UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC PROPERTIES OF NOVEL PYRAZOLE DERIVATIVES (TBBB47 & TBB47-A) ON HUMAN BREAST CANCER (MDA-MB-231) AND NORMAL MOUSE EMBRYONIC FIBROBLAST (NIH/3T3) CELLS

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR SCIENCES (BIOMEDICAL SCIENCE)

DEPARTMENT OF BIOMEDICAL SCIENCES
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ABSTRACT

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Introduction: Metastasis, a significant characteristic of malignant tumours, is one of the most life-threatening pathological events in breast cancer. According to GLOBOCAN, breast cancer is the most prevailing type of malignant neoplasms among women with over one million new cases each year. Advanced chemotherapy developed therapy-resistant to major subpopulations in the world. Thus, further development for safer and more effective drugs against breast cancer is needed. In search of synthetic chemotherapeutic drug substances that can inhibit the process of multistage breast cancer, we have investigated the effects of two different novel pyrazole derivatives that potentially have the minimal killing of normal cells by using low dose concentration. The pyrazole urea (GeGe3) has been suggested to be a novel blocker of MAPK and P13K pathways and is implicated in inhibiting physiological and tumor angiogenesis. Herein, we aimed to continue from the previous study to evaluate the cytotoxic properties of TBBB47 and TBB47-A, two novel compounds of pyrazole derivatives from pyrazole urea (GeGe3). **Methodology:** Cell viability and cytotoxic effects of TBBB47 and TBB47-A on MDA-MB-231 and NIH/3T3 cells were measured using MTT (*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*) assay. MDA-MB-231 and NIH/3T3 cells were grown in complete Dulbecco's modified eagle medium and cultured under humidified conditions. **Results:** From MTT assay, TBBB47 and TBB47-A showed no toxic effect at concentrations of 1.56µM, 3.12µM, 6.25µM, 12.5µM, 25µM, 50µM and 100µM after three incubation periods on both cell lines. TBBB47 and TBB47-A showed cytotoxic effects at high concentration (>200µM) for both cell lines. To compare between two cell lines, both compounds showed similar pattern of cytotoxic effects on MDA-MB-231 and NIH/3T3 cells. **Conclusion:** TBBB47 and TBB47-A show cytotoxic effects on human breast cancer (MDA-MB-231) and normal mouse fibroblast cells (NIH/3T3). For the compounds to show cytotoxic effects on human breast cancer (MDA-MB-231) and normal mouse fibroblast cells (NIH/3T3), longer incubation period is required.

Keywords: pyrazole derivatives, cytotoxic, cell viability, MDA-MB-231, NIH/3T3

ABSTRAK

SIFAT SITOTOKSIK TERBITAN PYRAZOLE (TBBB47 & TBB47-A) PADA SEL KANSER PAYUDARA MANUSIA (MDA-MB-231) DAN SEL NORMAL FIBROBLAS EMBRIONIK TIKUS (NIH/3T3)

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Pengenalan: Metastatik merupakan ciri signifikan pada tumor malignan yang merupakan salah satu kejadian patologi yang membahayakan nyawa dalam kanser payudara. Menurut GLOBOCAN, kanser payudara adalah sejenis neoplasma malignan yang paling kerap berlaku di kalangan wanita dengan lebih daripada satu juta kes baharu setiap tahun. Kemajuan dalam kemoterapi telah membawa kepada rintangan terapi terhadap subpopulasi utama di dunia. Oleh itu, pengembangan yang lebih lanjut untuk ubat yang lebih selamat dan berkesan amat diperlukan untuk melawan kanser payudara. Dalam pencarian unsur ubat kemoterapi sintetik yang dapat menghalang peringkat-peringkat kanser payudara, kami telah menyiasat kesan dua terbitan pyrazole baharu yang berpotensi mempunyai ciri-ciri mínima untuk membunuh sel normal dengan menggunakan kepekatan dos yang rendah. Urea pyrazole (GeGe3) telah disyorkan untuk dijadikan sebagai penghalang baru untuk laluan MAPK dan P13K serta terlibat dalam penyekatan fisiologi angiogénesis tumor. Di sini, kami berhasrat untuk menyambung kajian sebelumnya untuk menilai sifat sitotoksik TBBB47 dan TBB47-A, dua sebatian terbitan pyrazole baharu daripada urea pyrazole (GeGe3). **Metodologi:** Kebolehhidupan sel dan kesan sitotoksik TBBB47 dan TBB47-A terhadap sel MDA-MB-231 dan NIH/3T3 diukur dengan menggunakan asai MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromida). Sel MDA-MB-231 dan NIH/3T3 telah dikembangkan dalam medium DMEM dan dikultur dalam keadaan lembap. **Keputusan:** Menurut asai MTT, TBBB47 dan TBB47-A tidak menunjukkan kesan toksik pada kepekatan 1.56μM, 3.12μM, 6.25μM, 12.5μM, 25μM, 50μM dan 100μM setelah tiga tempoh pengeraman pada kedua-dua jenis sel tersebut. Untuk membandingkan antara dua jenis sel, kedua-dua sebatian menunjukkan corak kesan sitotoksik yang serupa pada sel MDA-MB-231 dan NIH/3T3. **Kesimpulan:** TBBB47 dan TBB47-A menunjukkan kesan sitotoksik terhadap sel kanser payudara manusia (MDA-MB-231) dan sel normal fibroblas embrionik tikus (NIH/3T3). Manakala, tempoh pengeraman yang lebih lama diperlukan untuk membenarkan sebatian menunjukkan kesan sitotoksik pada sel kanser payudara manusia (MDA-MB-231) dan sel normal fibroblas embrionik tikus (NIH/3T3).

Kata kunci: terbitan pyrazole, sitotoksik, kebolehhidupan sel, MDA-MB-231, NIH/3T3

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LIST OF ABBREVIATIONS

AJCC	America Joint Committee on Cancer
ANOVA	Analysis of variance
ANGPTL4	Protein angiopoietin- like-4
ATCC	American Type Cell Culture
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine serum albumin
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CDC	Centre of Disease Control
CO2	Carbon dioxide
CTC	Circulating tumor cells
CXCR4	Chemokine receptor 4
CXCL12	Chemokine 12 protein
DCIS	Ductal Carcinoma in situ
DF	Diluent factor
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
ER	Estrogen receptor
ERK	Extracellular receptor kinase
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FDA	Food and Drug Administration
fMLP	Formyl peptide receptor
GeGe3	Pyrazolyl-urea
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
HER-2	Human Epidermal Growth Factor receptor 2
HR	Hazard ratio
IC50	Inhibitory concentration (50)
IL-8	Interleukin-8
LHRH	Luteinizing hormone-releasing hormone
MTT	3-[4,5-dimethlthiazole-2-yl]-2,5-dephenyltetrazolium bromide
MAPK	Mitogen-activated protein kinase
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein
PDL-1	Programmed death-ligand 1
PKC	Protein kinase C
P13K	Phosphoinositide 3-kinase
PIGF	Placental growth factor
PR	Progesterone receptor
RS	Rate survival
RPM	Revolutions per minute
SEER	Surveillance, Epidemiology, and End Results
SEM	Standard Error of Mean
TBBB47	Pyrazole derivatives compound 1
TBB47-A	Pyrazole derivatives compound 2
TNBC	Triple Negative breast cancer
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Background

Over the years, breast cancer is the most frequent malignancies leading to cancer-related deaths in women worldwide . As stated by World Health Organization (WHO), breast cancer accounted for 24% of all cases of cancer and 15% of all cancer-related deaths in 2018, reaching 626,679 deaths in women. Advanced metastasis leads to poor curability is mainly the product of metastatic tumors spreading and infiltrating widely throughout the distant organ, resulting in incomplete resection of the tumor. Despite the tremendous progress in recognizing and treating breast cancer in recent years, it remains a significant health concern and faces major challenges. Treatment involves approaches to the locoregional such as surgery , radiation therapy and systemic therapy required resistance (Harbeck et al, 2019). Eventually, the existing therapies evoke damage to healthy tissues.

Metastasis, an important aspect in malignant tumors, is one of the most life-threatening pathological events (Xiao et al. 2019). One of the pre-requisites for cancer to metastasis is the capability of cells to undergo process of migration and invasion into the surrounding tissues. Antimetastatic therapies not only enhance the regression and invasion of tumors but also have some benefits over traditional chemotherapy. There are a few anticancer drugs currently being used for the treatment of breast cancer and have been approved by FDA. Such drugs inhibit tumor cell proliferation, hinder angiogenesis of tumors or improve immune function but very few of such drugs prevent cancer metastasis; however, certain adverse effects have been associated with their long-term use (Qian, Mei & Zhang, 2017). Additionally, that is not only the case where metastatic tumors are also immune to some cytotoxic agents. A search for a secure and novel antimigration agent is therefore highly justifiable.

Nowadays, pyrazole molecule have received more attention on their pharmacological properties. It consists of a five-membered heterocyclic compound particularly useful in therapeutic activities. Karrouchi et al., (2018) identified molecules of pyrazole as protein glycation inhibitors, anti-tuberculosis, antifungal, anticancer, antidepressant, anti-inflammatory, antibacterial, antioxidant and antiviral agents. Such synthesis of pyrazole-derived gives potent inhibitory effect on MCF-7 cell line, breast carcinoma cells (Karrouchi et al., 2018) . In short, the main aim is to develop a new effective anticancer medication with improved dose selectivity, increased therapeutic index and reduced side effects.

Inspired by literature studies and in continuation from previous effort , the cytotoxicity properties of two novel pyrazole derivatives TBBB47 and TBB47-A on breast cancer cell line, MDA-MB-231 cells were determined. This novel compound of pyrazole derivatives was noted for their antiangiogenic mechanism (Meta et al., 2017). All compounds used in this research were synthesized from the property of pyrazole molecule have; five-membered heterocycle which proved has an anticancer activity and potential to inhibit migration of breast cancer cells (Karrouchi et al., 2018). The cytotoxic event caused by novel pyrazole derivatives compound (TBBB47 and TBB47-A) were studied to investigate if the compounds were able to induce cytotoxic effect on breast cancer (MDA-MB-231) and normal mouse embryonic fibroblast (NIH/3T3) cells. This was accomplished by cell viability testing using methyl-thiazolyl-diphenyl-tetrazolium bromide (MTT) assay.

