Thesis for the Degree of Doctor of Philosophy

Cyperus amuricus Induces Cell Death in Human Hepatocellular Carcinoma Hep3B Cells

by

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(*Cyperus amuricus* 는 Hep3B 간암세포에서 세포사멸을 유도한다)

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by
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List of Abbreviations

3-MA	3-methyladenine
AMPK	Adenosine monophosphate-activated protein kinase
Apaf-1	Apoptotic protease activating factor-1
ATF6	Activating transcription factor 6
Atgs	Autophagy related genes
AVOs	Acidic vesicular organelles
C. amuricus	*Cyperus amuricus*
CDKIs	Cyclin-dependent kinases inhibitors
CDKs	Cyclin-dependent kinases
CHOP	C/EBP-homologous protein
DAPI	4',6-diamidino-2-phenylindole
DFF	DNA fragmentation factor
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescent
eIF2α	Eukaryotic initiation factor 2 (eIF2) α subunit
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRP78/BiP	Glucose-regulated protein 78
HCC	Hepatocellular carcinoma
IRE1α	Inositol requiring enzyme-1 α

LC3	Microtubule-associated protein1 light chain 3
mTOR	Mammalian target of rapamycin
PARP	Poly(ADP ribose) polymerase;
PBS	Phosphate-buffered saline
PERK	Protein kinase (PKR)-like ER kinase
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
Rb	Retinoblastoma proteins
tBid	Truncated Bid
TNF	Tumor necrosis factor
UPR	Unfolded protein response
WST-1®	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt

***Cyperus amuricus* Induces Cell Death in Human Hepatocellular Carcinoma Hep3B Cells**

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Abstract

Cyperus amuricus (*C. amuricus*), belongs to *Cyperaceae* family, has been widely used to treat astringent, diuretic, wound healing and other intestinal problems for centuries. Recent studies have demonstrated that *C. amuricus* retains potent pharmacological efficiency in anti-lipase, antioxidant and antineoplastic capabilities. However, the molecular mechanisms of *C. amuricus* on anticancer activities remain unclear. Therefore, the present study was carried out to investigate the precise mechanisms of *C. amuricus*-induced cell death in human hepatocellular carcinoma cells (HCC).

C. amuricus were comparably and significantly cytotoxic to Hep3B cells, but not to A549, HaCaT and HEK293 cells. *C. amuricus* obviously elicited G1 cell cycle arrest in Hep3B cells concomitant with the upregulation of $p21^{CIP1/WAF1}$, $p27^{KIPI}$ and $p16^{INK4a}$ proteins and the downregulation of cdc25A, cyclin D1 and cyclin E, CDK4 and 2 as well as E2F-1, phospho-Rb. *C. amuricus* also sequentially

activated the various caspases (cleaved of caspase-8, -9, -3, -7, and -6, and cleaved PARP) and increased ratio of pro-versus anti-apoptotic Bcl-2-related proteins, prompting the permeability change of mitochondrial membranes and the release of cytochrome *c* from mitochondria. Based on these results, *C. amuricus* induces apoptosis via the activation of both extrinsic death receptor- and intrinsic mitochondria-mediated pathways in HCC Hep3B cells.

The potentials of *C. amuricus* on endoplasmic reticulum (ER) stress-mediated apoptosis and G1 cell cycle arrest in Hep3B cells was further evaluated. *C. amuricus* profoundly triggered ER stress through the activation of unfolded protein response (UPR), leading to the alteration of the phosphorylation levels of ER sensors, the dissociation of GRP78/BiP, the reduction of p-PERK and the increase ATF6 and IRE1α. These consequences were accompanied by the increment of cytosolic Ca^{2+} levels and the activation of caspase-12 and CHOP, which could incite ER stress-induced apoptosis in *C. amuricus*-treated Hep3B cells. In addition, the effect of *C. amuricus*-mediated G1 arrest was clarified by the induction of ER chaperones on modulating cell cycle regulatory molecules. Consistent with the above results, *C. amuricus* is an efficient apoptosis-inducing agent for Hep3B cells, via the G1 arrest, ER stress and mitochondrial-dependent intrinsic pathways.

The detailed mechanism of *C. amuricus*-induced apoptosis associated with autophagy were next examined. During early exposure (3-12 h), *C. amuricus* induced autophagy via the accumulation of acidic vesicular organelle (AVO)-positive cells and the upregulation of Atg5-Atg12 conjugate, Atg7, Beclin-1, LC3-II and DAPK3 proteins. Interestingly, *C. amuricus* suppressed the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) and upregulated the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK), stimulating the activation of apoptosis. Especially, pre-treatment of 3-methyladenine (3-MA) blocked *C.*

amuricus-induced increase of Atg7, Beclin-1, LC3-II and AMPK phosphorylation, revealing the crosstalk between *C. amuricus*-induced apoptosis and autophagy.

Collectively, this is the first study indicating the effects of *C. amuricus* on cell cycle arrest, ER stress, apoptosis and autophagy in HCC Hep3B cells. It could be informative to elucidate the precise mechanism and biological efficacy of *C. amuricus* on cellular response in other cancer types to chemo-sensitization.

PART I

General Introduction

Chapter 1

General introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver accounting for 80%-90% of primary malignant liver tumors, and is currently the leading cause of death amongst cirrhotic patients [Parkin *et al*., 2000; Gospodarowicz *et al*., 2009].

Well-defined risk factors for hepatic carcinogenesis have been pointed out [Bosch *et al*., 1999]. The majority of HCC develops in the context of liver cirrhosis and this preneoplastic condition is the strongest predisposing factor [Colombo and Sangiovanni, 2003; Rahbari *et al*., 2011]. More than 80% of HCC arise in patients with chronic liver disease due to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [Bruix *et al*., 2004; Farazi and DePinho, 2006; Gospodarowicz *et al*., 2009]. Cirrhosis from non-viral causes such as dipsomania, primary biliary cirrhosis and hereditary hemochromatosis also contribute to a high risk of HCC. Furthermore, concomitant risk factors such as HBV, HCV infection as well as alcoholism, tobacco consumption, obesity or diabetes raise the relative risk of HCC pathology, as demonstrated in numerous human studies and further supported by animal models [Fong *et al*., 1994; Hassan *et al*., 2002; Rampone *et al*., 2009].

Nowadays, molecular pathology, genomic analysis and core signaling pathways have essentially provided a better understanding of the mechanisms of hepatic carcinogenesis and cancer progression and have supported to identify new molecular targets that are linked to the prognosis of HCC.

The present study aimed to explore the anticancer effects and the possible cell death pathways induced by *C. amuricus*, supporting the development of potential drugs and possible targeted therapies for liver cancer.

