5-2014

Investigation of Rice Bran Derived Anti-cancer Pentapeptide for Mechanistic Potency in Breast Cancer Cell Models

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

by

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Bachelor of Science in Food Science and Engineering, 2011

May 2014
University of Arkansas

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ABSTRACT

Bioactive peptides derived from food sources with anti-proliferative properties against cancer have drawn more attention in recent years. A pentapeptide derived from rice bran has shown anti-proliferative propertiesagainst human breast cancer cells. The objective of this study was to investigate the mechanistic action of the pentapeptide-induced apoptosis in breast cancer cell models (MCF-7 and MDA-MB-231). The growth inhibition activity of the pentapeptide was evaluated by MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assayand trypan blue assay in a dose- and time-dependent manner. The apoptotic properties of pentapeptide-induced apoptosis on cancerous breast cells were evaluated by morphological changes, DNA fragmentation, and caspases-3/7, -8,and -9 activities. The levels of molecular targets (p53, COX-2, TNF-α, Fas, Bax, Bcl-2, and ErbB-2) were evaluated by enzyme-linked immunosorbent assay (ELISA) kits. Pentapeptide showed growth inhibition activities on MCF-7 and MDA-MB-231 cells. Apoptotic features including morphological changes,DNA fragmentation, and caspases activation were observed in both cells lines after pentapeptide treatment. Decreased levels of COX-2, Bcl-2, and ErbB-2 and increased levels of p53, TNF-α, Fas, and Bax expression were detected after cells exposed to pentapeptide from 72 to 96 hr. The results suggest that the pentapeptide inhibits growth of human breast cancer cells by introducing apoptosis through a caspase-dependent pathway. The pentapeptide amplifies the death signal by down-regulating the expression of ErbB-2 in both cell lines and COX-2 in ER (Estrogen Receptor)-positive MCF-7 cells. This study provides insight on the molecular mechanism of action of the pentapeptide against breast cancer cells. After further animal and human clinic trial, the pentapeptide has the potentiality to be analternative strategy to current anti-cancer drugs.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor Prof. Navam Hettiarachchy for the continuous support of my Master's study and research, for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me throughout my research and writing of this thesis.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Kristen E Gibson, Dr. Mahendran Mahadevan,and Dr. Jackson Lay Jr., for their encouragement, insightful comments, and hard questions.

I appreciate being given access to the Biomass Center, University of Arkansas in orderto conduct cell line research.I thank my fellow labmates in the department of Food science: Dr. Ees Eswaranandam, Dr. Arvind Kannan, and Jay Rayaprolu, for the discussions and the technical support in the last two years. I specifically thank to Dr. Arvind Kannanand Madhuram Ravichandran for cell culture technique training.

Last but not the least, I would like to thank my family and friends for making this possible and for making me pursue a higher education of my interest.

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LIST OF ACRONYMS AND ABBREVIATIONS

The following table describes the significance of various abbreviations and acronyms used throughout the thesis. The page on which each one is defined or first used is also given.

Abbreviation	Meaning	Page
PER	Protein Efficiency Ratio	1
kDa	Kilodalton	2
HDRB	Heat Stabilized De-Fatted Rice Bran	9
ER	Estrogen Receptor	12
TNF-α	Tumor Necrosis Factor-Alpha	13
PCD	Programmed Cells Death	15
Caspase	Cysteine-Dependent Aspartate-Directed Proteases	16
Bcl-2	B-Cell Lymphoma 2	16
IAP	Inhibitor Of Apoptosis Proteins	16
NF-κB	Nuclear Factor-Kb	16
MAPK	Mitogen-Activated Protein Kinase	16
Cox-2	Cyclooxygenase	18
PG	Prostaglandin	20
MTS	3-(4,5-Dimethylthiazol-2-Yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2h-Tetrazolium, Inner Salt	28
HMEC	Human Mammary Epithelial Cells	28
ATCC	American Type Culture Collection	31
BPE	Bovine Pituitary Extract	31
FBS	Fetal Bovine Serum	31
EDTA	Ethylenediaminetetraacetic Acid	31
LSD	Least Significant Difference	35
TUNEL	Terminal Deoxynucleotidyl Transferase Dutp Nick End Labeling	45
PI	Propidium Iodide	45
CAD	Caspase-Activated Dnase	52
ELISA	Enzyme-Linked Immunosorbent Assay	65
FADD	Fas-Associated Death Domain	72
DISC	Death-Inducing Signaling Complex	74

INTRODUCTION

Rice (Oryza sativa) is one of the most important staple foods. In the United States, the production of rough rice was 265.9 million cwt in 2013 with 130.1 million cwt produced in the state of Arkansas (National Agricultural Statistics Service 2013). Rice bran is the aleurone layer of the brown rice kernel. During the production of white rice, bran is removed from the kernel by friction. Bran accounts for about 10% of rough rice (Parrado et al 2006).

Traditionally, rice bran is an underutilized component of rice primarily used as a livestock feed supplement and natural oil extraction source in food industry. Protein is an important component in rice bran. Rice bran protein accounts for 12-16% of total weight, along with dietary fiber (10-27%), and lipid (12-23%) (Wiboonsirikul et al 2008). Among all of the cereal bran derived proteins, rice bran protein has a unique nutritional value due to its high protein contentand high protein efficiency ratio (PER) of 2.0 to 2.5 compared with casein at 2.5 (Burks and Helm 1994).

In western countries, breast cancer is the leading cause of cancer death in women causing of over 39,990 deaths in 2013 (Cancer Facts & Figures 2013). Various treatments, such as chemotherapy, radiation, and immunotherapy have been applied to kill breast cancer cells by inducing apoptosis (Debatin 2000). Bioactive peptides derived from food sources with anti-cancer proliferation properties have increasingly drawn attention. Hernandez et al (2009) demonstrated that lunasin (i.e. a novel and promising chemopreventive peptide derived from soybean, wheat, barley, and other plant seeds) showed anti-breast cancer proliferative properties.In recent years, hydrolysates and peptides derived from rice bran protein have received considerable attention due to the discoveries of some specific anti-disease activities such as anti-oxidative, anti-mutagenic, and anti-carcinogenic properties (Adebiyi et al 2009;

Renuka et al 2007; Chanput et al 2009). Kannan et al (2010) isolated a pentapeptide from rice bran with potential anti-proliferation activity against several cancers in tissue culture. Those studies showed that anti-cancer proliferative agents derived from foods maybe promising new candidates for breast cancer therapy.

Kannan et al (2009, 2010) reported that rice bran hydrolysates (<5 kDa fraction) prepared by enzymatic hydrolysis showed 70-80% anti-proliferative activity on human breast cancer cells. A novel pentapeptide with a Glu-Gln-Arg-Pro-Arg sequence was purified from this fraction and showed nearly 80% anti-proliferation abilities on breast cancer cells lines (MCF-7 and MDA-MB-231) (Kannan et al 2010). However, the mechanism of action in breast cancer cell models remains to be investigated.

In the present study, the pentapeptide derived from rice bran with anti-proliferative activity against breast cancer celllines (MCF-7 and MDA-MB-231)was studied to determine its mechanistic action with following specific objectives: 1)to determine the anti-proliferative effect of pentapeptide on human breast cancer cell lines; 2) to investigate thepentapeptide-induced apoptotic featureson human breast cancer cell lines; 3) to investigate the apoptotic pathways by evaluating levels of molecular targets in pentapeptide treated cancerous breast cell lines.

LITERATURE REVIEW

Food as Medicine

According to the national vital statistics reports in 2013, the leading cause of death in the U.S. is heart disease, which accounted for 24.1% of total deaths, followed by malignant neoplasms (cancer), chronic lower respiratory diseases, and cerebrovascular diseases with percentages of 23.3%, 5.6%, and 5.3%, respectively (Kochanek et al 2013). Aging has become a global issue due to the rise of life expectancies and the improvement of medical conditions. Obesity is also a critical health issue throughout the world. Approximately one in six adults are obese, and more than 48.8% of adults in U.S. are overweight or obese (Overweight & Obesity 2013).

Munoz and Chavez (1998) reported that populations with plant food as a large proportion of their diets tend to have a lower incidence of cancer. Other studies also indicate that most diseases are preventable and could be minimized by developing a healthy lifestyle, which includes a proper diet and regular physical activity. Epidemiological studies have shown that some flavonoids and other polyphenols in wine or other alcoholic beverages, fruits, and vegetables can reduce the risks of heart diseases (St Leger et al 1979, Yano et al 1977, Knekt et al 1996). Another recent epidemiology study showed that high fiber and unsaturated fatty acids in fruits, vegetables, legumes, whole grains, and fish have protective roles in preventing different cancers and cardiovascular diseases. Arnason et al (1981) reported phytochemical constituents of plants native to eastern Canada and indicated that at least 105 plants are considered medically effective, based on their bioactive compounds such as monoterpenes, polyacetylenes, alkaloids, and astringent tannins. Those studies point to potentially significant applications of natural food compounds with pharmacological properties to be used as medicine. There is a growing interest in functional foods because of the promotion of healthy eating habits in public education. Based

on their own physical condition, consumers can optimize the healthiness of their diet by eating foods which have been formulated or fortified with specific health-promoting nutraceuticals. More research is needed in order to uncover the nutritional constituents and underlying mechanisms for potential health benefits.

Bioactive Compounds in Foods

"Bioactive compounds" are defined as food constituents with extranutritional value and are usually present in small quantities (Kris-Etherton et al 2002). Identification, isolation, and characterization of bioactive compounds in foods have been intensively studied. In recent years, impressive progress has been made not only in identifying novel bioactives, but also in configuring the biological mechanisms of bioactive compounds to reduce the risk of major chronic diseases. Table I lists some well known and intensively studied bioactive compounds from natural food sources such as fruits, vegetables, cereals, oils, and animal products.

Major bioactive compounds in plant foods include phytochemicals, phenolic compounds, flavonoids, and isoflavonoids, which have been proven to be effective agents with anti-carcinogenic properties (Kitts 1994). Another group of important plant bioactives is organosulfur compounds, which is mostly present in onions (ANiurn cepa L.) and garlic (Allium sativurn L.). Organosulfur compounds have shown several extranutritional properties such as anti-carcinogenicity, anti-mutagenicity, anti-thromboticity, and lipid-lowering activity (Wargovich and Goldberg 1985). Furthermore, numerous bioactives have also been found in, or have been produced from, animal products. Most of those are protein hydrolysates and peptides produced after enzymatic treatment or fermentation from protein-rich sources such as milk and fish (Zioudrou et al 1979; Yokoyama et al 1992). In addition, other bioactive compounds such as C-

9-, and C-12-octadecadienoic acid (CLA) with antioxidative and anticancer properties have been found in meats and dairy foods after heating (Ha et al 1987; 1989).

Table I. Bioactive Compounds in Fruits and Vegetables, Cereals, Oils, and Animal products[1]

Sources	Compound name
Fruits and vegetable	
Apples	Quercetin, epicatechin, chlorogenic acid, p-coumaric acid, phloridzin
Citus fruits	Naringenin, hesperetin, hesperedin, eriocitrin, naringin, meoeriocitrin, natrituin, p- coumaric acid, caffeic acid, ferulic acid
Grapes	Tannic acid, quercetin, procyanidines, other phenolics
Onion	Quercetin, myricetin
Carrots	Lignin, carotene
Tomato	Quercetin, lycopene, rutin, prunin
Garlic	S-Allyclcysteine, S- allylmercaptocysteine
Oils	
Cocoa	Catechin, epicatechin, chlorogenic acid
Soybean	Genistein, daidzein, glycitein, phenolic acids, tocopherols, peptides
Seame seed	Sesamol, sesaminol, tocopherol, sesamolinol
Cottonseed	Quercetin, rutin, kaempferol, gossypeti, heracetin, dihydroquercetin, quercetrin, isoquercetrin
Peanuts	Taxifolin
Creal crops	
Rice	Orizanol, isovitexin, cyanidine-3- O-
Wheat/wheat bran	β-D-glycopyranoside, pinoresinol, other phenolics
Barley	Flavanols (procyanidins); proanthocyanidins
Oat	Esters of caffeic and ferulic acids
Animal products	
Dairy products	Omega-3 fatty acids (conjugated linolenic acid)
Fish	Omega-3 fatty acids (alpha-linolenic acid)
Meat	Omega-3 fatty acids (conjugated linolenic acid)
Yogurt	Bacterial cultures

[1]Cited from Kris-Etherton et al (2002) and Pennington (2002)

Protein Hydrolysates and Peptides as Bioactives

Proteins, considered the most important building block for life, are also known to possess a wide range of nutritional properties. Proteins are one of the major energy sources and the primary source of amino acids, which are essential for growth and maintenance. Furthermore,

many dietary protein sources such as egg, dairy products, and seafood, contain compounds with specific biological properties that have health-promoting capabilities (Kitts 1993). Among the protein derived bioactive compounds, hydrolysates and peptides are major contributors to nutraceutical and functional properties (Hartman and Meisel 2007).

Protein hydrolysates have been used as amino acid carriers for protein digestion deficient individuals (Mannheim and Cheryan 1990). The hydrolysates and peptides derived from milk and egg have been most intensively studied. Generally, those functional peptides can only be activated by release them from the intact protein molecule during gastrointestinal digestion or food processing. After release, hydrolysates and peptides may exert bioactivities *in vivo* that may prevent cardiovascular diseases, decrease the risk of cancer, and exert inverse effects on nervous system degradation (Mölleret al 2008).

Along with the investigation of the bioactivity of protein hydrolysates, studies have focused on the characterization of a single peptide that contributes specific health properties. Bioactive peptides have special advantages because they: (1) are considered safe and healthy compounds since they are derived from natural foods; (2) are easily absorbed with high activity because of their low molecular weight; (3) are very stable in different situations; and 4) no hazardous immunoreaction result from their simple structures (Sarmadi and Ismail 2010). Generally, bioactive peptides are present in the range of 3 to 16 amino acid molecules predominately comprised of Val, Leu, Pro, His, Tyr, Glu, and Asp (Saiga et al 2003). Many studies have been conducted to prepare, identify, and characterize novel bioactive properties of peptides from plant or animal sources like peanut kernels (Hang et al 2010), rice bran (Kannan et al 2010, Revilla 2009), sun flower protein (Megias et al 2008), alfalfa leaf protein (Xie et al 2008), corn gluten meal (Li et al 2008), egg-yolk protein (Sakanaka et al 2006), milk-kefir and

soymilk-kefir (Liu et al 2005), medicinal mushroom (Wachtel et al 2004), casein (Suetsuna et al 2000), and buckwheat protein (Tang et al 2009). A long list of bioactive peptides with potential anticancer activity have been identified, isolated from enkephalin analogues (Daiichi and Yakuhin 1985; Scholar et al 1987), papaver somniferum pollen (Xu and Jin, 1998), antineoplastic protein (Ridge and Sloane 1996; Sloane 2002), and soy protein (Hernandez et al 2009).

Rice Bran as a Source of Bioactive Compounds

Rice bran

Rice (Oryza sativa) is one of the most important staple foods. It feeds more than half of the population around the world, especially in Asia, Africa, and Latin America. The demand for rice keeps increasing because of the steadily growing world population (Wang and Li 2004). In the United States, the production of raw rice in year 2013 was 265.9 million cwt with 130.1 million cwt of that was produced in the state of Arkansas (National Agricultural Statistics 2013). The structure of rice kernel is illustrated Figure 1 (Lasztity 1996). Rice bran is the hard outeraleurone layer of the brown rice kernel. During the whiting process, which applies friction to the grain surface, the bran is removed from the kernel and usually accounts for

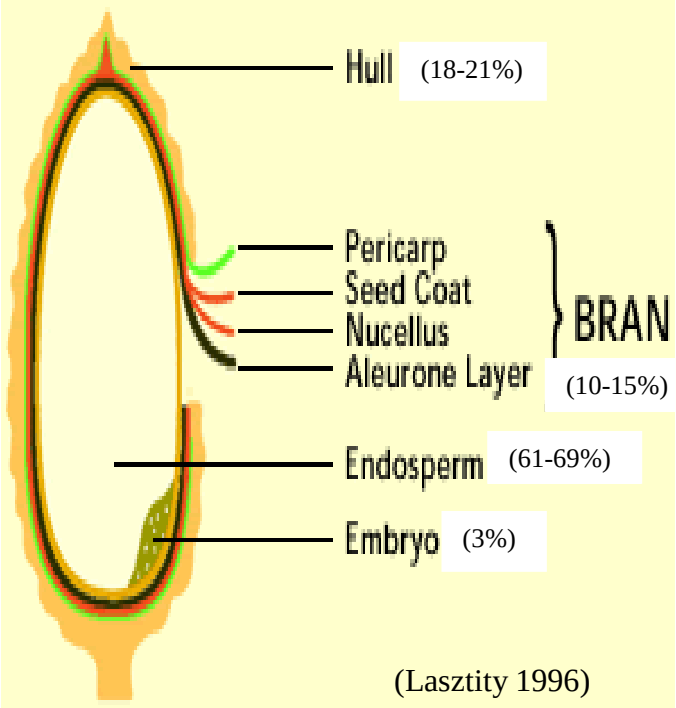

Figure I. Rice Kernel Structure

about 10% of raw rice weight. In Arkansas, nearly 13.1 million cwt of rice bran is produced each year (National Agricultural Statistics 2013).

Nutraceuticals from rice bran

Traditionally, rice bran is used as livestock feed supplement because of its high content of protein (12-15%), lipids (23%), and dietary fiber (27%) (Susan and Priscilla 2009). It also has been used as a natural oil extraction source in the food industry (Iqbal et al 2005). In terms of human nutrition, rice bran has drawn attention recently due to its potential health promoting properties. It contains high quality dietary fiber (cellulose, hemi-cellulose, pectin and lignin), which is proven to be effective in lowering the cholesterol in plasma, decreasing the incidence of obesity and diabetes, and preventing physical disorders associated with the intestinal system and coronary heart disease (Wiboonsirikul et al 2008). Other functional compounds include some well-known antioxidants such as polyphenolics (phenolic acids and flavonoids), α-tocopherols (vitamin E), tocotrienols, and oryzanols (Nam et al 2005; Xu et al 2001). Thus, rice bran is considered an inexpensive food ingredient with potential nutraceutical properties (Saunders et al 1990).

Rice Bran Protein, Hydrolysates & Peptides

Protein is one of the major components in rice bran comprising 12-15% of the total weight. Rice bran protein contains albumin, globulin, glutelin, and prolamin with percentages of 37%, 36%, 22%, and 5%, respectively (Tang 2002; Wiboonsirikul 2008). Rice bran protein has unique nutritional value compared to all the cereal bran derived proteins due to: (1) a high protein digestibility of more than 90%; (2) the highest content of lysine compared with other cereal bran proteins (Juliano et al 1985); (3) a high protein efficiency ratio (PER) of 2.0 to 2.5 compared casein with 2.5 PER; (4) a good source of hypoallergenic proteins, which may be used as a

suitable infant food ingredient and would add variety in protein sources for children with food allergies (Burks and Helm 1994).