1.2 Problem Statement

The problem statement for this study are:

- There is no study conducted on TBBB47 and TBB47-A on the human breast cancer (MDA-MB-231) and normal mouse embryonic fibroblast (NIH/3T3) cells.
- Prior to study the antimigration effect on the compound TBBB47 and TBB47-A, cytotoxicity study needs to be carried out.

1.3 Objectives

1.3.1 General Objective

The general objective of this study is to determine cytotoxic properties of TBBB47 and TBB47-A on human breast cancer (MDA-MB-231) and normal mouse fibroblast cells (NIH/3T3).

1.3.2 Specific Objectives

First specific objective is to determine the cytotoxic effect of TBBB47 and TBB47-A on human breast cancer cells (MDA-MB-231) using MTT assay. Secondly, to determine the cytotoxic effect of TBBB47 and TBB47-A on normal mouse embryonic fibroblast (NIH/3T3) using MTT assay. Last but not least, to compare the cytotoxic effect of TBBB47 and TBB47-A between MDA-MB-231 and NIH/3T3 cell lines.

1.4 Hypothesis

Hypothesis of this study is stated hereby GeGe3 novel pyrazole derivatives (TBBB47, TBB47-A) show cytotoxic properties on human breast cancer (MDA-MB-231) and normal mouse embryonic fibroblast (NIH/3T3) cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

2.1.1 Introduction to Breast cancer

Breast cancer is uncontrolled growth of malignant cancer cells at mammary gland. (Lukong, 2017). It is composed of three primary parts which are ducts, lobules and connective tissue. Lobules is the gland that produce the milk. Ducts are the tubes which convey milk to the nipple. Connective tissue comprises of both fibrous and fatty tissue surrounds everything. The type of cells that mostly give rise to breast cancer were originated from lobules and ducts. A limited proportion of breast cancers however grow from fat or fibrous tissue. The major prevalence types of breast cancer are invasive ductal carcinoma where the cancer cells migrate into other areas of the breast tissue out of the duct and invasive lobular carcinoma where cancer cells spread from the lobules to neighboring breast tissues (CDC, 2018) .

As breast cancer starts to migrate to other parts of the body, it is called metastasis. Harbeck et.al (2019) found that majority of driving on alteration of tumour evolution in primary breast cancer are preserved on metastatic sites. During metastasis development, tumour evolution from the primary tumour occur, showing different genetic and leads to rise in epigenetic evolution. On the molecular level, breast cancer can be classified as a heterogenous disease where molecular characterization is derived from activation of human epidermal growth factor-2 positive and negative (HER-2), activation of oestrogen and progesterone receptor, and *BRCA* mutations (Dagogo-Jack & Shaw, 2018).

According to Harbeck et al. 2019, Perou and Sorlie had mentioned that there are four subdivision of breast cancer. Such that are human epidermal growth factor receptor 2 (HER2) enriched without ER expression, luminal A and luminal B expressing the oestrogen receptor (ER). Tumour expressing ER and/or progesterone receptor (PR) are considered hormone receptor-positive breast cancers, whereas tumour that lack of all receptors ER, PR and does not overexpress HER2 protein is called triple negative breast cancer (TNBC). TNBC is the most aggressive among all of subtypes of breast cancer; it is difficult to treat, and more likely to spread in diagnosed patients. While about 50% of all TNBC patients respond to traditional chemotherapy, the efficacy of these therapies is limited by drug resistance growth. Women with TNBC have low prognosis and limited treatment options.

2.1.2 Stages of Breast cancer

The America Joint Committee on Cancer (AJCC) is an organization whom responsible to manage the staging of breast cancer. Such that, breast cancer was classified into five stages. For each stage, there is sub-stages in order to describe the invasiveness.

Stage	Description
Noninvasive	
0	No tumor or invasion to the basement membrane of the duct nor neighboring normal tissue; (DCIS)
Invasive	
IA	• Tumour size above two centimeters. • No spread outside of the breast. • There is no involvement of lymph node.
IB	• Micro groups of cancerous cells found in the lymph node (> 0.2mm less than 2mm) • Presence of tumor in the breast (<2cm)
IIA	• Absence tumor in the breast but presence of cancer (>2mm) found in one to three axillary lymph nodes • Tumor not larger than 2cm spread to axillary lymph node • Tumor not larger than 5 cm has not spread to axillary lymph node (HER2- negative ER-2 +, PR-) and classified as stage 1
IIB	• Tumor >2cm but less than 5cm, presence of micro breast cancer cells >0.2mm <2mm found in lymph nodes (HER2- +, ER +, PR+) • Tumor >2cm but <5cm; cancer spread to one to three axillary lymph nodes or to breastbone • Tumor >5cm not spread to axillary lymph nodes
IIIA	• No presence of tumor in the breast; cancer found in four to nine axillary lymph nodes or in breastbone. • Tumor >5cm; micro cancer cells >0.2mm but <2mm found in lymph nodes

	• Tumor >5cm; cancer spread to one to three axillary lymph nodes or breastbone. • Tumor >5cm (grade 2 stage of breast cancer)
IIIB	• Tumor spread to chest wall or skin of the breast and swelling or an ulcer • Cancer spread to up nine axillary lymph nodes or spread to lymph nodes near the breastbone. • Grade 3 (classified as stage IIA)
IIIC	• Cancer spread to ten or more axillary lymph nodes • Cancer spread to lymph nodes above or below collarbone • Cancer spread to lymph nodes near breastbone • Classified as stage IIIA. If the above mention is grade 2 (ER+, PR+, HER+/-)
Metastatic	
IV	• Cancer spread to distant organ such as lung, skin, bones and liver. • De novo at first diagnosis

(Lombardi et al., 2020; The American Joint Committee on Cancer, 2020)

2.1.3 Epidemiology of breast cancer

World Incidence of Breast Cancer

According to GLOBOCAN (2018), there were about 2 million cases of breast cancer each year. Worldwide, breast cancer accounts for 11.6% incidence rates of all cancers in the year 2018 with estimation 1 in 4 women will have breast cancer. Breast cancer is the most diagnosed cancer among women with the incidence rates at 24.2% and 15% mortality rates. Meanwhile, the mortality rates for breast cancer are 6.6% of all cancers regardless of genders, whereby 626,679 women died from breast cancer in the year 2018. Breast cancer is the most frequently diagnosed among the type of female cancer in most of the country (154 of 185) and the leading cause of death in over 10 countries (GLOBOCAN, 2018). The highest incidence rates of breast cancer occur in Western and Northern Europe while Fiji has the highest mortality rates of breast cancer

worldwide. Despite hereditary and genetic factors, non-hereditary conditions are the main drivers of the globally observed and interethnic occurrence variations.

Estimated new cases and mortality of all cancers in the year 2018

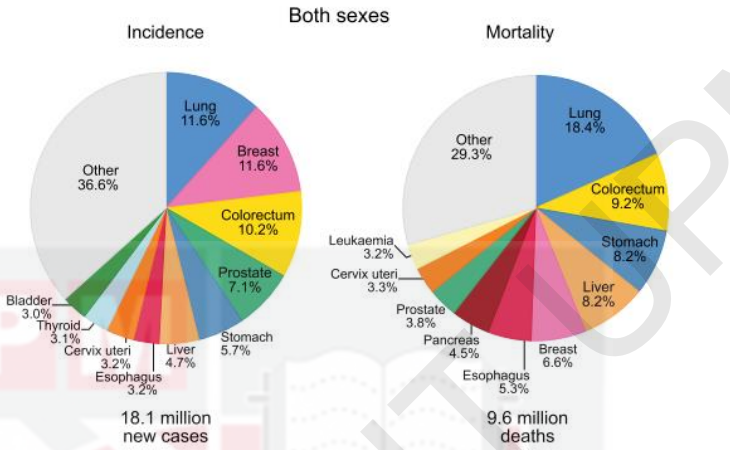

Figure 2.1: Pie chart of incidence and mortality of all cancers in the year 2018
(World Health Organization, 2020)

Breast Cancer Incidence in Malaysia

Among Malaysian women, breast cancer is the most common cancer that accounted for 32.7% of total female cancers (GLOBOCAN, 2018). Currently, Malaysia is following a Westernized diet that are high in sugar and fat, followed by sedentary lifestyle and experienced changes in reproductive characteristics, and breastfeeding rate. These are the risk factors of developing breast cancer (Tan et al., 2018). Regarding ethnicity, Chinese have the highest incidence of breast cancer (59.9/100000) followed by Indians (54.2/100000) and Malays (34.9/100000). A case control study conducted by Tan et al. found out Chinese have a low rate of breastfeeding. The short duration of breastfeeding may contribute to the increase of breast cancer incidence among Chinese in Malaysia compared with Indians and Malays. Their study also found that high intake of soymilk and soy products is associated with reduced risk of breast cancer, particularly among Asian women. To compare with Malays and Indians, Chinese consumed significantly less soy products ($p<0.001$). Besides that, breast cancer is the second leading cause of death among all types of cancer in Malaysia with 11% mortality rate.

Malaysia has been established cancer screening programme for breast, cervix uteri and colorectal cancers. Supporting evidence shows that cancer screening programme improve cancer survival. According to National Cancer Institute, Malaysia, a study conducted shows the overall 5 years rate survival (RS) for breast cancer was highest at stage I and stage II of diagnosis. However, survivals deteriorated at a faster rate for stage III and stage IV. By ethnicity, Chinese had the highest RS followed by Indians and Malays. The survivals were predominantly higher in women and younger age group. On the other hand, the hazard ratio (HR) in breast cancer was mainly highest in stage IV breast cancer (refer Appendix 1). Early diagnosis can increase the RS and lower the HR (Malaysian study on cancer survival, National Cancer Institute, Ministry of Health Malaysia, 2018).