1.1. *Cyperus amuricus* (*C. amuricus*)

Herbal medicines play an increasing role in primary health care systems among the population as synthetic anticancer remedies are beyond the reach of the common man due to the high cost factors and toxic side effects. Many plant-derived drugs have a vital role in cancer therapy which execute their therapeutic effects by inhibiting cancer activating enzymes and hormones, stimulating DNA repair mechanism, promoting production of protective enzymes inducing antioxidant action, killing tumors via programmed cell death and enhancing immunity [Sofowora *et al.*, 2013]. *Cyperus amuricus* (*C. amuricus*, Figure 1.1) is a monocotyledonous and multipurpose medicinal herb which belongs to *Cyperaceae* family. It is a perennial sedge with slender and scaly creeping rhizomes, fibrous base and arises singly from the triquetrous tubers with dense tuft of 10 to 60-cm culms. This delicate grass, growing in tropical, subtropical and temperate regions; is widespread in North America, Japan, Korea, and Russia (Far East) [Maximowicz and Mém, 1859]. A few studies have been recently commissioned to elucidate the pharmacological activities of *C. amuricus*. The dried whole plant of this herb, well-known as Chinese Amuersuocao or Korean Bangdongsani, has been traditionally prescribed for exerting astringent, diuretic, diaphoretic, desiccant, and cordial properties in folk medicine [Kakarla *et al.*, 2014]. Infusion of this grass has been commonly credited with treating wounds, tumors, piles, and other intestinal problems in Bangladesh [Rahmatullah *et al.*, 2009]. Previous phytochemical investigation on *C. amuricus* revealed the presence of three antioxidant phenolic components, including 3,4-dimethoxy benzoic acid, 4-hydroxybenzoic acid, and piceatannol which exhibited powerful free radical scavenging, especially against DPPH and superoxide anions [Lee *et al.*, 2008]. The steam distillation of *C. amuricus* also showed 57.3% inhibitory activity of pancreatic lipase *in vitro* [Sharma *et al.*, 2005].

Figure 1.1. Leaf and flower of *Cyperus amuricus* (*C. amuricus*)

1.2. Apoptosis

Apoptosis, programmed cell death, is a conserved mechanism in eukaryotes that occurs normally during physiological or pathological events, such as embryonic development, optimal functioning of the immune system, turnover of senescent or damaged cells, for the maintenance of tissue homeostasis and the prevention of tumor development [Vaux and Korsmeyer, 1999].

Morphological hallmarks of apoptosis exhibit cell shrinkage, plasma membrane blebbing, nucleosomal fragmentation, chromatin condensation (karyorrhexis) and formation of apoptotic bodies. These apoptotic bodies are then consumed by macrophages or neighboring cells without causing any inflammatory response and damage to the surrounding tissues [Kerr *et al.*, 1972].

The biochemical events that ultimately lead to these morphological features of apoptosis require the activation of a family of proteolytic enzymes known as caspases (cysteine-dependent aspartic acid-specific proteases) [Nicholson and Thornberry, 1997]. Caspases are highly specific proteases which cleave a multitude of proteins directly after aspartate residues in the short tetrapeptide motifs, these events ultimately lead to the cell demise that cleave proteins at specific protein residues**.** Under normal conditions, these proteins are housed within cells as zymogens and are activated by an N-terminal cleavage following a death stimulus. The hundreds of caspase substrates identified including, prosurvival proteins that are cleaved for inactivation, prodeath proteins that are cleaved for activation and some are considered to be innocent bystanders. Apoptotic caspases are subdivided into two major classes: initiator caspases (caspase-2, -8, -9, and -10) and executioner caspases (caspase-3, -6 and -7) [Timmer and Salvesen, 2006; Li and Yuan, 2008]. Generally, initiator caspases have a prerequisite for executioner caspase activation. Once active, initiator caspases require the formation of a multiprotein complex, including death-inducing signaling complex (DISC),

complex II, apoptosome, PIDDosome or ripoptosome [Tenev *et al*., 2011]. Executioner caspases can carry out cell death through proteolysis of anti-apoptotic proteins, cellular DNA repair machinery (such as poly(ADP-ribose) polymerase (PARP) and DNA-PK), disassembly of cell structure (nuclear and cellular morphology) and deregulation of structural proteins (such as actin, fodrin, lamin and gelsolin) [Enari *et al.*, 1998; Orth *et al.*, 1996; Rudel and Bokoch, 1997]

Apoptosis is regulated through the intrinsic mitochondria- or extrinsic death receptor-mediated pathway, and both pathways fundamentally converge to activate the downstream effector caspases (Figure 1.2) [Li and Yuan, 2008].

1.2.1. Intrinsic pathway

The mitochondrial-dependent intrinsic pathway is triggered by intracellular stress signals, such as ionizing radiation, chemotherapeutic drug treatment, growth factor withdrawal, adenosine triphosphate (ATP) depletion, oxidative stress or endoplasmic reticulum stress (ER stress). The intrinsic cellular stresses then aggregate at the mitochondrial membrane, resulting in mitochondrial membrane potential loss and mitochondrial outer membrane permeabilization (MOMP). The intrinsic pathway relies heavily on the Bcl-2 family of anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1) and pro-apoptotic members (Bax, Bak, Bid, Bad, Bim, Noxa, and Puma), and a shift in their balance may lead the cell towards apoptosis. Upon apoptosis, pro-apoptotic Bcl-2 proteins (Bak and Bax) activate, which after several conformational changes and oligomerization for the proteolipid pores formation in the mitochondrial outer membrane resulting in MOMP [Joza *et al.*, 2001; Danial *et al.*, 2007] and the cytosolic release of pro-apoptotic mitochondrial factors cytochrome *c*, apoptosis inducing factor AIF, endonuclease G EndoG and SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI)). Consequent to its cytoplasmic release, cytosolic cytochrome *c* together with apoptosis protease-activating factor-1 (Apaf-1) and

pro-caspase-9 forms the apoptosome, which main function is the activation of caspase-9. Activated capase-9 in turn catalyzes the activation of the downstream effector caspase-3, -6 and -7, that execute apoptotic changes in the cell, including cleavage of DFF40/45 (leading to DNA degradation [Widlak *et al.*, 2000] and PARP-1 (leading to its inactivation, and thus inhibiting its functions in DNA repair that given the circumstances could otherwise drain the cell of ATP). The cytoplasmic release of SMAC/DIABLO, on the other hand, inhibits the anti-apoptotic function of several members of the inhibitor of apoptosis (IAP) family (Survivin, Livin and XIAP), thereby derepressing caspase activation [Tamm *et al.*, 1998]. Following their mitochondrial release, AIF and EndoG relocate to the nucleus, where they mediate large-scale DNA fragmentation independently of caspases for apoptotic function [Kilbride and Prehn, 2013].

