THE EFFECTS OF VALPROIC ACID ON NAV1.5 GENE EXPRESSION IN MDA-MB-231 CELLS

by

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Dissertation submitted in partial fulfillment

of the requirements for the degree

of Bachelor of Health Sciences (Honours) (Biomedicine)

MAY 2016

ACKNOWLEDGEMENT

I would like to express my profound gratitude to my supervisor, Dr. Noor Fatmawati Mokhtar for giving me the opportunity to carry out my final year project under her supervision, and for her valuable guidance and motivation throughout the final year project. I would like to convey my heartfelt appreciation to postgraduate, Nur Sabrina Kamarulzaman for her continuous guidance and encouragement in this project.

Also, I would like extend my sincere thanks to my family and friends who have been a great support to me as well as to those involved directly or indirectly in completing this project. Above all, I praise God, the almighty for providing me with this immensely valuable experience and for successful completion of this project.

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LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	American Type Culture Collection
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
Ct	Cycle threshold
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
GABA	Gamma-aminobutyric acid
H^+	Hydrogen ion
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid

Na^+	Sodium ion
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
PR	Progesterone receptor
RNA	Ribonucleic acid
SEM	Standard error of mean
TAE	Tris-acetate-EDTA
TNM	Tumor, node and metastasis
TTX	Tetrodotoxin
VGSC	Voltage-gated sodium channel
VPA	Valproic acid
WHO	World Health Organization

KESAN-KESAN ASID VALPROIK TERHADAP EKSPRESI GEN NAV1.5 DALAM SEL-SEL MDA-MB-231

ABSTRAK

Sejak beberapa dekad yang lalu, penyelidikan barah payudara berkembang secara meluas dan banyak rawatan baru ditemui setiap tahun untuk meningkatkan kualiti hidup pesakit. Walau bagaimanapun, metastasis masih menjadi penyebab utama mortaliti kanser dan rawatan bersasarkan metastasis sedang diselidik. Saluran natrium bervoltan kardiak, Nav1.5 dan varian neonatalnya, nNav1.5 telah ditemui dalam sel kanser payudara manusia yang bermetastasis tinggi, MDA-MB-231 dan dikaitkan dengan metastasis. Kajian-kajian sebelum ini telah membuktikan kesan antikanser asid valproik terhadap beberapa jenis sel kanser. Dalam kajian ini, asid valproik iaitu sejenis antikonvulsi yang menyekat saluran natrium bervoltan, telah digunakan untuk mengkaji kesannya terhadap motiliti dan viabiliti sel MDA-MB-231 dan ekpresi gen Nav1.5 dan nNav1.5 dalam sel MDA-MB-231. Ujian fungsi seperti ujian MTT dan ujian motiliti sisian telah dijalankan untuk mengkaji viabiliti dan motiliti sel dan ujian molekular iaitu reaksi rangkaian polimerase 'real-time' telah dijalankan untuk mengkaji expresi gen Nav1.5 dan neonatal Nav1.5. Ujian MTT menunjukkan penurunan dalam viabiliti sel MDA-MB-231 secara kepekatan bergantung. Trend yang sama dilihat dalam ujian motiliti sisian di mana motiliti sel MDA-MB-231 berkurang. Pengurangan tingkah laku metastatik (motiliti sisian) diikuti dengan penurunan expresi gen Nav1.5 dan neonatal Nav1.5 dengan kepekatan asik valproik yang tertinggi. Daripada penemuan kajian ini, ia boleh disimpulkan bahawa asid valproik mempunyai

potensi yang tinggi untuk digunakan dalam rawatan kanser payudara agresif dan penyelidikan secara mendalam perlu dilakukan untuk mengkaji kesan-kesan lain anti-metastatik asid valproik dalam kanser payudara.

THE EFFECTS OF VALPROIC ACID ON NAV1.5 GENE EXPRESSION IN MDA-MB-231 CELLS

ABSTRACT

Over the past few decades, breast cancer researches have been ongoing and new treatments are being discovered every year to improve patients' quality of life. However, metastasis remains largely as the cause of cancer mortality and metastatic-targeted therapies are being studied. In the highly metastatic human breast cancer cell line, MDA-MB-231, cardiac voltage-gated sodium channel Nav1.5 and its neonatal splice variant, nNav1.5 were found to be overexpressed and strongly linked to metastatic behavior of the cells. Previous studies have proved valproic acid's anti-tumor effects in different types of cancer cells. In this study, valproic acid, an anticonvulsant known to inhibit voltage-gated sodium channels, was used to study its effects on MDA-MB-231 cell motility and viability as well as the gene expression of Nav1.5 and nNav1.5 in MDA-MB-231 cells. Functional assays such as MTT and lateral motility assays were conducted to study the viability and motility of the cells. For molecular assay, real-time polymerase chain reaction was employed to study Nav1.5 and neonatal Nav1.5 gene expression. MTT assay showed a decline in MDA-MB-231 cell viability with increasing concentrations of valproic acid. The same trend was observed in lateral motility assay, which showed reduction in motility of MDA-MB-231 cells. The suppression of metastatic behavior (lateral motility) was followed by down-regulation of Nav1.5 and neonatal Nav1.5 gene expression with the highest concentration of valproic acid. From the findings, it can be deduced that valproic acid has

great potential to be included in aggressive breast cancer therapy and further research should be done to investigate its other anti-metastatic properties in breast cancer.