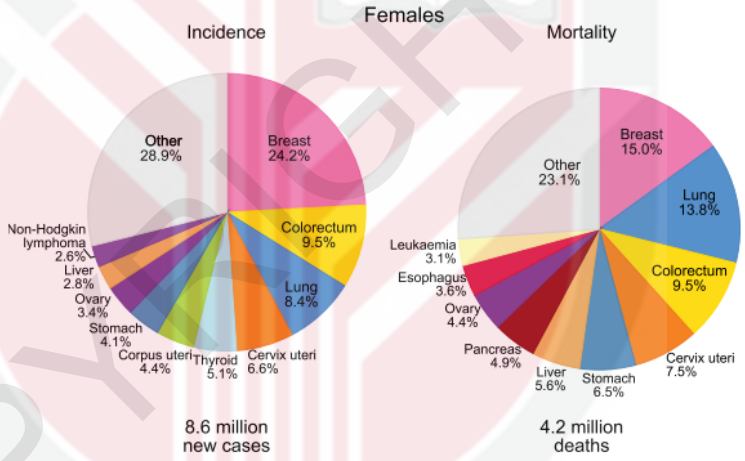

Figure 2.2 : Pie chart of incidence worldwide of all cancers, females, in the year 2018

(World Health Organization, 2020)

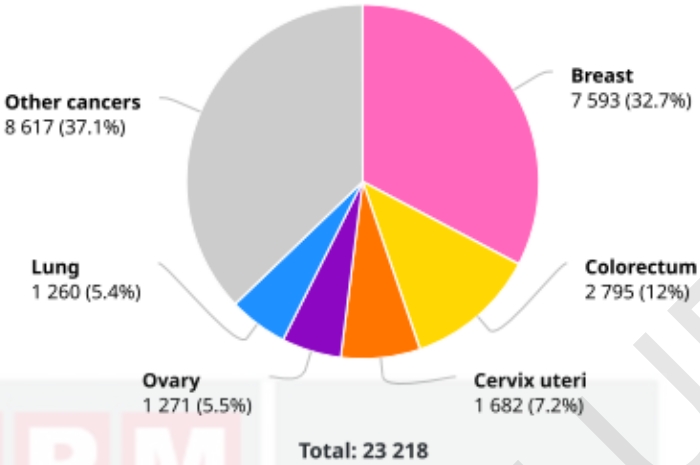

Figure 2.2 : Pie chart of incidence in Malaysia of all cancers, females, in the year 2018

(World Health Organization, 2020)

2.1.4 Metastasis of Breast cancer

The formation of tumour require cancer cells to undergo genetic mutations, adapt with their microenvironment, and induce angiogenesis sprouting that subsequently lead to metastasis (Hapach et al., 2019). In order to enhance patient survival, the underlying mechanisms of metastasis need to be understood to enable targeted intervention. Metastasis is an intricate process of cell dissemination where it involved a multi-step process of the invasion and migration of tumor cells from primary site to secondary location with subsequent colonization of these cells (Van Zijl et al, 2011).

The metastatic cascade involves sequential events which consists of five major steps: ***Local invasion*** *:* cancer cells start to spread by migrating to surrounding tissue. It continues its journey invading basement membrane and connective tissue. The cells then start to change structure from highly differentiated to undifferentiated. Epithelial-mesenchymal transition (EMT), is a transformation where it enables tumor cells to be able migrate and invade. In order to migrate, morphological protrusions are acquired

by tumor cells and responsive to migratory stimuli. After the timeline process of detachment from the primary tumor, cancer cells degrade the ECM. Surrounding tissues such as immune cells reacts to cancer cells invasion by developing inflammation.

Intravasation : The process of tumor cells migrating into vessels such as veins and lymphatic system. The same factors engaged in local invasion where secretion of proteases like matrix metalloproteinase. Through angiogenesis, it allows growing tumors for nutrients intake. The VEGF prominently released which is angiogenic factors that accomplish the process of intravasation.

Survival in the Circulation : circulating tumor cells (CTCs) require blood for a hostile environment. CTCs that presence in the blood has the chance to survive and produce distant metastasis. Tumor cell initiated a process called tumor cell-induced platelet aggregation by protecting themselves from stress and immune surveillance by secreting substances like cancer pro-coagulant. Moreover, this platelet surrounded the tumor cells helped tumor cells to arrive at distant organ.

Extravasation at Distant Organ Sites : Tumour that has formed emboli will blocks and bind to the endothelium of blood vessels in the target organ. In order to metastasized to other organ, CTCs have to escape the circulation a step called extravasation. During this process, cancerous cells secrete factors that enhance the vascular hyperpermeability for instance protein angiopoietin- like-4 (Angptl4). Furthermore, cancer cells able to gain access to certain organs by releasing chemokine factors for example expression of CXCR4 targeting surface organ that in turns lead to the expression of CXCL12.

Micrometastasis Formation : As CTCs arrived the secondary sites, cancer cells release growth factors which more likely providing nutrients for future metastasis. Such growth factors involved are inflammatory proteins, placental growth factor (PIGF) and many more. As a result, VEGF receptor will be recruited and premetastatic niche is formed.

Metastatic Colonization *:* After colonization of cancer cells to distant organ, tumour cells continue to proliferate and undergo angiogenesis to form metastatic tumor and micrometastases.

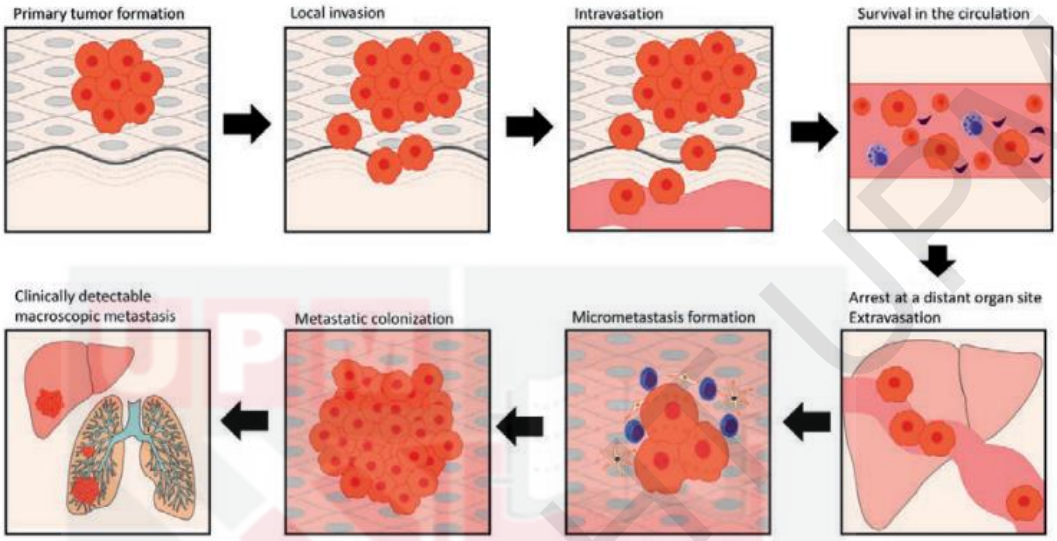

Figure 2.1.3 : Invasion-metastatic cascade of breast cancer

(Pachmayr et al. , 2017 ;Hapach et al. , 2019)

Therefore, there is a need in understanding the mechanism of metastasis in order to develop effective anticancer drug with minimal side effect. The current research focus on resistance of metastatic tumours are because it resistant to cytotoxic agents (Qian, Mei & Zhang, 2017). Blocking those measures can be an efficient risk-reduction technique. It accounts for 80% percent of diagnoses of breast cancer are due to the advancement of cancer to metastases to other parts of organ.

2.1.5 Risk factors of Breast cancer

According to Shah et al (2014), genetic factor, age, reproductive factor and environmental or lifestyle factor are the risk factors for developing breast cancer.

Age

One in twenty-six people will develop breast cancer at the age of 40 and above based on SEER database provided by United States.

Genetic factor

Those who have family member with breast cancer and has genetic mutation on *BRCA1* and *BRCA2* gene are significantly increase risk to develop breast cancer.

Reproductive factors

Statistically, women who had never give birth are prone to develop breast cancer (nulliparous) compared to parous women. Other than that, breastfeeding has shown protective effects against breast cancer.

Lifestyle

Alcohol intake more than 3 to 6 drinks per week are increased the risk to develop breast cancer. Nevertheless, those who have sedentary life but not doing any physical activity at least twice per week which in turn can be obese that lead to insulin resistance are the comorbidities that associated of developing breast cancer. Finally, radiation exposure mutated the gene in human body increased the risk to develop breast cancer.

2.1.6 Breast Carcinoma Cell Line, MDA-MB-231

For the purpose of this study, MDA-MB-231 cells line was used to achieve our objective as it commonly used to model late-stage breast cancer. This type of cancer cell line is highly invasive and lacks expression of oestrogen, progesterone, and HER 2 receptors. Prior to study about the antimigration effect of novel pyrazole derivatives, there is a need of using invasive metastatic tumour which is MDA-MB-231 cells.

2.1.7 Normal Mouse Fibroblast Cell Line, NIH/3T3

Normal mouse embryonic fibroblast was used in this study to compare cytotoxic effects between human breast cancer and normal fibroblast cells. In a study shown by Wang et al (2017), normal fibroblast has shown sensitivity towards pyrazoline derivatives. Therefore, we investigated novel pyrazole derivatives specifically TBBB47 and TBB47-A to determine inhibitory concentration (IC_{50}) on normal fibroblast cells, NIH/3T3.

2.2 Treatment Approaches of Breast Cancer

Researchers around the world have been investigating and striving to understand breast cancer and supporting those battling breast cancers by discovering an efficient form of treatment, but breast cancer treatment remains a huge obstacle to the medical field until today. Triple negative breast cancer cells have acquired resistance to clinical therapy and creates disparities in accessing diagnosis, treatment and management of breast cancer (Harbeck et al, 2019). Nevertheless, some chemotherapy drugs develop adverse effects which is outweigh the beneficial. Clinical management of breast cancer has shifted from based tumour burden to biologically-centred approaches as the molecular alteration drive the tumour to variation of breast cancer carcinogenesis. As for now, locoregional treatment and systemic therapy are the two main types of management of breast cancer (Harbeck, et al., 2019). Kingston and Johnson (2016) had suggested that in order to treat breast cancer, a research need to focus on the mechanism of resistance to develop new therapeutic approaches by targeting the pathways involved. According to National Cancer Institute, breast cancer treatment is following the stage of disease. There are six types standard treatment for breast cancer which have been clinically practiced (National Cancer Institute, 2020). This includes:

2.2.1 Surgery

Surgery to the breast purposely is to remove the cancer. The presence of cancer will be followed by remove the lymph nodes because it caused the spreading of cancer

cells. The three types of surgery involved are lumpectomy, total mastectomy, and modified radical mastectomy. Firstly, is lumpectomy where the operation involved remove cancer and some normal tissue Meanwhile, total mastectomy is the removal of whole breast and some part of the armpit that cancer has presented. Lastly, is modified radical mastectomy where whole removal of the breast including armpit area and chest wall muscles. At this stage, surgeon will give chemotherapy prior surgery in order to shrink the tumor. As tumor shrink, it will reduce the amount of area that need to be removed.

