ANTI-PROLIFERATIVE EFFECTS ON NEUROBLASTOMA CELL BY MANGOSTEEN EXTRACTS

A THESIS

BY

CHAKAPHAN MARTWANGSAENG

Presented in Partial Fulfillment of the Requirements for the

Master of Science in Molecular Biology

at Srinakharinwirot University

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AN ABSTRACT

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Neuroblastoma is an embryonal tumour of the sympathetic nervous system. Despite many advances during the past three decades, neuroblastoma has remained an enigmatic challenge to clinical and basic scientists. The mangosteen tree has been cultivated for centuries in tropical areas of the world. The tree is presumed to have originated in Southeast Asia. In Thailand, it was denoted to be the queen of fruits. The mangosteen rind, leaves and bark have been used as folk medicine for thousands of years. This study presents anti-proliferative effects of γ-mangostin on neuroblastoma cell line (Neuro-2A).

Apoptosis induction was determined by morphological changes, MTT assay for cell viability, time and dose response, Hoechst 33342 nuclear staining, DNA fragmentation and Western blot analysis of Bcl-2,Bax and PARP-1 in Neuro-2A cells after treatment with γ-mangostin.

The results indicated anti-proliferative effects of γ-mangostin on Neuro-2A cells with IC_{50} = 7.49 µg/ml. The antiproliferation was shown to be in a time and dose dependent fashion. Upon treatment of the cells with γ-mangostin, apoptotic cell death was detected as determined by morphological changes of neuronal cells including membrane blebbing and cell shrinkage. Chromatin condensation and DNA fragmentation were then detected. Western blot analysis showed decrease level of Bcl-2, induce Bax and cleaved PARP-1 expression upon treatment with γ-mangostin, indicating apoptosis induction.

ฤทธิ์เหนี่ยวนำการตายของเซลล์มะเร็งสมองโดยสารสกัดจากมังคุด

บทคัดย่อ

ของ

จักรพันธุ์ มาตย์วังแสง

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา

ตามหลักสูตรปริญญามหาบัณฑิต สาขาวิชาอณูชีววิทยา

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มะเร็งสมองเป็นก้อนเนื้อในระยะตัวอ่อนของระบบประสาทแบบซิมพาเทติก ถึงแม้ผ่านมาไม่น้อยกว่าสามทศวรรษ โรคมะเร็งสมองก็คงยังยากต่อการเข้าใจและอธิบายได้ จึงถือเป็นสิ่งท้าทายต่อการศึกษาทางด้านคลินิกและการค้นคว้าทางด้านวิทยาศาสตร์พื้นฐาน มังคุดจัดเป็นไม้ผลชนิดหนึ่งที่พบว่ามีการเพาะปลูกในแถบเขตร้อน เชื่อกันว่ามีต้นกำเนิดมาจากเขตเอเชียตะวันออกเฉียงใต้ ในประเทศไทยมังคุดได้รับการยกย่องให้เป็นราชินีแห่งผลไม้ ซึ่งส่วนของใบและเปลือกไม้ของมังคุดนั้น เคยถูกนำมาใช้ในการรักษาโรคต่าง ๆ เป็นเวลากว่าพันปี ในการศึกษานี้ได้แสดงถึงฤทธิ์เหนี่ยวนำการตายของสารแกมมา-แมงโกสทินในเซลล์มะเร็งสมอง (Neuro-2A)

กระบวนการตายของเซลล์แบบอะพอบโทซีสนั้นสามารถศึกษาได้จาก การเปลี่ยนแปลงรูปร่างของเซลล์ การวิเคราะห์เซลล์ที่ยังมีชีวิตโดยอาศัยกระบวนการทำงานของ MTT ผลของความสัมพันธ์ระหว่างความเข้มข้นของสารและเวลา การย้อมนิวเคลียสของเซลล์โดย Hoechst 33342 การแตกออกเป็นชิ้นๆ ของดีเอ็นเอ และ การวิเคราะห์โปรตีนโดยกระบวนการ Western blot ซึ่งเลือกศึกษาโปรตีน Bcl-2, Bax และ PARP-1 ในเซลล์ Neuro-2A หลังจาก เลี้ยงร่วมกับสารแกมมา-แมงโกสทิน

จากผลการทดลองได้แสดงให้เห็นถึงการยับยั้งการเจริญของเซลล์ Neuro-2A ที่เป็นผลมาจากสารแกมมา-แมงโกสทิน และได้ค่า IC_{50} เท่ากับ 7.49 ไมโครกรัมต่อมิลลิลิตร ซึ่งการยับยั้งการเจริญนี้ยังขึ้นอยู่กับความเข้มข้นของสารและเวลา จากการเลี้ยงเซลล์ร่วมกับสารแกมมา-แมงโกสทินได้แสดงลักษณะการตายของเซลล์แบบอะพอบโทซีส ซึ่งประกอบด้วย การเปลี่ยนแปลงรูปร่างของเซลล์ประสาท การผิดรูปร่างของเยื่อหุ้มเซลล์และการหดตัวของเซลล์ ทั้งนี้การขดตัวของดีเอ็นเอและการแตกออกเป็นชิ้นๆ ของดีเอ็นเอสามารถแสดงผลให้เห็นได้เช่นกัน ในส่วนของการวิเคราะห์โปรตีนโดยกระบวนการ Western blot พบว่า มีการลดลงของ Bcl-2 การแสดงออกมากขึ้นของ Bax และยังแสดง cleaved PARP-1 จากการเลี้ยงเซลล์ร่วมกับสารแกมมา-แมงโกสทิน ซึ่งแสดงถึงการเกิดกระบวนการตายของเซลล์แบบอะพอบโทซีส

The thesis titled

"Anti-proliferative effects on neuroblastoma cell by mangosteen extracts"

by

Chakaphan Martwangsaeng

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Molecular Biology of Srinakharinwirot University.

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Chakaphan Martwangsaeng

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ABBREVIATIONS

DNA	=	Deoxyribonucleic acid
kb	=	Kilobase pair
bp	=	Base pair
et al.	=	et alii (and other)
h	=	Hour
min	=	Minute
°C	=	Degree celsius
mg/ml or mg ml^{-1}	=	Miligram per milliliter
mM/ml or mM ml^{-1}	=	Millimolar per milliliter
g	=	Gram
mg	=	Milligram
µg	=	Microgram
ng	=	Nanogram
L	=	Liter
mL	=	Milliliter
µL	=	microliter
M	=	Molar
mM	=	Milimolar
µM	=	Micromolar
µmole	=	Micromole
nmole	=	Nanomole
nm	=	Nanometer
rpm	=	Rounds per minute

CHAPTER I

INTRODUCTION

Neuroblastoma is an embryonal tumor of the sympathetic nervous system, the commonest extracranial solid tumor of very early childhood. Neuroblastoma has a diverse pattern of presentation and a widely variable clinical course, ranging from tumors that spontaneously regress (stage 4S) or differentiate into benign ganglioneuromas, to those which follow an unrelenting, progressive course despite treatment, with an inevitably fatal outcome. Neuroblastoma accounts for 7–8% of malignant disease in children. The United Kingdom annual incidence is 6–8 person in every million children. Ninety percent of children diagnosed with neuroblastoma are less than 5 years old the peak incidence is in those aged less than 2 years. While neuroblastoma in adolescence and adulthood is rare. The distribution of cases is approximately equal between males and females. [1]

Neuroblastoma during youth is rare fewer than 10% of cases occur in patients greater than 10 years of age. The clinical behavior and biological characteristics of the disease during teenaged and mature person are thought to differ from those during child. [2] The management of neuroblastoma has relied on surgery, radiation therapy and chemotherapy. The latter plays a central role in multimodality treatment plans. [3]

Although surgery alone may be curative for low stage disease, and supportive care may be the only treatment indicated for the majority of 4S patients, chemotherapy plays a central role and is the predominant modality in the management of patients with neuroblastoma. Many agents over the years have been shown to be effective alone or in various combinations against neuroblastoma. [3]

Hereafter success relies on the advancement of new chemotherapeutic agents, evaluating different schedules and combinations, overcoming drug resistance, investigating new methods of bone marrow purging and transplantation, and the continued advancement of biologic and targeted therapy. [3]

The mangosteen (mangkut in Thai) tree has been cultivated for centuries in tropical areas of the world. The tree is inferential to have originated in Southeast Asia. Mangosteen tree has been generally found in Malay Peninsula, Myanmar, the Moluccas, Cambodia, Vietnam and Thailand mangosteen. [4]

Only about 25% of the total volume to edible portion of the fruit, whereas the remainder is tough, bitter pericarp which exudes a yellow resin (hence the term xanthones or yellow in Greek) The pericarp , leaves and bark of mangosteen have been using as traditional medicine

for thousands of years. The thick pericarp has been and is used for treating catarrh, cystitis, diarrhea, dysentery, eczema, fever, intestinal ailments, pruritis and other skin ailments. The mangosteen leaves are also used by some natives as teas to cure diarrhoea, dysentery, fever, and thrush. It is also known that concentrates of mangosteen bark can be used for genito-urinary afflictions and stomatosis.

To date, much evidence has been shown that consumption of mangosteen extracts can reduce the risk of cancer, heart disease, inflammation, immune system decline and brain dysfunction. The compounds have been shown to prevent cancer by many mechanisms, for examples, induction of apoptosis in many human cancer cell lines such as human leukemia HL60 cell, human colon cancer DLD-1 and COLO 205 cells

Objective

This study present the anti-proliferative effects of mangosteen extracts (γ-mangostin) on neuroblastoma cell line (Neuro-2A) by detecting morphological changes, % cell viability, time and dose effect of γ-mangostin on cell viability. The characteristics of apoptosis cell death which are chromatin condensation, DNA fragmentation, expression of Bcl-2, Bax and cleaved PARP-1 and also included.

CHAPTER II

LITERATURE REVIEW

2.1 Neuroblastoma

2.1.1Basic concepts

Neuroblastoma is the most common extracranial solid tumor in babyhood and childhood and can originate from any neural crest element of the sympathetic nervous system. These diverse injury may show a wide pattern of differentiation ranging from benign ganglioneuroma to partially differentiated ganglioneuroblastoma to highly malignant neuroblastoma. This unfathomable tumor is one of the rare human malignancies known to demonstrate spontaneous regression from an undifferentiated state to a completely benign cellular appearance. [5] The neuroblastoma accounts for 7–8% of malignant disease in children. The United Kingdom annual rate is 6–8 per million children. Ninety percent of children diagnosed with neuroblastoma are less than 5 years old; the peak rate is in those aged less than 2 years. Neuroblastoma in youth and adulthood is rare. The distribution of cases is approximately equal between males and females [6]

However, the majority of children older than 1 years of age at presentation with advanced disease will die from progressive disease. Treatments are based on the subtype classification which depend not only on the clinical and imaging stage of the disease but also on biological and biochemical parameters. [7]

When the injury is localized, the outcome has been generally favorable. However, the long-term survival for children with advanced disease has shown only marginal improvement despite aggressive multimodality therapy. Recent biologic and genetic characteristics have been identified, which, when added to classic clinical staging, has allowed accurate patient assignment to risk groups so that treatment strategies can be more effectively undertaken. [3]

2.1.2 History

German physician Rudolf Virchow was the first to describe an abdominal tumor in a child as a "glioma" in 1864. The characteristics of tumors from the sympathetic nervous system and the adrenal medulla were then noted by German pathologist Felix Marchand in 1891. [8] In 1901 William Pepper was described the distinctive presentation of stage 4S in infants (liver but no bone metastases). In 1910 James Homer Wright understood the tumor to originate from primitive neural cells, and named it neuroblastoma. [9]

2.1.3 Diagnosis

The diagnosis of neuroblastoma is based on the presence of characteristic histolopathological features of tumor tissue or the presence of tumor cells in a bone marrow aspirate or biopsy, accompanied by raised concentrations of urinary catecholamines. High-risk patients often have raised concentrations of serum lactate dehydrogenase, ferritin, or chromagranin, but these are relatively non-specific for the population as a whole and do not seem to be independently prognostic of outcome in light of modern biological co-variates. Tumor-specific genetic markers and histopathological assessment are crucial determinants of treatment planning, especially for children younger than 18 months. Tumor biopsies sufficient to provide enough materials for molecular genetic analyses are therefore highly encouraged at the time of diagnosis. [10]

2.1.3.1 Tumor biopsy and examination of bone marrow

Histopathology, microscopy and tumor biology (status of N-myc amplification, immunochemistry, cytogenetic analysis) of the primary tumor tissue is required to stratify patients into risk groups. Bilateral bone marrow aspirates and core trephine biopsies are required to exclude infiltration of bone marrow.