CHAPTER 1

INTRODUCTION

Over the last few decades, there has been great advancement in the development of effective treatments for breast cancer and improvement in the quality of life of women suffering from the disease. However, breast cancer tops as the most common cancer in women worldwide particularly in developing countries where most cases are diagnosed in late stages. The survival rates of breast cancer vary largely around the world and the low survival rates in less developed countries are due to the lack of diagnosis, early screening programmes and treatment facilities (World Health Organization, 2016). Based on a study conducted by Weigelt *et al.* (2005), it was shown that 90% of cancer deaths were resulted by metastases. Metastatic disease is invariably untreatable and there is a dire need to discover molecularly targeted anti-metastatic therapies.

The growing body of knowledge regarding metastatic processes has provided the basis for developing molecular targets against metastasis. Voltage-gated sodium channels (VGSCs) have been studied for the past few decades and they were found to promote the process of metastasis through various mechanisms. Therefore, they became the ideal target to control metastasis not only in breast cancer, but also in other types of cancer cells. Inhibitors of VGSCs are being discovered and many clinically available drugs are being repurposed to act on VGSCs. This can be a cost-effective measure to combat metastasis while saving time developing a new drug. Incorporating anti-metastatic drugs into clinical settings may help to reduce the mortality of patients succumbing to metastatic disease.

Rationale of study

The tetrodotoxin-resistant cardiac voltage-gated sodium channel, Nav1.5 has been shown to be highly expressed in MDA-MB-231 human breast cancer cells and strongly linked to its invasive and metastatic behavior. Previous studies have shown that treatments targeting VGSCs may help to inhibit metastasis of certain cancer cells. The anticonvulsant, valproic acid has been in cancer research in recent years and the effects on viability, motility and invasion of breast cancer cell lines were described in previous studies. Although its proposed mechanism of action on cancer cells was by inhibition of histone deacetylase, some studies also proved its role in blockade of voltage-gated sodium channels. Using valproic acid, inhibitions of gene expressions of voltage-gated sodium channel Nav1.5 and its neonatal splice variant were anticipated, subsequently reducing the VGSC-mediated metastasis MDA-MB-231 cells. Along with metastasis, the viability and motility of MDA-MB-231 cells were also expected to be suppressed.

Objectives

Main objective:

To study the effects of valproic acid (VPA) on Nav1.5 as well as neonatal Nav1.5 gene expression in MDA-MB-231 human breast cancer cells.

Specific objectives:

1. To determine the effects of valproic acid (VPA) on the viability of MDA-MB-231 cells.
2. To determine the effects of valproic acid (VPA) on the motility of MDA-MB-231 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a group of diseases characterized by uncontrolled division of abnormal cells in the body. It can involve any tissue of the body and named according to the organ that it affects. Cancers can be described by the type of cells that form them. For example, cancers arising from bone and soft tissues are known as sarcoma and those arising from epithelial cells are known as carcinoma. However, not all tumors are cancerous (National Cancer Institute, 2015). Tumors that are cancerous are known as malignant tumors where they can spread into or invade tissues whereas tumors that are non-cancerous are known as benign tumors and they do not spread into or invade tissues. Cancer cells can invade and destroy neighboring healthy tissues and organs. They may begin in one part of the body and spread to other areas through a process known as metastasis (National Health Service, 2014).

2.1.1 Hallmarks of cancer

There are six hallmarks of cancer. Unlike normal cells that control cell growth and release of growth factors, cancer cells are able to deregulate these signals and sustain chronic proliferation. Cancer cells are also able to elude tumor suppressors such as the p53 tumor-suppressor protein encoded by TP53 gene and Retinoblastoma protein (pRb) in human retinoblastoma. The third hallmark of cancer cells is the ability to resist apoptosis mainly by the loss of TP53 tumor suppressor function. Cancer cells also enable limitless replicative potential by maintaining their telomeres. 90% of cancer cells increase the production of

telomerase through which telomerases functions by adding telomeric DNA to the ends of chromosomes (Hanahan & Weinberg, 2011).

Sustained angiogenesis is another characteristic of cancer in which cancer cells are able to stimulate new blood vessel formation for their growth and survival. This provides oxygen, nutrients, growth factors and a pathway for tumor expansion, local invasion and dissemination. Besides that, cancer cells activate invasion and metastasis. Beginning with local invasion and intravasation into blood and lymphatic vessels, cancer cells transit though lymphatic and hematogenous systems and escape into parenchyma of distant tissues. There they form small nodules and finally grow into large tumors (Hanahan & Weinberg, 2011).

2.1.2. Causes of cancer

According to Anand *et al.* (2008), majority of cancers around 90% to 95% of cases are due to environmental factors and the remaining 5% to 10% of cases are due to inherited genetics. The common environmental factors that cause cancer mortality are diet and obesity, tobacco, infections, harmful radiations, stress, lack of physical activity and environmental pollutants. Specific chemicals which are linked to certain types of cancer are called carcinogens. Tobacco smoke contains over 70 carcinogens that include polycyclic aromatic hydrocarbons, lead, nitrosamines, arsenic, cadmium and vinyl chloride. 90% of lung cancer cases are caused by tobacco smoking (Kuper, 2002).

Cancer occurs when a cell undergoes genetic alterations, causing inability of the cell to correct the damages and undergo apoptosis. This produces an abnormal protein which provides information different compared to normal protein and cause cells to divide

uncontrollably and become cancerous. Mutations are normally corrected by the body. The most common cause of cancer is acquired or sporadic mutations. Smoking tobacco, high exposure to ultraviolet radiation, viruses and age cause these mutations and the risk increases with age. Since the mutation is not present in zygote, it is acquired later in life. In contrast with acquired mutations, germline mutations are less common and accounts for 5% to 10% of all cancers. They are inherited from parents to children. The mutation is present in egg or sperm that forms the child and once fertilization occurs, it creates a zygote where there are mutations present in all cells including germ cells (American Society of Clinical Oncology, 2016; Canadian Cancer Society, 2016).

