COMBINATION OF CAPSAICIN AND RESVERATROL INDUCES APOPTOSIS MEDIATED THROUGH DE NOVO LIPOGENESIS INHIBITION IN HEPATOCELLULAR CARCINOMA CELL LINE

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ABSTRACT

Various combination of chemotherapeutic drugs have been used for treatment of cancers in order to reduce toxicity in non-cancer cells. However, these therapeutic approaches still have nonspecific effect to cancer cell. New anticancer agents with selective to cancer cells have been remarkably considered. Many cancers exhibit overexpressed *de novo* lipogenesis (DNL) pathway while normal cell shows relatively less expression. DNL pathway exerts an important function for cancer growth and proliferation. Capsaicin and resveratrol, phenolic compounds from natural plant extracts, have strong anticarcinoma effects with lack of cytotoxic to normal cell. Therefore, present study aimed to focus on effects of combined capsaicin and resveratrol recipe on promotion of DNL inhibition leading to apoptosis in Hep G2 cells. Combined treatment increased cell apoptosis via reduction of mitochondrial membrane potential Furthermore, combined treatment decreased fatty acid synthase (FASN) expression. We therefore suggest that DNL pathway appears to target for apoptosis induction of combined treatment in Hep G2 cells. Taken together, combination of capsaicin and resveratrol exerts a potential and selective effect on cancer therapy through inducing apoptosis mediated via DNL pathway inhibition.

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CHAPTER I

INTRODUCTION

Rationale of the study

Hepatocellular carcinoma (HCC) is a primary cancer of hepatocytes that most commonly develops from alcoholic liver cirrhosis or chronic infection by hepatitis B Virus and hepatitis C virus (1). HCC is the fifth most prevalent malignant tumors but the second most leading cause of cancer death from worldwide. Chemotherapy such as doxorubicin is common to treat HCC. However, chemotherapy also has side effect to damage normal cells (2). Therefore, the new effective therapies based on selective approaches are challenged.

De novo lipogenesis (DNL) metabolic pathway synthesizes fatty acids from precursor carbon source, citrate. This pathway starts from citrate produced from tricarboxylic acid (TCA) cycle exits the mitochondrion and converted into acetyl-CoA by ATP-citrate lyase (ACLY). Acetyl-CoA carboxylase (ACC) acts on this acetyl-CoA yielding malonyl-CoA. Then, fatty acid synthase (FASN) produces the production of long chain saturated fatty acid (LCFAs), 16-carbon palmitate from malonyl-CoA (3). LCFAs can be modified to complex fatty acids that can be used as building blocks for cell membrane biosynthesis (4). Moreover, LCFAs can be converted to FA-acyl CoA by acyl-coA synthase (ACS). FA-acyl CoA, palmitate is subsequently transfer acyl group to L-carnitine by carnitine palmitoyltransferase-1(CPT-1) activity. Then, product, palmitoylcarnitine is transported to mitochondria for β-oxidation to provide energy (4-7). Nowadays, it has been reported that DNL pathway is focused to be a new target of therapeutic agent for cancer. Products of DNL, LCFAs is produced by overexpressed enzyme of DNL in various cancers (4). LCFAs are decreased by ACC inhibition can reduce proliferation and induces apoptosis in human glioblastoma cells (8). Thus, DNL pathway plays an important role for cancer proliferation and is a potential target for cancer therapy. Inhibition of LCFAs by FASN inhibitor causes malonyl-CoA accumulation leading to inhibits CPT-1 activity. Inhibition of CPT-1 can up-regulate ceramide level which induces pro-

apoptosis gene resulting to apoptosis in breast cancer cells (9). Moreover, decreased DNL by capsaicin doesn't have effect in normal cells (10). It suggests that this DNL pathway is a selective target for cancer therapy.

Both of capsaicin and resveratrol are major components in chili pepper and grapes, respectively (11-12). Both plants are widely planted and world widely consumed. Capsaicin (trans-8-methyl-N-vanillyl-6-non-enamide) has been shown to have several effects, including antioxidant, anti-inflammatory, and anti-obesity (11). Moreover, capsaicin has potent antitumor effect via apoptosis pathway in both *in vitro* and *in vivo* in several cancer models (13-15). However, the mechanisms underlying capsaicin-induced apoptosis have been proposed to involve many pathways, such as increasing intracellular Ca^{2+} (16), decreasing mitochondrial membrane potential ($\Delta\Psi m$) and increasing ROS generation (17). Recently, capsaicin has been reported to exhibit apoptosis via suppression of DNL pathway in Hep G2 cells (10). Resveratrol (3,5,4-trihydroxy-trans-stilbene) is compound that can induce apoptosis via many mechanisms, including increasing ROS generation and inducing AMP-activated protein kinase (AMPK) signaling pathway (18). It has been presented that apoptotic effect of capsaicin and resveratrol have not effect on normal cells, suggesting selective effect on cancer cells (10, 19-20). Combination of cancer therapeutic agents, including capsaicin and resveratrol have been growing interest because of their synergistic effect and reduce the risk of resistance and toxicity caused by using higher doses of single compounds (21-24). Research studies of co –treatment of FASN inhibitors, cerulenin and C75 have been reported that reduce chemotherapy resistance of docetaxel via extenuated HER -2/ neu (c- erb B-2) oncogene in breast cancer cells (25-26). Capsaicin combined with docetaxel has represented synergistic anticancer effect on inhibition of DNL via inhibited ACC by AMPK activation and PI3K/AKT/mTOR signaling inhibition in prostate cancer cells (27). Combination treatment of resveratrol and rapamycin has been shown that anticancer effect associated with DNL via inhibition of PI3K/AKT/mTOR signaling in of bladder cancer cells (28). Furthermore, the previous findings have shown the combination effect of capsaicin and resveratrol expressed synergistic anticancer effects on cell proliferation suppression of and induces apoptosis via ROS generation in many cancers (29-31), but their anticancer effect has not been shown on DNL. Thus, combined capsaicin and resveratrol

targeting suppression of DNL synthesis appears to be a selective and an attractive therapeutic strategy in cancers.

In the present study, we hypothesized that combination of capsaicin and resveratrol promotes DNL inhibition leading to inducing apoptosis in Hep G2 cells. The combined treatment has more efficiency to induce cancer death than each single treatment. Moreover, synergistic or additive anticancer efficiency of combined inhibition will be aimed to lower doses used of each single treatment.

Objectives

1. To investigate the effect of combination of capsaicin and resveratrol on cell proliferation and apoptosis in Hep G2 cells.
2. To investigate the effect of combination of capsaicin and resveratrol on de novo lipogenesis pathway in Hep G2 cells.
3. To investigate the effect of combination of capsaicin and resveratrol on ROS production in Hep G2 cells.

Research Scope

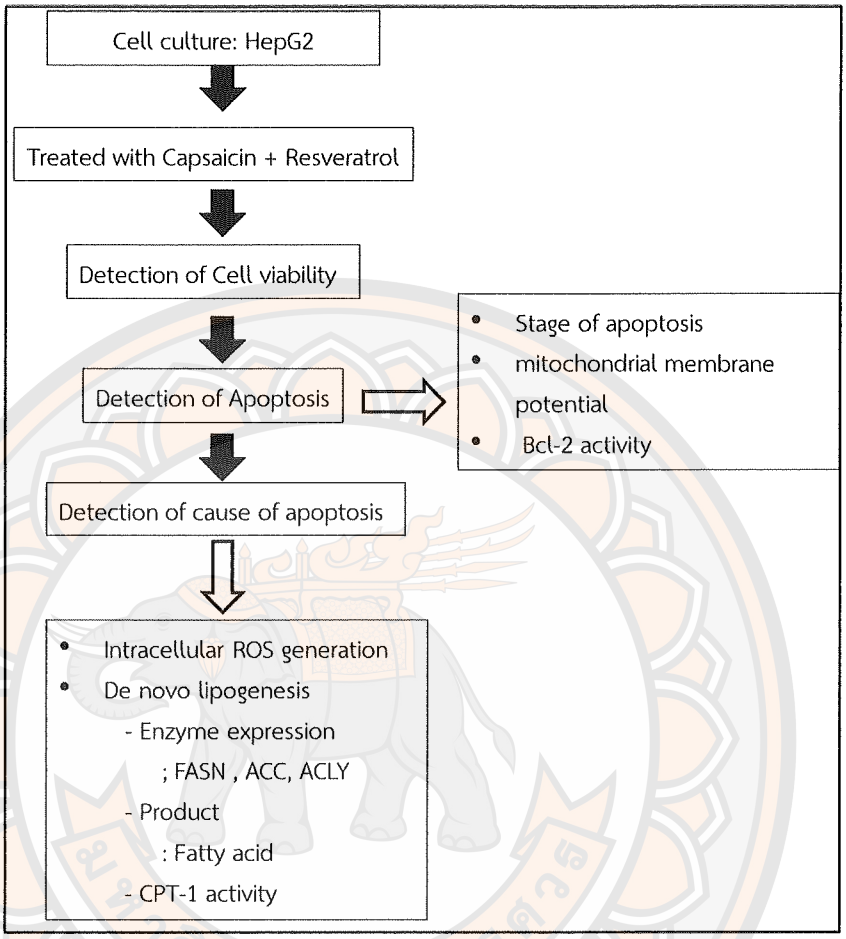

Figure 1 Scope of this study

Keywords

Capsaicin; Resveratrol; Apoptosis; *De novo* lipogenesis, Reactive oxygen species, Hep G2 cells

Research Hypothesis

Combination of capsaicin and resveratrol will inhibit DNL pathway leading to selectively inducing apoptosis in Hep G2 cells but not in primary human hepatocytes.

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Cancer

Cancer is a disease is exhibited DNA mutation leading to abnormal cell growth and uncontrolled cell proliferation. There are many risk factors that can induce DNA damage and DNA mutations such as tobacco, carcinogens, viral infections, excess bodyweight, etc. Growing up cancer can interfere with the work of normal cells in that organ and it also can invade and spread to other parts of the body through blood circulation and lymphatic system. As shown in figure 2, the worldwide cancer incidence is 17,036,900 new cases per year estimated number by world area. Cancer is a group of diseases that is the 2nd leading causes of death following cardiovascular disease in worldwide. Number of new cancer cases and cancer death in men have more number then women, as shown in figure 3 (32).

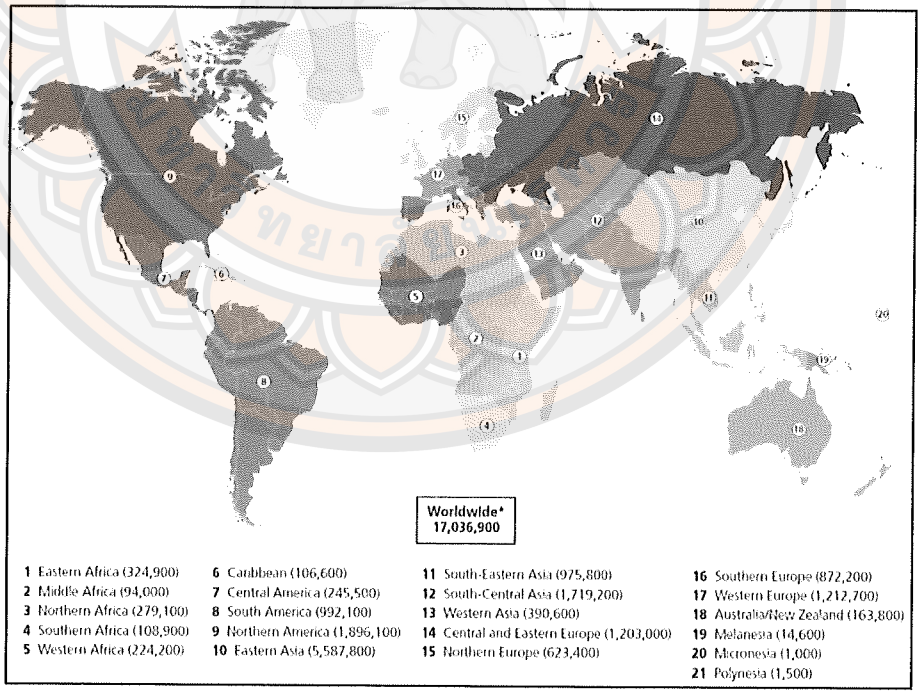

Figure 2 The world area of new cancer cases in 2018

Source: Society, 2019

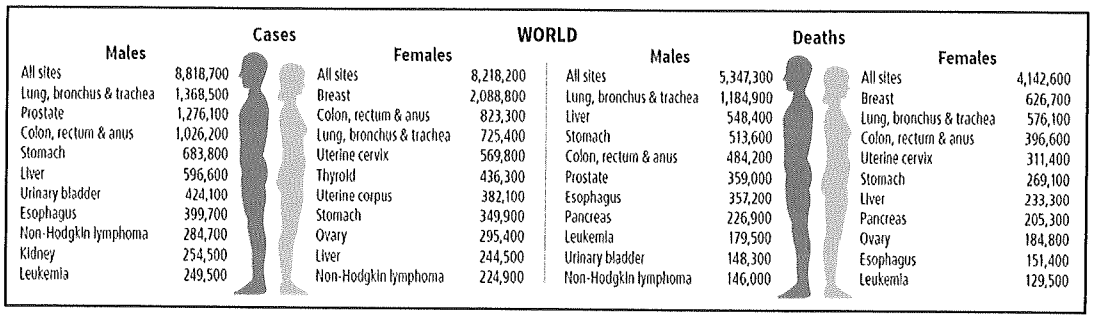

Figure 3 Number of new cancer cases and deaths in each sites of men and women

Source: Society, 2019

Liver cancer

Normally, liver is an organ is located at the right hand in the abdominal cavity, under a diaphragm and upper a stomach of body. Liver is one of the important organs that it has various functions. It gets blood of the hepatic portal vein that carries nutrients from the intestine to break down nutrients and metabolize drugs to form that the body uses and metabolizes toxic to nontoxic. Liver also products bile to break down fat in the small intestine. Liver produces red blood cells in 2 months of the fetus, globulin proteins, albumin proteins, and some substrates protein about blood clotting. Moreover, liver is a storage source of energy in glycogen form that balances glucose level via insulin and glucagon enzyme activity (33).