Although the high content of proteins present in rice bran, proteins are bound extensively to phytic acid (1.7%) and fiber (12%), leading to difficulty in proteins' separation and extraction using traditional solvents such as alkali, acid, salt, and alcohol, which results in low protein extraction yeilds. Thus, proteases have been used to enhance the solubility of rice bran proteins. Hamada (1999) reported that the protein extraction yield increased significantly after proper proteolysis with a degree of protein hydrolysis (DH) of 10%. Direct enzymatic hydrolysis has been applied to rice bran to produce a wide range of hydrolysates and peptides (Deslie et al 1988).

In recent years, the discoveries of novel hydrolysates and peptides from rice bran protein have received great attention. Those bioactive hydrolysates and peptides have proven specific anti-disease activities such as anti-oxidative, anti-mutagenic, and anti-carcinogenic properties (Adebiyi et al 2009; Renuka and Arumughan 2007; Chanput et al 2009; Kannan 2010).In Hettiarachchy's research program, rice bran hydrolysates were prepared by direct hydrolysis of heat stabilized de-fatted rice bran (HDRB). The peptides were separated and purified.A novel pentapeptide, with the sequence Glu-Gln-Arg-Pro-Arg was identified with inhibitory activities against the proliferation of MCF-7 and MDA-MB-231 human breast cancer cell lines (Hettiarachchy 2013; Kannan 2010). However, the mechanistic action of this peptide on breast cancer cells needs investigation.

Cancer

Cancer is one of the leading causes for global mortality in both men and women (Kaufman and Earnshaw 2000). In 2013, an estimated 16.6 million people were diagnosed with

cancer with the estimated deaths of 5.8 million (American Cancer Society 2013). It is estimated that deaths from cancer in 2015 and 2030 will increase to 9 million and 11.4 million, respectively (Word Health Organization 2013). Cancer, which accounts for nearly 1 in every 4 deaths, is the second leading cause of death in the U.S (American Cancer Society 2013).

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Cancer cells produce growth signals themselves, ignore cell death signals, replicate without limits, and invade normal tissues through basement membranes and capillary walls (Bhutia et al 2008). In addition, tumor cells and tumor-infiltrating host cells can exert immunosuppressive effects to invalidate the immune system (Bhutia 2008). If the spread is not controlled, it can result in death. Genetic and epigenetic factors contribute to the initiation of cancer in a normal cell. Those factors include both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposure to external factors and detectable cancer (American Cancer Society 2013).

Generally, traditional cancer therapies include surgery, radiation, hormone therapy, chemotherapy, biological therapy, and targeted therapy. However, those therapies are expensive and usually kill normal cells at the same time, causing side effects such as nausea, vomiting, loss of appetite, fever, and fatigue (Wu et al 2006). Therefore, there is an increasing need for further research in this area and for the pharmaceutical industry to develop more effective and less toxic drugs. In addition, there is a growing interest in the identification and characterization of natural antitumor agents derived from natural food sources to explore alternative and inexpensive therapies.

Breast Cancer & Bioactive Peptide

Breast cancer

Breast cancer is one of the leading causes of cancer related deaths and illnesses in the United States. In year 2013, 39,620 estimated deaths from breast cancer (females) were reported according to the Cancer Facts & Figures (2013). Approximately one-third of women with breast cancer develop metastases and ultimately die from the disease.

Chemotherapy, radiation therapy, and immunotherapy all rely heavily on apoptosis to kill breast cancer cells. However, cells can subsequently survive and gain resistance to these treatments leading to a more aggressive cell variant with an inclination to metastasize despite initially responding to the therapy (Simstein et al 2003). This chemo-resistance often results in the progression of breast cancers with hormone-independent, invasive, metastatic, anti-estrogen-resistant phenotype characteristics. The underlying mechanism of chemotherapy-resistant cancer cells is still unknown (Campbell et al 2001).

Cancer cell lines as *in vitro* models for cancer study

Diverse models are used for cancer studies including primary tumours, paraffin-embedded samples, cancer cell lines,xenografts, tumor primary cell cultures (Louzada et al 2012; Vergo-Gogola et al 2007; van Staveren et al 2009). Due to the difficulties of animal research including ethical concerns, cost, maintenance and relative inefficiency, genetic and DNA analysis and drug testing in cell culture system has been encouraged as alternative method for the study of disease. Cell line model is easy to manipulate and characterize at molecular level (van Staveren et al 2009). It has various advantages including easily to be handled, cultured as an unlimited self-replicating source in almost infinite quantities, research data could be highly replicable due to their relatively high degree of homogeneity. A fundamental understanding of

the characteristics of cell lines is indispensible for studyingmechanistic cellular pathways anddisclosing critical genes involved in various diseases.

The use of the appropriate *in vitro* model in cancer research is crucial for the investigation of genetic, epigenetic and cellular pathways.The model are also used for studying proliferation deregulation, apoptosis and cancer progression anddefining potential molecular markers and characterization of cancer therapeutics (Louzada et al 2007; Vergo-Gogola et al 2007; van Staveren et al 2009; Nakatsu et al 2005; Kao et al 2009). Cancer cell lines have been widely used for above research purposes.Their characterization shows that they are an excellent tool in the genetic approach for studying of the biological mechanisms involved in cancer (Louzada et al 2007). The use of the cell model for testing and development of new anticancer drugs andtherapiesis needed (Gazdar et al 2010;Pfragner et al 2009).

Human breast cancer cell lines: MCF-7 and MDA-MB-231

MCF-7, named after the place where it was first derived:Michigan Cancer Foundation-7, is a well-studied cell line that was first isolated in 1970 from the pleural effusion of a 69-year old Caucasian woman (Burdall et al 2003). Currently, MCF-7 is the most commonly used breast cancer cell line in the world. MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium (Holiday and Speirs 2011). These characteristics include the ability to process estrogen via estrogen receptors in the cell cytoplasm, which makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line. From the cell culture aspect, MCF-7 is easy to culture and is capable of forming the epithelial-like growsas monolayer in the flask. The growth of MCF-7 can be inhibited by inducing tumor necrosis factor alpha (TNF-α) and anti-estrogens, which makes MCF-7 a controllable cell line for specific human breast cancer research (Alkhalaf et al 2003).

Another commonly used cell line for *in vitro* human breast cancer research is MDA-MB-231. This cell linewas obtained from a patient in 1973 at M. D. Anderson Cancer Center (Holiday and Speirs 2011). One of the majordifferences between MCF-7 and MDA-MB-231 cells is that the MDA-MB-231 cell line has an invasive phenotype, which could be used as a model for study of breast cancer in metastasis status (Wang 2009). It has anepithelial-like morphology and appears phenotypically as spindle shaped cells (Sorlie et al 2009). Compared to MCF-7 cells, MDA-MB-231 cells grow much faster and are more resistant to drug therapies. For instance, reseveratrol is a potential chemopreventative agent in fighting human cancers. However, reseveratrol can only induce apoptosis in MCF-7 cellsbut not in MDA-MB-231 (Pozo-Guisado et al 2002).

Bioactive peptide

Recently, studies on both the preparation of small molecular therapeutics (like peptides) and their mechanisms of action in treating, preventing, and management of various diseases have drawn the attention of the scientific community. Among those peptides, food derived peptides with potential antitumor activity have drawn increasing attention and several of them have moved forward to clinical trials. In comparison with other chemotherapies, bioactive peptides possess the advantages of high affinity, strong target cells specificity, low toxicity, and high modification feasibility (Michael and Erkki 2004).

Potential sources of anticancer peptides are found in both animal and plant foods. Soybean is the primary plant source of anti-tumor peptides. Kim et al (2000) concluded that soybean protein hydrolysates showed inhibition abilities on cancer cell growth. The anti-proliferation abilities of soybean hydrolysates against cancer cells were also reported by Kops et al (1997) and Kuba et al (2003). The anticancer activities of individual soybean peptides were

also studied. The most well known soybean-derived peptide is called lunasin, which is a linker peptide with 43 amino acids that has shown outstanding carcinogenic suppressing capacities in both *in vitro* and *in vivo* models.

Apart from soybean, two other anticancer peptides have also been identified, purified, and characterized from wheat (Matsui et al 1999) and rice bran (Hettiarachchy 2013; Kannan et al 2010). Kannan et al (2009) prepared rice bran hydrolysates from heat stabilized de-fatted rice bran (HDRB) using Alcalase. The hydrolysates were then treated with a stimulated gastrointestinal (GI) juice and separated into fractions with different molecular weights (>50, 10-50, 5-10, and <5 kDa) using ultrafiltration. Those fractions were evaluated for multiple-site (human breast, colon, and liver cancer cell lines) anticancer activities and a <5 kDa fraction showed 70-80% growth inhibition activity on human breast cancer cells (MCF-7). After isolation and purification, a novel pentapeptide (EQRPR: Glu-Gln-Arg-Pro-Arg) was identified and showed inhibitory activities onMCF-7 human breast cancer cell lines (Kannan et al 2010). The structure of pentapeptide is shown as Figure II.

As for animal food sources, milk and fermented milk products are most intensively studied and are considered the major source of novel anticancer hydrolysates and peptides (Ganjam et al 1997; Manso et al 2002; Takeda et al 2004; Meisel 2004).

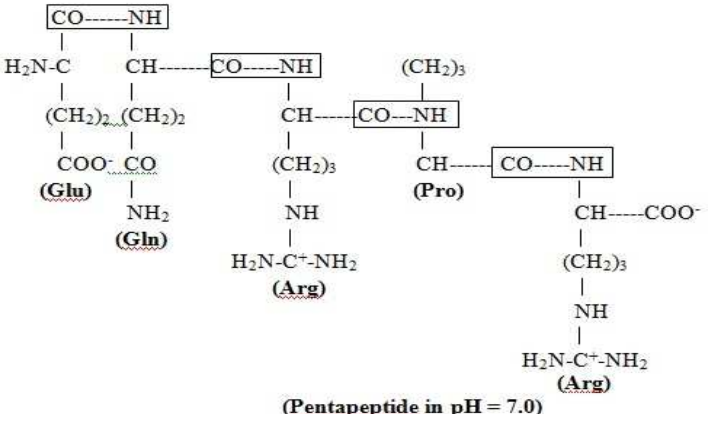

Figure II. Pentapeptide Structure

Other well-studied animal food peptides sources include fish (Lee et al 2004; Picot et al 2006), egg (Pellegrini et al 2004; Davalos et al 2004), and whey protein (Papenburg et al 1990). Despite

the advances in discovering novel bioactive peptides, future research should also focus on improving the efficiency of the peptide targets on cancer cells by delivering them at a desired location with appropriate dosage. The biological mechanism of each tumor-targeting peptide against specific cancer cell lines is neededfor further research before being used as therapeutics in humans.

Biomarkers & Possible Mechanism of Bioactive Peptide Against Breast Cancer

Apoptosis

Generally, physiological and pathological stimuli may trigger a process of genetically programmed cells death (PCD), otherwise known as apoptosis. The morphological changes during the apoptosis of cells include double-stranded DNA fragmentation, condensation of chromatin, cellular atrophy, and protrusion of the cell membrane (Earnshaw et al 1999). Apoptosis plays a critical role in the development of a variety of human diseases including cancer, immune system diseases, and neurodegenerative disorders. Apoptosis is a multi-step, multi-path cell-death program that is inherent in every cell of the body. In the process of tumorgenesis, the ratio of proliferated cell and apoptotic cells is altered and uncontrolled, followed by the invasion of tumor cells with metastatic potential (Olopade et al 1997).

In cancer treatment, anti-cancer agents such as chemotherapy and irradiation kills target cells primarily by inducing apoptosis. The steps are: 1) initiation of apoptosis; 2) activation of the caspases by a signal transduction cascade; and 3) proteolytic cleavage of cellular components. The general apoptosis pathway is initiated by various stimuli such as heat shock, viruses, toxic insults, cytokines, hormones, DNA damage, and growth factor deprivation (Earnshaw et al 1999). During the early stage of caspase cascade initiation which will lead to the final death of cell,

apoptosis is mediated by various downstream signaling factors including Bcl-2 family proteins, inhibitor of apoptosis proteins (IAP), nuclear factor-κB (NF-κB), and the mitogen-activated protein kinase (MAPK) family (Simstein et al 2003). Two major apoptotic mechanisms including the death receptor-dependent (Extrinsic) pathway and the mitochondria-dependent (Intrinsic) pathway involve the activation of caspase-8 and -9 as shown in Figure III (Cryns and Yuan et al 1998). The caspase-3 or -7 are activated to cleave various cytoplasmic or nuclear substrates including DNAse which lead to the typical morphological changes and DNA fragmentation in apoptotic cells (Degen et al 2000; Stroh and Schulze et al 1998; Casciola et al 1995). Caspase-8 is activated through the extrinsic pathway by interactions between ligands and death receptors. Caspase-9 is involved in the intrinsic pathway and activated due to the mitochondrial cytochrome c leakage (Kumar 1999).

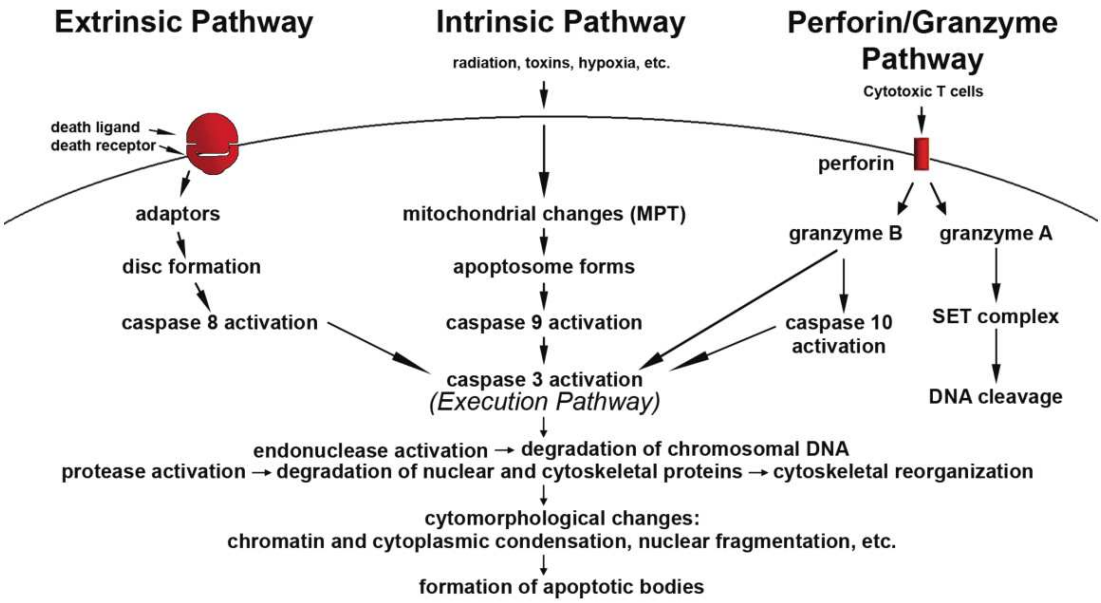

Figure III. Pathways of Cancer Cell Death via Apoptosis

Caspases

Caspases, a group of cysteine-dependent aspartate-directed proteases, plays the most important role in controlling cell apoptosis. They act as proenzymes consisting of a pro-domain and a catalytic protease domain. Caspases are subclassified as initiator caspases (e.g., caspase-8, -9, -10, and -12) and effector caspases (e.g., caspase-3, -6, and -7). The upstream initiator caspases activate the downstream effector caspases, which cleave other cellular proteins and lead to apoptosis (Okun et al 2008).

The caspase cascade is the primary feature of cell apoptosis. The caspase cascade may be activated by two pathways: the intrinsic pathway and the extrinsic pathway (Yamasaki-Miyamoto et al 2009). In the early stage of the intrinsic pathway, the mitochondria serve as the primary target for tumor necrosis factor (TNF)-induced cytotoxicity, which leads to the release of cytochrome C, followed by the activation of adaptor protein Apaf-1. The Apaf-1 activates the initiator caspase (procaspase-9), resulting in the activation of downstream effector caspases. In the extrinsic pathway, apoptosis is induced by a mitochondria-independent way. The death receptor proteins such as Fas, are activated, followed by cleavage of procaspase-8, and the initiation of the caspase cascade (Kim et al 2002).

Although the observation of the caspase cascade is considered the most important benchmark for the characterization of the potential efficiency of certain cancer treatment, there are differences in caspase expression between various cancer cell lines. The most unique feature of the MCF-7 breast cancer cell line is that caspase-3 is not expressed, due to the deletion of casp-3 gene (Kurokawa et al 1999). Liang et al (2002) reported that a sequential expression of caspases-9, -7, and -6 was observed, without the activation of caspase-3. Janicke et al (1998) concluded that caspase-3 activation contributes to some typical morphological changes of

apoptotic cells, such as DNA fragmentation, despite the fact that caspases-3 is not required for TNF-induced apoptosis. However, Yang et al (2001) found that the presence of caspase-3 increased MCF-7's apoptotic sensitivity. The study of pentapeptide-induced caspase cascade in apoptotic human breast cancer cells is the first step to understand its mechanistic potency in human breast cancer.

Biomarkers

As an important biological indicator of cancer status and progression of the physiological state of the cell at a specific time, biomarkers (Bcl-2 family proteins, TNF-α, Fas, and Cox-2) represent powerful tools for monitoring the course of cancer and gauging the efficacy and safety of novel therapeutic agents.

The Bcl-2 family proteins are considered vital control points of apoptosis due to the regulation of the release of cytochrome C and the activation of caspase-9. The subfamilies of Bcl-2 proteins include anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, Bfl- 1/A1, Bcl-W, Bcl-G) and pro-apoptotic proteins (Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-XS, Krk, Mtd, Nip3, Noxa, Bcl-B) (Danial 2007). Bax-like death factors such as Bax, Bak, and Bok/Mtd induce apoptosis by building channels on membranes for cytochrome C release. There are several possible theories to explain the mechanism of proapoptotic protein-induced cytochrome C release: 1) the formation of a new channel (Schlesinger et al 1997); 2) the enlargement of existing transition pore permeability (Tsujimoto and Shimizu 2000); and 3) the formation of supramolecular openings (Zamzami and Kroemer 2001). Anti-apoptotic proteins, generally classified as Bcl-2-like survival factors include Bcl-2, Bcl-xL, Bcl-w, A1/Bfl-1, Boo/Diva/Bcl-B and Mcl-1. These anti-apoptotic proteins are located on the outer mitochondrial membrane and protect the cells by forming heterodimers with pro-apoptotic proteins (Borner 2003). The interactions between Bcl-2

family proteins affect cellular sensitivity to apoptosis and the ratio of pro-apoptotic proteins and anti-apoptotic proteins determines the fate of cells. The Bcl-2 proteins also interact with caspases in a mitochondrial-independent pathway. For example, the caspase adapter Apaf-1 can be inactivated by binding with the anti-apoptotic protein Bcl-XL (Gross et al 1999). Many factors affect the levels of individual Bcl-2 proteins, such as the lineage of the cell, the activation of transcription factors, nonendogenous chemicals, and estrogen responsiveness.

TNF-α is a naturally occurring cytokine secreted by cells of the immune system and other systems (Feuerstein et al 1994). Although TNF-α is cytotoxic to some tumor cells, it is rarely cytotoxic to normal cells. This unique property has led to numerous studies of TNF as a chemotherapeutic agent and apoptosis-inducing agent (Erika et al 2006). TNF can activate both apoptotic pathways and survival pathways inside the cell. TNF-α has two receptors, p55 (TNFR1) and p75 (TNFR2), with most apoptotic pathways mediated through p55 (Story et al 1998). Although p55 expression is necessary for TNF to produce apoptosis, it is not sufficient by itself**.** Many death and survival genes, which are regulated by extracellular factors, are involved in apoptosis (Simstein et al 2003).

