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EFFECT OF ZINC ON ANOIKIS IN H460 LUNG CANCER CELLS

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แร่ธาตุสังกะสีมีบทบาทสำคัญในการควบคุมการทำงานของเซลล์ให้เป็นปกติ พบว่าภาวะการขาดสังกะสีมีความสัมพันธ์กับการเกิดและการดำเนินของโรคมะเร็งหลายชนิด อย่างไรก็ตามบทบาทของสังกะสีต่อการตายแบบอะนอยคิสและการแพร่กระจายของเซลล์มะเร็งยังไม่ทราบแน่ชัด การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลและกลไกการออกฤทธิ์ของสังกะสีต่อการตายแบบอะนอยคิสของเซลล์มะเร็งปอดชนิด เอช 460 ผลการศึกษาพบว่าสังกะสีในขนาดความเข้มข้นที่ไม่เป็นพิษต่อเซลล์มีผลทำให้เซลล์มะเร็งปอดชนิด เอช 460 มีความไวต่อการตายแบบอะนอยคิสเพิ่มขึ้น โดยมีการรอดชีวิตของเซลล์ลดลงและมีจำนวนเซลล์ที่เกิดอะพอพโทซิสเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) นอกจากนี้ยังพบว่า สังกะสีทำให้จำนวนและขนาดโคโลนีของเซลล์มะเร็งลดลง ซึ่งแสดงถึงฤทธิ์ของสังกะสีในการยับยั้งการเจริญของเซลล์มะเร็งปอดชนิด เอช 460 ในสภาวะไร้การยึดเกาะ สำหรับกลไกในการกระตุ้นการตายแบบอะนอยคิสในเซลล์มะเร็งของสังกะสีอาจเกี่ยวข้องกับการลดระดับของ phosphorylated protein kinase B (pAkt) และ caveolin-1 (Cav-1) ผลการศึกษาเหล่านี้แสดงว่า สังกะสีสามารถกระตุ้นการเกิดอะนอยคิสของมะเร็งปอดได้ ซึ่งข้อมูลที่ได้นี้อาจเป็นประโยชน์ในการพิจารณานำสังกะสีมาใช้ในการบำบัดรักษามะเร็ง เพื่อป้องกันการแพร่กระจายของเซลล์มะเร็งต่อไป

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CHALAMART PRAMCHU-EM: EFFECT OF ZINC ON ANOIKIS IN H460 LUNG CANCER CELLS. ADVISOR: ASST. PROF. KULWARA MEKSAWAN, Ph.D., CO-ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., 78 pp.

Zinc is an essential trace element that plays an important role in the normal function of cells. Zinc deficiency was found to be associated with the development and progression of various cancers; however, the role of zinc in cancer cell anoikis and metastasis was still unknown. The purpose of this study was to investigate the effect of zinc on anoikis in human lung cancer H460 cells. The results showed that non-toxic concentrations of zinc enhanced anoikis response in human lung cancer H460 cells with significant decreased cell viability and increased number of apoptotic cells ($p < 0.05$). Moreover, zinc significantly decreased the colony number and colony size of cells supporting the inhibitory effect of zinc on the anchorage-independent cell growth. The underlying mechanism of zinc-induced cancer anoikis may involve decrease in phosphorylated protein kinase B (pAkt) and caveolin-1 (Cav-1). These results indicate that zinc has an ability to sensitize lung cancer cells to anoikis, and these could be beneficial in developing strategies for cancer metastasis prevention.

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
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LIST OF ABBREVIATIONS

ANOVA	one-way analysis of variance
Akt	protein kinase B
APAF	apoptosis protease activating factor
Bax	B-cell lymphoma-2-associated X protein
Bcl-2	B-cell lymphoma-2 protein
Cav-1	caveolin-1
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ERK	extracellular signal-regulated kinases
et al.	et alibi, and others
FBS	fetal bovine serum
h	hour, hours
Mcl-1	myeloid cell leukemia-1 protein
mg/mL	milligram per milliliter
mM	millimolar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

nm	nanometer
OMM	outer mitochondrial membrane
PI3K	phosphoinositide 3-kinase
pAkt	phosphorylated protein kinase B
PI	propidium iodide
pERK	phosphorylated extracellular signal-regulated kinases
poly-HEMA	poly-2-hydroxylethylmethacrylate
PBS	phosphate buffered saline
p	*p* value
RPMI	Roswell Park Memorial Institute 1640 medium
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of mean
TBST	tris-buffer saline, 0.1% Tween 20
%	percentage
℃	degree Celsius
µL	microliter
µM	micromolar

CHAPTER I
INTRODUCTION

1.1 Background and Rationale

Zinc is an essential micronutrient found in all body organs and secretions. It generally presents in foods, and good sources of zinc are seafood, red meat, poultry, eggs, dairy products, grains, and cereals (Gropper, Smith, and Groff, 2005). Zinc plays a critical role in physiological activities which control normal cell function including cell growth, cell replication, membrane stability, brain development, reproduction, and fetal development. Zinc is a constituent of many proteins, such as catalytic enzymes and transcription factors. It is necessary for the activity of several enzymes involved in metabolic functions. In addition, many transcription factors that regulate DNA synthesis and RNA transcription require zinc to maintain the structural integrity (Gropper et al., 2005; Chasapis et al., 2012). In general, the normal range of zinc concentration in plasma or serum is 12-18 μM. However, these values can be lowered in the patients with zinc deficiency or many diseases, such as gastrointestinal disorders, alcoholism, chronic liver disease, renal disease, and others (Hajo, and Wolfgang, 2010). Zinc deficiency is related to pathological states, such as hair loss, growth retardation, delayed wound healing, impaired immune function and cancer development (Gropper et al., 2005).

Many studies suggested that the abnormal serum zinc levels in patients with several types of cancer were found. Serum zinc levels are reduced in patients with cancer of breast (Alam, and Kelleher, 2012), gallbladder (Gupta, Singh, and Shukla, 2005) and lung (Issell et al., 1981). It was found that dietary zinc deficiency is correlated with an increased risk of developing cancer and mortality (Leone et al., 2006; Epstein et al., 2011). Moreover, the association between zinc deficiency and an increased tumor size and progression of stage I and II in the patients with head and neck cancer was reported (Prasad et al., 1998). Based on these evidences, it is possible that abnormal serum zinc levels may play the role in the progression and severity of cancer.

Cancer is a leading cause of death worldwide. The major cause of cancer-related death is related with metastasis (Gupta, and Massagué, 2006). Cancer metastasis is a multistep biological process caused by detachment of cancer cells from the primary tumor to distant sites of the body. Metastatic cancer cells can survive and travel through the circulatory system followed by their adhesion to the target sites and establishment of the new tumors (Chaffer, and Weinberg, 2011; Hou et al., 2011). However, cancer metastasis has a barrier which is the cancer cells undergo a special type of apoptosis by loss of cell adhesion from the extracellular matrix (ECM), termed anoikis (Chiang, and Massagué, 2008; Paoli, Giannoni, and Chiarugi, 2013; Simpson, Anyiwe, and Schimmer, 2008). The regulation of anoikis is mediated by different pathways, which lead to the caspases activation, DNA fragmentation and cell death (Paoli et al., 2013).

Several evidence indicates that the anoikis process is triggered by the imbalance of the pro-apoptotic proteins such as Bax, Bid, Bim, and Bmf and the anti-apoptotic proteins such as Bcl-2, Bcl-XL and Mcl-1 in the Bcl-2 family (Cory, Huang, and Adams, 2003; Valentijn et al., 2003). In the absence of appropriate cell adhesion to ECM, pro-apoptotic Bax protein interacts and forms oligomers at the outer mitochondrial membrane (OMM). The oligomerization of Bax results in membrane permeabilization. Cytochrome c is released to the cytoplasm which finally regulated the activation of caspases (Chiarugi, and Giannoni, 2008; Paoli et al., 2013; Simpson et al., 2008). Moreover, lack of ECM-mediated cell contact stimulates anoikis by decreasing the pro-survival proteins, namely PI3K/Akt and ERK (Khwaja et al., 1997; Kennedy et al., 1999; Galante et al., 2009). Caveolin-1 (Cav-1) is an essential structural protein of caveolae in the plasma membrane. It has shown to be correlated with cancer progression and metastasis. The elevated Cav-1 levels promote survival and suppression of cancer cell anoikis by sustaining Akt activation (Li et al., 2003) and preventing Mcl-1 protein degradation (Chunhacha et al., 2012).

Recently, there has been increasing interest in effects of several natural products and nutrients on anti-cancer properties. Interestingly, natural products including curcumin (Pongrakhananon et al., 2010), imperatorin (Choochuay et al., 2013) and artonin E (Wongpankam et al., 2012) have been found to sensitize cancer cell anoikis via the decrease in anti-apoptotic proteins. In addition, the role of nutrients, especially vitamins and minerals, against cancer have been reported. It was found that

certain vitamin and mineral supplementations were related with decreased risk of cancer (Hercberg et al., 2004; Meyer et al., 2005). Among mineral supplementation, zinc showed the effect on cancer development (Epstein et al., 2011). The activity of zinc in cell culture model has been investigated. Recent study showed that zinc mediated apoptosis in prostate cancer cell via increased levels of Bax and decreased Bcl-2 expression (Ku et al., 2012). Although the anti-cancer activity of zinc was found through several mechanisms, the activity of zinc on cancer cell metastasis and related features are still unknown. The objective of this study was to investigate the effect of zinc on anoikis response in lung cancer cells. The information from this study may be beneficial for developing therapeutic strategies of cancer treatment and prevention.

1.2 Objectives of the Study

1) To investigate the effect of zinc on anoikis in non-small cell lung cancer H460 cells
2) To investigate the effect of zinc on the expression of anoikis related proteins, including Cav-1, pAkt/Akt, pERK/ERK, Mcl-1, Bcl-2, and Bax in non-small cell lung cancer H460 cells

1.3 Benefits of the Study

This study provides information on the role of zinc in mediating anoikis of cancer cells. The results of this study may be beneficial for further zinc use in developing therapeutic strategies to prevent cancer metastasis.

CHAPTER II
LITERATURE REVIEW

2.1 Zinc in Human Health

2.1.1 Role of zinc in human

Zinc is one of the most important micronutrients in the human body. In adult, the body generally contains approximately 2 to 4 grams of zinc, which is distributed throughout all organs, tissues and body fluids. Zinc is mainly stored in the brain, eyes, liver, kidneys, muscle, bones, and prostate. Zinc in food is in complex with amino acids which are part of peptides and proteins. The content of zinc in foods varies widely. Good sources of zinc are seafood, red meats, eggs, dairy products, grains, and cereals (Trumbo et al., 2001). According to the Dietary Reference Intake, the daily recommendations for zinc in adult men and women are set at 11 mg and 8 mg, respectively. The zinc recommendation during pregnancy or lactating women is 12 mg/day. The tolerable upper intake level of elemental zinc is 40 mg/day. The excessive zinc intake (225-450 mg of elemental zinc) can cause toxicity that leads to a metallic taste, nausea, vomiting, abdominal cramps, and bloody diarrhea (Gropper et al., 2005; Chasapis et al., 2012).

The sites of zinc absorption are mainly in the small intestine (duodenum, jejunum, and ileum). After zinc absorption, it may be used functionally, stored in the cell, or transported into the circulation for use by other tissues. Many compounds in

food may complex with zinc and inhibit its absorption, especially phytate, oxalate, fiber, and tannin. In addition, divalent cations and vitamins may inhibit zinc absorption and compete with zinc for binding ligands in the intestinal lumen and for transporters of the enterocytes (Ford, 2004; Tran et al., 2004). Zinc storage is found in all body organs as part of the protein thionein known as metallothionein. The concentrations of metallothionein in liver and red blood cell reflect the zinc status or stores. Zinc is mainly excreted through the gastrointestinal tract, kidneys and skin. The salivary glands, intestinal mucosa, liver, and pancreas are the major sources of endogenous zinc secreted into the gastrointestinal tract. Even though certain amount of zinc is reabsorbed, some also is excreted in the feces. In the kidneys, zinc filtered is reabsorbed by the tubules. A small amount of zinc is excreted in the urine. Other routes of zinc loss occur with exfoliation of skin and with sweating (Gropper et al., 2005).

The major biological roles of zinc are associated with the cellular homeostasis that control normal functions in human health. Zinc is a constituent of several proteins and requires for the activity of various enzymes. It is required for a structural integrity to the enzyme by binding directly to amino acid involved in the catalytic reaction. These enzymes are related with the metabolism of macronutrients. For example, carboxypeptidase A, a peptidase secreted by the pancreas to digest protein, is bound tightly with zinc to maintain its normal activity (Gropper et al., 2005).

Zinc plays an important role in physiological activities including cell growth, membrane stabilization, wound healing, cell-mediated immunity and generalized host defense. The role of zinc in cell growth is correlated with its potential in regulating protein synthesis. In gene transcription process, zinc is a necessary structural component of DNA-binding proteins or transcription factors known as zinc fingers. Several transcription factors that regulate gene expression appear to require zinc for maintaining structure and bind to DNA (Hajo, and Wolfgang, 2010). Furthermore, the essentiality of zinc on cell membrane is related to stabilization of membrane structure. The release of zinc from metallothionine is found to increase under oxidative stress condition. This process results in reduced cell membrane damage through scavenging free radical and maintaining a reduced state of phospholipids and thiol (SH) group (Chasapis et al., 2012).

2.1.2 Zinc deficiency and zinc supplementation

Zinc is essential in human health to maintain the normal physiological activities. Nowadays, zinc deficiency has been reported, and its prevalence was found in developing countries (Brown et al., 2002). It occurs as a result of malabsorption syndromes, liver cirrhosis, diabetes mellitus, renal diseases, hemolytic anemia, diarrhea, and malignancy. Moreover, zinc deficiency can be induced by inadequate zinc intake, high consumption of phytate, or increased losses of zinc (Wessells, and Brown, 2012). Clinical symptoms of zinc deficiency are associated with several

pathological states, such as skin change, delayed would healing, hair loss, growth retardation, brain development disorders, testicular atrophy, triggered arteriosclerosis, impaired immunity and development of cancer (Issell et al., 1981; Gupta et al., 2005). However, all of these symptoms may be reversible or preventable with zinc supplementation (Fortes et al., 1998; Bhandari et al., 2002; Prasad et al., 2008). Zinc supplementation is found in several forms, such as zinc oxide, zinc sulfate, zinc chloride, zinc gluconate, and zinc acetate. The amount of elemental zinc in each form is different. For example, the percentages of elemental zinc in zinc chloride, zinc sulfate and zinc gluconate are 48, 23 and 14.3, respectively (Gropper et al., 2005).

Zinc deficiency has been reported to be correlated with many chronic illnesses, such as diabetes mellitus (Chasapis et al., 2012). It was found that serum zinc levels in both type-1 and type-2 diabetic patients were significantly lower than those in healthy controls. Moreover, zinc supplementation in type-2 diabetic patients resulted in elevated serum zinc levels and reduced HbA1c (Al-Maroof, and Al-Sharbatti, 2006). Lack of zinc can lead to delayed wound healing. Lansdown et al. (2007) found that patients with chronic leg ulcers had low serum zinc levels and abnormal zinc metabolism. Moreover, the authors also suggested that zinc sulfate might be beneficial in leg ulcer treatment, and the topical administration of zinc appeared to be superior to oral supplementation because of its potential in decreasing superinfections and necrotic material.