2.2.2 Radiation therapy

One of the types of cancer treatment which use high energy (X-ray) to kill cancer cells. The two types involved in radiation therapy are external and internal radiation therapy. The external radiation includes use of machine that sends radiation to the cancer in the body meanwhile internal where involved insertion of radioactive substance near cancer cells by using needles, wires or catheters. Such example of internal radiation therapy is Strontium-89 where it has been used to relieve bone pain that causes by the breast cancer. However, all these types of radiation therapy are depending on the type and stages of the breast cancer.

2.2.3 Chemotherapy

Chemotherapy is a drug treatment that uses chemicals to stop the growth of cancer cells at faster rate either by killing on inhibit the migration of cancer cells. Drug administered through oral (mouth), intravenous or intramuscular injection or by inhalation and goes to the bloodstream are called systemic chemotherapy whereas drug injected directly at specific organ such as breast is called regional chemotherapy. This are some of the drugs approved by FDA to treat and prevent breast cancer:

Drug used to prevent breast cancer:

- Raloxifene Hydrochloride
- Tamoxifen Citrate

Drug used to treat breast cancer:

- Atezolizumab
- Cyclophosphamide
- Doxorubicin Hydrochloride
- Exemestane
- Fulvestrant
- Herceptin (Trastuzumab)
- Lapatinib Ditosylate

2.2.4 Hormone therapy

Hormone therapy is a process of hormone removal to blocks the action of cancer cells from growing. Some hormones facilitated breast cancer growth such as estrogen where this hormone produced by the ovaries. In order to stop them, surgery or radiation need to be conducted to stop estrogen from working where this process is called ovarian ablation. Some other hormone therapy includes for the treatment of breast cancer are:

- Tamoxifen – given to the patients that having localized breast cancer but may develop endometrial cancer if not monitored.
- LHRH agonist – commonly give to premenopausal woman who had just been diagnosed with hormone receptor positive (HER+) breast cancer.
- Aromatase inhibitor- administered to postmenopausal woman who have diagnosed with hormone receptor positive (HER+) breast cancer. This drug types such as letrozole and exemestane decrease estrogen level.

2.2.5 Targeted therapy

It is a drug treatment which attack specific cancer cells without harm normal cells. Some of the targeted therapy are:

- Monoclonal antibodies – such as trastuzumab and pertuzumab which obstruct the effects of the growth factor protein of HER2 that sends signal growth to breast cancer cells. They are neoadjuvant and an adjuvant therapy used to treat early-stage HER2+ breast cancer.
- Tyrosine kinase – for instance are lapatinib and neratinib. They have a same as monoclonal antibodies.
- PARP inhibitors- such that are olaparib and talazoparib. It is used to treat patients with *BRCA1* or *BRCA2* gene mutations and HER2- breast cancer. These drugs can also treat triple-negative breast cancer (TNBC).

2.2.6 Immunotherapy

This treatment use patients' immune system to fight against cancer. Such substances are modified in the laboratory to boost body's natural defenses against cancer. For instance, is immune checkpoint inhibitor therapy where it involved PD-1 protein that bind to PDL-1 and allow the T cells to kill the cancer cells.

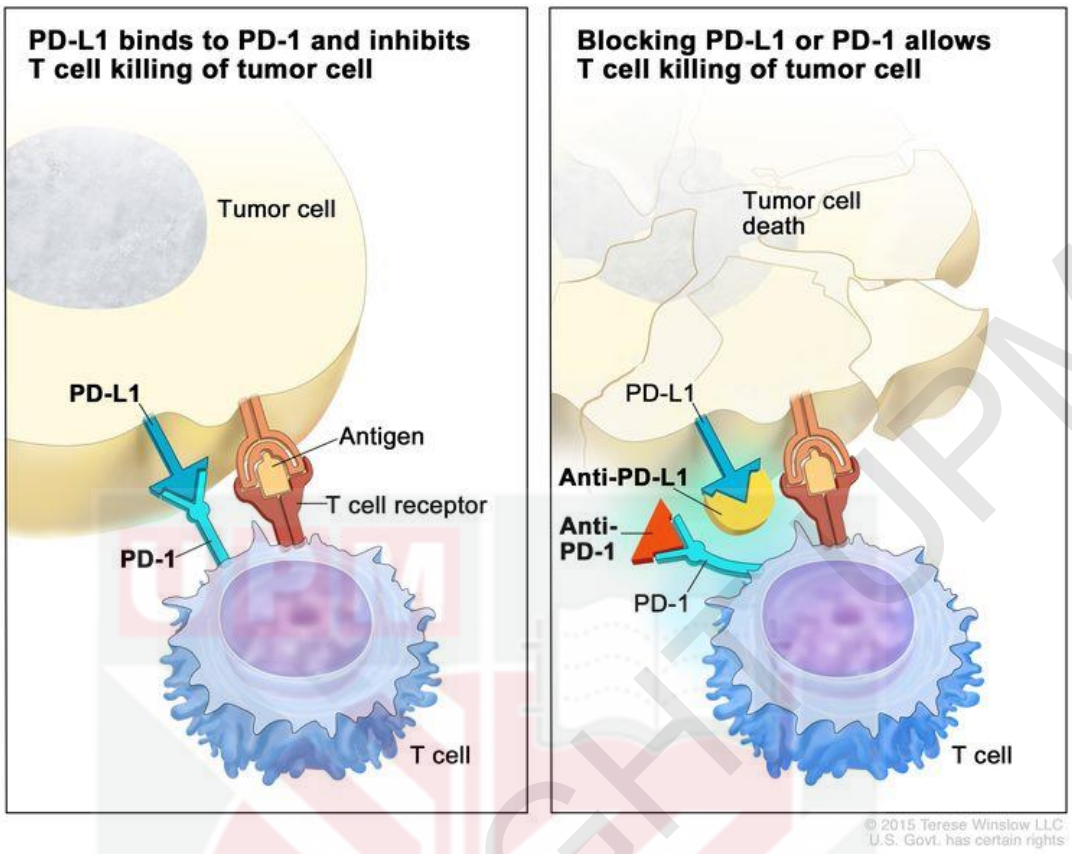

Figure 2.2.6 Immunotherapy PD-1 inhibitor has been clinically practiced treating breast cancer.

(National Cancer Institute, 2020)

However, these six types of therapy majorly produce side effects to the patients with breast cancer such as heart failure and hemorrhage.

2.3 Pyrazole

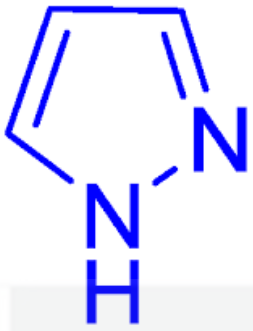

Figure 2.3 Structure of Pyrazole

(Karrouchi et al, 2018)

Pyrazole, one of a class of heterocyclic series organic compounds characterized by a ring structure in adjacent positions composed of three carbon atoms and two nitrogen atoms (Karrouchi et al, 2018). In a pharmacological study reported by Liu et al (2019) proved that pyrazole molecule exhibit many bioactivities such as anti-cancer, anti-microbial, anti-convulsant, anti-inflammatory and anti-depressant. Nevertheless, combination with other substituents at heterocycles ring of pyrazole has interesting effects as anticancer agents such as pyrazole combination with platinum(III) has shown significant inhibitory effects on induce cell cycle arrest and apoptosis in breast cancer cells (Czarnomysy et al, 2018). Due to this, investigation of pharmacological effects of pyrazole on varied cell type is still in continuation as it brings promising anticancer therapy.

2.3.1 Pyrazole-urea (GeGe3)

Angiogenesis plays a key role in tumour development as required for rapid growth and metastasis, hence targeting angiogenesis is a promising therapeutic strategy for the treatment of breast cancer (Dewangan et al., 2019). In a literature studies by Meta et al., 2017 found that parental compound named pyrazole-urea (GeGe3) has a potent blocker of angiogenesis in MAPK and P13K pathways where these pathways play a key role on tumor growth and angiogenesis . According to Meta et al (2017) shown that pyrazole molecules are able to inhibit the chemotaxis of neutrophils induced by both IL-8 and fMLP (IC_{50} at the sub nanomolar level). From studies carried out at the intracellular level it seems that these molecules inhibit phosphorylation of different targets such as PKC, p38 and ERK, very important agents in anti-inflammatory and autoimmune diseases.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

MDA-MB-231, human breast cancer cells and NIH/3T3, normal mouse embryonic fibroblast cells were purchased from American Type Cell Culture (ATCC), USA. The 0.25% Trypsin-EDTA, Dulbecco's Modified Eagle's medium (DMEM), , and penicillin-streptomycin (pen strep) were purchased from Gibco (Rockford II, USA). Fetal bovine serum (FBS) was purchased from Merck (Darmstadt, Germany). The novel Pyrazole derivatives (TBBB47 and TBB47-A) were obtained from Department of Pharmacy, University of Genoa (Italy). Bovine serum albumin (BSA) and Trypan blue were purchased from Sigma Aldrich (St. Louise MO, USA). 3-[4,5-dimethlthiazole-2-yl]-2,5-dephenyltetrazolium bromide (MTT) was purchased from Invitrogen (Waltham, Massachusetts, USA). Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS) were obtained from Amresco (Solon, Ohio).

3.2 Methods

3.2.1 Culturing cells

3.2.1.1 Cell Thawing

MDA-MB-231, human breast cancer cells and NIH/3T3, normal mouse embryonic fibroblast cells were cryopreserved in DMEM medium containing 10% DMSO in cryovial and stored in -80ºC freezer. The cryopreserved cells were thawed at 37ºC water bath within 1 minute. Then, the cell suspension was transferred into a 15mL centrifuge tube comprising 4mL of pre-warmed DMEM media before being centrifuged at RPM 1200 for 4 minutes at 4ºC. After centrifugation, the supernatant was discarded, and the cells were suspended in 1mL DMEM media. Lastly, the 1mL cell suspension was transferred into a new culture flask containing 4mL of DMEM

media. The cells were maintained in 5% carbon dioxide (CO2) humidified incubator at 37 ºC.