Liver cancer is cancer originating from liver with incidence is 596,600 new cases in men and 548,400 new cases in women per year. The violence of liver cancer is 2nd in men, 6th in women leading causes of death with 548,400, 233,300 cases, respectively (32, 34). Their type can be divided from cell types following: HCC is the most common liver cancer that is a beginning from hepatocyte, accounting for approximately 70% of all liver cancer, cholangiocarcinoma is bile duct cancer that is developed from cell of bile duct that it is type accounted for approximately 10-20 % of all liver cancer following HCC, liver angiosarcoma is rare liver cancer type that it have a beginning from blood vessels of liver, and hepatoblastoma is very rare type that develops in children with cells is similar to fetal liver cells (35).

Hepatocellular carcinoma (HCC)

HCC is predominantly primary malignancy that accounted approximately for 65% of all liver cancer and it is common in males with a male: female ratio of 2.4 in its worldwide distribution. The age of HCC patients is usually between 30 and 50 years. HCC incidence is predominant in Asian countries including China, Mongolia, Sub-Saharan Western, Eastern Africa and particular Southeast Asia (36-37). Thai Association for the Study of the Liver (THASL) has been shown HCC is common found 1st in men and 3th in women in Thailand which 80-90 percent of HCC have cirrhosis. The survival trend is 5-year in HCC patients that it has improved by > 60% from 1975. It is divided 2 pattern: HCC start from single tumor that growing up and then spread to other part of liver, and HCC start from many tumor nodules. This pattern is common caused by developed from chronic liver damage and cirrhosis (36).

Infection with hepatitis B virus or hepatitis C virus is the most common risk of HCC. Hepatitis B virus with genotype A-H is double stand DNA virus. Hepatitis B virus can transfect via blood transfusion, injection and sex. Hepatitis B virus is cause of chronic hepatitis leading to cirrhosis and develop to HCC in 8-10 years. Infection of Hepatitis B virus is only risk factor of HCC without evidence of cirrhosis. Hepatitis C virus is single standard RNA virus with various genotype. The 80% of patient develop to chronic hepatitis and 20% to cirrhosis. Hepatitis C virus infection develop to HCC with the evidence of cirrhosis. Alcohol consumption increase risk of Hepatitis C virus developing HCC. The both of hepatitis B and C virus infection increase risk of cirrhotic patient to develop to HCC. Cirrhosis is scarring of the liver form infection of hepatitis B and C virus and excessive drinking that it can developed to be HCC. Moreover, diabetes and obesity are nonalcoholic fatty liver disease which fat accumulation in the liver. Diabetes mellitus affects to glucose metabolism in liver and can enhance to liver cell damage, chronic hepatitis and HCC. Aflatoxins, cigarette smoking, metabolic and genetic disorders may increase the risk of developing HCC (38).

HCC may have symptom of liver cirrhosis that liver decompensated including; ascites, condition of abdomen have fluid buildup, splenomegaly that enlargement of the spleen with size or weight. Portal hypertension condition of high pressure in portal vain that is blood vessel that carries blood from other organ to

detoxifies chemicals and metabolizes drugs, Jaundice condition that patient have yellow of skin, eyes and mucous membrane which it is caused by high level of bilirubin in body Hepatocellular carcinoma develop mild to modulate patient may have pain around upper abdomen and eating food less than usual, fatigue, weight loss, and diarrhea, patient have mass around the upper abdomen, and severe patient will have intraperitoneal hemorrhage, tumor rupturing, leading to bleeding into the peritoneum and moreover, severe symptom of patient that not able to clean toxic and toxic travel to brain leading to impaired memory and may have loss of consciousness that is called hepatic encephalopathy (39).

HCC do not appear symptom in early stage. Therefore, who have a high risk to be HCC should enter into a surveillance program. The surveillance program is ultrasound examination every six months. People who doesn't enter into surveillance program. They have Alpha-Fetoprotein Tumor, AFT level may indicate liver cancer. Doctors will usually order a computed tomography (CT) scan is an x-ray test give images of your liver tumors about the size, shape, and location of any tumors, or Magnetic resonance imaging (MRI) like CT scans but use radio waves and strong magnets to look detailed images of soft tissues. MRI can help show that cancer spread to other parts of the body to detect HCC. Biopsy, it take sample of liver tissue to study under microscope to confirm HCC by needed is passed skin to liver tissue, it has risk of invasive biopsy. In addition, bone scan for look metastasis of cancer (40).

HCC treatments have many treatment forms, as shown in figure 4 including patients presenting with very early (stage 0) and early-stage diseases (stage A), which represent 20–30%, are suitable for curative treatments such as resection, liver transplantation, or local ablation with percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA). Patients with intermediate stage (stage B) receive a survival benefit from transarterial chemoembolization (TACE). Patients with advanced HCC that consists of macroscopic vascular invasion (portal vein invasion), extrahepatic spread (lymph nodes and metastatis) or cancer-related symptoms (performance status 1–2) have a proven first line treatment with sorafenib. Patients with terminal stage (stage D), which represents that 10–20% receive symptomatic treatment (1).

However, resection treatment is limited that it has high recurrence. It has been reported that many patients get this treatment will have a recurrence of HCC elsewhere in liver. In addition, resection also has limitation for HCC patient caused by cirrhosis which it will not tolerate to resection surgery. The liver transplantation is one option for HCC patient has less 5 cm tumor nodule without invasion. Symptomatic treatment, chemotherapy is drug treatment that uses powerful chemicals to kill fast-growing cells in your body that it has several side effects by it can damage both cancer and normal cells. Moreover, current treatment of HCC still is difficult with high resistance and poor prognosis which usually found at the late stage (41).

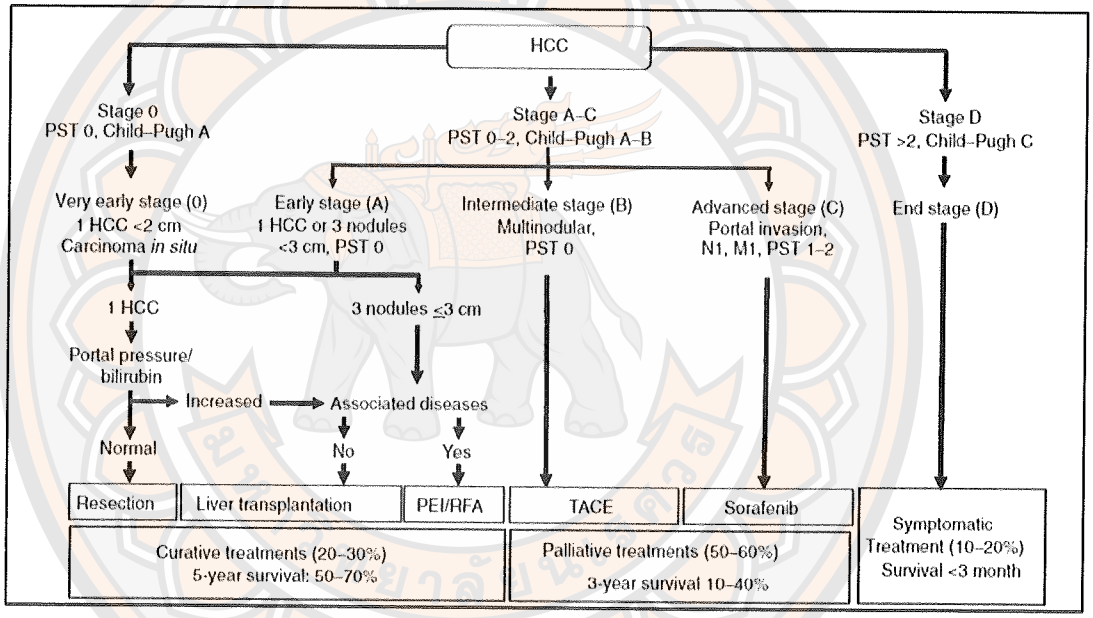

Figure 4 Treatment of HCC in various stages

Source: Au, & Frenette, 2015

Hallmarks of cancer

Hallmarks of cancer include sustaining proliferation signaling, evading growth suppressors, resisting cell apoptosis, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, promoting inflammation of tumor, gene mutation, evading immune destruction, and reprograming energy metabolism, as shown in figure 5 (42).

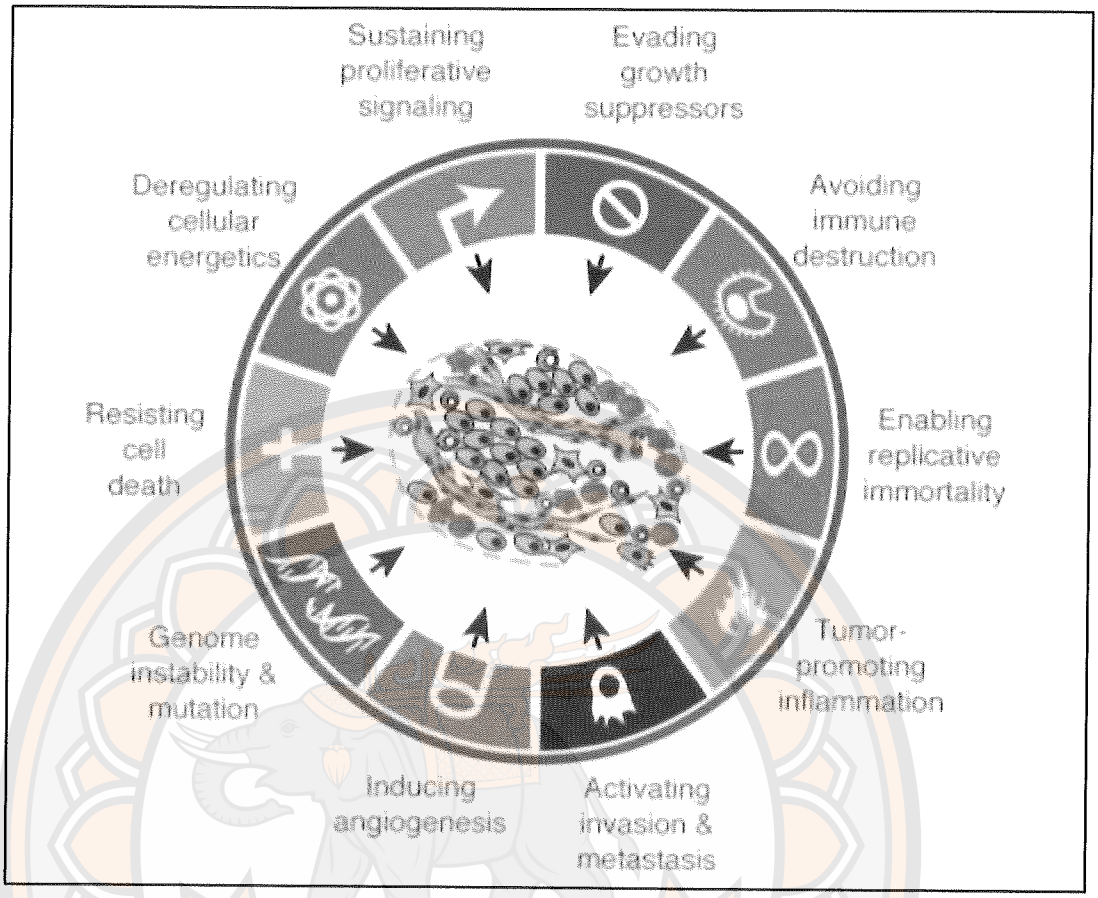

Figure 5 Hallmarks of cancer

Source: Hanahan, & Weinberg, 2011

Energy production of normal cell

Normal cells of body have processing to synthesize adenosine triphosphate, ATP from uptake nutrients (sugar, lipid, protein) to support cellular activity. Glucose, single molecule of sugar that is main energy source of body. Glucose is absorbed at small intestine enter to blood circulation. Glucose is uptake to cell by insulin hormone activity. Insulin hormone is synthesized by endocrine cells, beta cells in the regions of islets of Langerhans of the pancreas. Insulin hormone release to blood circulation when beta cells are induced from glucose level is high in blood. Mechanism of insulin hormone is it will bind and activate tyrosine kinase receptor located at cell membrane. Then active tyrosine kinase receptor will enhance the PI3K/AKT/mTOR signaling

pathway resulting translocation of glucose transporter to membrane and uptake glucose into cytosol. Glucose in cytosol are entered to glycolysis pathway for starting ATP generation. Glycolysis pathway, a glucose is converted to pyruvate 2 molecule. The pyruvate enter into mitochondria and then it is oxidized to acetyl-coA by pyruvate dehydrogenase complex (PDC) located in mitochondria. If cell has abundant oxygen, acetyl-coA enter to Krebs cycle, (citrate acid cycle) to produce ATP and the reduced electron carriers (nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide, ($FADH_2$)). NADH and $FADH_2$ are used to generate ATP by oxidative phosphorylation (OXPHOS) occur in mitochondria cristae. Total yield of ATP synthesis is 38 ATP/ glucose 1 molecule. The overall reaction can be expressed in figure 6 (43-44). In normal cells, OXPHOS contributes to 70% of the ATP-generating metabolism while fatty acid synthesis is exclusively generated from exogenous transported fatty acids derived from nutritional consumption (45).