Many researchers have suggested that zinc and other antioxidant minerals prevent cellular damage in the retina. These may delay the progression of age-related macular degeneration (Age-Related Eye Disease Study Research Group, 2001; Evans, and Lawrenson, 2012). Moreover, zinc deficiency may lead to complications in pregnant women, such as risk of premature labor and miscarriages, inhibited fetal brain development, lowered birth weight, and decreased immunity of both mother and baby (Shah, and Sachdev, 2001). Deficiency of zinc in children may affect the growth retardation, intellectual development, and reproductive system. Nevertheless, zinc supplementation is a successful management of these illnesses (Brown et al., 2002). Furthermore, zinc supplementation has been very beneficial in patients with Wilson's disease due to its ability to compete with copper for binding sites (Czlonkowska, Gajda, and Rodo, 1996). Brewer et al. (1998) stated that zinc acetate has the effectiveness in the long-term treatment of Wilson's disease patients.

For immune system, Fortes et al. (1998) reported that zinc supplementation enhanced the cell-mediated immune response in healthy older population. Many studies suggested that the deficiency of zinc was a risk factor for susceptibility to diarrhea, pneumonia and other infection in children and elderly in developing countries (Girodon et al., 1997; Bahl et al., 1998; Bhutta et al., 2000; Brooks et al., 2005; Meydani et al., 2007). A randomized placebo-controlled trial conducted in New Delhi, India found that zinc supplementation (10-20 mg/day of elemental zinc) for 4 months in children aged 6 to 30 months decreased the incidence of severe and prolonged

diarrhea (Bhandari et al., 2002). In addition, the result of the study in elderly population found that normal serum zinc in elderly was related to a reduced number of antibiotic prescriptions, and a lower incidence and duration of pneumonia (Meydani et al., 2007).

The association between zinc supplementation and common cold treatment has been investigated. In a randomized placebo-controlled trial, the duration and severity of cold symptoms in 50 subjects with common cold who received a zinc acetate lozenge (13.3 mg of zinc) every 2-3 h while awake for as long as they had cold symptoms were significantly reduced compared with those in controls (Prasad, 2008). In addition, several researchers suggested the beneficial effects of zinc lozenges, nasal sprays, and nasal gels on duration and severity of cold symptoms (Turner, and Cetnarowski, 2000; Eby, and Halcomb, 2006; Singh, and Das, 2011).

2.2 Cancer

Cancer is a genetic disease in which abnormal cells aggressively proliferate without control. Cancer cells can invade the surrounding tissues and spread to other distant organs. The major types of cancer include lung cancer, breast cancer, prostate cancer, colon cancer, and lymphoma. Cancer has become a major public health problem worldwide with approximately 8.2 million cancer related deaths in 2012 (Siegel, Naishadham, and Jemal, 2013). According to the Bureau of Health Policy and Strategy, Ministry of Public Health Thailand, cancer is the first leading cause of death in 2012, with 98.5 per 100,000 population rates of death (Figure 1) (Ministry of Public

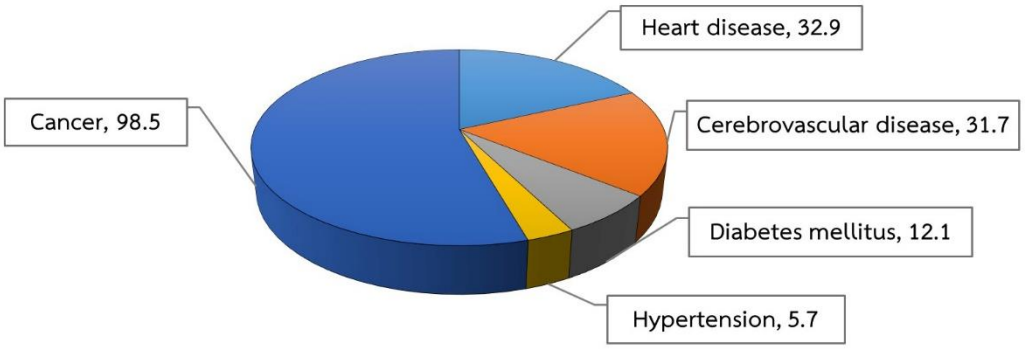

Figure 1 Death rates of leading causes of death in Thailand, 2012 (Ministry of Public Health, 2012)

Health, 2012). The most life-threatening pathological events and over 90 percent of the clinical death of cancer patients are associated with metastasis (Chaffer, and Weinberg, 2011).

2.2.1 Origins of cancer

Human normal cells generally grow and divide to produce new cells in order to keep the normal function. When the body found damaged cells, the programmed cell death is stimulated resulting in cell death and the news cells were produced. However, sometimes the regulation of this process is abnormal, and the genetic materials of cells such as deoxyribonucleic acid (DNA) become damaged or changed, producing mutations. Under normal condition, the normal cells have an ability to repaired or eliminate the damaged DNA. In contrast, the cancer cells are not repaired but they have the same damaged DNA in the new cells. Abnormal cells that

lose control of their normal growth and replication processes form a mass of tissue called tumor. Benign tumors are not cancerous. They could be removed and do not spread to other organs. In contrast, malignant tumors can invade nearby tissues and disseminate through other organs (Bertram, 2000; Steeg, 2006). The capabilities of cancer to develop the malignant growth have been shown through various mechanisms, for example, self-sufficiency in growth signals, resistance of programmed cell death, insensitivity to antigrowth signals, unlimited replication, sustained angiogenesis, and tissue invasion and metastasis (Hanahan, and Weinberg, 2000).

2.2.2 Causes and treatments of cancer

Causes of cancer involve approaching exogenous and endogenous factors for a long period of time. There are different types of exogenous factors, such as tobacco, infectious organisms, radiation, industrial chemicals, and carcinogenic agents in food that lead to cancer development. Several endogenous factors are also found to be associated with cancer, such as inherited genetic mutations, oxidative stress, inflammation, hormones, and immune conditions. Cancer treatment depends on type and stage of cancer, age, and health status of the patients. Cancer patients generally receive a combination of therapies and palliative care. Treatments include surgery, chemotherapy, radiation, immunotherapy, hormone therapy, and targeted therapy (Souhami, and Tobias, 1995; Bertram, 2000).

2.2.3 Types of cancer

Cancer is a group of diseases that affects many different tissues and types of cells. They can be grouped by their tissues of origin. The most common type of cancer is carcinomas formed by epithelial cells that cover the inside and outside surfaces of the body. Subtypes of carcinomas include adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma. Cancers that are originated in bone, cartilage, blood vessels, muscle, or other connective or supportive tissue are called sarcomas. In addition, cancers that generate in blood-forming tissue of the bone marrow are termed leukemias. These cancer types do not form solid tumors, but large numbers of abnormal white blood cells are enhanced in the blood and bone marrow. Lymphomas are cancers that begin in lymphocytes (T cells or B cells) initiated in lymph nodes and tissues in immune system. Adenomas are cancers that arise in pituitary gland, thyroid, adrenal gland, and other glandular tissues (Souhami, and Tobias, 1995).

2.2.4 Metastasis

Cancer metastasis is a complicated multistep process caused by dissemination of cancer cells from their primary sites to secondary sites. The steps of metastasis are followed by the cancer cell detachment from extracellular matrix (ECM) or neighboring cells, migration, invasion, and extravasation to the circulatory system. Metastatic cells can survive and travel through the circulatory system followed by their

adhesion to the target sites in order to initiate new tumors (Figure 2). To obtain the oxygen and nutrients for tumor growth, cancer cells have a potential to stimulate the formation of new blood vessels, called angiogenesis (Steeg, 2006). The ability of cancer cells to metastasize depends on their individual properties, such as the original location, the ability to penetrate the walls of lymphatic or blood vessels, and the properties of the host defense mechanism. The main sites of cancer metastasis are lung, liver, brain, and bone. Malignancies, which can result in secondary deposits in these organs, include lung, breast, prostate, and melanoma (Gabriel, 2007). The type and frequency of the symptoms in patients with metastasis are different. It depends on the size and location of secondary tumors. For example, brain metastasis can cause a variety of symptoms, such as nausea and vomiting, memory loss, personality and behavioral changes, and seizure while bone metastasis likely causes pain and bone fractures. Treatments of metastasis can be treated with systemic therapy, surgery, radiation therapy, or a combination therapy (Chiang, and Massagué, 2008). Since metastasis remains the major cause of mortality in cancer patients, the knowledge regarding the mechanisms of metastasis and tumor progression are important for design the new therapeutic approaches to reduce mortality (Chiarugi, and Giannoni, 2008; Chaffer, and Weinberg, 2011).

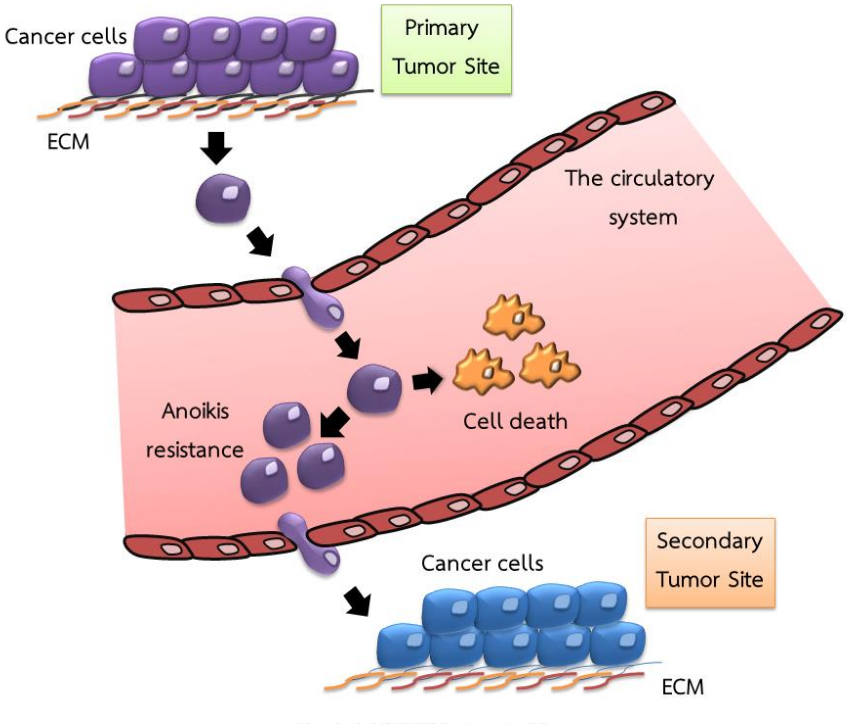

Figure 2 The steps of metastasis

2.2.5 Anoikis process

Anoikis is an apoptotic process initiated by losing contact from the ECM or neighboring cells due to the lack of the normal survival signal regulation. The execution of anoikis can be driven by various pathways, which merge into caspases activation, DNA condensation and cell death (Chiarugi, and Giannoni, 2008; Paoli et al., 2013). In general, both normal and cancer cells including epithelial cells and solid tumor cells must receive adhesion-dependent signals from the interaction between cell adhesion molecules, namely integrin and ECM to survive. Activation of these survival signals can activate pro-survival proteins, such as phosphoinositide 3-kinase (PI3K)/Akt and ERK and stimulate the expressions of anti-apoptotic proteins in Bcl-2 family, whereas the expressions of pro-apoptotic proteins are suppressed (Khwaja et al., 1997; Galante et

al., 2009) (Figure 3). This survival activation results in cell survival. However, when the cells detach from ECM, they lose their normal survival signals from integrin-ECM adhesion resulting in anoikis induction and cell death (Chiarugi, and Giannoni, 2008; Paoli et al., 2013).

During anoikis process, death of the detached cells is stimulated. Expression of survival proteins, namely Akt and ERK proteins is inhibited. The inhibition of survival protein expression leads to decreased activity of anti-apoptotic proteins and increased activity of pro-apoptotic proteins. Pro-apoptotic proteins form oligomers in the outer mitochondrial membrane (OMM), creating channels and thus causing its permeabilization. Disruption of OMM results in the release of cytochrome c. Then, cytochrome c induces apoptosome complex formation of caspase-9 and the apoptosis protease activating factor (APAF), resulting in the effector caspase-3 activation and anoikis promotion as shown in Figure 4 (Chiarugi, and Giannoni, 2008). Generally, after loss of cell adhesion, low aggressive cancer cells undergo anoikis, whereas metastatic cancer cells tend to resist anokis by activating survival signaling or inhibiting apoptotic pathway, which are called anoikis resistance (Simpson et al., 2008; Paoli et al., 2013). Caveolin-1 (Cav-1), a structural protein of caveolae in the plasma membrane was found to play a role in anoikis resistance. Many studies suggested that the up-regulation of Cav-1 promoted cell survival and prevent anoikis of cancer cells through sustaining survival protein activation and preventable anti-apoptotic protein degradation (Figure 5) (Li et al., 2003; Chunhacha et al., 2012).