1.2.2. Extrinsic pathway

Otherwise, the death receptor-dependent extrinsic pathway is mediated through the binding of receptor specific cytokines (such as TNF-related apoptosis-inducing ligand TRAIL, FasL/CD95L and tumor necrosis factor TNFα) to death receptors (TNFR1, TRAILR1, Fas) on the cell surface. This leads to clustering of death receptors, intracellular recruitment of adaptor proteins (Fas-associated death domain FADD and tumor necrosis factor receptor type 1-associated death domain TRADD) and pro-caspase-8 or -10, thereby giving rise to the death inducing signaling complex (DISC) and subsequent activation of the latter caspases [Nagata *et al.*, 1997]. Caspase-8 or -10 then activate the downstream effector caspase-3 as well as the intrinsic pathway though cleavage of Bid, eventually leading to apoptotic cell death.

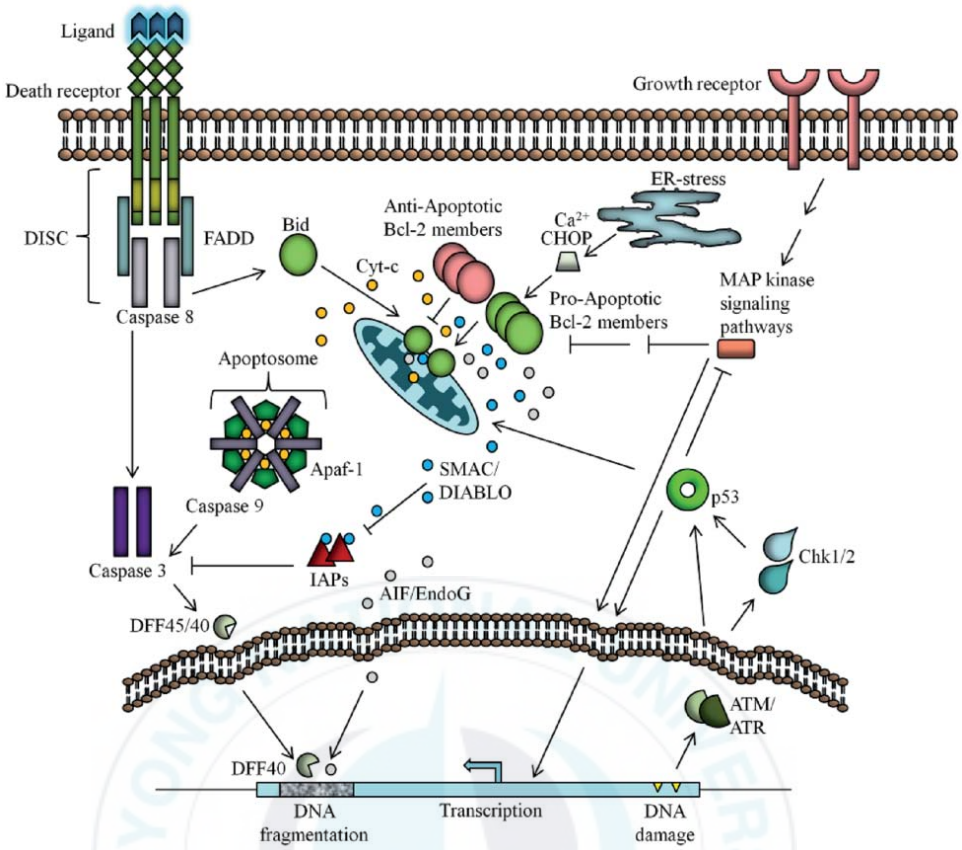

Figure 1.2. Simplified overview of apoptosis. Initiation of apoptosis can occur through the binding of an extracellular ligand to death receptors (extrinsic pathway), or through the mitochondrial permeabilization (intrinsic pathway) as a response to DNA damage or intracellular stress, and lead to the activation of initiator- and executioner caspases [Li and Yuan, 2008].

1.3. Autophagy

Autophagy (presently synonymous with macroautophagy) is an evolutionarily preserved mechanism in the eukaryotic cells to maintain homeostasis. Autophagy can be triggered by different stimuli: nutrient starvation, cytotoxic drug and metabolic stress. Autophagy has two important functions. One is the degradation of accumulated cellular proteins and unneeded/damaged organelles as a mean of recycling cellular building blocks and maintaining cellular homeostasis and integrity [Levine and Klionsky, 2004]. Another function of autophagy as a self-digestion process is the recycling of cellular contents during metabolic stress [Marino and Lopez-Otin, 2004]. Prolonged activation of autophagy due to continuous metabolic stress can contribute towards cell death. In this case, autophagy is seen as an accelerator rather than effector in non-apoptotic route of programmed cell death under normal physiological conditions. Dying cells very often display the accumulation of autophagosomes and autophagy is considered to be an important mediator of the clearance of cell corpses [Amelio *et al.*, 2011].

The morphological features elicited in autophagy include cell membrane blebbing, partial chromatin condensation with no DNA laddering, cytosolic vacuolization, degradation of Golgi, polyribosomes and endoplasmic reticulum. Briefly, autophagy is initiated as a flattened vesicle in the cytoplasm which grows up to a cup shaped isolation membrane called phagophore which sequesters the selected organelles and cytoplasmic materials. Eventually, this membrane closes to a double membrane autophagosome. The autophagosome then fuses with the lysosome forming an autolysosome which degrades the sequestered substances. Lysosomal hydrolases then degrade the intracellular material for energy [Fleming *et al.*, 2011].

1.3.1. Genes regulating autophagy

A highly conserved group of genes are involved in mediating autophagy, namely the autophagy related genes (Atg), which replaces the previous nomenclature of APG (autophagy), AUT (autophagy) and CVT (cytoplasm-to-vacuole targeting) pathways into one nomenclature. The best characterized Atg genes include Atg3, Atg 4, Atg5, Atg7, Atg10, Atg12, the microtubule associated protein 1 light chain 3 (LC3) (yeast homologue Atg8) and Beclin-1 (yeast homologue Atg6) [Tanida *et al.*, 2004; Edinger *et al.*, 2004].