2.1.3.2 Urinary catecholamines

Neuroblastoma cells can secrete catecholamines and their metabolites, including homovanillic acid, vanillylmandelic acid and dopamine. A random urine sample sent for measurement of concentrations of homovanillic acid, vanillylmandelic acid is useful because they are likely to be elevated.

2.1.3.3 Ferritin and lactate dehydrogenase

Neuroblastoma cells produce ferritin; concentrations are often elevated in advanced disease. Concentrations of lactate dehydrogenase are also often raised in advanced neuroblastoma, possibly due to a high turnover of cells; it may be a marker of more aggressive disease.

2.1.3.4 Imaging

CT can provide detailed information regarding composition, location, size and relation to surrounding structures (e.g. lymph nodes) of the primary tumor. MRI is preferred to evaluate the spinal cord if paraspinal tumors are suspected. ^{123}Iodine-metaiodobenzylguanidine (^{123}I-MIBG): imaging of the whole body using ^{123}I-MIBG is a very specific method to detect tumor masses, metastases and involvement of bone marrow because greater than 90% of neuroblastomas selectively concentrate ^{123}I-MIBG. ^{123}I-MIBG can be used to assess therapy response and prognosis. ^{99}mTc-diphosphosphonate scintigraphy is used if ^{123}I-MIBG is negative. [1]

2.1.4 Signs and symptoms

The symptoms of neuroblastoma are often vague in primary stage cause difficulty to diagnosis. Fatigue, loss of appetite, fever, and joint pain are common. Symptoms depend on primary tumor locations and metastases if present:

In the abdomen, a tumor may cause a swollen belly and constipation.

A tumor in the chest may cause breathing problems.

A tumor pressing on the spinal cord may cause weakness and thus an inability to stand, crawl, or walk.

Bone lesions in the legs and hips may cause pain and limping.

A tumor in the bones around the eyes or orbits may cause distinct bruising and swelling.

Infiltration of the bone marrow may cause pallor from anemia.

Neuroblastoma often expand to other parts of the body before any symptoms are apparent and 50 - 60% of all neuroblastoma cases show metastases.

The most common location for neuroblastoma to originate (i.e. the primary tumor) is on the adrenal glands. This occurs in 40% of localized tumors and in 60% of cases of widespread disease. Neuroblastoma can also develop anywhere along the sympathetic nervous system chain from the neck to the pelvis. Frequencies in different locations include: neck (1%), chest (19%), abdomen (30% non-adrenal), or pelvis (1%). In rare cases, no primary tumor can be discerned .[1]

2.1.5 Presentation

Presentation of neuroblastoma can show in various ways. The location of the tumor, along with the presence or absence of dissemination, leads to a wide spectrum of symptoms and signs. One third of infants with neuroblastoma have a primary mediastinal tumor; adrenal or abdominal primaries are much more common in older children. Other tumor sites include the neck and pelvis; a distinct primary tumor is not identified in some cases.

2.1.5.1 Mediastinal and cervical tumors

Patients with thoracic involvement can present with obstructive symptoms because the tumor encroaches upon or invades surrounding structures, including the trachea, oesophagus or superior vena cava cause respiratory distress or stridor, dysphagia and superior vena cava obstruction, respectively. Some tumors may be discovered incidentally on chest radiography; lesions within the neck or cervical region can present as a palpable mass or Horner's syndrome.

2.1.5.2 Paraspinal tumors

Up to 15% of patients have paraspinal tumors, which can present with compression of the spinal cord due to extension into the neural foramina. [11] This is an oncological emergency and should be suspected in a patient with leg weakness, back pain, bladder or bowel dysfunction or sensory disturbance. [12] Radiotherapy, chemotherapy or surgery may be required to relieve compression of the spinal cord .[12]

2.1.5.3 Tumors in the abdomen or pelvis

Presentation can be with abdominal distension and pain secondary to a mass, or with problems related to excretion.

2.1.5.4 Metastatic disease

Tumor cells diffuse via the bloodstream to lymph nodes, bone and bone marrow; spread to the brain and lungs may also occur. Children with disseminated disease are often unwell and can present with anorexia, lethargy, bone pain, fever with anaemia and thrombocytopenia secondary to invasion by neuroblastoma cells into bone marrow. Periorbital ecchymoses and proptosis are signs of retrobulbar deposits from neuroblastoma cells disseminating to the bony orbits.

2.1.5.5 Paraneoplastic phenomena

Some patients present as a result of paraneoplastic phenomena (e.g. due to production of vasoactive intestinal peptides by neuroblastoma cells, resulting in sweating, chronic watery diarrhea and hypertension). [13] Opsoclonus-myoclonus is characterized by ataxia and rapid, abnormal movements of the eyes, and affects 2–4% of patients. It is thought to be immune-mediated. Therapeutic strategies have included corticosteroids, adrenocorticotrophic hormone, chemotherapy or immunoglobulin, with varying degrees of success. Improvement is often seen after treatment of the underlying neuroblastoma.[1]

2.1.6 Staging of Neuroblastoma

The most common staging in neuroblastoma is based on the International Neuroblastoma Staging System (INSS) [14]This system uses the clinical stage, radiographic and nuclear medicine results, surgical findings, and bone marrow examination. In general, the staging is defined as follows:

Stage 1: Localized tumor without regional lymph node involvement.

Stage 2: Unilateral tumor with either incomplete gross resection or ipsilateral nodal involvement.

Stage 3: Tumor that crosses the midline or has contralateral nodal involvement.

Stage 4: Tumor disseminated to distant lymph nodes, bone, bone marrow, liver, etc.

Stage 4S: Special category: age _1 year, localized primary tumor, dissemination only to liver, skin, or bone marrow. [7]Stage 4S disease is seen in infants with a small localized tumor, where dissemination has occurred to bone marrow, liver or skin, resulting in hepatomegaly and subcutaneous nodules. Massive hepatomegaly secondary to intrahepatic neuroblastoma can give rise to respiratory compromise, more commonly seen in infants less than 2-month-old. [15] Involvement of bone marrow is less than 10%. Stage 4S tumors often regress spontaneously and the overall the prognosis is very good.[1]

Although international agreement on staging (INSS) has been used, the need for an international consensus on risk assignment has also been recognized in order to compare similar cohorts in results of studies. Beginning in 2005, representatives of the major pediatric oncology cooperative groups have met to review data for 8,800 neuroblastoma patients treated in Europe, Japan, USA, Canada, and Australia between 1990 and 2002. This task force has proposed the International Neuroblastoma Risk Group (INRG) classification system. Retrospective studies revealed the high survival rate of 12-18 month old age group, previously categorized as high-risk, and prompted the decision to reclassify 12-18 month old children without N-myc (also commonly referred to as MYCN) amplification to intermediate risk category. [16]

The new INRG risk assignment classify neuroblastoma at diagnosis based on a new International Neuroblastoma Risk Group Staging System (INRGSS):

Stage L1: Localized disease without image-defined risk factors.

Stage L2: Localized disease with image-defined risk factors.

Stage M: Metastatic disease.

Stage MS: Metastatic disease "special" where MS is equivalent to stage 4S.

The new risk stratification will be based on the new INRGSS staging system, age (dichotomized at 18 months), tumor grade, N-myc amplification, unbalanced 11q aberration, and ploidyinto four pre-treatment risk groups: very low, low, intermediate, and high risk. [1]

2.1.7 Biological Markers

The biological hallmarks of neuroblastoma relate to acquired genetic abnormalities, some of which have significant prognostic features. The main genetic markers are amplification of MYCN, ploidy changes (DNA content), and partial deletions of chromosome 1 and 11 and gains of chromosome[17-24] These markers are useful for risk stratification of patients at presentation and allow more informed selection of the most appropriate and intensive treatments. This is beneficial in sparing patients with low- and some intermediate-risk disease, the deleterious effects of chemotherapy, radiotherapy, and bone marrow transplantation. [7]

2.1.8 Biochemical

Biochemical markers associated with poor prognosis include high serum levels of lactate dehydrogenase, neuron-specific enolase, and ferritin levels. Urinary catecholamines are not

predictive of outcomes. However, low vanillylmandelic acid and high homovanillic acid levels have been associated with shortened survival. [25]

2.2 Apoptosis

2.2.1 Background

Apoptosis is of greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This likeness emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance. [26]

2.2.2 Introduction of apoptosis

Apoptosis has long been identified as an evolutionarily conserved process of active cell elimination during development.Its phenotypic features include DNA fragmentation and

chromatin condensation, cell shrinkage, and formation of apoptotic bodies, which are cleared by phagocytosis without initiating a systemic inflammatory response. The execution of apoptosis requires novel gene expression and protein synthesis. Apoptosis has evolved as an intricate and critical mechanism for balancing cell proliferation and for the active remodelling of tissues during development.

John FR Kerr studied ischemic liver damage and identification of apoptosis under pathological settings dates back to the 1960s. He observed a novel cell death phenotype that was morphologically distinct from classical necrosis. Dying hepatocytes in the ischemic penumbra were found to have shrunk to form small round masses of cytoplasm containing condensed nuclear chromatin. These dying cells were taken up by neighboring hepatocytes and phagocytes without initiating a broader inflammatory response. This phenomenon was also recognized in normal rat livers. This distinct type of cell death, temporarily named 'shrinkage necrosis', was also found to occur in cancer and during normal development. The term 'apoptosis' was subsequently coined to replace shrinkage necrosis, and has later been used interchangeably with programmed cell death, albeit loosely, because of similar requirements for genetic programming and new protein synthesis, as well as morphological similarities .[27] Cell death, along with differentiation and growth, is a fundamental aspect of the life cycle of a eukaryotic cell, the control of cell number is the result of the balance between cell loss and gain. The molecular mechanisms leading to the controlled removal of cells in tissues by apoptosis are not fully understood. It is clear that under physiological conditions the process is active, requires energy and the induction/activation of specific genes have led to the identification of several genes needed for the completion of the cell death program. These genes have been classified into specific functional groups that play distinct roles within the cell death program. The first group of genes includes permissive elements which specify which cells will undergo apoptosis. The second group comprises elements whose induction or down-regulation initiates the apoptosis pathway. A third set of genes includes effector elements required for killing and the subsequent disposal and degradation of cellular remnants. Genes with functional homology to some of those defined in *C. elegans* have been described in mammals: *Bcl-2* and *ted-9*, *caspases* and *ted-3*. These genes, grown into families of homologous genes, together with additional important regulatory elements, are at the center of intense research efforts to dissect the molecular mechanisms of the death machinery. [28]

2.2.3 The apoptotic process

The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. Those morphological changes are a consequence of molecular characteristic and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles. [29] Apoptosis is in contrast to the necrotic mode of cell -death in which the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue.[26] Apoptotic cells display distinctive morphology during the apoptotic process, and this can be seen in Figure 1. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton (A). Chromatin breakdown in the nucleus often leads to nuclear condensation (B). Cell continue to shrink (C), packaging each other into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The end stages of apoptosis are often characterized by the appearance of membrane blebs (D) or blisters process. Small vesicles called apoptotic bodies are also sometimes observed (D, arrow).

