer. The estimates in recent years are that the incidence rate increases by 0.5% per year, and the death rate goes down by generally 1% per year (American Cancer Society, 2022). The severity is still nonnegligible.

General breast cancer

SEER Stage	5-year Relative Survival Rate
Localized*	99%
Regional	86%
Distant	29%
All SEER stages combined	90%

TNBC

SEER Stage	5-year Relative Survival Rate
Localized	91%
Regional	65%
Distant	12%
All stages combined	77%

Figure 1-5: 5-year relative survival rate in terms of breast cancer. Left: most types of breast cancer; Right: triple-negative breast cancer (TNBC) (American Cancer Society)

It is routinely found that for most breast cancer patients there is expression of progesterone receptor (PR), estrogen receptor (ER), and elevation of HER2 (Brenton et al., 2005) (**Figure 1-6**). These markers can help classify breast tumors into hormone receptor positive cancer, HER2 elevated cancer, and the type of cancer that does not reveal any of these features. The latter type is named as triple-negative breast cancer (TNBC) because of the fact that none of these markers are produced (Chavez et al., 2010). There is a big advantage when treatments are applied to tumors that either produce hormones or enhance HER2 level. Targeted agents can be used to interrupt production and action of hormones when treating tumors in which PR and ER are rich. Tumors where HER2 is amplified can be treated by inhibiting HER2 (Brenton et al., 2005). Whereas TNBC is mainly treated by elaborately designed chemotherapy.

Considering the detriment TNBC could cause deaths and the difficulty in TNBC treatment, an appropriate and effective chemotherapy is deadly needed, which will be discussed throughout chapters.

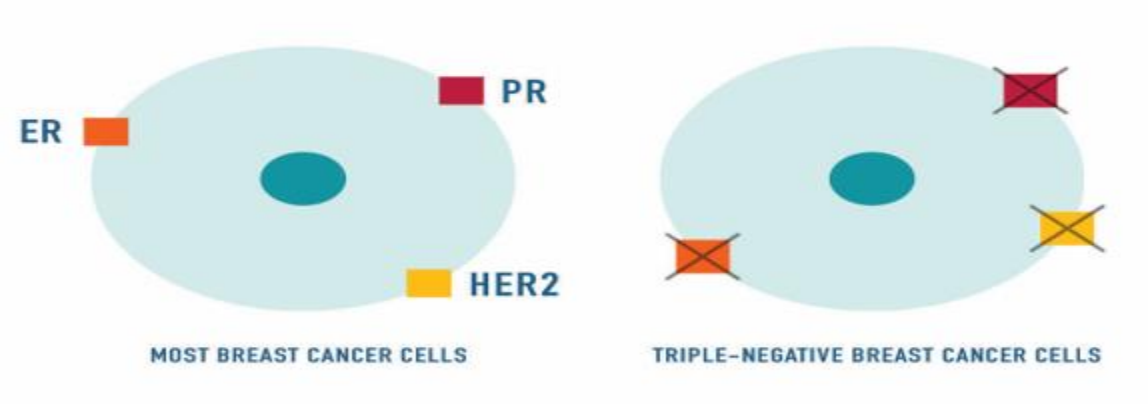

Figure 1-6: Difference in receptor sites between most breast cancer cells and TNBC cells (National Cancer Institute, 2021)

1.5.2 Pancreatic Cancer

Pancreas plays an important role in metabolism regulation. It can secrete digestive enzymes for small intestine as well as produce various hormones such as insulin. Pancreatic cancer is one of the most aggressive and fatal cancer types. When cancer cells grow in pancreas, they significantly affect pancreas function and cause severe problems. They are often developed in human pancreas with nearly no recognizable symptoms, until they reach the late stage when the tumors have successfully metastasized to distant organs. **Figure 1-7** shows the 5-year relative survival of different cancer types. The top three cancer types with leading survival are prostate, thyroid, and testis cancer, possessing 98%, 98%, and 95% overall survival rate, respectively.

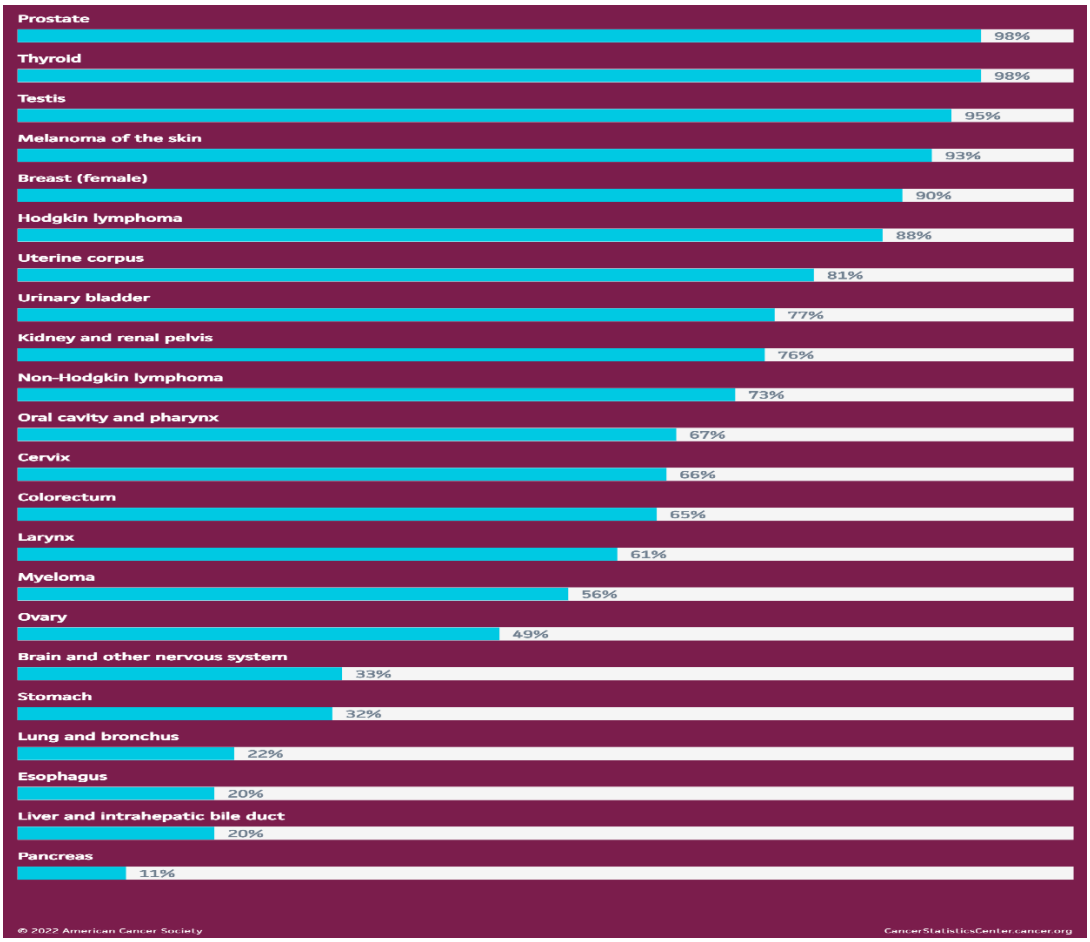

Figure 1-7: 5-year survival rate by cancer type (American Cancer Society, 2022)

SEER Stage	5-year Relative Survival Rate
Localized	42%
Regional	14%
Distant	3%
All SEER stages combined	11%

Figure 1-8: 5-year relative survival rate of pancreatic cancer (American Cancer Society, 2022)

On the other hand, pancreas cancer is on the very end of **Figure 1-7**, with only 11% survival rate. The data demonstrates that pancreatic cancer has already become the biggest challenge for cancer research. Even if abundant studies were conducted, little progress has been made. During the past 5 years, overall possibility for pancreatic cancer survival remains to be 11% (**Figure 1-8**). When the cancer has not propagated outside pancreas (localized stage), there is around 42% survival rate. When the cancer has metastasized to nearby lymph nodes and structures (regional stage), the survival rate reduces to 14%. If pancreatic tumors already spread to different organs such as liver, bones, lungs, etc. (distant stage), the survival possibility is estimated to be only 3% (American Cancer Society, 2022). Therefore, pancreatic cancer study is urgent and impactful, and thus it will be discussed in detail hereinafter.

1.6 Cisplatin's Antitumor Activity – Giving Insights to Further Chemotherapy

Cisplatin has been used as a chemotherapeutic agent to kill tumors for a long period. It not only exhibits decent ability in killing cancer, but also gives good insights to later design of other chemotherapies. In this section, understanding of Cisplatin and its antitumor mechanism will be discussed in detail.

1.6.1 Cisplatin

Cisplatin, as mentioned in previous section, is a commonly used cytotoxic drug. It is included in the platinum complex family that can cause cross linking of DNA. Its structure is

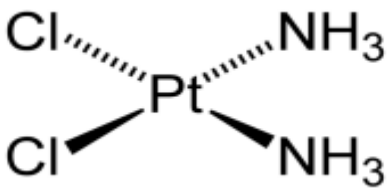

Figure 1-9: Cisplatin structure

shown in **Figure 1-9**. Cisplatin was first synthesized in 1844 (Peyrone, 1844), whereas it was approved for clinical use to treat cancer in 1978. Cisplatin was so widely used for various types of cancer that it was even considered as an era of Cisplatin at that time (Hanna & Einhorn, 2014). However, the praise was stopped

shortly after people found Cisplatin could cause side effects that may also lead to severe symptoms. Cisplatin contains platinum, a heavy metal element, which could induce nephrotoxicity including renal failure and chronic dysfunctions (Schreiner & Maher, 1965). The heavy metal can easily attach to proteins, which causes platinum accumulation in organs especially in kidney, since the excretion of Cisplatin is mainly achieved in kidneys. It is proposed that high dose of Cisplatin can result in nausea, vomit, hair drop, hearing loss, immunosuppression, and other symptoms (Ana-Maria & Dietrich, 2011). However, if dose is reduced, the antitumor effect will also become lower. The side effects significantly limit the use of Cisplatin in the clinic.

1.6.2 DNA Modification Caused by Cisplatin

DNA is considered as the major target when Cisplatin goes into human body. Much evidence shows that guanine bases are easiest for Cisplatin to attack (Baik, Friesner, & Lippard, 2003; Robins, 1973); (Eastman, 1982). While a portion of Cisplatin reacts with biomolecules on the pathway to nucleus after Cisplatin treatment, others reach nuclear DNA and mainly form covalent bonds with guanine bases. Research shows that about 60% to 65% intra-strand 1,2-GG

will be formed for Cisplatin when it attacks DNA, while other linkages include AG strands, GG inter-strand, etc. (Fichtinger-Schepman, 1985). After different linkages are formed, the structure of DNA will be vastly changed by those cross links which will consequently resist DNA replication and transcription. Finally, apoptosis will be activated leading to cell death.

1.6.3 Antitumor Mechanism – Dissociative Electron Transfer (DET) Reaction

To better understand the antitumor mechanism of Cisplatin, it is necessary to review the function of prehydrated electrons. Prehydrated electrons are produced by ionization of water caused by ionizing radiation. If prehydrated electrons are not captured by a transition molecule, they will quickly get solvated by the surrounding water molecules to form hydrated electrons in about 540 femtoseconds (Wang, Luo & Lu, 2008). The hydrated electrons are stable, whereas the prehydrated electrons are very active. Within this short time period before it is transformed to hydrated electrons, prehydrated electrons can react with halogen species in the drug (Cisplatin) and dissociate the drug (Cisplatin) into fragments (Lu, 2020) (**Figure 1-10**). These resultant radical fragments can effectively induce DNA damage by attacking bases in DNA and causing chemical bond breaks, and consequently cancer cells will be eliminated (Wang, Nguyen, Lu, 2009). The DET reaction also explains why there is a priority of guanine bases being attacked, since guanine is likely to act as an electron donor compared to other bases (Lu, Kalantari, & Wang, 2007). Hence, the combination of chemotherapy (Cisplatin) with radiation therapy drastically enhances the efficiency in killing cancer cells.

If there is no radiation applied, there will be no prehydrated electrons since the ionization of water can hardly happen in this situation. However, there are still weakly-bounded electrons in cells, especially rich in cancer cells and TME. These weakly-bounded electrons are similar to prehydrated electrons in aspect of occurrence of DET reaction, thus DET reaction can still happen even without ionizing radiation. However, much faster DET reaction will occur under ionizing radiation since more reactive electrons are activated, which unravels the radiosensitizing effect of Cisplatin (Lu, 2007).

$$e_{pre}^{-} + Pt(NH_3)_2Cl_2 \rightarrow [Pt(NH_3)_2Cl_2]^{\bullet -} \rightarrow Pt(NH_3)_2Cl^{\bullet} + Cl^{-}$$

$$e_{pre}^{-} + Pt(NH_3)_2Cl \rightarrow [Pt(NH_3)_2Cl]^{\bullet -} \rightarrow Pt(NH_3)_2{}^{\bullet} + Cl^{-}$$

Figure 1-10: DET reactions of Cisplatin (Zhang & Lu, 2021)

1.7 Femtomedicine

Although Cisplatin exhibits good efficiency in antitumor effect, its cytotoxicity to normal cells and risk of suffering from severe symptoms significantly limit its clinical use. The finding of the DET mechanism of Cisplatin has offered an opportunity of discovering new anticancer agents. Therefore, the novel non-platinum-based halogenated molecules were proposed by Dr. Lu for the purpose of more safely and effectively killing cancer. Through an innovative femtomedicine (FMD) approach, a new class of novel anti-cancer targeting compounds (called FMD compounds) have been discovered (Lu, Zhang, Ou, Wang, & Warrington, 2015).