Autophagy Related Genes (Atgs): With the exception of Beclin-1, all of the aforementioned genes are involved in the conjugation of Atg12 to Atg5 and the modification of LC3. Both of these modifications are required for autophagy to occur. Processing of Atg12 first involves its activation by Atg7 (an E1-like enzyme), its transfer to Atg10 (E2-like enzyme), and finally its conjugation to Atg5). The conjugated Atg12-Atg5 then forms what is known as an autophagosomal precursor, which designates the point of origin of the isolation membrane. Later, after the formation of the isolation membrane, Atg12-Atg5 dissociates from the membrane [Tanida *et al.*, 2004].

The second modification involves LC3 (yeast homologue Atg8), which has two forms: LC3-I is cytoplasmic and LC3-II is membrane bound. Pro-LC3 is converted to LC3-I by autophagin (Atg4) and undergoes further processing to LC3-II during an ubiquitin-like modification involving the E1-like enzyme Atg7 and E2-like enzyme Atg3 [Tanida *et al.*, 2004]. During the last step of LC3 processing, LC3-II is conjugated to phosphatidylethanolamine (PE), which anchors the protein into both the inner and outer membrane of preautophagosomes and autophagosomes. Increased level of LC3-II is an indicative of the extent of autophagosome formation in the cell; therefore LC3-II is commonly used as a marker of autophagy. Once the

autophagosome matures to an autolysosome, the lysosomal hydrolytic enzymes degrade the LC3-II on the inner membrane [Tanida *et al.*, 2004].

Beclin-1: Another important protein identified as a regulator of autophagy is Beclin-1, which was first discovered as a Bcl-2 binding partner through a yeast two hybrid screen. Beclin-1 is a coiled-coil, 60 kD protein that complexes with the class III phosphatidylinoside 3-kinase complex (PI3K) and localizes to the membrane of the trans-Golgi network. Beclin-1 and class III PI3K function to initiate autophagosome formation and assist other autophagy proteins in localizing to the membrane of the preautophagosome [Pattingre *et al.*, 2005]. Pattingre *et al.* (2005) suggested that under nutrient rich conditions Beclin-1 is bound to Bcl-2 and autophagy is inhibited. In contrast, under starvation conditions Bcl-2 dissociates from Beclin-1 and autophagy is induced [Pattingre *et al.*, 2005].

1.3.2. Signaling pathways regulating autophagy

Autophagy is regulated by several complex signaling pathways including nutrient related pathways (TOR and Ras/PKA), insulin/growth factor pathways, phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) pathways, energy signaling (AMPK), stress response (ER stress, hypoxia, oxidative stress) and infection (Figure 1.3) [Yang and Klionsky, 2010].

Phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling is one of the key regulatory pathways in inducing autophagy since it acts as the major sensor of nutrients, energy and growth factors [Levine and Kroemer, 2008; Wang *et al.*, 2012]. Under nutrient rich condition, PI3K gets activated and phosphorylates Akt which in turn phosphorylates and inhibits tuberous sclerosis protein 2 (TSC2). In the absence of activated TSC2, Ras homolog enriched in brain (RheB) is phosphorylated and activated which in turn will activate mammalian target of rapamycin (mTOR). mTOR inhibits the initiation of autophagy by phosphorylating and repressing ULK1. Under harsh metabolic

conditions (starvation, hypoxia), PI3K-mTOR pathway is inhibited, allowing for the autophagic machinery to be activated. It was recently found that Akt can directly phosphorylate Beclin-1 independently of mTOR thereby inhibiting autophagy and promoting oncogenesis [Wang *et al.*, 2012].

In recent years, it was discovered that glucose is the primary energy source for mammalian cells and autophagy activities are modulated in accordance with glucose availability. The initiation of autophagy process is regulated by 5' AMP-activated protein kinase (AMPK), an energy sensor in the cells. AMPK is a serine tyrosine kinase which can sense the level of adenosine monophosphate (AMP) and ATP. Increased AMP: ATP ratio can trigger the activation of AMPK by the serine threonine kinase liver kinase B1 (LKB1). Activated AMPK phosphorylates and inhibits mTOR thus allowing for the initiation of autophagy [Bell *et al.*, 1990; Hardie *et al.*, 2011].

1.3.3. Connection between apoptosis and autophagy

There is an extensive interplay between apoptosis and autophagy. One convergence point is Beclin-1. Beclin-1 has a BH3-only protein domain which can bind with anti-apoptotic Bcl-2 family members [Kang *et al.*, 2011]. Bcl-xL binds to the BH3-domain of Beclin-1 and inhibits the starvation-induced autophagy. Inhibition of Bcl-2 expression by small interfering RNA in MCF-7 breast cancer cells also lead to autophagic cell death [Akar *et al.*, 2008]. Additionally, Bcl-2, which is found in the mitochondria and ER inhibits apoptosis, but only ER-localized Bcl-2 functionally inhibits autophagy by preventing calcium release from the ER, resulting in the subsequent activation of mTOR and the repression of autophagy.

Another convergence point is Atg5. In an independent study, Atg5 protein has been documented to interact with Fas associated protein with death domain (FADD) to induce apoptosis. Atg5 can be cleaved by calpains to a 24kD fragment which

then acts as BH3-only protein interacting with Bcl-xL, allowing for the activation of Bax and the induction of apoptosis [Yousefi *et al.*, 2006]. These studies suggest that autophagy and apoptosis are regulated in a similar manner.

Nevertheless, apoptosis and autophagy occasionally occur in a mutually exclusive manner. Caspases, effectors of the apoptotic pathways, have been shown to cleave and inactivate Beclin-1, and the resulting suppression of Beclin-1 increases apoptosis in HeLa cervical cancer cells. In a less drastic scenario, camptothecin (CPT) treatment in breast cancer cells results in autophagy activation and delayed apoptosis. In this context, autophagy maybe activated to protect cells from dying (apoptosis) [Morselli *et al.*, 2009; Janku *et al.*, 2011].

Thus, autophagy and apoptosis share a common panel of regulators, and the decision to activate either or both of these cellular processes is governed by the specific post-translational modification and the cellular localization of these proteins. Both autophagy and apoptosis can occur together to enhance cell killing, or in a mutually exclusive manner. The decision to activate either of these pathways is most likely governed by the health status of the cell, or perhaps the nature of the stimuli.