3.2.1.2 Cell culture

MDA-MB-231 cells and NIH/3T3 cells were cultured in T25 culture flask containing DMEM medium and were maintained at 37ºC in 5% carbon dioxide (CO2) humidified incubator until they reached 80%-90% confluence. The medium was changed every 2 days to maintain the proliferation of the cells and ensured the cells were healthy.

3.2.1.3 Subculture of cells

The cells were sub-cultured into new culture flask with ratio 1:3 to increase the capacity for cells proliferation when the cells reach from 80%-90% confluence. The DMEM media was discarded from the flask and 2mL PBS was used to wash any debris. In order to detach the cells from the flask, 1mL of trypsin were added and the cells were incubated at 37ºC for 2 minutes. After 2 minutes of incubation, 2mL of media were added into the flask to stop the activity of trypsin on the cells. Together with the 1mL of trypsin, 3mL of cell suspension was transferred into 15mL centrifuge tube. The cell suspension was centrifuged at 4ºC with RPM 1200 for 4 minutes. After centrifugation, the supernatant was discarded, and the pellet was resuspended with 1mL media. Following 1:2 ratio, the cells suspension was divided into 2 different T75 new culture flasks containing 9mL media each and was incubated at 37ºC.

3.2.1.4 Cell viability

MDA-MB-231 cells and NIH/3T3 cells were obtained from the flask by detaching the cells using trypsin as mentioned in section 3.2.1.3. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 1mL media. In performing cell count, 50µL of cells suspension was diluted in 50µL of 0.4% trypan blue. Then, 10µL of the dilution was loaded on the haemocytometer and by using

microscope the cells were counted manually. The total number of the cells was determined by using this formula:

$$Total\ number\ of\ cells = \frac{A+B+C+D}{4} \times 10^4 \times 2^{DF}$$

Where A,B,C and D represent the number of cells counted from the four corner quadrants of the haemocytometer and DF is the diluent factor.

The cells viability was determined using this formula:

$$Viability(\%) = \frac{No\ of\ living\ cells}{Total\ no.of\ cells} \times 100$$

3.2.1.5 Seeding optimization

In particular, seeding optimization were carried out with the aim of obtaining optimum cell seeding density in the 96-well plate for the MTT assay. The optimization test is essential because it helps to exclude false negative results by establishing the range of optimum density for continuous development and stimulates further proliferation. First of all, different seeding density of cells were seeded in DMEM media for 24,48 and 72 hours. The cell seeding density seeded in 96-well plate were in range from $1x10^5$ to $1x10^6$ which were one folded dilution.

Following 24, 48- and 72-hours incubation, 20µL of MTT solution (5mg/mL) were added into each well and were incubated for 4 hours. After 4 hours incubation, the media containing MTT solution was discarded, and 100µL of 100% DMSO was added. At wavelength 570nm, the absorbance readings for each sample were measured by using microplate reader (UVM 340, ASYS Hitech GmbH, Austria) and were analysed with Microsoft Excel.

3.3 MTT Assay for Cytotoxicity Testing

MTT assay was a colorimetric assay. This assay was used to access the proliferation and the viability of cells. It was determined by the colour changes of the 3-[4,5-dimethlthiazole-2-yl]-2,5-dephenyltetrazolium bromide (MTT) solution from yellow to the purple formazan. This colour changes formed by the action of mitochondrial enzyme, succinate dehydrogenase, that present in the living cells. In this experiment, MTT tests were performed to evaluate cell viability following treatment with novel pyrazole derivatives (TBBB47 and TBB47-A).

Both cell lines were seeded in the 96-well plate at density of $1x10^5$ cell/well in 100µL of DMEM media and were incubated for overnight. After 24 hours, the cells were treated with different concentrations of TBBB47 and TBB47-A which were two-fold diluted from 0µM to 200µM and were incubated for 24, 48 and 72 hours. Following time point, 20µL of MTT (5mg/mL) was added and incubated in the dark at 37ºC for 4 hours in 5% carbon dioxide (CO_2) humidified incubator.

Following 4 hours, the formazan precipitates were formed in each well. The precipitates were dissolved with 100µL of DMSO and the absorbance was measured at 570nm for each well using reference background 630nm. For analysing the results, percentage of cell viability was plotted against concentration (µM).

3.4 Treatment

3.4.1.1 TBBB47 and TBB47-A Preparation

TBBB47 and TBB47-A were dissolved in DMSO as it has limited solubility in aqueous solution. Molecular weight of TBB47-A is 392.43 mol/g; molecular weight of compound 2 is 442.43. Stock concentration (100µM) of TBBB47 and TBB47-A were prepared by dissolving 6.1 mg of compound in required amount of by using the equation below:

$$Volume\ of\ DMSO = \frac{6.1\ \text{mg}}{Molecular\ Weight\ of\ Compound\ \times 100\mu\text{M}}$$

Next, the stock was kept in freezer (-20ºC). The stock was prepared in order to prepare different concentration of pyrazole derivatives for further experiments.

3.4.1.2 Study Design

In this study, there were 2 groups which included:

- **Negative control group (N):** untreated cells which to examine the MDA-MB-231 and NIH/3T3 cells condition.
- **Treatment group:**
  - 200µM to 1.5625µM : TBBB47 and TBB47-A pre-treated MDA-MB-231 cells and NIH/3T3 cells, respectively.

CHAPTER 4

RESULTS

Prior to the anti-migration assay, the toxic concentration of pyrazole derivatives (TBBB47 and TBB47-A) needed to be determined. Hence, the MTT assay was performed to evaluate the cytotoxic effect of TBBB47 and TBB47-A on MDA-MB-231 and NIH/3T3 cells at different concentration. This is preliminary screening testing; therefore, TBBB47 and TBB47-A concentrations were two-fold diluted from 200µM to 1.5625µM. The percentage of cell viability of MDA-MB-231 cells and NIH/3T3 cells following treatment with various concentration (200µM-1.5625µM) of TBBB47 and TBB47-A were determined.

4. 1 Cytotoxic effect of Novel Pyrazole derivatives TBBB47 on MDA-MB-231 cells

Figure 4.1.1 showed that TBBB47 compound was toxic towards MDA-MB-231 cells with IC_{50} values 127µM, 131µM, and 162µM after three incubation periods, respectively. There is significantly reduced cell viability following TBBB47 treatment at 200µM. On the other hand, any concentration below than 127µM did not show any significant changes on cell viability of MDA-MB-231 cells.

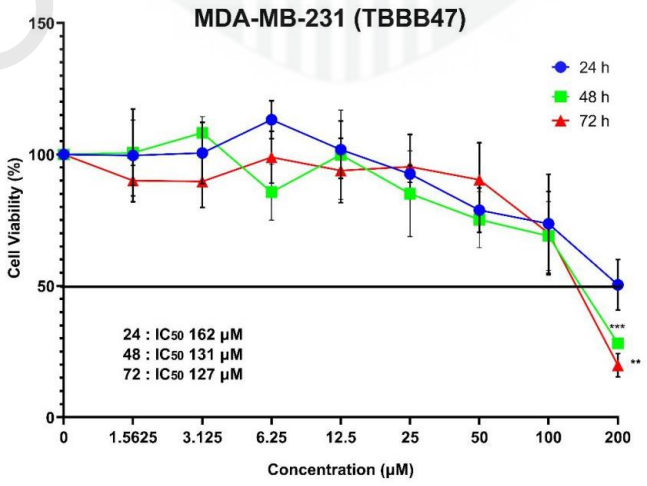

Figure 4.1.1: Cytotoxic effects of TBBB47 on MDA-MB-231 cells via MTT assay. Cells were treated with different concentration of TBBB47 (1.5625Mm to 200μM) on treatment group for three different time point. Mean data expressed as ± SEM based three replicates experiments. Results were analysed by two-way ANOVA and followed by Dunnett's post hoc test. The mean marked with **,*** were significantly different with *p*-value less than 0.01, 0.001 respectively as compared to control group.

Figure 4.1.2 showed that TBBB47 has cytotoxic effects on MDA-MB-231 cells at dose and time-dependent manner. As the graph showed increase incubation periods will increase the cytotoxic effects of TBBB47 towards MDA-MB-231 cells.

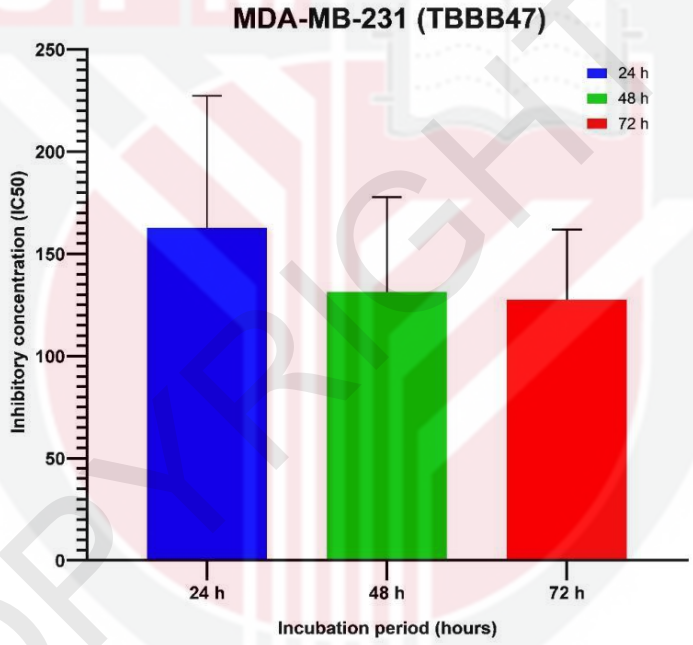

Figure 4.1.2: IC_{50} graph of TBBB47 on MDA-MB-231 cells via MTT assay. Cells were treated with TBBB47 at three different time point. Mean data expressed as ± SEM based on three replicates. Results were analysed by one-way ANOVA and followed by Dunnett's post hoc test.