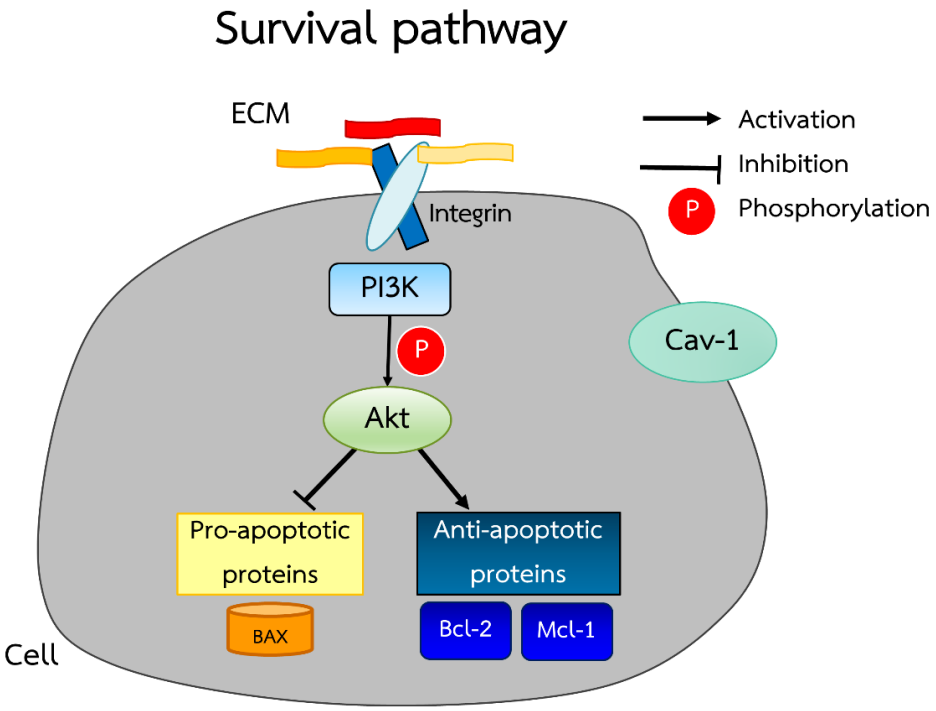

Figure 3 Activation of survival pathway in both normal and cancer cells

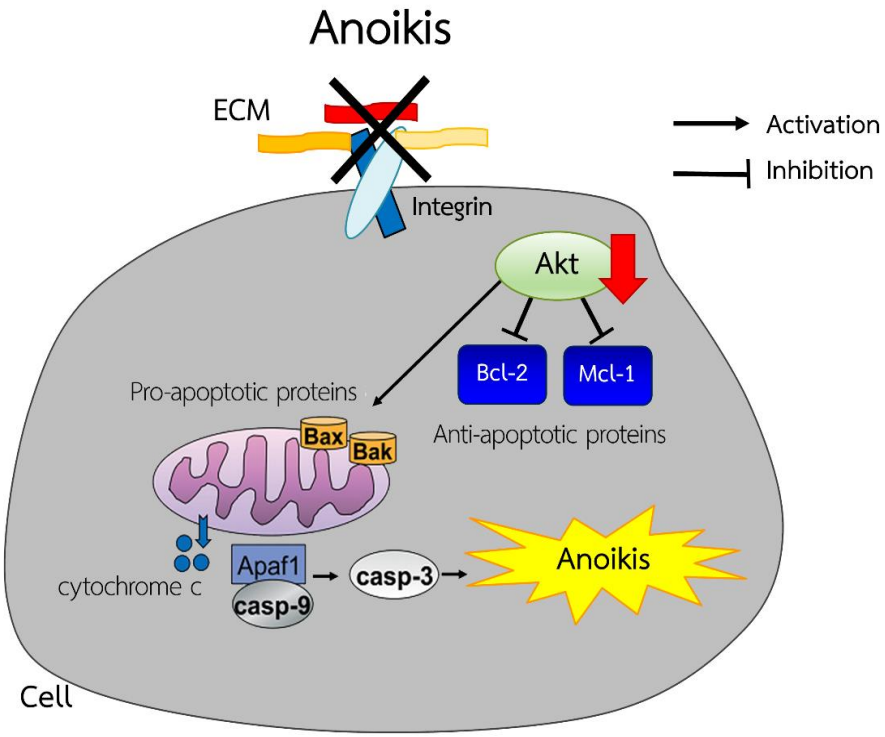

Figure 4 Activation of anoikis process

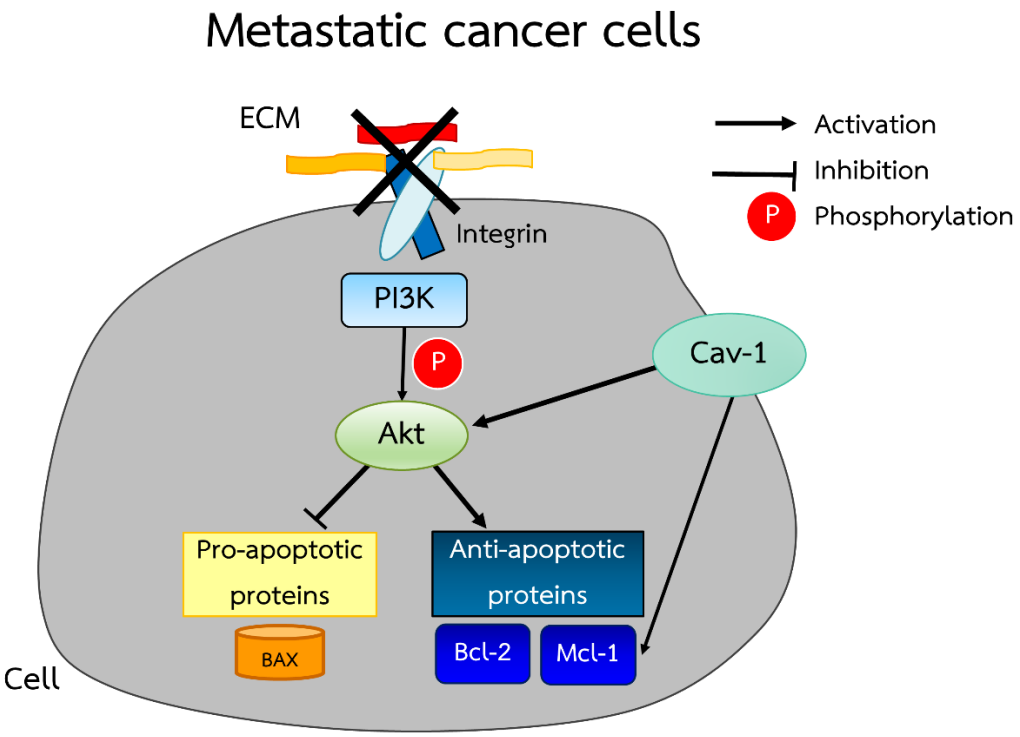

Figure 5 Anoikis resistance of metastatic cancer cells

2.2.5.1 Role of anoikis-associated proteins

2.2.5.1.1 Bcl-2 family proteins

The Bcl-2 family proteins are the key in anoikis process. They can be divided into three groups depending on their functions. The first group is the anti-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2), Bcl-XL and myeloid cell leukemia sequence 1 (Mcl-1). The other two groups are pro-apoptotic proteins including the multi-domain pro-apoptotic proteins (Bax, Bak, and Bok) and the pro-apoptotic BH3-only proteins (Bid, Bad, Bim, Bik, Bmf, Noxa, Puma, and Hrk) (Cory et al., 2003). The anti-apoptotic Bcl-2 family proteins prevent cell death by forming heterodimer with pro-apoptotic Bax protein, and thus inhibiting the oligomerization of Bax and Bak at OMM. Therefore, mitochondrial membrane integrity can be maintained and pore formation can be prevented. In contrast, the increase in pro-apoptotic BH3-only proteins can induce anoikis by inactivating the function of Bcl-2, thus freeing the pro-apoptotic BH3-only protein to stimulate the formation of Bax-Bak oligomer (Chiarugi, and Giannoni, 2008).

After cell detachment, survival protein is activated, which subsequently alters the levels of pro-apoptotic proteins and anti-apoptotic proteins in Bcl-2 family (Placzek et al., 2010). The increased levels of pro-apoptotic proteins, such as Bax and Bak have been found during anoikis process (Rosen et al., 1998; Liu et al., 2006; Owens et al., 2009). Bcl-2 overexpression has been shown to prevent anoikis in

suspended cancer cell lines (Pongrakhananon et al., 2010). Likewise, an anti-apoptotic Mcl-1 protein may also determine the sensitivity of cells to anoikis. The increased level of endogenous or overexpression of Mcl-1 was shown to inhibit anoikis response in many cancers (Woods et al., 2007; Boisvert-Adamo et al., 2009).

2.2.5.1.2 Pro-survival proteins

Several kinase/phosphatase signaling molecules have been implicated in anoikis as central regulators. Protein kinase B (Akt) is serine/threonine kinase and a major signal transducer downstream of activated phosphoinositide 3-kinase (PI3K). The PI3K/Akt pathway is an critical driver of cell proliferation, cell growth and cell survival (Dummler, and Hemmings, 2007). Akt activation enhances cell survival via several mechanisms, such as phosphorylating the pro-apoptotic protein Bad in order to inhibit cytochrome c release from mitochondria (Khwaja et al., 1997), stimulating the anti-apoptotic Bcl-2 protein release (Datta et al., 2000) and preventing the caspase-9 activation (Cardone et al., 1998). Many studies suggested that PI3K/Akt signaling pathway activation resulted in anoikis resistance (Khwaja et al., 1997; Davies et al., 1999; Sakamoto, and Kyprianou, 2010). Similarly, Diaz-Montero, Wygant, and McIntyre, (2006) reported that activated Akt overexpression was found in anoikis-resistant human osteosarcoma cells. In neoplastic cells, the activated PI3K/Akt pathway inhibited proteasomal degradation of Mcl-1, then Bim expression could not activate Bax (Woods et al., 2007). Moreover, in anoikis condition of human mammary

epithelial (HMEC) cells and MCF 10A cells, the up-regulation of Akt suppressed the expression of Bmf (Schmelzle et al., 2007).

2.2.5.1.3 Caveolin-1 (Cav-1)

Caveolin (Cav-1) is an essential structural protein component of the plasma membrane, termed caveolae. It plays an important role in cellular processes, including cell signaling and cell transport (Razani, Woodman, and Lisanti, 2002; Parton, and Simons, 2007). Various studies indicated that up-regulation of Cav-1 was correlated with an increased metastatic potential and mortality rate in cancer patients (Yang et al., 1998; Kato et al., 2002; Joo et al., 2004). The high level of Cav-1 was found in numerous multidrug resistant cancer cells, such as adriamycin-resistant MCF-7 breast adenocarcinoma cells, HT-29-MDR colon carcinoma cells, vinblastine-resistant SKVLB1 ovarian carcinoma cell, and taxol-resistant A549-T24 lung carcinoma cells (Lavie, Fiucci, and Liscovitch, 1998; Yang et al., 1998). Nowadays, the role of Cav-1 on anoikis resistance is widely interested. Elevated expression of Cav-1 increased cell survival and clonal growth under serum-free condition in prostate cancer cells (Tahir et al., 2001). Likewise, in human breast cancer MCF-7 cells, overexpression of Cav-1 was found to increase cell survival in anchorage-independent growth condition (Fiucci et al., 2002). The relationship of Cav-1 and Akt survival protein has been investigated. Li et al. (2003) stated that the up-regulation of Cav-1 could promote prostate cancer cell survival by sustaining Akt level. Furthermore, Cav-1 overexpression can prevent

Mcl-1 degradation through the ubiquitin-proteasome pathway causing anoikis resistance in lung cancer cell (Chunhacha et al., 2012).

2.3 Role of zinc in cancer

It has been known that zinc is a necessary component to maintain normal function of cells. The relationships of zinc and clinical conditions including cancer have been investigated. Epidemiological studies found that zinc deficiency was related to an increased risk of cancer development and mortality (Leone et al., 2006; Epstein et al., 2011). Generally, the normal range of zinc concentration in plasma is 12-18 μM (78-117 μg/dL). However, these values can be lowered in the patients with zinc deficiency or other diseases including cancer (Hajo, and Wolfgang, 2010). The levels of zinc in serum and malignant tissues of patients with various types of cancer are different. Interestingly, the serum zinc levels are reduced in patients with cancer of breast, gallbladder, and lung (Alam, and Kelleher, 2012; Gupta et al., 2005; Issell et al., 1981). Moreover, zinc deficiency was associated with increased tumor size and overall stage of the head and neck cancer patients (Prasad et al., 1998).

Both innate and adaptive immunity are main mechanisms to eliminate cancer cells in human body. Researchers suggested that zinc plays an important role in normal development and functions of neutrophils, macrophages, natural-killer cells, and cell-mediated immune response (Prasad, 2009; John et al., 2010). Zinc deficiency affects the functions of phagocytosis, intracellular killing and cytokine generation. Many

studies indicated that zinc deficiency caused thymic and lymphoid tissue atrophy and lead to reduced antibody and cell-mediated responses in both animals and humans (Beck et al., 1997; Fraker et al., 2000; Prasad, 2008). Zinc deficiency suppressed the function of T and B lymphocytes and impaired cell-mediated immune response in zinc-deficient mice (Shankar, and Prasad, 1998). Doerr et al. (1998) demonstrated that nearly 65% of head and neck cancer patients were zinc deficient based on the concentration of zinc in lymphocytes and granulocytes. In addition, these patients were found to have impaired T helper 1 cell function and decreased NK cell activity.

In an experimental human model, the healthy male subjects aged 20-45 years received a hospital diet containing zinc in the dose of 12 mg/day for 4 weeks, and then they were given a zinc-restricted diet (zinc 3-5 mg/day) for 28 weeks. The result showed that the levels of zinc in lymphocytes and granulocytes of the subjects who received 3 mg of dietary zinc during the zinc-restricted period were decreased. Moreover, the production of interferon (IFN)-γ, interleukin (IL)-2, tumor necrosis factor (TNF)-α generation, and NK-cell activity were significantly declined (Prasad, 2000). It has been known that TNF-α is an essential pro-inflammatory cytokine playing a role in the defense mechanism against cancer. The association between zinc and TNF-α has been investigated. The patients with type-2 diabetes mellitus and metabolic syndrome were supplemented with zinc sulfate (30 mg/day of elemental zinc) for 8 week compared with placebo group. The result showed that zinc-supplemented patients had

significantly increased transmembrane TNF-α expression on monocytes (Meksawan, Sermsri, and Chanvorachote, 2014). Based on this research, it is possible that zinc supplementation may be beneficial in the decreased risk of cancer.

Oxidative stress is a critical factor involved with several chronic diseases including cancer. Zinc also has an ability to protect the oxidation damage by decreasing the production of reactive oxygen species (ROS) and the inflammatory cytokines (Ho et al., 2001; Uzzo et al., 2002; Prasad, 2008). Nowadays, there has been growing interest in effect of zinc in terms of anti-cancer activity. Zinc has been shown to suppress the invasive abilities of prostate cancer cells (Ishii et al., 2004). Moreover, Ku et al. (2012) demonstrated that zinc mediated prostate cancer cell apoptosis via the induction of Bax and the decrease of Bcl-2 proteins. However, there is no study investigating the role of zinc in anoikis of cancer cells.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell culture

The human non-small cell lung cancer H460 cells, derived from the pleural fluid of lung, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human keratinocyte HaCaT cells were obtained from Cell Lines Service (Heidelberg, Germany). H460 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI), while HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All medium were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin and streptomycin (Gibco, Gaitherburg, MA, USA). Cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C.

3.1.2 Reagents

Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, propidium iodide (PI), dimethy sulfoxide (DMSO), poly-2-hydroxylethylmethacrylate (poly-HEMA) phosphate buffered saline (PBS) and agarose were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibody for pAkt, Akt, pERK, ERK, Cav-1, Bcl-2, Bax, Mcl-1, and β-actin and anti-rabbit secondary antibody were obtained from Cell Signaling (Danvers, MA, USA).

3.2 Experimental design

The study was designed as shown in Figure 6. Zinc sulfate is widely use as zinc supplementation; therefore, this form was used in the study. The concentrations of zinc sulfate were prepared in various concentrations which were equivalent to elemental zinc of 0 to 150 μM (0-975 μg/dL). To eradicate the direct effect of zinc, the cytotoxic effects of zinc on non-small cell lung cancer H460 cells in attached condition were investigated. Cells were treated with zinc at the concentrations of 0, 1, 5, 10, 25, 50, 100, and 150 μM at 37°C for 24 h, and cell survival was determined by MTT assay. To clarify the safety of zinc in normal human cells, the cytotoxicity in normal keratinocyte HaCaT cells were investigated. HaCaT cells were incubated with various concentrations of zinc (0, 1, 5, 10, 25, 50, 100, and 150 μM) at 37 °C for 24 h, and cell survival was examined by MTT assay. Non-toxic concentrations of zinc were used in further experiments.