1.7.1 Discovery of Femtomedicine (FMD) Compounds

The main difference of femtomedicine compounds (FMDs) compared from cisplatin is that there is an aromatic ring instead of the heavy metal--platinum. There are many benefits for the new FMDs. The first benefit is that it is non-platinum-based so it is less toxic to normal cells than Cisplatin; second, the aromatic ring is more nucleotide-like, therefore easier to intercalate into DNA; third, the DET reaction of FMDs can occur, similar to the mechanism of Cisplatin (**Figure 1-11**). A femtomedicine compound can either attract one or two prehydrated (weakly-bounded) electrons and produce radicals that result in DNA damage as mentioned hereinbefore. Fourth, the newly discovered FMD compounds are essentially non-polar so they can easily cross the cellular membrane. With the basic molecular structure of diamino benzene, 6 different compounds in a family have been discovered as effective anticancer targeting agents (**Figure 1-12**) (Lu, Zhang, Ou, Wang, & Warrington, 2015; Wang et al., 2016). They all contain halogen species as the reactive groups. Compound A, B, and C possess two halogen atoms, while compound D, E, and F possess one. Although their molecular structures are nearly the same, they could exhibit different characteristics. Herein, compound B and compound C are the main focus, and the evaluation and comparison of the two compounds will be discussed through various experiments.

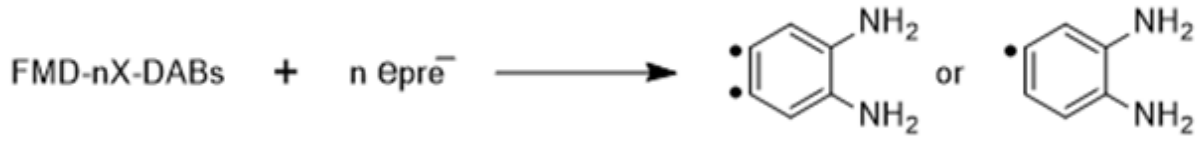

Figure 1-11: DET reactions of FMD compounds. (n equals to 1 or 2; X refers to I, Cl, Br) (Lu, Zhang, Ou, Wang, & Warrington, 2015; Wang et al., 2016)

1.7.2 Selective Cytotoxicity

Although killing cancer cells is a desired target for any types of treatments, it is usually hard to maintain viability of normal cells simultaneously. However, it is suggested that FMD compounds may eliminate cancer cells while sustaining the life of normal cells. As mentioned in previous section, cancer cells have a more reductive intracellular environment and hypoxia occurs particularly in TME. Oxygen molecules are potent rivals against FMD compounds since they tend to compete for electrons and be transformed to superoxide. Therefore, with the competition of oxygen molecules, there are less electrons available for the DET reaction with FMD compounds, and thus the efficiency of the drug becomes lower in normal cells. For cancer cells, in contrast, the reductive intracellular and hypoxic TME will lead to a much more effective DET reaction efficiency and therefore a preferential anti-cancer efficacy of FMD compounds (Lu et al., 2015).

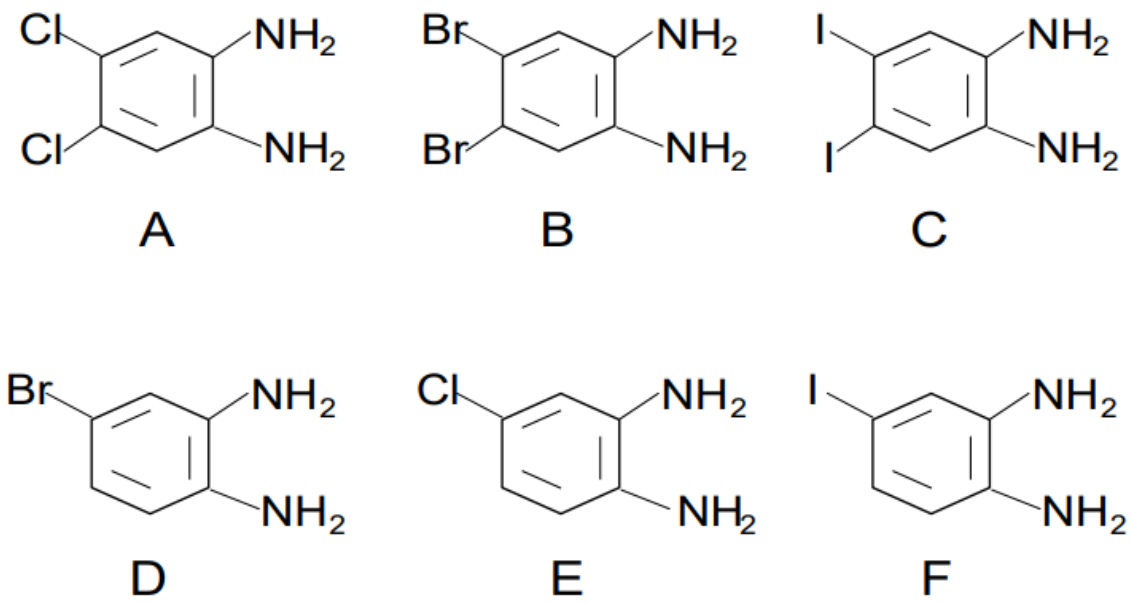

Figure 1-12: Chemical structures of non-platinum-based FMD compounds (Lu et al., 2015)

A: (4,5-)dichloro-(1,2-)diamino-benzene (4,5-dichloro-1,2-phenylenediamine), FMD-2Cl-DAB;

B: (4,5-)dibromo-(1,2-)diamino-benzene (4,5-dibromo-1,2-phenylenediamine), FMD-2Br-DAB;

C: (4,5-)diiodo-(1,2-)diamino-benzene (4,5-diiodo-1,2-phenylenediamine), FMD-2I-DAB;

D: bromo-(1,2-)diamino-benzene, FMD-1Br-DAB;

E: chloro-(1,2-)diamino-benzene, FMD-1Cl-DAB; and

F: iodo-(1,2-)diamino-benzene, FMD-1I-DAB.

Chapter 2

In Vitro Cytotoxicity Test on FMD through MTT Assay

2.1 MTT Assay

MTT, chemically as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a popular agent in examination of metabolic activity of living cells (Grela, Kozłowska, & Grabowiecka, 2018). MTT assay can be applied relying on enzymatic reduction of lightly colored tetrazolium salt to the corresponding colored formazan that can give signals under spectrophotometer (Grela et al., 2018).

Tetrazolium salts are a big family that have been involved in many protocols related to cell viability and growth from the 20th to the 21st century (Bernas & Dobrucki, 2000). They routinely possess 4 nitrogen atoms in a tetrazole ring and are colorless in general, whereas intense color can be observed when they are reduced to formazan (Berridge, Herst, & Tan, 2005). MTT is one of the commonly used tetrazolium salts, whose application was first evaluated by Mossman (1983). It was suggested that MTT can be reduced by NADH dependent oxidoreductases mainly in living cells with active mitochondria (Stockert, Blázquez-Castro, Cañete, Horobin, & Villanueva, 2012). **Figure 2-1** illustrates the transformation from soluble yellow (light) MTT to its insoluble purple formazan. A reagent that can dissolve formazan will be added subsequently before taken for spectrophotometric detection. Acid isopropanol was first suggested as the formazan solvent by Mosmann (1983), however, it was proved not perfect soon after. Some other proposed solvents were also denied because of their unsatisfied stability. Ethanol, for example, dissolves formazan well, while it leads to protein precipitation that interrupts final measurement (Mosmann, 1983).

Alkaline dimethyl sulfoxide (DMSO) (H. Wang, Wang, Tao, & Cheng, 2012) and sodium dodecyl sulfonate (SDS) based mixtures (Young, Phungtamdet, & Sanderson, 2005) were subsequently discovered as the best solvents for formazan while exhibiting minimum side effects. In this chapter, SDS supplemented with hydrochloric acid (HCl) are used in related experiments.

MTT assay is widely used in cell biology due to its high efficiency in cell viability examination and drug evaluation. It is claimed as the simplest and cheapest tests among all vitality estimations in aspect of equipment and reagents used (Grela et al., 2018). It also gives relatively fast result compared with traditional plate counts under appropriate execution (Brambilla, Ionescu, Cazzaniga, Edefonti, & Gagliani, 2014; Dalai, Pakrashi, Kumar, Chandrasekaran, & Mukherjee, 2012). Therefore, it is of great importance that MTT assay will be properly conducted.

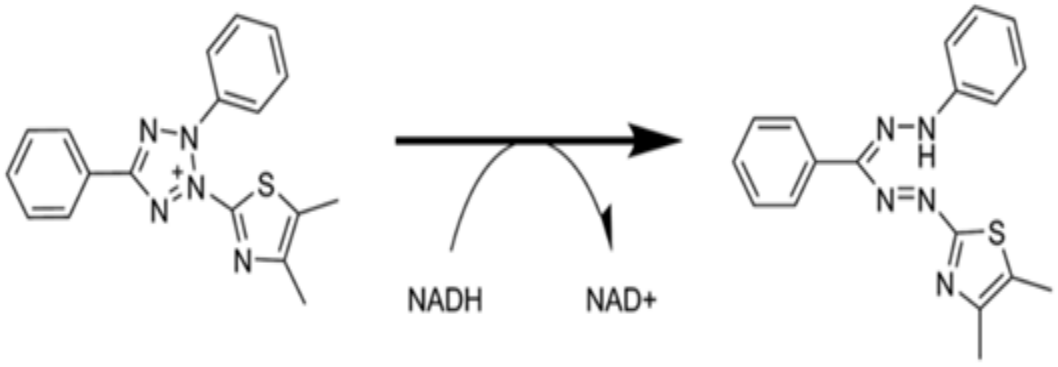

Figure 2-1: Process of enzymatic reduction from MTT to its insoluble formazan

2.2 Experimental Procedure of MTT Assay

The main steps of MTT assay include seeding, treatment (addition of drugs), addition of MTT related reagents, and spectrophotometric detection.

To properly seed cells, 5000 cells per well are uniformly distributed into 96-well transparent tissue culture plate (BD Falcon™, Corning Inc., NY, USA). After seeding, the plate will be placed into appropriate incubator depending on cell lines. Overnight incubation is necessary since the time is required for cells to be fully attached. Observation under microscope is an optional way to guarantee cell adherence is complete.

A series of calculation regarding the amount of drug addition needs to be done before beginning of treatment. A small portion of drug is extracted and diluted for better accuracy. Cells in each well are treated by specific concentration of drugs (compound B or compound C). At least 3 replicates for each concentration are required in each experiment in case that error may occur. After treatment, 96-well plate are placed into incubator for typically 48 hours.

MTT stock solution is normally prepared by adding MTT powder into phosphate buffer saline (PBS) for a final concentration of 5mg/mL. After stirring, MTT solution is sterilized by 0.22 μm filter because MTT is sensitive to microorganism. When used in experiments, the stock solution is diluted to 1/10 by adding appropriate colorless growth medium (without phenol red). When treatment is done, MTT working solution (1/10 concentration of stock solution) is added to each well containing cells. However, removal of previously added growth medium with drug must

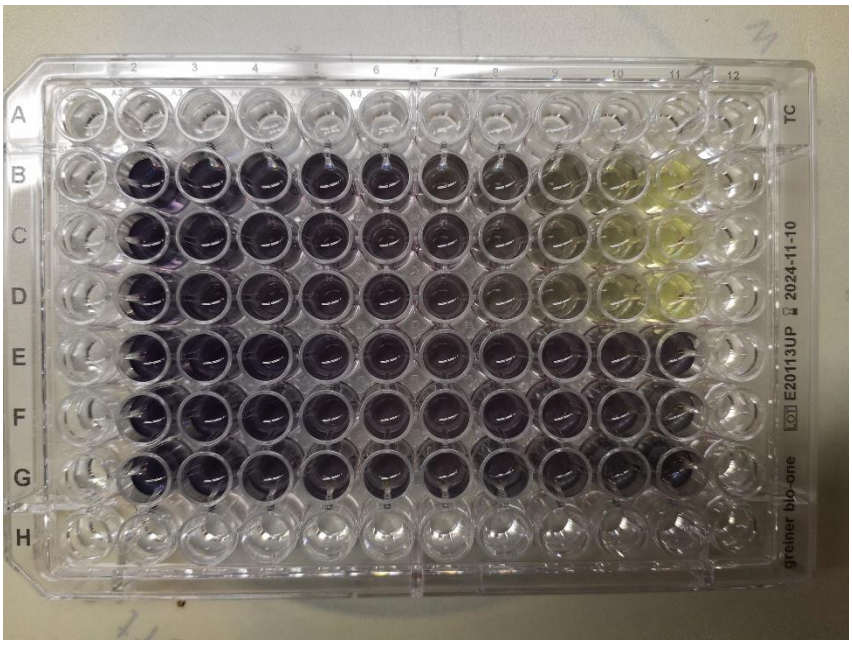

Figure 2-2: A sample for MTT assay. Rows B-D: 5000 cells seeded in each well treated by different concentration of drug in each column. Three replicates are set within each column. Rows E-G: 5000 cells seeded in each well without drug treatment. The darker the color in each well, the more formazan crystals are yielded. The last column from row B to row D shows that there is nearly no formazan formed.

be carefully done prior to addition of MTT, since the colored medium can cause difference in absorbance after use of spectrophotometer. The culture plate is then placed into incubator for around 4 hours, which offers time for reaction of MTT to happen. After that, SDS mixture (0.1 g/mL SDS powder in 0.01 M HCl) is added to each well for solubilization of formazan crystals produced by reaction in last step. The final sample needs to be incubated at least 4 hours for complete solubilization.