4.2 Cytotoxic effect of Novel Pyrazole derivatives TBB47-A on MDA-MB-231 cells

Figure 4.2.1 showed that TBB47-A compound was toxic towards MDA-MB-231 cells with IC_{50} values 79µM, 102µM, and 124µM after three incubation periods, respectively. There are significantly reduced cell viability following TBB47-A treatment at 100µM for 48- and 72-hour post incubation while 200µM for 24, 48- and 72-hours post-incubation. On the other hand, any concentration below than 79µM did not show any significant changes on cell viability of MDA-MB-231 cells.

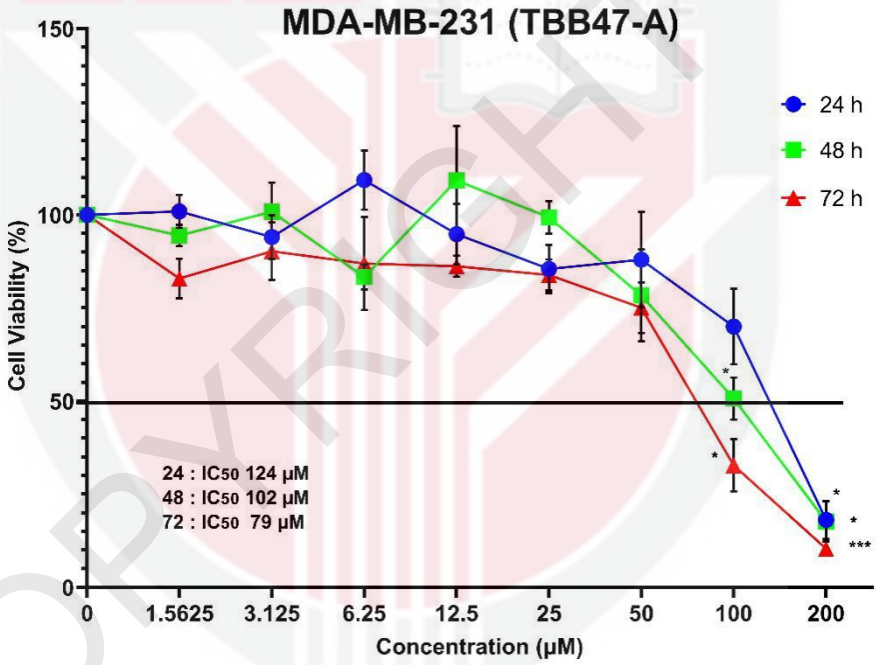

Figure 4.2.1: Cytotoxic effects of TBB47-A on cell viability MDA-MB-231 cells via MTT assay. Cells were treated with different concentration of TBB47-A (1.5625µM to 200µM) on treatment group for three different time point. Mean data expressed as ± SEM based on three replicates experiments. Results were analysed by two-way ANOVA and followed by Dunnett's post hoc test. The mean marked with *, **,*** were significantly different with *p*-value less than 0.05, 0.01, and 0.001 respectively as compared to control group.

Figure 4.2.2 showed that TBB47-A has cytotoxic effects on MDA-MB-231 cells at dose and time-dependent manner. As the graph showed increase incubation periods will increase the cytotoxic effect of TBB47-A towards MDA-MB-231 cells.

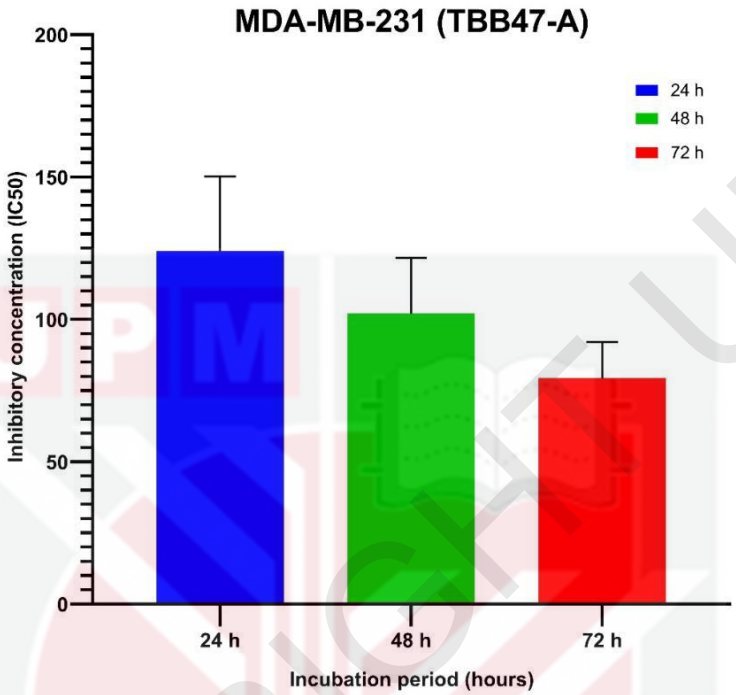

Figure 4.2.2: IC_{50} graph of TBB47-A on cell viability MDA-MB-231 via MTT assay. Cells were treated with TBB47-A at three different incubation periods (24, 48 and 72 hours), respectively. Mean data expressed as ± SEM based on three replicates experiments. Results were analysed by one-way ANOVA and followed by Dunnett's post hoc test.

4.3 Cytotoxic effect of Novel Pyrazole derivatives TBBB47 on NIH/3T3 cells

Figure 4.3.1 showed that TBBB47 compound was toxic towards NIH/3T3 cells with IC_{50} values 124µM, and 130µM after 48- and 72- hours of incubation period, respectively. Meanwhile, no cytotoxic effect shown after 24- hour incubation period with IC_{50} more than 200µM. There is significantly reduced cell viability following TBBB47 treatment at 200µM for 48- hour post-incubation. On the other hand, any

concentration below than 124µM did not show any significant changes on cell viability of NIH/3T3 cells.

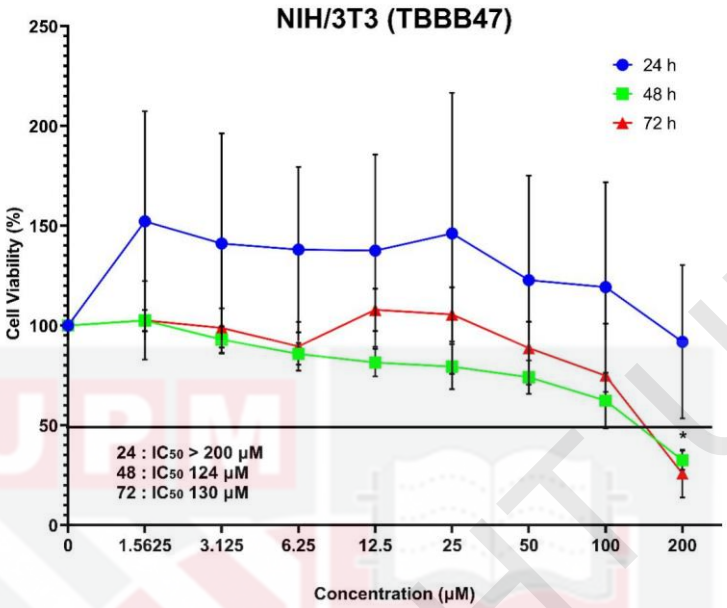

Figure 4.3.1: Cytotoxic Effects of TBBB47 on cell viability of NIH/3T3 via MTT assay. Cells were treated with different concentration of TBBB47 (1.5625Mm to 200µM) on treatment group for 24, 48 and 72 hours. Data was expressed as mean ± SEM based on three replicates experiments. Results were analysed by two-way ANOVA and followed by Dunnett's post hoc test. The mean marked with * was significantly different with *p*-value less than 0.05 as compared to control group.

Figure 4.3.2 showed that TBBB47 has cytotoxic effects on NIH/3T3 cells at dose and time-independent manner. As the graph shown gradually increase on the IC_{50} after 24-hour incubation but the IC_{50} decrease after 48-hour incubation. Following 72-hour post-incubation , there is slightly increase in the mean of inhibitory concentration (IC_{50}) .

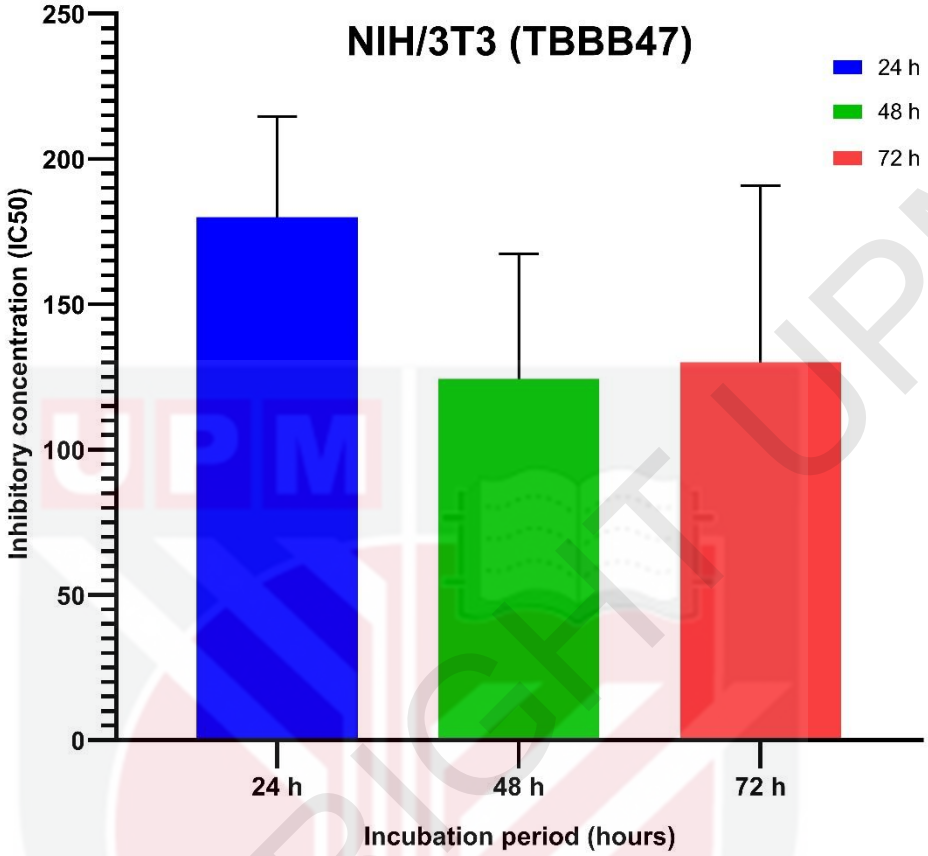

Figure 4.3.2: IC_{50} graph of TBBB47 on cell viability NIH/3T3 cells via MTT assay. Cells were treated with TBBB47 at three different time point. Data was expressed as mean ± SEM based on three replicates (three independent experiments). Results were analysed by one-way ANOVA and followed by Dunnett's post hoc test.