To determine the effects of zinc on H460 cells in detached condition, cells were detached into a single-cell suspension in RPMI serum free medium and seeded in poly-HEMA-coated plates. Suspended cells were treated with non-toxic concentrations of zinc and cell survival was examined by anoikis assay. To investigate mode of cell death in response to zinc treatment, apoptotic cell death was detected by nuclear staining assay. Cells were incubated with zinc for various times and then stained with Hoechst 33342 and PI. The apoptotic cells having condensed chromatin

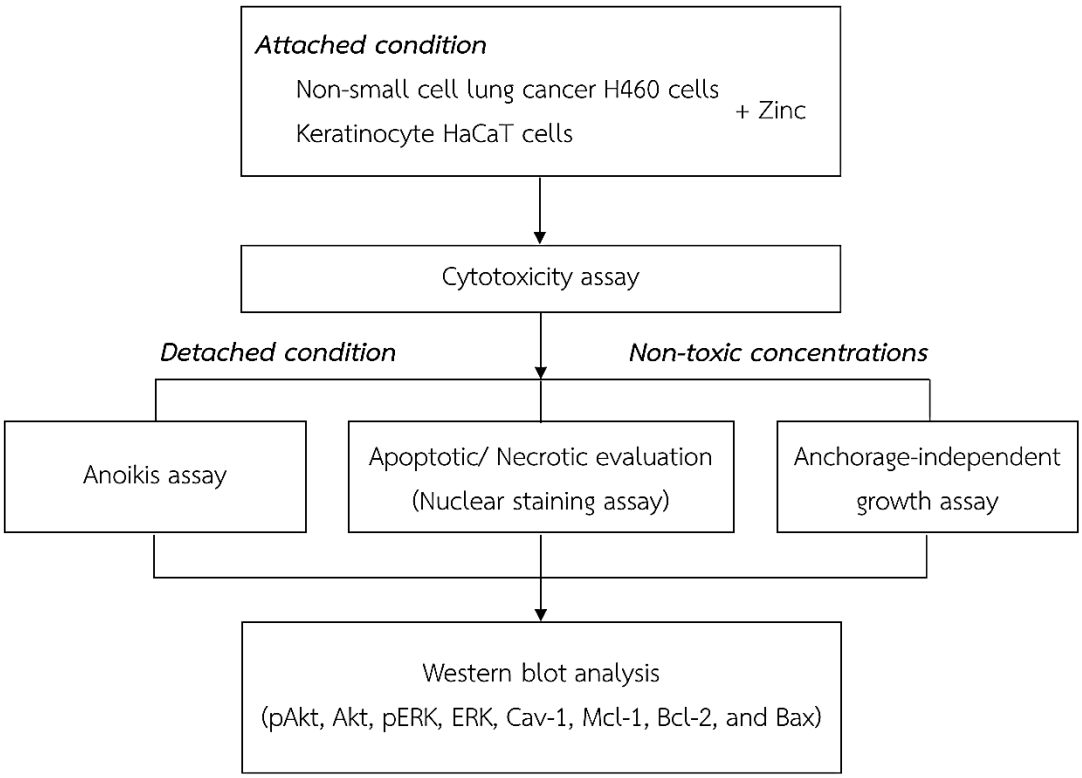

Figure 6 Experimental design of the study

and/or fragmented nuclei were stained by Hoechst, and only necrotic cells were stained by PI.

The effect of zinc on cell growth in detached condition was examined by anchorage-independent growth assay. Cells were suspended in RPMI complete medium with zinc and 0.33% agarose. After 2 weeks of incubation, colony number and colony size were visualized and scored under a light microscope. To further evaluate the mechanism by which zinc sensitizes H460 cells anoikis, the levels of anoikis regulatory proteins were determined by western blot analysis. Cells were seeded in poly-HEMA-coated plates and treated with non-toxic concentrations of zinc. After

specific treatments, cells were subjected to perform western blot analysis and evaluated the expression of anoikis-associated proteins.

3.3 Methods

3.3.1 Sample preparation

To investigate the effect of zinc on H460 cells, zinc sulfate was dissolved in phosphate buffer saline (PBS), as described by Ku et al. (2012) to prepare zinc concentrations ranging from 0 to 150 μM.

3.3.2 Cytotoxicity assay

Cell viability was determined by MTT assay. To measure cell density, H460 and HaCaT cells were counted by automated cell counter (Bio-Rad, USA) and adjusted density to $1x10^5$ cells/mL. Then, the cells were seeded in 96-well plate and incubated with various concentrations of zinc at 37 ℃ for 24 h. The treated cells were added with 100 μL of MTT (0.4 mg/mL) at 37 ℃ for 4 h. Then, the MTT solution was removed, and 100 μL of DMSO was added to dissolve the formazan crystal giving a purple color. The optical density (OD) of MTT product was measured at 570 nm using a microplate reader (Wallac Model 1420, USA). As viable cells can convert yellow MTT solution to purple formazan by mitochondrial reductase, the absorbance of formazan solution is referred to amount of living cells. The OD ratio of treated and the non-treated control

value was calculated and presented as the percentage of viable cells (Freshney, 2010b).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

3.3.3 Anoikis assay

Six-well tissue culture plates were coated with 200 µL of poly-HEMA (6 mg/mL in 95% ethanol) and left to evaporate overnight in a laminar flow hood at room temperature. H460 cells were pre-treated with non-toxic concentrations of zinc for 24 h at 37 ℃. Then the cells were trypsinized into a single cell suspended in the RPMI serum free medium and seeded in poly-HEMA-coated plates at the density of $1x10^5$ cells/mL. The detached cells were treated with non-toxic concentrations of zinc and incubated at 37 ℃ for various times. After specific times of incubation, the cell survival was determined by MTT assay, following the protocol of Pongrakhananon et al. (2010). Briefly, cells were seeded in 96-well plate and incubated with 100 µL of MTT (0.4 mg/ml) for 4 h at 37 ℃. The absorbance of MTT product was measured at 570 nm using a microplate reader. The OD ratio of treated and the non-treated control value was calculated and presented as the percentage of viable cells.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

3.3.4 Nuclear staining assay

Apoptosis and necrosis were investigated by Hoechst 33342 and PI co-staining (Freshney, 2010a). Hoechst 33342 dye is a cell permeable nucleic acid stain that emits blue fluorescence when bound to dsDNA. The bright red fluorescence PI dye, on the other hand, is excluded from live and apoptotic cells and is incorporated into necrotic cells where membrane integrity is lost. After pre-treatment with zinc at non-toxic concentrations for 24 h, H460 cells ($1x10^5$ cells/mL) were seeded in poly-HEMA-coated plates and treated with non-toxic concentrations of zinc at 37 ℃ for various times. After specific treatment, cells were stained with 10 μM of Hoechst 33342 and 5 μg/ml of PI for 30 min at 37 ℃ and analyzed under a fluorescence microscope using blue filter for Hoechst 33342 and red filter for PI. The apoptotic cells having condensed chromatin and/or fragmented nuclei were stained by Hoechst, while the DNA of membrane damaged cells were stained by PI and were considered as necrotic cells. Fluorescence in the cells was visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

$$\text{Apoptotic cells (\%)} = \frac{\text{Apoptotic cells}}{\text{Total cell number}} \times 100$$

$$\text{Necrotic cells (\%)} = \frac{\text{Necrotic cells}}{\text{Total cell number}} \times 100$$

3.3.5 Anchorage-independent growth assay

The anchorage-independent cell growth was examined by the colony formation assay in soft agar, as described by Koleske, Baltimore and Lisanti (1995). H460 cells were suspended in RPMI containing 10% FBS and 0.33% low melting temperature agarose and treated with zinc at the various concentrations, and then 250 µL of zinc-treated H460 cells ($1x10^3$ cells/mL) were plated in a 24-well plate over a 500 µL layer of solidified RPMI with 10% FBS and 0.6% agarose. The cells were fed every 3 days with 250 µL of completed RPMI media containing various concentration of zinc. The survival colonies were visualized and scored under a light microscope (Olympus IX51 with DP70) after 2 weeks of zinc treatment at 37 °C.

$$\text{Relative colony number} = \frac{\text{Colony number of treatment}}{\text{Colony number of control}}$$

$$\text{Relative colony size} = \frac{\text{Diameter of treatment}}{\text{Diameter of control}}$$

3.3.6 Western blot analysis

The expressions of proteins associating anoikis process (pAkt, Akt, pERK, ERK, Cav-1, Mcl-1, Bcl-2, and Bax) were determined by Western blot analysis (Burnette, 1981). After specific treatments, the cells were incubated in lysis buffer containing 20 mM Tris-HCL (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and commercial protease inhibitor mixture (Roche Molecular Biochemicals,

Basel, Switzerland) at 4 °C for 30 min. Cell lysates were collected and determined for protein content using the BSA protein assay kit (Pierce, Rockford, USA). Proteins of each sample were denatured by heating at 95 °C for 5 min with Laemmli loading buffer and subsequently loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred onto nitrocellulose membranes (Bio-Rad, USA). The membranes were blocked in 5% skim milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM sodium chloride, 0.1% Tween 20) for 1 h at room temperature, and then incubated with appropriate primary antibodies at 4 °C overnight. Membranes were washed three times with TBST for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The immune complexes were detected by chemiluminescence (SuperSignal West Pico; Pierce, Rockford, USA) and quantified using the densitometry software (ImageJ software, National Institute of Health, USA). ß-actin was used as a loading control in each treatment.

3.3.7 Statistical analysis

All data are presented as the means ± the standard error of mean (SEM) of independent triplicate experiments and normalized with the non-treated control. Differences between groups were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test at a significant level of $p < 0.05$.

CHAPTER IV
RESULTS

4.1 Cytotoxic effect of zinc on non-small cell lung cancer H460 cells and keratinocyte HaCaT cells in attached condition

The cytotoxic effect of zinc in non-small cell lung cancer H460 cells and keratinocyte HaCaT cells were examined by MTT assay. In attached condition, cells were treated with zinc at the concentrations of 0, 1, 5, 10, 25, 50, 100, and 150 μM at 37°C for 24 h, and cell survival was determined. Figure 7A showed that significant cytotoxic effects of zinc in H460 cells were found at the concentrations of 100 and 150 μM with the approximately 77% and 75% of cells remaining viable, respectively (Table 1). There were no significant effects on H460 cells viability at the concentrations less than 100 μM. Moreover, to clarify the safety of zinc in normal human cells, the cytotoxicity in normal keratinocyte HaCaT cells were investigated. HaCaT cells were incubated with various concentrations of zinc (0, 1, 5, 10, 25, 50, 100, and 150 μM). After 24 h, the results showed no significant change in cell viability in normal keratinocyte cells treated with zinc (Figure 7B, Table 2). These results suggested that the non-toxic concentrations of zinc in H460 cells were less than 100 μM and these concentration ranges would be used in further experiments.

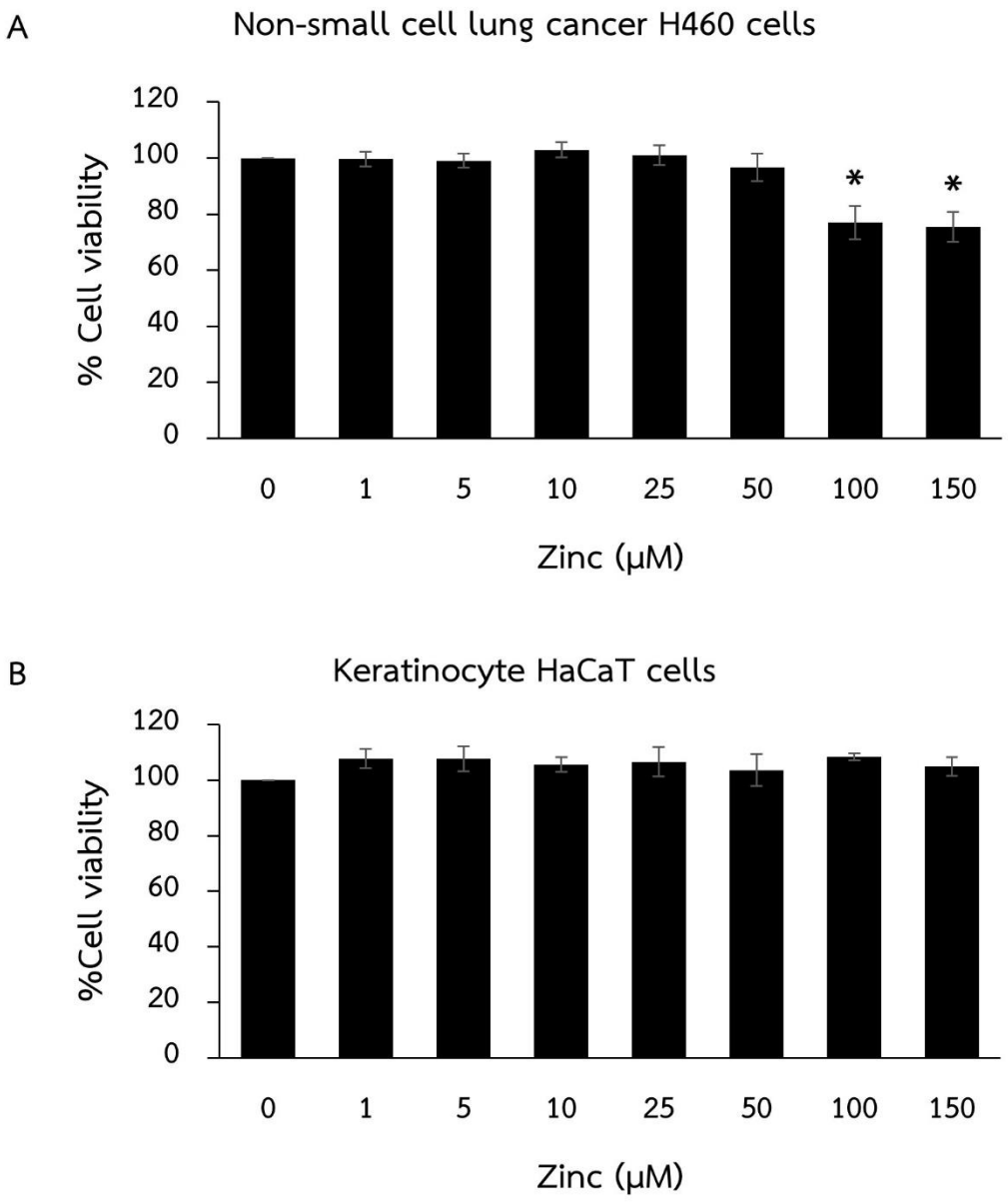

Figure 7 Cytotoxic effect of zinc on (A) non-small cell lung cancer H460 cells and (B) keratinocyte HaCaT cells in attached condition

Table 1 The percentage of H460 cell viability in attached condition determined by MTT assay after treatment with various concentration of zinc

Zinc (μM)	% Cell viability
0	100.00 ± 0.00
1	99.59 ± 2.61
5	99.05 ± 2.58
10	102.95 ± 2.78
25	100.96 ± 3.52
50	96.72 ± 4.93
100	77.01 ± 5.93*
150	75.49 ± 5.38*

The data are presented as mean ± SEM of independent triplicate experiments. *$p < 0.05$ versus non-treated control

Table 2 The percentage of HaCaT cell viability in attached condition determined by MTT assay after treatment with various concentration of zinc

Zinc (μM)	% Cell viability
0	100.00 ± 0.00
1	107.77 ± 3.46
5	107.62 ± 4.53
10	105.56 ± 2.63
25	106.59 ± 5.26
50	103.62 ± 5.72
100	108.33 ± 1.36
150	104.88 ± 3.29

The data are presented as mean ± SEM of independent triplicate experiments. *$p < 0.05$ versus non-treated control

4.2 Effect of zinc on anoikis in non-small cell lung cancer H460 cells

To study the effect of zinc on cell anoikis, H460 cells were pre-treated with zinc at non-toxic concentrations (0, 5, 10, 25, and 50 μM) at 37 °C for 24 h. Then the cells were trypsinized into single cell and cultured in the poly-HEMA-coated plates containing non-toxic concentration of zinc. Cell survival was observed at 0, 3, 6, 9, and 12 h after detachment by MTT assay. Interestingly, the detached cells treated with zinc were reduced in a dose-dependent manner. Figure 8 showed that after detachment, viability of H460 cells decreased over time, and a significant reduction of live cell was first detected at 3 h after cell detachment at the concentrations of 10, 25 and 50 μM. With 5 μM of zinc, the number of viable H460 cells decreased to approximately 51%, 50%, and 38% of viable cells in control condition at 6, 9, and 12 h of detached condition, respectively. The results showed that 10 μM of zinc could reduce the number of detached cells to approximately 58%, 48%, 39%, and 29% of viable cells in control condition at 3, 6, 9, and 12 h after detachment, respectively, while 25 μM of zinc could decrease the survival of detached cells to approximately 51%, 43%, 35%, and 28% of viable cells in control condition at 3, 6, 9, and 12 h after detachment, respectively. In addition, zinc at concentration of 50 μM could reduce viable cells at 3, 6, 9, and 12 h after detachment to approximately 43%, 36%, 29%, and 24% of viable cells in control condition, respectively (Table 3).