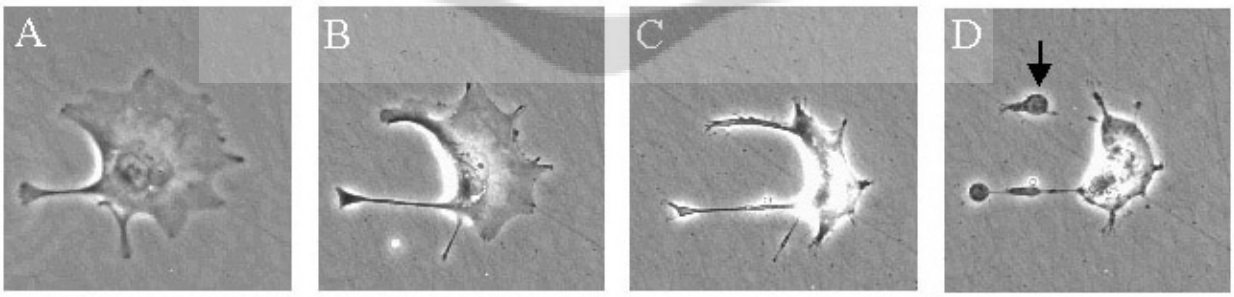

Figure 1 Morphology of an apoptosic trophoblast cell as captured by time-lapse microscopy (images taken over a 6 hour period). (Figure from [30])

The alternative to apoptotic cell death is necrosis, which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death. But since necrosis refers to the degradative process that occurs after cell death, it is considered

by some to be an inappropriate term to describe a mechanism of cell death. Oncosis is therefore used to describe a process that leads to necrosis with karyolysis and cell swelling whereas apoptosisleads to cell death with cell shrinkage, pyknosis, and karyorrhexis. Therefore the terms “oncoticcell death” and “oncotic necrosis” have been proposed as alternatives to describe cell death that is accompanied by cell swelling, but these terms are not widely used at this time.[31,32]

Apoptosis includes cellular shrinking, chromatin condensation and marinating at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosis without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation, as can be seen in Figure 2

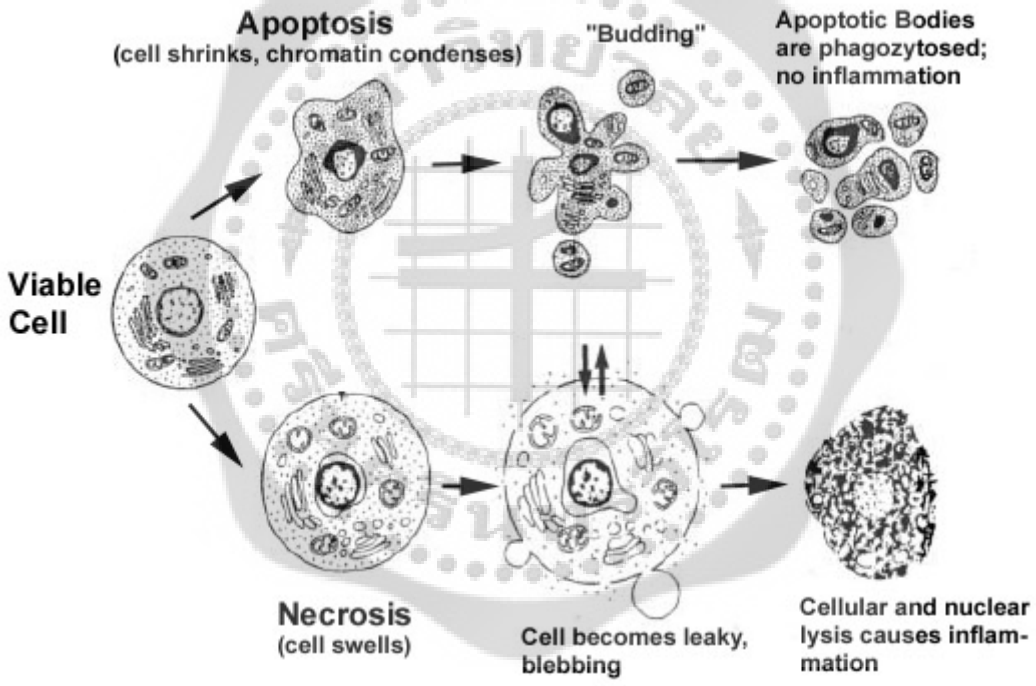

Figure 2 Hallmarks of the apoptotic and necrotic cell death process. (Modified from Van Cruchten S. 2002 [33])

2.2.4 Mechanisms of apoptosis

The pathways of apoptosis are extremely complicated, where energy dependent flow of molecular events takes place. Mainly two types of apoptotic pathways such as intrinsic and extrinsic (or death receptor) have been well described that involve a number of proteins. Both intrinsic as well as extrinsic pathways of apoptosis are associated and influence each other. [34]

2.2.5 Mediators of apoptosis

2.2.5.1 PARP

More than 40 years ago, an unusual type of post-translational modification, poly(ADP-ribosyl) ation, was discovered in eukaryotic cells. Although barely detectable in resting cells, poly(ADP-ribosyl)ation was dramatically and transiently induced in cells assaulted with DNA damaging agents. In the late 1980s, the highly conserved gene encoding the protein responsible for this activity, poly(ADP-ribose) polymerase (PARP), was isolated and ten years of intensive characterization ensued. Only recently, as a result of a combined approach incorporating cell biological, biochemical and knockout mouse data, has a cohesive framework for understanding PARP function emerged. Indeed, this protein plays a pivotal role in maintaining genome integrity through modulation of multiple cellular responses (including base excision repair, necrosis and apoptosis) in answer to genotoxic stress.[35, 36]

2.2.5.1.1 Multiple members of a PARP family

One of the first clues indicating the presence of multiple PARPs was the observation of ARP activity in mouse embryo fibroblasts derived from PARP-1-knockout mice.[37] At about the same time, resulting from several different lines of research, four new PARPs were identified: PARP-2, PARP-3, vault-PARP(VPARP) and tankyrase .(Figure 3a) [38] Strikingly, the homology between these proteins is limited to the C-terminal half of PARP-1, termed here the PARP homology domain, with no relationship outside this region. The PARP homology domain was initially defined as the catalytic domain by limited proteolysis of PARP-1. [39] This highly conserved domain was further refined as a 40-kDa fragment sufficient for catalytic activity and the crystal structure of this region (PARP-1, aa 654–1014) was solved.[40, 41] Structure analysis revealed a domain comprising secondary structure units (multiple β strands and one α helix; Figure 3b) that form a cavity known as the NAD^+-binding fold, a tertiary structure that is conserved in bacterial ADP-ribosylating toxins. [42] Alignment of the catalytic domains of the five PARPs (Figure 3b) shows that the amino acid identities are clustered within these structural units, with all the amino acids involved in NAD^+ binding and catalysis conserved. Of the five PARPs, tankyrase contains the smallest PARP domain comprising solely of this highly conserved tertiary structure. The finding that recombinant tankyrase functions as a PARPin vitro [43] defines this minimal domain as sufficient for PARPactivity.

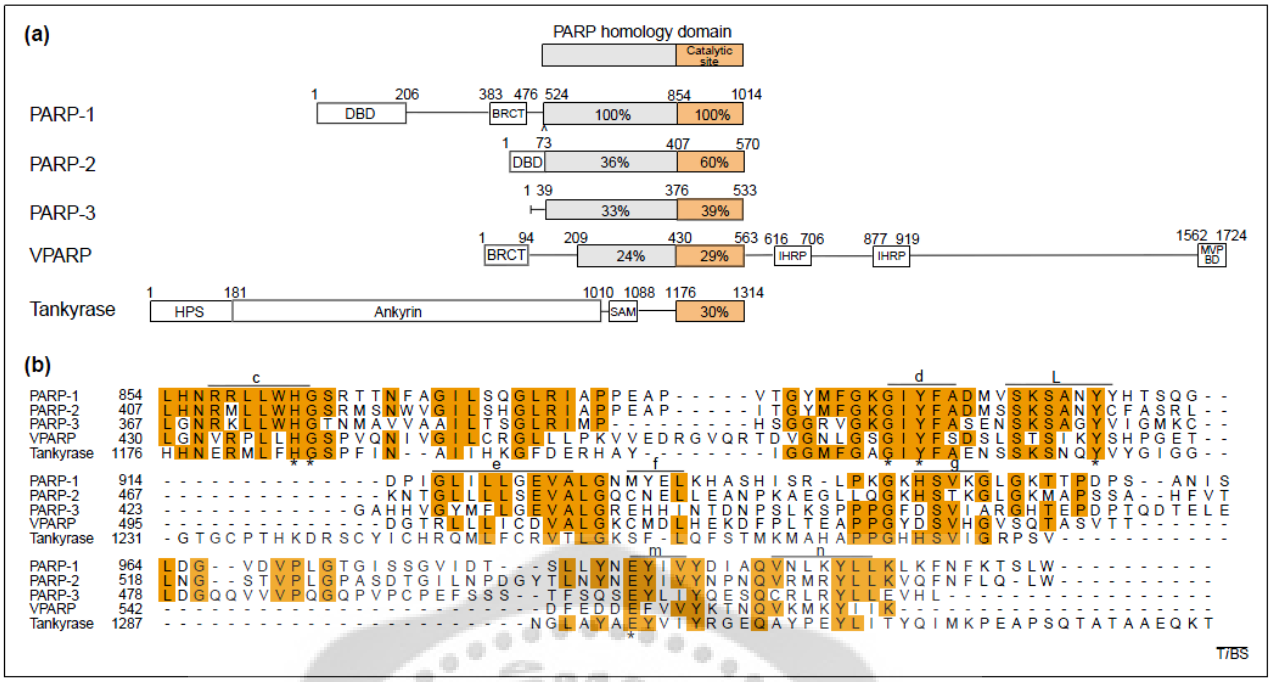

Figure 3 (a) Schematic primary structures of the five members of the poly(ADP-ribose) polymerase (PARP) family. Percentages indicate identity to PARP-1. (^) indicates the start site for sPARP-1. (b) Alignment of the catalytic domains of the five human PARPs: PARP-1 (GenBank no. M32721), PARP-2 (GenBank no. AJ236912), PARP-3 (GenBank no. AF083068), VPARP (GenBank no. AF158255) and tankyrase (GenBank no. AF082556). Amino acid identities between three or more PARPs are shaded. The secondary structures indicated by lines are based on the crystal structure. β strands are indicated by c, d, e, f, g, m, n and L indicates an αhelix. Asterisks indicate positions conserved in bacterial ADP-ribosylating toxins. Abbreviations: BRCT, BRCA1 C-terminal domain; DBD, DNA-binding domain; HPS, region containing homopolymeric runs of His, Pro and Ser; IHRP, inter-α-trypsin inhibitor family heavy-chain-related protein; MVP BD, major vault protein binding domain; SAM, sterile α module; sPARP-1, short PARP-1; VPARP, vault PARP. Figure from [27, 44]

PARP-1 and DNA damage responses PARP-1 is a nuclear protein comprised of three functional domains (Figure 4). The amino-terminal DNA-binding domain contains two zinc fingers that are important for the binding of PARP-1 to single-strand breaks and double-strand breaks (DSBs). [45, 46] A third zinc finger was recently described and found to be dispensable for DNA binding, but is important for coupling damage-induced changes in the DNA-binding domain to alterations in PARP-1 catalytic activity. [47, 48] In the central automodification domain, specific glutamate and lysine residues serve as acceptors of ADP-ribose moieties, thereby allowing the enzyme to poly(ADP-ribosyl)ate itself . [49, 50] Interestingly, this domain also

comprises a BRCA1 carboxy-terminal (BRCT) repeat motif, a protein–protein interaction domain that is found in other components of the DNA damage response pathway. The presence of this motif raises the possibility that unexplored protein–protein interactions involving this domain might also play an important part in PARP-1 biology. Finally, the C-terminal catalytic domain sequentially transfers ADP-ribose subunits from NAD+ to protein acceptors, thereby forming pADPr. [51] Studies in various model systems have implicated PARP-1 in multiple processes, all of which involve DNA-related transactions. After the induction of certain types of DNA damage, including nicks and DNA DSBs, PARP-1 is rapidly recruited to the altered DNA and its catalytic activity increases 10- to 500-fold, resulting in the synthesis of protein-conjugated long branched pADPr chains 15 to 30 sec after damage. [46, 52]

Owing to the size and large negative charge of pADPr (which is twice the charge density of DNA), the addition of pADPr interferes with the functions of modified proteins,such as histones, topoisomerase I and DNA protein kinase (DNA-PK). [53] Notably, however, the bulk of pADPr is attached to PARP-1. Once formed, this polymer could recruit hundreds of other proteins. [54- 58] Some of these recruited proteins — typified by XRCC1, the scaffolding protein that assembles and activates the DNA base excision repair (BER) machinery [59- 60] — bind directly to pADPr, whereas others are indirectly recruited because they interact with pADPr-binding proteins. At the same time, formation of pADPr diminishes the affinity of PARP-1 and histones for DNA, providing a mechanism for removing PARP-1 from damaged DNA and for the local modulation of chromatin compaction. [55, 61 -63] *In vitro* studies suggest that removal of PARP-1 provides access for repair proteins [64] and suppresses further pADPr synthesis [65] . Further polymer growth is also antagonized by two enzymes that hydrolyse pADPr, poly(ADP-ribose) glycohydrolase (PARG) and, possibly, the ADP-ribose hydrolase ARH3. [66 -67] ADP-ribosyl protein lyase, which cleaves the link between the first ADP-ribose and modified amino acids, has been described in rat tissues [68 ,69] and might also function in human cells. The concerted action of these enzymes removes pADPr from PARP-1, restoring its ability to recognize DNA strand breaks and initiate a new round of damage signalling.