4.4 Cytotoxic effect of Novel Pyrazole derivatives TBB47-A on NIH/3T3 cells

Figure 4.4.1 showed that TBB47-A compound was toxic towards NIH/3T3 cells with IC_{50} values 39µM, and 79µM after 48- and 72- hour incubation periods, respectively. Meanwhile, no cytotoxic effect shown after 24- hour incubation period with IC_{50} more than 200µM. There is significantly reduced cell viability following TBB47-A treatment at 200µM for 48- and 72-hours post-incubation. On the other hand, any concentration below than 39µM did not show any significant changes on cell viability of NIH/3T3 cells.

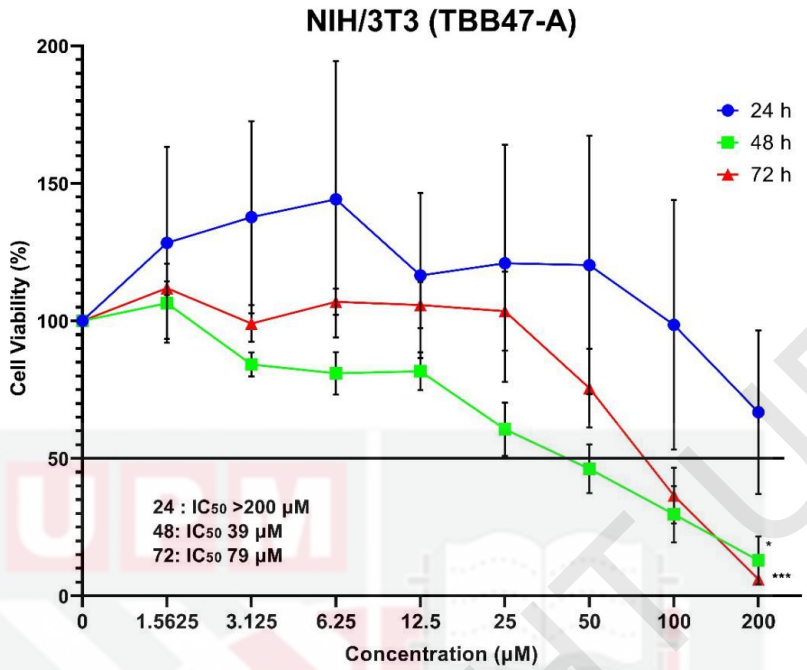

Figure 4.4.1: Cytotoxic Effects of TBB47-A on cell viability of NIH/3T3 via MTT assay. Cells were treated with different concentration of TBB47-A (1.5625Mm to 200µM) on treatment group for 24, 48 and 72 hours. Mean data expressed as ± SEM based on three replicates experiments. Results were analysed by two-way ANOVA and followed by Dunnett's post hoc test. The mean marked with *,*** was significantly different with *p*-value less than 0.05 and 0.001 respectively as compared to control group.

Figure 4.4.2 showed that TBB47-A has cytotoxic effects on NIH/3T3 cells at dose and time-independent manner. As the graph shown gradually increase on the IC_{50} after 24-hour incubation but the IC_{50} decrease after 48-hour incubation. Following 72-hour post-incubation , there is increase in the mean of inhibitory concentration (IC_{50}) .

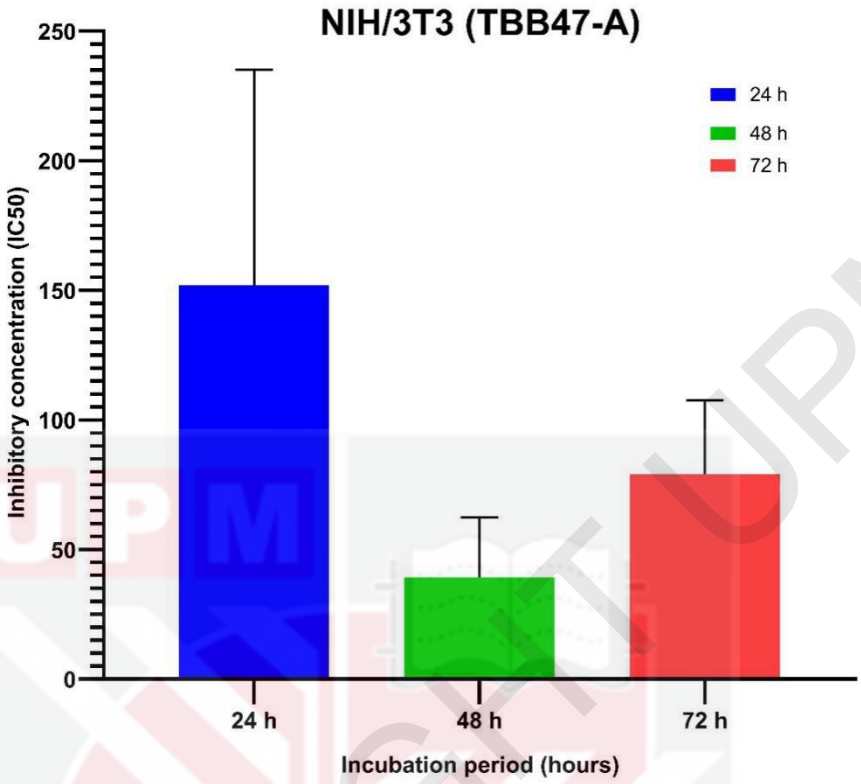

Figure 4.4.2: IC_{50} graph of TBB47-A on cell viability NIH/3T3 cells via MTT assay. Cells were treated with TBB47-A at three different time point. Mean data expressed as ± SEM based on three replicates experiments. Results were analysed by one-way ANOVA and followed by Dunnett's post hoc test.

4.5 Comparison TBBB47 cytotoxic effects between MDA-MB-231 cells and NIH/3T3 cells

Figure 4.5.1,4.5.2 and 4.5.3 showed the comparison between cell lines that has cytotoxic effect after treatment with TBBB47. There is no significant difference between MDA-MB-231 cells and NIH/3T3 cells indicating that TBBB47 showed similar cytotoxic effects towards MDA-MB-231 cells and NIH/3T3 cells. For both cell lines, both showed reduced in cell viability towards higher concentration (>200µM).

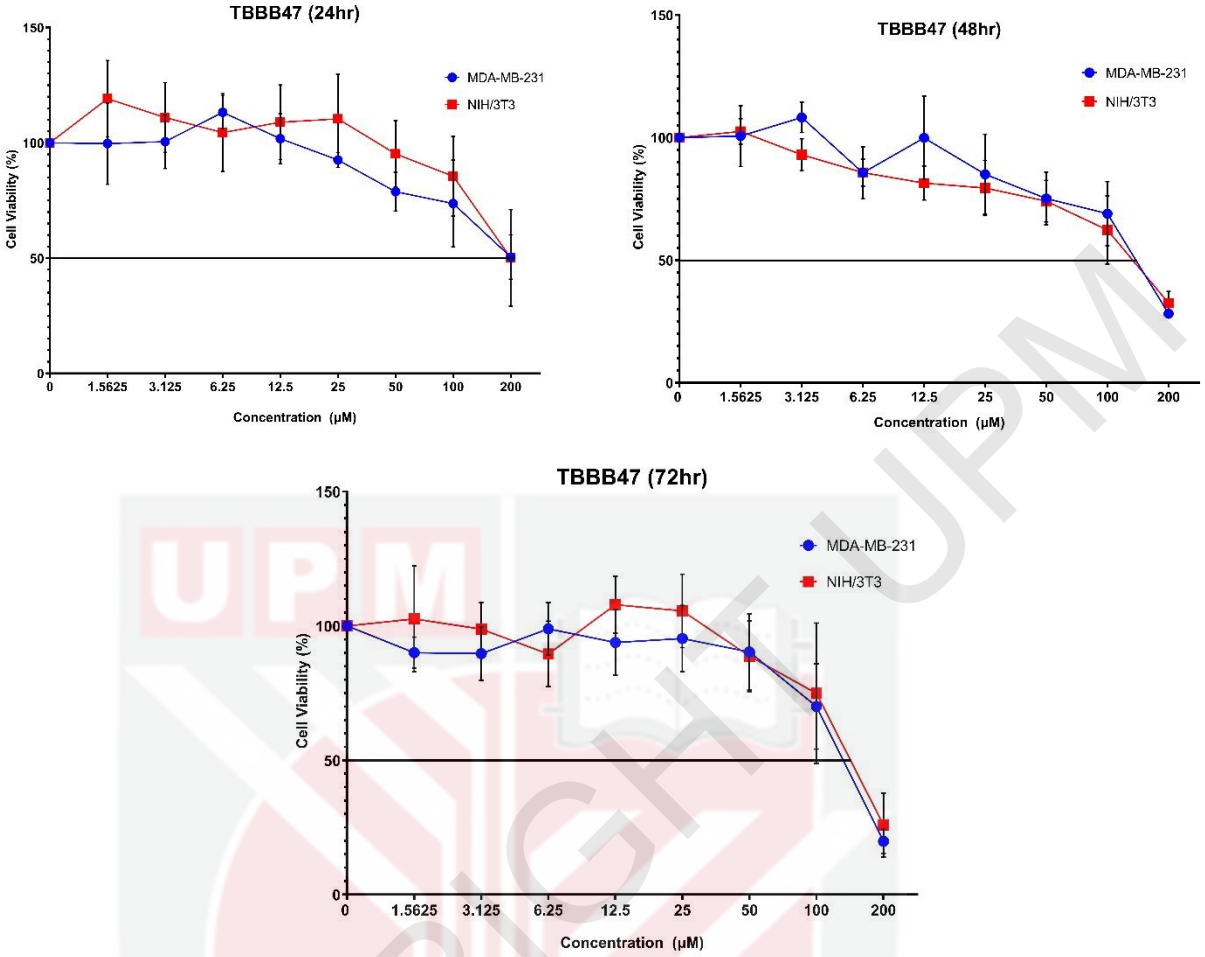

Figure 4.5.1, 4.5.2 and 4.5.3 Effect of TBBB47 on cell viability between two cell lines MDA-MB-231 and NIH/3T3 cells. Cells were treated with TBBB47 at three different incubation periods (24,48 and 72 hour). Mean data expressed as ± SEM based on three replicates experiments. Results were analysed by paired t-test.