Fas, also named Apo-1 or CD95, is a member of the TNF and nerve growth factor (NGF) receptor (Keane et al 1996). Fas is located on the cell surface and is activated by its ligand (Fas L) binding. Fas then forms the death-inducing signaling complex (DISC), which finally leads to the activation of effector caspases resulting in apoptosis in cells (Chopin et al 2002; Chen and Lin 2004). Studies have reported that anticancer drugs activate apoptosis in breast cancer cells by inducing the expression of death receptor ligands, such as Fas (Fulda et al 1998). In recent years, there is a growing interest in studying chemotherapies by targeting specific death receptors such as Fas, to induce apoptosis in human cancer cells. The advantage of this idea is that the

mechanism of death receptor induced apoptosis is based on the direct activation of caspase machinery (Ashkenazi and Dixit 1998). Therefore, it is necessary to evaluate the expression of Fas and FasL levels in apoptotic human breast cancer cell lines induced by the pentapeptide.

Cyclooxygenase (COX), generated from free arachidonic acid, is an important enzyme in charge of formation of prostaglandin (PG) H2 from arachidonic acid and is expressed in the prostanoid biosynthetic pathway. It has received considerable attention due to its multiple roles in human cancer development, including stimulation of cell growth, angiogenesis, cell invasiveness, and increasing the production of mutagens. On the other hand, COX-2 also inhibits apoptosis and suppresses the immune system. There is clear evidence that the overexpression of COX-2 enhances the production of several PGs that regulate various physiological processes (Brown and DuBois 2005). The effect of COX-2 on cell growth results from the increased production of PGE_2 and PGF_2, which contributes to cell proliferation (Bandopadhyay et al 1987). Meanwhile, on the side of apoptosis, there is sufficient evidence that COX-2 suppresses apoptosis by changing the cellular levels of anti-apoptotic and pro-apoptotic proteins (Devendra et al 2004). For example, COX-2 stimulates the activation of Akt, which is responsible for inactivation of pro-apoptotic proteins (Lin et al 2001). Further along in cancer development, COX-2 also stimulates the invasion of cancerous cells to other tissues, which increases cancer metastasis. In addition, PGE_2 induces inverse effects on the activity of natural killer cells such as T and B cells. Evaluation of COX-2 levels in pentapeptide treated human breast cancer cells may improve our understanding of the underlying mechanism of pentapeptide induced human breast cancer cell apoptosis.

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CHAPTER 1

Anti-proliferative effect of pentapeptide on human breast cancer cell models (MCF-7 and MDA-MB-231)

Ruiqi Li, Navam Hettiarachchy, Mahendran Mahadevan

Abstract

Bioactive peptides derived from food sources with anti-proliferative properties against cancer have drawn more attention in recent years. Studies have focused on the characterization of peptides from natural sources that contribute specific health benefits. A pentapeptide derived from rice bran has shown anti-proliferative properties against human breast cancer cell lines (MCF-7 and MDA-MB-231). The objective of this study was to investigate the anti-proliferative activities of this pentapeptide on growth of breast cancer cell models (MCF-7 and MDA-MB-231). The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was used to evaluate the growth inhibition activities of the pentapeptide in a dose- (10, 50, 100, 200, 400, 500, and 1000 μg/mL) and time-dependent manner (24, 48, 72, and 96 h). The trypan blue exclusion assay was used to determine the cell viabilities of both breast cancer cell lines and noncancerous breast cells (HMEC) after pentapeptide treatment. Decreases in growth of MCF-7 and MDA-MB-231 cells were observed after treatment with the pentapeptide. The maximum inhibitory activities were found on MCF-7 (90.9%) and MDA-MB-231 (87.0%) after incubating with the pentapeptide for 72 and 96 hrs, respectively. The results suggested that the pentapeptide inhibits the growth of human breast cancer cell lines in a dose- and time-dependent manner and has no effects on the growth of normal breast cell line.

Introduction

Rice (Oryza sativa) is considered the primary staple food for more than half of the population of the world. In the United States, the state of Arkansas ranks first in the production of rough rice (125.9 million cwt), which accounted for nearly 47.9% of national total production (262.5 million cwt) in 2013(National Agricultural Statistics Service 2013). Rice bran is the hard outside aleurone layer of the brown rice kernel. Bran is removed from the kernel by friction during the production of white rice and it accounts for about 10% of rough rice (Parrado et al 2006).

Traditionally, rice bran is underutilized as a livestock feed supplement and natural oil extraction source due to its high content of protein (12-15%), lipids (23%), and dietary fiber (27%) (Susan and Priscilla 2009). It contains a high content of dietary fiber and antioxidants such as polyphenolics (phenolic acids and flavonoids), α-tocopherols (vitamin E), tocotrienols, and oryzanols. These compounds have proven their bioactivities including lowering the cholesterol, decreasing the incidence of obesity, diabetes, intestinal system disorders, and coronary heart diseases.

Commercially, the rice bran is heat stabilized to prevent hydrolysis and oil oxidation during extraction. The resulting defatted (removal of 15-20% oil) by-product is called heat-stabilized defatted rice bran (HDRB).HDRB contains approximately 18.5-20.9% protein, 25.2-25.6% total fiber, 15.3-16.8% starch, 1.5-2.0% lipid, 2.0-2.5% total phenolics, 1.73-2.28% phytic acid, 10.5-12.0% water, and 9.0-10.4% ash (Hettiarachchy 2009).HDRB contains increased percentages of fiber (35-48%) that can be used in fiber-dense food products. It also contains higher protein content (15-20%) that can be extracted with dilute alkali and enzyme treatment to produce a 50-60% bran protein concentrate(Mazza 1999).

As a protein rich plant source, rice bran protein derived hydrolysates and peptides have received considerable attention as potential anti-oxidative, anti-mutagenic, and anti-carcinogenic agents (Adebiyi et al 2009; Renuka and Arumughan 2007; Chanput et al 2009; Kannan et al 2010). Enzymatic hydrolysis by pancreatic enzymes, especially trypsin, is considered the most effective method to produce value-added bioactive peptides (Möller et al 2008). In the preparation of bioactive hydrolysates and of peptides derived from rice bran, direct enzymatic hydrolysis has been widely used due to its advantage in breaking down the extensive bonding between proteins and phytic acid (1.7%) and fiber (12%).

Cancer is one of the leading causes for global mortality in both men and women (Kaufman and Earnshaw 2000). Breast cancer is one of the leading causes of cancer related deaths and illnesses in the United States. 39,620 estimated deaths for breast cancer (females) were reported in 2013 (Cancer Facts & Figures 2013). Generally, traditional cancer therapies include surgery, radiation, hormone therapy, chemotherapy, biological therapy, and targeted therapy. However, those therapies result in side effects such as nausea, vomiting, loss of appetite, fever, and fatigue (Wu et al 2006). In addition, these treatments may select cancer cells that can subsequently survive, which lead to a more aggressive cell variant with an inclination to metastasize (Simstein et al 2003).

Kannan et al (2009) prepared rice bran hydrolysates from HDRB by hydrolysis using Alcalase. The hydrolysates were then treated with stimulated gastrointestinal (GI) juice and separated into fractions based on their molecular weight (>50, 10-50, 5-10, and <5 kDa) using ultrafiltration. Those fractions were evaluated for multiple-site (human breast, colon, and liver cancer cell lines) anticancer activities and the <5 kDa fraction showed 70-80% growth inhibition activity on human breast cancer cells (MCF-7). After peptide purification and isolation, a novel

pentapeptide with a sequence Glu-Gln-Arg-Pro-Arg was identified and showed inhibitory activities against the proliferation of MCF-7 human breast cancer cell lines (Kannan et al 2010).

The objective of this study was to determine the anti-proliferative effect of the pentapeptide on human breast cancer cell models (MCF-7 and MDA-MB-231). The growth inhibition properties of pentapeptide against breast cancer cells was evaluated by MTS assay and Trypan blue assayin a time- and dosage-dependent manner.

Materials and Methods

Materals. Pentapeptide (amino acids sequence: EQRPR: Glu-Gln-Arg-Pro-Arg) was purchased from Biomatik LLC. (Wilmington, Delaware, USA). Human breast cancer cell lines (MCF-7: ATCC HTB-22^{TM} and MDA-MB-231: ATCC HTB-26^{TM}) and normal human mammary epithelial cells (HMEC: ATCC PCS-600-010^{TM}) were purchased from ATCC (American Type Culture Collection). The Bovine Pituitary Extract (BPE), Eagle's Minimum Essential Medium, serum free medium, fetal bovine serum (FBS), insulin, trypan blue powder, and trypsin-EDTA solution were purchased from Sigma (MO, USA). The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]one solution kit was obtained from Promega Corporation (Madison, WI, USA).

Methods.

Cell culture. The human breast cancer cell lines (MCF-7 and MDA-MB-231) were selected as breast cancer cell models in this research. MCF-7 was derived in the Michigan Cancer Foundation-7 in 1973 from pleural effusion in a breast cancer patient. MCF-7 cells are useful for *in vitro* breast cancer studies due to their capacity to process estrogen via estrogen receptors in the cell cytoplasm, which makes the MCF-7 cell line an estrogen receptor (ER) positive control

cell line. The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Center. MDA-MB-231 breast cancer cells have an epithelial-like morphology, which appear phenotypically as spindle shaped cells. The difference between the two cell lines is that MCF-7 is an ER-positive breast cancer line derived from an in situ carcinoma, meaning that the cancerous cells had not yet invaded surrounding tissues; however, the MDA-MB-231 is an ER-negative breast cancer line derived from a metastatic carcinoma. Selection of both cell lines helps in better illustrating and understanding the mechanism of pentapeptide-induced growth inhibition on human breast cancer. For evaluating the growth effects of normal human breast cells caused by pentapeptide, the HMEC (Human Mammary Epithelial Cells) which were derived from normal adult mammary glands, was selected.

For cell culture method, the human breast cancer cell lines (MCF-7 and MDA-MB-231) were grown in the recommend conditions (ATCC, Manassas, VA, USA). The MCF-7 cells were cultured in a 75 cm^2 flask with Eagle's Minimum Essential Medium supplemented with 0.01mg/mL bovine insulin and 10% FBS. The HMEC was cultured with serum-free medium supplied with insulin (5.0 µg/mL) and BPE (70 µg/mL). Then, the cells were grown in a humidified 5% CO_2 incubator at 37°C for about 7 days and reached 80 to 90% of fully confluent (approximately 5 x 10^6 cells) before cells were collected for further assays. The MDA-MB-231 cells were cultured in a canted neck 75 cm^2 flask (to maintain the inside atmosphere in the flask) with Leibovitz's L-15 Medium including 10% FBS and incubated at 37°C in an incubator with 95% relative humidity.

Cell growth inhibition assay (MTS). The cytotoxic effects of pentapeptide on the breast cancer cell lines (MCF-7 and MDA-MB-231) and noncancerous human breast cancer cell (HMEC)

were evaluated by the MTS assay (Kannan et al 2010). MTS assay is a colorimetric method for determining the number of viable cells after a certain treatment. The principle of this assay is that the MTS are reduced to formazans by the cytochrome systems (in the presence of Lactate Dehydrogenase) of viable cells, and the blue color developed is a direct measure (at 490 nm) of the viability of the cells. The amount of colored product formed is proportional to the number of living cells. In this study, the cell viabilities of human breast cancer cell lines and HMEC were determined using MTS assay after pentapeptide treatment. Cells (MCF-7, MDA-MB-231, and HMEC) were cultured using respective methods described above and collected from a 75 cm^2 flask using trypsin-EDTA digestion. The cells were re-suspended in the media and the cell densities were determined using a hemocytometer. Then, cells were diluted with the media (Eagle's Minimum Essential Medium and Serum Free Medium) to reach a density of 2.5 x 10^4cells/mL and transferred into a 96-well plate with 200 µL per well. The cells (5 x 10^3 cells/well) were allowed to attach for 24 hr before removing the spent media. The pentapeptide was dissolved using media at various concentrations (10, 50, 100, 200, 400, 500, and 1000 µg/mL), media alone (negative control), and genistein at 400 µg/mL (positive control) were added to the respective well with 200 µL of final volume and incubated for various time periods (24, 48, 72, and 96 h). All treatments (including negative and positive controls) were conducted in triplicates within three wells in each 96-well plate. Genistein was selected as positive control because it is well-recognized as an anti-breast cancer agent and it is very effective at an optimized concentration of 400µg/mL (Hsieh et al 1998; Kannan et al 2008; Kannan et al 2010). The MTS one solution reagent was added to each well (40 µL/well) and the plate was incubated in the dark (to prevent discoloration of MTS reagent) at 37°C for 1 hr to allow color development. The absorbances of samples were measured at 490 nm using a microplate reader

after adding 10% SDS to each well for reaction termination. The cytotoxic effect of the pentapeptide was expressed as a relative percentage of cell survival rate calculated as follows:

Relative survival rate (%) = A_{490}Sample ×100 / A_{490}Negative control

A_{490}Negative control: Absorbance at 490 nm of cells cultured with media alone; A_{490}Sample: Absorbance at 490 nm of cells treated with pentapeptide or genistein at various conditions.

Cell viability assay (Trypan blue dye exclusion). Trypan blue assay is a dye exclusion method that determines the number of dead/live cells by using trypan blue dye to stain dead cells in blue and counted under a microscope (VWR® Inverted Microscope) using a hemocytometer. Since live cells with intact cell membranes cannot be stained due to the membrane selectivity of compounds (Trypan blue dye does not penetrate membranes), the live cells could be also counted using a hemocytometer. In this study, cells (MCF-7, MDA-MB-231, and HMEC) were cultured as monolayer and grown for 3 days on 24-well flat-bottom plates. The pentapeptide at various concentrations (10, 50, 100, 200, 400, 500, 1000 µg/mL) in media were added to each well and treated for various times (24, 48, 72, and 96 hr). Genistein (400 µg/ml) in media and media alone were used as positive and negative controls, respectively. The spent media was removed by aspiration and cells were trypsinized from the plates using trypsin-EDTA solution to detach cells from the cell culture substratum. The trypsinized cells were transferred into a 15 mL centrifuge tube and re-suspended using 10 mL fresh media. Then, 100 µL of cell suspension was stained by 100 µL of0.5% Trypan blue dye.

Data Analysis. All the treatments in MTS and Trypan blue assay were conducted in triplicates and the values were reported as means ± standard deviation of three determinations. The

cytotoxic effects of pentapeptide on human breast cancer cell lines and normal breast cells were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS 9.2 2000, SAS Institute Inc., Cary, N.C., U.S.A.). The Fisher's protected least significant difference (LSD) test was conducted to separate the means at $P \leq 0.05$.

Results and Discussion

To determine the growth inhibitions of the pentapeptide on human breast cancer cell lines, the MTS assay and Trypan blue assay were used to evaluate the anti-proliferative activities after pentapeptide (50, 100, 200, 400, 500, and 1000 µg/mL) treatment for various times (24, 48, 72, and 96 h). The Figure 1.1and 1.2 shows that pentapeptide decreased the survival of MCF-7 and MDA-MB-231 cells in a time-dependent manner compared to negative controls (cells cultured using media alone). The maximum inhibitory activities were found on MCF-7 (90.9%) and MDA-MB-231 (87.0%) after incubation with pentapeptide for 96 hrs. A relatively lower growth reduction on MDA-MB-231 cells suggested it was more resistant to pentapeptide treatment than MCF-7 cells. No significant ($p > 0.05$) decreases in survival of pentapeptide treated HMEC (normal breast cell line) were observed, which suggested that the pentapeptide has no cytotoxic effect on normal human breast cells (Figure 1.3).

The Figure 1.4 shows that the pentapeptide at various concentrations inhibited the growth of both cell lines in a dose-dependent pattern. The maximum growth inhibitions were achieved after treatment with pentapeptide at the highest concentration of 1000 µg/mL on MCF-7 (90.9%) and MDA-MB-231 (87.0%). Significant ($p < 0.05$) decreases in survival of pentapeptide treated MCF-7 and MDA-MB-231 cells were observed compared to negative controls. The anti-proliferative activities of pentapeptide on both breast cancer cell lines were increased along with

the increasing dosages of pentapeptide. There were no significant differences ($p > 0.05$) in growth inhibitions of pentapeptide at dosages of 1000 µg/mL and 500 µg/mL on MCF-7 cells. These results suggest that the growth inhibition activity of pentapeptide was saturated at a concentration of 500 µg/mL on MCF-7 cell line. The pentapeptide had shown relatively higher anti-proliferative activities on MCF-7 cells at each concentration than on MDA-MB-231 cells (Figure 1.4). This result could be explained by the characteristics of two human breast cancer cell lines. MDA-MB-231 is more resistant to anti-cancer treatment since it is derived from a cancerous site that was in metastatic status (late stage of cancer) (Awad et al 2000). Figures 1.4 and 1.5 shows the cell viabilities of pentapeptide treated MCF-7, MDA-MB-231, and HMEC determined using Trypan blue assay. These results suggested that pentapeptide inhibits the growth of cancerous human breast cell lines in a time- and dose-dependent pattern while pentapeptide has no anti-proliferative activity on HMEC cells.

Both results determined by MTS assay and Trypan blue assay have the similar trends which shown that the pentapeptide inhibits the growth of both human breast cancer cell lines, however, the survival (%) of cells treated with pentapeptide determined by trypan blue assay are relatively higher than the results evaluated by MTS assay. This difference could be explained by the natural of two assays. MTS assay determine the cells survival percentage by evaluating the levels of dehydrogenase in the mitochondria of the cells, while the Trypan blue assay differentiates the alive or died cells through the integrity or damage of cell membrane. It is highly possible that the death of breast cancer cells start from the damage of mitochondria after the pentapeptide treatment then gradually damage the integrity of the cell membrane. Thus, the survival (%) of cells determined by MTS is lower than the results evaluated by Trypan blue assay.

These findings show the anti-proliferative activities of pentapeptide on growth of human breast cancer cell lines (MCF-7 and MDA-MB-231), which signify that the rice bran derived pentapeptide could be a potential anti-breast cancer agent. Previous research has shown that the pentapeptide caused growth inhibitions on several cancer cell lines including Caco-2, MCF-7, HepG-2, and A549 (Kannan et al 2010). In this study, media alone and genistein (400 µg/mL) were used as negative and positive controls. Genistein causes growth inhibition on human breast cancer cells via apoptosis and was used as a positive control (Fioravanti et al 1998). In this study, the pentapeptide results in anti-proliferative activities on MCF-7 (90.9%) and MDA-MB-231 (87%) may also be due to apoptosis.

In recent years, bioactive peptides are emerging as a novel alternative for anti-cancer drugs. They exert functions through regulated protein–protein or protein–DNA interactions (Borghouts et al 2005). The bio-activities of functional peptides are based on their amino acid composition and sequence (Kannan et al 2010).Generally, those bio-active peptides consist of 5 to 40 amino acids and exhibit multifunctional activities including anti-oxidative, immunomodulatory, ACE-inhibitory, hypocholesterolemic, anti-microbial, and anti-cancer activities (Meisel and FitzGerald 2003; Kim et al 2008). Studies have shown that short peptides with 2 to 5 amino acids were produced from natural sources and possess various biological activities (Saiga et al 2003; Parkash et al 2002; Nagaoka et al 2001; Suetsuna et al 2000).