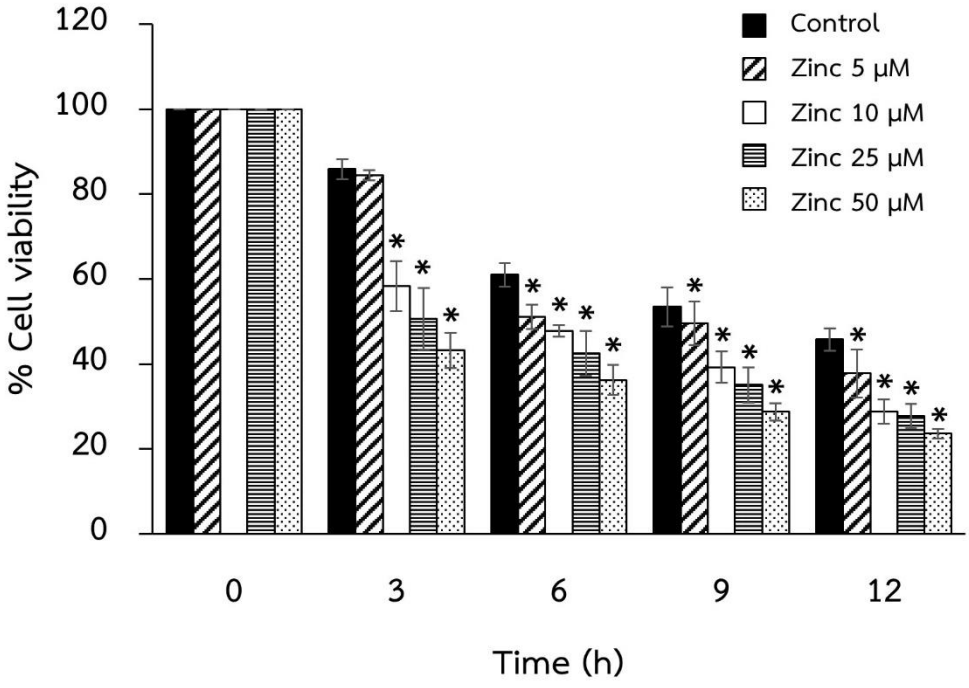

Figure 8 Effect of zinc on cell viability of H460 cells in detached condition

Table 3 The percentage of H460 cell viability in detached condition determined by MTT assay after treatment with non-toxic concentrations of zinc

Zinc (μM)	**% Cell viability**				
	0 h	**3 h**	**6 h**	**9 h**	**12 h**
0	100.00 ± 0.00	85.88 ± 2.29	61.05 ± 2.80	53.47 ± 4.57	45.82 ± 2.64
5	100.00 ± 0.00	84.47 ± 1.23	51.14 ± 2.89*	49.58 ± 5.14*	37.79 ± 5.64*
10	100.00 ± 0.00	58.32 ± 5.90*	47.77 ± 1.35*	39.28 ± 3.73*	28.84 ± 2.88*
25	100.00 ± 0.00	50.68 ± 7.27*	42.57 ± 5.28*	35.08 ± 4.20*	27.76 ± 2.92*
50	100.00 ± 0.00	43.21 ± 4.11*	36.26 ± 3.58*	28.75 ± 1.98*	23.62 ± 1.14*

The data are presented as mean ± SEM of independent triplicate experiments. *$p < 0.05$ versus non-treated control

To confirm the apoptosis response of the cells, Hoechst 33342 and PI co-staining assay was performed. Results indicated that the reduction of cell viability was mainly due to anoikis, as indicated by the increase in cells with chromatin condensation and intense nuclear fluorescence. Figure 9A and 9B showed that after 12 h of cell detachment, the percentages of apoptotic cells were significantly increased to approximately 37, 43 and 52 at the zinc concentration of 10, 25 and 50 μM, respectively. Likewise, at 24 h after cell detachment the apoptotic cells were approximately found 50%, 53% and 58% in response to zinc treatment at 10, 25 and 50 μM, respectively (Table 4), while the necrotic cells were only slightly detectable by nuclear PI fluorescence in this condition. These results suggested that apoptosis was the primary mode of cell death induced by detachment of H460 cells.

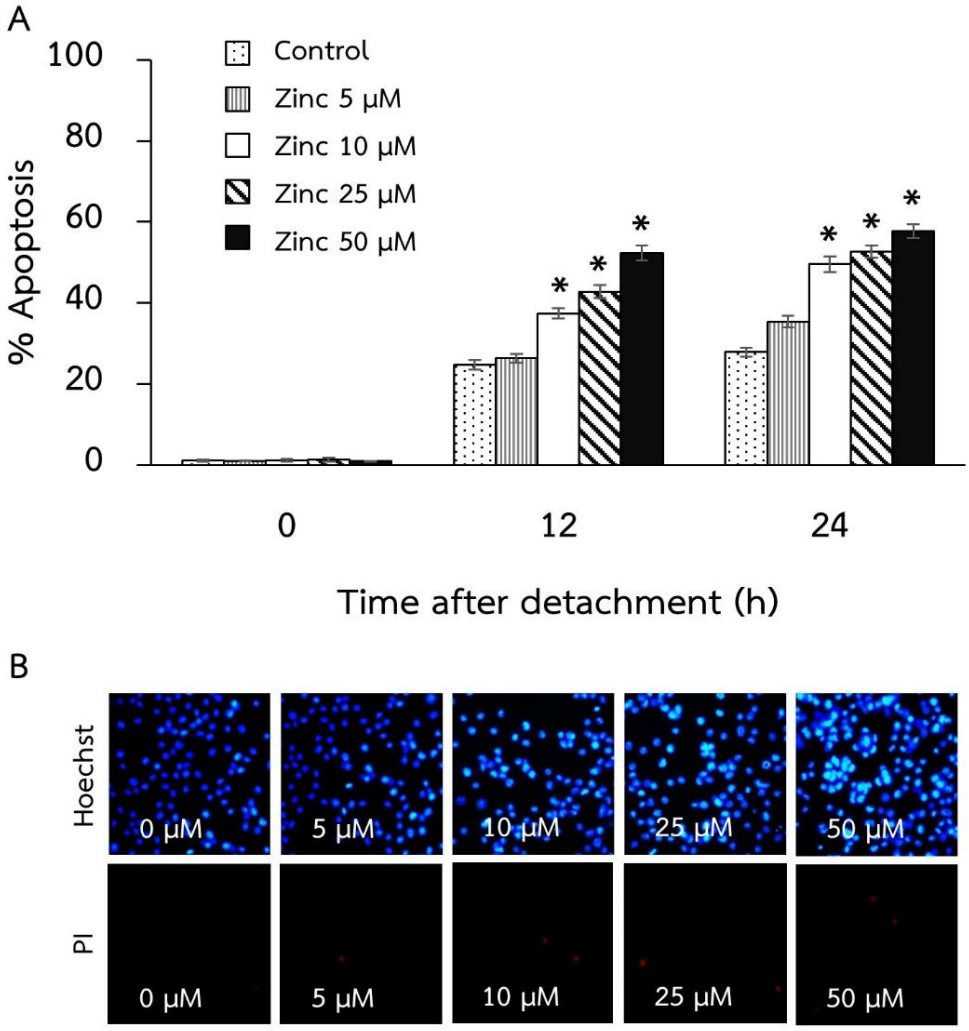

Figure 9 Effect of zinc on mode of cell death of H460 in detached condition

Table 4 The percentage of apoptotic H460 cells detected by Hoechst 33342/PI co-staining assay after treatment with non-toxic concentrations of zinc in detached condition

Zinc (μM)	% Apoptosis		
	0 h	**12 h**	**24 h**
0	1.10 ± 0.26	24.71 ± 1.19	27.85 ± 1.09
5	1.03 ± 0.05	26.32 ± 1.13	35.38 ± 1.44
10	1.18 ± 0.34	37.41 ± 1.31*	49.57 ± 1.91*
25	1.25 ± 0.48	42.80 ± 1.63*	52.65 ± 1.56*
50	0.93 ± 0.07	52.32 ± 1.85*	57.73 ± 1.73*

The data are presented as mean ± SEM of independent triplicate experiments. *$p < 0.05$ versus non-treated control

4.3 Effect of zinc on anchorage-independent growth in non-small cell lung cancer H460 cells

An important characteristic of cancer metastasis is the growth of cancer cells in the anchorage-independent condition. The supportive data regarding the effect of zinc on anchorage-independent growth of H460 cells were examined by colony formation assay. H460 cells were prepared into single cell suspension, and then subjected to soft agar. Cells were treated with non-toxic zinc concentrations (0, 5, 10, 25, and 50 μM). Relative colony number and relative colony size were scored and analyzed after 2 weeks of zinc treatment. Figure 10A and 10B showed that the colony number and colony size of H460 cells were significantly decreased after zinc treatment in a dose-dependent manner as compared to non-treated control. In particular, zinc at the concentration of 50 μM significantly reduced colony size as compared to non-treated control and 5 μM of zinc conditions (Figure 10B, Table 5). Figure 10C showed the photographs of colonies visualized under a light microscope. These results indicated that zinc treatment had ability to inhibit the growth of H460 cells in the anchorage-independent condition.

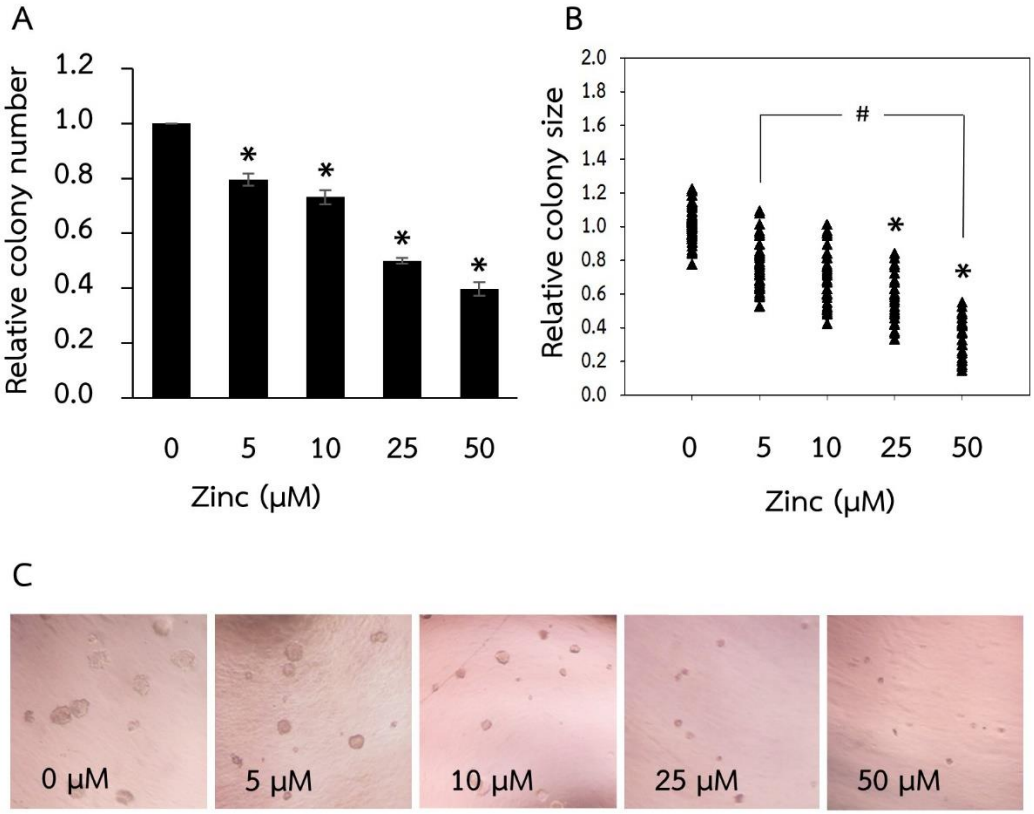

Figure 10 Effect of zinc on anchorage-independent growth in H460 cells

Table 5 The relative colony number and size of H460 cells in anchorage-independent growth assay analyzed under a light microscope after 2 weeks

Zinc (μM)	Relative colony number	Relative colony size
0	1.00 ± 0.00	1.00 ± 0.00
5	0.80 ± 0.02*	0.74 ± 0.08
10	0.73 ± 0.03*	0.61 ± 0.07
25	0.50 ± 0.01*	0.49 ± 0.07*
50	0.40 ± 0.02*	0.38 ± 0.06*, #

Values of relative colony number are the mean ± SEM of independent triplicate experiments.

Values of relative colony size are the diameter of colony of independent triplicate experiments.

*$p < 0.05$ versus non-treated control

#$p < 0.05$ versus zinc 5 μM

4.4 Effect of zinc on expression of anoikis-associated proteins in non-small cell lung cancer H460 cells

The possible mechanisms of zinc involving in cancer cell anoikis were determined focusing on the expression of anokis-associated proteins, namely Cav-1, pAkt/Akt, pERK/ERK, Mcl-1, Bcl-2, and Bax. H460 cells after pre-treatment were cultured in poly-HEMA-coated plates and exposed with non-toxic concentrations of zinc, and then the cells were evaluated by western blot analysis. Figure 11A and 11B showed that zinc significantly decreased Cav-1 and pAkt levels in a dose-dependent manner as compared to non-treated control, while there were no effect on pERK/ERK level (Table 6). The effects of zinc on apoptosis regulatory proteins, including Mcl-1, Bcl-2, and Bax under the same treatment condition were also investigated. Mcl-1, Bcl-2, and Bax protein expression were unchanged during anoikis condition (Figure 12A and 12B, Table 7). The results indicated that zinc treatment could sensitize H460 cell anoikis through down-regulation of Cav-1 and pAkt expression.