The last step of MTT assay is to perform a spectrophotometry detection of the sample. The density of formazan can be measured by the SpectraMax microplate reader (Thermo Scientific, Mississauga, ON, Canada) at the absorbance of 570 nm since the optical density (OD) is directly proportional to the number of dissolved formazan crystals. Therefore, cell viability or drug

cytotoxicity can be determined by the absorbance value of treated wells compared with that of corresponding untreated wells. The calculation can be presented by:

$$\text{Survival rate} = [\text{OD(treatment group)}/\text{OD(control group)}] \times 100\%$$

Error bars for MTT assay in final diagrams present the standard deviation of all replicates within same concentration.

It is necessary to notice that adherent cells are mainly used for MTT assay since there is a medium removal step in the experimental procedure. For suspension cells, other protocol should be considered.

2.3 Cell Lines

As mentioned in previous chapter, both pancreatic cancer and TNBC severely endanger human life. Hence, two pancreatic cancer cell lines (PANC-1 and BXPC-3 from ATCC) and one TNBC cell line (MDA-MB-231 from ATCC) were mainly investigated. **Figure 2-3** shows micrographs of different cell lines. The difference in cell characteristics and morphology can be clearly seen. PANC-1 and BXPC-3 are the frequently used cell lines of pancreatic cancer. PANC-1 is pancreatic ductal carcinoma which exhibits poor differentiation but strong ability to metastasize, while BXPC-3 is pancreatic adenocarcinoma which does not have the ability to metastasize and exhibits moderate to poor differentiation (Kim, Han, Min, Jin, Yi, & Kim, 2014). PANC-1 possesses relatively higher motility than BXPC-3, and PANC-1 cells tend to individually

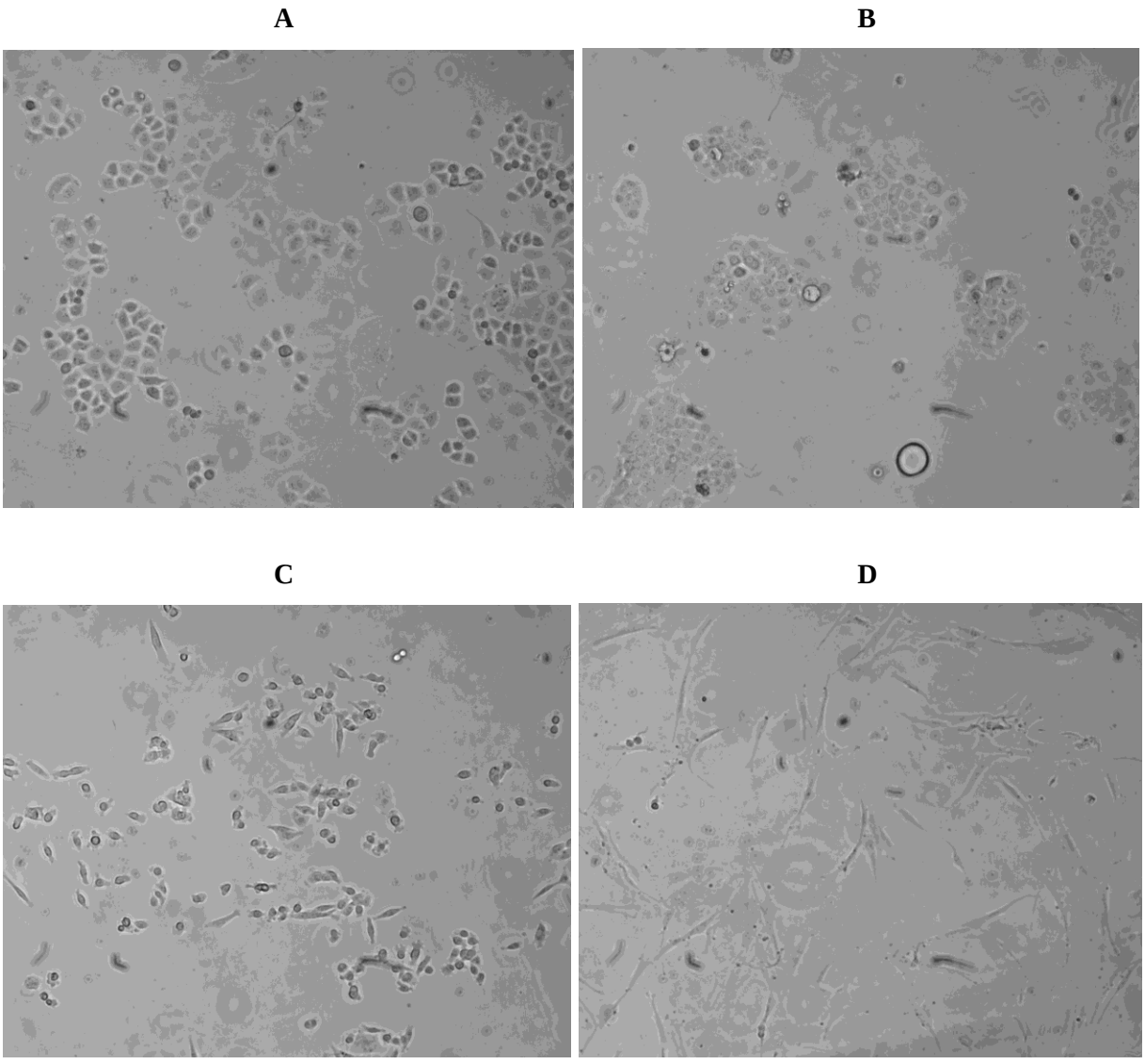

Figure 2-3: Micrographs of different cell lines used in experiments. A: PANC-1 (pancreatic cancer); B: BXPC-3 (pancreatic cancer); C: MDA-MB-231 (TNBC); D: GM05757 (normal cells)

live in culture plates, whereas existence of BXPC-3 cells is usually found in various clusters as shown in **Figure 2-3**. These pictures are consistent with the documented images in the ATCC. MDA-MB-231 is one of the most commonly used cell lines in studying TNBC. It has strong aggressiveness and invasiveness, and it is poorly differentiated. It displays endothelia-like morphology (Harrell et al., 2014) and possesses stellate projections which can usually bridge adjacent cell colonies (Kenny et al., 2007). GM05757 is a normal fibroblast cell line that is particularly susceptible to cytotoxic drug. It is widely used in various research for examination of drug toxicity to normal cells in comparison to multiple cancer cells (Ito et al., 2009; Meng et al., 2005; Khosravifarsani et al., 2019).

All the cell lines were stored in a liquid nitrogen tank. Cells taken from liquid nitrogen tanks were thawed and then performed at least two passages before use in any experiments in order for obtaining more reliable results. PANC-1 and BXPC-3 were cultured in the medium mainly with Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640. The complete growth medium (directly used in culture) was prepared by adding 10% fetal bovine serum (FBS) for nutrients supplement and 1% Penicillin-Streptomycin (PS) for protection from contamination into corresponding base medium. Both PANC-1 and BXPC-3 were cultured in the water-contained incubator with 5% carbon dioxide and 95% air. The base medium for MDA-MB-231 was Leibovitz's L-15. In order for culture use, L-15 medium additionally contained 10% FBS. MDA-MB-231 was kept in water-contained incubator with 100% air. GM05757 was cultured using Minimal Essential Medium (MEM) supplemented with 10% FBS and 1% PS, cultured in carbon

dioxide incubator same as PANC-1 and BXPC-3. All cells were kept in the proper incubator at 37 °C and were not taken out except for experimental use to maintain their cellular activity.

2.4 Experimental Results

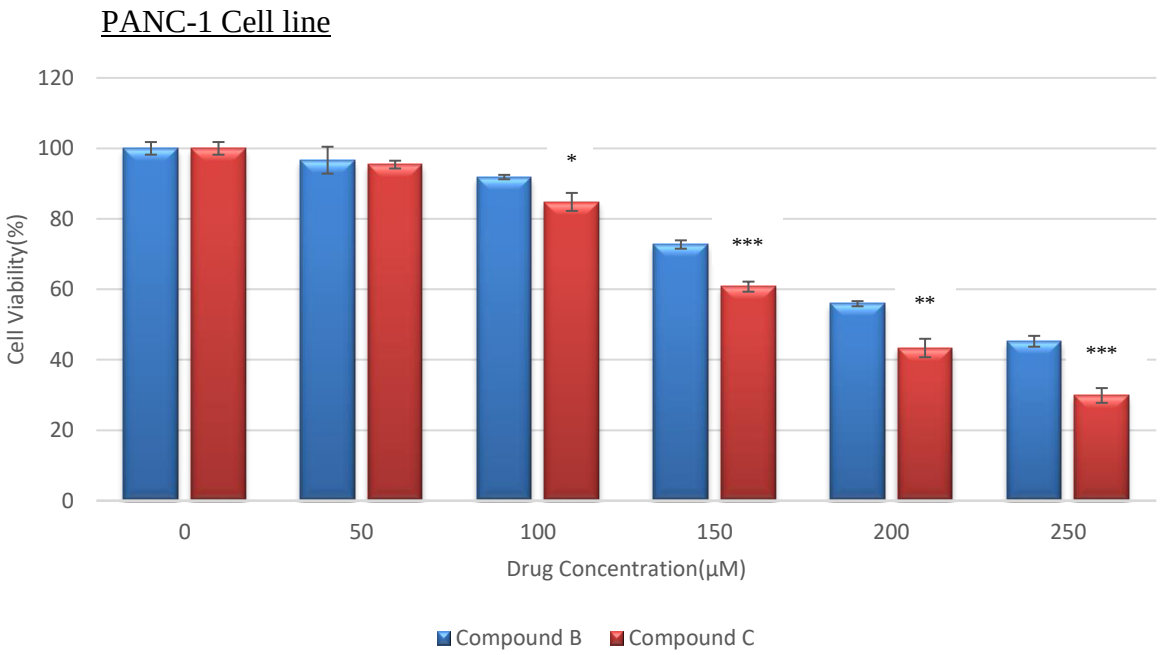

Figure 2-4: Effect of compound B and compound C on PANC-1 cell line examined through MTT assay (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

Human pancreatic cancer cell line (PANC-1) was treated by compound B and compound C with the concentration from 0 μM to 250 μM. As shown in **Figure 2-4**, the cell viability gradually decreases from 100% to slightly over 40% as the concentration increases when treated by compound B, whereas cell viability drops to around 30% at the 250 μM concentration of compound C. In comparison, both drugs reveal cytotoxicity to PANC-1 cells and show minimum difference

within 50 μM concentration, while compound C exhibits significantly higher efficiency in killing PANC-1 cells and overmatches compound B at higher concentration.

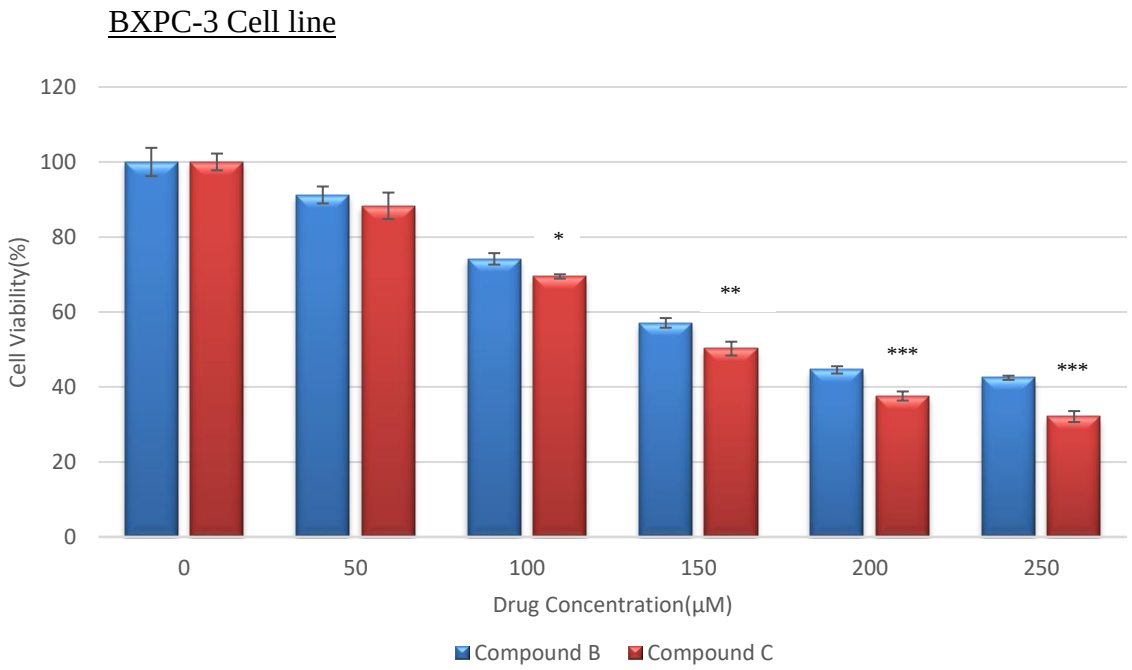

Figure 2-5: Effect of compound B and compound C on BXPC-3 cell line examined through MTT assay (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

Figure 2-5 shows the result of MTT assay on another pancreatic cancer cell line: BXPC-3. At the concentration of 250 μM, compound B kills around 60% cells, while more than 70% BXPC-3 cells can be eliminated by compound C. Compared with PANC-1, BXPC-3 cells are more susceptible to both drugs at lower concentration. On the other hand, the overall killing efficiency remains high since most BXPC-3 cells die after treatment, especially for treatment using compound C.