In the present study, the pentapeptide with a sequence of Glu-Gln-Arg-Pro-Arg (EQRPR) showed significant anti-breast cancer activities. The major advantages could be explained by the size and composition of amino acids. Shorter peptides (less than 10 amino acids) could exposethe functional groups more efficiently due to their higher solvent accessibility (Kannan et al 2010). Low molecular sized bioactive peptides with 3 to 5 amino acids have been isolated and

purified from soy protein, wheat germ, and whey protein respectively (Kim et al 2007; Korhonen and Pihlanto 2003; Sannier et al 2000). The Pro and Arg in the pentapeptide are thought to be major contributors to its anti-cancer activities. David and Ramamoorthy (2008) concluded that anti-microbial peptides such astritrpticin, lactoferricins, and indolicidin are rich in positively charged residue such as Arg. PR-39 is a linear peptide isolated from procine small intestine. It exerts anti-cancer activities and is rich in Arg and Pro (Agerberth et al 1991). In addition, the presence of proline may increase the pepetide's accessibility to solvent due to its predominance in turns of a polypeptide chain. Kim et al (2008) isolated an anticancer peptide from soy protein with a high percentage of Pro (22%). High frequency occurrences of proline residue are also observed in other bioactive peptides and assume to play an indispensible roleon bioactivity of the peptide (Andrushchenko et al 2006).

Conclusion

In summary, the results determined using MTS assay and Trypan blue assay showed that the pentapeptide significantly inhibited the growth of MCF-7 and MDA-MB-231 cells in a time- and dose-dependent manner.The cancer cell death induced by the pentapeptide may be caused by membrane disruption through apoptosis due to its unique amino acids composition. Additionally, pentapeptide has no cytotoxity effect on normal human breast cells, which suggests its potential application in inhibiting cancer cells without harming the growth of normal cells.

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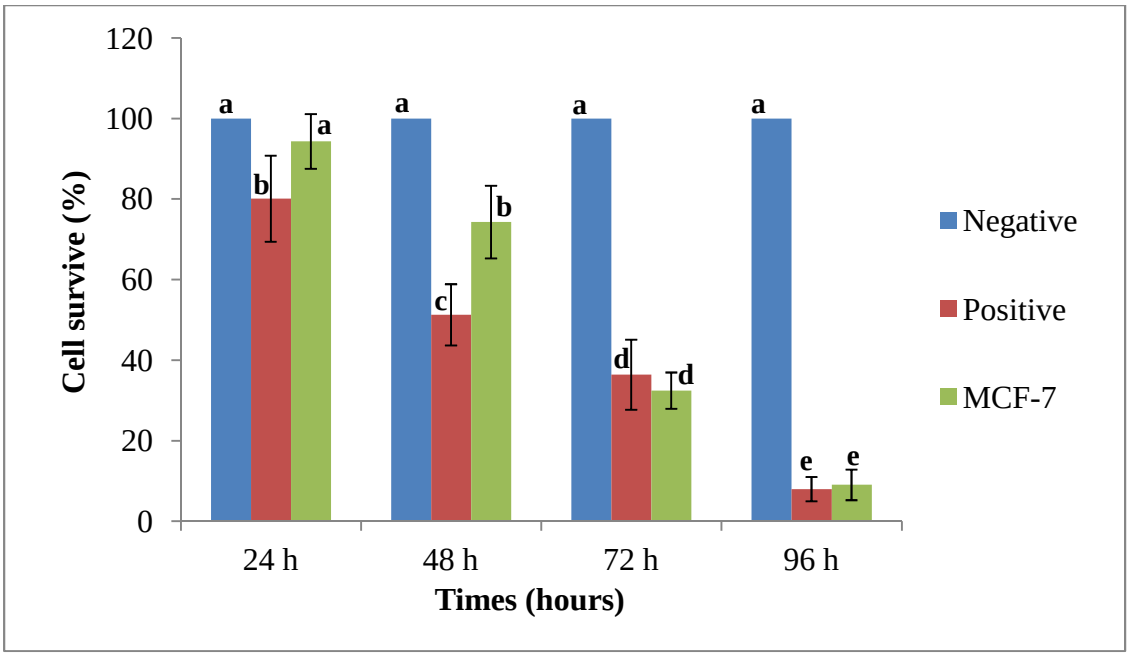

Figure 1.1. Growth inhibition effects of pentapeptide (1000 µg/mL) on human breast cancer cell line (MCF-7) after various incubation times (24, 48, 72, and 96 hours) determined using MTS assay.

The MCF-7 cellswere cultured with media alone and genistein (400 µg/mL) are considered negative and positive controls, respectively. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

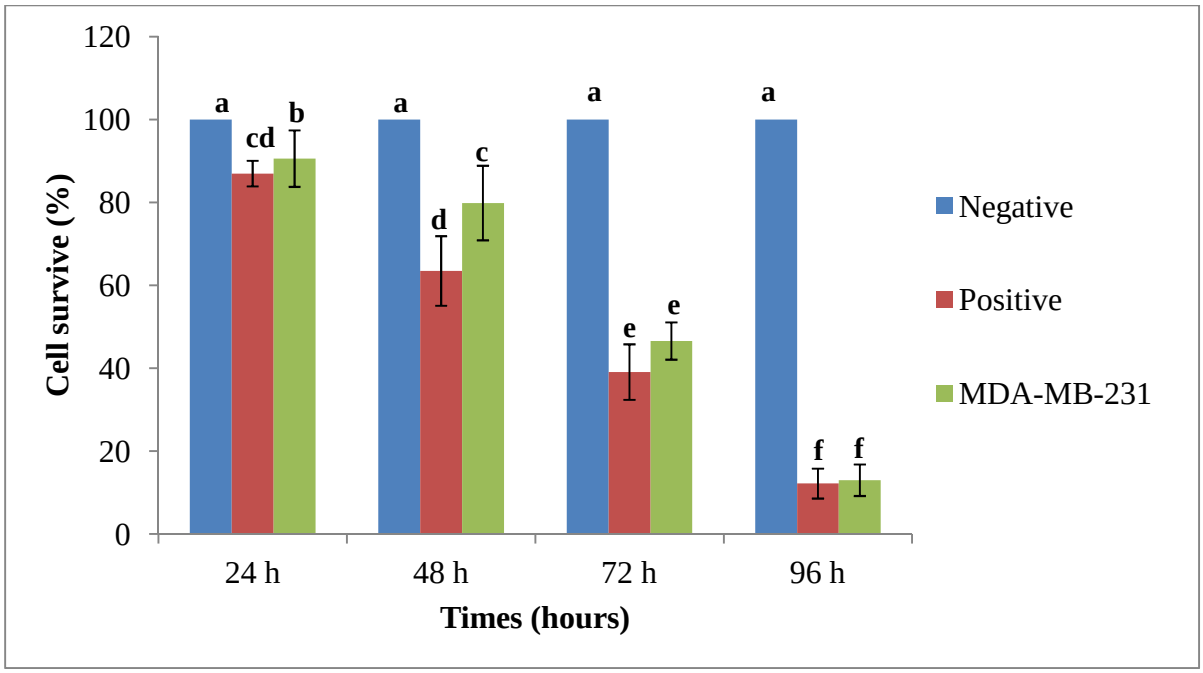

Figure 1.2. Growth inhibition effects of pentapeptide (1000 µg/mL) on human breast cancer cell line (MDA-MB-231) after various incubation times (24, 48, 72, 96 hours) determined using MTS assay.

The MDA-MB-231 cells were cultured with media alone and genistein (400 µg/mL) are considered negative and positive controls, respectively. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

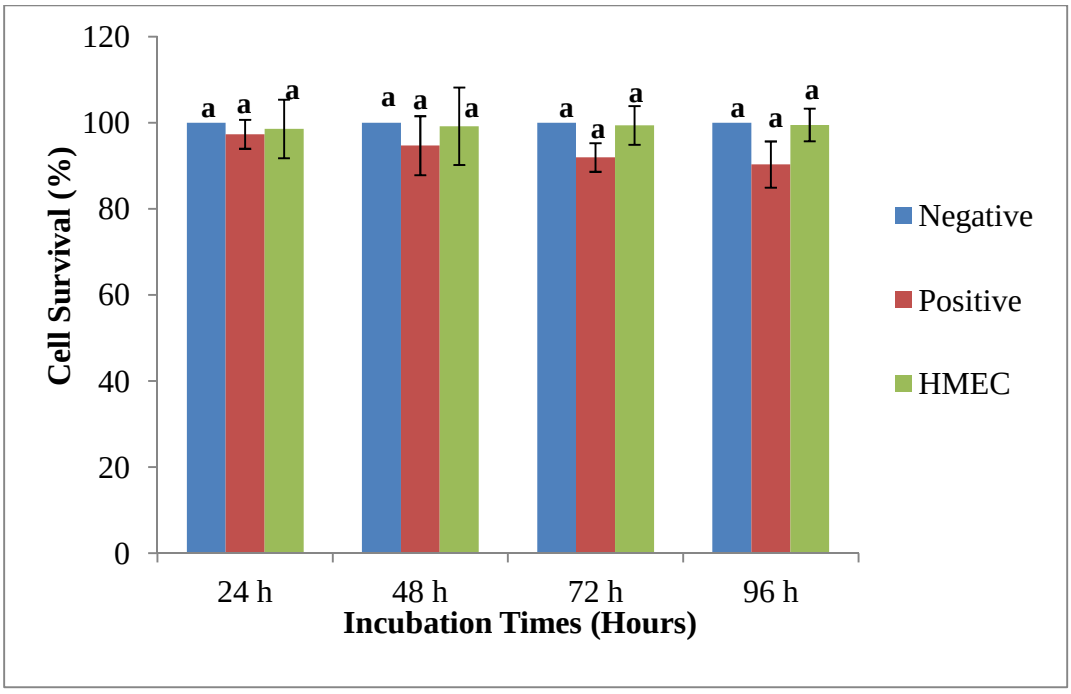

Figure 1.3. Effects of pentapeptide (1000 µg/mL) on human noncancerous breast cells (HMEC) after various incubation times (24, 48, 72, 96 hours) determined using MTS assay. The MDA-MB-231 cells were cultured with media alone and genistein (400 µg/mL) are considered negative and positive controls, respectively. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

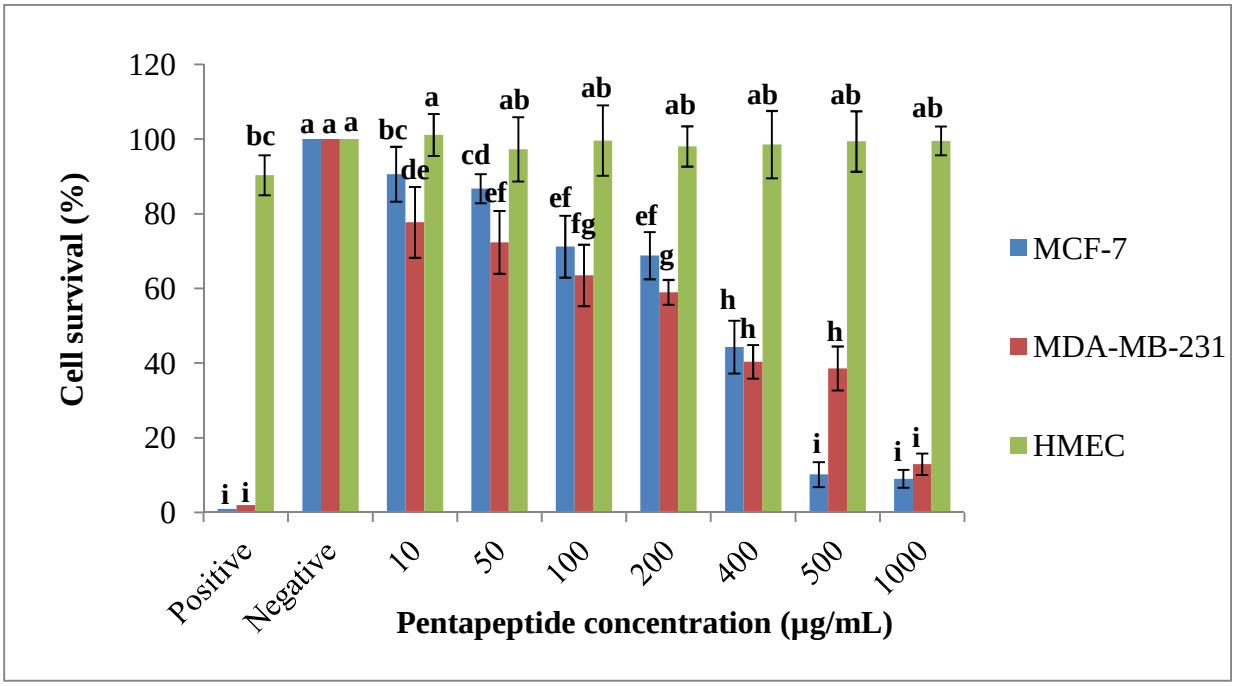

Figure 1.4. The growth inhibition effects of pentapeptide at various concentrations (10, 50, 100, 200, 400, 500, 1000 µg/mL) on MCF-7, MDA-MB-231, andhuman noncancerous breast cells (HMEC) after 96 hours incubation determined using MTS assay.

The cell lines were cultured with media alone and genistein (400 µg/mL) are negative and positive controls. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

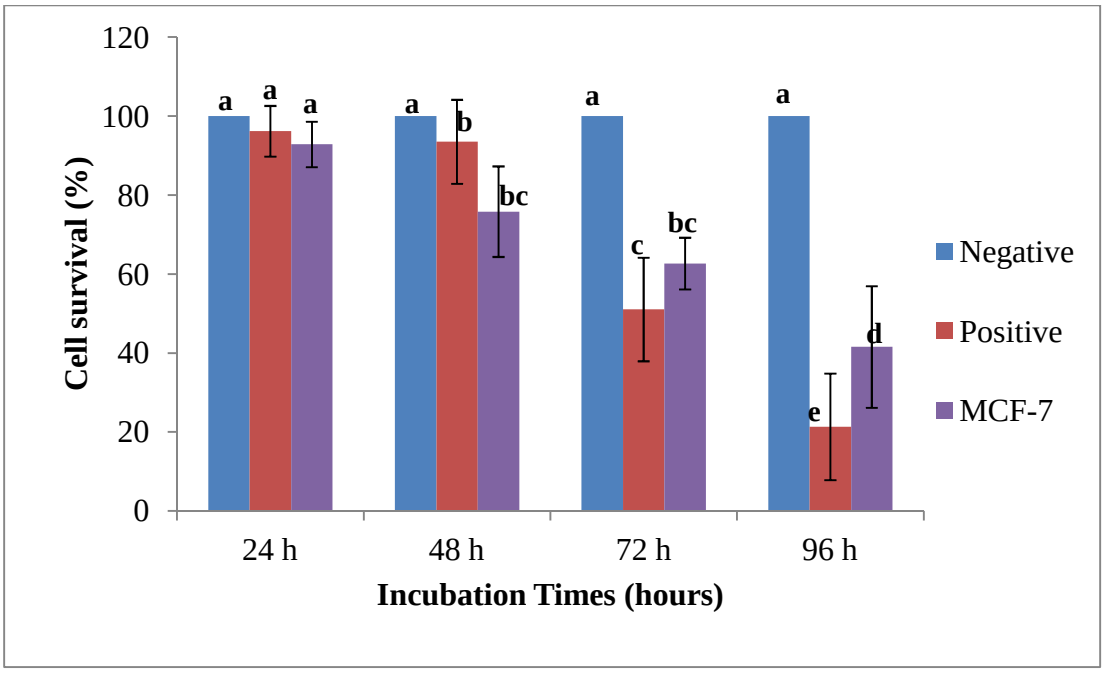

Figure 1.5. Survival (%) of pentapeptide (1000 µg/mL) treated human breast cancer cell line (MCF-7) after various incubation times (24, 48, 72, and 96 hours) determined using Trypan blue assay.

The MCF-7 cellswere cultured with genistein (400 µg/mL) and media alone are considered positive and negative controls, respectively. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

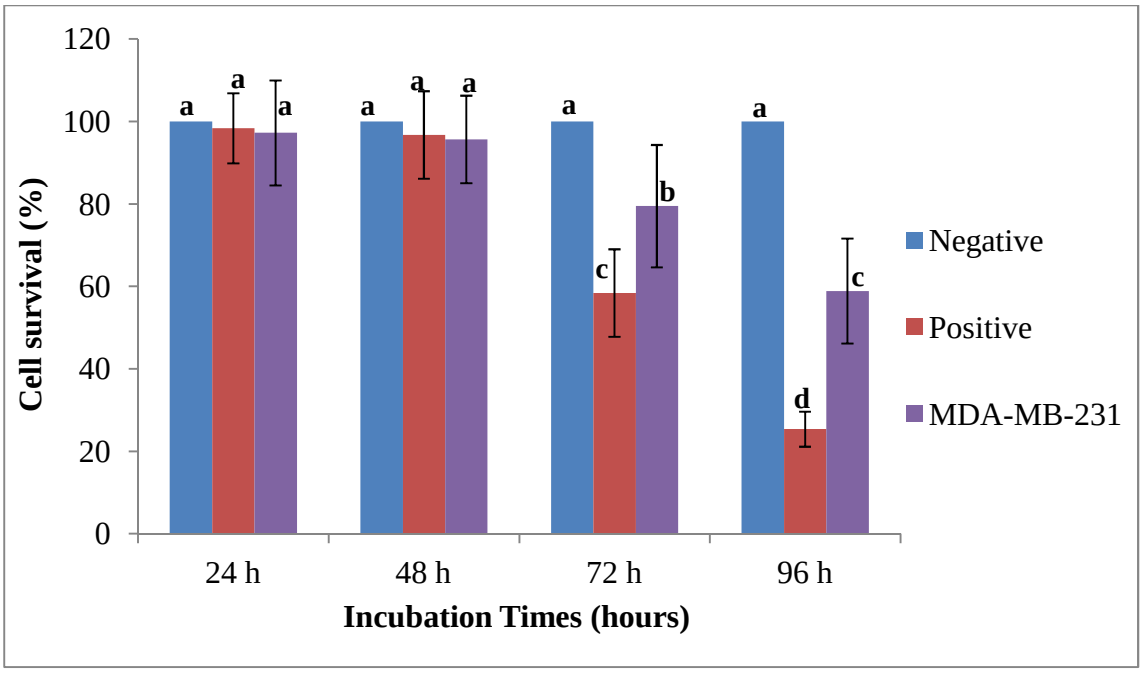

Figure 1.6. Survival (%) of pentapeptide (1000 µg/mL) treated human breast cancer cell line (MDA-MB-231) after various incubation times (24, 48, 72, and 96 hours) determined using Trypan blue assay.