Although pADPr has a half-life of seconds to minutes, the consequences of pADPr metabolism on cellular homeostasis can persist long after PARP-1 and the hydrolases have acted. Polymer synthesis consumes substantial amounts of NAD^+ and pADPr cleavage generates large amounts of AMP, leading to activation of the bioenergetic sensor AMP-activated protein kinase (AMPK) [70-71] .Therefore, the various consequences of PARP-1 activation reflect the collective effects of pADPr synthesis on PARP-1 substrates, binding of various proteins to pADPr, changes in cellular NAD^+ (and ATP) levels during pADPr synthesis

and changes in AMP levels owing to pADPr degradation (FIG. 1). In conditions that cause excessive DNA damage, such as post-ischaemic damage in the heart or brain, PARP-1 hyperactivation produces high levels of pADPr at the expense of NAD^+ and ATP, which become depleted and induce death by necrosis or apoptosis .

In addition to its role in BER described above, PARP-1 is involved in several other nuclear processes. The observation that rapid recruitment of mitotic recombination 11 (MRE11) and ataxia telangiectasia-mutated (ATM), crucial components of the homologous recombination machinery, to DNADSBs is dependent on pADPr synthesis [52 ,72] suggests that PARP-1 acts as a facilitator of homologous recombination. Studies in rodent and chicken cells indicate that recruitment of MRE11 to help restart stalled replication forks is also dependent on PARP-1.[73-75] Additional *in vitro* studies in rodent and human cells have implicated PARP-1 in non-homologous end joining (NHEJ). [76- 78] Consistent with these various roles in DNA damage responses, *PARP-1–/–* mice demonstrate heightened sensitivity to DNA-damaging agents, particularly alkylating agents and ionizing radiation. PARP-1 might also regulate transcription by modulating chromatin structure, altering DNA methylation patterns, acting as a co-regulator of transcription factors and interacting with chromatin insulators.[79, 80] Chromatin immunoprecipitation experiments demonstrate that PARP-1 is generally associated with actively transcribed genes, at which it is postulated to regulate histone H1 binding to chromatin. [81] Moreover, gene expression profiling in *PARP-1–/–* mouse cells[82] and human breast cancer cells treated with *PARP-1* short-hairpin RNAs (shRNAs) [83] reveal that PARP-1 loss or downregulation alters the expression of many genes involved in cell cycle control and stress response, including p53. Collectively, these observations implicate PARP-1 in transcription as well as in multiple aspects of the DNA damage response.

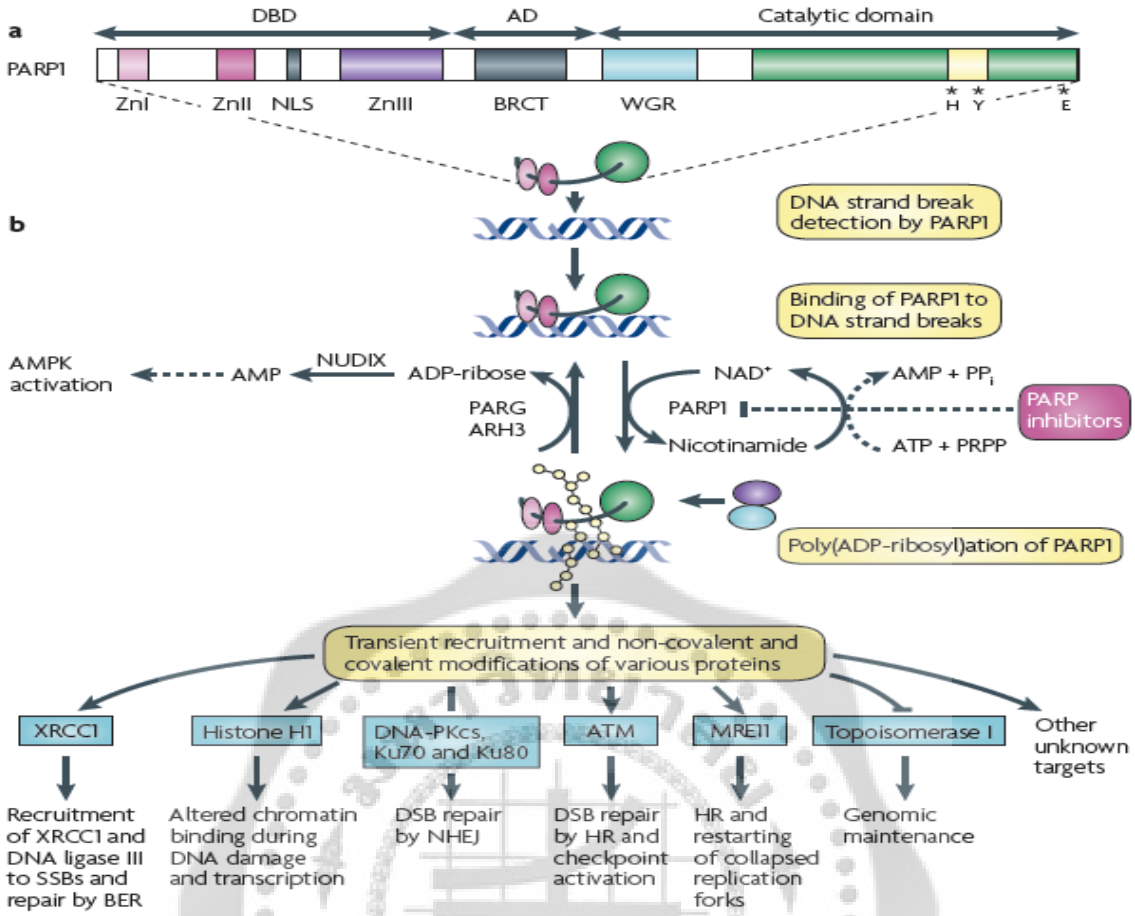

Figure 4 Structural and functional characteristics of PARP-1.

In figure 4 (a) Poly(ADP-ribose) polymerase 1(PARP-1) is shown with its DNA-binding (DBD), automodification (AD) and catalytic domains. The PARP signature sequence (yellow box within the catalytic domain) comprises the sequence most conserved among PARPs. Crucial residues for nicotinamide adenine dinucleotide (NAD+) binding (histidine; H and tyrosine; Y) and for polymerase activity (glutamic acid; e) are indicated.

(b) Consequences of PARP-1 activation by DNA damage. Although not shown to simplify the scheme, PARP-1 is active in a homodimeric form. PARP-1 detects DNA damage through its DBD. This activates PARP-1 to synthesize poly(ADP) ribose (pADPr; yellow beads) on acceptor proteins, including histones and PARP-1. Owing to the dense negative charge of pADPr, PARP-1 loses affinity for DNA, allowing the recruitment of repair proteins by pADPr to the damaged DNA (blue and purple circles). Poly(ADP-ribose) glycohydrolase (PARG) and possibly ADP-ribose hydrolase 3 (ARH3) hydrolyse pADPr into ADP-ribose molecules and free pADPr. ADP-ribose is further metabolized by the pyrophosphohydrolase NUDiX enzymes into AMP, raising AMP:ATP ratios, which in turn activate the metabolic sensor AMP-activated protein kinase (AMPK). NAD^+ is replenished by the enzymatic conversion of nicotinamide into NAD^+ at the expense of

phosphoribosylpyrophosphate (PRPP) and ATP. Examples of proteins non-covalently (pADPr-binding proteins) or covalently poly(ADP-ribosyl)ated are shown with the functional consequences of modification. It is important to note that many potential protein acceptors of pADPr remain to be identified owing to the difficulty of purifying pADPr-binding proteins *in vivo*. PARP inhibitors prevent the synthesis of pADPr and hinder subsequent downstream repair processes, lengthening the lifetime of DNA lesions. ATM, ataxia telangiectasia-mutated; BeR, base excision repair; BRcT, BRcA1 carboxy-terminal repeat motif; DNA-PKcs, DNA-protein kinase catalytic subunit; DsB, double-strand break; HR, homologous recombination; NHeJ, non-homologous end joining; NLs, nuclear localization signal; PPi, inorganic pyrophosphate; ssB, single-strand break; Zn, zinc finger. [84]

2.2.5.2 Bcl-2 and Bax

The *Bcl-2* (B-cell lymphoma-2) gene was discovered at the t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas, where its transcription becomes excessively driven by the immunoglobulin heavy chain gene promoter and enhancer on chromosome 14. [85-87] One key early discovery that introduced a new paradigm for carcinogenesis was that overexpression of *BCL-2* does not promote cell proliferation as most previously discovered oncogenes do; rather, overexpression of *BCL-2* inhibits cell death. [88] Apoptosis has now been widely accepted as a prominent tumor-suppression mechanism. Mutations in certain oncogenes that result in the activation of cell proliferation, such as deregulated *MYC* expression, require a second mutation to inhibit the apoptosis machinery so that tumor promotion can proceed efficiently. [89, 90] Thus, the combined overexpression of *BCL-2* and *MYC* synergize potently in the development of lymphomas and certain other types of cancer. [91] It has also become clear that, beyond roles in cancer, Bcl-2 and other members of the family are essential for an array of apoptosis program, including developmentally programmed cell death, tissue turnover and host defense against pathogens.

2.2.5.2 .1 Bcl-2 family proteins have opposing apoptotic activities.

Bcl-2 family members have classically been grouped into three classes. One class inhibits apoptosis (Bcl-2, BCL-XL, BCL-W, MCL1, BCL-B (also known as BCL-2L10) and A1 (also known as BCL-2A1), whereas a second class promotes apoptosis (Bax, BAK and BOK (also known as MTD)). A third divergent class of BH3-only proteins (BAD, BIK (also known as BLK or NBK), BID, HRK (also known as death protein-5 (DP5)), BIM (also known as BOD), BMF, NOXA and PUMA (also known as BBC3)) has a

conserved BH3 domain that can bind and regulate the anti-apoptotic BCL-2 proteins to promote apoptosis (Figure 5).