The two main genes that play a critical role in cancer are proto-oncogenes and tumor suppressor genes. Proto-oncogenes are a group of genes that encode for proteins involved in cell growth and regulation but once they are mutated, they cause normal cells to become cancerous. The mutated proto-oncogenes are known as oncogenes manifest increased cell division, decreased cell differentiation and inhibition of apoptosis. Tumor-suppressor genes encode proteins for inhibition of cell proliferation. The loss-of-function mutations in tumor-suppressor genes lead to cancer formation (Chial, 2008; Lodish *et al.*, 2000).

Apoptosis is programmed cell death where cells commit suicide when they are no longer needed by activation of intracellular death program. A cell that undergoes apoptosis dies without damaging the neighboring cells, in contrast to necrosis which is potentially damaging, dangerous and leads to inflammatory response. Apoptosis is mediated by caspases which are proteolytic enzymes. Caspases exist as precursors in every cell and are activated by other caspases. They trigger cell death by cleavage of proteins in cytoplasm and nucleus of cells (Alberts *et al.*, 2002).

2.1.3. Symptoms of cancer

The general symptoms of cancer are unexplained weight loss and pain, a new lump, prolonged cough, bleeding and loss of appetite. These symptoms may indicate cancer but they may also manifest due to other health conditions. The specific signs and symptoms caused by cancer will vary depending on the body part affected. Cancer may also cause symptoms like fever, breathlessness and extreme tiredness as well as large or irregular moles (National Health Service, 2014).

2.1.4. Statistics

According to World Health Organization (2015), in 2012, there was 14.1 million of new cancer cases reported worldwide. The number of deaths due to cancer was 8.2 million and in 2012, the most common cancers reported globally were lung cancer (13%), breast cancer (12%), colorectal cancer (10%), prostate cancer (8%), stomach cancer (7%), liver cancer (6%) and cervical cancer (4%) (Figure 2.1). Lung, stomach, liver and colorectal cancer are the most common causes of cancer mortality worldwide where lung cancer accounts for the most cancer deaths.

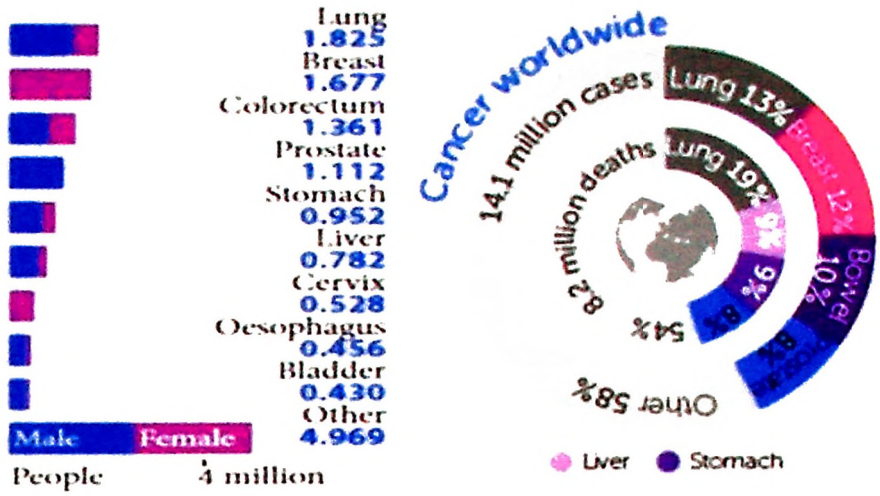

Figure 2.1: **Rate of incidence of cancer worldwide in 2012.** Number of new cases in millions (left). Percentage of new cases (right) (World Health Organization, 2014)

2.1.5. Metastasis

Metastasis is the spread of cancer from the primary site to other parts of the body. Cancer metastasis is largely responsible for the mortality and morbidity of cancer. Usually, metastases occur in the late stages of cancer and common sites of metastasis are lungs, liver, brain and bones. The metastatic cascade is divided into three stages, invasion, intravasation and extravasation. At the stage of invasion, the dissociation of malignant tumor cells from the primary mass is due to the loss of cell-to-cell adhesion capacity. Endopeptidases called matrix metalloproteinases (MMP) are released to degrade extracellular matrix and basement membrane for the cells to breach the extracellular matrix and escape. The cells invade surrounding stroma due to changes in cell-matrix interaction. Angiogenesis occurs for the tumor cells to obtain nutrients and remove waste products. In intravasation stage, the nearby blood vessels provide passage for detached cells to

metastasize to distant areas. They interact biochemically with endothelial cells and adhere strongly to endothelial cells. The tumor cells then penetrate the endothelial cells and basement membrane into the lumina of blood vessels. The cells survive in the vasculature during their transport and arrest at distant organ sites. They extravasate into the parenchyma of distant tissues and form micrometastases. This process is followed by proliferation of the cells, forming macroscopic and clinically detectable metastases (Tracey *et al.*, 2013; Valastyan & Weinberg, 2011).

2.2. Breast cancer

Breast cancer is a type of malignant tumor that develops from breast tissue. If left untreated, it may spread to the other parts of the body. The disease mostly occurs in women, but can occur in men to a lesser extent.