The MDA-MB-231 cells were cultured with genistein (400 µg/mL) and media alone are considered positive and negative controls, respectively. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

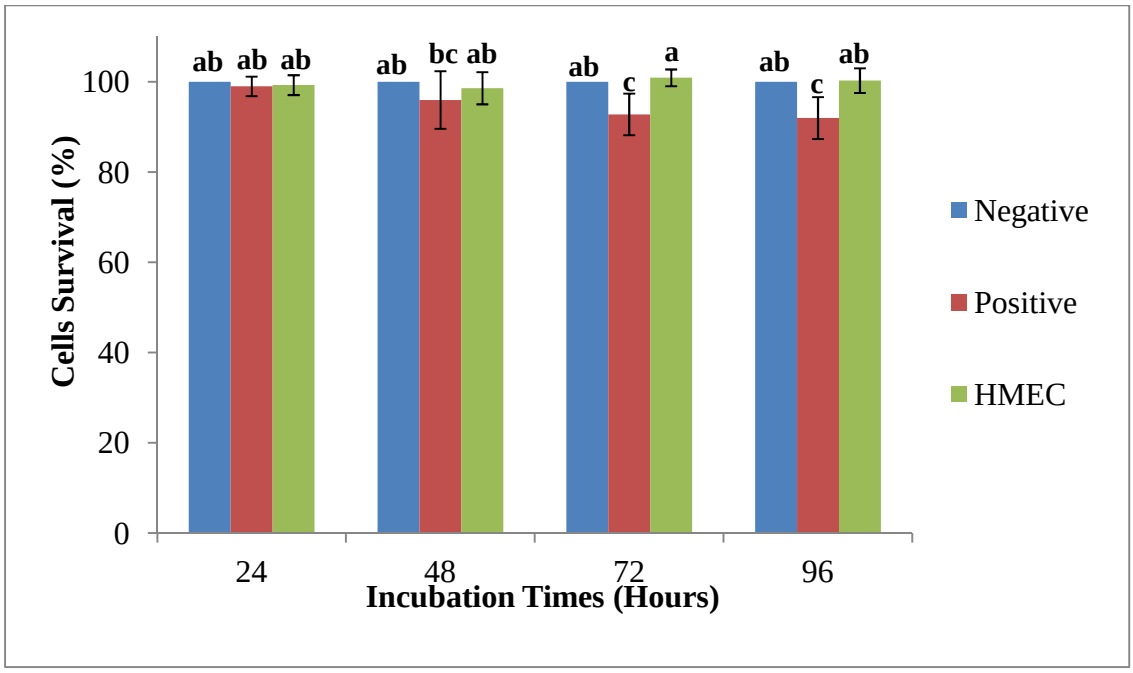

Figure 1.7. Survival (%) of pentapeptide (1000 µg/mL) treated human noncancerous breast cells (HMEC) after various incubation times (24, 48, 72, and 96 hours) determined using Trypan blue assay.

The HMECcells were cultured with genistein (400 µg/mL) and media alone are considered positive and negative controls, respectively. The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

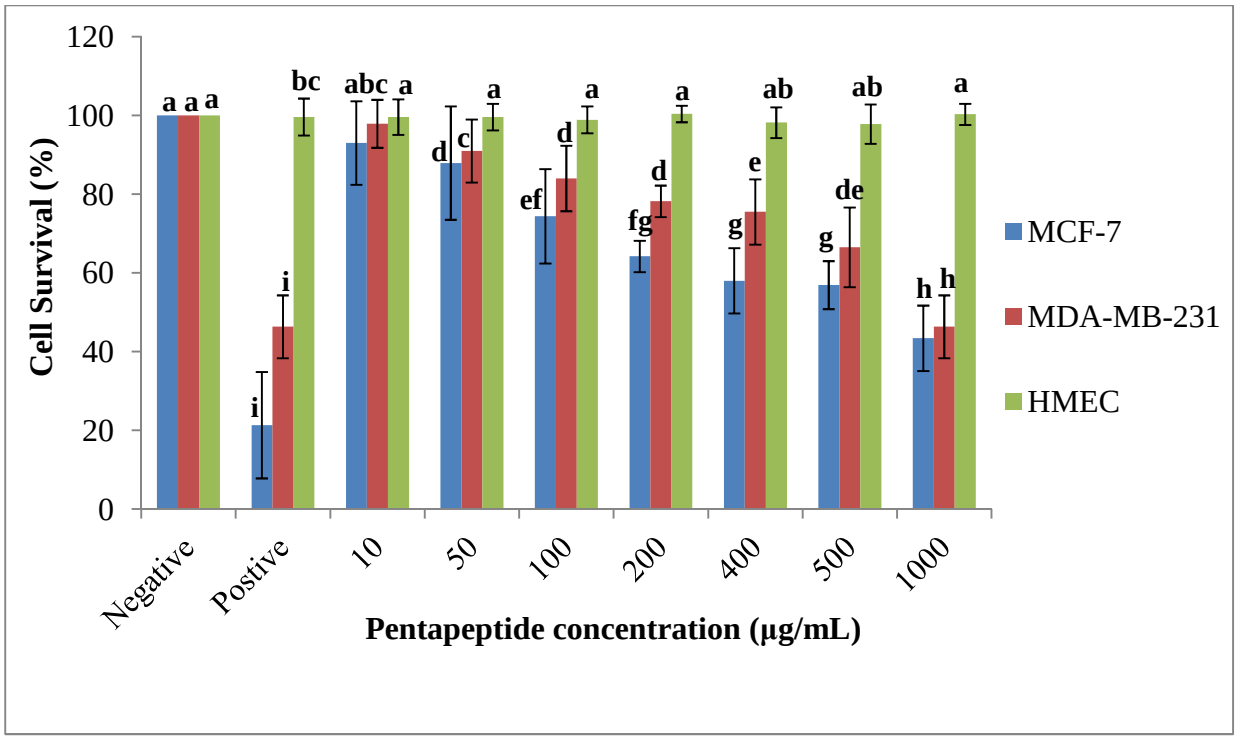

Figure 1.8. Survival (%) of pentapeptide treated (10, 50, 100, 200, 400, 500, 1000 µg/mL) MCF-7, MDA-MB-231, and HMEC cells after 96 hours incubation determined by Trypan blue assay.

The cellswere cultured with genistein (400 µg/mL) and media alone are positive and negative controls, respectively.The triplicate wells in each 96-well plates were run in duplicates. Values are means ± standard deviations of three determinations (n=6). Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

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04/22/2014

Dear Sir/Ma'am,

This is to inform you that Ruiqi Li is the first author of the paper and completed more than 51% of the work of the manuscript submitted to International Journal of Biomedical Research.

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CHAPTER 2

The apoptotic features of the pentapeptide treated human breast cancer cell lines (MCF-7 and MDA-MB-231)

Ruiqi Li, Navam Hettiarachchy, Mahendran Mahadevan

Abstract

Studies have focused on the characterization of peptides from natural sources that contribute specific health benefits. A pentapeptide derived from rice bran has shown anti-proliferative properties on human breast cancer cell lines (MCF-7 and MDA-MB-231). The objective of this study was to investigate the apoptotic features of pentapeptide-induced cell death in human breast cancer cell models (MCF-7 and MDA-MB-231). Morphological changes, DNA fragmentation, and increased caspases-3/7, -8, and -9 activities are considered hallmarks of cell death due to apoptosis. In this study, morphological changes of pentapeptide treated MCF-7 and MDA-MB-231 cells were observed using phase contrast microscopy (magnification: 200x). Apoptotic cells with fragmented DNA were observed using a TUNEL-based detection kit. The levels of caspases-3/7, -8, and -9 activities were determined using a luminescent assay. Distinct morphological changes including cell floating and shrinkage, nucleic blebbing, and the display of granular apoptotic bodies were observed in both cell lines after pentapeptide treatment. A typical pattern of fragmented DNA (significant green color over orange propidium iodide (PI) counter-staining under confocal microscopy) was also observed. Significant ($p < 0.05$) high levels of activated caspase-3/7, -8, and -9 were also determined in pentapeptide treated MCF-7 and MDA-MB-231 cells. These results suggest that the pentapeptide inhibits the growth of human breast cancer cells by introducing apoptosis.

Introduction

Apoptosis, also named programmed cell death (PCD), is a genetically regulated process that happens in every cell and is initiated by physiological and pathological stimuli. In normal tissue, the initiation of apoptosis is triggered by various pro-apoptotic stimuli. Then, the molecular executioner machinery of apoptosis is activated, which induce apoptotic changes in the nuclei (Saraste and Pulkki 2000). After the execution of apoptosis, the features of apoptosis become evident and observable. These features include morphological changes, DNA fragmentation, and activations of caspases, and have been recognized as hallmarks of apoptosis (Saraste and Pulkki 2000). The morphological changes during the apoptosis of cells include condensation of chromatin, cellular atrophy, and blebbing (Earnshaw et al 1999). In the process of tumorgenesis, the ratio of proliferated cells and apoptotic cells and the machinery of apoptosis are altered and uncontrolled.

Caspases are a group of cysteine-dependent aspartate-directed proteases which play an important role in controlling cell apoptosis. During early stages of apoptosis, the activation of initiator caspases by a signal transduction cascade will lead to the cleavage of cellular components, which finally results in the PCD. Caspases are subclassified as initiator caspases (caspase-8, -9, -10, and -12) and executer caspases (caspase-3, -6, and -7). The caspase cascade could be activated by two pathways: the intrinsic pathway and the extrinsic pathway (Yamasaki-Miyamoto et al 2009). In early stage of intrinsic pathway, the mitochondria serves as the primary target for TNF-induced cytotoxicity, which leads to the activation of adaptor protein Apaf-1, followed by the activation of the initiator procaspase-9. In the extrinsic pathway, apoptosis is induced by a mitochondria-independent sequence involving death receptor proteins such as Fas, and the cleavage of procaspase-8 (Kim et al 2002).

The observation of caspase cascade is considered one of the most important benchmarks for the characterization of the efficiency of certain cancer treatment. In human breast cancer cells, the non-expression of caspase-3 is the most unique feature of MCF-7 cell line (Kurokawa et al 1999). Liang et al (2002) reported that a sequential expression of caspases-9, -7, and -6 was observed. Thus, the study of the pentapeptide-induced caspase cascade in apoptotic human breast cancer cells is the first step to reveal its underlying mechanistic potency in human breast cancer.

Our results have shown that pentapeptide induced cell death in human breast cancer cell lines (MCF-7 and MDA-MB-231). However, it is still unclear whether the cell death is triggered by apoptosis. Hence, the objective was to study the apoptotic characteristics of pentapeptide-induced cell death of human breast cancer cell lines. These apoptotic features including morphological changes, DNA fragmentation, and increased levels of activated caspase-3/7,-8, and -9were observed and evaluated.

Materials and Methods

Materals. Human breast cancer cell lines (MCF-7 and MDA-MB-231), Leibovitz's L-15 Medium, and Eagle's Minimum Essential Medium were purchased from ATCC. Bovine insulin, fetal bovine serum (FBS), Trypan blue, and genistein were purchased from Sigma (St. Louis, MO, USA). Fluorometric DNA fragmentation detection kit III (F-dUTP) was obtained from PromoKine, Germany. Caspase assay kits for caspase-7, -8, and -9 activities were purchased from Promega Cop. (Madison, WI, USA).

Methods.

Cell culture. In Chapter 1, the research has proven that the pentapeptide showed anti-proliferative activities on human breast cancer cell lines (MCF-7 and MDA-MB-231), while having no cytotoxicity on normal human breast cell line (HMEC). Thus, only the mechanisms of pentapeptide-induced growth inhibition on human breast cancer cell lines (MCF-7 and MDA-MB-231) were investigated in Chapter 2 and Chapter 3. The human breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured following steps described in Chapter 1, Methods-Cell culture.The MCF-7 cells were cultured in a 75 cm^2 flask with Eagle's Minimum Essential Medium supplied with 0.01mg/mL bovine insulin and 10% FBS. Then, cells were grown in a humidified 5% CO_2 incubator at 37°C for around 7 days and reached 80 to 90% fully confluent (around 5 x 10^6 cells) before being collected for further assays. The MDA-MB-231 cells were cultured in a canted neck 75 cm^2 flask (for maintaining the inside atmosphere in the flask) with Leibovitz's L-15 Medium including 10% FBS and incubated at 37°C in a humidified incubator.

Observation of morphological changes. Cells (MCF-7 and MDA-MB-231) were trypsinized from a 75 cm^2 flask and the cells number was determined by a hemocytometer under microscopy (magnification: 200x). Then, the cells were transferred into a 96-well plate with a density of 5×10^3 cells per well. After 24 hours incubation, the spent media was removed and cells were treated with pentapeptide (1000 µg/ml) and incubated for 96 hrs. Genistein (400 µg/mL) and media alone were used as positive and negative controls, respectively. The cellular morphological changes were observed using phase contrast microscopy at a magnification of 200x(Akter et al 2012). Photos of the cells treated with pentapeptide, genistein, and media were taken using a using AmScope Color Digital Camera (MD 1000-CCD). Photos of cells grown in

media with the normal morphology were considered negative controls. The morphological changes of apoptotic cells include the shrinkage of cells, blebbing nuclei, and floating cells (Allen et al 1997).

DNA fragmentation. The detection of DNA fragmentation in pentapeptide treated human breast cancer cells was determined by fluorometric DNA fragmentation detection kit III (F-dUTP) following the company protocol (PromoKine, Germany). Cells (MCF-7 and MDA-MB-231) were cultured following the same method described above and treated with pentapeptide for 72 hrS. The cells cultured with media were used as controls. All the cells were fixed using 1.0% (w/v) paraformaldehyde in 1 x phosphate buffered saline (PBS) and ice-cold 70% (v/v) ethanol. Then, the cells were stained with Staining Solution supplied by the company (PromoKine, Germany) and treated with propidium iodide/RNase solution. The Nikon Laser-scanning confocal microscope (Plant Pathology at University of Arkansas) was used to detect the DNA fragmentations in apoptotic cells.

Assay for caspases activities. The activities of caspase-3/7, -8, and -9 were determined by caspase-Glo®assay kit following the company protocol (Promega Corp., USA). The caspase-3/7, -8,and -9 kits reagents were equilibrated to room temperature before use and prepared following the instruction. The cells were cultured and transferred into the white-walled 96-well plate with a density of 5 x 10^3 per well and allowed to attach for 24 hours. Then, the cells were treated with pentapeptide (1000 µg/mL) for 72 and 96 hours. One hundred µl of Caspase-Glo® reagent was added into each well and the contents were gently mixed contents using a plate shaker at 300

rpm for 30 seconds, followed by 2 hours incubation at room temperature. The luminescence of each sample was measured using a plate-reading luminometer.

Data Analysis. All the treatments in assays were conducted in triplicates and the values were reported as means ± standard deviation. The levels of activated caspases in pentapeptide treated human breast cancer cell lines were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS 9.2 2000, SAS Institute Inc., Cary, N.C., U.S.A.). The Fisher's protected least significant difference (LSD) test was conducted to separate the means at $P \leq 0.05$.

Results and Discussion

Observation of morphological changes. Generally, abnormal and unhealthy cells such as cancerogenic cells are removed through apoptosis (Xia et al 1999). Apoptosis could be recognized by several biological features including morphological changes (loss of cell volume and mitochondrial depolarization), DNA fragmentation, and caspases activation (Saraste and Pulkki 2000; Mgbonyebi et al 1999).The morphological changes are considered one of the most remarkable features of apoptotic cells because cell death via apoptosis exhibits a very distinctive pattern (Ziegler and Groscurth 2004). Thus, the morphological changes are the unique features of apoptotic cell death induced by various cell anti-proliferative agents. The prominent morphological changes of apoptotic cells including the change of cell shape, cells round up, and detached from each other. One of the major difference in cell morphology between apoptosis and necrosis is that the cells undergoing apoptosis still have compact organelles while the cytoplasmic ultrastructure of cells undergoing necrosis significantly increases with damaged plasma and internal membranes (Baba 2009). Thus, in order to evaluate whether the

pentapeptide-induced cell death was caused by apoptosis, the morphological changes of pentapeptide-treated MCF-7 and MDA-MB-231 cells were observed using a phase contrast microscopy (magnification: 200) and compared with the untreated controls. After 72 hrs of pentapeptide (1000 µg/mL) treatment on MCF-7 and MDA-MB-231 cells, the morphological changes (pointed out by arrows) including cell floating,shrinkage, and nucleic blebbingwere observed (Figures 2.1b and 2.1d); however, the control groups without pentapeptide treatment shown no detectable morphological changes (Figures 2.1a and 2.1c).

In the current study, the pentapeptide not only induced typical morphological changes in MCF-7 and MDA-MB-231 cells due to apoptosis but also extensive detachment of cells from the cell culture plates. Post 72 hours pentapeptide treatment, significant amounts of detached MCF-7 and MDA-MB-231 cells were observed compared to controls (Figure 2.1). Wen et al (1997) suggesting that integrins on the cell membrane are able to interact with extracellular matrix (ECM) components such as fibronectin, collagen, and vitronectin and results in the detachment of cells from substratum. The disruption of integrin-mediated signal transduction inducing by disassociation of integrins with ECM compounds leads to the inactivation of focal adhesion kinase which detaches the cells from the culture surface and apoptotic cell death (Fukai et al 1998). Our observations of pentapeptide treated MCF-7 and MDA-MB-231 cells suggest that human breast cancer cells undergo apoptosis and detach from cell culture substratum (Figure 2.1 b and d). The pentapeptide may induce the cell death via uncoupling of integrin-mediated signaling and/or disruption of cell-matrix interactions (Mgbonyebi et al 1999). These observations agreed with previous studies that suggesting the detachment of cells and changes of morphology are evidence of cell death induced by apoptosis (Hossain et al 2012; Hu et al 2010; Diaz-Ruiz et al 2001; Senaratne et al 2000; Mgbonyebi et al 1999). In addition, the pentapeptide

inducesapoptosis in cells and leads to the detachment on MDA-MB-231 cells suggesting that it could be a very promising in preventing/inhibiting the initiation/growth of metastatic cancer cells since the adhesion is crucial for cancer metastasis (Wong et al 1998).

Pentapeptide Induced DNA fragmentation in MCF-7 and MDA-MB-231 Cells. In the above section (observation of morphological changes), our data suggest that pentapeptide induced MCF-7 and MDA-MB-231 cell death may be caused by apoptosis. However, both morphological and biochemical features must be justified to confirm that cell death has occurred via apoptosis (Bortner et al 1995).Wyllie and Kerr (1972; 1980) proposed that DNA fragmentation is involved in cell apoptosis, showing that fragmented DNA with 180 to 220 bp lengths is observed before cell death. Afterwards studies and publications have proven that this form of DNA fragmentation is very widely observed in apoptosis of various cells and tissues under different circumstances (Bortner et al 1995). Thus, the degradation of internucleosomal DNA, the best-characterized biochemical benchmark, is evaluated in current study as the definitive apoptotic marker (Saraste and Pulkki 2000; Allen et al 1997).