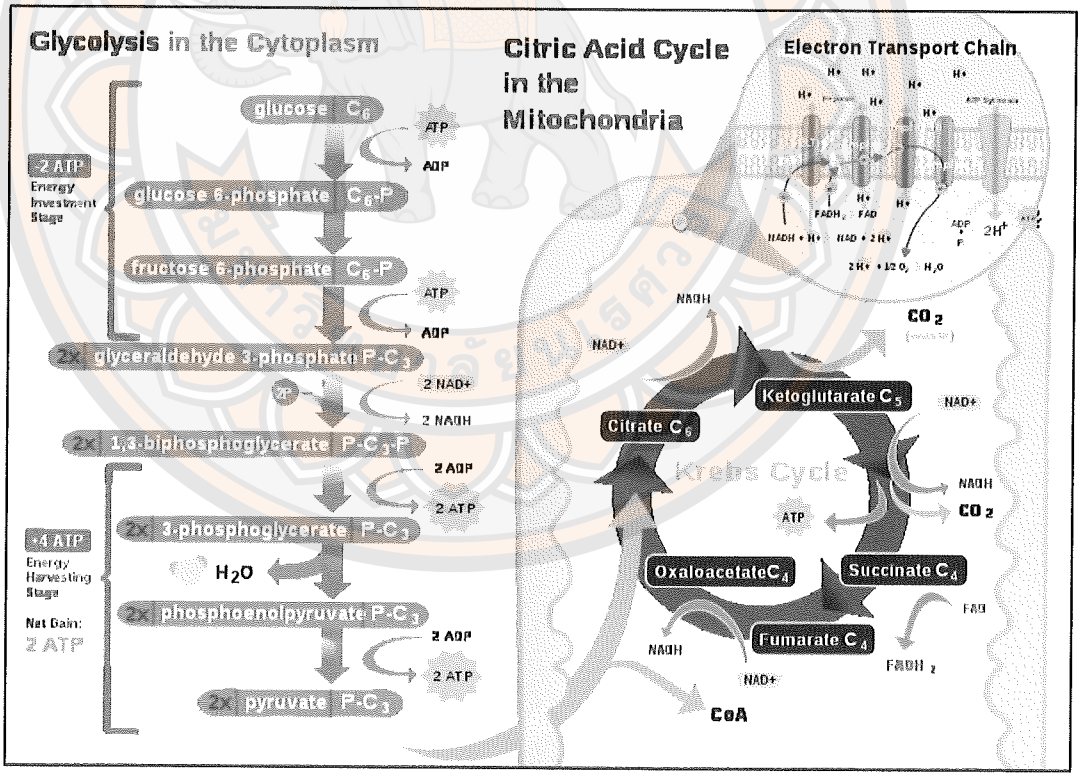

Figure 6 The overall reaction of glucose metabolism of normal cell

Source: Cheng, Geng, Cheng, & Guo, 2018

Energy metabolism in cancer

Cancer cells up-regulate the hypoxia inducible factors (HIFs) to control the expression of transformed genes of glycolysis and OXPHOS pathways that is caused by reduction of vascular supply and the deprived environment of nutrition (46). Cancer is shown reprograming of major energy metabolism from oxidative phosphorylation (OXPHOS) to rely on aerobic glycolysis that is called Warburg effect. This leads to cancer have up-regulation of glycolysis via expression of glucose transporters (GLUT) membrane pumps involved in glucose absorption, and up-regulation lactic acid fermentation via increased expression of lactate dehydrogenase A subunit that promotes the metabolic shift of pyruvate transformation to lactate for energy although in those cancer cells present abundant oxygen that called aerobic glycolysis. The lot of pyruvate from metabolism of glucose in glycolysis pathway enter into lactic acid fermentation approximately for ~85% and enter into mitochondria matrix approximately for ~5%, as shown in figure 7 (47).

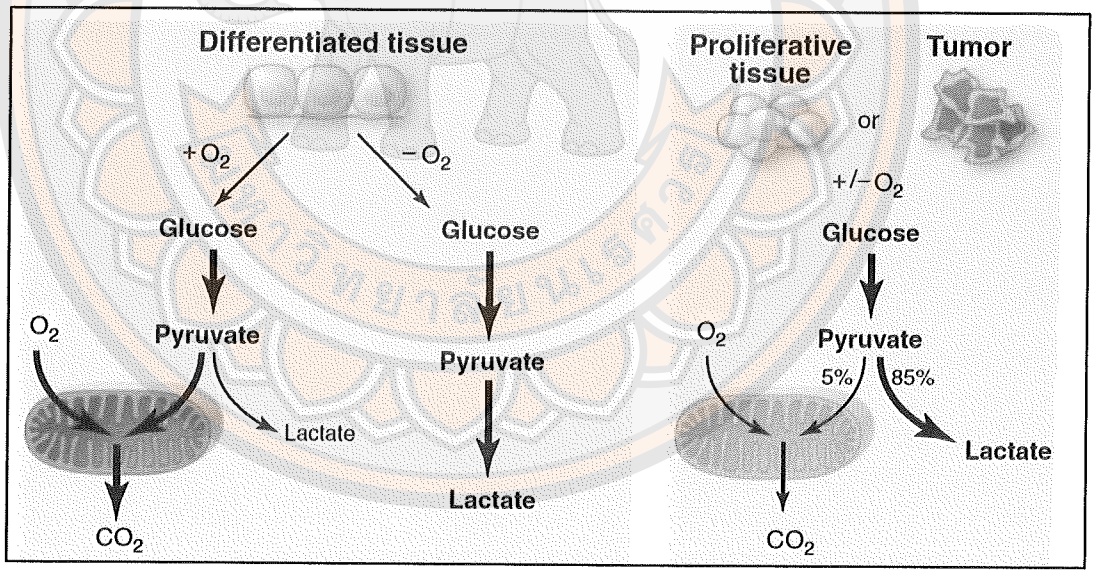

Figure 7 The Warburg effect

Source: Zhang, Zhang, Hu, & Tam, 2015

In normal cells, OXPHOS contributes to 70% of the ATP-generating metabolism while fatty acid synthesis is exclusively generated from exogenous transported fatty acids derived from nutritional consumption. In contrast, the alteration of the metabolic pathway of those cancers, the translocation of the carbons from OXPHOS, citrate is entered to be precursor for the de novo synthesis of LCFAs in DNL pathway that is up-regulated to synthesize biomolecules to support rapid proliferation in cancers that becomes predominant for controlling the cellular function (47). Citrate is transported from the CTP that it is increased in cancer (48) and then it is converted to acetyl-CoA by ACLY. Inhibition of citrate transporter decrease activity of DNL to produce LCFAs contributing suppressed proliferation and induced apoptosis in HCC (49). ACC acts on this acetyl-CoA yielding malonyl-CoA. Then, FASN produces the production of LCFAs from substrates, acetyl-CoA, malonyl-CoA, and a reducing agent NADPH. The most abundant LCFAs from DNL is palmitatic acid, 16-carbon palmitate from malonyl-CoA (3). LCFAs can be modified to complex fatty acids that can be used as building blocks for cell membrane biosynthesis (4). LCFAs can be converted to FA-acyl CoA by ACS. FA-acyl CoA, palmitate is subsequently transfer acyl group to L-carnitine by CPT-1 activity. Then, product, palmitoylcarnitine is transported to mitochondria for fatty acid oxidation (FAO), β-oxidation to provide energy (figure 8) (7). Cancer has been showed that fatty acid oxidation (FAO) in mitochondria is important for its survivals. The CPT-1 inactivation by CPT-1 knockdown and etomoxir treatment can inhibits cell growth and ATP production through repressing FoxO transcription factors in ovarian cancer both in vitro and in SCID mice (50). The enzymes in the DNL pathway are up-regulated or constitutively expressed in most types of cancer cells (51). The high expression of FASN has been reported in lipogenic tissue, such as the liver. However, in most normal tissues has been shown that the expression and activity of FASN is low expression and undetectable (45). Several studies have demonstrated that suppression of FASN promotes apoptosis in cancer cells but it is unable to suppress proliferation of normal cells (52-53). Therefore, DNL pathway is focused to be a selective target of therapeutic agent for cancer. Previous studies of capsaicin on DNL, It has been shown weather capsaicin suppressed *de novo* synthesis of fatty acid through selectively inhibited lipogenic enzyme FASN activity and expression of protein which correlated

with apoptosis in HCC cell lines (10). Capsaicin induces TRPV1 receptor that inhibits DNL in Hep G2 cells through CaMKKβ/AMPK activation, resulting AKT/mTOR and SREBP1 inhibition (54-55). The major lipogenic genes, ACLY, ACC and FASN, were all significantly overexpressed both mRNA and protein and they were suppressed by resveratrol in ductal carcinoma both *in vitro* and *in vivo*. These study also confirm that SREBP1 inhibition by resveratrol is upstream pathway of these lipogenic genes regulation and induce cell death with silenced SREBP1 gene (56-57).

Many studies has reported that effect of LCFAs inhibition induced apoptosis in cancer. Inhibition of activity of DNL to produce LCFAs and induce apoptosis in hepatocellular carcinoma (49). Blocking FASN enhances cell growth arrest and apoptosis in breast cancer (58-59). Decreased LCFAs induce alteration of lipid composition of membrane that reduces proliferation and induces apoptosis in the glioblastoma, lung, astrocytoma, and leukemia cancer cell (8, 60). FASN inhibition by cerulenin and orlistat lead to induce mitochondria-dependent apoptosis via direct NADPH accumulation not ROS generation which is confirmed which FAS inhibition still induce apoptosis in breast cancer without ROS generation by NADPH oxidase inhibitor and ROS scavenger in breast cancer cells (61). The mechanism of inhibition FASN can induce apoptosis via decreasing LCFAs lead to high accumulation of malonyl-CoA which involved inhibition of the b-oxidation of DNL by suppressing CPT-1 transported palmitoyl-CoA into the mitochondrial matrix for β-oxidation (10, 62). Elevated ceramide is generated from palmitoyl-CoA accumulation in cytoplasm lead to enhancing apoptosis (63). Elevated ceramide is generated from palmitoyl-CoA accumulation in cytoplasm following CPT-1 activity inhibition lead to enhancing apoptosis by inducing pro-apoptotic genes DAPK2 and BNIP3 expression in breast cancer cells (53, 64) and colon cancer cells (63). Ceramide accumulation can lead to suppression of Bcl-2 anti-apoptotic genes leading to cell metabolism impairment and apoptosis (65-66). Disruption of mitochondrial menbrane is activated by oligomerization of Bax. Normally, it is inhibited to oligomerization with interacting Bcl-2 (67). Decreased Bcl-2 protein level lead to reduce interaction between Bax and Bcl-2 and increase apoptosis (68). Bax tanslocates to insert mitochondrial membrane and increase mitochondrial permeabilization resulting reduced $\Delta\Psi m$. The $\Delta\Psi m$ reduction enhances cytochrome c that localized between the inner and outer mitochondrial

membranes is released to cytosol and then it combines with Apaf–1 and procaspase-9 to produce apoptosome. Then, activated caspase-9 cleaves caspase-3 followed by apoptosis (4).

The signaling pathways play an important on DNL pathway

Cancer has been found overexpression of receptor tyrosine kinases (RTKs) several human cancers (69). RTKs can activate PI3K/ AKT/ mTOR signaling pathway lead to up-regulation of glycolysis via activating glucose transporter (GLUT) and hexokinase 2 enzyme (70). PI3K/ AKT/ mTOR signaling also can enhance the SREBPs resulting to overexpression of DNL in cancer (71). It has been shown that SREBPs is transcription factor of important enzyme DNL pathway. SREBPs present in 3 from including SREBP1a, SREBP1c and SREBP2 protein binding with SREBP cleavage-activating protein (SCAP). SREBP/ SCAP complex is inactive by SCAP binding Insig located at endoplasmic reticulum. Glucose can activate dislocation of SREBP/ SCAP from Insig. SREBP/ SCAP translocate from endoplasmic reticulum (ER) to Golgi apparatus. Then proteinase in Golgi will cleave N-terminal domain active form of SREBP. Then N-terminal domain active enter into nucleus to induce protein transcription of ACC, ACLY and FASN important enzyme in DNL (72-73). It has reported that SREBPs have overexpressed and associated with growth in many cancers including prostate cancer, breast cancer and liver cancer (74-76). The proposed pathways of signaling of DNL in cancer cell can summarize in the below at figure 8 (44).

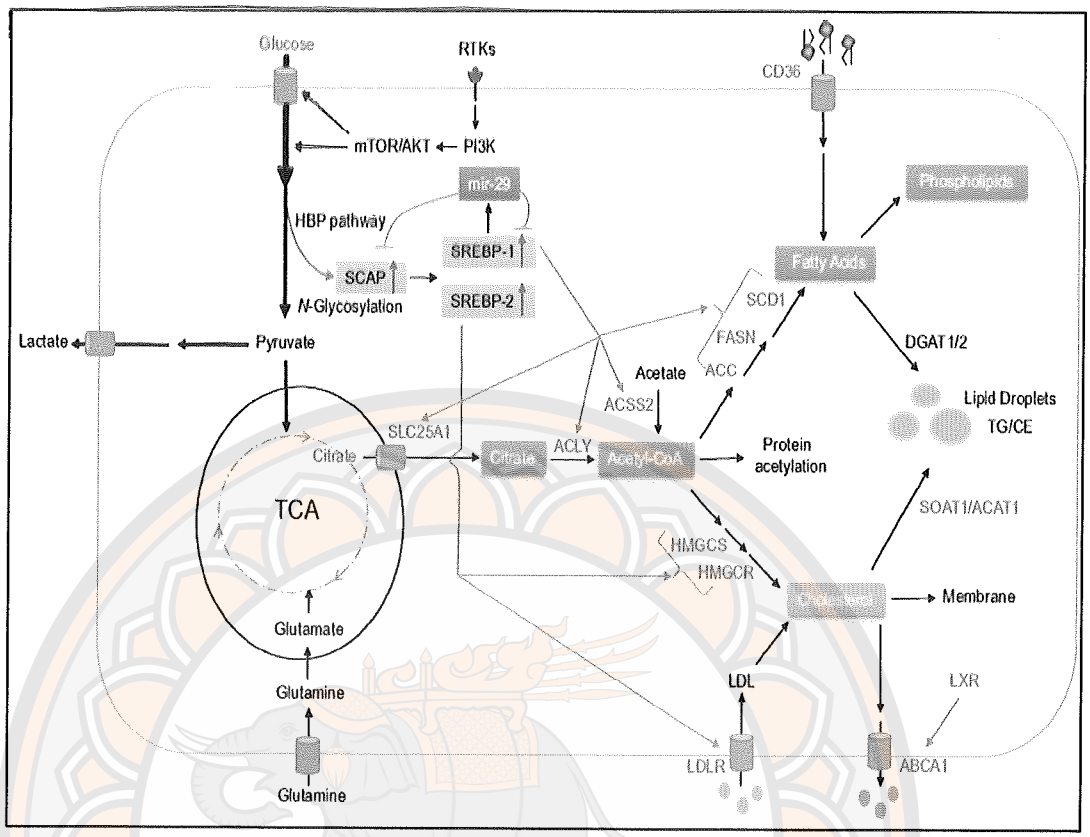

Figure 8 The signaling pathways of DNL in cancer cell

Source: Cheng et al., 2018

Mechanism of cell death

The paths of cell death are apoptosis, necrosis, and autophagy. These are different characteristics of cell morphology and biochemical properties. They can be explained in the below picture, as shown in figure 9.