2.2.1. Breast structure and function

The breast is composed of glandular, ductal, connective and adipose tissue. Fats and lobules are embedded in fibrous tissue and makes up mammary glands, which are the accessories for reproduction in women but rudimentary and functionless in men. Lobules are milk producing glands and ducts are the milk passages that connect the lobules and nipples. Fats and connective tissues protect and give shape to the breasts while areola contains sweat glands which secrete moisture as lubricant during breastfeeding (Figure 2.2). The breast's main function is to produce, store and release milk for breastfeeding. The hormone estrogen is involved in breast development and stimulation of the growth of milk ducts. Progesterone prepares the breasts for lactation and stimulates the lobules (Canadian Cancer Society, 2016).

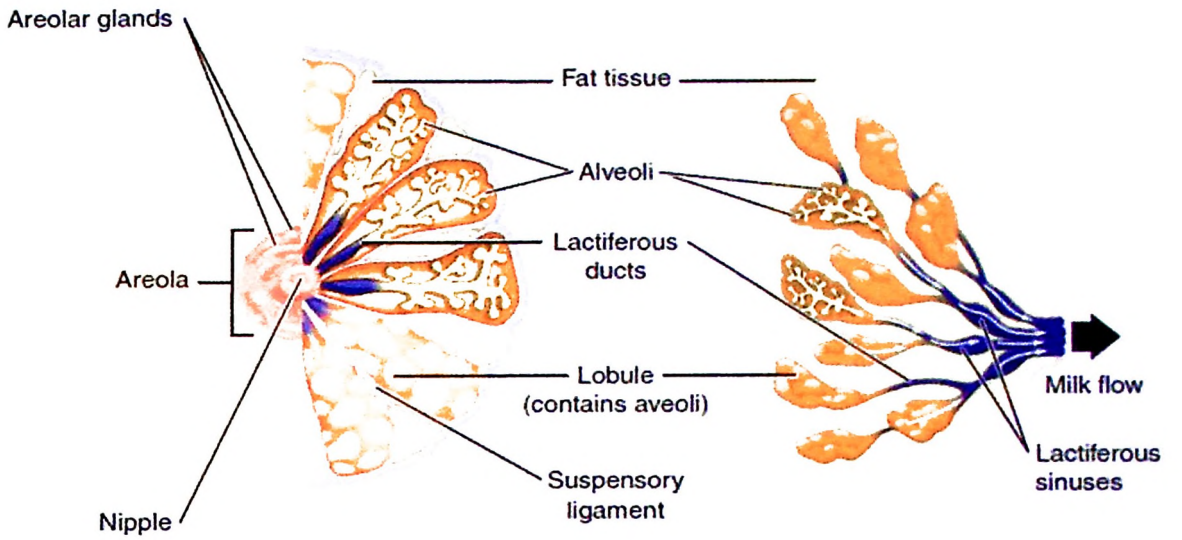

Figure 2.2: **The anatomy of breast** (OpenStax College, 2016)

2.2.2. Classification of breast cancer

Breast cancer can be classified according to several aspects such as histopathology, stage, grade, receptor status and DNA-based classification. Normally, breast cancer is classified according to its histopathology which is based on the characteristics seen under light microscope. It can be categorized into lobular carcinomas and ductal carcinomas. Lobular carcinomas are cancers developing from milk ducts and ductal carcinomas are cancers developing from lobules of the breast. *Carcinoma in situ* is low grade cancer cells or precancerous cells within breast tissue that stays in the place of origin and do not spread to the neighboring tissues. Invasive carcinoma or infiltrative carcinoma is where the cells invade into surround tissues (American Cancer Society, 2014).

The grading of breast cancer relies on similarity of breast cancer cells to normal breast tissue. This classifies the cancer as well differentiated (low-grade), moderately differentiated (intermediate-grade) and poorly differentiated (high-grade). The grading criteria are tubule formation, nuclear polymorphism and mitotic count. Lower-grade tumors are treated less aggressively whereas high-grade tumors are treated more aggressively (American Cancer Society, 2014).

The staging system that is usually used for breast cancer is called tumor, node and metastasis (TNM). It takes into account the size of tumor, whether the cancer has invaded into lymph nodes and whether the cancer has spread to other parts of the body. Stages 0, I, IIA, IIB, IIIA, IIIB, IIIC and IV describes the tumor according to the criteria of staging (Canadian Cancer Society, 2016). Another classification of breast cancer is based on receptor status. Estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) are stained using immunohistochemistry technique, identified and often used for therapeutic and prognostic decision making. There are 8 combinations of receptor subtypes and triple negative (ER/PR-, HER2-) breast cancer has been found to have the worst overall and survival prognosis because they do not respond to hormonal therapies and HER2-targeted therapies due to absence of these receptors (Onitilo *et al.*, 2009).

The traditional DNA classification of breast cancer was based on the theory in which the cells that divide quickly have worse prognosis. Other criteria include presence of certain proteins such as Ki-67 and percentage of cells in the S phase. Modern classifications are mainly on the ploidy of cancer cells and gene patterns.

2.2.3. Statistics

Breast cancer is the most commonly diagnosed cancer among women in 140 of 184 countries worldwide. It represents one in four of all cancers in women. Since 2008, incidence of worldwide breast cancer has increased by more than 20% and mortality has increased by 14%. 1 in 8 women is expected to develop breast cancer during their lifetime. It is estimated that around 522000 women died from breast cancer in 2012 worldwide, with mortality rates varying around the world. When diagnosed early, around all women with breast cancer survive for five years or more compared to 3 in 20 women when diagnosed in late stage. Breast cancer in men is rare and the risk of getting breast cancer is about 1 in 1000 (World Health Organization, 2016).