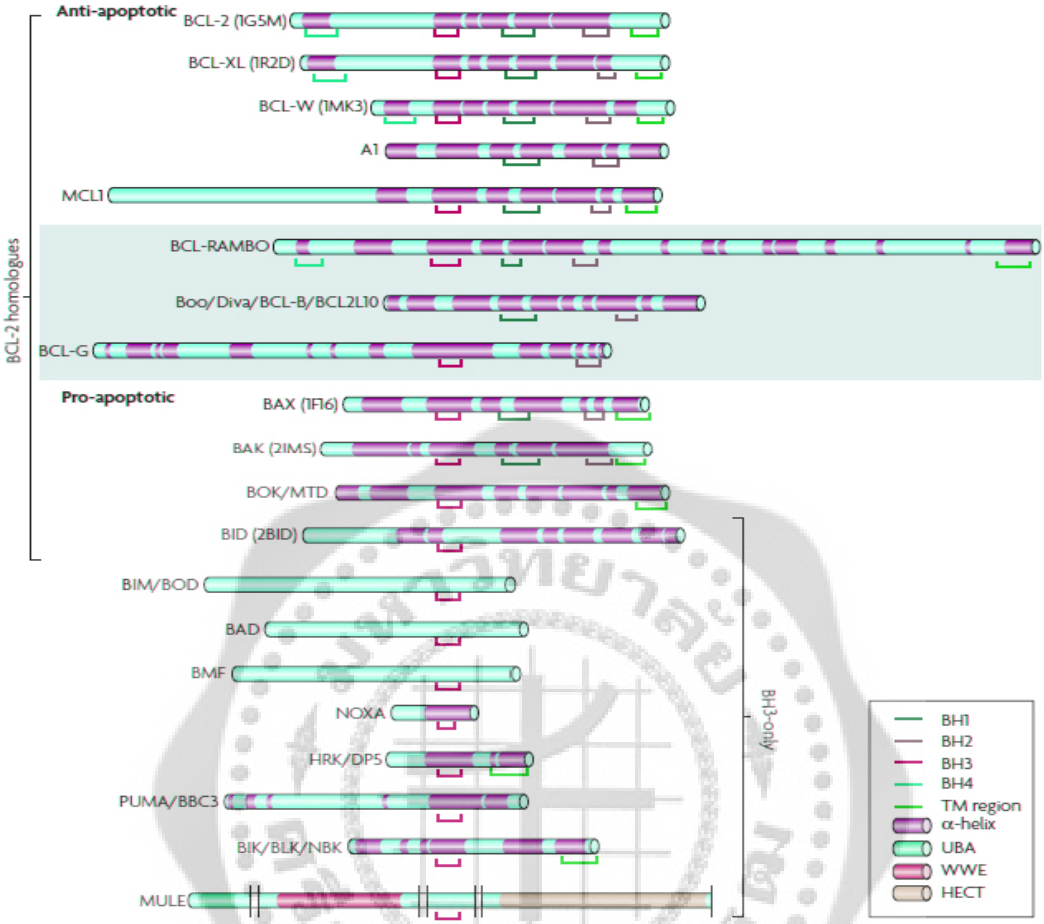

Figure 5 Sequence alignment of core BCL-2 family proteins and BH3-only proteins (modified From Youle RJ. 2008 [92])

It appears that the pro-apoptotic family members BAX and BAK are crucial for inducing permeabilization of the outer mitochondrial membrane (OMM) and the subsequent release of apoptogenic molecules (such as cytochrome *c* and DIABLO (also known as SMAC)), which leads to caspase activation. The anti-apoptotic family members, such as BCL-2 and BCL-XL, inhibit BAX and BAK. Recent evidence indicates that BH3-only proteins de-repress BAX and BAK by direct binding and inhibition of BCL-2 and other anti-apoptotic family members. [93] By contrast, an opposing model postulates direct activation of BAX and BAK by some BH3-only proteins (specifically BIM, tBID and PUMA). [94] (Figure 6). BAX and BAK promote caspase activation by their effects on mitochondria. Either directly or indirectly, these two pro-apoptotic BCL-2 family members induce the release of proteins from the space between the inner and outer mitochondrial membranes. [95] This process of mitochondrial outer membrane permeabilization (MOMP) results in the release of cytochrome *c* and other soluble proteins into

the cytosol. Although it is commonly thought that BAX and BAK form pores in membranes, the biochemical nature of such pores and how anti-apoptotic Bcl-2 family proteins might regulate them remains a key and controversial issue in the field of cell death.[96] At the same time as cytochrome *c* release (or immediately before), BAX and BAK induce mitochondria to fragment into more numerous and smaller units, which suggests connections between mitochondrial division processes and the functions of the Bcl-2 family.[97]

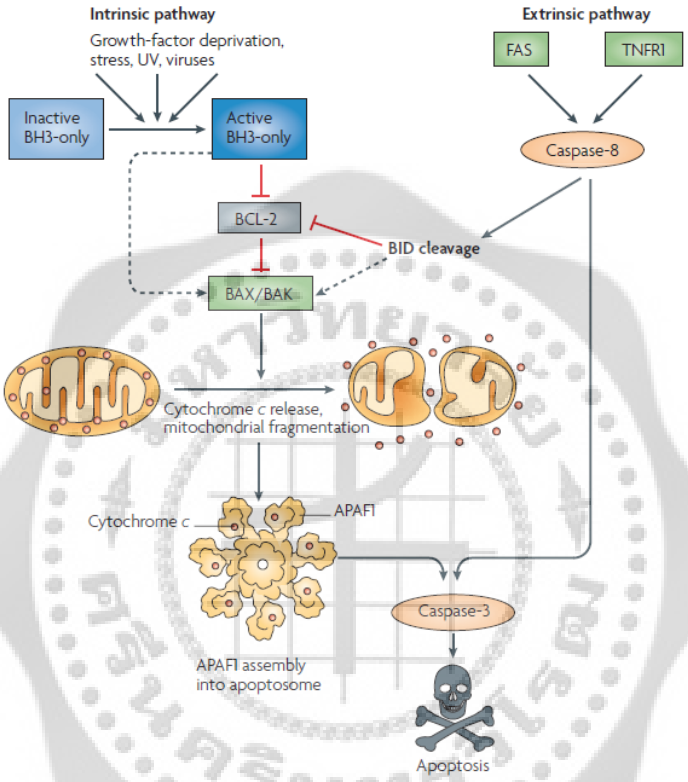

Figure 6 Scheme depicting intrinsic and extrinsic pathways of apoptosis. (Figure from Youle RJ 2008 [92])

Apoptosis can be induced by cell surface receptors, such as Fas and tumor necrosis factor receptor-1 (TNFR1) (extrinsic pathway, right), or by various genotoxic agents, metabolic insults or transcriptional cues (intrinsic pathway, left). The intrinsic pathway starts with BH3-only protein induction or post-translational activation, which results in the inactivation of some Bcl-2 family members. This relieves inhibition of BAX and BAK activation, which in turn promotes apoptosis. Some BH3-only proteins, such as BIM and PUMA, may also be able to activate BAX and/or BAK (as shown by the dotted line). Once activated, BAX and BAK promote cytochrome *c* release and mitochondrial fission, which leads to the activation of APAF1 into an apoptosome and activates caspase-9 to activate caspase-3. Caspases in turn cleave a series of substrates, activate DNases and orchestrate the demolition of the cell. The extrinsic pathway can bypass the mitochondrial step and activate caspase-8 directly, which leads to caspase-3 activation and

cell demolition. The BCL-2 family regulates the intrinsic pathway and can modulate the extrinsic pathway when cleavage of BID communicates between the two pathways. [92]

2.3 γ-Mangostins

2.3.1 Overview

The tropical fruit name mangosteen has been cultivated for centuries in tropical areas of the world. The tree is presumed to have originated in Southeast Asia or Indonesia and has largely remained indigenous to Malay Peninsula, Myanmar, Thailand, Cambodia, Vietnam, Laos and the Moluccas (Figure 7A). The inner pulp of the mangosteen fruit is highly praised as one of the best tasting of all tropical fruits. The scientific name is *Garcinia mangostana*. The entire fruit is typically 2.5-7.5 cm in diameter, roughly the size of a tangerine (Figure 7B). The rind (or skin) of the fruit is 0.6-1.0 cm thick and contains a purplish pigment. The inner pulp consists of four to eight juicy, white-colored segments (fruit portion, Figure 7B). The edible portion of the fruit comprises only about 25% of the total volume, whereas the remainder is tough, bitter pericarp which exudes a yellow resin (hence the term xanthones or yellow in Greek)(Figure 7B). The mangosteen rind, leaves and bark have been used as folk traditional medicine for thousands of years. The thick mangosteen rind has been and is used for treating catarrh, cystitis, diarrhea, dysentery, eczema, fever, intestinal ailments, pruritis and other skin ailments. The mangosteen leaves are also used by some natives in teas and for diarrhea, dysentery, fever, and thrush. It is also known that concentrates of mangosteen bark can be used for genito-urinary afflictions and stomatosis. [4]

Figure 7 The *Garcinia mangostana* Linn tree (A), the appearance of mangosteen fruit (B) (modified from Akao Y. 2008) [4]

2.3.2 Chemical structure

Mangosteen has been shown to contain a variety of secondary metabolites such as prenylated and oxygenated xanthones (98).[106] The xanthones have been isolated from pericarp, whole fruit, bark, and leaves of mangosteen. α-, β- and γ-mangostin, garcinone E, 8-deoxygartanin and gartanin are the most studied xanthones. In this study we use γ-mangostins. The chemical structure of γ-mangostin is shown in figure 8

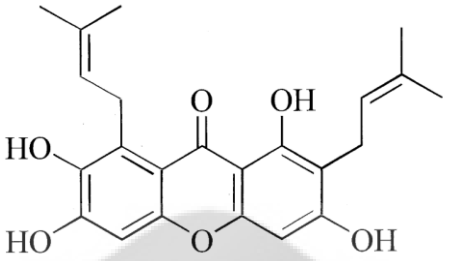

Figure 8 The chemical structure of γ-mangostin $C_{23}H_{24}O_6$ Molecular Weight: 396.4107 [99]

2.3.3 Pervious report about γ-mangostins

The previous report has indicated a potent anti- proliferative activity of γ-mangostin from the *Garcinia mangostana* inhibit inhibitor-kappaB kinase activity and decrease lipopolyaccharide-induced cyclooxygenase-2 gene expression in C6 rat gioma, [100] inhibit of 5-hydroxytryptamine2A receptors in 5 fluoro-α-methyltryptamine-induced head twitch responses of mice [101] , Anti-inflammatory in lipopolysaccharide (LPS)-stimulated RAW 264.7 cell [102] increases serotonin 2A/2C, muscarinic, histamine and bradykinkn erceptor mRNA expression [103], prevent lipopolysaccharade-mediated inflammation and insulin resistance in primary cultures of human adipocytes, [104]

In addition to iduce apoptosis in cancer cells, γ-mangostin from thull of hull *Garcinia mangostana* on human malignant glima cells [105] , increased the cell cycle arrest in G(1) phases and induced apoptosis in human melanoma cells, induce cell cycle arrest and apoptosis in human colon DLD-1 cells.[106]

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals used in this study were analytical grade and were listed alphabetically as follows.

Name of chemicals	Company
Acrylamide	BioRad
Agarose gel	BMA product
Ammonium persulfate	USB
Boric acid	Sigma
Bovine serum albumin	USB
Bromophenol blue	Sigma
Calcium chloride ($CaCl_2$)	Sigma
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma
Dimethylsulfoxide (DMSO)	Amresco
Minimum Essential Medium (MEM)	Gibco BRL
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Ethidium bromide	Sigma
Fetal bovine serum (FBS)	PAA
Glycerol	Sigma
Glycine	Sigma
HEPES (N-[2-hydroxyethyl] piperazine-N' [2-ethanesulfonic acid]	USB
Hoechst 33342	Invitrogen
2-Mercaptoethanol	Sigma
Magnesium chloride ($MgCl_2.6H_2O$)	Sigma
Methanol	Merck
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)	Sigma
Penicillin/streptomycin	PAA
Potassium chloride (KCl)	Merck
Potassium phosphate (KH_2PO_4)	Merck
Propidium iodide	Sigma
Sodium chloride (NaCl)	Merck

Sodium citrate	Biorad
Sodium dodecyl sulfate (SDS)	Merck
Sucrose	Merck
TEMED (N,N,N',N'-Tetramethylethylene diamine)	USB
Tris ([Tris Hydroxymethyl] aminomethane)	Sigma
Triton-x 100	Phamacia
Trypan blue	Sigma
Trypsin	PAA
Tween-20	Phamacia

3.1.2 Instruments

Name of instrument	**Company**
Centrifuge-HSC 10K	Vant
Centrifuge-MIKRO 20	Hettich
Centrifuge ICE centra-4R	Hettich
CO_2 incubator (water-jacketed)	NuAire
FACScan flow cytometer	Becton Dickinson
Fluorescence microscope	Olympus
Inverted microscope	Olympus
Lamina flow-Nu-440	NuAire
Western blot apparatus	BioRad
Shaking-Mini Rocker MR-I	Biosan
Microplate reader-Multiskan EX	Thermo electron

3.2 Cell culture

The neuroblastoma cell line, Neuro-2A, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell line was maintained as a monolayer in MEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The medium was refreshed every 2-3 days. After about 90% of confluence, the cultured cells were detached with 0.25% trypsin-EDTA and sub-cultured. Cells were used in the study are in the exponential phase of growth and at passage 5^{th} to 30^{th} after thawing.

3.3 γ-Mangostin extraction and isolation

γ-Mangostin was obtained from Assoc. Prof. Dr. Sunit Suksamrarn, Department of Chemistry, Faculty of Science, Srinakharinwirot University. Mangosteen fruit (*G. mangostana*)

was collected from Kombang District, Chanthaburi Province, Thailand in April 2007. γ-Mangostin was dissolved in DMSO at the stock concentration of 100 µg/mL and further diluted to the desired working concentration before use.