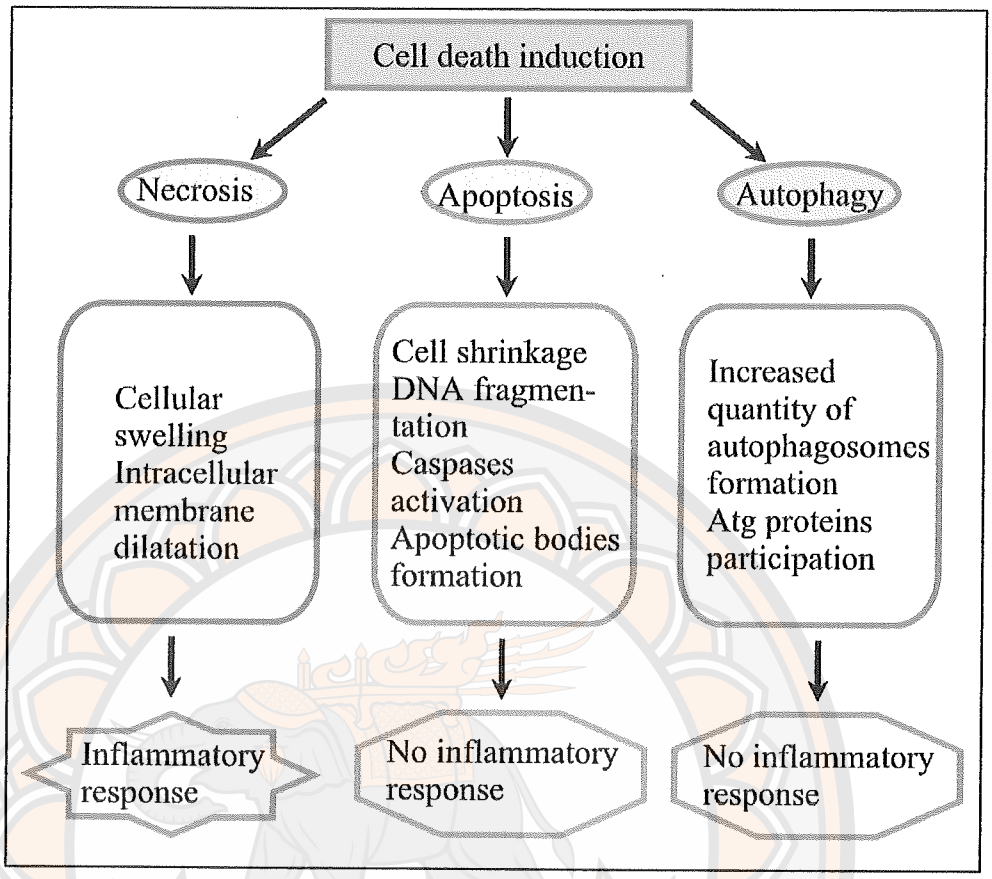

Figure 9 Path of cell death

Source: Jan, & Chaudhry, 2019

Apoptosis pathway

Apoptosis pathway is the most common of program cell death without inflammatory response and controlled mechanism of the development of an organism. They can be divided to 2 pathway of activation including extensive pathway and intensive pathway.

The extrinsic pathway is triggered when TRAIL binds to TRAIL R1 or TRAIL R2. Receptor dimerization, along with the subsequent oligomerization and clustering of the receptors, leads to the recruitment of the adaptor protein Fas-associated protein with death domain (FADD). FADD allows the recruitment of the inactive pro-caspase-8 or caspase-10 via a shared death effector domain (DED) leading to the formation of the death-inducing signaling complex (DISC). Depending

on the cell type, apoptosis activation through the extrinsic pathways may or may not depend on the intrinsic pathway. For example, in type I cells, upon DISC activation, sufficient caspase-8 is activated and, in turn, directly activates the effector caspases (caspase-3, -6, -7) leading to the execution of apoptosis FLICE-inhibitory protein (c-FLIP) shares structural homology with pro-caspase-8 and possesses a death effector domain that lacks protease activity. In specific conditions, its structure allows c-FLIP to be recruited to the DISC where it inhibits the processing and activation of pro-caspase-8. Although many isoforms of c-FLIP have been identified, only three are expressed in human cells. They consist of two short variants, c-FLIPS and c-FLIPR, and a long splice variant, c-FLIPL. Both c-FLIPL and c-FLIPS contain two DEDs and compete with pro-caspase-8 for association with FADD. Depending on the level of c-FLIPL expression, its function at the DISC will vary. When present in high amounts, c-FLIPL will exert an anti-apoptotic effect at the DISC. When present in low amounts, it may heterodimerize with caspase-8 at the DISC and promotes apoptosis. The c-FLIP is thus seen as a major inhibitor of the extrinsic pathway of apoptosis. In so-called type II cells, less caspase-8 is activated at the DISC and efficient apoptosis requires further signal amplification via the intrinsic or mitochondrial pathway. This is achieved by caspase-8-mediated Bid cleavage to generate a truncated form of Bid (tBid) which subsequently engages Bax/ Bak to activate the mitochondria.

The intrinsic pathway is usually triggered in response to DNA damage, hypoxia or oncogene overexpression. As a sensor of cellular stress, p53 is a critical initiator of the intrinsic pathway. In response to cellular damage, p53 translocates from the cytoplasm to the nucleus where it promotes the transcription of pro-apoptotic members of the Bcl-2 family. Pro-apoptotic Bcl-2 family members Bax and Bak form pores in the outer mitochondrial membrane causing the release of cytochrome c and other apoptogenic factors such as apoptosis inducing factor (AIF) and SMAC/DIABLO into the cytoplasm. The released of cytochrome c, along with apoptosis protease activating factor-1 (APAF-1) and pro-caspase-9 form the apoptosome. Within the apoptosome, clustered pro-caspase-9 gets activated and cleaves downstream effector caspases, leading to the hallmark of apoptosis. The release of SMAC/ DIABLO from the mitochondria promotes apoptosis by binding to

and neutralizing members of the family of inhibitor of apoptosis proteins (IAPs), which can

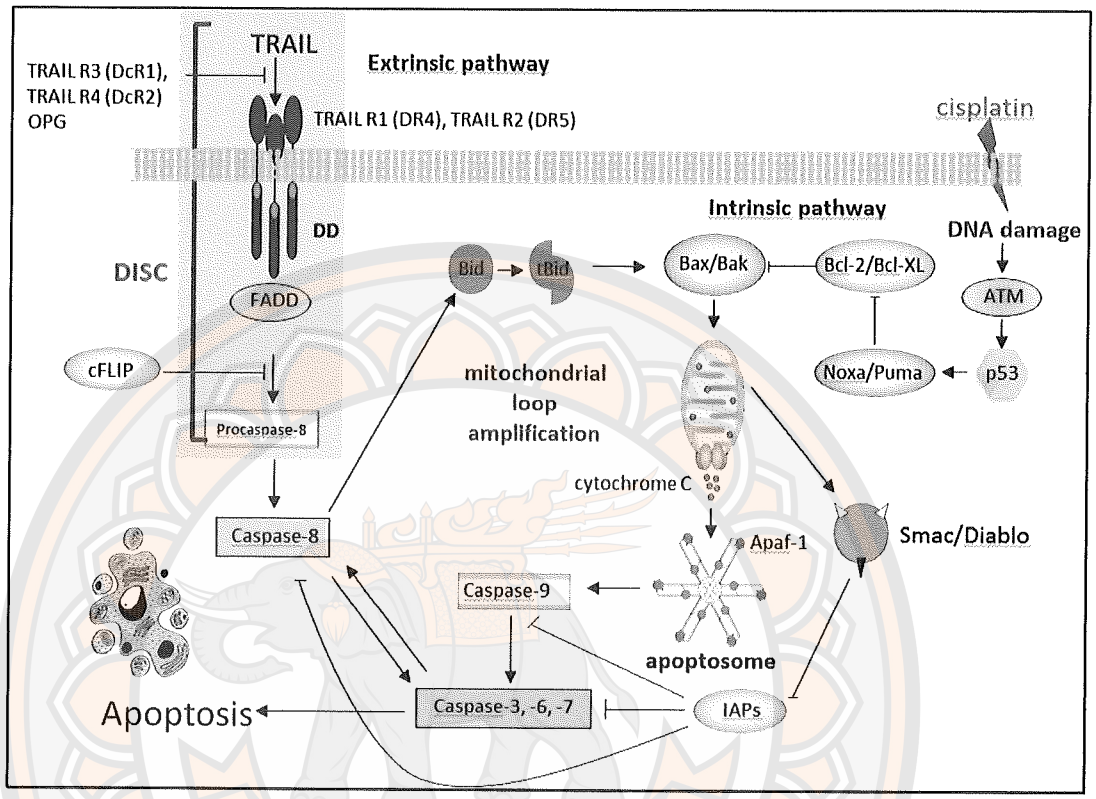

Figure 10 Apoptosis pathway

Source: Jan, & Chaudhry, 2019

Block caspase-3 activity through its baculovirus IAP repeat domains. Although the extrinsic and intrinsic pathways are activated by different mechanisms, these two pathways are interconnected. In type II cells, activated caspase-8 cleaves pro-apoptotic Bcl-2 family member Bid to form truncated Bid (tBid), which can then interact with Bax/ Bak. This interaction increases the release of cytochrome c from the mitochondria.

Thus, Bid provides a connection between extrinsic and intrinsic pathways (so called mitochondrial amplification loop). The reasons that determine whether tumor cells rely on type I or II signaling are not well understood but resistance has been attributed to dysfunction of different steps in the TRAIL-induced apoptosis pathway

and/or elevation of survival signals. In particular, it has been proposed that the levels of c-FLIP and XIAP relative to caspase-8 and SMAC/ DIABLO might be important determinants. The apoptosis pathways can be summarize in figure 10 (77-79).

Capsaicin

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is chemical compound group of capsaicinoids that can be found in plants of the capsicum family. Capsaicin is the most compound in chili pepper (contains 89% of capsaicin in placenta) (11). Capsaicin is natural extract that it can be extracted from chili pepper by many methods. There are solvent extraction, ultrasonic assisted extraction, microwave assisted extraction and etc. Capsaicin first was isolated from paprika and cayenne by Thresh in 1876 and was resolved molecular structure by Nelson and Dawson in 1923. Chemical formula of capsaicin is $C_{18}H_{27}NO_3$ (figure 11) and molecular weight is 305.40 g/mol. Capsaicin are colerless, hydrophobic, lipophilic, crystalline and odorless alkaloid (80).

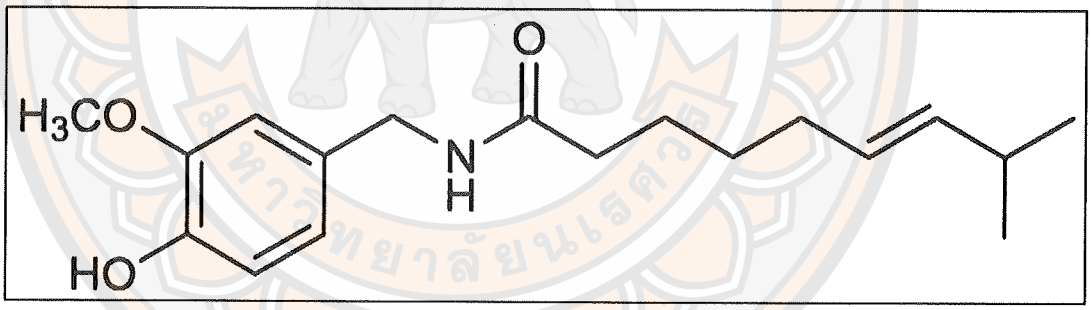

Figure 11 Chemical structure of capsaicin

Source: Chapa-Oliver, & Mejia-Teniente, 2016

Capsaicin is commonly and frequently consumed worldwide that is spice and food additive and it is also a drug for traditional medications. Over the past decades, research studies have been shown properties and applications of capsaicin are antioxidant, anti-inflammatory and anti-obesity as well as anticancer property (81). Anticancer effects of capsaicin are interested to study in various cancer including nasopharyngeal cancer, prostatic cancer , liver cancer, colorectal cancer, lung cancer,

and gastric cancer , while leaving normal cells unharmed(80, 82). Capsaicin mechanism induced cancer death are proposed in many pathway leading induce apoptosis in over 40 distinct cancer cell lines in both in vitro and in vivo cancer models (82). The mechanisms underlying of capsaicin-induced apoptosis has been proposed multiple signaling pathways and depend upon cell types. TRPV1 channel containing six transmembrane helices per subunit (S1 to S6) is expressed on sensory neurons as responsible to pain reception from temperatures >43 °C, low extracellular pH, protons, variety of endogenous lipids termed endovanilloids including capsaicin (figure 12) (83). Activated TRPV1 by capsaicin lead cations influx into call through the TRPV1 pore located in a hydrophobic stretch between transmembrane segments 5 and 6. Then cations influx into cell resulting action potentials are generated. These channels are more selectively with Ca^{2+} than Na^{+}, ratio 9.6:1 (83). Capsaicin directly bind to the TRPV1 and TRPV6 receptors. They are nonselective cation-channels overexpressed and related to enhance apoptosis in human cancer cells such as brain cancer, lung cancer , pancreatic cancer, breast cancer, skin cancer, renal cancer compared to normal cells (15, 84-88), including HCC (89). Capsaicin bind TRPV1 lead to induce apoptosis in cancer via multiple mechanisms, including ROS production such as superoxide and hydrogen peroxide that impairment of calcium homeostasis in ER and then leads to the reduction of $\Delta\Psi m$, activation of pro- apoptotic Bax, suppression of anti-apoptotic Bcl-2, and induction downstream caspase-3-activated apoptosis (90). Capsaicin similarly bind TRPV6 triggers apoptosis via calcium influx that leads to stimulate calpains, apoptotic regulating factor as a family of calcium-dependent intracellular cysteine proteases in human small cell lung cancer (SCLC) (85). Interesting, capsaicin has been shown in cancer models that it can increase TRPV1 and TRPV6 expression and this could potentially lead to excessive increase of intracellular calcium and trigger apoptosis (15). In contrast, it has been found that capsaicin induce apoptosis without TRPV1 receptor. They demonstrated that ROS generation and apoptosis induced by capsaicin is not a consequence of vanilloid receptor and calcium signaling by pretreatment of cells with BAPTA-AM, which chelate cytosolic free calcium, did not prevent ROS generation or apoptosis in transformed T-cells (91). Capsaicin also induced apoptosis which is proposed in DNL pathway. Capsaicin has been shown to induce apoptosis thought suppresses FASN

expression of Hep G2 in vitro model (10). It has been showed that the combination of capsaicin with resveratrol caused synergistic anticancer efficiency (92).