2.2.4. Signs and symptoms

The initial noticeable symptom of breast cancer is a lump that feels different from the other parts of the breast. The lumps might be also found in lymph nodes located in the armpits. Other symptoms include one breast becoming lower and larger, skin dimpling or puckering, change in nipple position or shape or nipple becoming inverted. Thickening of the skin is called orange peel skin or *peau d'orange*. Besides that, tenderness and rashes on or around nipple, milky or bloody discharge from nipple, constant swelling and pain beneath armpit and breast may also indicate breast cancer (Watson, 2008).

2.2.5. Risk Factors

The potential modifiable risk factors associated with breast cancer are physical inactivity, alcohol consumption, weight gain after age of 18, obesity, long-term heavy smoking, use of menopausal hormone therapy and night shift work that disrupts sleep pattern. The non-

modifiable factors that potentially cause increased breast cancer risk include personal or family history of breast or ovarian cancer, older age, type 2 diabetes, inherited mutations in *BCRA1* and *BCRA2* or other breast cancer susceptibility genes and benign breast conditions. Besides that, high-dose chemotherapy to the chest at young age, high bone mineral density and high breast tissue density, long menstrual history, having first child after the age of 30, high levels of sex hormones, recent use of oral contraceptives and never having children also contribute to non-modifiable risk factors of breast cancer (American Cancer Society, 2016).

2.3. MDA-MB-231

MDA-MB-231 is a highly metastatic breast cancer cell line established from a pleural effusion of a 51 year old woman with metastatic breast cancer. The cell line was obtained in 1973 at M. D. Anderson Cancer Center. It is a breast adenocarcinoma which is triple negative for estrogen receptor, progesterone receptor and HER2 and has epithelial-like morphology that appears phenotypically as spindle-shaped. The cells have invasive phenotype *in vitro* (American Type Culture Collection, 2014; Holliday & Speirs, 2011).

2.4. Voltage-Gated Sodium Channels (VGSCs)

2.4.1. Structure and function

Voltage-gated sodium channels are heteromeric macromolecular protein complexes that contain a pore-forming α-subunit and smaller non-pore-forming β-subunits (Figure 2.3). The α-subunit has four domains (DI – DIV) and each domain has six membrane-spanning

segments (S1 – S6). The highly conserved S4 segment functions as voltage sensor. Each α-subunit is approximately 260 kDa. The β-subunits are type 1 transmembrane glycoproteins with extracellular N-terminus and cytoplasmic C-terminus of approximately 35 kDa each (Catterall, 2000).

The α-subunit contains nine isoforms, Nav1.1-Nav1.9 which are encoded by genes *SCN1A-SCN11A* and there are four β subunits, β1-β4 which are encoded by genes *SCN1B-SCN4B*. Both the α-subunit and β-subunits are believed to interact with other signaling molecules which enable adjustments of the channel's activity. The β-subunits isoforms Nav1.1 to Nav1.9 are proteins and vary in functional form of α-subunit. A unique α-subunit Na_X was recently recognized. First classification of sodium channels categorized them into two groups, either sensitive or resistant to the potent puffer fish neurotoxin, tetrodotoxin (TTX) (Savio-Galimberti *et al.*, 2012).

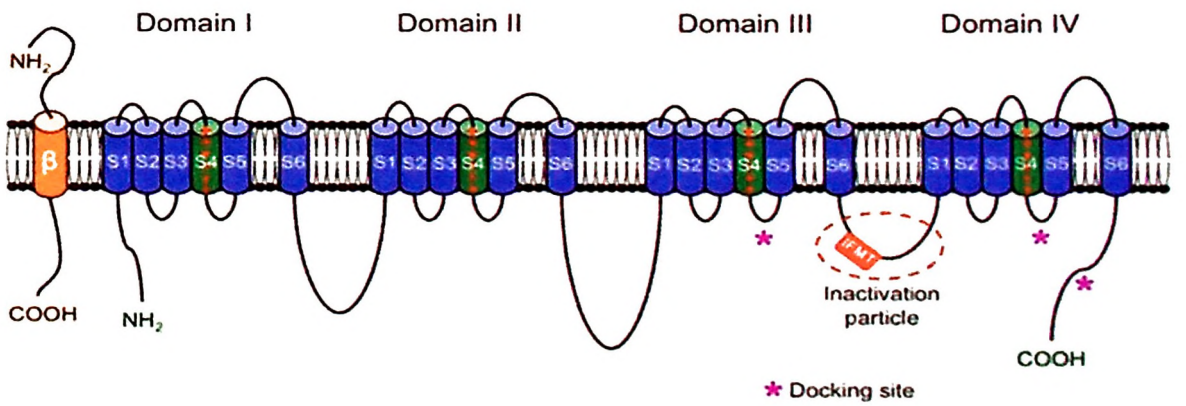

Figure 2.3: **Schematic representation of voltage-gated sodium channel** (Savio-Galimberti *et al.*, 2012).