The nuclear DNA is the genetic material that is crucial for producing cellular proteins; the cleavage of DNA into fragments represents the most severe damage to the cell (Zhang and Xu 2002).This process occurs after the cells are attacked by various apoptotic stimuli. The chromosomal DNA is cleaved by nucleases in a caspase-dependent manner. One apoptotic nuclease is DNase (CAD, caspase-activated DNase),which requires Ca^{2+} and Mg^{2+} and is inhibited by Zn^{2+} (Peitsch et al 1992).The initiator caspases such as caspase-8 and -9 are activated and subsequently activate downstream executor caspases including caspases-3, -6, and -7. The CAD is activated by dissociating from ICAD (inhibitor of CAD) due to the cleavage of

downstream caspases. The cleavage of CAD on DNA is responsible for the fragmented genetic materials, which results in the biochemical changes that characterize apoptotic cell death (Nagata 2000).This phenomenon can be analyzed by agarose gel electrophoresis, which measures DNA fragmentation in nuclear extracts showing the typical "DNA-ladder" configuration. Another method to measure DNA fragmentation is based on the labeling of free 3-hydroxyl ends of the fragmented DNA with the fluorescein-12-dUTP under the terminal deoxynucleotidyl transferase (TdT) catalyzing (Rosl 1992; Gavrieli et al 1992). Sgonc and Gruber (1998) also suggest that the TdT-mediated dUTP nick end-labeling (TUNEL)-based method is highly recommended for characterization of apoptotic cells, simultaneous determination of cell markers, and apoptosis.

In the current study, a TUNEL-based kit was used to detect the DNA fragmentation in pentapeptide treated apoptotic MCF-7 and MDA-MB-231 cells. The TdT catalyzes the fluorescein-12-dUTP incorporation located at the free 3'-hydroxyl ends of the fragmented DNA. Confocal microscopy was used to observe the fluorescein-labeled fragmented DNA from the stained apoptotic cells which showing green fluorescence at 520 nm against an orange red propidium iodide (PI) counter-stain (fluorescence at 620 nm). After MCF-7 and MDA-MB-231 cells were treated with pentapeptide (1000 µg/mL) for 72 hr, the DNA fragmentation assay was performed and the results revealed a typical fragmented DNA pattern (Figures 2.2b and d) compared to negative controls (Figures 2.2a and c). This is in agreement with results by Sgonc and Gruber (1998), which the apoptotic cells showed a green-stained nuclei after being labeling by TUNEL technique. Other studies also observed fragmented DNA in various cancer cell lines after treatment with different anti-cancer agents including fucoidan, melatonin, ganoderma lucidum extract, epinecidin-1, roscovitine, and wortmannin (Yamasaki-Miyamoto et al; 2009; Mediavilla et al 2002; Hu et al 2002; Chen et al 2009; Mgbonyebi et al 1999; Hossain et al 2012).

These results combined with results describing morphological changes suggest that pentapeptide is able to induce human breast cancer cell death via apoptosis.

Pentapeptide induces apoptosis in human breast cancer cells (MCF-7 and MDA-MB-231) through the caspases-dependent pathway. To verify the role of caspases in pentapeptide-induced growth inhibition on human breast cancer cells, the levels of caspase-3/7, -8, and -9 were evaluated after appropriate treatments. Genistein (400 μg/mL) was used to induce apoptosis in MCF-7 and MDA-MB-231 cells and was considered the positive control. Significantly increased ($p< 0.05$) levels of active caspase-3/7 fragments were detected in both cell lines in the presence of pentapeptide (1000 μg/mL) from 72 to 96 hr (Figure 2.3). MDA-MB-231 cells have also shown comparatively higher levels of caspase-3/7 than MCF-7. Significantly increases ($p< 0.05$) in levels of activated caspase-8 and caspase-9 were detected at 72 and 96 h after the pentapeptide treatment compared to the controls (MCF-7/MDA-MB-231 cultured with media). Pentapeptide induced significant ($p< 0.05$) increases in relative caspase-8 intensity in MCF-7 (from 5.2 to 7.7 folds) and MDA-MB-231 (from 3.8 to 6.9 folds) compared to the untreated cells (Figure 2.4). The caspase-9 was also significantly ($p< 0.05$) activated after the pentapeptide treatment in MCF-7 (from 2.2 to 2.7 folds) and MDA-MB-231 (from 2.0 to 2.2 folds) cells (Figure 2.5).

Caspase cascade is generally recognized as a hallmark of cells undergoing apoptosis. Caspases are group of cysteine proteases dividing into two sub-categories including effector caspases (such as caspase-3 and -7) and initiator caspases (such as caspase-8 and -9) (Tao et al 2007). Two major apoptotic mechanisms including death receptor-dependent and mitochondria-dependent pathways involve the activation of caspase-8 and -9, respectively (Cryns and Yuan et

al., 1998). The caspase-3 or -7 are activated to cleave various cytoplasmic or nuclear substrates including DNAse which lead to the characteristic morphological changes and DNA fragmentation (Degen et al 2000; Stroh and Schulze et al 1998; Casciola et al 1995). Figure 2.3 indicates that the caspase-3/7 activities in cells treated with pentapeptide increased significantly ($p< 0.05$) compared to control treatments with medium alone. Since MCF-7 is a caspase-3-deficient breast cancer cell line, the results suggest that effector caspase-7, a sub-family member of caspase-3, plays indispensible role in MCF-7 cells during apoptosis. Previous studies have shown its role in apoptosis of caspase-3-deficient MCF-7 cells, (Hu et al., 2001; Janicke et al., 1998; Twiddy et al., 2006). The activations of caspase-3/7 were observed in both cell lines after treatment with pentapeptide from 72 and 96 hrs which is consistent with the resulting morphological changes and the DNA fragmentation. Therefore, the results suggested that pentapeptide-induced MCF-7 and MDA-MB-231 cells death were involved in a mechanism of caspase-dependent apoptosis.

To further investigate the features of caspase-dependent apoptosis in pentapeptide treated human breast cancer cells, the caspase-8 and caspase-9 levels in MCF-7 and MDA-MB-231 cells were evaluated. In the current study, the activated caspase-8 and -9 in cells reacted with aminoluciferin-labeled substrates and the relative concentrations were determined by measuring the luminescence intensities. Caspase-8 is activated through extrinsic pathway by interactions between ligands and death receptors. Caspase-9 is involved in intrinsic pathway and activated due to the mitochondrial cytochrome c leakage (Kumar 1999). The results showed that pentapeptide activates caspase-8/-9 and induced apoptosis in MCF-7 and MDA-MB-231 cells (Figures 2.4 and 2.5). These results suggest that pentapeptide triggers apoptosis via a combination of intrinsic and extrinsic pathways.

Conclusion

In conclusion, by evaluating the apoptotic features of pentapeptide treated human breast cancer cells (MCF-7 and MDA-MB-231) including morphological changes, fragmented DNA, and activation of caspases, the results in the current chapter demonstrated that the pentapeptide induces cell death in human breast cancer cell lines through apoptosis. The pentapeptide may be a potential anti-breast cancer agent or future alternative for current drug therapies when the molecular mechanisms are better characterized.

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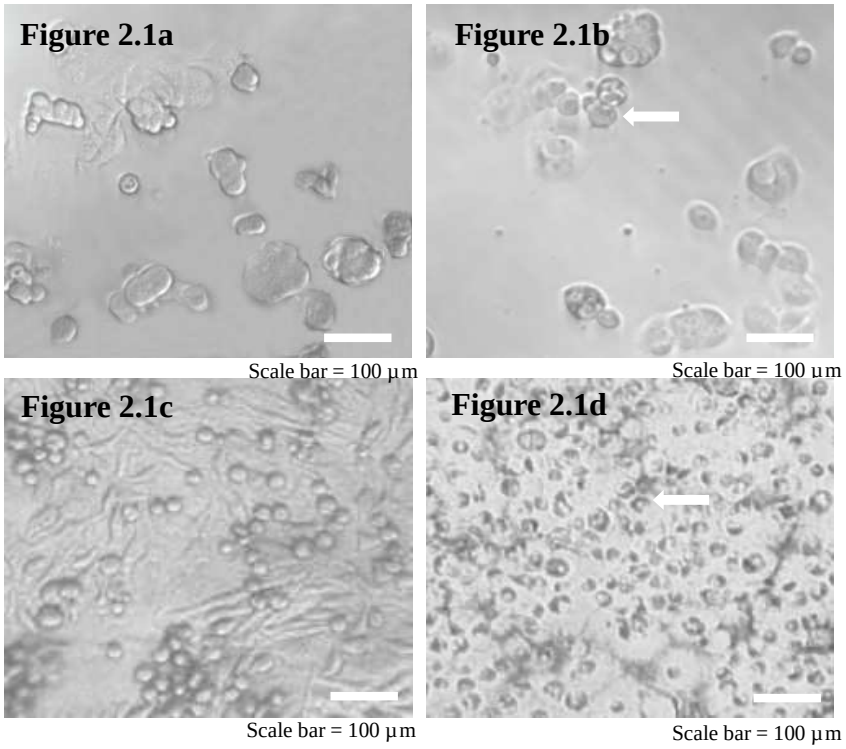

Figure 2.1 (a,b,c,d). Effect of pentapeptide on the morphology of MCF-7 and MDA-MB-231 cells.

Figure 2.1a.Morphology of MCF-7 cells cultured with media and observed after 72 hr incubation (control).

Figure 2.1b.Morphology of MCF-7 cultured in the presence of pentapeptide (1000 µg/mL) and observed after 72 hr incubation

Figure 2.1c.Morphology of MDA-MB-231 cells cultured with media and observed after 72 hr incubation (control).

Figure 2.1d.Morphology of MDA-MB-231 cells cultured in the presence of pentapeptide (1000 µg/mL) and observed after 72 hr incubation.

All cell morphology were observed using a phase contrast microscopy (magnification: 200) and photos were taken using AmScope Color Digital Camera (MD 1000-CCD).

All arrows point to the retracted and round cells which were undergoing apoptosis.

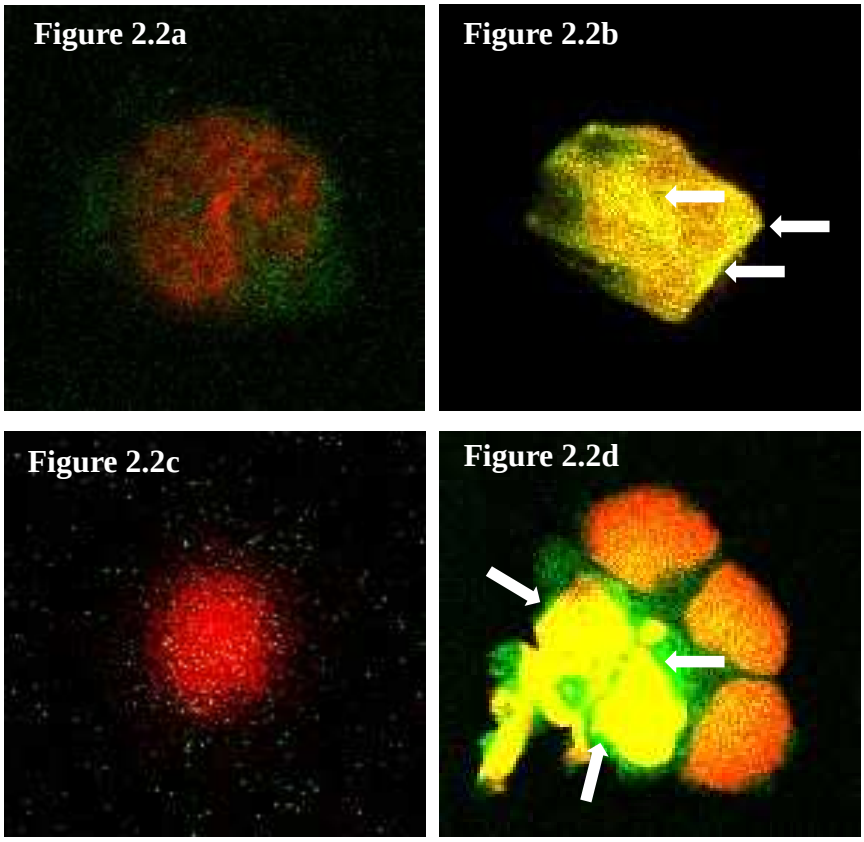

Figure 2.2 (a,b,c,d). DNA Fragmentation of pentapeptide treated MCF-7 and MDA-MB-231 cells.

Figures 2.2a.MCF-7 cells grown in media and observed after 72 hr.

Figures 2.2b.MCF-7 cultured in the presence of pentapeptide (1000 µg/mL) and observed after 72 hr incubation.

Figures 2.2c.MDA-MB-231 cells grown in media and observed after 72 hr.

Figures 2.2d.MDA-MB-231 cultured in the presence of pentapeptide (1000 µg/mL) and observed after 72 hr incubation.

All cells were stained using a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based DNA fragmentation kit and observed using a confocal microscope (Ex/Em = 488/520 nm for FITC/green/apoptotic, and 488/623 nm for PI/red/rhodamine).

All arrows point to the areas emit green light which reflect the fragmented DNA.

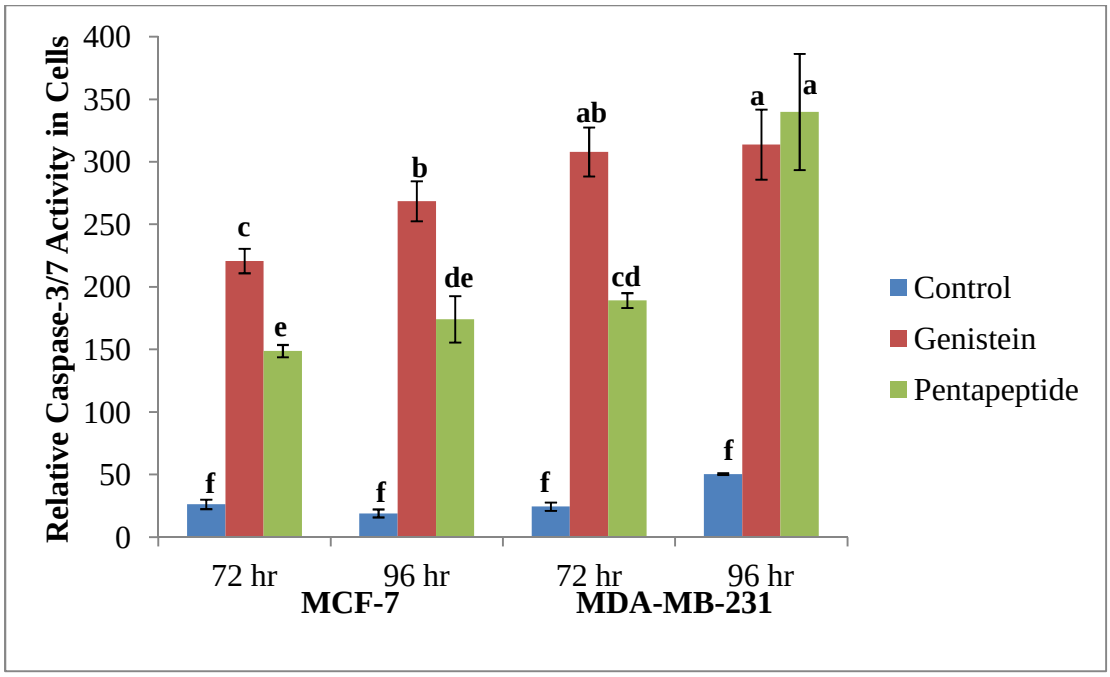

Figure 2.3. The relative intensity of activated caspase-3/7 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive control, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

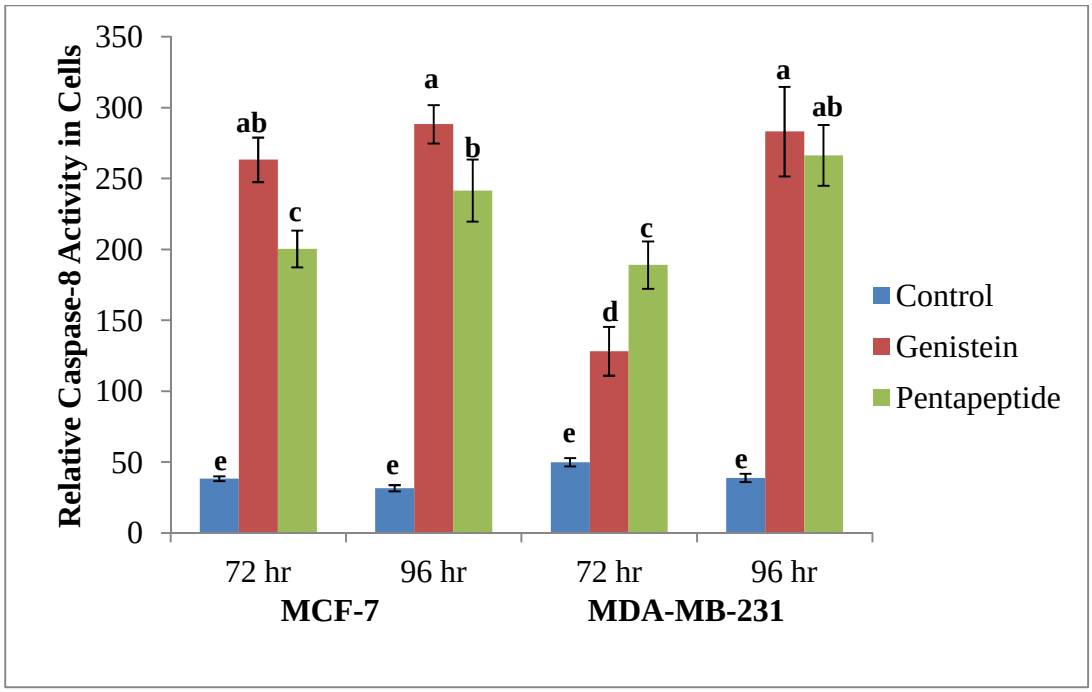

Figure 2.4. The relative intensity of activated caspase-8 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively.Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

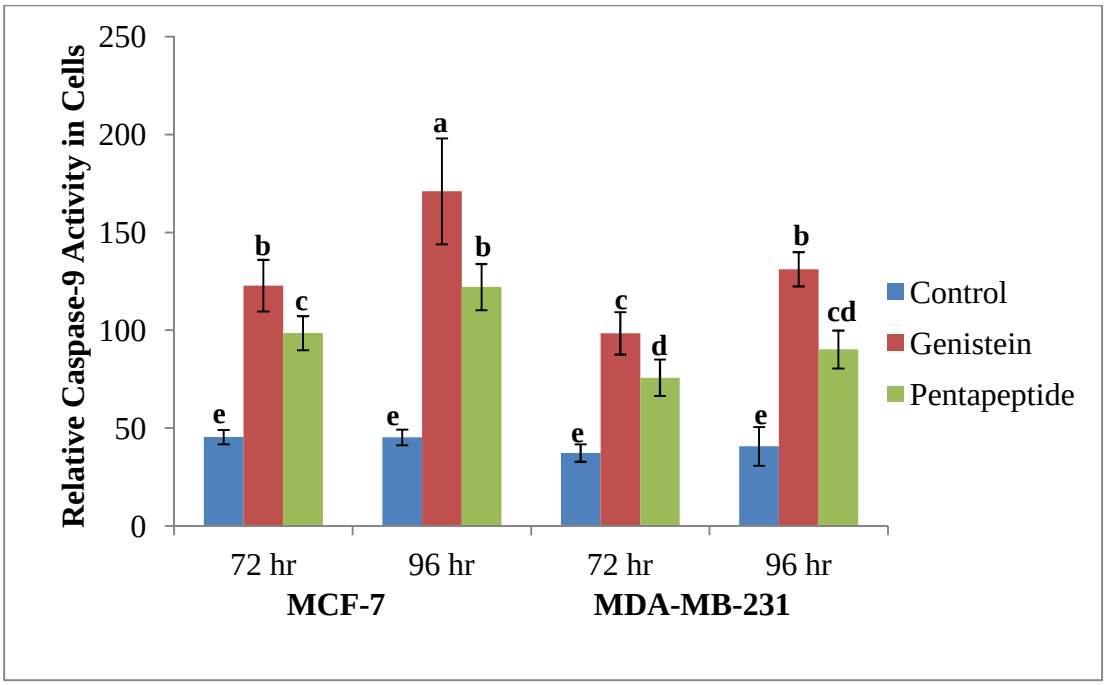

Figure 2.5. The relative intensity of activated caspase-9 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

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04/22/2014

Dear Sir/Ma'am,

This is to inform you that Ruiqi Li is the first author of the paper and completed more than 51% of the work of the manuscript submitted to International Journal of Biomedical Research.