MDA-MB-231 Cell line

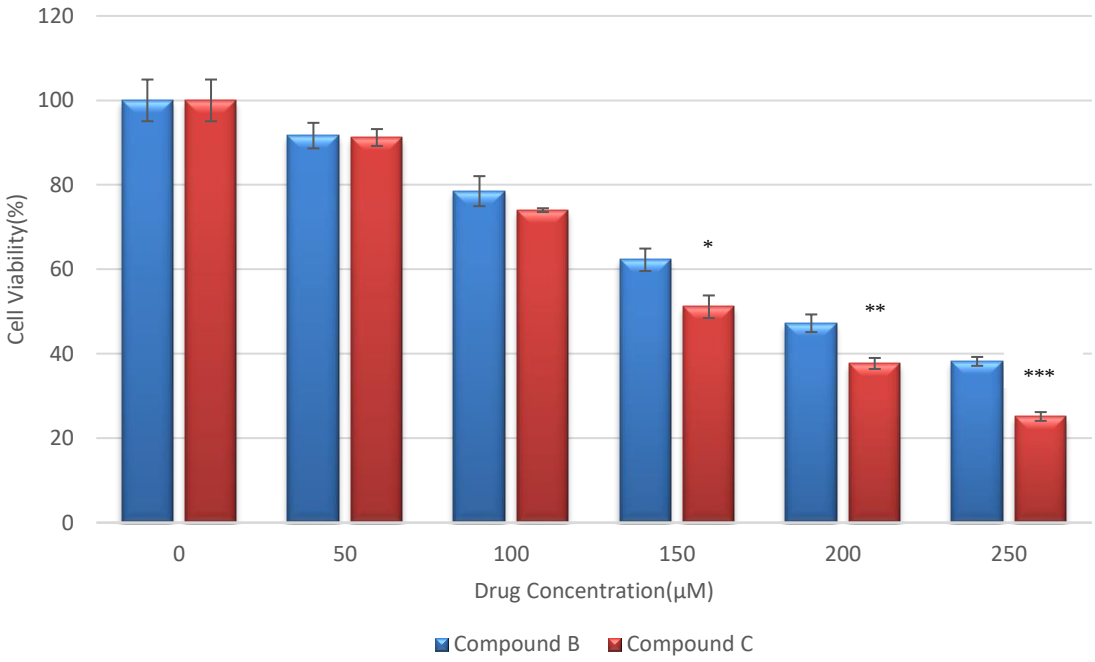

Figure 2-6: Effect of compound B and compound C on MDA-MB-231 cell line examined through MTT assay (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

A human breast cancer (TNBC) cell line MDA-MB-231 was also investigated through MTT assay (**Figure 2-6**). With treatment of compound B, the survival rate decreases from 100% to 40%; with treatment of compound C, the survival rate drops below 25%. Similar to pancreatic cancer cell lines, compound B effectively kill MDA-MB-231 cells, while effect of compound C is even better. At the concentration of 50 μM, compound B exhibits similar killing efficiency but surpassed by compound C at higher concentration.

GM05757 Cell line

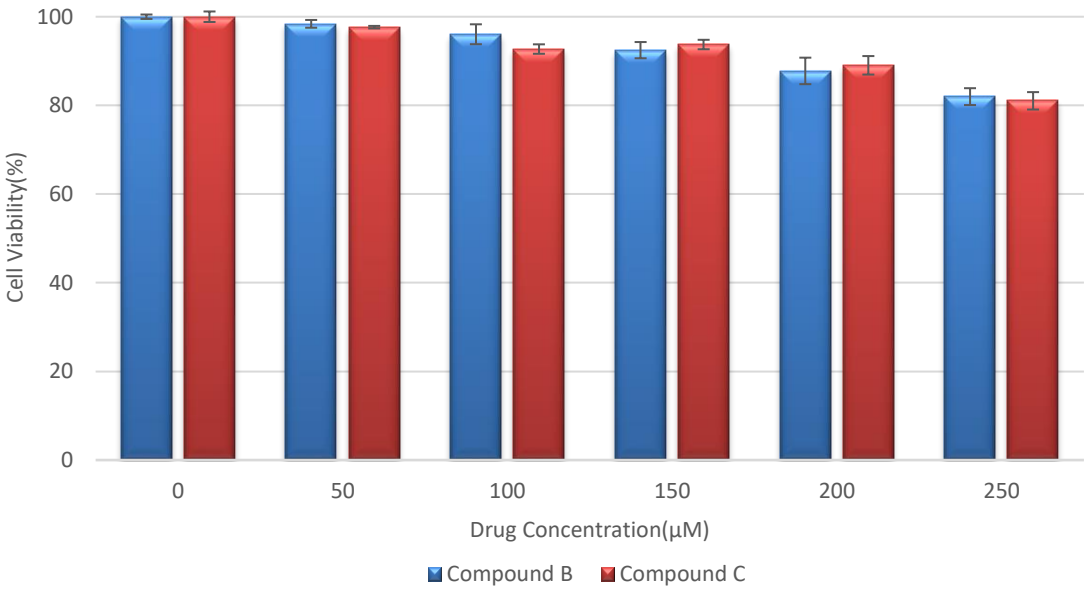

Figure 2-7: Effect of compound B and compound C on GM05757 cell line examined through MTT assay

Besides cancer cell lines, a normal human fibroblast cell line (GM05757) was treated by various concentration of FMD compounds similarly. However, the result is completely different. The increase of drug concentration shows little impact to the normal fibroblast cells. At the highest concentration applied to this experiment (250 μM), cell viability remains higher than 80%. The result demonstrates that GM05757 is resistant to both compound B and compound C. In addition, difference between the impact of compound B and compound C is insignificant, which indicates that both drugs exhibit similar level of cytotoxicity to normal cells

2.5 Conclusion

MTT assay is a fast and convenient way to examine cell viability and drug cytotoxicity. In this chapter, two representative pancreatic cell lines (PANC-1 & BXPC-3), one TNBC cell line (MDA-MB-231) are evaluated and compared with the human normal fibroblast cell line (GM05757). The results show that cell viability for all 3 cancer cell lines significantly decreases when drug concentration increases, whereas normal cells remain their viability even at high concentration. These results indicate that both drugs exhibit good efficiency in killing both TNBC and pancreatic cancer cells without significant toxic effects to the normal cells. Furthermore, compound C shows a better efficacy to kill cancer cells than compound B, meaning that compound C is a more potent antitumor agent.

However, more tests and assays are needed before drawing a final conclusion. In next chapter, clonogenic assay will be introduced, and the result of clonogenic assay on different cell lines will be discussed in detail.

Chapter 3

In Vitro Reproductive Examination on FMD through Clonogenic Assay

3.1 Clonogenic Assay

Clonogenic assay was first established by Puck and Marcus (1956) who published a paper describing the method to test the ability of cells for colony formation. Up to now, the clonogenic assay has been used for over 60 years and become a common technique in cell biology (Rafehi et al., 2011). In this chapter, mechanism, procedure, and experimental result of clonogenic assay will be presented. Similarly, pancreatic cell lines (PANC-1 & BXPC-3), TNBC cell line (MDA-MB-231), and normal fibroblast cell line (GM05757) were investigated through clonogenic assay, and their performance will be illustrated.

Clonogenic assay (also called colony formation assay) is an *in vitro* cell survival assay which tests the ability of a single cell to grow into a colony (Franken et al., 2006). It is proposed that a colony defined in this assay needs to consist of at least 50 cells. Other clusters containing less than 50 cells will be neglected since the reproducibility is then considered as limited. It is easily known that cells are dead when they lose their regular activities, or their membranes and cellular structures have been damaged. However, another important characteristic indicating cell death is reproducibility. Cells that no longer divide are classified into dead cells. The loss of reproductive ability is called reproductive death (Stoddart, 2011). Therefore, the clonogenic assay can clearly identify cell death based on the examination of colony formation.

Clonogenic assay is initially the technique to examine cell reproductive death with treatment of ionizing radiation, but it is also used for test of cytotoxic agents (Franken et al., 2006). It is known that cancer cells routinely have the ability to unlimitedly reproduce. This brings necessity to the application of clonogenic assay and makes it widely implemented in cancer study because the feature of cancer cells can be well determined. It is a more clinically relevant assay than the MTT assay. Thus, it is widely used in cancer research, though it is more time-consuming than the MTT assay and typically takes about 2 weeks for each test.

3.2 Cell Line and Preparation

The cytotoxicity and therapeutic efficiency of FMD drugs (compound B & compound C) were evaluated by testing reproductive integrity through clonogenic assay. PANC-1, BXPC-3, MDA-MB-231 were investigated in comparison with GM05757 to examine whether drugs exhibit selective cytotoxicity to cancer cells.

All cell lines were passaged more than twice after being taken out from liquid nitrogen tank until their growth conditions were good for experimental use. Culture media and incubation methods were the same as those described in the previous chapter.

3.3 Experimental Procedure

The procedure of clonogenic assay includes initial handling of cells, seeding, treating, observation (optional), staining (fixing), and counting (**Figure 3-1**).

Cells were first rinsed by PBS and then harvested through trypsinization. Trypsin is potentially toxic to cells so it should be neutralized by growth medium right after cells are detached. After centrifuged, the supernatant was discarded, and the cells were thoroughly mixed in proper growth medium until ready for counting. Cells counting was particularly critical in clonogenic assay since any small error in this step might generate large differences when obtaining the experimental results. Therefore, pipetting up and down were necessary to get homogeneous cell suspension.

Instead of 96-well culture plates, dishes with appropriate size were used since the dishes offered more space for cells to grow individually. Cell suspension was diluted into desired seeding concentration, and cells were evenly seeded on dishes. Again, at least 3 replicates were set for each drug concentration.

Similarly, treatment was made after 1-day incubation when cells attached to dishes but had not started to replicate. Since clonogenic assay is very sensitive, and it mimics the body metabolism after taking drugs, so the drug should be removed in a short time, typically within 1 day. After that, the cells were maintained in fresh growth medium for conventionally one to three weeks giving time for the single cell to grow into sufficiently large colony. In this project, 14 days of incubation time was implemented empirically. During the incubation time, cells can be observed

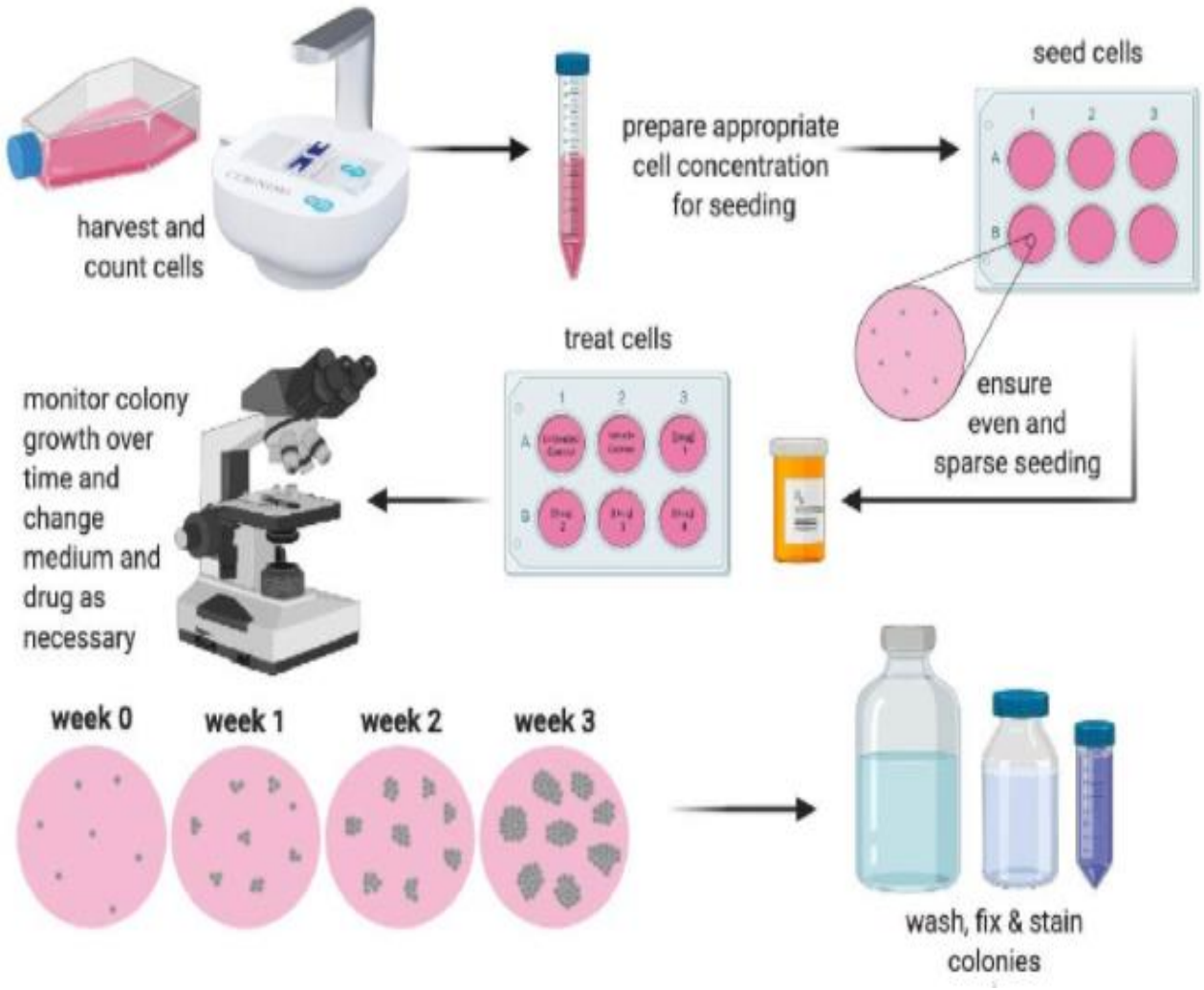

Figure 3-1: A summary of the experimental procedure of clonogenic assay. Cells are harvested, seeded, treated, fixed, stained, and counted (Jenna, 2020)

using microscope to ensure nothing abnormal exists. If the number of cells seeded in each dish was large, regular removal of medium and addition of fresh one might be required.