TTX-resistance is characteristic for isoforms Nav1.5, Nav1.8 and Nav1.9 whereas Nav1.1, Nav1.4, Nav1.6 and Nav1.7 are sensitive to TTX in nanomolar concentrations (Waszkielwicz *et al.*, 2013). Voltage-gated sodium channels have been discovered to play an important role in excitable cells such as neurons and cardiomyocytes where they are responsible for action potential initiation and conduction. Certain excitability-related pathologies such as neuropathic pain, cardiac arrhythmia and epilepsy are linked to the abnormalities in functions of voltage-gated sodium channels (Mantegazza *et al.*, 2010).

2.4.2. Relationship with metastasis

Apart from excitable cells, voltage-gated sodium channels are also found to be expressed in non-excitable cells such as glial cells, osteoblasts, fibroblasts, endothelial, immune cells with unknown role (Diss *et al.*, 2004). Surprisingly, VGSCs were found in various carcinomas, such as breast cancer (Brackenbury *et al.*, 2007; Fraser *et al.*, 2005) and prostate cancer (Diss *et al.*, 2004). A unique fast inward sodium current (I_{Na}) was found in MDA-MB-231 cells which was missing in weakly invasive MDA-MB-468 and MCF-7 cells and it was suggested that I_{Na} was involved in invasive activity of MDA-MB-231 through its participation in regulation of intracellular sodium homeostasis (Roger *et al.*, 2003). VGSCs expression was significantly up-regulated in metastatic human breast cancer cells and their activity potentiated cellular directional motility, invasion and endocytosis (Fraser *et al.*, 2005). VGSC β subunits have been detected in prostate, breast, lung and cervical cancers (Brackenbury, 2012; Patel & Brackenbury, 2015). According to Brackenbury (2012), VGSC expression has been found to be the highest in breast, bowel and prostate cancers.

2.4.3. Nav1.5 and neonatal Nav1.5

Nav1.5 is an isoform of voltage-gated sodium channel that is tetrodotoxin-resistant. It is found primarily in cardiomyocytes, responsible for cardiac action potential and impulse propagation through the heart. It mediates fast influx of sodium ions across the cell membrane, causing depolarization. *SCN5A* is the gene that encodes Nav1.5 and it is a highly conserved gene (Catterall, 2014). Nav1.5 has been shown to influence Src kinase activity, cortactin phosphorylation (Y421) and polymerization of actin filaments which in turn promotes the acquisition of invasive morphology in MDA-MB-231 cells and acts as central regulator of invadopodia formation (Brisson *et al.*, 2013).

Gao *et al.* (2009) deduced that the expressions of Nav1.5 mRNA and protein were significantly increased in MDA-MB-231 compared to lowly metastatic MCF-7 cells and the invasive property of MDA-MB-231 was potentiated by Nav1.5 by stimulating secretion of proteolytic enzyme matrix metalloprotease (MMP-9). In highly metastatic MDA-MB-231 human breast cancer cells, the activity of Nav1.5 acts on the invasion of extracellular matrix by increasing the activity of Na^+/H^+ exchanger type 1 (NHE-1) in caveolae, subsequently enhancing proton efflux and proteolysis of acidic cysteine cathepsins B and S which are released extracellularly. Cysteine cathepsins are related to papain which are involved in intracellular degradation of endocytosed proteins, antigen presentation, bone resorption and enzyme or hormone maturation (Brisson *et al.*, 2011; Gillet *et al.*, 2009). In a study carried out by Busco *et al.* (2010), NHE1 was shown to be localized in invadopodia, causing acidification and extracellular matrix degradation.

The neonatal splice variant of Nav1.5 was found to be predominant VGSC in metastatic breast cancer, *in vitro* and *in vivo* (Fraser *et al.*, 2005). Neonatal Nav1.5 is primarily accountable for the invasive behavior manifested by voltage-gated sodium channels in MDA-MB-231 cells and aiming for reduction in neonatal Nav1.5 activity may help in clinical management of breast cancer (Brackenbury *et al.*, 2007). The molecular difference between the adult and neonatal isoforms of Nav1.5 is a stretch of 7 amino acids (Figure 2.4). Compared to the adult isoform of Nav1.5, the neonatal variant demonstrated depolarized threshold of activation and voltage to peak, much slower kinetics of activation and inactivation, around 50% greater transient charge sodium influx, slower recovery from inactivation and larger persistent sodium currents (Djamgoz & Onkal, 2010; Onkal & Djamgoz, 2009)

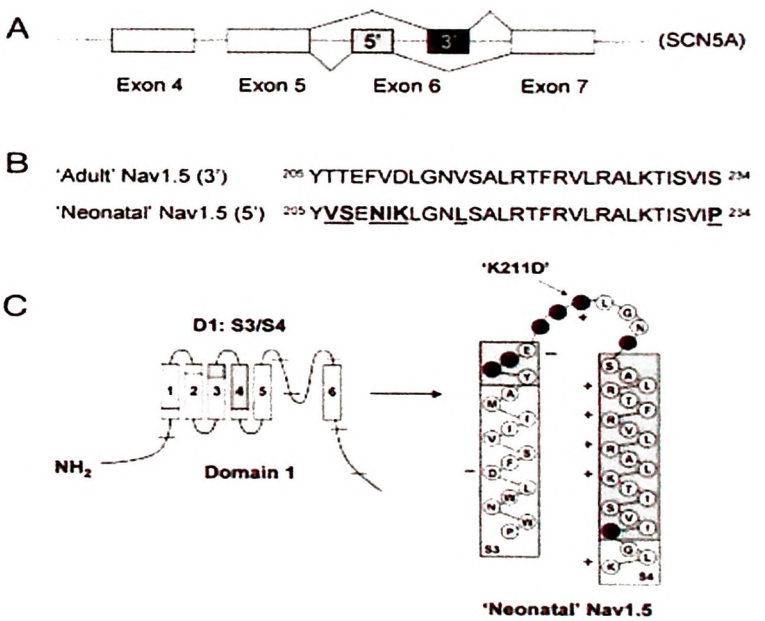

Figure 2.4: **Differences between nNav1.5 and Nav1.5.** A. Schematic diagram of *SCN5A* gene showing 5'- (neonatal) and 3'- (adult) exon 6. B. Altered amino acids in bold. C. Predicted locations of difference in amino acids in D1:S3. (Onkal & Djamgoz, 2009)