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CHAPTER 3

Evaluation of molecular targets in pentapeptide-induced apoptotic pathways in human breast cancer cell models (MCF-7 and MDA-MB-231)

Ruiqi Li, Navam Hettiarachchy, Mahendran Mahadevan

Abstract

Bioactive peptides derived from food sources with anti-proliferative properties against cancer have drawn more attention in recent years. A pentapeptide derived from rice bran was shown its anti-proliferative activities on human breast cancer cell lines via apoptosis. Evaluating the levels of various critical molecular targets was considered an effective mean to understand the underlying pathways involved in cell apoptosis after certain anti-cancer treatments. Thus, in this chapter, the objective was to investigate the apoptotic pathways of pentapeptide-induced apoptosis in breast cancer cell models (MCF-7 and MDA-MB-231) by determining the expression of various biomarkers. The levels of molecular targets (p53, COX-2, TNF-α, Bax, Bcl-2, Fas, and erbB-2) were evaluated by ELISA kits. Significant decreases in levels ($p < 0.05$) of COX-2 and increases in levels ($p < 0.05$) of p53 were detected after treatment with pentapeptide from 72 to 96 h. Significant ($p < 0.05$) decreases in the levels of Bcl-2 and erbB-2 and increases in the levels of TNF-α and Bax were also detected after pentapeptide treatment from 72 to 96 h. The results suggest that pentapeptide inhibits growth of human breast cancer cells by introducing apoptosis through a caspase-dependent pathway. Pentapeptide stimulated the levels of p53 in both cells lines and may also suppress the COX-2 in ER-positive breast cancer cells such as MCF-7 by regulating estrogen synthesis. The pentapeptide also amplifies apoptotic signals by down-regulating the expression of ErbB-2.

Introduction

Studies have proven that various downstream signaling factors including the nuclear factor-κB (NF-κB), the Fas, the inhibitor of apoptosis proteins (IAP), and the mitogen-activated protein kinase (MAPK) family can mediate the process of apoptosis (Simstein et al 2003). As important biological indicators of cancer status and progression for the physiological state of the cell at a specific time, those molecular targets represent powerful tools for monitoring the course of cancer and gauging the efficacy and safety of novel therapeutic agents.

TNF-α is a naturally occurring cytokine secreted by cells of the immune system and other systems. This unique property has led to numerous studies of tumor necrosis factor (TNF) as a chemotherapeutic agent and apoptosis-inducing agent. TNF can activate both apoptotic pathways and survival pathways inside the cell. TNF-α has two receptors, p55 (TNFR1) and p75 (TNFR2), with most apoptotic pathways mediated through p55.

Fas, a member of the TNF, is located on the cell surface and is activated by its ligand (Fas L) binding (Keane et al 1996). Fas forms death-inducing signaling complex (DISC), which finally leads to the activation of effecter caspases (Chopin et al 2002; Chen and Lin 2004). Studies have reported that anticancer drugs activate apoptosis in breast cancer cells by inducing the expression of death receptor ligands, such as Fas ligand (FasL) (Fulda et al2001).

The Bcl-2 family proteins are considered a vital control point of apoptosis by regulating the release of cytochrome C and the activation of caspase-9. The subfamilies of Bcl-2 proteins include anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, Bfl- 1/A1, Bcl-W, Bcl-G) and pro-apopotic proteins (Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-XS, Krk, Mtd, Nip3, Noxa, Bcl-B) (Danial 2007). Bax-like death factors such as Bax, Bak, and Bok/Mtd induce apoptosis by building the channels on membrane for cytochrome C release. Anti-apoptotic proteins, are

generally classified as Bcl-2-like survival factors. These anti-apoptotic proteins are located on the outer mitochondrial membrane and protect the cells by forming heterodimers with pro-apoptotic proteins (Borner 2003). The interactions between Bcl-2 family proteins affect cellular sensitivity to apoptosis and the ratio of pro-apoptotic proteins and anti-apoptotic proteins determines the fate of cells. The Bcl-2 proteins also interact with caspases in a mitochondrial-independent pathway and play an important role in the activation of caspase-9 (Gross et al 1999).

Cyclooxygenase (COX) is an important enzyme in charge of formation of prostaglandin H2 from arachidonic acid. It has received considerable attention due to its multiple roles in human cancers development. The effect of COX-2 on cell growth results from the increased production of PGE_2 and PGF_2, which contributes to the cell proliferation (Bandopadhyay et al 1987). In addition, the COX-2 suppresses the apoptosis by changing the cellular levels of antiapoptotic and proapoptotic proteins. In a further step of cancer development, COX-2 also stimulates the invasion of cancerous cells to other tissues.

The purpose of this study is to quantify the levels of molecular targets (p53, COX-2, TNF-α, Fas, Bax, Bcl-2, and ErbB-2) in pentapeptide treated human breast cancer cells. This study provides insight on the molecular mechanism of action and apoptotic pathways of the pentapeptide against breast cancer cell lines and some preliminary results on it therapeutic/drug-like property.

Materials and Methods

Materals. Human breast cancer cell lines (MCF-7 and MDA-MB-231), Leibovitz's L-15 Medium, and Eagle's Minimum Essential Medium were purchased from ATCC. Bovine insulin, fetal bovine serum (FBS), Trypan blue, and genistein were purchased from Sigma (St. Louis, MO, USA). The human ELISA kits for determining levels of p53, Bcl-2, and ErbB-2 were

supplied by Abcam Plc. (Cambridge, MA, USA). The human COX-2 and Bax ELISA kits were supplied by Enzo Life Science Inc. (Farmingdale, NY, USA). The TNF-α and Fas human ELISA kits were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and RayBiotech Inc. (Norcross, GA, USA), respectively.

Methods.

Cell culture. The human breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured following steps described in Chapter 1, Methods-Cell culture. The MCF-7 cells were cultured in a 75 cm^2 flask with Eagle's Minimum Essential Medium supplied with 0.01mg/mL bovine insulin and 10% FBS. Then, cells were grown in a humidified 5% CO_2 incubator at 37°C for around 7 days and reached 80 to 90% fully confluent (around 5 x 10^6 cells) before collecting cells for further assays. The MDA-MB-231 cells were cultured in a canted neck 75 cm^2 flask (to maintain the inside atmosphere in the flask) with Leibovitz's L-15 Medium including 10% FBS and incubated at 37°C in a humidified incubator.

Determination of levels of p53, COX-2, TNF-α, Fas, Bax, Bcl-2, and ErbB-2. The levels of TNF-α, Bax, Bcl-2, COX-2, p53, Fas, and ErbB-2 in pentapeptide-treated MCF-7 and MDA-MB-231 cells were determined using respective human ELISA kits (sensitivity: range 1.0 to 70 ng/mL) following the company protocols under same principle. Cells were cultured and treated with pentapeptide (1000 µg/mL) for 72 and 96 hr. This concentration was used in all experiments that determining the levels of molecular targets. The cell lysates were prepared using lysis buffer and placed in 96-well plates coated with monoclonal detective antibodies and incubated for 2 hr. The unbound materials were removed by washing buffer. Horseradish

peroxidase conjugated streptavidin was added to bind to the antibodies in respective wells and the plate was then incubated for 1 hr. The substrate reagent was added to each well and the absorbances were measured at 450 nm using a microplate reader. The concentrations of p53, COX-2, TNF-α, Bax, Bcl-2, Fas, and ErbB-2 were determined by standard curves plotted using standard proteins.

Data Analysis. All the treatments in the ELISA assays were conducted in triplicates. The data of levels of p53, COX-2, TNF-α, Fas, Bax, Bcl-2, and ErbB-2were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS 9.2 2000, SAS Institute Inc., Cary, N.C., U.S.A.). The Fisher's protected least significant difference (LSD) test was conducted to separate the means at $P \leq 0.05$.

Results and Discussion

Expression of p53 and COX-2 in pentapeptide treated human breast cancer cell lines. To further investigate the possible mechanisms of pentapeptide-induced apoptosis in MCF-7 and MDA-MB-231 cells, the expressions of p53 (a tumor suppressor) and COX-2 (potential target for prevention and treatment of breast cancer) were assessed using ELISA assays in the presence of the pentapeptide (1000 μg/mL). Significantly ($p< 0.05$) increased levels of p53 were observed after 72 and 96 hrs of incubation with pentapeptide in both cell lines (Figure 3.1). However, the pentapeptide showed significant ($p< 0.05$) down-regulation on COX-2 levels in MCF-7 cells while no significant ($p> 0.05$) decreased levels of COX-2 in MDA-MB-231 were observed (Figure 3.2).

In an attempt to identify the possible mechanism of apoptosis in MCF-7 and MDA-MB-231 cells in response to pentapeptide, the expressions of p53 and COX-2 were evaluated after pentapeptide treatment. It is widely known that p53 is a tumor suppressor gene involving in cell-cycle regulation and the induction of apoptosis by mediating the ratio of Bax (pro-apoptotic protein) to Bcl-2 (anti-apoptotic protein) (Mercer et al 1992; King and Cidlowsky 1998). The p53 protein helps in repairing the damaged DNA to cause G1 arrest and avoid the cancerous cell proliferation. Studies have suggested that the up-regulation of the p53 protein is in accordance with growth inhibition of cancerous cells when exposed to various anti-cancer agents (Chopin et al 2002; Cui et al 2007; Sun 2006; Wang et al 2004; Kuo et al 2005). In our study, the expression of p53 increased significantly ($p< 0.05$) in pentapeptide treated MCF-7 and MDA-MB-231 cells and suggests that pentapeptide up-regulates the activated p53 which may either trigger the onset of DNA repair or induces the apoptosis via mitochondrial-dependent pathway by regulating the levels of downstream molecules such as Bax, Bcl-2, and Fas. Thus, further investigation is needed to indentify the actual role of p53 in pentapeptide-mediated apoptosis in MCF-7 and MDA-MB-231 cells.

COX-2 (Cyclooxygenase-2) is being intensively evaluated as a pharmacologic target for both the prevention and treatment of cancer. Studies have shown that high expression of prostaglandin (PG) detected in many types of cancers and enhanced PG synthesis may contribute to carcinogenesis via stimulation of cancer cell proliferation (Howe and Dannenberg 2002). Since the primary role of COX-2 in PG production as a critical synthase, studies has been carried out to design COX-2 inhibitors can be effective in treating breast tumors (Singh and Lucci 2002). Other studies also suggest that COX-2 over-expression may result in the suppression of pro-apoptotic proteins such as Bax and Bcl-xL during apoptosis (Wang and DuBois 2004). In our

research, the pentapeptide displayed a down-regulating effect on COX-2 level in MCF-7 cells; while the COX-2 level remained unchanged in a MDA-MB-231 cell line. These results suggested that pentapeptide suppress the level of COX-2 which leads to the cell growth inhibition via decreasing of estrogen levels in an Estrogen Receptor (ER)-positive MCF-7 cell line; however, pentapeptide has no effect on regulating COX-2 level in an ER-negative MDA-MB-231 cell line. This result is agree with the previous study by Harris et al (1999), who suggested that PGs may also stimulate proliferation indirectly via increased estrogen biosynthesis in breast tissue.

Pentapeptide induced apoptosis is promoted via a TNF-α and Fas-dependent pathway and the activation of caspase-8. To assess whether a death receptor-mediated pathway is involved in pentapeptide-induced apoptosis, the TNF-α and Fas protein levels were determined in MCF-7 and MDA-MB-231 cells after exposure to the pentapeptide. TNF-α levels increased significantly ($p < 0.05$) after pentapeptide treatment for 72 and 96 hrs compared to negative controls (cell lines cultured only with fresh media) (Figure 3.3). Even though MCF-7 has shown significantly ($p < 0.05$) lower TNF-α concentrations than in MDA-MB-231 cells, there were more folds of TNF-α activated in MCF-7 (3 folds) than in MDA-MB-231 cells (1.3 to 1.5 folds) after incubation in the presence of pentapeptide (1000 µg/mL) for 72 and 96 hrs. The positive control, genistein resulted in significant ($p < 0.05$) increases in Fas levels in MCF-7 and MDA-MB-231 cells, and significant ($p < 0.05$) Fas increases were observed in pentapeptide treated cells in a time-dependent manner (Figure 3.4).

TNF and Fas are considered members of the death domain receptors and they activate caspases through their death receptor-activator complexes (Ashkenazi and Dixit 1998). Studies

have demonstrated that MCF-7 cells are sensitive to TNF-induced apoptosis and that TNF-α is an effective inducer of apoptosis in MCF-7 cells (Chopin et al 2004; Burow et al 1999; Burow et al 1998; Cai et al 1997; Tewari and Dixit 1995; Jeoung et al 1995). The TNF- and Fas-induced apoptotic pathway activated via the interaction of TNF-receptor (TNFR)-associated death domain (DD), leads to the recruitment of Fas-associated death domain (FADD) (Rath et al. 1999). In turn, the FADD binds pro-caspase-8 and the activated caspase-8 triggers a protease cascade leading to apoptosis in cells (Degterev et al 2003). Studies have shown some anticancer agents such as ellipticine, caffeic acid phenethyl ester, doxorubicin, and camptothecin initiate the apoptosis via increasing Fas expression and recruiting death domain receptors such as FADD (Kuo et al 2005; Watabe et al 2004; Boesen et al 1999; Fulda et al 1997). In this study, pentapeptide treatment induced the increases in levels of TNF-α, Fas, and Caspase-8 in MCF-7 and MDA-MB-231 cells. These data suggest that the pentapeptide stimulated apoptosis by inducing death receptor-mediated pathway.

The mitochondrial-dependent pathway is activated by changing the Bax/Bcl-2 ratio involving the activation of caspase-9. To determine whether the mitochondrial apoptotic pathway is involved in pentapeptide induced apoptosis in MCF-7 and MDA-MB-231 cells, the expression of anti-apoptotic protein (Bcl-2) and pro-apoptotic protein (Bax) were investigated for indicated times (72 and 96 hr) after pentapeptide (1000 µg/mL) treatment. Significant ($p < 0.05$) increases in Bax expression and decreases in Bcl-2 levels were observed in pentapeptide treated MCF-7 and MDA-MB-231 cells after 72 and 96 hr compared to the untreated cells (Figures 3.5 and 3.6). The expressions of activated Bax increased significantly ($p < 0.05$) in MCF-7 and MDA-MB-231 cells with 2.6 to 5.5 folds and 11.5 to 22.5 folds, respectively.

Furthermore, more decreases in Bcl-2 level in MDA-MB-231 (from 6.5 to 16.8 ng/mL) than MCF-7 (from 11.0 to 12.1 ng/mL) were observed along with prolonged incubation time from 72 to 96 hr.

The mitochondrial-mediated apoptotic pathway is activated through the disruption of mitochondrial membrane potential leading to the change of permeability by the opening of transition pores and release of cytochrome c (Shim et al 2007; Schrivastava et al 2006; Kallio et al 2005;). Then, caspase-9 activates effector caspase-3, -7, and -6, which results in the activation of subsequent apoptosis (Pirnia et al 2002). The Bcl-2 family proteins, including pro-apoptotic proteins and anti-apoptotic proteins, play a curial role in determining the ultimate fate of cells and the ratio of Bax to Bcl-2 is a critical element in apoptosis regulation. The pro-apoptotic protein (such as Bax, BAD, Bak, and Bok) inserts into the mitochondria membrane and release the cytochrome c via forming a large channel, whereas the anti-apoptotic proteins including Bcl-2, Bcl-xL, and Bcl-w could prevent this process (Kirkin et al 2004).

Previous studies have demonstrated the importance of Bax/Bcl-2 ratio in regulating the mitochondria-mediated apoptotic pathway in human breast cancer models (Shim et al 2007, Cui et al 2007; Pozo-Guisado et al 2005; Hu et al 2002; Leung and Wang 1999). Our data showed that pentapeptide treatment increased the expression of Bax while down-regulating the Bcl-2 levels, which happened along with the increased levels of caspase-9 and apoptosis in MCF-7 and MDA-MB-231 cells. Therefore, these results clearly indicate that the mechanism of pentapeptide-induced apoptosis by mitochondrial-mediated pathway involves the mitochondrial membrane potential change via controlling the Bax/Bcl-2 ratio.

Pentapeptide down-regulates the ErbB-2 expression in human breast cancer cell models. To study the possible role of ErbB-2 in pentapeptide induced apoptosis in human breast cancer cell lines, the ErbB-2 levels in MCF-7 and MDA-MB-231 were determined using ELISA assay. The pentapeptide lead to significant ($p < 0.05$) decreases in levels of ErbB-2 in both cell lines. Further, more down-regulating effects on ErbB-2 levels in MCF-7 cells (from 1.4 to 2.0 folds) than MDA-MB-231 (from 1.1 to 1.6 folds) were observed after pentapeptide treatment (Figure 3.7). ErbB-2 is a member of receptor tyrosine kinases. Its over-expression results in the resistance to apoptosis which may lead to the failure of chemotherapy in cancer cells (Roskoski 2002). The high expression of ErbB2 promotes cell growth by activation of Akt (a serine/threonine kinase), which inactivates pro-apoptotic proteins such as Bax, Bad, and caspase-9 through phosphorylation (Zhou and Hung 2003). Previous studies suggest that down-regulation of ErbB-2 could efficiently suppress the breast tumor cell growth (Wang et al 2007; Xia et al 2006; Lee et al 2002; Daly et al 1997). The results of MCF-7 and MDA-MB-231 cells treated with pentapeptide suggest that pentapeptide may enhance the apoptotic signal by suppressing the expression of ErbB-2.

Conclusion

There are two apoptotic pathways involved in pentapeptide-induced apoptosis in human breast cancer cell lines. In one pathway, the apoptosis is activated after the increased Bax/Bcl-2 ratio leads to the release of cytochrome c in mitochondria-mediated pathway. In the second pathway, pentapeptide induces apoptosis via death receptor-mediated pathway by stimulating the expression of TNF-α, followed by forming the death-inducing signaling complex (DISC) through Fas expression and the activation of caspase-8. The pentapeptide may amplify apoptotic signals by down-regulating the expression of ErbB-2. Pentapeptide stimulated the levels of p53

in both cells lines and may also suppressed the COX-2 in ER-positive breast cancer cells such as MCF-7 by regulating the estrogen synthesis. The findings in this study indicate the potential therapeutic value of this pentapeptide and further research in animal tumor models is necessary to confirm its anti-cancer activity *in vivo*.