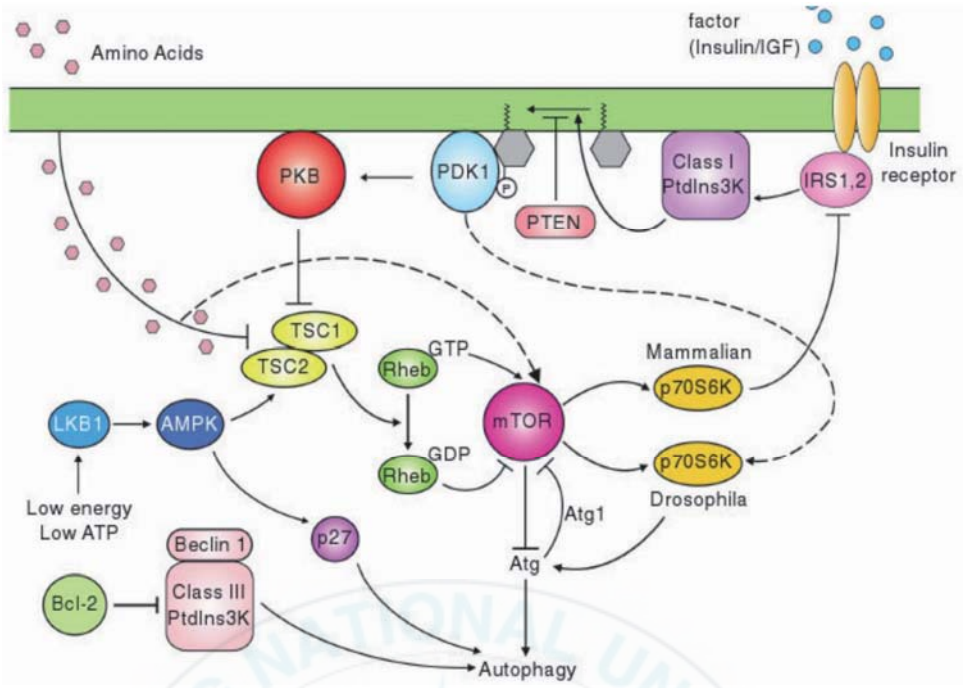

Figure 1.3. Regulation of autophagy in mammalian cells. Autophagy occurs at a basal level and can be induced in response to environmental signals including nutrient, energy deprivation and also microbial pathogens. Insulin regulates growth by binding to the insulin receptor, causing activation of class I PI3K signalling and phosphorylation of plasma membrane lipids. The regulation of autophagy is complex and far from understood. The best characterized regulatory pathway includes class I PI3K and mTOR, which act to inhibit autophagy. The class III PI3K/Vps34 is needed for activation of autophagy. mTOR activity is probably regulated in part through feedback loops to prevent insufficient or excessive autophagy [Yang and Klionsky, 2010].

1.4. Endoplasmic reticulum (ER) stress

Endoplasmic reticulum (ER) is a key organelle in the regulation and secretion of all proteins. After translation of linear proteins, these nascent peptides enter into the ER and become mature in this organelle. The ER ensures proper folding and post-translational modifications of entered proteins before these proteins are transported to Golgi.

The lumen of ER is an environment with very high Ca^{2+} concentration. Ca^{2+} are continuously transported into the ER by active transport through calcium ATPases. The lumen with high Ca^{2+} concentration is a suitable environment for formation of disulfide bonds of proteins. Thanks to such oxidative environment and presence of calcium-dependent chaperone proteins in the ER, protein folding is done within the ER and folded proteins are transported out [Xu *et al.*, 2005].

ER works in a dynamic fashion. The flux into the ER is not always at the same level, changing according to the cells' programs. Physiological state of the cell and environmental conditions are the main factors affecting the dynamic situation of the ER. ER always tries to keep protein mechanism well maintained. Therefore, protein folding capacity is adjusted in order to retain high fidelity. To maintain this homeostasis, ER requires sensors to determine the physiological conditions in the ER and signaling to regulate and maintain the homeostasis. However, homeostasis cannot be always well-maintained, resulting in an imbalance between the unfolded protein load in the ER and the effectiveness of ER machinery working on handling the situation. This imbalance is called endoplasmic reticulum stress (ER stress). The pathway reconciling the stress response and ER homeostasis is called unfolded protein response (UPR). Presence of such a sensing mechanism and regulation was detected in a study showing that increasing the unfolded protein load of ER results in increased expression of ER lumen chaperones [Ron and Walter, 2007].

1.4.1. Unfolded protein response (UPR)

Disturbances in the ER's homeostatic environment disrupts the protein folding machinery and results in an accumulation of unfolded proteins in the ER lumen, thus sensing the stress and activating the unfolded protein response (UPR). The UPR's primary aim is to sustain cell survival by attenuating protein synthesis and restoring cellular homeostasis via the activation of a cascade of transcription factors which regulate expression of genes encoding for chaperones, components of the ER-associated degradation (ERAD) system and components of the autophagy machinery**.**

The UPR is orchestrated by three ER-transmembrane transducers: inositol requiring enzyme-1 (IRE1), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6), which are maintained in an inactive state through association with the ER chaperone, glucose regulated protein 78 kDa (GRP78/BiP or HSPA5) under physiological conditions (Figure 1.4). Upon ER stress, unfolded proteins accumulate in the ER lumen resulting in the dissociation of Grp78 from PERK, IRE1 and ATF6, subsequently activating the UPR [Reddy *et al.*, 2003].

PERK: PERK is a type I ER transmembrane protein with serine/threonine kinase activity. Its N-terminus is in the ER lumen, involved in the regulation of its dimerization, and is kept inactive through interaction with Grp78, while the C terminus is cytosolic and harbors its autophosphorylation sites and the kinase domain. Upon release of Grp78, PERK is activated similar to IRE1 by oligomerization of PERK and trans-autophosphorylation of its cytoplasmic domain [Kouroku *et al.*, 2007]. In addition to its autophosphorlyation function, PERK also phosphorylates eukaryotic translation initiation factor-2-alpha (eIF2α) at Ser51 site. Ser51 phosphorylation of eIF2α results in inhibition of eIF2α re-cycling by guanine nucleotide exchange factor eIF2B. Lack of eIF2B functioning on eIF2α decreases the amount of active GTP-bound form of eIF2α. As a result of less active eIF2α,

general translation initiation is halted, thereby reducing the amount of newly synthesized proteins. Activation of the PERK arm not only signals through reduction of translation, but also regulates activation of some downstream targets. For instance, activating transcription factor-4 (ATF4) and nuclear factor κB (NFκB) are activated at translational and post-translational levels via PERK signaling [Ron and Walter, 2007]. In the pro-survival response of the UPR, ATF4 acts as an essential player in activating another important transcription factor C/EBP homologous protein (CHOP), which is also called growth arrest, DNA damage inducible protein-34 (GADD34) and ER oxidase-1 (ERO1) [Marciniak *et al.*, 2004]. CHOP is reported to downregulate Bcl-2 [McCullough *et al.*, 2001] and upregulate transcription of certain BH3-only proteins [Puthalakath *et al.*, 2007]. This event favors Bax/Bak activation which leads to MOMP and initiation of the intrinsic apoptotic pathway. Furthermore, CHOP knockout mice show lower rates of apoptosis in response to ER stress [Oyadomari *et al.*, 2002]. Although CHOP is thought to be a major factor in determining cell fate in response to ER stress it is clear that other factors are also involved.