After 14-days incubation, medium in dishes were removed, and all dishes were carefully washed using PBS. Then PBS was removed, and 2 mL of the mixture of 6% glutaraldehyde (fixative) and 0.5% crystal violet (staining material) was added to dishes. In order for complete staining, at least 30 minutes was needed before removal of the crystal violet mixture. Then stained colonies were carefully rinsed using tap water, and all dishes were placed in air at room temperature until they became dry.

The final step of clonogenic assay was counting. All colonies within each dish were counted and recorded. Normally, cell clusters with less than 50 cells can be distinguished from other colonies by their size. However, it is sometimes hard to determine by naked eyes. In this case, microscope can be used to observe those cell clusters. When cell counting was finished, the first step for analysis was to calculate the plating efficiency (PE). PE is the fraction of colonies formed without treatment. PE should be determined for each experiment, since it may be influenced by any small changes. The surviving fraction (SF) was then calculated by the fraction of colonies formed with treatment divided by PE. The results were plotted with surviving fraction on y-axis and drug concentration on x-axis.

$$PE = \frac{No.\,of\ clonoies\ formed\ without\ treatment}{No.\,of\ cells\ seeded} * 100\%$$

$$SF = \frac{No.of\ clonoies\ formed\ after\ treatment}{No.of\ cells\ seeded * PE} * 100\%$$

In general, clonogenic assay needs to be done couples of times to determine the optimal cell number seeded for each drug concentration. If the number of cells seeded is too small, error will be large; if the number of cells seeded is too large, different colonies may bridge each other which makes trouble for cell counting. Since there are at least 3 replicates for each drug concentration, PE and SF are obtained by averaging the fractions within each concentration, and thus the error bar indicates the standard deviation of these fractions.

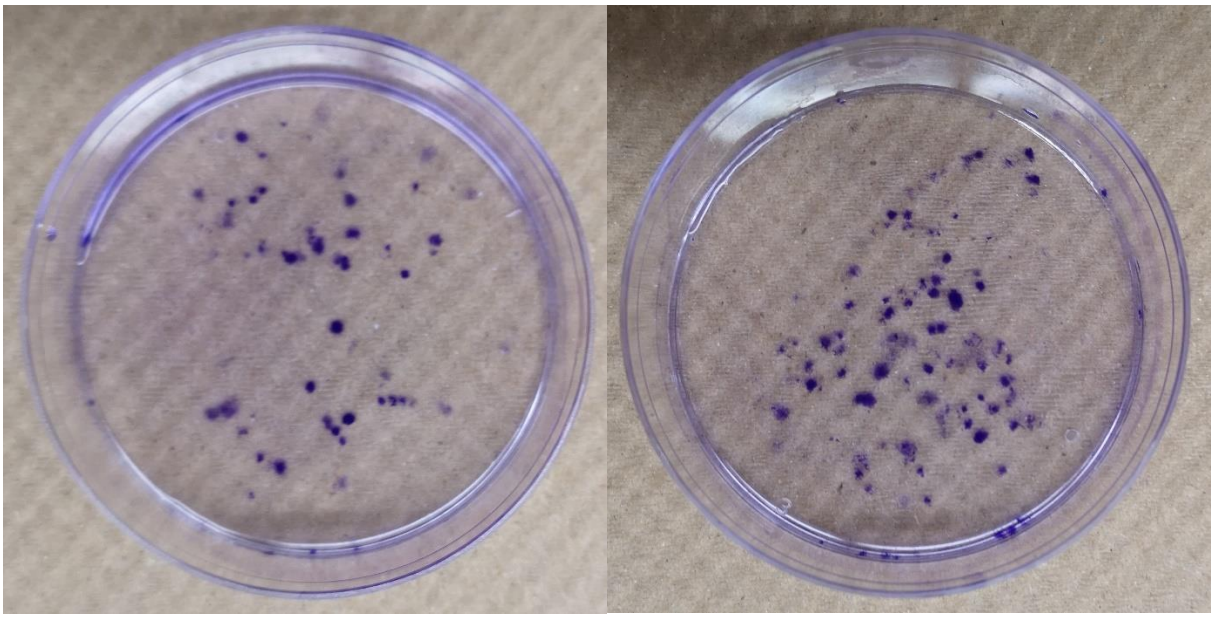

Figure 3-2: Two sample dishes with colonies fixed and stained. Left: lower density; Right: higher density

3.4 Experimental Results

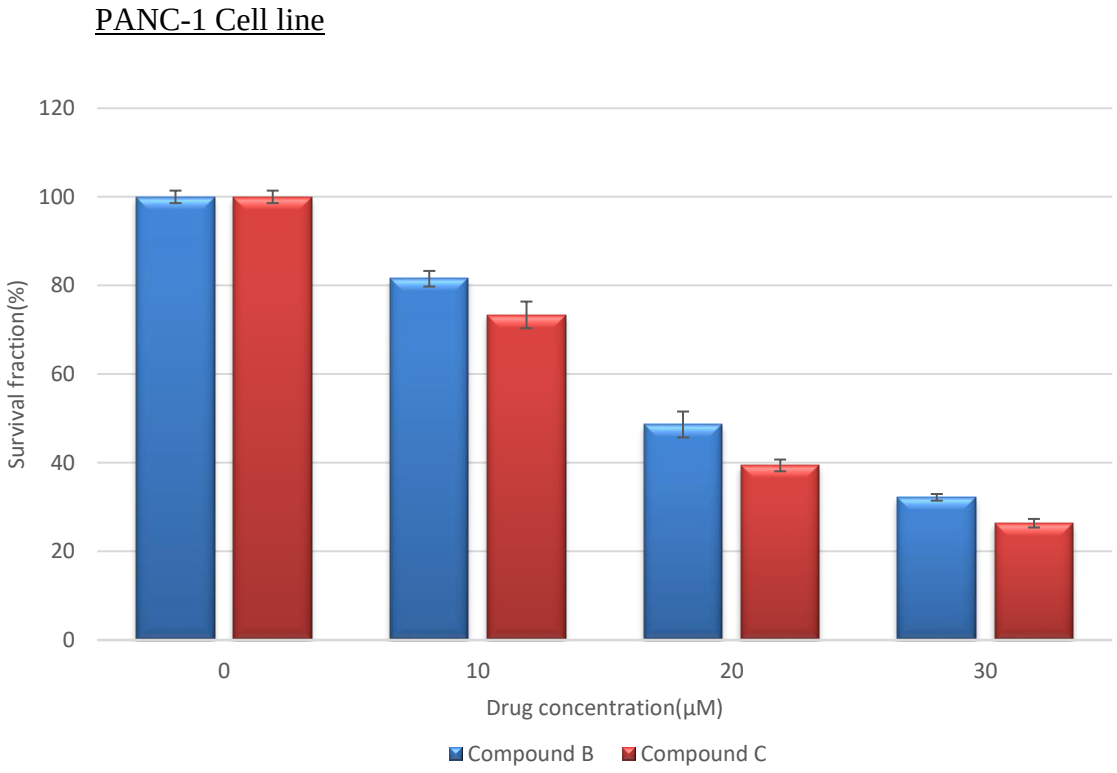

Figure 3-3: Effect of compound B and compound C on PANC-1 cell line examined through clonogenic assay

PANC-1 cells were treated by compound B and compound C with the concentration from 0 μM to 30 μM. The drug concentration of clonogenic assay is significantly smaller than that of MTT assay because clonogenic assay is more sensitive. Single cell in each dish is very susceptible to drugs even at low concentration. As shown in **Figure 3-3**, both compounds have the ability to limit PANC-1 cells proliferation. At 30 μM, less than 40% cells are able to form colonies after treatment of compound B, while compound C restricts the survival fraction of PANC-1 cells to

around 25%. Additionally, the limitation to reproducibility of PANC-1 cells exerted by compound C is about 10% higher than that of compound B.

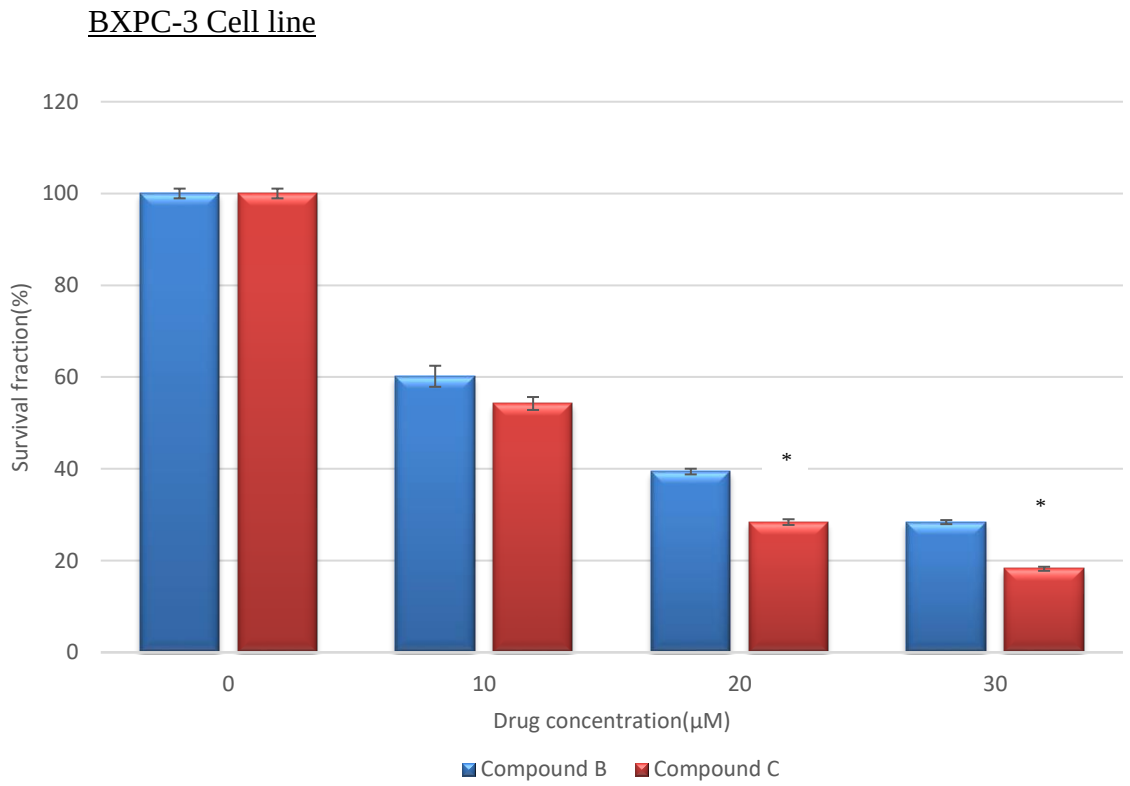

Figure 3-4: Effect of compound B and compound C on BXPC-3 cell line examined through clonogenic assay (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

It is also found that both drugs have strong influence on survival fraction of BXPC-3 cells. At 10 μM, the survival fraction already decreases to below 60% showing that BXPC-3 cells are particularly susceptible to FMD compounds even at extremely low concentration. When the drug concentration increases to 30 μM, the proliferation rate keeps decreasing. This result is consistent

with result of MTT assay which indicates that a relatively low concentration of FMD compounds may even exhibit high efficiency against BXPC-3 cell line compared with PANC-1.

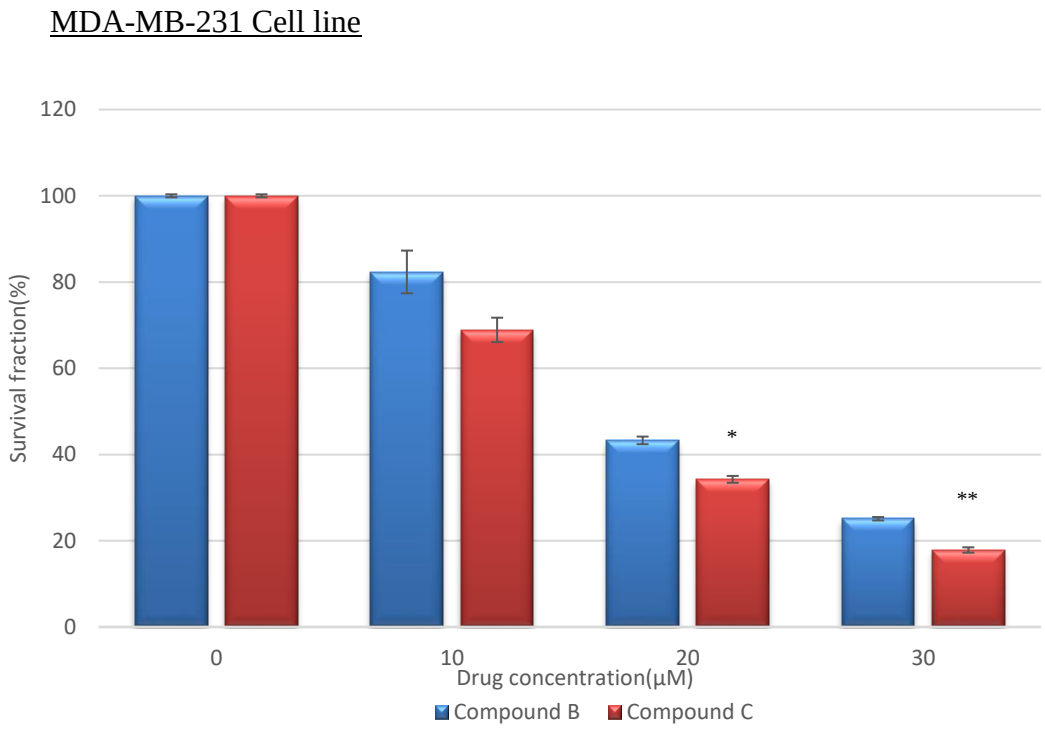

Figure 3-5: Effect of compound B and compound C on MDA-MB-231 cell line examined through clonogenic assay (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

Figure 3-5 shows the effect of FMD compounds on MDA-MD-231 cells. Similarly, both drugs can efficiently reduce the reproducibility to around 20%. Furthermore, compound C is about 10% more effective.