2.5. Valproic acid

2.5.1. Background

Valproic acid, also known as valproate, sodium valproate and divalproex sodium is a chemical derived from valeric acid which is naturally produced by *Valeriana officinalis* plant. It is a branched short-chain fatty acid. Valproic acid is generally used as first-line anticonvulsant agent and also used for treating depression, absence seizures, tonic-clonic seizures, complex partial seizures, juvenile myoclonic epilepsy, schizophrenia, migraine headaches, seizures related to Lennox-Gastaut syndrome and mood stabilizer. It can be given orally or intravenously (American Society of Health-System Pharmacists, 2016). Valproic acid alters the activity of gamma-aminobutyrate (GABA) by increasing synthesis of GABA, inhibiting GABA transaminobutyrate and degradation of GABA. It also reduces N-Methyl-D-Aspartate-mediated excitation and blocks voltage-gated sodium, calcium and potassium channels (Chateauvieux *et al.*, 2010). Other effects of valproic acid include inhibition of protein kinase C and decreased turnover of brain arachidonic acid (Jann *et al.*, 2016).

2.5.2. Valproic acid and cancer therapy

Valproic acid was found to interfere with multiple processes in cancer cells which includes induction of apoptosis and differentiation, inhibition of angiogenesis, invasion, tumour-associated inflammation and proliferation as well as stimulation of anti-cancer immunity (Michaelis *et al.*, 2007). Recently, valproic acid has been found to inhibit histone deacetylase (HDAC) which made a breakthrough in cancer therapy. The inhibition of

histone deacetylase promotes formation of decondensed chromatin which is an active form for transcription and promotes the expression of genes. The proteins that are produced by the genes are found to be crucial in cellular activity, cell cycle control, DNA repair, differentiation and apoptosis (Chateauvieux *et al.*, 2010). Valproic acid seems to exhibit its anti-tumor activity through the inhibition of histone deacetylase rather than blockade of VGSC activity (Koltai, 2015). However, studies found that valproic acid inhibits voltage-gated sodium channels in use-dependent manner (McLean & Macdonalds, 1986; Golan *et al.*, 2011). According to Waszkielewicz *et al.* (2013), anticonvulsants such as valproic acid and phenytoin act on voltage-gated sodium channels. Large *et al.* (2009) reported that valproic acid reduced sodium currents in recombinant human Nav1.2 channels in brain.

Grabarska *et al.* (2014) concluded that valproic acid treatment on larynx cancer cells has resulted in suppression of proliferation and migration, increase in apoptosis and cell cycle arrest. Moreover, valproic acid potentiates anticancer activity of cisplatin on larynx cancer cells at low doses. Montgomery *et al.* (2004) reported reduction in ovarian cancer cell line proliferation with valproic acid. Other clinical studies have also demonstrated the chemotherapeutic properties of valproic acid in treatment of acute myeloid leukemia and myelodysplastic syndromes (Kuendgen & Gattermann, 2007) as well as solid breast and cervix tumors (Duenas-Gonzalez *et al.*, 2008). As for breast cancer, Li *et al.* (2012) proved that valproic acid inhibits MDA-MB-231 cells migration by up-regulating *NM23H1* expression which is a metastatic suppressor gene. Surprisingly, valproic acid was found to be able to induce estrogen-sensitive phenotype in the ER-negative MDA-MB-231 cells, which may open up chances of using anti-estrogen therapy on these cells (Fortunati *et al.*, 2008). The growing body of evidence that shows valproic acid's effects on various types of

cancer cells proved that valproic acid has other anti-cancer and anti-metastatic attributes that can be uncovered.

CHAPTER 3

METHODOLOGY

3.1 Cell line

MDA-MB-231, the highly aggressive human breast cancer cells were acquired from American Type Cell Culture (ATCC). The cells were observed under phase-contrast microscope (Plate 3.1).