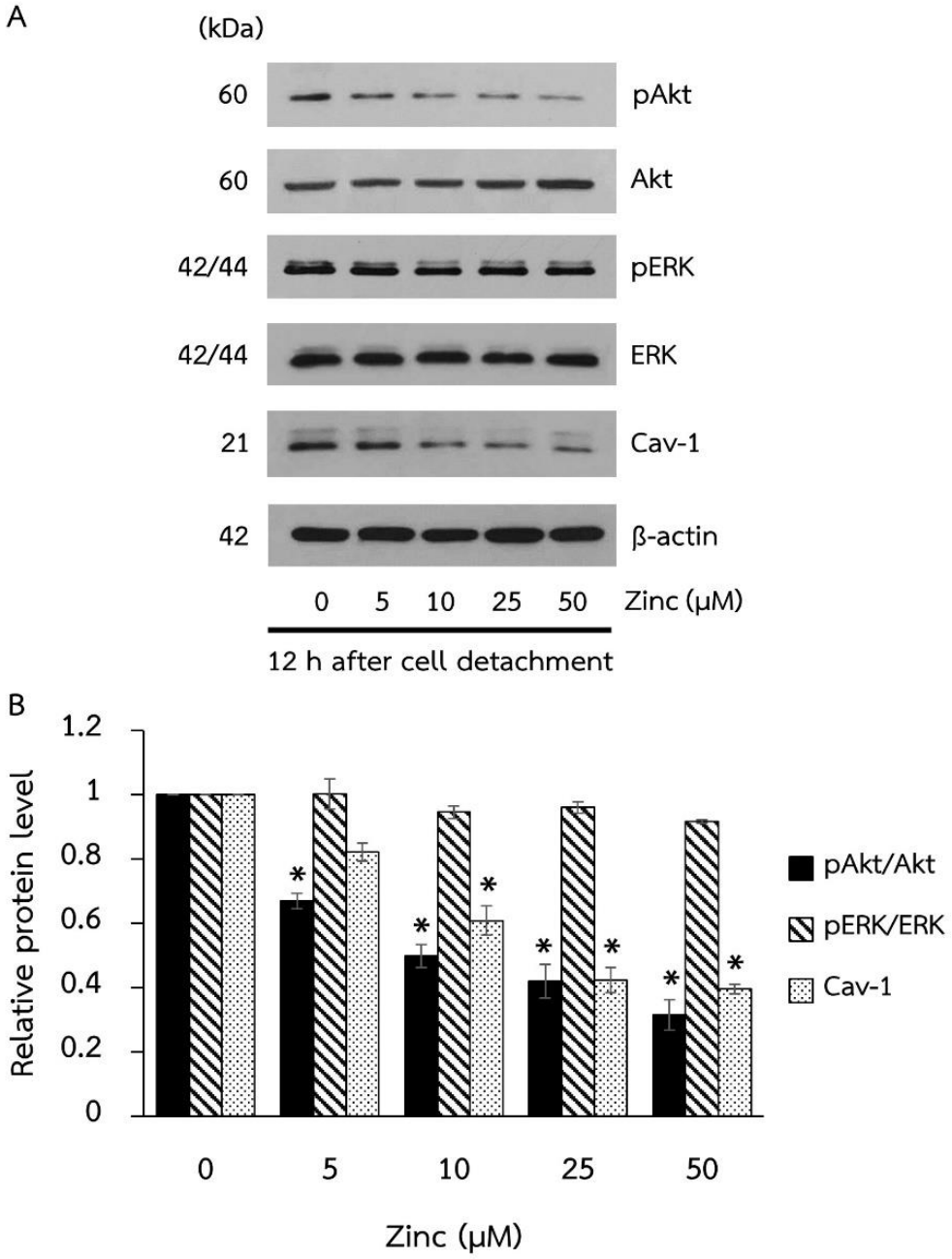

Figure 11 Effect of zinc on expression of survival associated proteins in H460 cells

Table 6 The relative of survival associated protein in H460 cells determined by Western blot analysis after treatment with non-toxic concentration of zinc at 12 h after detachment

Zinc (μM)	Relative protein level		
	pAkt/Akt	pERK/ERK	Cav-1
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
5	0.67 ± 0.02*	1.00 ± 0.05	0.82 ± 0.03
10	0.50 ± 0.04*	0.95 ± 0.02	0.61 ± 0.05*
25	0.42 ± 0.05*	0.96 ± 0.02	0.42 ± 0.04*
50	0.31 ± 0.05*	0.92 ± 0.01	0.40 ± 0.02*

The data are presented as mean ± SEM of independent triplicate experiments. *$p < 0.05$ versus non-treated control

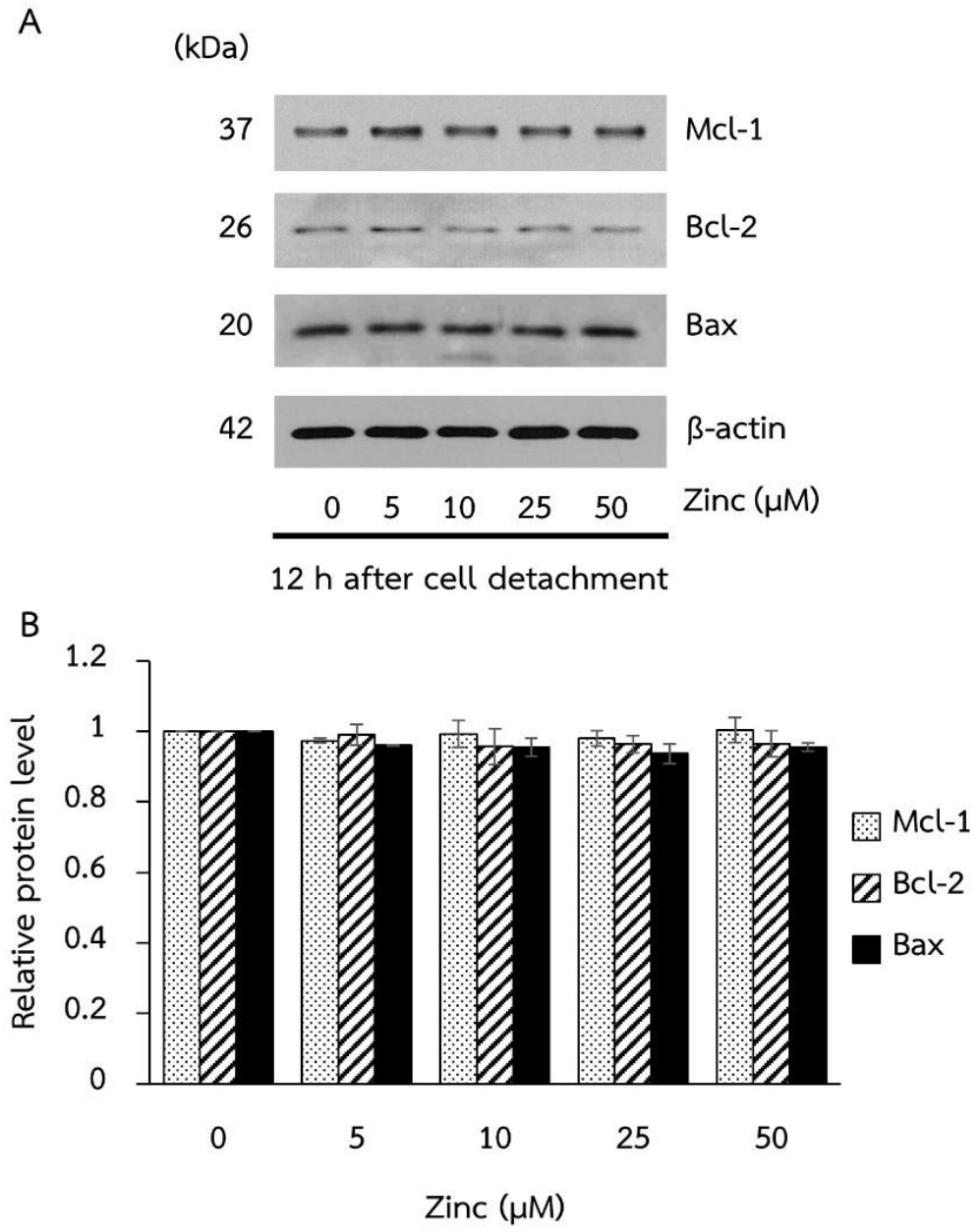

Figure 12 Effect of zinc on expression of anoikis-associated proteins in H460 cells

Table 7 The relative of anoikis-associated protein in H460 cells determined by Western blot analysis after treatment with non-toxic concentration of zinc at 12 h after detachment

Zinc (μM)	Relative protein level		
	Mcl-1	Bcl-2	Bax
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
5	0.97 ± 0.01	0.99 ± 0.03	0.96 ± 0.00
10	0.99 ± 0.04	0.96 ± 0.05	0.96 ± 0.03
25	0.98 ± 0.02	0.96 ± 0.02	0.94 ± 0.03
50	1.00 ± 0.04	0.96 ± 0.04	0.96 ± 0.01

The data are presented as mean ± SEM of independent triplicate experiments. *$p < 0.05$ versus non-treated control

CHAPTER V
DISCUSSION AND CONCLUSION

This study was proposed to investigate the effect of zinc on anoikis in non-small cell lung cancer H460 cells. The effects of zinc on the expression of anoikis related proteins, including Cav-1, pAkt/Akt, pERK/ERK, Mcl-1, Bcl-2, and Bax were also examined in this cancer cells.

The present study demonstrated for the first time that zinc possibly negatively regulate the survival and growth of detached cancer cells. The non-toxic concentration of zinc could sensitize the detached lung cancer cells to anoikis. Indeed, lung cancer has been shown to be a leading cause of cancer mortality, worldwide (Siegel et al., 2013). Moreover, the high mortality rate in this cancer was shown to be strongly associated with the presence of metastasis (Steeg, 2006). Thus, this novel effect of zinc may benefit the use as well as the development of new strategies for cancer metastasis prevention.

Based on previous studies, the cytotoxic effect of zinc in prostate cancer cells, LNCaP and PC-3 cells were found with the doses of 75 and 250 μM, respectively (Ku et al., 2012), while the cytotoxic effect in Human B cell Burkit Lymphoma Raji cells was found with the dose of 100 μM (Mehr, 2011). In the present study, the toxic concentration of zinc in H460 cells was found at the dose of 100 μM. Therefore, zinc concentrations of less than this dose were used in further experiments to eradicate

the direct cytotoxic effect of zinc which can overwhelm the anoikis response in this cancer cell model. Moreover, to clarify the safety of zinc in normal human cells, the cytotoxicity in normal keratinocyte HaCat cells were also determined. The result showed that zinc had no cytotoxic effect on normal keratinocyte cells.

During the detached condition, it was found that H460 cells treated with non-toxic concentrations of zinc significantly decreased cell viability due to anoikis in dose dependent manner. Anoikis is an apoptotic cell death that is characterized by specific morphological changes, including cell shrinkage, membrane blebbing, DNA condensation and fragmentation (Chiarugi, and Giannoni, 2008; Paoli et al., 2013). To ensure that H460 cells underwent anoikis after zinc treatment, the apoptotic cell death and DNA condensation were observed by Hoechst 33342 and PI co-staining. After cell detachment, the percentage of apoptosis cells after treatment with zinc was increased significantly in dose dependent manner. In addition, the necrotic cell death was found only slightly detectable in this study. Therefore, it was confirmed that zinc sensitized apoptosis after detachment by DNA condensation and fragmentation.

The underlying mechanism of zinc for anoikis sensitization was found to be via down-regulating the expression of Cav-1 and phosphorylated Akt (Figure 13). It is well recognized that the kinase/phosphatase signaling molecules, such as PI3K/Akt and ERK has been implicated in anoikis as central regulators, and the increased cellular survival pathway Akt is accounted for chemotherapeutic resistance as well as anoikis resistance in cancer (Kennedy et al., 1999). The up-regulation of pAkt was found in anoikis

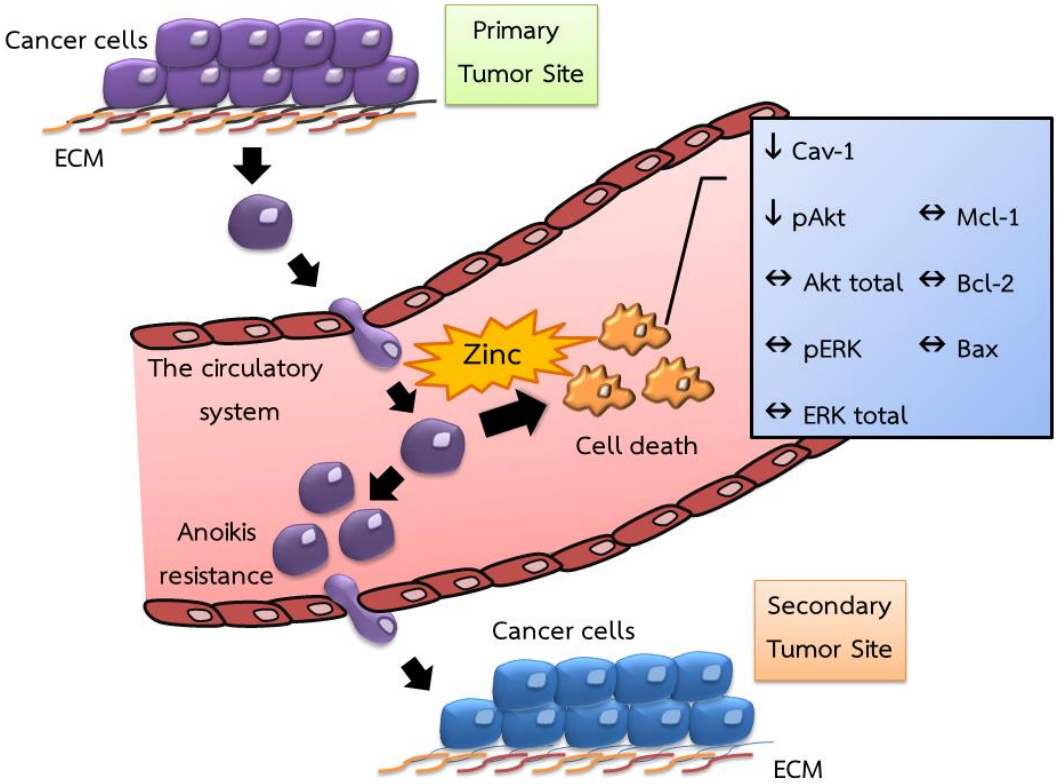

Figure 13 The schematic representation of role of zinc in anoikis sensitization

resistance in human osteosarcoma cells (Diaz-Montero et al., 2006). Akt activation enhances cell survival through preventing the cytochrome c release from mitochondria by phosphorylation the pro-apoptotic protein Bad (Khwaja, 1999), causing the release of anti-apoptotic protein Bcl-2 (Datta et al., 2000) and inhibiting the caspase-9 activity (Cardone et al., 1998). It was reported that the activation of pro-survival ERK protein leads to inhibition of the anoikis process. Overexpression of ERK was found to increase anoikis resistance in pancreatic cancer cell lines, MIA-PaCa-2 and BxPC-3 through phosphorylation of the anti-apoptotic protein Bcl-2 (Galante et al., 2009). In this study, the effect of zinc on pERK and ERK expression were investigated. The results showed

that the non-toxic concentration of zinc had no effect on pERK/ERK level, but significantly decreased the level of activated Akt protein. Similarly, Pengpaeng, Sritularak and Chanvorachote (2015) found that dendrofalconerol A sensitized lung cancer cells to anoikis via down-regulating the expression of activated Akt protein.