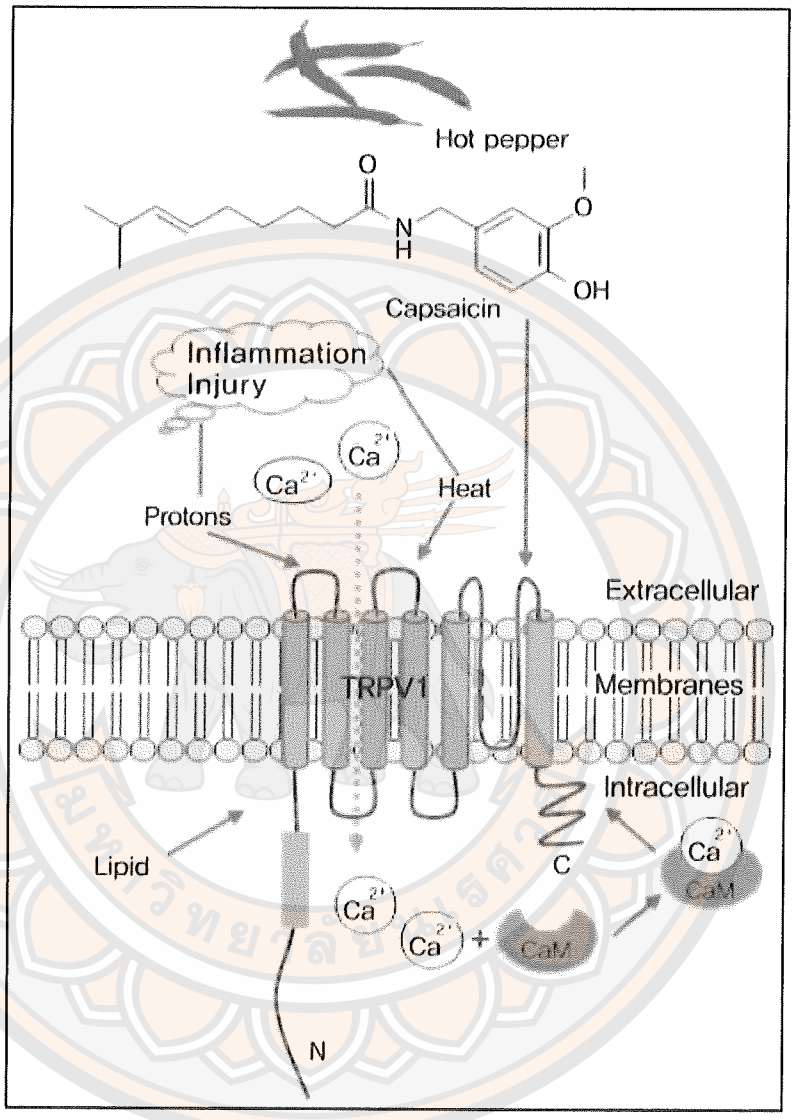

Figure 12 TRPV1 receptor of capsaicin

Source: Yang, & Zheng, 2017

Resveratrol

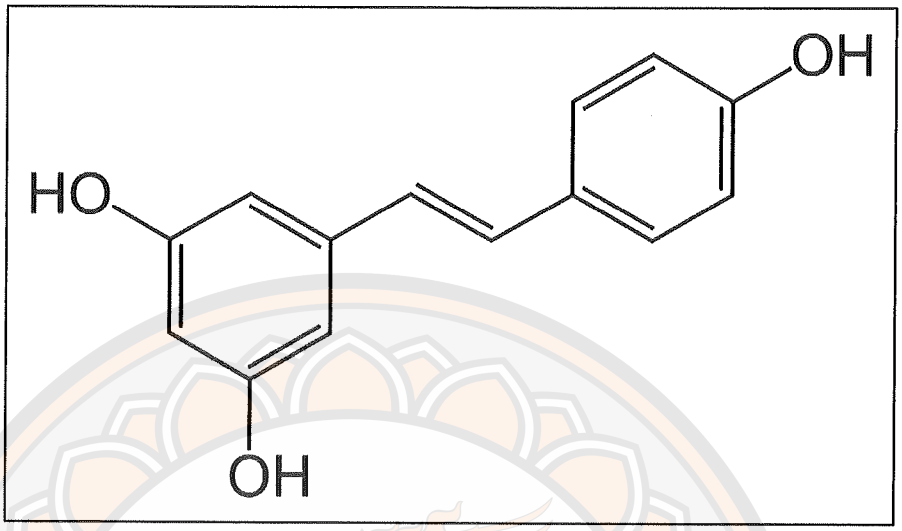

Figure 13 Chemical structure of resveratrol

Source: Berretta et al., 2020

Resveratrol (3,5,4-trihydroxy-trans-stilbene) is plant compound which acts like antioxidant agent. These compound can be found in red wine, some berries, grapes and peanuts. The source of resveratrol is mostly in skins and seeds of grapes and berries which are the top source of resveratrol. Resveratrol is natural polyphenol with stibene compound, its structure consists of 2 phenolic rings and double styrene bond form to 3,5,4'-Trihydroxystilbene and its molecular weight is 228.25 g/mol (figure13). Resveratrol expresses two form of chemical structure which are cis- and trans- isomer. However, trans-isomers is stable nature form and more appears than cis isomer form. The many studies of resveratrol has been shown properties of decreasing blood pressure, increasing lifespan, protecting brain, increasing insulin sensitivity and suppressing growth and inducing cell death in cancer both in vitro and animal model. Nevertheless, its high dose may affect to increase bleeding. Moreover, these compound also are safe well for the most people (12, 93). Many studies on resveratrol has been shown that has anticancer effect in various cancers such as colon cancer, liver cancer, bone cancer, skin cancer, prostate cancer and so forth (93). Resveratrol is

compound that can induce apoptosis via many mechanisms, including increasing ROS generation and inducing AMPK signaling pathway (18).

It has been presented that apoptotic effect of capsaicin and resveratrol have not effect on normal cells, suggesting selective effect on cancer cells (10, 19-20). Thus, combined capsaicin and resveratrol targeting suppression of DNL synthesis appears to be a selective and an attractive therapeutic strategy in cancers.

Reactive oxygen species (ROS)

ROS are free radical containing oxygen in molecule such as superoxide (O_2^-), peroxyl radical (ROO^-) and hydroxyl radical (OH^-). These molecule contain unpaired electron that they are reactive oxidized substances with molecular biological inner cells such as lipid, protein and DNA leading damaged molecules and causes many abnormalities within the human body such as cancer, cardiovascular diseases and human aging. Cells have ROS production in first O_2^- form. Mitochondria is main source of O_2^- that is generated via electron transport chain at complex-I and complex-III. Additionally, peroxisomes, plasma membrane, and cytosol have enzyme that produce O_2^- (figure 14). Normally, cells have low ROS level and have mechanism of ROS regulation including enzyme, antioxidant, and some vitamin (94).

The intracellular ROS level is elevated in cancer cells which is caused by increased metabolism to support rapid growth of cancer, same as antioxidant and cellular detoxification program are enhanced to scavenge and neutralize ROS to against oxidative damage (95). ROS levels below the ROS threshold or balance with antioxidant play an important role in promote tumorigenesis, metastasis, and angiogenesis in cancer cells but excessive accumulation of ROS at levels above the ROS threshold, ROS trigger apoptotic signals (96). It is not surprising that several natural dietary bioactive compounds that cause increased ROS levels have been shown to selectively target cancer cells. Several anticancer agents increase ROS production leading cause of apoptosis that was proposed in many pathways (96-97). Both capsaicin and resveratrol have been presented that they increase ROS production followed by a disruption of mitochondrial membrane potential and subsequent apoptosis in in many cancers (96, 98). Increasing ROS production was proposed in many pathways (97).

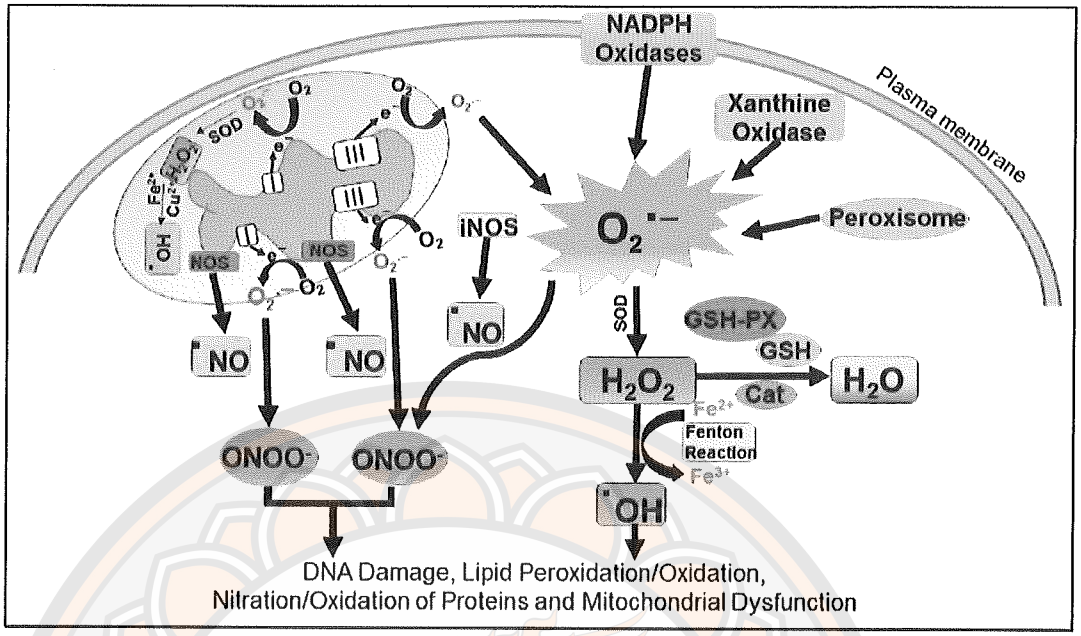

Figure 14 Generation of reactive oxygen species (ROS) in the cell

Source: Kaushal, Chandrashekar, & Juncos, 2019

Capsaicin induces ROS in cancer

Capsaicin induce superoxide production through activating NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) as enzyme complex on plasma membrane. They confirmed through blocking NADPH oxidase activity by inhibitors (diphenylene iodonium, apocynin and neopterine), and then treated with capsaicin, their result shown that superoxide generation and apoptosis were inhibited in Hep G2 cells (79). Capsaicin induce ROS production via blocking activities of electron transport chain complex-I and complex-III as major sites for ROS generation in pancreatic cancer cells. They confirm through electron transport chain inhibition by depleted mitochondrial DNA in BxPC-3 cells and they found that these cells had no significance of ROS generation and apoptosis in capsaicin treatment as same as control without treatment. Their results further demonstrate that capsaicin treatment not only inhibit the enzymatic activity and expression of SOD, catalase and glutathione peroxidase but also reduce glutathione level as compared to controls, resulting in severe mitochondrial damage leading to apoptosis in pancreatic cancer

cells *in vivo* (99). Capsaicin inhibits the plasma membrane NADH-oxidoreductase (PMOR) electron transport chain, causing an increase in ROS level and subsequent disruption of the mitochondrial membrane potential and apoptosis in transformed T-cells. They demonstrated that ROS generation and apoptosis induced by capsaicin is not a consequence of vanilloid receptor and calcium signaling by pretreatment of cells with BAPTA-AM, which chelate cytosolic free calcium, did not prevent ROS generation or apoptosis (91). Interestingly, binding of capsaicin to the TRPV1 results in an increase in intracellular Ca^{2+} level and activation of the apoptotic pathway(96). Capsaicin also induce ER stress and ROS via inducing TRPV1 channel lead to Ca^{2+} influx (14, 100). ER stress as unfolded protein response can mediate accumulation of ROS such as hydrogen peroxide (H_2O_2) (101). Ca^{2+} can enhance ROS generation form inhibiting complex-I and complex-III in electron transport chain (102).

Resveratrol induces ROS in cancer

Resveratrol elevates the intracellular ROS level which is cause of apoptosis, they confirmed with ROS scavenger N-acetyl cysteine (NAC) can suppress apoptosis in resveratrol treatment in human colon cancer cells (98). Resveratrol can induce ROS generation via selectively increased NAPDH oxidase-5 (Nox5) mRNA expression in non-small cell lung cancer (NSCLC) cells (103). Resveratrol increases hypoxia-inducible factors (HIFs) as transcription factors that respond to a decrease in available oxygen and increase in many cancers. Their elevation by resveratrol direct induced ROS production and apoptosis which its result was reversed after HIF-1a knockdown (104). Resveratrol also can inhibit TIGAR (TP53-induced glycolysis and apoptosis regulator) protein expression as responsible as maintaining glutathione (GSH) levels. Overexpression TIGER increased glutathione levels and reduced intracellular ROS level and apoptosis after resveratrol treatment and its result reversed in TIGAR silencing in human non-small cell lung cancer cells (105).

Co-treatment of resveratrol and capsaicin increased ROS production and led to significant tumor reduction in xenograft mouse preclinical model (106). Co-treatment of resveratrol and capsaicin increased ROS, nitric oxide (NO) production in colon cancer cells (107).

ROS causes damage cellular proteins, lipids and nucleic acids, which can lead to induce cell apoptosis (96). Capsaicin-induced ROS generation can lead to hydrolysis membrane sphingophospholipids that release and accumulation of phosphocholine as well as, more importantly, ceramide .Moreover, ROS can induce apoptosis through various signal pathways, including activation of JNK/ P38 mitogen-activated protein kinases (MAPKs) in prostate cancer (65), induced ER stress (108).

CHAPTER III

METHODOLOGY

Cell culture and treatment

Human hepatocellular carcinoma (Hep G2) cells were obtained from Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan) (JCRB1054). Cells were cultured in Eagle's Minimum Essential Medium (EMEM) (Corning, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, MA, USA) and 1% penicillin/ streptomycin solution (100 IU/ mL of penicillin and 100 µg/ml of streptomycin (Gibco, MA, USA) under a humidified 5% CO_2 at 37°C in 95% humidified incubator. Every subculture, cell morphology and numbers were recorded. To ensure the normal growth of cells and to gain the consistent of results, the use of cells exceeding ten subculture passages was avoided. Mycoplasma contamination was detected by staining with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (D1306, Molecular Probes, Thermo Fisher Scientific, USA) and visualized with a fluorescence microscope (Axio Observer A1, Carl Zeiss MicroImaging GmbH, Germany).