IRE1: Other arm of the UPR signals through IRE1. IRE1 is a type I ER transmembrane protein containing a serine/threonine kinase domain and an endoribonuclease. There are two IRE1 isomers in humans, IRE1α and IRE1β. IRE1α is ubiquitously expressed, whereas IRE1β expression is restricted to the epithelial cells of the intestine and the lungs. IRE1 is the most conserved branch of the UPR, and has been suggested to play a role in processes such as development, metabolism, immunity, inflammation and neurodegeneration [Kaufman *et al.*, 2010]. IRE1was firstly identified in yeast and found to be encoded by IRE1 gene. This gene encodes an ER transmembrane protein with a luminal and a cytoplasmic domain. Cytoplasmic domain of IRE1 consists of a kinase activity, which is triggered by activation of ER stress signal coming from the luminal part. Following the activation signal, IRE1 activates itself by oligomerization and trans-13

autophosphorylation of the kinase domains [Zhou *et al.*, 2006]. Activation of the kinase domain of IRE1 also activates its other functional response, endonucleolytic cleavage. The substrate of this endonucleolytic activity is a transcription factor Hac1 in yeast [Mori *et al*., 1996], and Xbox binding protein 1 (XBP1) in metazoans. IRE1 dependent transcriptional regulation works through its endonucleolytic activity to cleave and remove an intron from XBP1 mRNA. This cleavage makes XBP1 transcription factor active by leading spliced XBP1 mRNA to translation. Apart from activation of XBP1 by endonucleolytic cleavage, XBP1 expression is also controlled at the transcription level during UPR. XBP1 mRNA levels are increased as UPR is induced. Functional XBP1 protein works as a transcription factor at the promoter sites of some genes having role in endoplasmic reticulum associated protein degradation and transport of unfolded proteins out of ER. Furthermore, with accompany of NF-Y, XBP1 binds to two different types of cis-acting elements, which are ER stress enhancer and UPR element [Yoshida *et al*., 2006]. On the other hand, IRE1 is responsible for activation of kinases having role in inflammation and cell death machinery in association with TRAF2. Downstream of IRE1, TRAF2 activates kinase Ask1 and stress induced Jun N-terminal kinase (JNK) [Urano *et al*., 2000], as well as caspase-12 of apoptotic pathway [Yoneda *et al*., 2001].

ATF6: The third arm of UPR involves ATF6. ATF6 is a type II transmembrane receptor and a member of the leucine zipper protein family that is synthetized as an ER membrane-tethered precursor, with its C terminal domain located in the ER lumen and its N-terminal DNA-binding domain facing the cytosol 21. There are two isoforms of ATF6, ATF6α and β [Ye *et al*., 2000]. In resting cells, ATF6 is found tethered to ER membrane as an inactive precursor. Upon ER stress, ATF6 is dissociated from GRP78/BiP, then transported from ER membrane to Golgi apparatus. At the Golgi, the 90 kDa ATF6 protein undergoes two protease cleavages, which are processed by site 1 protease (S1P) and by site 2

protease (S2P), respectively. After cleavage, remaining active ATF6 fragment (50 kDa) translocates into the nucleus to act as an activate transcription factor for the UPR target genes, including XBP1 and CHOP/GADD153 [Haze *et al.*, 1999]. When UPR signal is activated, IRE1 and ATF6 arms of UPR act cooperatively in a way that while ATF6 increases the transcription of XBP1, IRE1 undertakes endonucleolytic cleavage and activating role of XBP1 to trigger expression of target alarm genes.

1.4.2. ER stress and cell death

Accumulation of excess unfolded proteins in the ER triggers UPR to somehow overcome this unfavorable situation. Three main factors of UPR control activities of some key regulators functioning in the cell death response. While the decision of cells' survival or death relies on the activities of these key UPR elements, actual factor behind this scenario is dependent on how severe the cell experiences a stress in the ER and how persistent the stress is.

ER stress might contribute to apoptosis through some key apoptotic regulators, such as Bax and Bak. This pathway is generally thought to be regulated by the balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins. If this balance favors the pro-apoptotic Bcl-2 family proteins, mitochondrial permeabilization will occur and trigger the release of mitochondrial factors and the formation of apoptosome, leading to the intrinsic apoptosis pathway. In the process, the central role of caspase-9 is mobilized to execute apoptosis in response to ER stress; however, recent studies have also suggested that caspase-2 may play a role in inducing MOMP in certain cell models upstream of caspase-9 activation [Gupta *et al.*, 2010]. ER stress mediated cell death might also signal through IRE1 branch of UPR. In this case, caspase-12 activation with the help of TRAF2 triggers death in mice [Nakagawa *et al.*, 2000]. Paralog of mice caspase-12 in human, caspase-4 is also associated with the ER functioning, possibly performing a similar role of its

paralog in mice [Hitomi *et al*., 2004]. Furthermore, IRE1 mediated Ask1 activation leads to JNK activation, resulting in the phosphorylation of JNK targets. Among these targets, phosphorylation of Bcl-2 inhibits its anti-apoptotic activity, whereas phosphorylation of Bim results in activation of its pro-apoptotic function. At the transcription factor level, ER stress induced apoptosis is regulated by CHOP (GADD153). CHOP can repress the expression of anti-apoptotic Bcl-2, resulting in the promotion of cell death [McCullough *et al*., 2001]. In brief, at least two of the three branches of UPR have already been detected as factors in ER stress induced cell death. Therefore, cell death response is likely to be resulted from the cooperative role of different UPR signaling elements. Even though the primary goal of UPR is to alleviate the stress level in the ER, if the improper folding process in the ER is excessive or persistent, UPR response aims directing cells to death, typically apoptosis ([Xu *et al*., 2005].