GM05757 Cell line

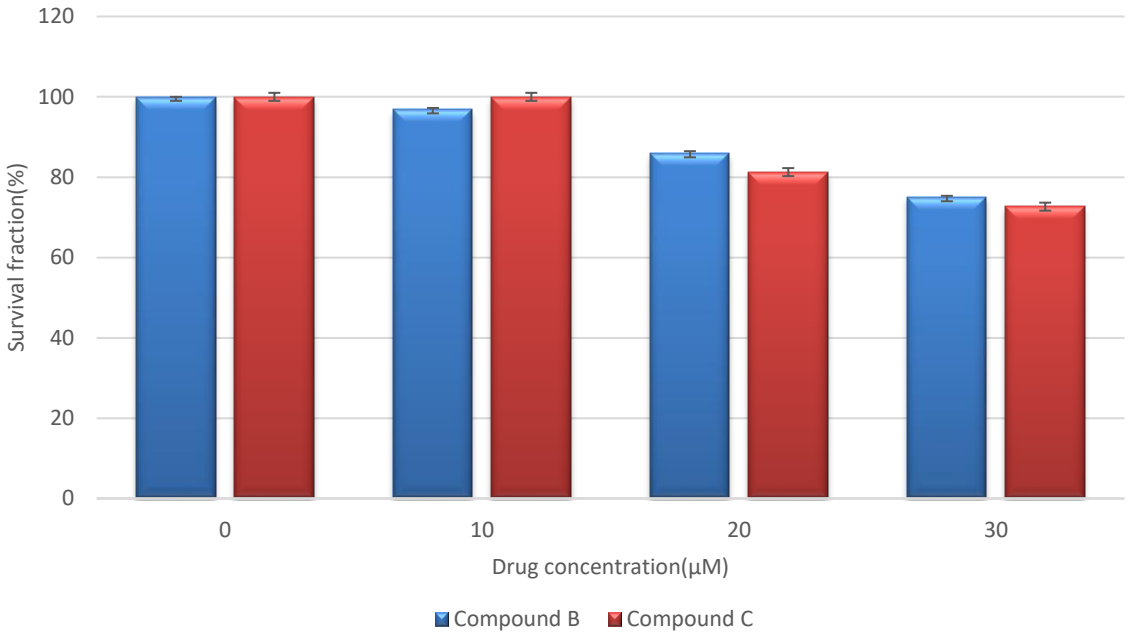

Figure 3-6: Effect of compound B and compound C on GM05757 cell line examined through clonogenic assay

Different from pancreatic and TNBC cells, normal fibroblast cell (GM05757) is much less susceptible to compound B and compound C. At 10 μM, GM05757 almost remains its proliferation rate the same as that of the control group. Even at 30 μM, the survival fraction is more than 75% for both compounds. In addition, the impact exerted by compound B is similar to the impact of compound C towards the normal cells.

3.5 Conclusion

Clonogenic assay is a superior technique in examining reproducibility by determining the colony-forming ability. Pancreatic cell lines (PANC-1 & BXPC-3), TNBC cell line (MDA-MB-231), and normal fibroblast cell line (GM05757) were investigated through clonogenic assay in this chapter. While both compound B and compound C generate good efficiencies in limiting reproducibility of all 3 kinds of cancer cells, they cause much smaller effectson the normal cells. Again, when comparing compound B with compound C, the overall efficiency of compound C is higher, which is consistent with the result obtained through MTT assay.

Although great advantage can be achieved, clonogenic assay is more complex and time-consuming than MTT assay. Therefore, the combined use of both assays will generate more useful results, which are also more clinically relevant.

In next chapter, caspase-3/7 green detection technique will be introduced, which focuses on study of cell apoptosis and morphology when chemotherapeutic treatment is applied.

Chapter 4

In Vitro Apoptosis Measurement through Caspase-3/7 Green Detection

4.1 Caspase-3/7 Green Detection

Caspase-3/7 green detection is a useful technique to provide nuclear morphology and measure caspase-3/7 activity that is closely relevant to cell apoptosis. The apoptotic cells can be observed using the fluorescent microscope, and images can be taken using the software NIS-Elements. Cell lines used for this examination include PANC-1, BXPC-3, MDA-MB-231, and GM05757. In this chapter, principle and procedure of caspase-3/7 green detection as well as the obtained images and analysis will be described.

Apoptosis (programmed cell death) is a specific regulated process of cell death which plays a crucial role in many regular processes ranging from fetal development to immune system regulation (Carrasco, Stamm, & Patel, 2003). Apoptosis can be morphologically identified by chromatin condensation, nuclear fragmentation, and loss of mitochondrial inner transmembrane potential. Consequently, these apoptotic cells break into membrane-surrounded fragments which will be cleaned by phagocytosis (Green & Reed, 1998). After caspases are activated, the recognition sites are normally marked by 3 to 4 amino acids followed by an aspartic acid residue, with occurrence of cleavage after the aspartate (Alnemri, 1997; Cryns & Yuan, 1998). It is suggested that cell deaths caused by many chemotherapeutic drugs are due to apoptotic modulation, which brings interest in apoptosis study for researchers (Lopez & Tait, 2015).

Caspase-3/7 green detection is based on the reagent, a fluorogenic substrate, provided by CellEvent™. This reagent contains a conjugation of nucleic acid binding dye with a four amino acid peptide (DEVD). Initially before the activation of caspases, the reagent is non-fluorescent because the peptide hinders the dye's ability to bind to DNA. However, when cells are modulated to become apoptotic, caspase 3 and 7 will be activated, and they can cleave the DEVD peptide and release the dye. Consequently, the dye is free and able to bind to DNA generating bright fluorescence. Therefore, fluorescence of apoptotic cells can be observed using the microscope (at excitation/emission maximum of 502/530 nm) and fluorescence images can be obtained by the NIS-Elements software.

In order to better determine the fraction change of apoptotic cells, all nuclei can be stained by Hoechst 33342 which is generally used to stain live cells. Hoechst 33342 can bind to AT enriched regions of the minor grove in DNA and can be observed by using the fluorescent microscope (with excitation around 350 nm).

With application of caspase-3/7 green detection, apoptotic nuclei are stained green which can be clearly distinguished from other cells. Furthermore, the dyes can help observe nuclear condensation since the normal nuclei are often spherical, whereas shrunken nuclei usually appear in apoptotic cells.

4.2 Experimental Procedure

Caspase-3/7 green detection requires the following steps: seeding, treating, addition of CellEvent™ reagent, and images taking.

Seeding and treating were similar to the steps in MTT assay in chapter two. 5000 cells per well were seeded in 96-well black culture plate. Black culture plate rather than transparent culture plate, however, was used in this assay because indoor lighting was limited which was helpful for obtaining clearer images. Cells were treated by compound B or compound C after they attached to the plate. Then, 48 hours incubation time was applied.

Before adding CellEvent™ reagent, medium with drugs were removed from culture plate. The working solution was prepared by adding proper amount of CellEvent™ reagent into growth medium making the concentration to be 2 to 8 μM. In this experiment, 3 μM was implemented since it was proved to be able to generate bright fluorescence after couples of tests had been done. The working solution was added to each well containing cells. 1 μg/ml of Hoechst 33342 was also added to each well to stain all live cells as a contrast of apoptotic cells, and then the plate was put back into the incubator for half an hour.

During the last minutes of incubation, the mercury arc lamp was turned on for minutes to be ready for later use. The lamp is important because it can offer light source for fluorescence microscopy. When the camera was connected with the microscope, the software NIS-Elements provided by Nikon could be used for images capture. Cell lines treated by different concentrations of drugs were captured as images in order. At least two images per well were taken for later analysis. One was green detection image indicating apoptotic cells taken at excitation wavelength

around 500 nm. The other image was taken at excitation wavelength around 350 nm showing all live cells. Two images were subsequently merged into one image so that the percentage of apoptotic cells were clearly shown.

Note that a long time of image taking process may cause loss of fluorescence, which leads to inaccuracy in image analysis. Hence, the images should be obtained in a fast pace. Although Hoechst 33342 stains cells blue, thy were manually colored as red using NIS-Elements for clear distinction between cells and dark background. Bar plots were obtained based on the percentage change of apoptotic cells. At least 3 replicates for each drug concentration were set, and the error bars indicated standard deviation of the data.

4.3 Experimental Results

Pancreatic cell lines (PANC-1 & BXPC-3), TNBC cell line (MDA-MB-231), and normal fibroblast cell line (GM05757) were investigated through caspase-3/7 green detection. Treatment was given at concentrations of 0 μM, 50 μM, 100 μM, 150 μM of compound B or compound C, respectively. Following images (from **Figure 4-1** to **Figure 4-4**) are representatives obtained by merging the two images stained by CellEvent™ reagent and Hoechst 33342 within each well. Green spots indicate apoptotic cells labelled by green detection reagent, while red spots indicate live cells labelled by Hoechst 33342.

As shown in **Figure 4-1**, apoptotic and live PANC-1 cells can be clearly distinguished. Image A indicates the control group without addition of drugs. There is only one green spot on that image meaning the occurrence of apoptosis is extremely low in the normal condition. In fact, the percentage of apoptotic cells is below 1% without any treatment. Image B-D are treated by compound B, which shows an increment of apoptotic fraction with the increase of drug concentration. On the other hand, image E-G are treated by compound C, which also shows consistent increase but with a higher rate. In image G, there are more than 60% cells become apoptotic demonstrating the high cytotoxicity to PANC-1 cells is exerted through induction of apoptosis.

Green detection of BXPC-3 and MDA-MB-231 is shown in **Figure 4-2** and **Figure 4-3**, respectively. Consistently, with the increase of drug concentration, percentage of apoptotic cells also increases. Furthermore, compound C exhibits higher ability to induce apoptosis.

On the contrary**, Figure 4-4** presents the apoptotic fraction of GM05757 does not change much when treated by different drug concentrations. The images illustrate that apoptosis of the normal cell line is nearly independent of FMD drugs, which proves little cytotoxicity of FMD compounds is exerted to normal cells. Besides, there is no condensation appearing in images of GM05757 cell line, inferring the nuclei of the cells are in good condition.

The bar plots (from **Figure 4-5** to **Figure 4-8**) collect the apoptotic fraction from each image of 4 different cell lines. It is clearly seen that apoptosis induced for all cancer cell lines is significantly higher when treated by compound C than compound B. Whereas there is no such impact to the normal cell line (GM05757). Additionally, the apoptotic fraction for GM05757 remains to be lower than 5% showing that FMD compounds exhibit minimal influence on normal cells.

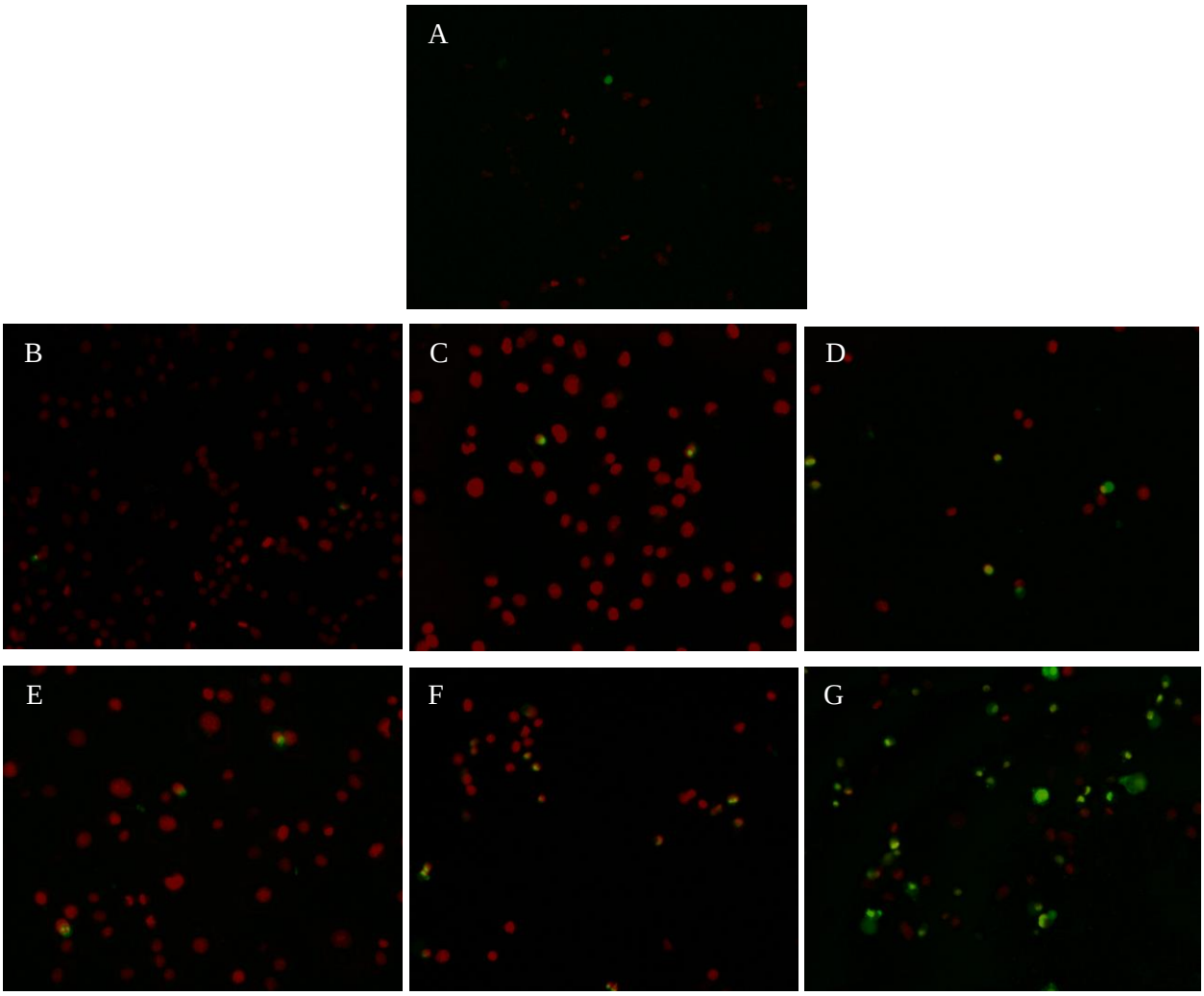

Figure 4-1: Representative merged images for PANC-1 cell line. A: control group; B-D: treated by 50, 100, 150 µM compound B, respectively; E-G: treated by 50, 100, 150 µM compound C, respectively. Green spots indicate apoptotic cells labelled by green detection reagent; red spots indicate live cells labelled by Hoechst 33342