Determination cell viability by MTT analysis

Hep G2 cells at a density of 3×10^4 cells/ well were seeded in 96-well plates (SPL Life Sciences Inc., Korea). After 24 hours of adherence, cells were treated with different concentrations of capsaicin (Sigma-Aldrich, Darmstadt, Germany), resveratrol (Sigma-Aldrich, Darmstadt, Germany), and their combinations for 24 hours. After treatments, 5 mg/ml in phosphate buffer solution (PBS) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco, USA) was added to each well and incubated at 37 °C for 4 hours. Then, the crystals of formazan were dissolved in 150 µl DMSO and the absorbance of each well was measured at 595 nm in a microplate reader (Synergy HT Multi-Mode, BioTek Instruments, Inc, USA).

Evaluation of apoptosis by flow cytometry

Hep G2 cells at a density of 1×10^6 cells were seeded in 35 mm culture dish for 24 hours. Then, cells were treated with capsaicin, resveratrol, and their combinations at the indicated IC_{50} concentrations. After 24 hours of treatment, cells were harvested, washed with PBS, resuspended in annexin binding buffer, and then double stained with Alexa Fluor 488 annexin V and PI (Life Technologies, Invitrogen, NY, USA) for 15 minutes. Apoptotic cells were analyzed by FACSCalibur flow cytometry (Becton Dickinson (BD)) and calculated by CellQusetPro software (Mac OS 9, BD Biosciences, USA).

Determination of $\Delta\Psi m$ by flow cytometry

Hep G2 cells at a density of 3×10^5 cells of were seeded in 24-well plate for 24 hours. Then, cells were treated with combination of capsaicin and resveratrol at the indicated concentration. After 24 hours of treatment, cells were harvested, washed with PBS and then incubated with 5,5′,6,6′- tetrachloro-1,1′,3,3′- tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Life Technologies, Thermo Scientific, NY, USA) at 37°C and 5% CO_2 for 45 minutes. The $\Delta\Psi m$ in Hep G2 cells were detected by FACSCalibur flow cytometry and the data were analyzed using CellQuset Pro software.

Detection of Bcl-2 activity

Hep G2 cells at a density of 1×10^6 cells were seeded in culture dish 35 mm for 24 hours. Cells were treated with combination of 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours. Bcl-2 activity was measured by flow cytometer using Muse Bcl-2 activation dual detection Kit (MCH200105; EMD Millipore, Germany). Briefly, After 24 hours of treatment, cells were harvested, washed with PBS, fixed with fixation buffer, permeabilized with permeabilization buffer, incubated with antibody cocktail (anti-phospho-Bcl-2(Ser70), Alexa Fluor 555, and anti-Bcl-2, PECy5) at room temp for 30 minutes, and resuspend in 1x assay buffer. Then samples were detected Bcl-2 levels by Muse Cell Analyzer (Merck Millipore, Germany).

Analysis protein expression by western blot assay

Hep G2 cells at a density of 2×10^6 cells were seeded in culture dish 60 mm for 24 hours. Cells were treated with combination of 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours. Cells were harvested, washed with PBS and extracted protein by Mammalian Protein Extraction Reagent (MPER) (Thermo Scientific, Rock ford, IL, USA). Lysate protein concentrations were quantified by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rock ford, IL, USA). Proteins were separated by electrophoresis on 8% SDS-PAGE and were transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with rapid block solution (AMRESCO, Solon, OH, USA), membranes were incubated with primary antibodies anti-fatty acid synthase (FASN) (Abcam, Biomes Diagnostic CO., Ltd, Thailand), anti- Acetyl-CoA carboxylase (ACC) (Merck Millipore, Germany), and anti-ATP-citrate lyase (ACLY) (Cell Signaling Technology Inc., USA) at 4 °C overnight. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-Rabbit IgG secondary antibody (Life Technologies, invitrogen, NY, USA) for 2 hours. The blots were visualized using the Novex ECL chemiluminescent substrate reagent kit (Life Technologies, invitrogen, NY, USA). The protein bands were detected using CCD camera (ImageQuant LAS 4000).

Detection of long chain free fatty acid level

Hep G2 cells at a density of 1×10^6 cells were seeded in culture dish 35 mm for 24 hours. Cells were treated with combination of 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours. The intracellular long chain fatty acid was detected by free fatty acid bioassay kit (US Biological; Life Sciences). The manufacturer's protocol was followed. Long chain free fatty acids are changed to CoA derivatives that were oxidized with concomitant generation of fluorescencesignal. Briefly, after treated, cells were harvested, homogenized with chloroform-Triton-X 100 (1% Triton-X 100 in pure chloroform). The supernatant was collected after centrifugation. The air drying techniques were used to remove chloroform and then the dried pellet was dissolved with fatty acid assay buffer and detected by fluorometry at Ex/Em 535/590 nm with microplate reader.

Detection of ROS production by flow cytometry

Hep G2 cells at a density of $3x10^5$ cells were seeded in 24-well plate for 24 hours. Then, cells were treated with combination of capsaicin and resveratrol at the indicated concentration. After 24 hours of treatment, cells were harvested, washed with PBS and then incubated with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Life Technologies, Thermo Scientific, NY, USA) at 37°C and 5% CO_2 for 30 minutes. ROS level in Hep G2 cells was detected by FACSCalibur flow cytometry and the data were analyzed using CellQuset Pro software.

Detection of CPT-1 activity

CPT-1 activity was quantified by spectrophotometric method as described previously (109). Briefly, Hep G2 cells at a density of 2×10^6 cells were seeded in culture dish 60 mm. After 24 hours, cells were treated with combination of 0.3 mM capsaicin and 0.1 mM resveratrol. Cells were harvested, washed PBS and lyzed in Tris-Hcl buffer (pH 7.4) containing 1 mM EDTA and 0.25 M sucrose. Then, the samples were centrifuged at 500 g, at 4 °C for 10 minute. The supernatant was then centrifuged at 15000 g, at 4 °C for15 minute to collect mitochondria. Mitochondrial protein concentrations were quantified mixed with 100 mM Tris buffer pH 8.0 containing, 0.1% Triton X-100, 1 mM EDTA, 0.5 mM DTNB, and 0.01 mM palmitoyl CoA. L-carnitine at1.25 mM was added and measured O.D. at 412 nm using microplate reader.

CHAPTER IV

RESULTS

Capsaicin, resveratrol, and the combination of capsaicin and resveratrol decreased viability of Hep G2 cells

The cytotoxic effect of capsaicin, resveratrol and the combination of capsaicin and resveratrol in Hep G2 cells was evaluated after 24 hours of incubation using MTT assay as shown in figure 15. Capsaicin (figure 15A), resveratrol (figure 15B), and their combination (figure 15C) significantly inhibited Hep G2 cell viability in a dose-dependent manner. Treated cells with capsaicin and resveratrol gave IC50 values of 0.4 mM and 0.2 mM, respectively. The combination of half IC50 dose of capsaicin and resveratrol showed a 50% inhibition effect on cell viability, while half IC50 dose of capsaicin or resveratrol mono-treatment casused less than 50% cytotoxic effect compared with 100% of vehicle treated with 0.2% DMSO. The cytotoxic test for 24 hours of Hep G2 cells with half IC50 dose of capsaicin at 0.2 mM combined with vary dose of resveratrol at 0.025-0.4 mM or half IC50 dose of resveratrol at 0.1 mM combined with vary dose of capsaicin at 0.05-0.5 mM showed a dose-dependent characteristics. To enhance cell viability inhibition greater than 50%, the combination of capsaicin at 0.3 mM and resveratrol at 0.1 mM caused 60% cytotoxic effect. Thus, reducing dose used, a combination of capsaicin and resveratrol exhibited an effective on growth inhibition in Hep G2 cells.

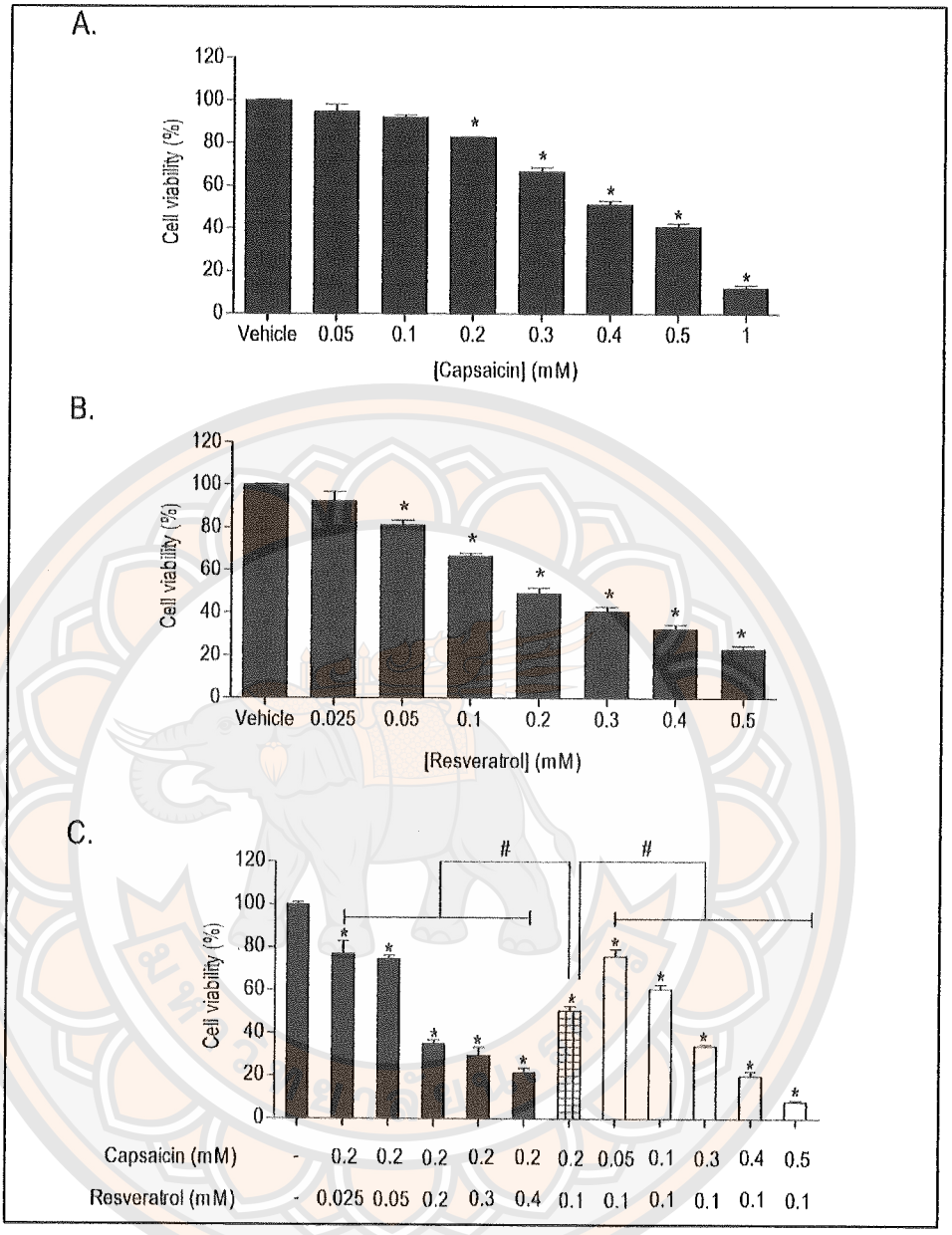

Figure 15 Effect of capsaicin and resveratrol on cell viability of Hep G2 cells after exposed with different concentations and incubated for 24 hours

MTT assay was performed and expressed an inhibition with percentage of cell viability compared with 100% of vehicle control which was treated with 0.2% DMSO without capsaicin or resveratrol treatment. The results were performed in three independent experiments with triplicates and presented as means ± SEM, n = 3, *$p<0.05$.

Capsaicin, resveratrol, and the combination of capsaicin and resveratrol induced apoptosis in Hep G2 cells

To determine the cytotoxic effect thought apoptosis induction, Hep G2 cells treated with the combination of 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours induced apoptosis at the early and late stages to 11.2% and 37.4%, respectively, compared with the vehicle control group with 4.7% and 4.5% of early and late apoptotic stages, respectively, as shown in figure 16A and 16B. Apoptosis was confirmed with significant reducing $\Delta\Psi m$ to 54.0 % compared with the vehicle control following treatment with the combination 0.3 mM capsaicin and 0.1 mM resveratrol, as shown in figure 16C. Decreased Bcl-2 activity, anti-apoptotic proteins that protect apoptosis through direct bind with pro-apoptotic Bax in intrinsic apoptotic pathway preventing the formation of the mitochondrial pore, induces apoptosis in cancer cells (110-111). Figure 16D showed that the combination combination of 0.3 mM capsaicin and 0.1 mM resveratrol potentially decreased Bcl-2 activity to 17.66 % compared with the vehicle control.

The combination of capsaicin and resveratrol suppressed fatty acid synthesis in Hep G2 cells

Overexpression of lipogenic enzymes in the DNL pathway are focused to be a new therapeutic target for cancers (51-52, 112-113). The present study determined an inhibition of the DNL promoting apoptosis in Hep G2 cells. Protein expression levels including ACLY, ACC, and FASN treated with the combination of 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours remained the same as in the vehicle control group while the level of intracellular fatty acid was significantly reduced, as shown in figure 17A, 17B and 17C. We suggest that the combination of capsaicin and resveratrol targets the DNL pathway causing apoptosis in Hep G2 cells.