It has been known that metastatic cancer cells have developed several mechanisms to prevent anoikis involving the alterations of Bcl-2 family protein expression (Placzek et al., 2010). However, this study found that levels of Bcl-2, Mcl-1, and Bax were unchanged after zinc treatment. Likewise, recent studies demonstrated that natural products, curcumin and dendrofalconerol A sensitized cancer cells to anoikis with no effect on Mcl-1 and Bax protein (Pongrakhananon et al., 2010; Pengpaeng et al., 2015). It is possible that the potential of zinc to sensitize non-small cell lung cancer H460 cells to anoikis may involve other mechanisms which required the further study to clarity these mechanisms.

The highly aggressive cancer and metastatic process have been linked with the survival and growth in the anchorage-independent condition (Zhu et al., 2001). Several murine experimental models demonstrated that anchorage dependent cell lines of breast cancer, such as BT483, Hs578T, MDA-MB-361, and ZR75 had metastatic potential to various distant sites (Mori et al., 2009). The results of colony formation assay in the present study suggested that zinc suppressed the growth of lung cancer cells in the anchorage independent condition as the colony number and size were significantly decreased in the zinc-treated cells. These results supported that zinc had a potential

in anoikis sensitization in lung cancer cells and may have a potential in inhibition of cancer metastasis.

Moreover, this study provided the information that zinc suppressed Cav-1 expression. Several studies suggested an important role of Cav-1 in anoikis resistance. Cav-1 expression associates with cancer metastasis and poor prognosis in cancer patients (Yang et al., 1998; Kato et al., 2002; Joo et al., 2004). Previous studies showed that Cav-1 produced by prostate cancer cells increased viability and clonal growth under serum-free condition (Tahir et al., 2001). In MCF-7 human breast cancer cells, the up-regulation of Cav-1 was found to enhance cell survival in anchorage-independent growth condition (Fiucci et al., 2002). In lung cancer, Cav-1 inhibited anoikis by maintaining the activated Akt level (Li et al., 2003) and the anti-apoptotic Mcl-1 level (Chunhacha et al., 2012). Also, Cav-1 was shown to increase migration and invasion of the lung cancer cells (Luanpitpong et al., 2010; Sanuphan et al., 2013; Chanvorachote, Pongrakhananon, and Chunhacha, 2014). This present study indicated a mechanism of zinc in sensitizing anoikis and reducing cancer metastasis by suppressing this metastasis regulatory protein.

In conclusion, the present study suggested that zinc sensitized non-small cell lung cancer H460 cells to anoikis through down-regulation of Cav-1 and pAkt protein levels. This novel finding could be beneficial for further use of zinc in anti-metastasis therapy and cancer metastasis prevention.

REFERENCES

Age-Related Eye Disease Study Research Group 2001. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins c and e, beta carotene, and zinc for age-related macular degeneration and vision loss: Areds report no. 8. Archives of Ophthalmology, 119, 1417-1436.

Al-Maroof, R.A., and Al-Sharbatti, S.S. 2006. Serum zinc levels in diabetic patients and effect of zinc supplementation on glycemic control of type 2 diabetics. Saudi Medical Journal, 27, 344-350.

Alam, S., and Kelleher, S.L. 2012. Cellular mechanisms of zinc dysregulation: A perspective on zinc homeostasis as an etiological factor in the development and progression of breast cancer. Nutrients, 4, 875-903.

Bahl, R., Bhandari, N., Hambidge, K.M., and Bhan, M.K. 1998. Plasma zinc as a predictor of diarrheal and respiratory morbidity in children in an urban slum setting. The American Journal of Clinical Nutrition, 68, 414S-417S.

Beck, F.W., Prasad, A.S., Kaplan, J., Fitzgerald, J.T., and Brewer, G.J. 1997. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. The American Journal of Physiology, 272, E1002-1007.

Bertram, J.S. 2000. The molecular biology of cancer. Molecular Aspects of Medicine, 21, 167-223.

Bhandari, N., Bahl, R., Taneja, S., Strand, T., Mølbak, K., Ulvik, R.J., et al. 2002. Substantial reduction in severe diarrheal morbidity by daily zinc supplementation in young north indian children. Pediatrics, 109, e86.

Bhutta, Z.A., Bird, S.M., Black, R.E., Brown, K.H., Gardner, J.M., Hidayat, A., et al. 2000. Therapeutic effects of oral zinc in acute and persistent diarrhea in children in

developing countries: pooled analysis of randomized controlled trials. The American Journal of Clinical Nutrition, 72, 1516-1522.

Boisvert-Adamo, K., Longmate, W., Abel, E.V., and Aplin, A.E. 2009. Mcl-1 is required for melanoma cell resistance to anoikis. Molecular Cancer Research, 7, 549-556.

Brewer, G.J., Dick, R.D., Johnson, V.D., Brunberg, J.A., Kluin, K.J., and Fink, J.K. 1998. Treatment of Wilson's disease with zinc: XV long-term follow-up studies. Journal of Laboratory and Clinical Medicine, 132, 264-278.

Brooks, W.A., Santosham, M., Naheed, A., Goswami, D., Wahed, M.A., Diener-West, M., et al. 2005. Effect of weekly zinc supplements on incidence of pneumonia and diarrhoea in children younger than 2 years in an urban, low-income population in Bangladesh: randomised controlled trial. The Lancet, 366, 999-1004.

Brown, K.H., Peerson, J.M., Rivera, J., and Allen, L.H. 2002. Effect of supplemental zinc on the growth and serum zinc concentrations of prepubertal children: a meta-analysis of randomized controlled trials. The American Journal of Clinical Nutrition, 75, 1062-1071.

Burnette, W.N. 1981. “Western Blotting”: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analytical Biochemistry, 112, 195-203.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., et al. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science, 282, 1318-1321.

Chaffer, C.L., and Weinberg, R.A. 2011. A perspective on cancer cell metastasis. Science, 331, 1559-1564.

Chanvorachote, P., Pongrakhananon, V., and Chunhacha, P. 2014. Prolonged nitric oxide exposure enhances anoikis resistance and migration through epithelial-

mesenchymal transition and caveolin-1 upregulation. BioMed Research International, 2014, 941359.

Chasapis, C., Loutsidou, A., Spiliopoulou, C., and Stefanidou, M. 2012. Zinc and human health: an update. Archives of Toxicology, 86, 521-534.

Chiang, A.C., and Massagué, J. 2008. Molecular basis of metastasis. New England Journal of Medicine, 359, 2814-2823.

Chiarugi, P., and Giannoni, E. 2008. Anoikis: a necessary death program for anchorage-dependent cells. Biochemical Pharmacology, 76, 1352-1364.

Choochuay, K., Chunhacha, P., Pongrakhananon, V., Luechapudiporn, R., and Chanvorachote, P. 2013. Imperatorin sensitizes anoikis and inhibits anchorage-independent growth of lung cancer cells. Journal of Natural Medicines, 67, 599-606.

Chunhacha, P., Pongrakhananon, V., Rojanasakul, Y., and Chanvorachote, P. 2012. Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. American Journal of Physiology, 302, C1284-C1292.

Cory, S., Huang, D.C., and Adams, J.M. 2003. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene, 22, 8590-8607.

Czlonkowska, A., Gajda, J., and Rodo, M. 1996. Effects of long-term treatment in Wilson's disease with D-penicillamine and zinc sulphate. Journal of Neurology, 243, 269-273.

Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B., et al. 2000. 14-3-3 Proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Molecular Cell, 6, 41-51.

Davies, M.A., Koul, D., Dhesi, H., Berman, R., Mcdonnell, T.J., Mcconkey, D., et al. 1999. Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. Cancer Research, 59, 2551-2556.

Diaz-Montero, C.M., Wygant, J.N., and Mcintyre, B.W. 2006. PI3-K/Akt-mediated anoikis resistance of human osteosarcoma cells requires Src activation. European Journal of Cancer, 42, 1491-500.

Doerr, T.D., Marks, S.C., Shamsa, F.H., Mathog, R.H., and Prasad, A.S. 1998. Effects of zinc and nutritional status on clinical outcomes in head and neck cancer. Nutrition, 14, 489-495.

Dummler, B., and Hemmings, B.A. 2007. Physiological roles of PKB/Akt isoforms in development and disease. Biochemical Society Transactions, 35, 231-235.

Eby, G.A., and Halcomb, W.W. 2006. Ineffectiveness of zinc gluconate nasal spray and zinc orotate lozenges in common-cold treatment: a double-blind, placebo-controlled clinical trial. Alternative Therapies in Health and Medicine, 12, 34-38.

Epstein, M.M., Kasperzyk, J.L., Andrén, O., Giovannucci, E.L., Wolk, A., Håkansson, N., et al. 2011. Dietary zinc and prostate cancer survival in a Swedish cohort. The American Journal of Clinical Nutrition, 93, 586-593.

Evans, J.R., and Lawrenson, J.G. 2012. Antioxidant vitamin and mineral supplements for slowing the progression of age-related macular degeneration. Cochrane Database of Systematic Reviews, 2, CD000254.

Fiucci, G., Ravid, D., Reich, R., and Liscovitch, M. 2002. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene, 21, 2365-2375.

Ford, D. 2004. Intestinal and placental zinc transport pathways. Proceedings of the Nutrition Society, 63, 21-29.

Fortes, C., Forastiere, F., Agabiti, N., Fano, V., Pacifici, R., Virgili, F., et al. 1998. The effect of zinc and vitamin A supplementation on immune response in an older population. Journal of the American Geriatrics Society, 46, 19-26.

Fraker, P.J., King, L.E., Laakko, T., and Vollmer, T.L. 2000. The dynamic link between the integrity of the immune system and zinc status. The Journal of Nutrition, 130, 1399S-1406S.

Freshney, R.I. 2010a. Chapter 20 Quantitation. Culture of Animal Cells : A Manual of Basic Technique and Specialized Applications. 6 ed. Hoboken, N.J.: Wiley-Blackwell.

Freshney, R.I. 2010b. Chapter 21 Cytotoxicity. Culture of Animal Cells : A Manual of Basic Technique and Specialized Applications. 6 ed. Hoboken, N.J.: Wiley-Blackwell.

Gabriel, J. 2007. The Biology of Cancer, London, John Wiley & Sons Ltd.

Galante, J.M., Mortenson, M.M., Bowles, T.L., Virudachalam, S., and Bold, R.J. 2009. ERK/BCL-2 pathway in the resistance of pancreatic cancer to anoikis. Journal of Surgical Research, 152, 18-25.

Girodon, F., Lombard, M., Galan, P., Rbrunet-Lecomte, P., Monget, A.A., J., Preziosi, P., et al. 1997. Effect of micronutrient supplementation on infection in institutionalized elderly subjects: a controlled trial. Annals of Nutrition and Metabolism, 41, 98-107.

Gropper, S.S., Smith, J.L., and Groff, J.L. 2005. Microminerals. Advanced Nutrition and Human Metabolism. 4th ed. California: Wadsworth Publishing.

Gupta, G.P., and Massagué, J. 2006. Cancer metastasis: Building a framework. Cell, 127, 679-695.

Gupta, S., Singh, S., and Shukla, V. 2005. Copper, zinc, and Cu/Zn ratio in carcinoma of the gallbladder. Journal of Surgical Oncology, 91, 204-208.

Hajo, H., and Wolfgang, M. 2010. The regulatory and signaling functions of zinc ions in human cellular physiology. Cellular and Molecular Biology of Metals. CRC Press.

Hanahan, D., and Weinberg, R.A. 2000. The hallmarks of cancer. Cell, 100, 57-70.

Hercberg, S., Galan, P., Preziosi, P., Bertrais, S., Mennen, L., Malvy, D., et al. 2004. The su.vi.max study: A randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. Archives of Internal Medicine, 164, 2335-2342.

Ho, E., Quan, N., Tsai, Y.-H., Lai, W., and Bray, T.M. 2001. Dietary zinc supplementation inhibits NF-κB activation and protects against chemically induced diabetes in CD1 mice. Experimental Biology and Medicine, 226, 103-111.

Hou, J.M., Krebs, M., Ward, T., Sloane, R., Priest, L., Hughes, A., et al. 2011. Circulating tumor cells as a window on metastasis biology in lung cancer. American Journal of Pathology, 178, 989-996.

Ishii, K., Otsuka, T., Iguchi, K., Usui, S., Yamamoto, H., Sugimura, Y., et al. 2004. Evidence that the prostate-specific antigen (PSA)/Zn2+ axis may play a role in human prostate cancer cell invasion. Cancer Letters, 207, 79-87.

Issell, B.F., Macfadyen, B.V., Gum, E.T., Valdivieso, M., Dudrick, S.J., and Bodey, G.P. 1981. Serum zinc levels in lung cancer patients. Cancer, 47, 1845-1848.

John, E., Laskow, T.C., Buchser, W.J., Pitt, B.R., Basse, P.H., Butterfield, L.H., et al. 2010. Zinc in innate and adaptive tumor immunity. Journal of Translational Medicine, 8, 118-118.

Joo, H.J., Oh, D.K., Kim, Y.S., Lee, K.B., and Kim, S.J. 2004. Increased expression of caveolin-1 and microvessel density correlates with metastasis and poor prognosis in clear cell renal cell carcinoma. BJU International, 93, 291-296.

Kato, K., Hida, Y., Miyamoto, M., Hashida, H., Shinohara, T., Itoh, T., et al. 2002. Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Cancer, 94, 929-933.

Kennedy, S.G., Kandel, E.S., Cross, T.K., and Hay, N. 1999. Akt/protein kinase B Inhibits cell death by preventing the release of cytochrome c from mitochondria. Molecular and Cellular Biology, 19, 5800-5810.

Khwaja, A. 1999. Apoptosis: Akt is more than just a Bad kinase. Nature, 401, 33-34.

Khwaja, A., Rodriguez-Viciana, P., Wennström, S., Warne, P.H., and Downward, J. 1997. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. The EMBO Journal, 16, 2783-2793.

Koleske, A.J., Baltimore, D., and Lisanti, M.P. 1995. Reduction of caveolin and caveolae in oncogenically transformed cells. Proceedings of the National Academy of Sciences, 92, 1381-1385.

Ku, J.H., Seo, S.Y., Kwak, C., and Kim, H.H. 2012. The role of survivin and Bcl-2 in zinc-induced apoptosis in prostate cancer cells. Urologic Oncology: Seminars and Original Investigations, 30, 562-568.

Lansdown, A.B.G., Mirastschijski, U., Stubbs, N., Scanlon, E., and Ågren, M.S. 2007. Zinc in wound healing: Theoretical, experimental, and clinical aspects. Wound Repair and Regeneration, 15, 2-16.

Lavie, Y., Fiucci, G., and Liscovitch, M. 1998. Up-regulation of caveolae and caveolar constituents in multidrug-resistant cancer cells. Journal of Biological Chemistry, 273, 32380-32383.

Leone, N., Courbon, D., Ducimetiere, P., and Zureik, M. 2006. Zinc, Copper, and Magnesium and risks for all-cause, cancer, and cardiovascular Mortality. Epidemiology, 17, 308-314.

Li, L., Ren, C.H., Tahir, S.A., Ren, C., and Thompson, T.C. 2003. Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site

interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Molecular and Cellular Biology, 23, 9389-9404.