4.6 Comparison TBB47-A cytotoxic effects between MDA-MB-231 cells and NIH/3T3 cells

Figure 4.6.1,4.6.2 and 4.6.3 showed the comparison between cell lines that has cytotoxic effect after treatment with TBB47-A. There is no significant difference between MDA-MB-231 cells and NIH/3T3 cells indicating that TBB47-A showed similar cytotoxic effects towards MDA-MB-231 cells and NIH/3T3 cells. For both cell lines, both showed reduced in cell viability towards higher concentration (>200µM).

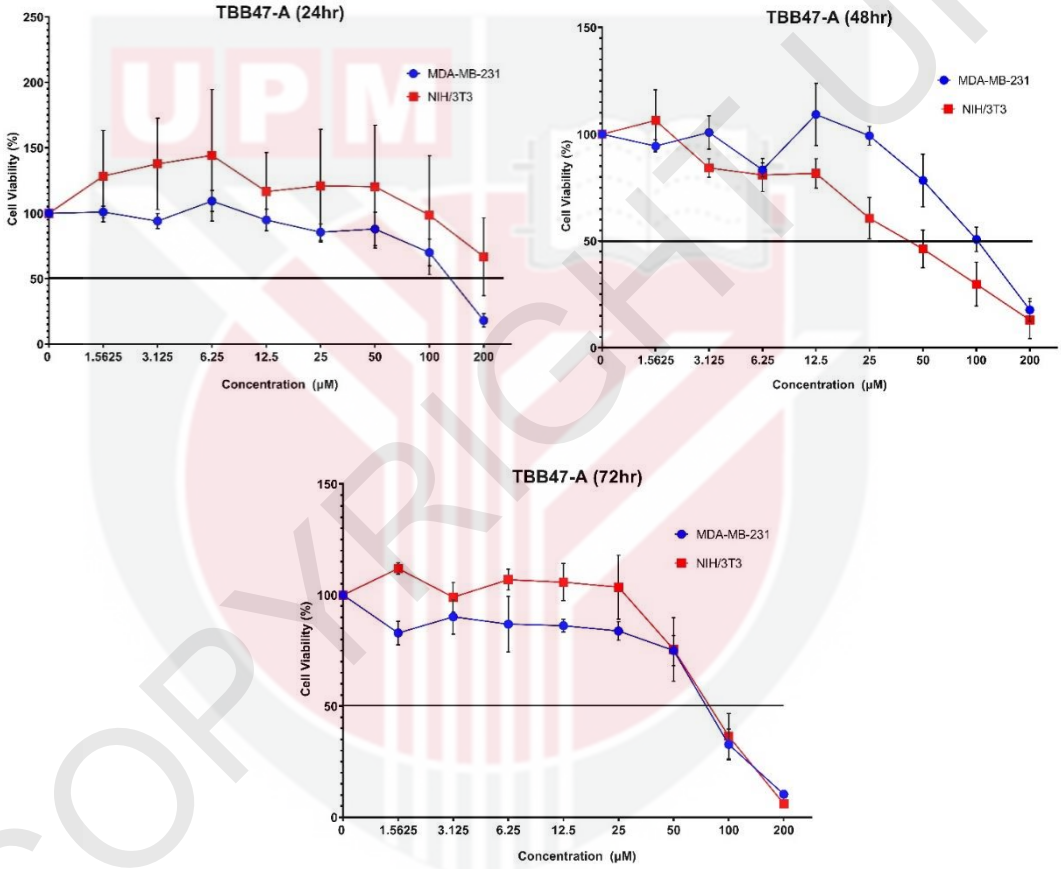

Figure 4.6.1, 4.6.2 and 4.6.3 Effect of TBB47-A on cell viability between two cell lines MDA-MB-231 and NIH/3T3 cells. Cells were treated with TBB47-A at three different time point. Mean data expressed as ± SEM based on three replicates experiments. Results were analysed by paired t-test.

CHAPTER 5

DISCUSSION

Metastasis is a complex process that can be triggered by many different factors. One of the factors is migration and invasion where it plays a major role enhancing the cancer cells to migrate and metastasized to distant organ. Migration was initiated by the nutrients provided from the formation of new blood vessels which is so called angiogenesis. Due to this, vascular endothelial growth factor receptor (VEGFR-2) such as VEGF, bFGF, PIGF, and IL-8 has a significant role in angiogenesis, tumor suppression, and metastasis (Saleh et al., 2020). This angiogenic factors activate MAPKs and P13K to induce migration and proliferation and survival of the endothelial cells that are involved in angiogenesis. However, over the last 30 years, it has been apparent that failures inhibiting the migration of cancer cells to distant organ. Therefore, targeting the ligand, VEGF could be promising cancer therapy approaches (Meta et al., 2017).

TBBB47 and TBB47-A were derived from parental compound named pyrazolyl-urea (GeGe3). This parental compound GeGe3 was found to be an angiogenesis inhibitor where it blocks the activation of MAPK and P13K signalling pathways in human umbilical vein endothelial cells (HUVEC) migration. To prove the novel compound has an antiangiogenic drug, Meta et al., 2017 suggested to further tested GeGe3 on different tumor cell lines to verify it inhibitory action. Previous study has shown GeGe3 as promising antiangiogenic compound. In this study, prior conducting antimigration assay, the novel pyrazole derivatives TBBB47 and TBB47-A were investigated for their cytotoxic effects on the human breast cancer (MDA-MB-231) and normal mouse embryonic fibroblast (NIH/3T3) cells.

MTT assay was performed to assess the cytotoxic effects of the compounds and to determine the half-maximal (50%) inhibitory concentration (IC_{50}) of TBBB47 and TBB47-A on MDA-MB-231 and NIH/3T3 cells. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition in vitro. In a study conducted by Wang et al (2017) reported that pyrazole derivatives has cytotoxic

selectivity between cancerous cells and normal fibroblast cells with concentration ranging from 23μM to 100μM. Therefore, this study confirmed previous reports by Wang et al (2017) that some pyrazole derivatives shown less cytotoxicity to normal cells .

According to the figure 4.1.1 and 4.3.1, TBBB47 concentration as low as 79μM showed cytotoxic effects on human breast cancer and normal cells. TBBB47 concentration range from 0 to 70μM were not had any significant effects to the both cell lines. Pyrazole derivatives treatment on MCF-7 cells was cytotoxic at lower concentration with IC_{50} values range from 0.01 to 15.54μM in the study by Karrouchi et al (2018). However, in this study TBBB47 pyrazole derivatives was toxic at concentration as low as 200μM due to significant on reduced 50% population of cell viability MDA-MB-231 and NIH/3T3 cells. Thus, it determines that pyrazole derivatives cytotoxic effects were varied with the cell type.

According to the figure 4.2.1 and 4.4.1, TBB47-A concentration as low as 100μM showed significant cytotoxic effects on human breast cancer. Meanwhile, TBB47-A was toxic at concentration as low as 200μM due to significant on reduced 50% population of cell viability on NIH/3T3 cells. TBB47-A concentration range from 0 to 30μM were not had any significant effects to the both cell lines. There is no significant difference shown on figure 4.5 and 4.6 for showing cytotoxic effects on both cell lines. Therefore, TBBB47 and TBB47-A has similar cytotoxic effects on MDA-MB-231 and NIH/3T3 cells.

According to the IC_{50} graph of MDA-MB-231 cells, both compounds increased cytotoxic effects when increased the incubation periods. This shown that both compounds showed cytotoxic effects on human breast cancer at time-and dose-dependent manner. Meanwhile, the IC_{50} graph of NIH/3T3 cells, both compounds showed cytotoxic effects on human breast cancer at time-and dose-independent manner. As studied by Cook et al (2018), each compound has different chemical structures that determining dose-dependent effects, and the thresholds of the compound effects on different cell lines physiology.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.0 Conclusion

TBBB47 and TBB47-A show cytotoxic effects on human breast cancer (MDA-MB-231) and normal mouse fibroblast cells (NIH/3T3). For the compounds to show cytotoxic effects on human breast cancer (MDA-MB-231) and normal mouse fibroblast cells (NIH/3T3), longer incubation period is required.

6.1 Future Recommendations

Although the objectives of this study have been achieved, there are some recommendation which could be used to improve and further understand about the effects of novel pyrazole derivatives (TBBB47 and TBB47-A) on human breast cancer (MDA-MB-231) and normal mouse embryonic fibroblast cells (NIH/3T3) cells. These recommendations included:

- Repeat experiment to confirm the data obtained.
- Morphology study should be performed in order to determine apoptotic cell death caused by the compounds on human breast cancer (MDA-MB-231).
- Scratch assay should be conducted to investigate the anti-migration effect of TBBB47 and TBB47-A on human breast cancer (MDA-MB-231) cells.

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APPENDICES

Drug	**IC_{50} (μM) MDA-MB-231**		
	24 hrs	48 hrs	72 hrs
TBBB47	200	146	140
TBB47-A	138	104	79

Table 2. Inhibitory concentration (IC50 μM) for different time point (24, 48 and 72 hours) of incubation period on breast cancer cells (MDA-MB-231).

Drug	**IC_{50} (μM) NIH/3T3**		
	24 hrs	48 hrs	72 hrs
TBBB47	>200	140	150
TBB47-A	>200	43	82

Table 3. Inhibitory concentration (IC50 μM) for different time point (24, 48 and 72 hours) of incubation period on normal fibroblast cells (NIH/3T3).

Drugs	MDA-MB-231	NIH/3T3
TBBB47	162 μM ± 19.079	96.66 μM ± 48.419
TBB47-A	107 μM ± 17.098	41.66 μM ± 23.

Table 3. Inhibitory concentration (IC_{50}) average mean of each compound between MDA-MB-231 and NIH/3T3 cells.