3.4 Morphological changes

Examination of morphological changes indicating induction of Neuro-2A differentiation. Cells were cultured in monolayer in 96 well plates, seeded at a density of 1×10^4 cells/well and allowed to grow for 24 h. These cells were then treated with culture media containing γ-mangostin at various concentrations (0, 0.5 and 5 µg/mL) for 24 h before being examined under the light microscope.

3.5 Cell proliferation and cell viability assay

This study used MTT for analysis of cell proliferation and cytotoxictiy of γ-mangostin. MTT assay is a colorimetric assay for measuring the activity of dehydrogenase enzymes that reduce yellow tetrazolium MTT to purple formazan in the mitochondria of living cells. A solubilization solution (usually either DMSO, an acidified ethanol solution or a solution of the detergent SDS in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring the absorbance at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The maximum absorption wavelength is dependent on the solvent employed.

Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and allowed to grow for 24 h. Cells were then treated with γ-mangostin at various concentrations of 0.005, 0.05, 0.5, 5, 50, and 500 µg/mL, whereas the control group was treated with DMSO. After incubation for 24 h, 100 µl of 0.5 mg/mL MTT solution was added to each well and the plate was further incubated for 2 h at 37°C. The supernatant was aspirated and 100 µl of DMSO was added to each well to solubilize water insoluble purple formazan crystals. The absorbance at 550 nm was measured using a microplate reader (Multiskan EX, Thermo electron corporation, Finland) and the inhibitory concentration 50% (IC_{50}) value was calculated using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

3.6 Time and dose response

Effect of γ-mangostin on the cell viability was further analyzed by using a trypan blue exclusion method. Cells were seeded in a 24-well plate at 1×10^5 cells/well and incubated for 24 h. Then, the cells were treated with indicated concentrations (0, 2.5, 5, 7.5, 10 and 20 μg/mL) of γ-mangostin for 3, 6, 9, 12 and 24 h. Cells were then harvested by trypsinization, stained with trypan blue and counted with hemocytometer. Cell survival was expressed as percentage of viable cells of treated cells to control cells. Cells were treated in triplicates and the experiments were repeated three times.

3.7 Detection of nuclear morphology

Nuclear morphology can be examined by Hoechst staining. The Hoechst stains are fluorescence dye used for labeling DNA and RNA and observed by fluorescent microscopy and flow cytometry techniques. Hoechst 33258 and Hoechst 33342 are the most used of these stains. They have the ability to bind to DNA molecules, causing the nuclei and mitochondria to appear fluorescence under ultraviolet light.

Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. Hoechst 33342 binds preferentially to adenine-thymine (A-T) regions of DNA. This stain binds into the minor groove of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye: base pair ratios.

Neuro-2A cells were seeded at a density of 1×10^6 cells/well in a 6-well plate and allowed to grow for 24 h. Then cells were treated with 20 μg/mL γ-mangostin for 0, 3 and 6 h. After trypsinization, cells were washed with 1X PBS once and stained with 3 μg/mL of Hoechst 33342 for 15 min. Stained cells were washed with PBS once and examined using fluorescent microscope with an ultraviolet filter.

3.8 DNA fragmentation analysis

DNA fragmentation is one of the biochemical hallmarks of apoptosis. The DNA fragmentation can be analyzed using agarose gel electrophoresis to demonstrate a “ladder” pattern. On the other hand, necrosis is characterized by random DNA fragmentation which forms a “smear” on agarose gel.

3.8.1 Genomic DNA extraction

Neuro-2A cells (1×10^6 cells/well) were seeded in 6-well plates and treated with 20 μg/mL γ-mangostin for 0, 12, 24 h. Both floating and attached cells were collected by

trypsinization, washed once with PBS and centrifuged at 13,000 × *g* for 5 min at room temperature. Supernatant was removed. Cell pellet was collected to extract DNA using a Genomic DNA extraction kit (Gentra Puregene cell kit) according to the manufacturer's instructions. Briefly, 300 µl of cell lysis solution was added and mixed by pipetting up and down. Then 1.5 µl RNase A was added to the cell lysate, mixed by inverting the tube 30 times and incubated at 37°C. After that, 100 µl protein precipitation solution was added and vortexed vigorously for 20 s at high speed. Then centrifuged at 13,000 × *g* for 1 min. Supernatant was transferred to a new tube and 300 µl isopropanal was added. Then mixed by inverting gently 50 times and centrifuged at 13,000 × *g* for 1 min. Supernatant was discarded and allowed DNA to air dry at room temperature for 10-15 min. Finally, 20 µl DNA hydration solution was added to the tube containing the pellet and incubated at 65°C for 1 h to dissolved the DNA.

3.8.2 Agarose gel electrophoresis

A 1.7% agarose gel electrophoresis was used to visualize DNA fragmentation. Preparation of 1.7% agarose gel is as followed; 1.7 g of agarose and 100 µl of 1X TBE buffer (89 mM Tris; pH 8.3, 89 mM boric acid, 2 mM EDTA) were added into a flask. Then heated to melt the agarose and poured into a horizontal gel support. After the gel was solidified, 20 µl of DNA sample was loaded into each lane and was electrophoresed at 100 V for 2 h. The gel was stained with ethidium bromide for 15 min. DNA fragment was visualized with UV light as DNA laddering of 180-200 bp and a photograph was taken.

3.9 Protein immunoblot analysis

The immunoblotting procedure is as follows. Proteins are transferred form an electrophoresis gel to a membrane surface. The transferred proteins become immobilized on the surface of the membrane in pattern that is an exact replica of the gel. Unoccucpied protein-binding site on the membrane are saturated to prevent non-specific binding of antibodies. This step is called either "blocking". The blot is probed for the protein of interest with a specific primery antibody. The blot is probed second time. The second prob is an antibody that is specific for the primery antibody type and is conjugated to a detectable enzyme. The site of the protein of interest is thus tagged with an enzyme through the specificities of the primary and secondary antibodies.

3.9.1 Sample preparation

To prepare samples for gel electrophoresis, cells need to be lysed to release the proteins of interest. Neuro-2A cells were incubated for different times in the presence or absence of 20 µg/mL γ-mangostin, harvested and washed once with iced cold PBS. Then,

1.5×10^{6} cells were lysed for 30 min on ice in 50 µl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% Triton X-100) containing complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysate was centrifuged at 19064.136 × *g* at 4°C for 30 min. The supernatant was transferred to a new tube and the protein content was determined before loading into the gel.

3.9.2 Protein determination

The concentration of protein in the cell lysate was determined by using Bio-Rad protein assay (Bio-Rad Laboratories, USA) which based on the method of Bradford's. Bovine serum albumin (BSA) was used as standard protein. To determine the protein concentration, BSA was diluted with distilled water to 1, 0.5, 0.25, 0.125, 0.0625 and 0 mg/mL. The cell lysate was also diluted to 1/10. After that, 100 µl of each standard protein and samples were mixed with 900 µl of dye solution and incubated at room temperature for 5 min. The protein concentration was determined at 595 nm by using a spectrophotometer.

3.9.3 Preparation of SDS-PAGE

The gels consist of acrylamide, bisacrylamide, SDS, and a Tris-HCl buffer with adjusted pH. In general, two-part discontinuous gels are used. The samples are loaded onto the upper portion (stacking gel), which has a low acrylamide concentration, low pH, and low resolving ability. When samples run through the stacking gel, all proteins are concentrated into a narrow band. That narrow band then enters the lower portion (separating gel) that separates proteins by size.

3.9.4 Separating gel

The acrylamide concentration chosen for the separating gel depends on the sizes of proteins to be separated. Smaller proteins are resolved at higher acrylamide concentrations and vice versa. The separating gel was prepared according to table 1. The separating gel solution was loaded to about 2 cm below the top of the glass plates. A thin layer of butanol was overlaid onto the surface of separating gel to prevent oxygen exposure to the gel and made gel surface to smooth. The separating gel was allowed to polymerize for 40 min.

Table 1 Separating gel preparation (0.375 M Tris, pH 8.8), for 1 gel of MINI PROTEAN II Bio-Rad

Stock solution	12%
dH_2O	2.17 mL
1.5 M Tris-HCl, pH 8.8	1.25 mL
10% SDS	50 µl
40% acrylamide	1.5 mL
10% ammonium persulfate	25 µl
TEMED	2.5 µl
Total volume	**5 mL**

Calculated volumes required per gel slab

Spacer thickness	Volume (mL)
0.5 mm	2.8
0.75 mm	4.2
1.0 mm	5.6
1.5 mm	8.4

3.9.5 Stacking gel

After the separating gel was polymerized, butanol was removed by washing with distilled water. The stacking gel was prepared according to Table 2. After pouring the gel, the comb was inserted to the gel and let it polymerized for 20 min.

Table 2 Stacking gel preparation (4% gel, 0.125 M Tris-HCl, pH 6.8)

Stock solution	**Volume (per 1 gel)**
dH_2O	1.52 mL
1.0 M Tris-HCl, pH 6.8	0.25 mL
10% SDS	20 µl
40% acrylamide	0.196 mL
10% ammonium persulfate	10 µl
TEMED	2 µl
Total volume	**2 mL**

3.9.6 Running condition

Twenty five µg protein of each sample were mixed with 5X sample buffer (60 mM Tris-HCl; pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue) and boiled for 5 min. Protein samples were loaded into the gel. The gel was run at 100 V for 1 h in running buffer containing 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS. After that, the gel was transferred onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation, USA) for 2 h at 100 V with the use of a Mini Trans-Blot Cell® (Bio-Rad). Transfer buffer contains 0.025 M Tris-HCl, 0.192 M glycine**, and 20% methanol.**

3.9.7 Protein detection

After protein transferring, the membrane was blocked with TBST (10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. The blots were incubated overnight at 4°C with primary antibody; rabbit polyclonal Bax and PARP rabbit monoclonal anti- Bcl-2 antibody (all diluted 1:3000, from Cell signaling Technology, Danvers, MA). To remove excess primary antibody, membranes were washed in TBST for 10 min 3 times followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase (diluted 1:10000) for 1 h at room temperature. To remove excess secondary antibody, washing step was required as for primary antibody. Immunoreactive protein bands were detected by chemiluminescent using enhanced chemiluminescence reagent (ECL) and exposed to X-ray film (both from Pierce, Rockford, IL).

3.9.8 Reprobing

The membrane can be used to detect other proteins or reprobe with other primary antibodies for 3-4 times. To remove the previous primary antibody, the membrane was

incubated with the Restore Western Blot Stripping buffer (Pierce, Rockford, IL) at 37°C for 15 min. The membrane was washed with TBST for 10 min 3 times. After that, continuing with the same step from blocking the membrane with 5% non-fat milk in TBST for 1 h at room temperature. In this work, the membrane was reprobed with β-actin antibody (Santa Cruz) at 1:5000 as the marker for equally loaded amount of proteins.

CHAPTER IV

RESULTS

4.1 γ-Mangostin can induce apoptosis in neuroblastoma cells, Neuro-2A Effect of γ-mangostin on the morphological changes

Examination the morphological changes to evaluate effect of γ-mangostin on Neuro-2A cells, under the light microscope (Figure 9). After 24 h of treatment with γ-mangostin at various concentrations (0, 0.5 and 5 μg/mL) the result showed morphological changes, some cells shrank and showed toxicity, cell quantity and density became lower when compared with control.

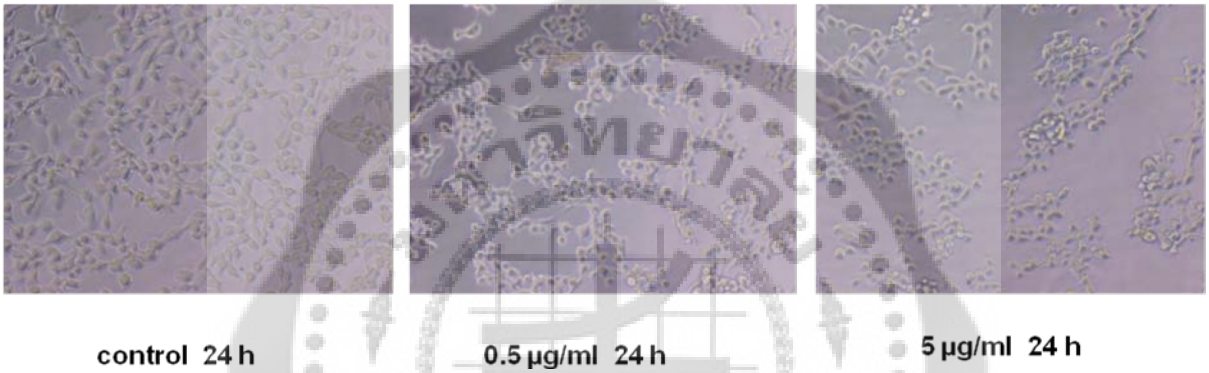

Figure 9 The morphological changes of Neuro-2A treated with 0.5% DMSO (control) and 0.5, 5 μg/mL γ-mangostin for 24 h.