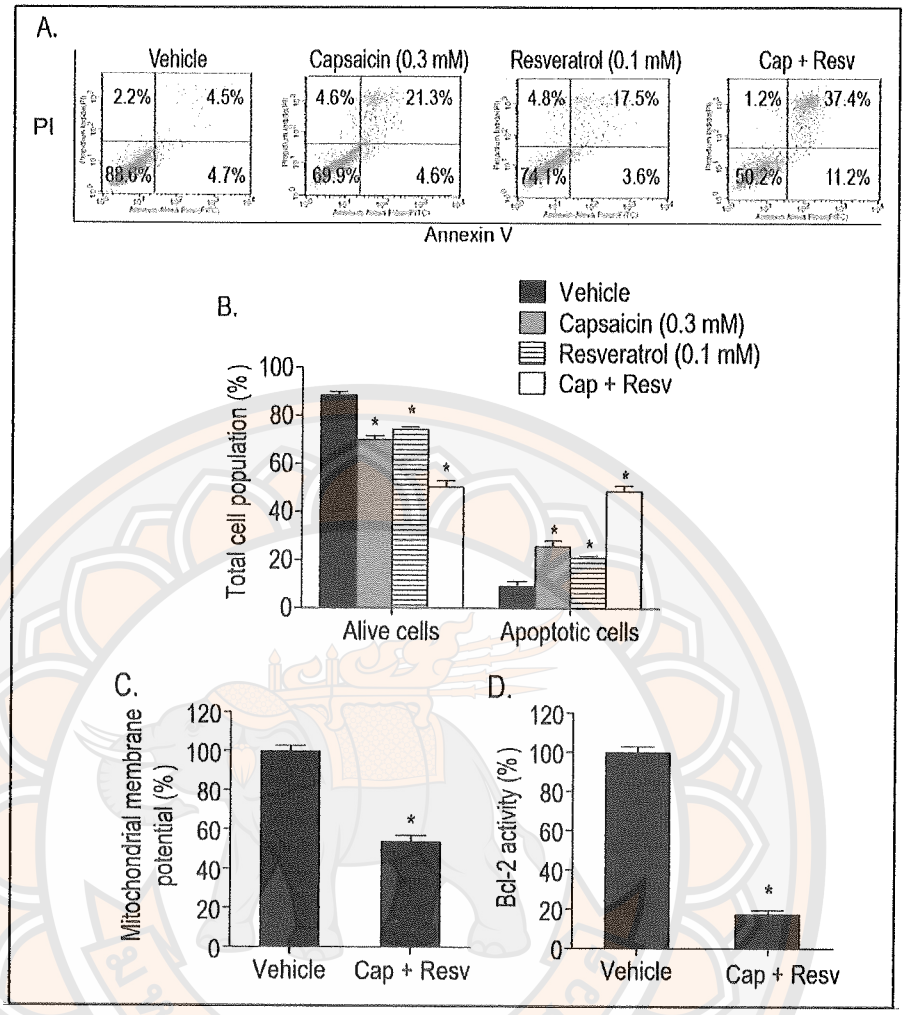

Figure 16 The combination of capsaicin and resveratrol effect on apoptosis in Hep G2 for 24 hours

Apoptosis analysis was performed by flow cytometer. (A) Cell apoptotic distribution (B) The calculated percentages of total cells were shown by bar diagram of cell viable, and early plus late apoptotic cells. The mitochondrial membrane potential ($\Delta\Psi m$) analysis determined by JC-1staining and detected by flow cytometry. (C) The calculated percentages of the loss of $\Delta\Psi m$ is shown in a bar graph. (D) Bcl-2 activity was quantified by flow cytometry using Bcl-2 phosphorylation relative to total Bcl-2 expression levels is shown in a bar graph. Data compared with 100% of vehicle control which was treated with 0.2% DMSO without capsaicin or resveratrol treatment. The results were performed in three independent experiments with triplicates and presented as means ± SEM, n = 3, *$p<0.05$.

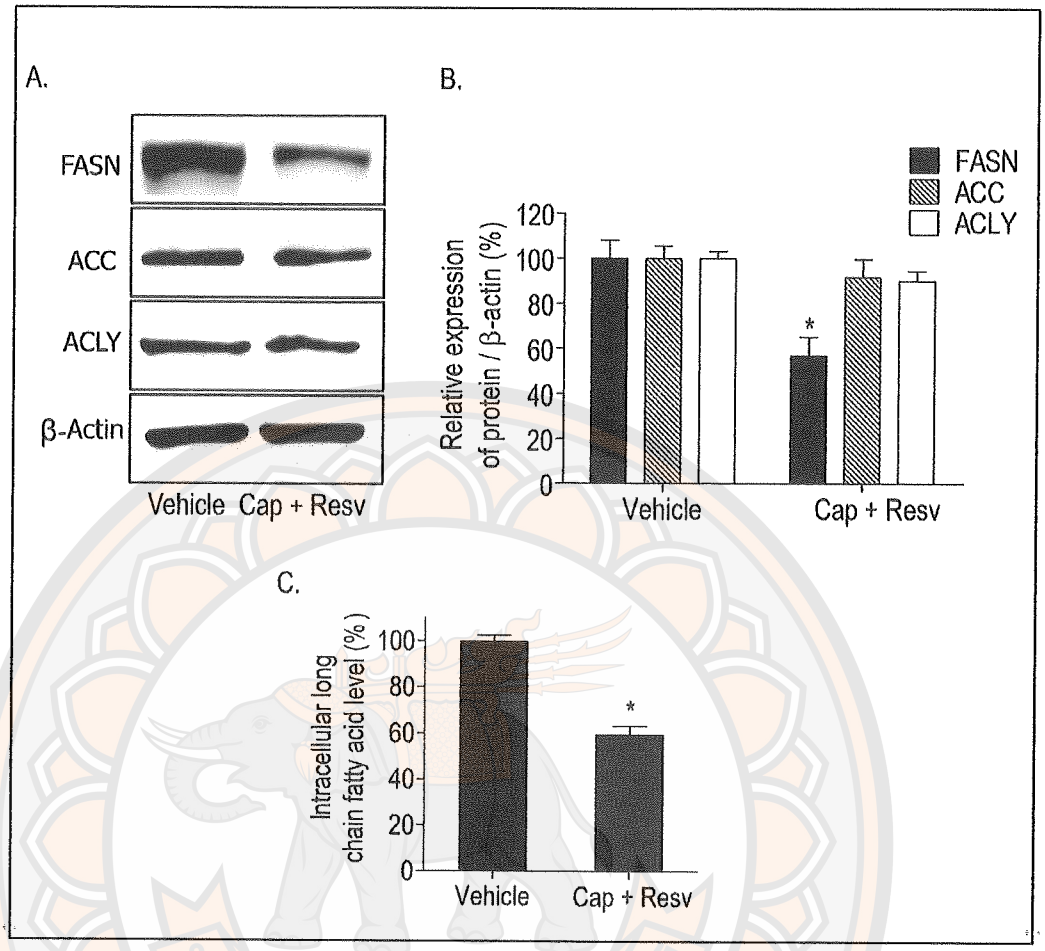

Figure 17 The effect of the combination of capsaicin and resveratrol on DNL enzyme and product after incubated for 24 hours in Hep G2 cells

Representative immunoblotting assay was performed to detect the expression of of FASN,ACC, andACLYproteins. β-actin was used to normalize equal amount and intensity of protein bands. (A) The of lipogenic proteins expression was detected by immunoblotting technique and visualized using a CCD camera. (B) Quantified density values were obtained and expressed as a percentage of β-actin/protein ratio. The level of intracellular long chain fatty acid were quantified as described in methods. (C) The calculated percentages of the intracellular long chain fatty acid is shown in a bar graph. Data compared with 100% of vehicle control which was treated with 0.2% DMSO without capsaicin or resveratrol treatment. The results were performed in three independent experiments with triplicates and presented as means ± SEM, n = 3, *$p<0.05$.

The effect of combination of capsaicin and resveratrol on Malonyl-CoA decreased by TOFA

5-Tetradecyloxy-2-fuoric acid (TOFA) inhibits Acetyl-CoA carboxylase-α (ACC) that changed acetyl-CoA to Malonyl-CoA. To study the apoptosis mechanism of combination of capsaicin and resveratrol. Apoptosis was detected by flow cytometry using JC-1 staining which detected ΔΨm. Hep G2 cells were treated with TOFA for 1 hour before cells were treated with combination of capsaicin and resveratrol for 24 hours. Figure 18A showed that only TOFA decreased percentages of ΔΨm to 96.96 %, combination of capsaicin and resveratrol decreased percentages of ΔΨm to 55.57 % and combination of capsaicin, resveratrol and TOFA decreased percentages of ΔΨm to 55.44 %. This result found that combination of capsaicin, resveratrol inhibited fatty acid synthesis which was major cause of apoptosis in Hep G2 cells.

The combination of capsaicin and resveratrol decreased CPT-1 activity that leads to inducing apoptosis in Hep G2 cells

Inhibition of fatty acid synthesis in DNL pathway produces an accumulation of malonyl-CoA that consequently decreases CPT-1 activity, resulting in inducing apoptosis in Hep G2 cells (62). The present study determined effect of the combination of capsaicin and resveratrol on CPT-1activity. Hep G2 cells were treated with combination of 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours. CPT-1 activity was quantified by spectrophotometric method. CPT-1 activity was decreased to 66 % compared with 100% of vehicle treated with 0.2% DMSO, as shown in figure 18B. The result suggested that inhibition of fatty acid synthesis decreases CPT-1 activity, leading to exert apoptotic induction in Hep G2 cells.

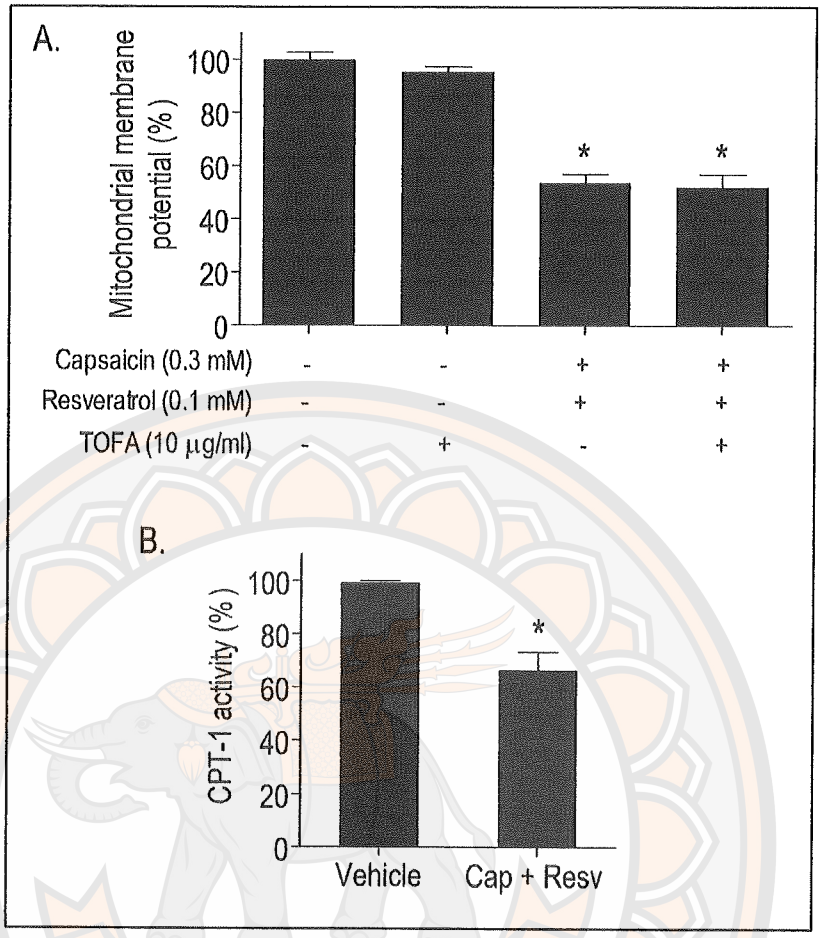

Figure 18 Effect of combination of capsaicin and resveratrol on ΔΨm and activity of CPT-1 after incubated for 24 hours in Hep G2 cells

(A)The loss of ΔΨm was determined by JC-1staining and detected by flow cytometry. Cells were treated with 10 μg/ml of TOFA only for 24 hours, combined capsaicin and resveratrol for 24 hours, pretreated with 10 μg /ml of TOFA for 1 hour before treated with combination of capsaicin and resveratrol for 24 hours. (B) Percentage of CPT-1 activity was determined in cells treated with combined capsaicin and resveratrol is shown in a bar graph. The data compared with 100% of vehicle control with 0.2% DMSO without capsaicin or resveratrol treatment. The results were performed in three independent experiments with triplicates and presented as means ± SEM, n = 3, *$p<0.05$.

The combination of capsaicin and resveratrol increased ROS generation in Hep G2 cells

Increased level of ROS production induced apoptosis. To determine effect of combination of capsaicin and resveratrol on increased of ROS level. ROS production was determined using fluorescent dye CM-H2DCFDA which was changed intracellular to green fluorescent product DCF by ROS. ROS analysis was performed by flow cytometry and compared with control cells treated with 0.2% DMSO. Figure 19A showed that Hep G2 cells treated with combined 0.3 mM capsaicin and 0.1 mM resveratrol for 24 hours increased percentages of ROS production to 172 % compare with 100% of control. We confirmed that ROS was one cause of apoposis with measuring $\Delta\Psi m$ by flow cytometry. Figure 19B showed that Hep G2 cells were treated with combined plus N-acetylcysteine (NAC), inhibition of ROS found that percentages of $\Delta\Psi m$ increased to 91 % when compared to the combination of capsaicin and resveratrol only with percentages of $\Delta\Psi m$ were 50%. Thus, combined capsaicin and resveratrol induces apoptosis through of increased of ROS production in Hep G2 cells.