The ability of ER stress to trigger autophagy has now been shown in several studies [Kouroku *et al*., 2007]. The UPR and autophagy both function to relieve cellular stress and reinstate homeostatic environment. Autophagy has also been shown to be a destructive process under certain cellular stress conditions. Studies have shown that in conditions where the intrinsic pathway is compromised ER stress can result in a form of autophagic cell death with features of necrosis [Ullman *et al*., 2008]. More recent studies are suggesting that the autophagosome may be acting as a platform for the formation of a death inducing signaling complex, similar to that found at the plasma membrane, resulting in a form of autophagy mediated apoptosis [Young *et al*., 2012]. The PERK-eIF2α branch of UPR was shown to mediate polyglutamine induced LC3 conversion and thus induce autophagy [Kouroku *et al*., 2007]. The IRE1 arm of the UPR pathway was also shown to induce autophagy through JNK/TRAF2/JNK and thus modulating Beclin 1 function and expression [Urano *et al*., 2000]. Regulation of the mTOR pathway is one of the mechanisms that link ER stress and autophagy. ER is the

major calcium storage site of the cell and Ca^{2+} release due to ER stress into the cytosol may activate autophagy through several mechanisms. Ca^{2+}calmodulin dependent kinase kinase β (CaMKKβ) is activated upon an increase in the cytoplasmic Ca^{2+} which activates AMPK and thus inhibits mTOR. Another Ca^{2+} activated kinase is aforementioned DAPK.

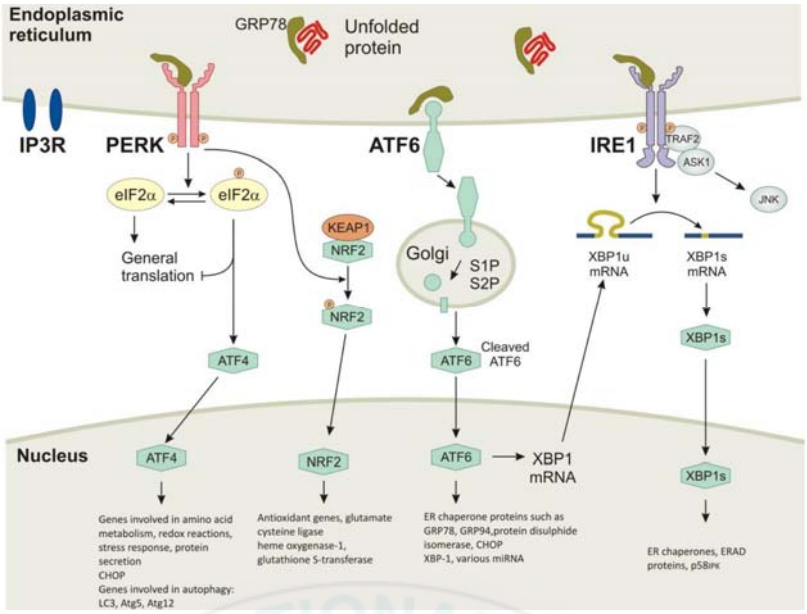

Figure 1.4. The accumulation of unfolded proteins in the ER lumen results in the dissociation of Grp78 from the three UPR sensors PERK, ATF6 and IRE1. Following Grp78 dissociation PERK dimerizes and autophosphorylates, activating its cytosolic kinase domain. PERK phosphorylates EIF2α inhibiting general protein synthesis and facilitating/permitting non canonical translation of ATF4 mRNA. Active PERK also phosphorylates NRF2 resulting in its dissociation from KEAP1, allowing NRF2 to translocate to the nucleus. Activation of ATF6 leads to its translocation to the Golgi where it is processed by site 1 and site 2 proteases (S1P and S2P) into an active transcription factor which results in the transcription of XBP1 mRNA. Activation of IRE1 results from its dimerization and autophosphorylation in a manner similar to PERK. IRE1 contains an endoribonuclease domain which processes unspliced XBP1 mRNA. Spliced XBP1 (XBP1s) mRNA is translated into an active transcription factor. IRE1 also possesses a kinase domain that recruits TRAF2 and ASK1 leading to the activation of JNK [Deegan *et al*., 2013].

1.5. Cell cycle

In order to propagate, mammalian cells must undergo a process of self-replication, in which a cell produces an exact copy of its genetic material and itself. This process is a highly orchestrated event that is tightly regulated by a series of checkpoints to insure transfer of the genetic material and survival of the daughter cells. The series of events that occur in a cell that lead up to cell division is collectively known as the cell cycle, which can be broken down into four distinct phases: Gap 1 (G1), DNA Synthesis (S), Gap 2 (G2) and Mitosis (M) (Figure 1.5) [Hanahan and Weinberg, 2011].

1.5.1. Cell cycle regulation

Progression through the different phases of the cell cycle is directional and tightly regulated. Transition to the next phase only occurs with the correct assembly and activation of cyclin and cyclin dependent kinase (CDKs) complexes. The CDKs form the catalytic subunit of the complex, and are stably expressed throughout the cell cycle. Cyclins, on the other hand, are expressed in a phase-specific manner, and function as the regulatory subunit of the hetrodimer. The oscillating expressions of the different cyclins are achieved by their timely synthesis and ubiquitin-mediated proteolysis, resulting in phase specific cyclin-CDK combinations (Figure 1.5). In order to become fully active, the assembled cyclin-CDK complex needs to be phosphorylated by a CDK-Activating Kinase (CAK) [Fisher and Morgan, 1994].

In response to extracellular signals (such as growth factors) cyclin D is produced in the early stages of the G1 phase [Sherr and Roberts, 1999]. Cyclin D binds to CDK4 and CDK6, forming active cyclin-CDK complexes that in turn phosphorylate the retinoblastoma susceptibility protein (Rb). Upon phosphorylation, Rb dissociates from its binding partner E2F, thereby activating

the latter transcription factor [Weinberg *et al.*, 1995]. Activated E2F can then transcribe various genes encoding proteins that are necessary for the transition to S phase; these include cyclin E, cyclin A and DNA polymerases [Johnson and Schneider-Broussard, 1998]. Activation of the cyclin E-CDK2 complex leads the cell from G1 to S phase. Cyclin E is slowly degraded during S phase, and its partner CDK2 now binds to cyclin A which allows the cell cycle to progress to late S phase. CDK1 is then activated by A-type cyclins at the later stages of S phase, which contributes to driving the cell towards mitosis [Furuno *et al.*, 1999]. At the onset of mitosis, CDK1 forms a complex with cyclin B which drives the cell through the final stages of the cell cycle.

CDK activity is also regulated by two families of CDK inhibitors (CDKIs): Inhibitor of kinase 4 (INK4) proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) and the CDK interacting protein/kinase inhibitory protein (Cip/Kip) family ($p21^{Cip1/WAF}$, $p27^{Kip1}$ and $p57^{Kip2}$). Additionally, activity of CDK1 and CDK2 may be restricted by inhibitory phosphorylation on the Tyr15 and Tyr14 residues mediated by Myt1 and Wee1. The latter inactivating phosphorylations can again be removed by the cdc25s.