4.2 Effect of γ-mangostin on cell viability

As shown in Figure 10, MTT assay was performed at various concentrations of 0.005, 0.05, 0.5, 5, 50, and 500 μg/mL of γ-mangostin for 24 h. The result showed that γ-mangostin decreased cell viability of Neuro-2A cells as demonstrated by the decrease in the formation when compared with control. The IC_{50} value (the concentration producing 50% inhibition of cell growth) was estimated using the software GraphPad Prism and shown to be 7.49 μg/mL.

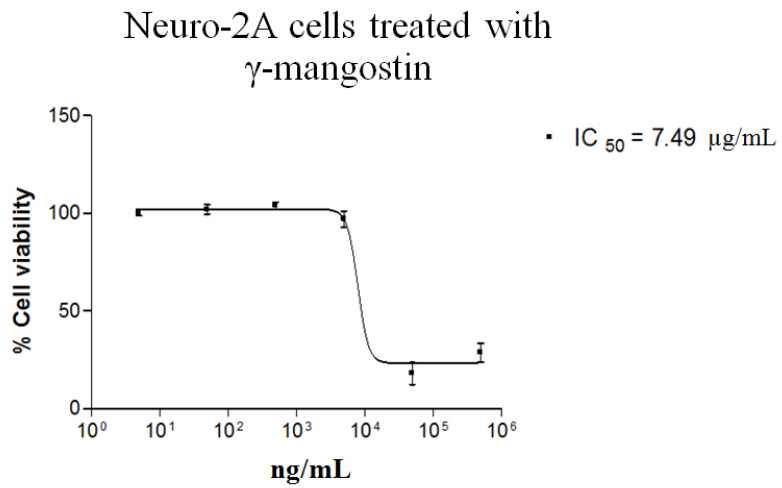

Figure 10 % Cell viability of Neuro-2A treated with 0.5% DMSO (control) and 0.005, 0.05, 0.5, 5 50, and 500 µg/mL γ-mangostin for 24 h and assessed by MTT assay showed IC_{50} = 7.49 µg/mL.

4.3 Time and dose response

After examined the toxicity of γ-mangostin on Neuro-2A cells, time and dose response was determined using trypan blue exclusion method by incubating the cells with 0, 2.5, 5, 7.5, 10 and 20 µg/mL of γ-mangostin for 3, 6, 9, 12 and 24 h. The data indicated time and dose dependent. (Figure 11)

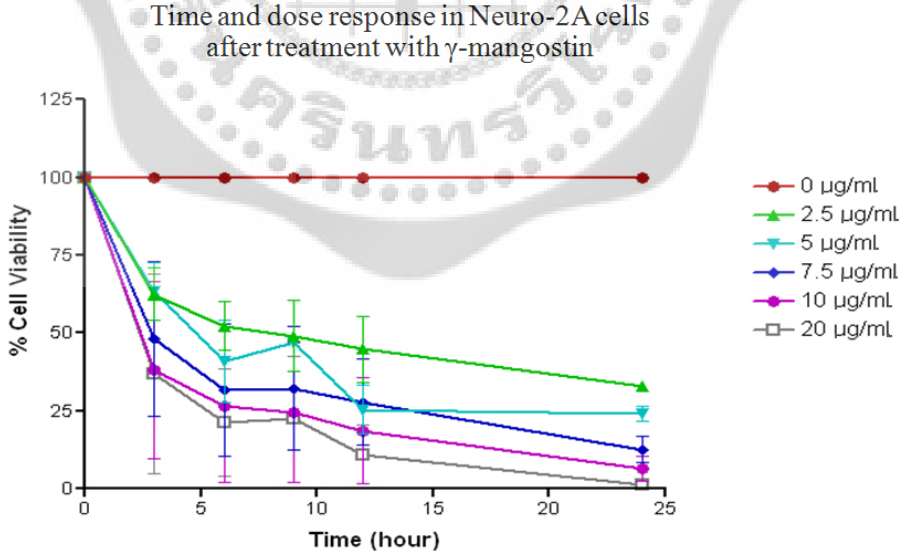

Figure 11 Time and dose response were determined using γ-mangostin at various concentrations (0, 2.5, 5, 7.5, 10 and 20 µg/mL) for 3, 6, 9, 12 and 24 h and stained by trypan blue before being examined under the light microscope.

4.4 Effect of γ-mangostin on the nuclear morphological changes

To examine the involvement of apoptosis in the antiproliferative effects of γ-mangostin on Neuro-2A, we carried out Hoechst 33342 nuclear staining (with 20 μg/mL γ-mangostin for 0, 3 and 6 h) and observed under fluorescent light microscope the result clearly showed chromatin condensation and nuclear fragmentation. On the other hand, we examined cell morphological changes by observing the cells under white light microscope and membrane blebbing was clearly observed. (Figure 12)

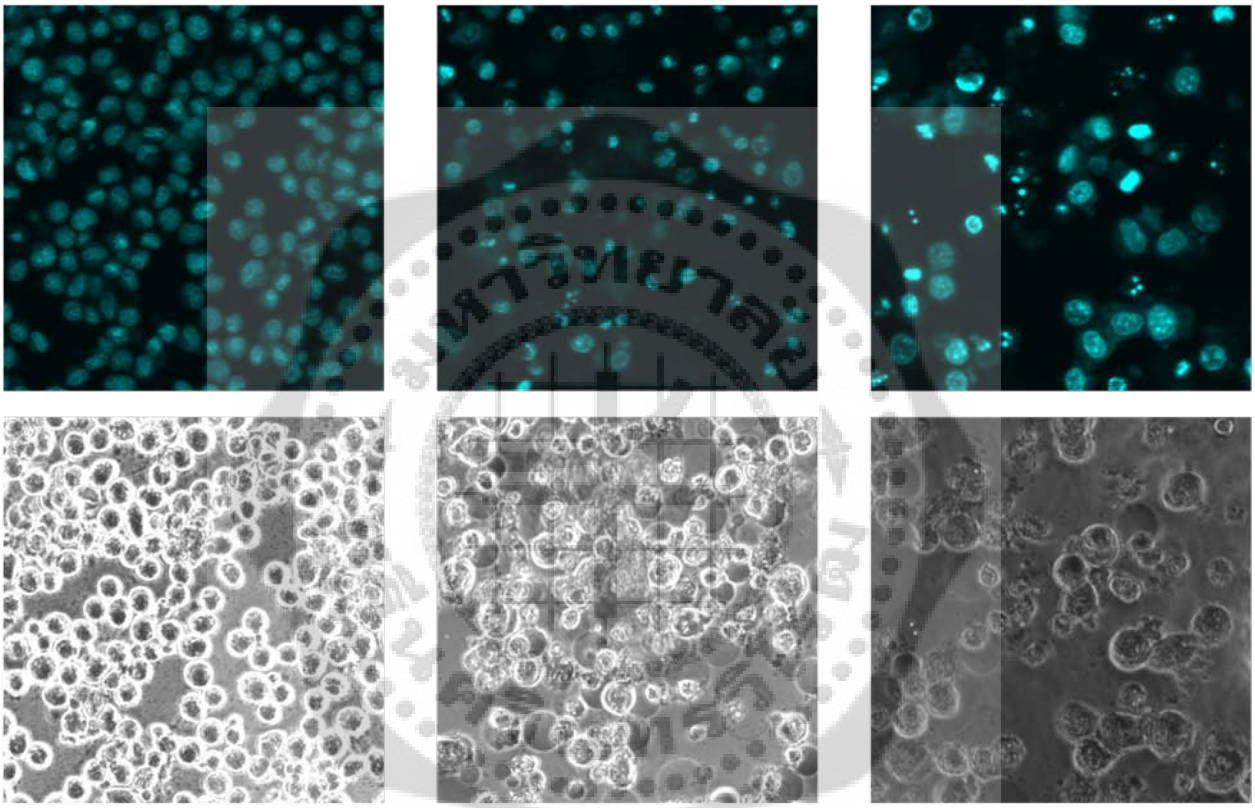

Figure 12 Chromatin condensation and nuclear fragmentation as observed by fluorescent light microscope (upper row), membrane blebbing by white light microscope (lower row) of Neuro-2A cells treated with γ-mangostin at 20 μg/mL for 0, 3 and 6 h.

4.5 DNA fragmentation

To investigate whether γ-mangostin actually induced DNA fragmentation, genomic DNA was isolated from Neuro-2A cells previously exposed to 0.5% DMSO for 24 h (control) and 20 μg/mL of γ-mangostin for 12 and 24 h and then electrophoresed. The results showed apoptotic intra-nucleosomal chromatin cleavage by endonucleases activity into multiple fragments of 180 bp producing thereby a typical DNA laddering. Results clearly showed that

DNA from γ-mangostin treated cells exhibited such fragments typical of apoptosis whereas control untreated cells did not provide ladders. Thereby, it is possible that γ-mangostin causes apoptosis of Neuro-2A cells (Figure 13).

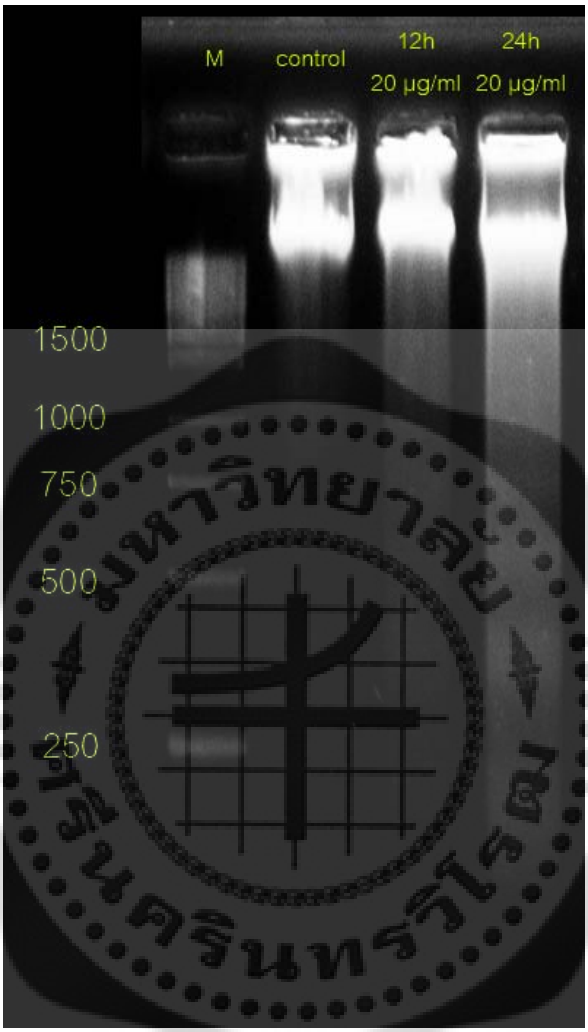

Figure 13 DNA fragmentation, cells were treated with 0.5% DMSO for 24 h (control) and with 20 µg/mL γ-mangostin for 12 and 24 h then determined by agarose gel electrophoresis.