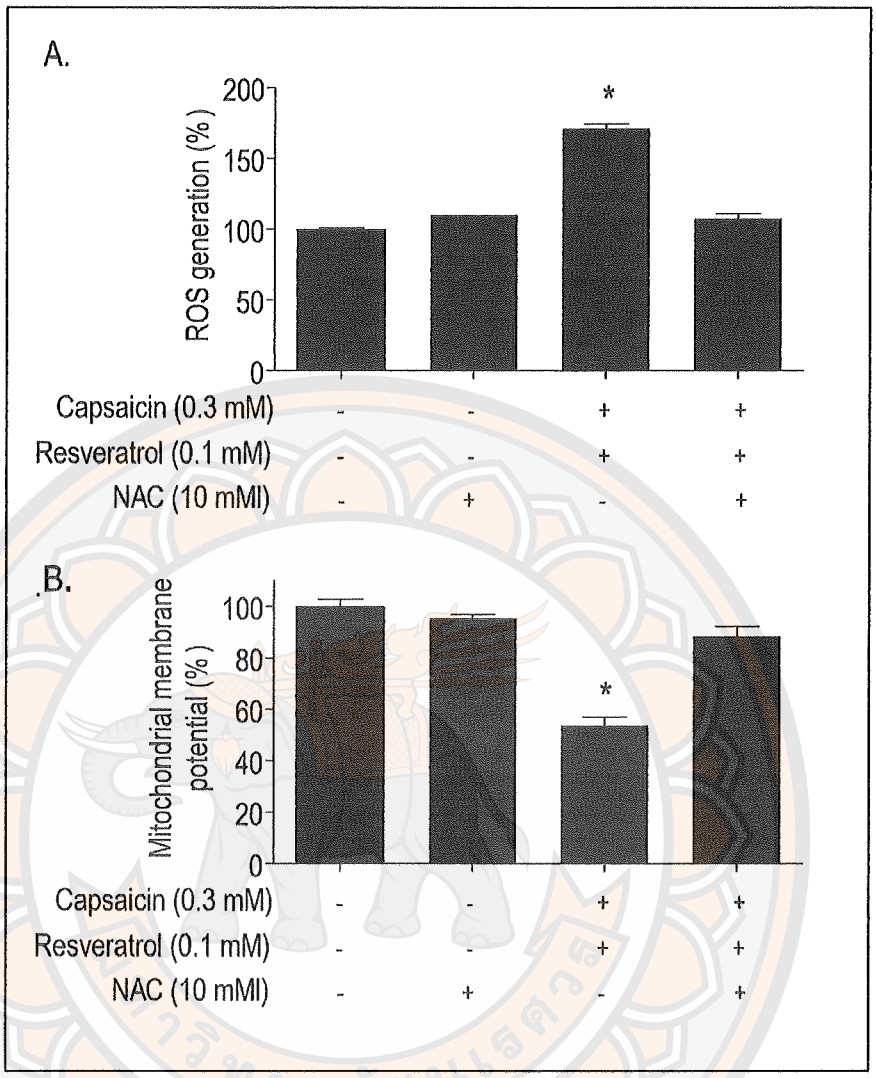

Figure 19 The combination of capsaicin and resveratrol induced apoptosis through increased ROS generation in Hep G2 cells for 24 hours

Cells were then stained with CM-H2DCFDA and ROS analysis was performed by flow cytometry. (A)The calculated percentages of ROS were shown by bar diagram. The $\Delta\Psi m$ was determined by JC-1 staining and detected by flow cytometry. Cells were treated with NAC and combination of capsaicin and resveratrol. (B) The calculated percentages of $\Delta\Psi m$ were shown by bar diagram. Data compared with 100% of vehicle control which was treated with 0.2% DMSO without capsaicin or resveratrol treatment. The results were performed in three independent experiments with triplicates and presented as means ± SEM, n = 3, *$p<0.05$.

CHAPTER V

DISCUSION

Over the past few years, various dietary polyphenol compounds including capsaicin and resveratrol have been recognized as one of the promising candidates for the therapeutic effectiveness of cancers (96, 114-115). The present finding showed more effectiveness of the combination of capsaicin and resveratrol than a monotreatment on reduced cell viability and induced mitochondria-dependent apoptosis in Hep G2 cells. We found that combination treatment enhanced apoptosis through suppression of fatty acid production by decreased FASN expression. Importantly, malonyl-CoA accumulation following inhibiting fatty acid synthesis was found to be not major causes of combined capsaicin and resveratrol treatment-induced apoptosis. We showed that reduction of CPT-1 which induced apoptosis did not depend on only an enhanced malonyl-CoA accumulation after this co-treatment. In addition, we found that apoptosis induction by combination treatment of capsaicin and resveratrol also was associated with enhanced ROS generation in Hep G2 cells.

DNL is the common biochemical process of fatty acids synthesis in the liver that converts excess carbohydrate to fatty acids, which are stored to provide energy in triacylglycerols (TGs) form (116). Previous finding has shown the up-regulated expression of DNL pathway recognized as a hallmark of cancer which is focused to be a new target of the therapeutic agent for cancers (7). Several steps are required to convert carbons from citrate to bioactive fatty acids. Intermediates from OXPHOS, citrates as a precursor for fatty acid synthesis are transported from the mitochondria to cytosol via mitochondrial citrate transporter (CTP) (48-49). ACLY act as produces precursor for FA synthesis by converting citrate to acetyl-CoA. Then malonyl-CoA are produced from acetyl-CoA by ACC enzyme. Subsequently, FASN produces the production of LCFAs from acetyl-CoA, malonyl-CoA and NADPH precursors. The common saturated LCFAs, palmitates are modified to form complex fatty acids by stearoyl-CoA desaturase (SCD1) enzyme converts to monounsaturated fatty acids (MUFAs), palmitoleic acid. MUFAs is precursor for the formation of phospholipid

that used as building blocks for cell membrane biosynthesis (4, 66). Furthermore, LCFAs are established as long chain acyl-CoA to mitochondrial fatty acid oxidation (FAO) by the formation of thioester bond to coenzyme A, catalyzed by ACS (4). Long chain acyl-CoA is subsequently transfer acyl group to L-carnitine by CPT-1 activity. Then, this product, palmitoylcarnitine is transported to mitochondria for FAO to provide energy (7). Enzymes in DNL pathway are generally low levels in normal tissue, exception of the liver tissue and fatty acids requirement for cells is generally provided via dietary fatty acids.

Several studies have reported that theses lipogenic enzyme are up-regulated or constitutively expressed in most types of cancer cells and are focused to be a new target of therapeutic agent for cancers (51-52, 112-113). Previous study has reported that ACLY depletion-suppressed cancer proliferation and induce apoptosis was associated with down-regulation of CPT-1 and TG accumulation via decreasing elongation of fatty acids prostate cancer cells (117). ND-646, ACC inhibitor suppresses fatty acid synthesis and tumor growth contributed apoptosis in preclinical models of non-small cell lung cancer cells (118). Depletion of SCD1 by siRNA results to reduce cell proliferation and induce apoptosis mediated through activating ER stress by induced CHOP transcription factor in H1299 human lung cancer cells (119). Chemical compound agents of FASN inhibitors, included cerulenin and C75, binding at KS domain and orlistat, binding at TE domain of FASN have been reported that are potent to block fatty acid synthesis contributed apoptotic cancer deaths (120-124). The natural polyphenols agent as potent therapeutics for cancers, such as EGCG, quercetin, curcumin and ginkgolic acid have been revealed to inhibit fatty acid synthesis through inhibiting FASN expression, leading apoptosis in cancers (121). In addition, capsaicin and resveratrol have been shown that inhibits fatty acid synthesis related with decreased FASN expression and selectively induces apoptosis in cancers without effect on normal cells (10, 56-57).

Recently, combinations of cancer therapeutic agents, including capsaicin and resveratrol have been growing interest because of their synergistic effect and reduce the risk of resistance and toxicity caused by using higher doses of single compounds (21-24). Research studies of co –treatment of FASN inhibitors, cerulenin and C75 have been reported that reduce chemotherapy resistance of docetaxel via extenuated

HER -2/ neu (c- erb B-2) oncogene in breast cancer cells (25-26). Capsaicin combined with docetaxel has represented synergistic anticancer effect on inhibition of DNL via inhibited ACC by AMPK activation and PI3K/AKT/mTOR signaling inhibition in prostate cancer cells (27). Combination treatment of resveratrol and rapamycin has been shown that anticancer effect associated with DNL via inhibition of PI3K/AKT/mTOR signaling in of bladder cancer cells (28). Furthermore, the previous findings have shown the combination effect of capsaicin and resveratrol expressed synergistic anticancer effects on cell proliferation suppression of and induces apoptosis via ROS generation in many cancers (29-31), but their anticancer effect has not been shown on DNL. Based on the collective evidence that capsaicin and resveratrol suppress fatty acid production, our research finding showed combination effect of capsaicin and resveratrol treatment on fatty acid synthesis inhibition via decreasing FASN expression in Hep G2 cells.

In previous studies, deprivation of fatty acid synthesis by capsaicin promotes the elevation of malonyl-CoA accumulation to be the major cause of apoptosis in HCC cells (10, 125). Increased malonyl-CoA following reduction of fatty acid level inhibit CPT-1 activity act to bring fatty acid to β-oxidation. Elevated ceramide is generated from palmitoyl-CoA accumulation in cytoplasm following CPT-1 activity inhibition lead to enhancing apoptosis by inducing pro-apoptotic genes DAPK2 and BNIP3 expression in breast cancer cells (53, 64) and colon cancer cells (63). In contrast, we found that ACC inhibitor, TOFA decreases malonyl-CoA cannot relive the effect of combination treatment inducing apoptosis in Hep G2 cells. Thereby, molonyl-coA accumulation maybe not a major cause of combination treatment of capsaicin and resveratrol-induced apoptosis in Hep G2 cells. Apart from malonyl-CoA, PPARα activating CPT-1 genes also involves in lipid metabolism of human (126-128). A previous report has demonstrated that blockade of LCFAs synthesis by ACLY depletion inhibits activity of PPARα, leading to inhibiting CPT-1 expression (117). In the presence of a low level of malonyl-CoA production by suppressing ACLY, inhibiting CPT-1 expression thus contributes apoptosis in cancer cells. `Instead of inhibiting PPARα, decreased LCFAs by C75, inhibitor of FASN induces PPARα-regulated expression of CPT-1 gene (129). Thus, we propose that co-treatment of

capsaicin and resveratrol decrease CPT-1 activity induce apoptosis following a suppression of fatty acid may be independent of malonyl-CoA in Hep G2 cells.

The transcription factors SREBP1c has an important role in DNL via the up-regulation of lipogenic gene transcriptions, such as ACLY, ACC, and FASN in lipogenic tissues and cancers (130-131). Capsaicin inhibits fatty acid content through inhibiting SREBP1c contributed apoptotic cell death in HCC cells (132). Resveratrol suppresses FASN expression by SREBP-1c depletion leading apoptosis in breast cancers both *in vitro* and *in vivo* (56-57). The most cancers have been shown the high activation of SREBP-1c which plays an important role in promoting cell growth and survival. SREBP-1c is activated by the PI3K/AKT/mTOR oncogenic pathway signaling (73, 131, 133-134). Capsaicin directly inhibits PI3K/AKT/mTOR signaling pathway leading to inhibition of SREBP-1c activity and contributed to apoptotic death in NPC-TW01 cancer cells (20) and HCC cells (132). Apart from PI3K/ AKT signaling pathway, inhibition of SREPP1 by capsaicin treatment is directly inhibited through activating TRPV1/CaMKKβ/AMPK signaling lead to lipogenesis inhibition contributed apoptotic cell death in HCC cells (132). Resveratrol has been shown to inactivate PI3K/AKT signaling by elevating expression of SIRT1 protein HCC cells (135) and BMP7 protein in colon cancer cells (136). Thus, combination effect of capsaicin and resveratrol treatment associated with upstream signaling pathway of fatty acid synthesis leading to apoptosis in HepG2 cells.

ROS are free radicals derived from molecular oxygen containing unpaired electron which significant damage to cell structures. Normally, cells have mechanism to maintain ROS homeostasis by enzyme, antioxidant, and some vitamin to scavenge and neutralize ROS to against oxidative damage. Mitochondria is main source of ROS, superoxide radicals (O_2^-) generated at complexes I and III of electron transport chain. Additionally, peroxisomes, plasma membrane, and cytosol have enzyme that produce O_2^- (94). Previous research found that increased ROS production as a common phenotype of cancer cells due to they have high metabolic rate in mitochondrial, endoplasmic reticulum, and cell membranes more than normal cells. Previous finding suggested that moderate concentration of ROS play an important role in promoting tumorigenesis, metastasis, angiogenesis and survival in cancer cells (95, 137).

However, according to several public data indicated that excessive accumulation of ROS promote cancer cell death. Several natural dietary bioactive compounds including capsaicin and resveratrol cause increased ROS levels and subsequent apoptosis in cancer cells (10, 96, 98, 105, 138-139). A previous study has demonstrated capsaicin enhances ROS production via activating NADPH oxidase activity and induces apoptosis in HCC cells. NADPH oxidase as enzyme complex facing the extracellular space of plasma membrane generates superoxide (O_2^-) (140). In pancreatic cancer, capsaicin induces ROS production via blocking the activity of electron transport chain complex-I and complex-III, resulting in severe mitochondrial damage leading to cell apoptosis both in vitro and in vivo(99). Resveratrol has been reported that can increases ROS generation via increasing NAPDH oxidase-5 (Nox5) mRNA expression in non-small cell lung cancer (NSCLC) (103). Resveratrol also enhances ROS production via activating transcription factors a hypoxia-inducible factors (HIFs). After resveratrol treatment in HIF-1a knockdown decreased ROS production leading to decreasing prostate cancer cells death (17). Moreover, reduction of antioxidant level involved accumulation of intracellular ROS level. Recent studies, capsaicin and resveratrol have been shown that they reduce glutathione and contributed apoptosis in NSCLC and HCC cells, respectively (99, 105). In addition, it has been shown the synergistic effect of co-treatment of resveratrol and capsaicin increases ROS production and induces apoptosis in colon cancer cells (107), and promotes significant tumor reduction in pancreatic tumor xenograft mouse model (106). In present study, we showed that the combination treatment of capsaicin and resveratrol significantly enhanced elevation of ROS generation and loss of $\Delta\Psi m$ contributed to apoptosis in Hep G2 cells. This effect of combination treatment was prevented by NAC inhibitor of ROS. Therefore, our findings suggest that ROS contributed to the increased apoptosis caused by the combination treatment of capsaicin and resveratrol in Hep G2 cells.

ROS-mediated cancer cell apoptosis has been revealed in many mechanisms. Elevated ROS levels by capsaicin can hydrolyze sphingophospholipids membrane to produce ceramide. The accumulation of ceramide induced apoptosis via activating pro-apoptotic protein in prostate cancer cells (65). A previous study has reported that elevation of ROS following capsaicin treatment exerts apoptotic induction via

disruption of mitochondria which leads to cytochrome c releasing into the cytosol and cleavage of caspase-9 and caspase-3 in pancreatic cancer cells (99). Additionally, ROS can also induce apoptotic protein via activation of JNK/ERK mitogen-activated protein kinases (MAPKs) pathway in leukemic cells (77) and activation of ER stress in nasopharyngeal carcinoma cells (108). Moreover, recent study has found ROS-contributes apoptosis associate with DNL inhibition. The obviously elevated ROS generation promotes a downstream inhibitory effect on the DNL pathway and induced apoptosis in HepG2 cells by capsaicin treatment (10). ROS also selectively suppressed DNL through activating AMPK pathway in non-small cell lung cancers (NSCLC) (141). Therefore, we suggest that ROS production promoting apoptosis may associate with inhibition of the DNL pathway after combined treatment of capsaicin and resveratrol in Hep G2 cells.

In summary, our result indicated that co-treatment of capsaicin and resveratrol has a portential cancer therapy through the DNL inhibition. Besides, our results obtained from the present study will provide an opportunity for further development of more potential cancer therapy as well as cancer prevention caused by metabolic disorder. (125)

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