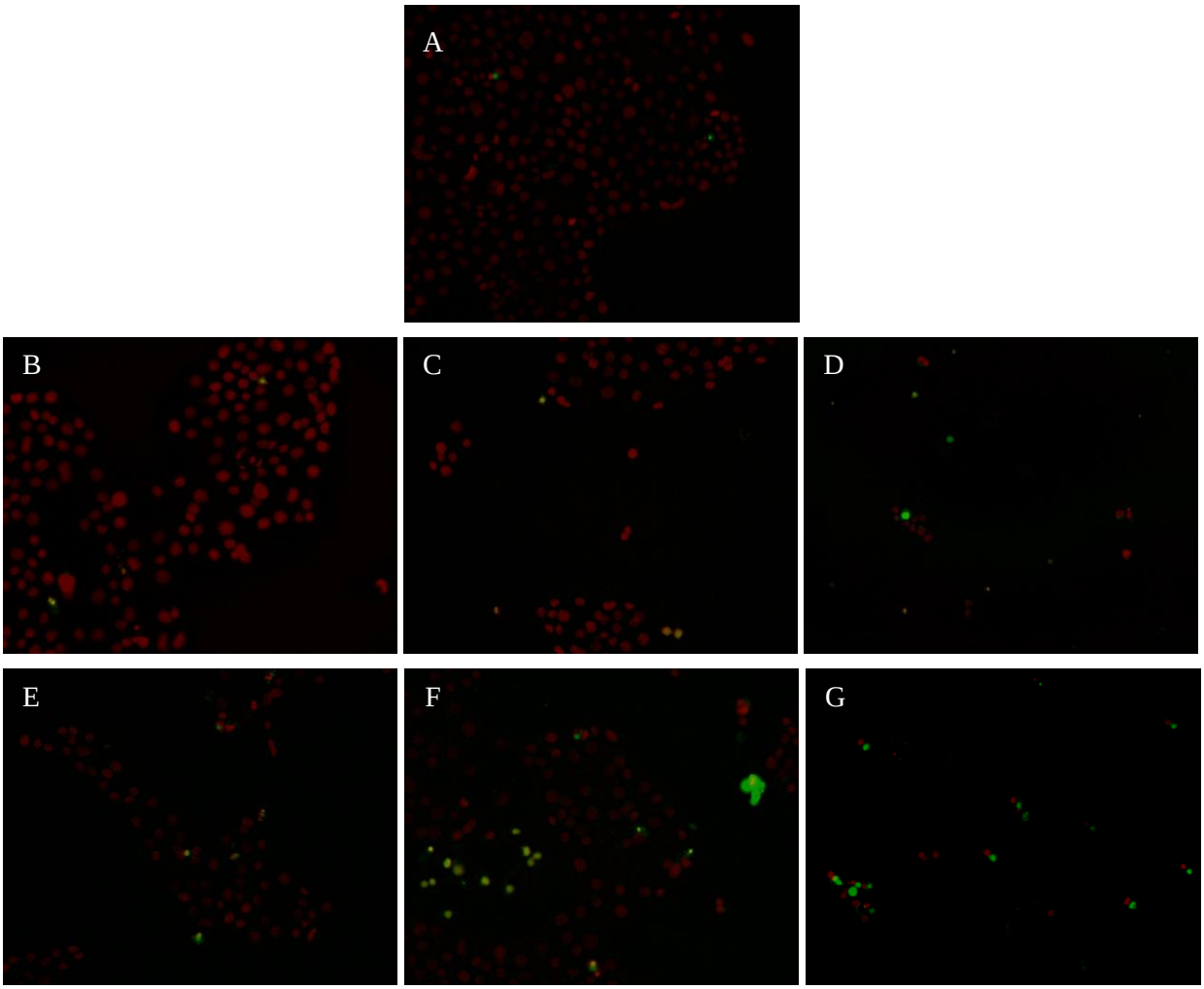

Figure 4-2: Representative merged images for BXPC-3 cell line. A: control group; B-D: treated by 50, 100, 150 μM compound B, respectively; E-G: treated by 50, 100, 150 μM compound C, respectively. Green spots indicate apoptotic cells labelled by green detection reagent; red spots indicate live cells labelled by Hoechst 33342

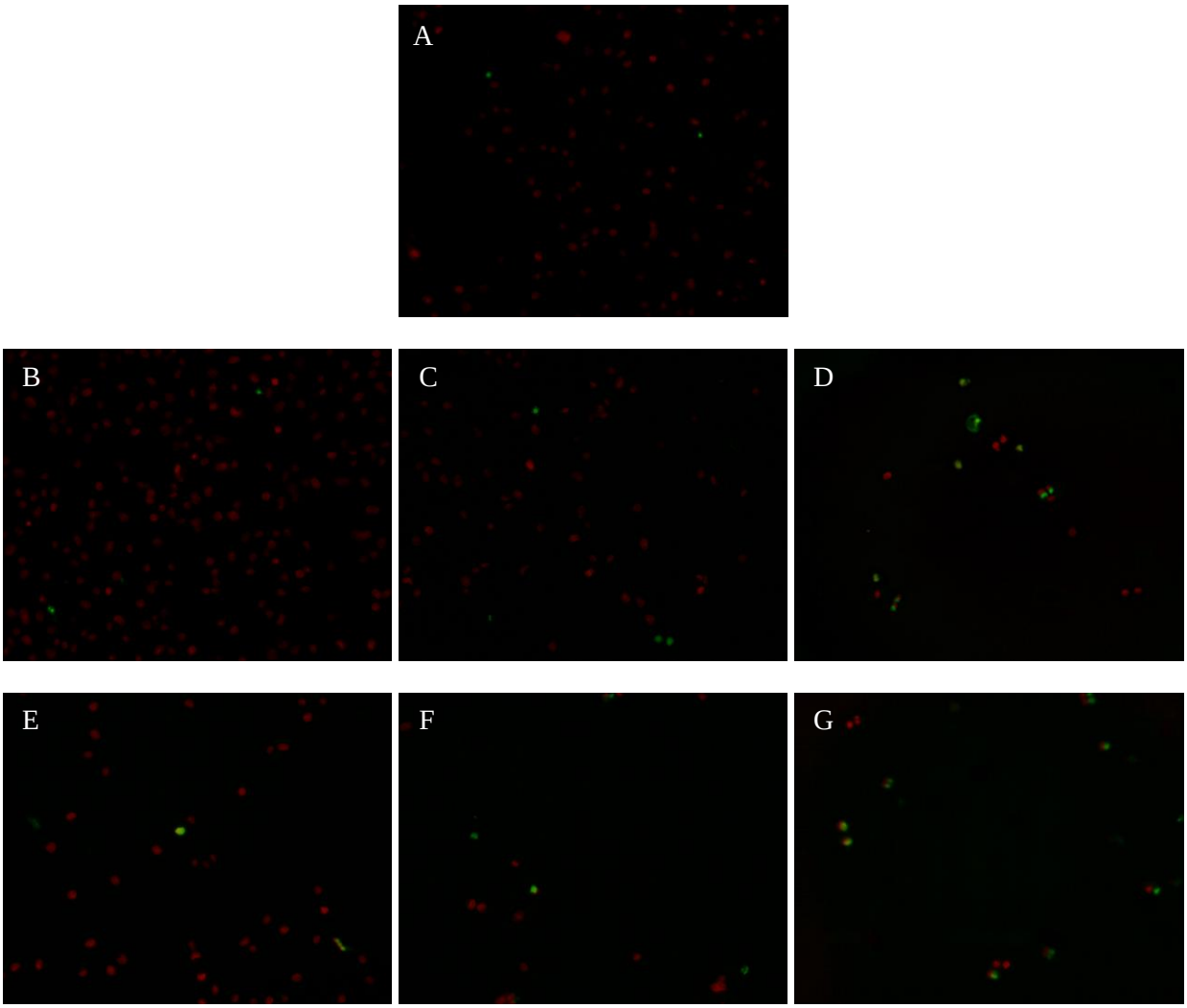

Figure 4-3: Representative merged images for MDA-MB-231 cell line. A: control group; B-D: treated by 50, 100, 150 μM compound B, respectively; E-G: treated by 50, 100, 150 μM compound C, respectively. Green spots indicate apoptotic cells labelled by green detection reagent; red spots indicate live cells labelled by Hoechst 33342

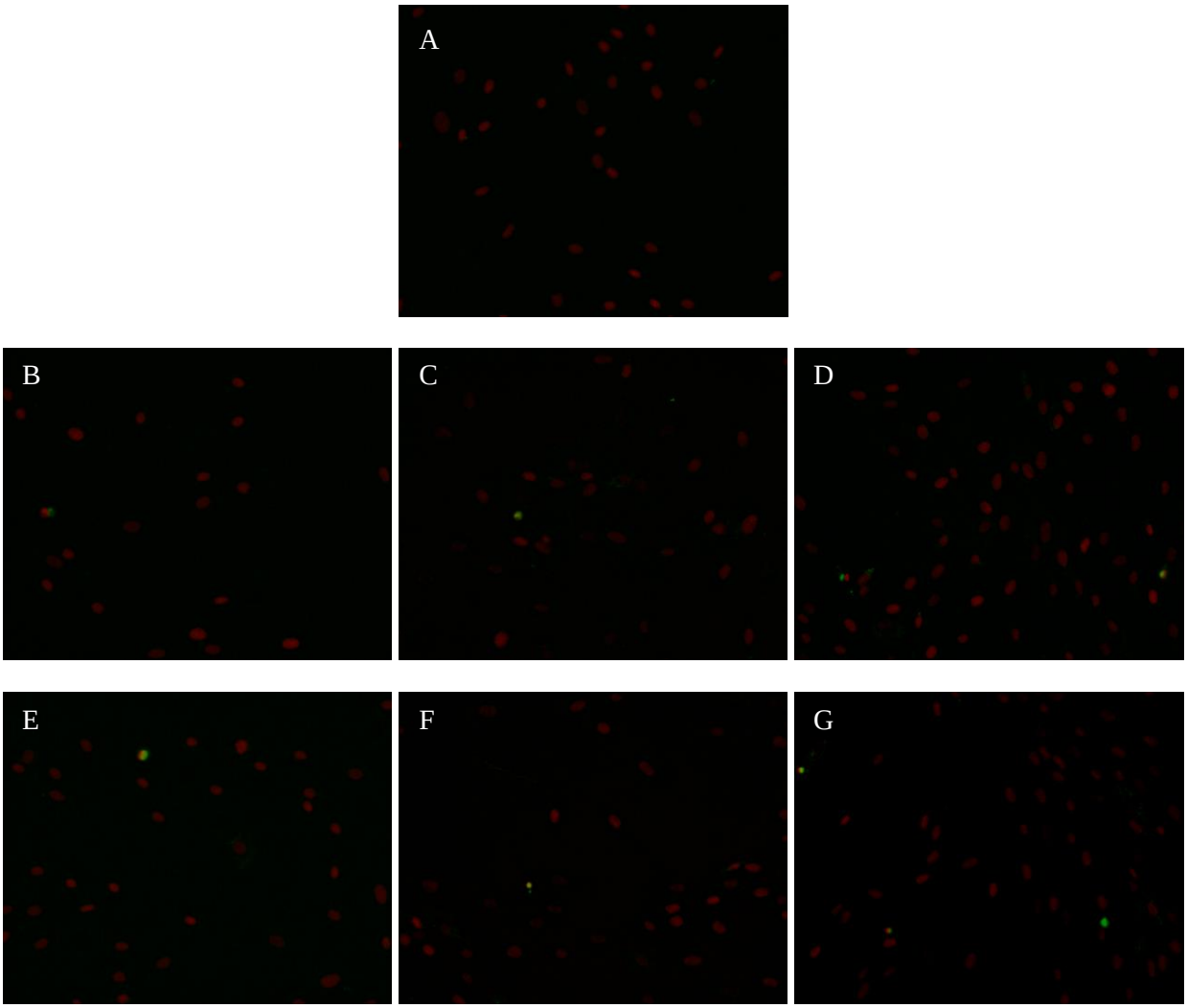

Figure 4-4: Representative merged images for GM05757 cell line. A: control group; B-D: treated by 50, 100, 150 μM compound B, respectively; E-G: treated by 50, 100, 150 μM compound C, respectively. Green spots indicate apoptotic cells labelled by green detection reagent; red spots indicate live cells labelled by Hoechst 33342