4.6 Effect of γ-mangostin on the expression of Bcl-2, Bax and cleaved PARP-1 proteins

Expressions of Bcl-2, Bax and cleaved PARP-1 when Neuro-2A cells were treated with γ-mangostin could be investigated by protein immunoblotting analysis (0.5% DMSO for 9 h (control) and 20 µg/mL γ-mangostin for 6 and 9 h). The results showed that treatment of Neuro-2A cells with 20 µg/mL γ-mangostin induced the increase in Bax protein level, decreased Bcl-2 (Figure 14) and cleaved PARP-1 expressions (Figure 15). The ratios of Bax, Bcl-2 and cleaved PARP-1 to β-tubulin were calculated by densitometer

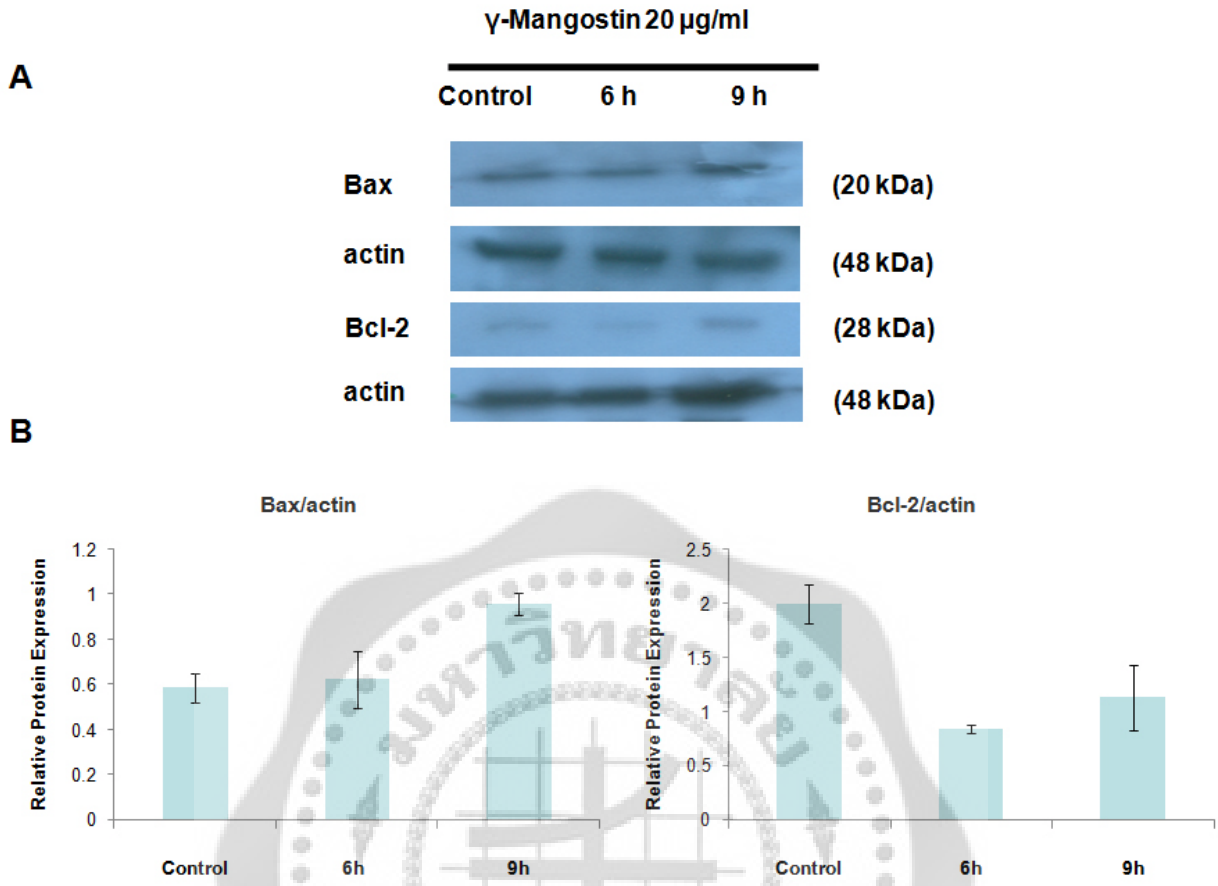

Figure 14 Effect of γ-mangostin on expressions of Bax and Bcl-2 in Neuro-2A cells. Cells were treated with 20 μg/mL γ-mangostin for 6 h and 9 h and examined by Western blot analysis. β-Actin was used as the internal control.

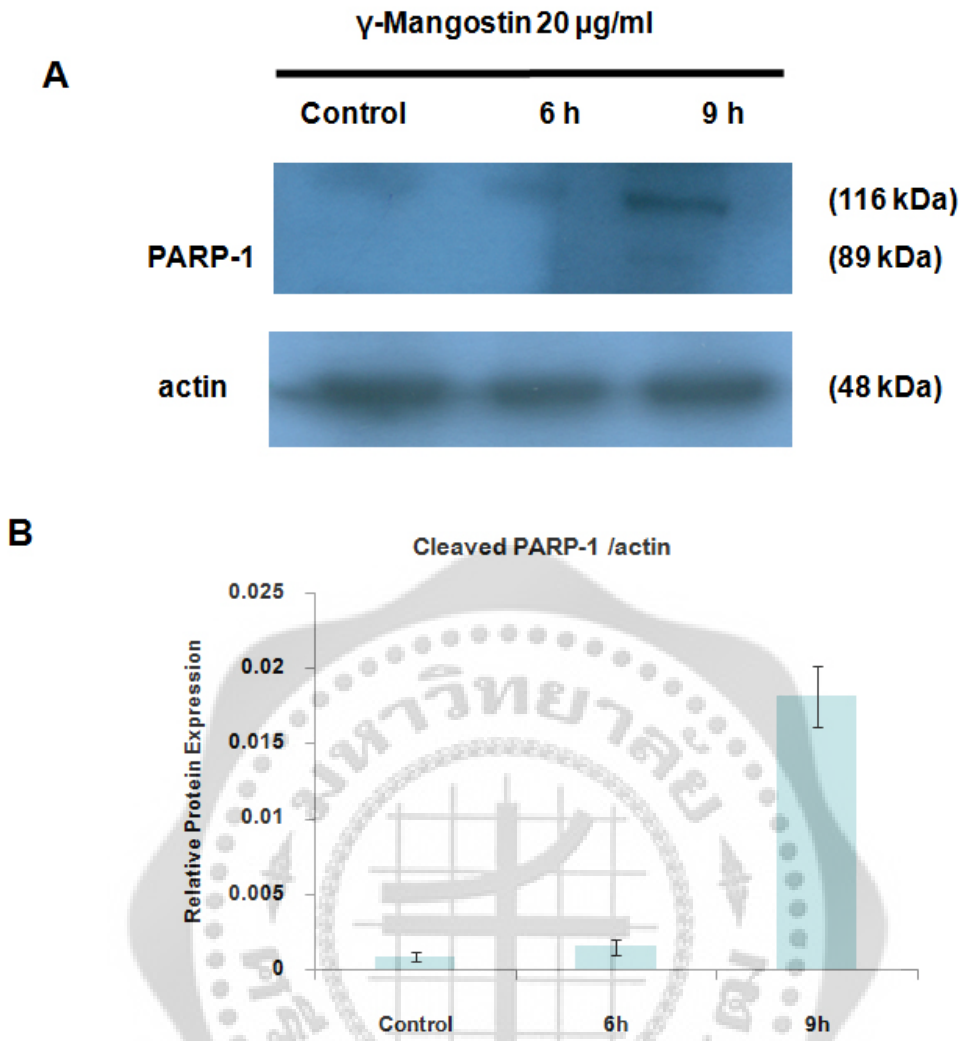

Figure 15: Effect of γ-mangostin on expression of cleaved PARP-1 in Neuro-2A cells. Cells were treated with 20 μg/mL γ-mangostin for 6 h and 9 h and examined by Western blot analysis. β-Actin was used as the internal control.

CHAPTER V

DISCUSSION

The mangosteen rind, leaves and bark have been used as folk traditional medicine for thousands of years.[4] This is the first study showing toxicity of γ-mangostin on Neuro-2A cells as detected by morphological changes upon treatment with 0.5 μg/mL γ-mangostin for 24 hours , which is similar to treatment with staurosporine (SSP). The results showed that γ-mangostin showed higher toxicity to Neuro-2A than SKNSH human neuroblastoma cells that needed 20 μg/mL Betulinic acid (BA) for 72 hours [107]. In addition, GeO_2 induced cell death in Neuro-2A at concentration 800 μM for 24 hours [108],which is higher than that of γ-mangostin. The study in colon cancer cells (DLD-1) with γ-mangostin [106] also showed of apoptosis induction, which is correlated to our study.

Apoptosis induction indicated by cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies [33]. This study showed nuclear condensation by Hoechst 33342 staining (with 20 μg/mL γ-mangostin for 0, 3 and 6 h). This results indicated that γ-mangostin induced nuclear condensation at lower concentration and shorter time than cells treated with GeO_2 (800 μM for 48 hours) [108]. The study of DNA fragmentation in Neuro-2A also implied that γ-mangostin induced DNA fragmentation (20 μg/mL for 12 and 24 hours) at lower concentration and shorter time than cells treated with colon cancer, DLD-1, cells treated with γ-mangostin at concentration 20 μM for 72 hours [106]

In addition, this study found the expression of pro-apoptosis Bax and anti-apoptosis Bcl-2 proteins, indicating apoptosis induction via intrinsic pathway. We found that γ-mangostin can induce Bax which is pro-apoptosis protein and affect to decrease level of Bcl-2 which is anti-apoptosis protein, resulting in cytochrome c releasing. The cytochrome c will then combine with other proteins forming apoptosome, leading to activated caspase 3 that will cleave PARP-1 (DNA repairing protein), causing DNA fragmentation as illustrated in Figure 16. This work is preliminary results for therapeutic drug development in the future.

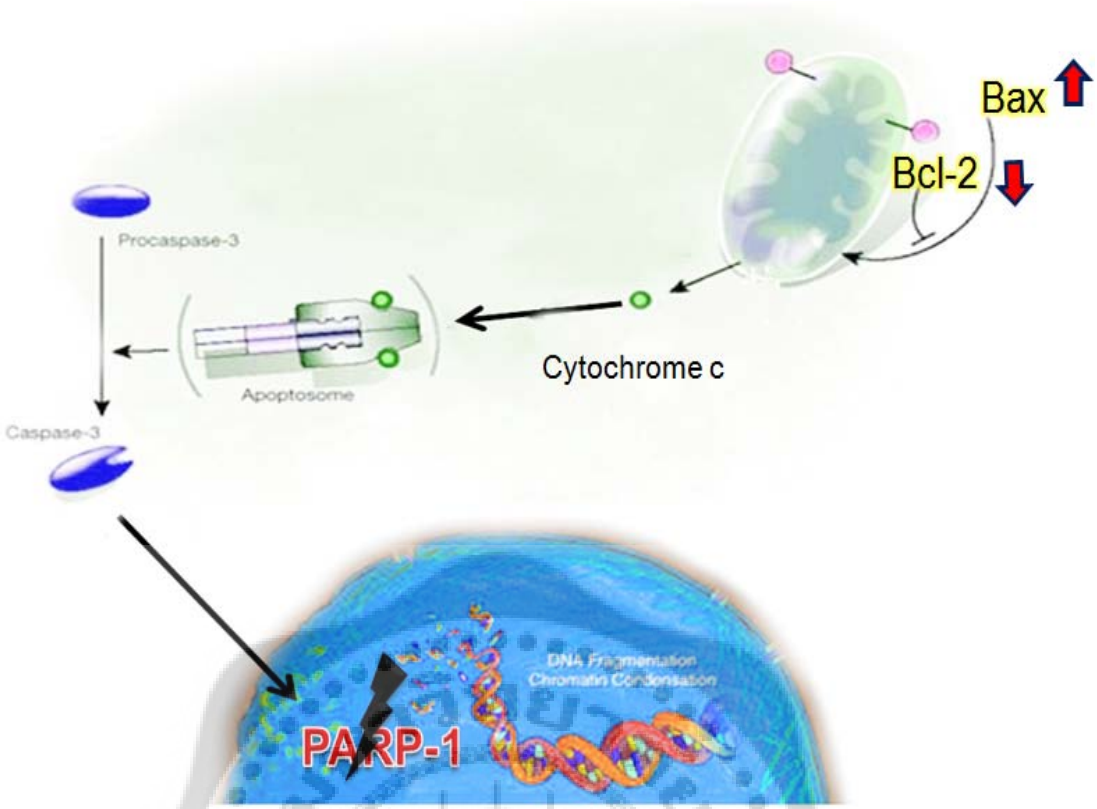

Figure 16 Apoptosis induction by γ-mangostin in Neuro-2A cells

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