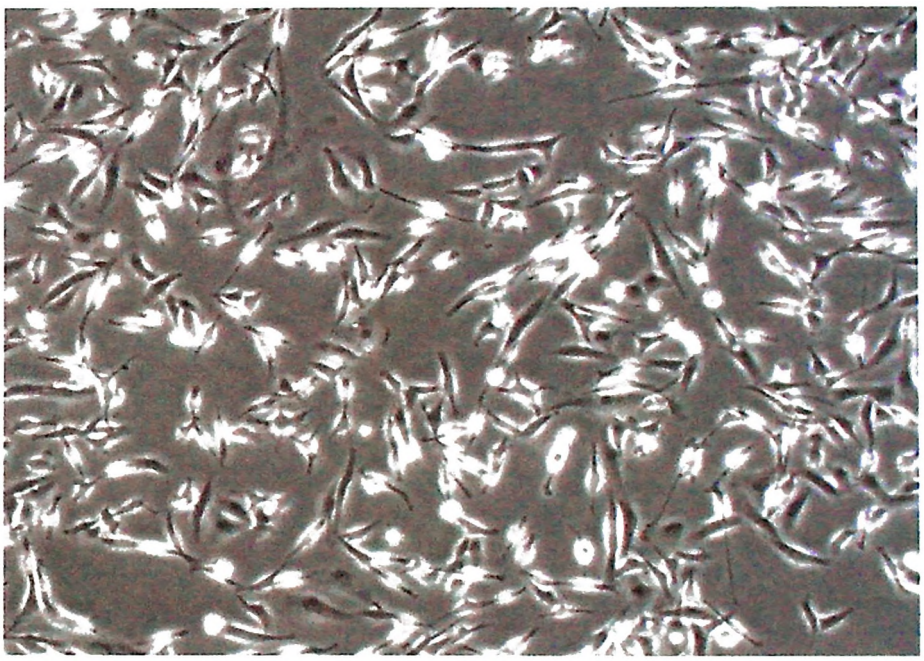

Plate 3.1: Photomicrograph of MDA-MB-231 cells (400x).

3.2. Aseptic techniques

The experiments and procedures were carried out under aseptic and sterile environment to avoid contamination of culture and reagents as well as equipments. Personal protective equipments like sterile gloves and lab coat were used. The working areas such as biosafety

cabinet type 2 and bench were disinfected with 70% ethanol prior to work and after work. The pipette tips and glassware brought into the biosafety cabinet were sterilized in autoclave. The cells, reagents and pipette tips were discarded in a beaker containing bleaching solution.

3.3. Preparation of Complete Medium

500ml of low glucose Dulbecco's Modified Eagle Medium (Nacalai Tesque, Japan) was supplemented with 25ml of 5% Fetal Bovine Serum and 10ml of 4mM L-glutamine (Life Technologies, USA). 5ml of 1% Penicillin/Streptomycin (Life Technologies, USA) was then added to the solution.

3.4. Subculture and Freezing of MDA-MB-231 Cells

3.4.1. Subculture of Cells

The MDA-MB-231 human breast cancer cells were maintained in complete medium in CO_2 incubator. 1ml of trypsin (Life Technologies, USA) was added to detach the cells from the culture dish and the dish was incubated for three minutes in CO_2 incubator. 1ml of complete medium was added to inactivate the trypsin. The cells were mixed and transferred into a falcon tube. The falcon tube was centrifuged at 1.5 rpm for three minutes. The supernatant was discarded and 1ml of Phosphate Buffer Solution was added into the falcon tube. The tube was centrifuged again at 1.5 rpm for three minutes and the supernatant was discarded. The cells were then mixed with 1ml of complete medium. 5ml of complete medium was added into a new 100mm culture plate. The mixed cells were added into the 100mm culture plate uniformly and spread. The culture dish was labeled with cell name,

passage number and date and incubated at 37°C in a humidified 5% CO_2 incubator until use.

3.4.2. Freezing of Cells

The cells not to be used anymore were pipetted into labeled cryovials. Cell reservoir with DMSO (Nacalai Tesque, Japan) was added into the cryovials and kept at 4°C for 15 minutes. Then, the cryovials were kept at -20°C for 1 hour and later at -80°C until use.

3.5. Cell plating

The 150mm culture dish containing subcultured cells was taken out from the incubator and the medium was discarded. 5ml of trypsin was added to detach the cells from the bottom and the culture dish was kept in CO_2 incubator for three minutes. 5ml of complete medium was added to inactivate the trypsin and the cells were mixed and transferred to a falcon tube. The falcon tube was centrifuged at 1.5 rpm for three minutes and the supernatant was discarded. 1ml of Phosphate Buffer Saline was added and centrifuged at 1.5 rpm for three minutes. The supernatant was discarded and mixed with 1ml of complete medium. 10µl of cells was loaded into haemocytometer and cell count was done under the microscope. The required volume of cell suspension for the assay is calculated using formula:

$$\frac{\text{Number of cells required to plate per ml}}{\text{Total number of cells in 4 gridded areas} \div 4 \times 10^4}$$

3.6. Preparation of Valproic Acid

Valproic acid was prepared in three concentrations. Using formula $M_1V_1 = M_2V_2$, the amount of valproic acid and complete medium required was calculated. 6.6mg of valproic acid (InvivoGen, USA) was weighed and dissolved in 4ml of complete medium to make 8mM of valproic acid. The solution was filtered using sterile filter into 1.5ml Eppendorf tube. 1.87ml of the 8mM valproic acid was pipetted into 0.63ml of complete medium to make 6mM of valproic acid. 0.67ml of the solution was pipetted into 1.33ml of complete medium to make 2mM of valproic acid. Valproic acid was prepared freshly before each assay and stored in -20°C until use.

3.7. Functional assays

3.7.1. MTT assay

For MTT assay, 96-well plate was used and the assay was conducted in triplicates. 3×10^4 of cells were plated each well. 100µl of complete medium was added into each well and incubated overnight. The next day, the complete medium was discarded and 100µl of valproic acid was added for each concentration. 100µl of complete medium only was added for control. After 24 hours of treatment, the medium was discarded and replaced with 100µl of complete medium. 10µl of 12mM MTT solution (Invitrogen, USA) was added into each well and 10µl of MTT solution was added to 100µl of complete medium only as negative control. The plate was incubated for 4 hours. After 4 hours, 85µl of medium was removed from each well and 50µl of DMSO was added to each well to inactivate the MTT solution. The plate was incubated for 10 minutes and the absorbance was read at 540nm using ELISA reader.