Liu, Z., Li, H., Derouet, M., Berezkin, A., Sasazuki, T., Shirasawa, S., et al. 2006. Oncogenic Ras inhibits anoikis of intestinal epithelial cells by preventing the release of a mitochondrial pro-apoptotic protein Omi/HtrA2 into the cytoplasm. Journal of Biological Chemistry, 281, 14738-14747.

Luanpitpong, S., Talbott, S.J., Rojanasakul, Y., Nimmannit, U., Pongrakhananon, V., Wang, L., et al. 2010. Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1. Journal of Biological Chemistry, 285, 38832-38840.

Mehr, H.R. 2011. Evaluation of cytotoxic effect of zinc on Raji cell-line by MTT assay. Iranian Jornal of Toxicology, 4, 390-396.

Meksawan, K., Sermsri, U., and Chanvorachote, P. 2014. Zinc supplementation improves anticancer activity of monocytes in type-2 diabetic patients with metabolic syndrome. Anticancer Research, 34, 295-299.

Meydani, S.N., Barnett, J.B., Dallal, G.E., Fine, B.C., Jacques, P.F., Leka, L.S., et al. 2007. Serum zinc and pneumonia in nursing home elderly. The American Journal of Clinical Nutrition, 86, 1167-1173.

Meyer, F., Galan, P., Douville, P., Bairati, I., Kegle, P., Bertrais, S., et al. 2005. Antioxidant vitamin and mineral supplementation and prostate cancer prevention in the SU.VI.MAX trial. International Journal of Cancer, 116, 182-186.

Ministry of Public Health. 2012. Death rates of leading causes of death in Thailand [Online]. Available: http://bps.ops.moph.go.th/Healthinformation/index.htm [Accessed January 30, 2014].

Mori, S., Chang, J.T., Andrechek, E.R., Matsumura, N., Baba, T., Yao, G., et al. 2009. An anchorage-independent cell growth signature identifies tumors with metastatic potential. Oncogene, 28, 2796-2805.

Owens, T.W., Valentijn, A.J., Upton, J.P., Keeble, J., Zhang, L., Lindsay, J., et al. 2009. Apoptosis commitment and activation of mitochondrial Bax during anoikis is regulated by p38MAPK. Cell Death and Differentiation, 16, 1551-1562.

Paoli, P., Giannoni, E., and Chiarugi, P. 2013. Anoikis molecular pathways and its role in cancer progression. Biochimica et Biophysica Acta, 1833, 3481-3498.

Parton, R.G., and Simons, K. 2007. The multiple faces of caveolae. Nature Reviews: Molecular Cell Biology, 8, 185-194.

Pengpaeng, P., Sritularak, B., and Chanvorachote, P. 2015. Dendrofalconerol A sensitizes anoikis and inhibits migration in lung cancer cells. Journal of Natural Medicines, 69, 178-190.

Placzek, W.J., Wei, J., Kitada, S., Zhai, D., Reed, J.C., and Pellecchia, M. 2010. A survey of the anti-apoptotic Bcl-2 subfamily expression in cancer types provides a platform to predict the efficacy of Bcl-2 antagonists in cancer therapy. Cell Death and Disease, 1, e40.

Pongrakhananon, V., Nimmannit, U., Luanpitpong, S., Rojanasakul, Y., and Chanvorachote, P. 2010. Curcumin sensitizes non-small cell lung cancer cell anoikis through reactive oxygen species-mediated Bcl-2 downregulation. Apoptosis, 15, 574-585.

Prasad, A.S. 2000. Effects of zinc deficiency on immune functions. The Journal of Trace Elements in Experimental Medicine, 13, 1-20.

Prasad, A.S. 2008. Clinical, immunological, anti-inflammatory and antioxidant roles of zinc. Experimental Gerontology, 43, 370-377.

Prasad, A.S. 2009. Zinc: role in immunity, oxidative stress and chronic inflammation. Current Opinion in Clinical Nutrition and Metabolic Care, 12, 646-652.

Prasad, A.S., Beck, F.W.J., Bao, B., Snell, D., and Fitzgerald, J.T. 2008. Duration and severity of symptoms and levels of plasma interleukin-1 receptor antagonist, soluble tumor necrosis factor receptor, and adhesion molecules in patients with common cold treated with zinc acetate. Journal of Infectious Diseases, 197, 795-802.

Prasad, A.S., Beck, W.J., Doerr, T.D., Shamsa, F.H., Penny, H.S., Marks, S.C., et al. 1998. Nutritional and zinc status of head and neck cancer patients: an interpretive review. Journal of the American College of Nutrition, 17, 409-418.

Razani, B., Woodman, S.E., and Lisanti, M.P. 2002. Caveolae: From cell biology to animal physiology. Pharmacological Reviews, 54, 431-467.

Rosen, K., Rak, J., Jin, J., Kerbel, R.S., Newman, M.J., and Filmus, J. 1998. Downregulation of the pro-apoptotic protein Bak is required for the ras-induced transformation of intestinal epithelial cells. Current Biology, 8, 1331-S1.

Sakamoto, S., and Kyprianou, N. 2010. Targeting anoikis resistance in prostate cancer metastasis. Molecular Aspects of Medicine, 31, 205-214.

Sanuphan, A., Chunhacha, P., Pongrakhananon, V., and Chanvorachote, P. 2013. Long-term nitric oxide exposure enhances lung cancer cell migration. BioMed Research International, 2013, 186972.

Schmelzle, T., Mailleux, A.A., Overholtzer, M., Carroll, J.S., Solimini, N.L., Lightcap, E.S., et al. 2007. Functional role and oncogene-regulated expression of the BH3-only factor Bmf in mammary epithelial anoikis and morphogenesis. Proceedings of the National Academy of Sciences, 104, 3787-3792.

Shah, D., and Sachdev, H.P. 2001. Effect of gestational zinc deficiency on pregnancy outcomes: summary of observation studies and zinc supplementation trials. The British Journal of Nutrition, 85, 101-108.

Shankar, A.H., and Prasad, A.S. 1998. Zinc and immune function: the biological basis of altered resistance to infection. The American Journal of Clinical Nutrition, 68, 447S-463S.

Siegel, R., Naishadham, D., and Jemal, A. 2013. Cancer statistics, 2013. CA: A Cancer Journal for Clinicians, 63, 11-30.

Simpson, C.D., Anyiwe, K., and Schimmer, A.D. 2008. Anoikis resistance and tumor metastasis. Cancer Letters, 272, 177-185.

Singh, M., and Das, R.R. 2011. Zinc for the common cold. The Cochrane Database of Systematic Reviews, 16, CD001364.

Souhami, R., and Tobias, J. 1995. Cancer and its Management, Massachusetts, Blackwell Science.

Steeg, P.S. 2006. Tumor metastasis: Mechanistic insights and clinical challenges. Nature Medicine, 12, 895-904.

Tahir, S.A., Yang, G., Ebara, S., Timme, T.L., Satoh, T., Li, L., et al. 2001. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. Cancer Research, 61, 3882-3885.

Tran, C.D., Miller, L.V., Krebs, N.F., Lei, S., and Hambidge, K.M. 2004. Zinc absorption as a function of the dose of zinc sulfate in aqueous solution. The American Journal of Clinical Nutrition, 80, 1570-1573.

Trumbo, P., Yates, A.A., Schlicker, S., and Poos, M. 2001. Dietary reference intakes. Journal of the American Dietetic Association, 101, 294-301.

Turner, R.B., and Cetnarowski, W.E. 2000. Effect of treatment with zinc gluconate or zinc acetate on experimental and natural colds. Clinical Infectious Diseases, 31, 1202-1208.

Uzzo, R.G., Leavis, P., Hatch, W., Gabai, V.L., Dulin, N., Zvartau, N., et al. 2002. Zinc inhibits nuclear factor-κB activation and sensitizes prostate cancer cells to cytotoxic agents. Clinical Cancer Research, 8, 3579-3583.

Valentijn, A.J., Metcalfe, A.D., Kott, J., Streuli, C.H., and Gilmore, A.P. 2003. Spatial and temporal changes in Bax subcellular localization during anoikis. The Journal of Cell Biology, 162, 599-612.

Wessells, K.R., and Brown, K.H. 2012. Estimating the global prevalence of zinc deficiency: Results based on zinc availability in national food supplies and the prevalence of stunting. Plos One, 7, e50568.

Wongpankam, E., Chunhacha, P., Pongrakhananon, V., Sritularak, B., and Chanvorachote, P. 2012. Artonin E mediates MCL1 down-regulation and sensitizes lung cancer cells to anoikis. Anticancer Research, 32, 5343-5351.

Woods, N.T., Yamaguchi, H., Lee, F.Y., Bhalla, K.N., and Wang, H.-G. 2007. Anoikis, initiated by Mcl-1 degradation and Bim induction, is deregulated during oncogenesis. Cancer Research, 67, 10744-10752.

Yang, G., Truong, L.D., Timme, T.L., Ren, C., Wheeler, T.M., Park, S.H., et al. 1998. Elevated expression of caveolin is associated with prostate and breast cancer. Clinical Cancer Research, 4, 1873-1880.

Zhu, Z., Sanchez-Sweatman, O., Huang, X., Wiltrout, R., Khokha, R., Zhao, Q., et al. 2001. Anoikis and metastatic potential of cloudman S91 melanoma cells. Cancer Research, 61, 1707-1716.

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table A-1 The percentage of H460 cell viability for each experiment in attached condition determined by MTT assay after treatment with various concentration of zinc

Zinc (μM)	% Cell viability		
	N1	N2	N3
0	100.00 ± 0.12	100.00 ± 0.03	100.00 ± 0.02
1	100.13 ± 0.03	96.10 ± 0.01	102.52 ± 0.01
5	100.97 ± 0.05	96.02 ± 0.02	100.47 ± 0.01
10	96.11 ± 0.03	100.04 ± 0.02	112.72 ± 0.02
25	102.23 ± 0.05	93.43 ± 0.02	107.21 ± 0.04
50	107.79 ± 0.02	81.57 ± 0.04	100.82 ± 0.01
100	83.85 ± 0.04	73.88 ± 0.01	73.30 ± 0.06
150	83.29 ± 0.05	75.68 ± 0.02	67.51 ± 0.02

The data are presented as mean ± SD.

Table A-2 The percentage of HaCaT cell viability for each experiment in attached condition determined by MTT assay after treatment with various concentration of zinc

Zinc (μM)	% Cell viability		
	N1	N2	N3
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1	102.82 ± 0.01	107.71 ± 0.06	112.80 ± 0.03
5	107.09 ± 0.01	115.10 ± 0.07	100.68 ± 0.02
10	105.00 ± 0.01	107.71 ± 0.04	103.98 ± 0.03
25	106.00 ± 0.01	115.20 ± 0.03	98.92 ± 0.04
50	103.00 ± 0.00	112.96 ± 0.04	94.92 ± 0.06
100	98.34 ± 0.02	112.31 ± 0.03	114.24 ± 0.03
150	103.55 ± 0.01	112.21 ± 0.03	98.90 ± 0.01

The data are presented as mean ± SD.

Table A-3 The percentage of H460 cell viability for each experiment in detached condition determined by MTT assay after treatment with non-toxic concentrations of zinc

Zinc (μM)	% Cell viability														
	0 h			3 h			6 h			9 h			12 h		
	N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	88.23 ±0.02	83.65 ±0.01	85.75 ±0.00	62.87 ±0.03	64.04 ±0.01	58.66 ±0.00	53.10 ±0.01	60.07 ±0.02	50.94 ±0.00	44.03 ±0.01	48.85 ±0.01	43.89 ±0.00
5	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	83.54 ±0.03	84.02 ±0.01	85.86 ±0.00	49.90 ±0.01	57.70 ±0.01	45.81 ±0.00	46.67 ±0.01	55.52 ±0.03	46.56 ±0.00	31.31 ±0.01	41.61 ±0.01	40.43 ±0.00
10	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	43.07 ±0.01	70.13 ±0.01	61.78 ±0.00	41.83 ±0.01	57.76 ±0.01	43.73 ±0.00	32.92 ±0.01	45.10 ±0.01	39.83 ±0.00	25.82 ±0.01	31.55 ±0.01	29.14 ±0.00
25	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	39.95 ±0.02	61.85 ±0.01	50.22 ±0.00	34.43 ±0.01	50.37 ±0.01	42.91 ±0.00	30.49 ±0.01	36.04 ±0.01	38.72 ±0.00	24.44 ±0.01	29.93 ±0.01	28.90 ±0.00
50	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	36.47 ±0.01	49.49 ±0.01	43.67 ±0.00	35.04 ±0.01	40.28 ±0.01	33.45 ±0.00	23.44 ±0.01	36.57 ±0.01	26.24 ±0.00	22.95 ±0.01	24.94 ±0.01	22.97 ±0.00

The data are presented as mean ± SD.

Table A-4 The percentage of apoptotic H460 cells for each experiment detected by Hoechst 33342/PI co-staining assay after treatment with non-toxic concentrations of zinc in detached condition

Zinc (μM)	% Apoptosis								
	0 h			12 h			24 h		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	0.91	1.40	29.00	23.83	21.30	32.00	25.00	26.54
5	1.00	1.09	1.01	30.00	25.00	23.97	43.00	28.43	34.71
10	1.00	0.98	1.57	37.00	38.54	36.69	46.00	52.70	50.00
25	1.00	0.95	1.80	41.00	51.54	35.87	61.00	45.39	51.56
50	1.00	0.87	0.91	43.00	63.97	50.00	65.00	55.31	52.87

Table A-5 The relative colony number and size of H460 cells for each experiment in anchorage-independent growth assay analyzed under a light microscope after 2 weeks

Zinc (μM)	Relative colony number			Relative colony size		
	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.00	1.00	1.00
5	0.80	0.78	0.82	0.77	0.66	0.81
10	0.71	0.73	0.76	0.61	0.53	0.68
25	0.49	0.49	0.51	0.56	0.42	0.50
50	0.38	0.43	0.39	0.45	0.35	0.36

Table A-6 The relative of survival associated protein in H460 cells for each experiment determined by Western blot analysis after treatment with non-toxic concentration of zinc at 12 h after detachment

Zinc (μM)	Relative protein level								
	pAkt/Akt			pERK/ERK			Cav-1		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5	0.69	0.69	0.73	0.96	1.02	1.06	0.85	0.80	0.85
10	0.56	0.52	0.49	0.93	0.94	0.97	0.68	0.57	0.60
25	0.48	0.38	0.46	0.96	0.98	0.95	0.48	0.41	0.42
50	0.42	0.25	0.32	0.92	0.92	0.93	0.42	0.41	0.38

Table A-7 The relative of anoikis-associated protein in H460 cells for each experiment determined by Western blot analysis after treatment with non-toxic concentration of zinc at 12 h after detachment

Zinc (μM)	Relative protein level								
	Mcl-1			Bcl-2			Bax		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5	0.97	0.99	0.97	0.96	0.96	0.95	0.97	0.98	1.03
10	1.02	0.95	1.01	0.98	0.94	0.98	0.93	0.98	1.03
25	0.98	0.96	1.01	0.94	0.92	0.98	0.98	0.94	1.02
50	1.03	0.96	1.00	0.96	0.96	0.97	0.99	0.96	0.92

VITA

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