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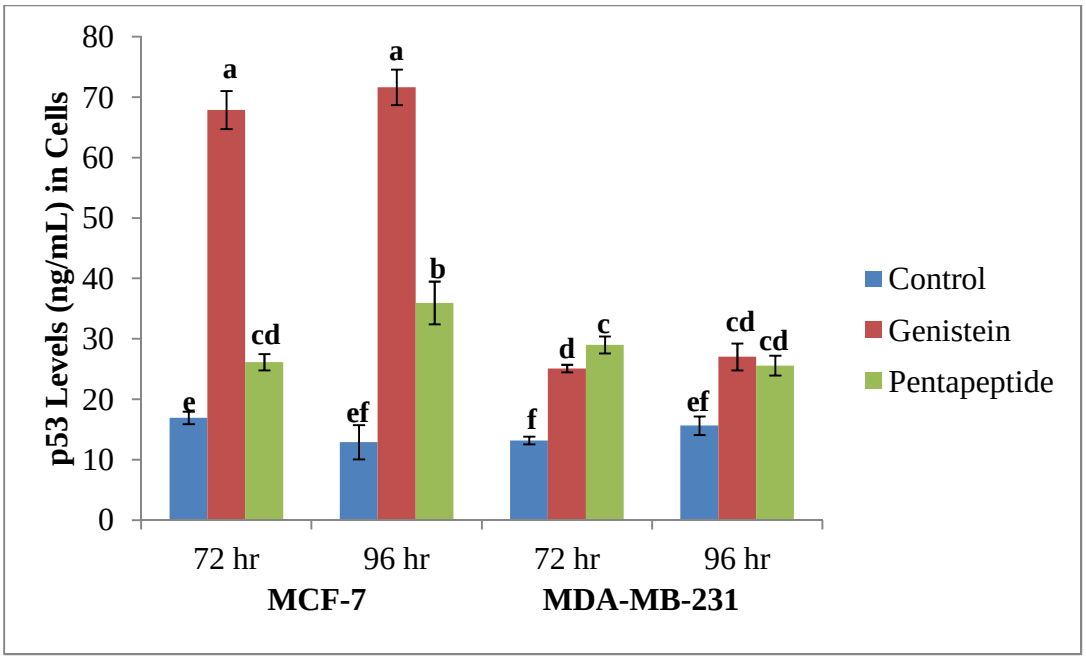

Figure 3.1. The levels of p53 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive control, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

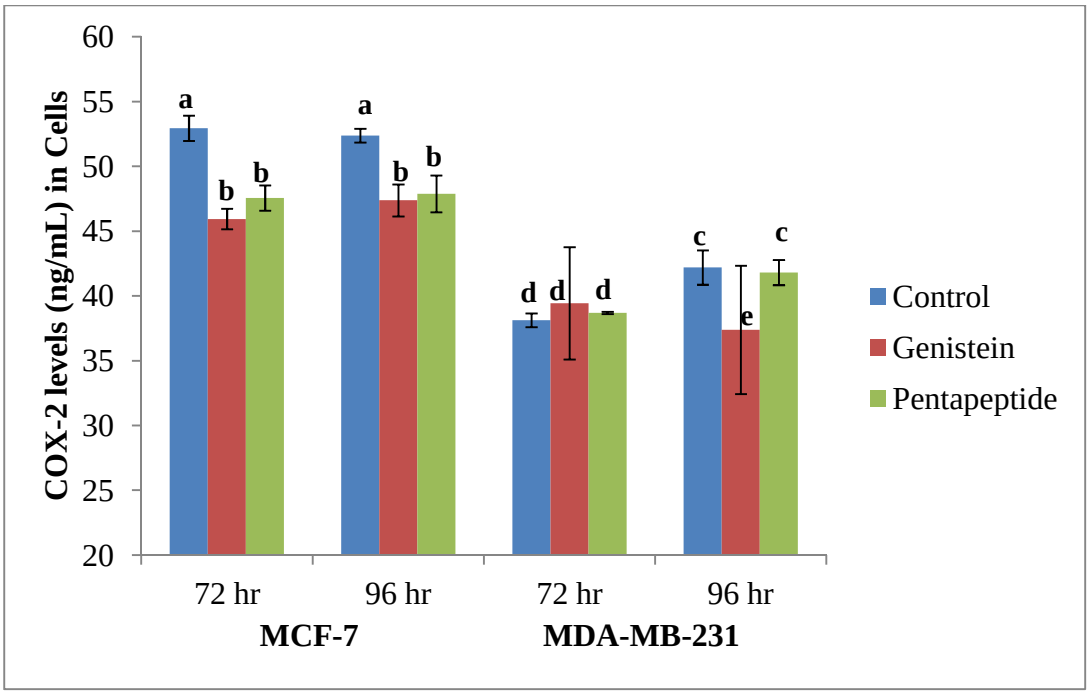

Figure 3.2. The levels of COX-2 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive control, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

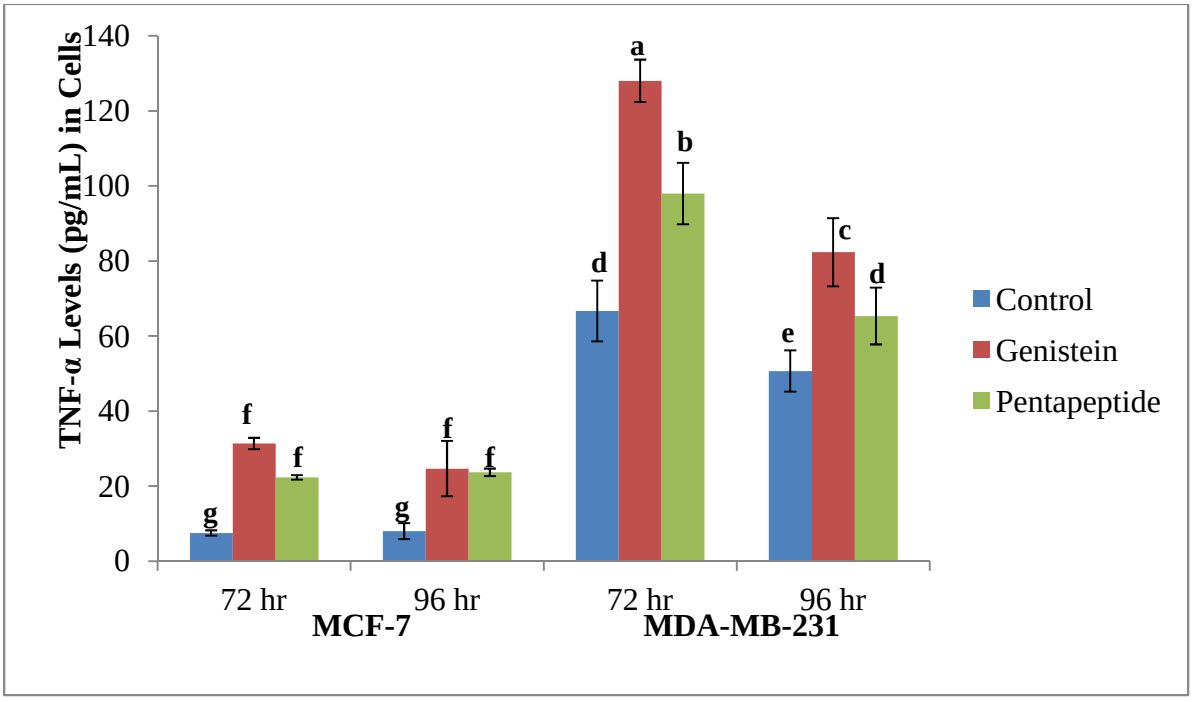

Figure 3.3. The levels of TNF-α in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

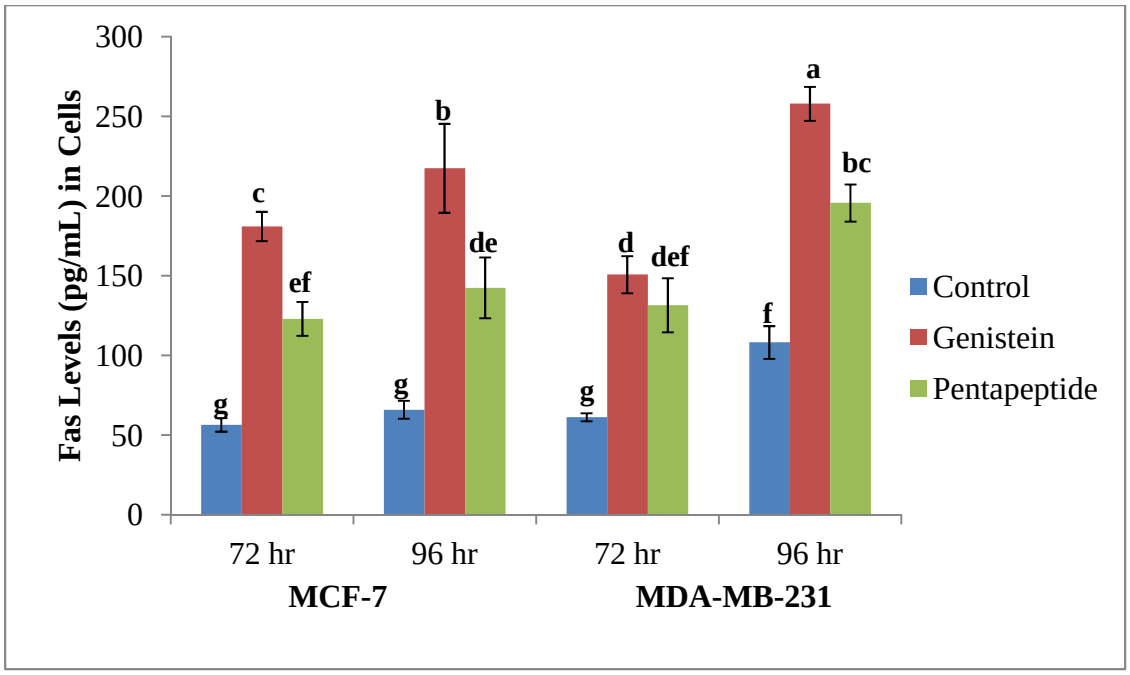

Figure 3.4. The levels of Fas in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

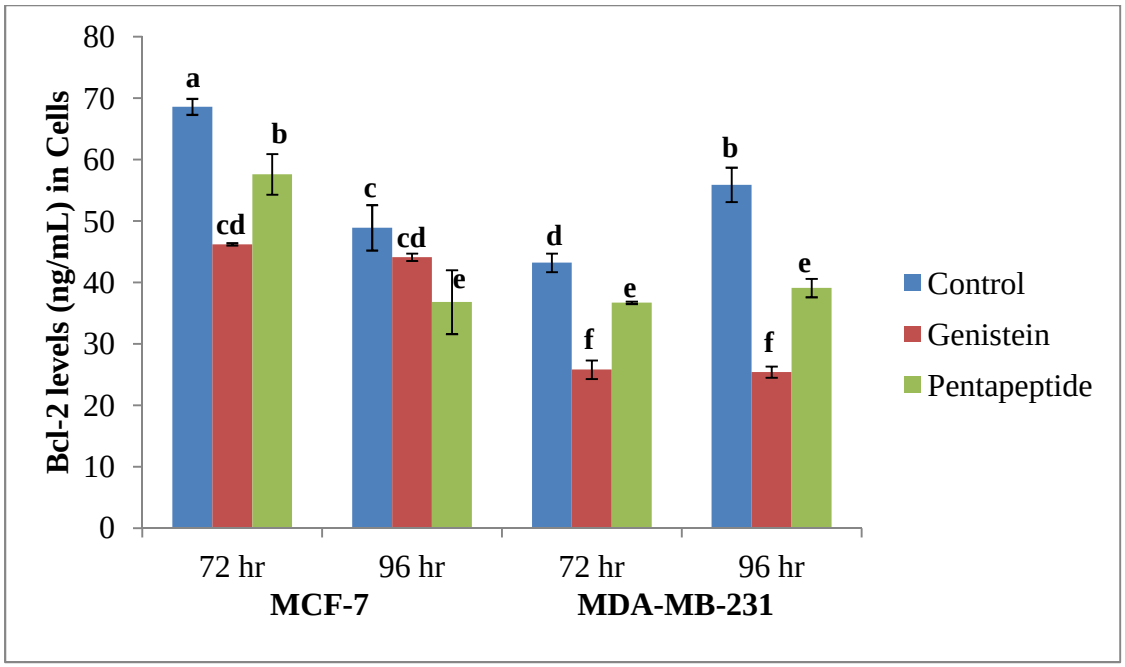

Figure 3.5. The levels of Bcl-2 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

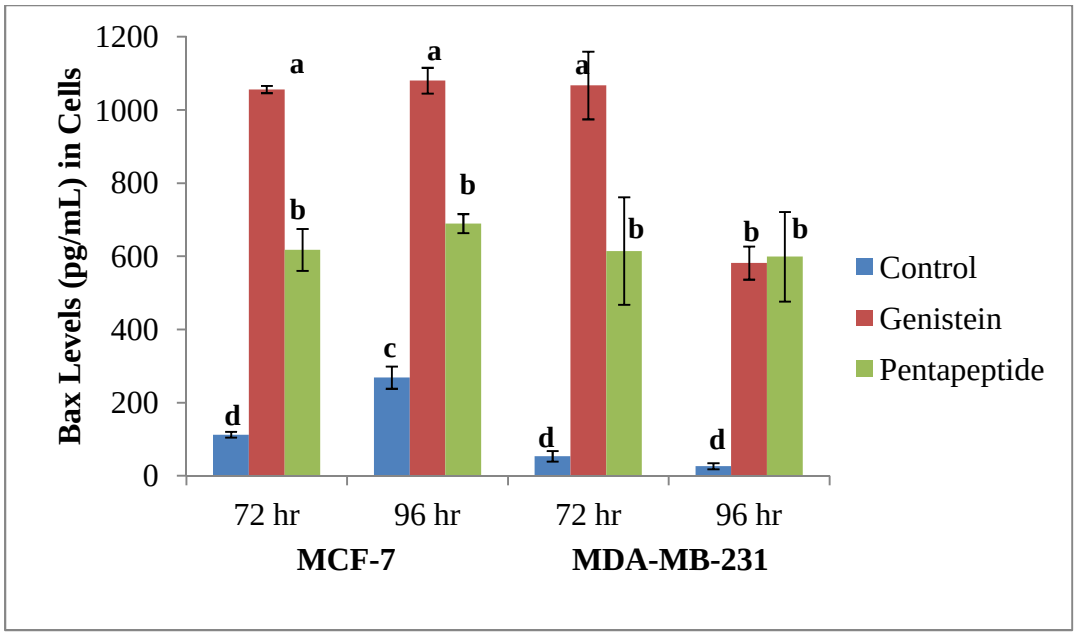

Figure 3.6. The levels of Bax in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

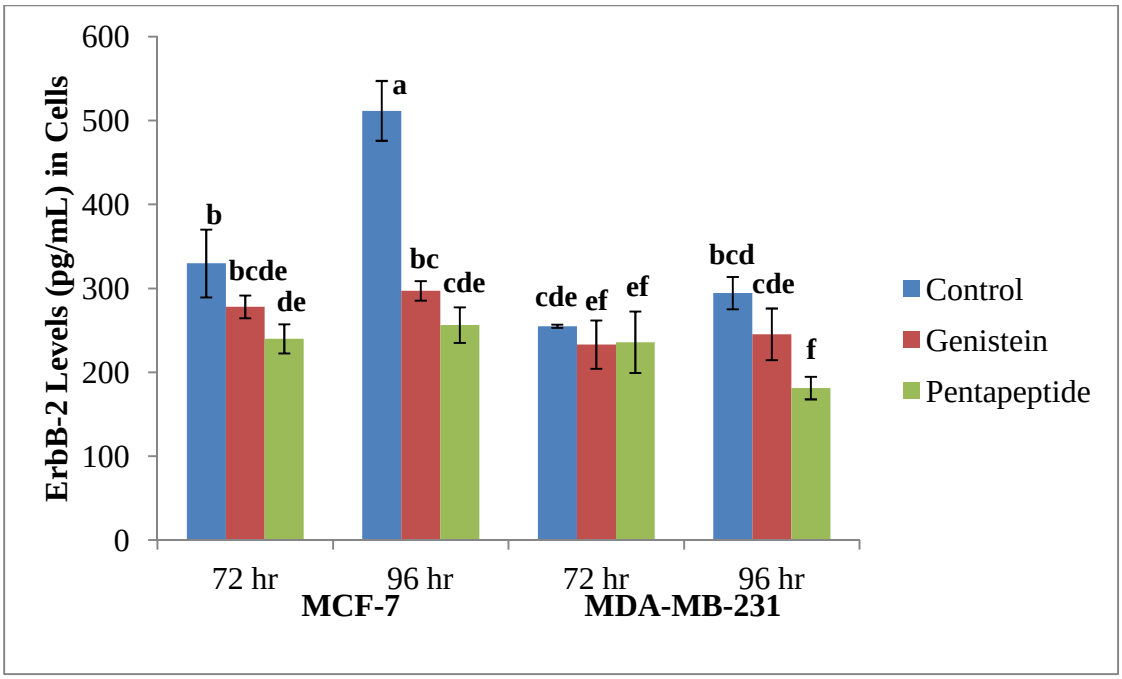

Figure 3.7. The levels of ErbB-2 in pentapeptide (1000 µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hours.

The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

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04/22/2014

Dear Sir/Ma'am,

This is to inform you that Ruiqi Li is the first author of the paper and completed more than 51% of the work of the manuscript submitted to International Journal of Biomedical Research.

Navam Hettiarachchy, Ph.D (Biochemistry)
Postgraduate Diploma in Human Nutrition
University Professor
IFT Fellow

CONCLUSION

This study demonstrated that a pentapeptide with a sequencing of EQRPR shows anti-tumor activities on human breast cancer cells (MCF-7 and MDA-MB-231) through apoptosis, but has no cytotoxic effect on normal human breast cells (HMEC). There are at least two apoptotic pathways involved in pentapeptide-induced apoptosis. In one pathway, the apoptosis is activated after the increased Bax/Bcl-2 ratio which leads to the release of cytochrome c in mitochondria-mediated pathway. In the second pathway, the pentapeptide may induce apoptosis via death receptor-mediated apoptotic pathway by stimulating the expression of TNF-α, followed by forming the death-inducing signaling complex (DISC) through Fas expression, which results in the activation of caspase-8. The pentapeptide stimulated the levels of p53 in both cells lines and may suppress the COX-2 in ER-positive breast cancer cells such as MCF-7 by regulating the estrogen synthesis. The pentapeptide may also amplify apoptotic signals by down-regulating the expression of ErbB-2. The findings in this study indicate potential therapeutic value of pentapeptide and further research in animal tumor models is necessary to confirm its anti-cancer activity *in vitro*. The impact of this study provides information on cell specific pathways affected by this pentapeptide and could open avenues for the use of the pentapeptide as a nutraceutical diet/drug for breast cancer prevention. In addition, this research provides insight on the molecular mechanism of action of the pentapeptide against human breast cancer cells and results on its potential drug-like property. The production of this pentapeptide is less expensive than general cancer drug and could be a promising alterative strategy to current expensive anti-cancer therapies.