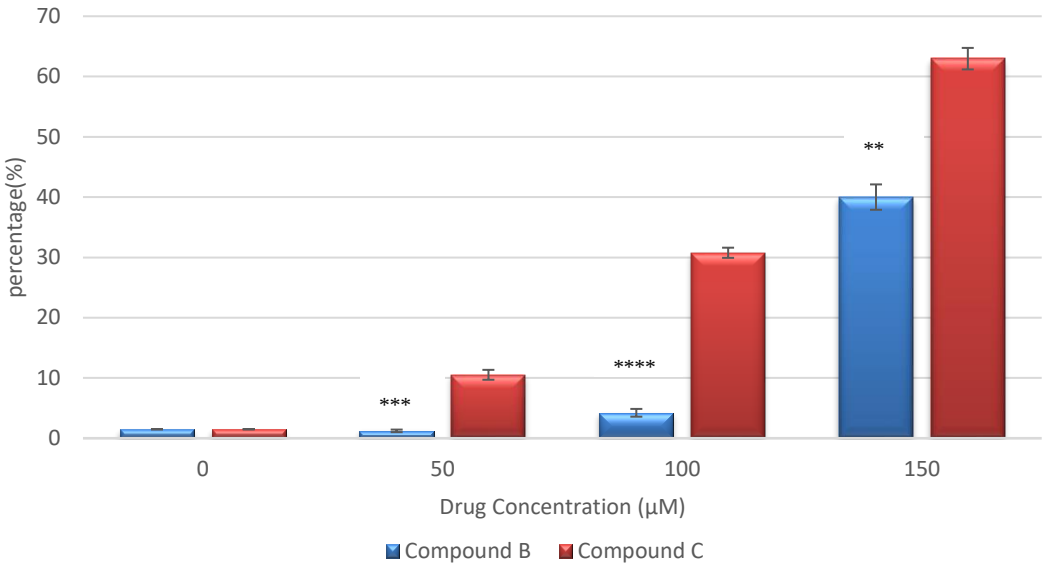

Figure 4-5: Apoptotic fraction of PANC-1 cell line with treatment of compound B or compound C examined through caspase-3/7 green detection (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

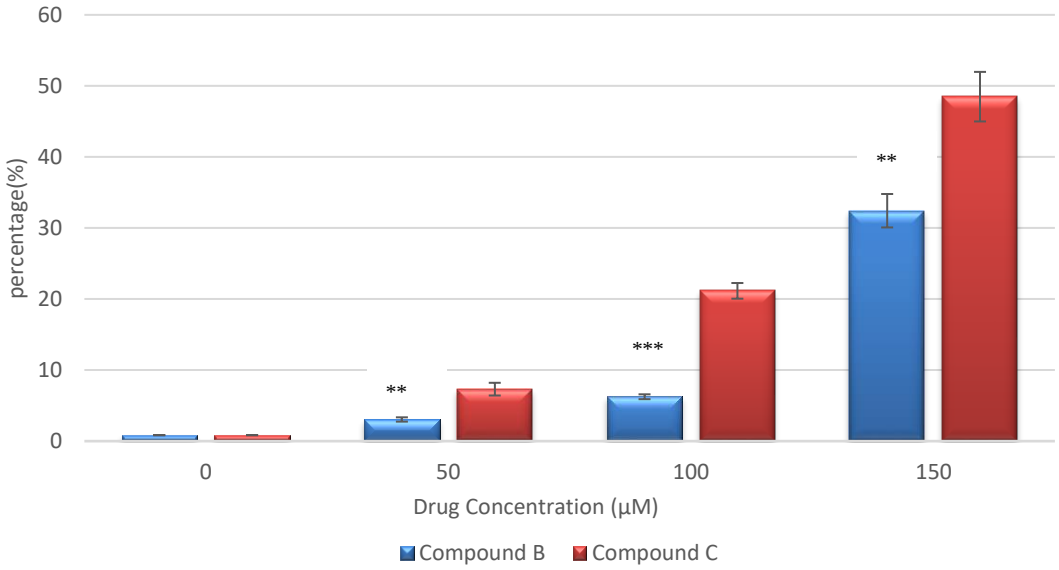

Figure 4-6: Apoptotic fraction of BXPC-3 cell line with treatment of compound B or compound C examined through caspase-3/7 green detection (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

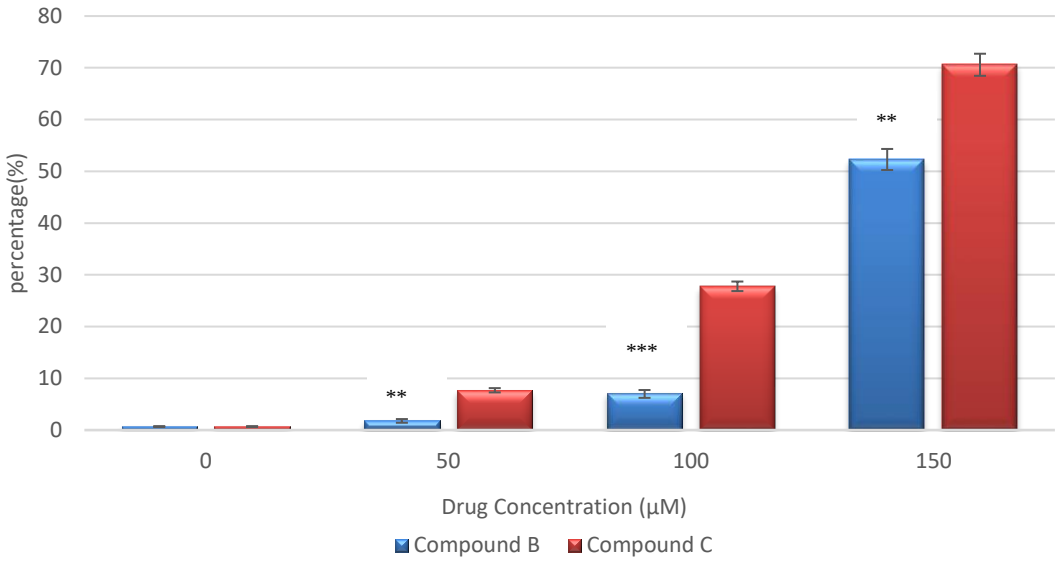

Figure 4-7: Apoptotic fraction of MDA-MB-231 cell line with treatment of compound B or compound C examined through caspase-3/7 green detection (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, obtained from student t tests)

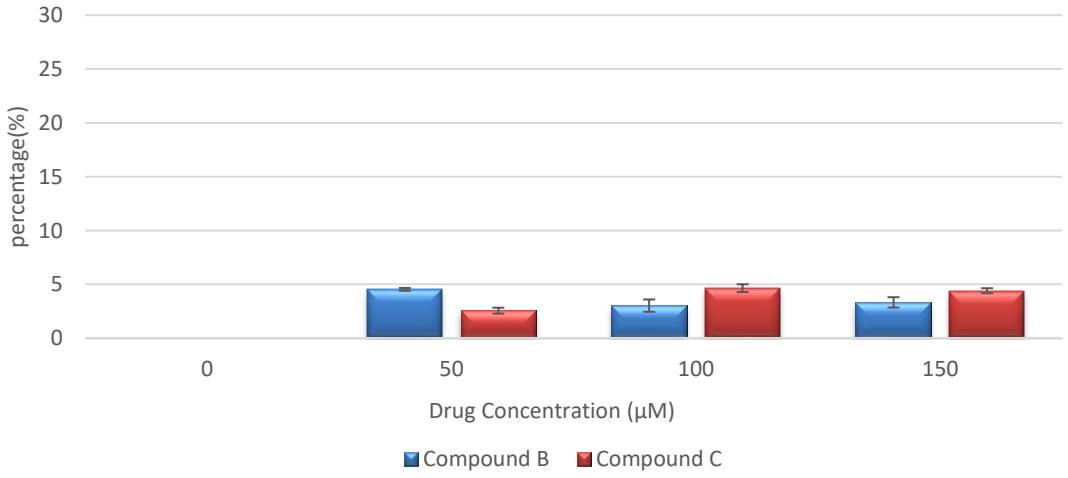

Figure 4-8: Apoptotic fraction of GM05757 cell line with treatment of compound B or compound C examined through caspase-3/7 green detection

4.4 Conclusion

In this chapter, caspase-3/7 activation level of PANC-1, BXPC-3, MDA-MB-231, and GM05757 cell lines is determined through caspase-3/7 green detection technique. The caspases activation caused by cytotoxicity of compound B and compound C can be quantified by the percentage of apoptotic cells.

The result infers that apoptosis can be largely induced by both compounds, while compound C exhibits higher efficiency in this effect. In contrast, minimal impact to the normal cell line GM05757 is seen, verifying that FMD compounds can exert their cytotoxicity mainly to cancer cells, while generate few side effects to normal cells. In addition, shrink of nuclei appears in cancer cells with high drug concentration applied, whereas no existence of nucleic condensation in GM05757 is observed, which offers another evidence that cancer cells are selectively killed by FMD compounds through apoptosis induction.

Chapter 5

Conclusion and Discussion

Cancer is a threatening disease for human's life, which causes more than 10 million death each year. It has become the second leading cause of death over the world and taken the first place in Canada recently. Because of the high mortality rate, cancer has been continuously studied over the past centuries, and many diagnosis modalities and treatments have been proposed and implemented. Although modern techniques are developing, some types of cancer are extremely hard to eliminate, especially for pancreatic cancer and triple-negative breast cancer (TNBC). While TNBC is the most threatening cancer for women, pancreatic cancer can endanger both men and women with only 11% average survival rate. An effective therapy is therefore highly needed for patients suffering from those cancers.

A new family of femtomedicine compounds have been discovered as novel chemotherapeutic targeting agents. This discovery was made on the basis of the mechanistic study of Cisplatin which causes considerable damage to cancer cells based on DET reaction, through an innovative femtomedicine approach. Then FMD compounds were proposed to be more reliable based on their features that they are non-platinum-based, more nucleotide-like for intercalation into DNA, DET reaction supportive, and non-polar for easily crossing the membrane. More importantly, it is known that Cisplatin exhibits severe side effects since it contains platinum, a heavy metal element that can cause nephrotoxicity and other dysfunction. FMD compounds, in contrast, possess an aromatic ring instead of platinum, which are proposed to show minimum cytotoxicity to normal cells.

To evaluate the effect of FMD compounds, Goetze et al. (2019) concluded that one of the FMD compounds, 2Br-DAB (compound B), was tested to be effective in eliminating pancreatic cancer cells, and this compound was not toxic *in vivo*. This brings evidence that FMD compounds can exert the effects same as proposed earlier. However, more study on 2Br-DAB (compound B) is needed. Additionally, efficiency of other FMD compounds in the series and their impact to other types of cancer remained unknown. Hence, in this study, evaluation and comparison of 2Br-DAB (compound B) and 2I-DAB (compound C) are experimentally completed on pancreatic and TNBC cell lines.

The three *in vitro* assays were implemented to evaluate the FMD compounds. The cytotoxicity of these compounds was first examined through the MTT assay. The results show that both pancreatic and TNBC cells were increasingly killed when the treatment dosage was increased, while no significant drug resistance was discovered. Furthermore, compound C exhibited higher efficiency in killing pancreatic and TNBC cells than compound B. In contrast, the minimal impact on the normal cell viability showed FMD compounds cause much less cytotoxicity to normal cells. Clonogenic assay was completed to test the cell reproducibility. The high proliferation capacity of GM05757 inferred that both FMD compounds were safe for use *in vitro*. On the contrary, the reproducibility for all cancer cell lines was significantly decreased, indicating the consistent cytotoxicity effect exerted by FMD compounds. The Caspase-3/7 green detection offers cell morphology and apoptosis analysis. By merging the images (stained live cells and apoptotic cells), the apoptotic fraction was found to be higher when higher concentration of drugs was applied.

Additionally, compound C was shown to be able to induce more apoptosis for cancer cells than compound B, while induced less than 5% apoptosis for GM05757 which was similar to the effect of compound B. Furthermore, condensed nuclei were seen after images were captured for cancer cells, whereas no such feature was found in GM05757. Nucleic condensation is considered as a necessary process during the pathway to apoptosis. Therefore, this further confirmed that the cytotoxicity effect exerted by FMD compounds were resulted from apoptosis induction, which offered a clear explanation on cancer cell death.

Demonstrated on multiple assays conducted in this study, two of the FMD compounds (compound B & compound C) are proved to exert potent antitumor effect on both pancreatic cancer and triple-negative breast cancer (TNBC). Through these *in vitro* tests, compound C has been demonstrated to exhibit higher antitumor efficiency than compound B. Before FMD compounds can be effectively utilized in the clinic, more study is necessary in future research. For example, *in vivo* experimental results are highly needed to examine whether effects of both compounds are consistent with *in vitro* tests. Overall, the discovered targeted chemotherapy using FMD compounds as novel anti-cancer targeting agents could potentially generate a large impact in the clinical treatment of multiple cancers, especially the most hard-to-treat cancers such as pancreatic cancer and TNBC exemplified in this thesis study. Therefore, further preclinical studies and future clinical trials will be of great interest.

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