1.5.2. Cell cycle checkpoints

Cell cycle checkpoints are regulatory pathways that control the order and timing of transitions and may stop cell cycle progression under unfavorable conditions. These signal transduction pathways may respond to both extrinsic and intrinsic factors, and malfunctions of such checkpoints have been implicated in tumorigenesis [Elledge *et al.*, 1996]. Four major checkpoints are found; G1/S, Intra S, G2/M and the spindle assembly checkpoint (Figure 1.5).

Although this process occurs hundreds of times, cancer cells can arise through the accumulation of many mutations in either the checkpoint genes or proliferation related genes in a cell. Together, these abnormalities disrupt the normal checks and

controls that regulate the cell cycle, leading to uncontrolled cellular proliferation or cancer. Since cancer is a genetic disease, recent advances in molecular, proteomic, and cellular biology has been used to uncover these abnormalities so that drugs can be developed to target mis-behaving proteins and eliminate the problematic cells. However, current therapeutics often have many side effects, and as a result lead to a poorer quality of life for patients [Hanahan and Weinberg, 2011].

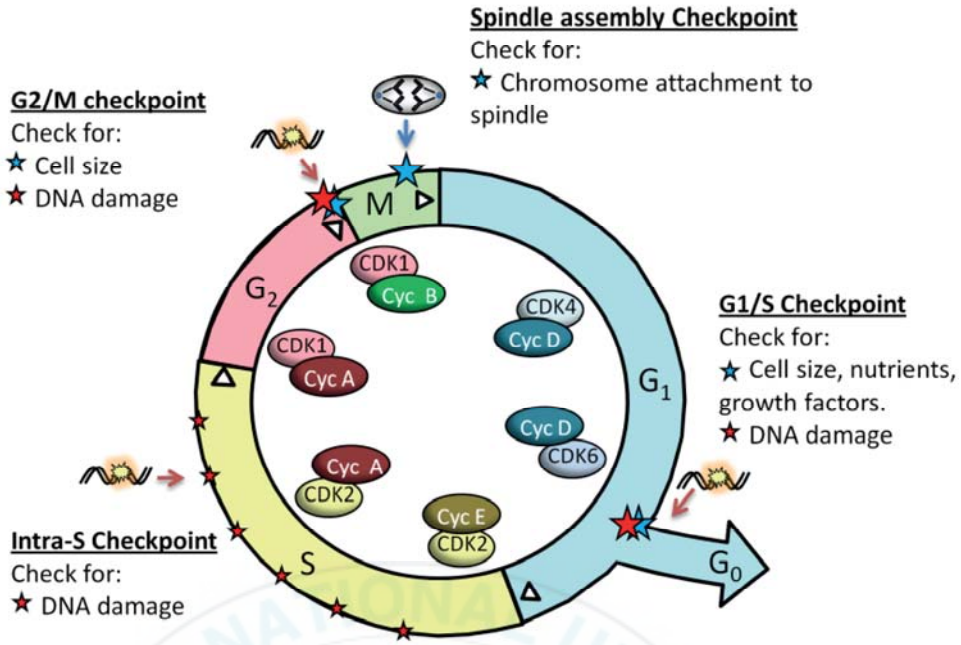

Figure 1.5. Overview of the cell cycle and its checkpoints. The cell monitors internal and external conditions, as indicated under the respective checkpoints, and may halt the cell cycle under unfavorable circumstances. The red stars signify DNA damage checkpoints, and the blue stars are checkpoints where the cell monitors external or internal conditions (as indicated in the figure). Overview of the cyclin-CDK complexes present indifferent stages of the cell cycle (inner circle) [Hanahan and Weinberg, 2011].

1.6. Aims of the present study

In order to investigate the antitumor effects and cytotoxic activities of herbal medicine *C. amuricus* on liver cancer and elucidate the potential cell death pathways in *C. amuricus*-treated HCC Hep3B cells, the following aims were defined:

Chapter 2 focuses on the induction of apoptosis and G0/G1 arrest by *C. amuricus* in HCC Hep3B cells via the extrinsic death receptor- and intrinsic mitochondria-mediated pathways.

Chapter 3 focuses on the induction of G1 arrest and mitochondrial-mediated apoptosis by *C. amuricus* in HCC Hep3B cells via endoplasmic reticulum stress.

Chapter 4 focuses on the induction of apoptosis and autophagy by *C. amuricus* in HCC Hep3B cells via AMPK and PI3K/Akt/mTOR signaling pathways.

All of the results support that *C. amuricus* induce cell cycle arrest, ER stress, apoptosis and autophagy in HCC Hep3B cells.

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PART II

Cyperus amuricus Induces Cell Death in Human Hepatocellular Carcinoma Hep3B Cells

Chapter 2

Induction of apoptosis and G0/G1 cell cycle arrest in Hep3B cells by *Cyperus amuricus*

2.1. Abstract

Cyperus amuricus (*C. amuricus*) is one of the most common herbs in Oriental folk medicine for exerting astringent, diuretic, wound healing and other intestinal problems. However, little is known about the molecular mechanism of *C. amuricus* on anticancer activity. In the present study, the underlying mechanism of the anticancer effect of *C. amuricus* were elucidated. The methyl alcohol extract from the whole plant of *C. amuricus* exhibited cytotoxicity against Hep3B cells, but not against A549 and HaCaT cells. Consistent with an acceleration of the sub-G1 phase, downregulation of cdc25A, cyclin D1 and cyclin E, CDK4 and 2 as well as E2F-1, phospho-Rb, with concomitant of upregulation of $p21^{CIP1/WAF1}$, $p27^{KIPI}$ and $p16^{INK4a}$ proteins, as evidenced by the appearance of cell cycle arrest, were detected in *C. amuricus*-treated Hep3B cells. Additionally, the sequential activation of various caspases (cleaved of caspase-8, -9, -3, -7, and -6, and cleaved PARP) and the changed expression of other proteins related to the apoptosis pathway were observed after *C. amuricus* exposure. An increment in the pro-apoptotic proteins (Bim, tBid, Bax and Bak) and a reduction of anti-apoptotic protein (Bcl-2) regulate Hep3B cell death by controlling the permeability of mitochondrial membranes and the release of cytochrome *c* from mitochondria into the cytosol with Apaf-1 after *C. amuricus* treatment. This is the first study indicating the potential of *C. amuricus* as a complementary agent for prevention and treatment of human liver cancer.

2.2. Introduction

Cancers are a group of diseases characterized by uncontrolled cell growth and spread. Primary liver cancer, especially hepatocellular carcinoma (HCC), is the fifth most common malignancy with more than 500,000 new cases diagnosed every year, and the third leading cause of cancer death with a mortality-to-incidence ratio exceeding 0.9 in the world [Llovet *et al.*, 2003; Ferlay *et al.*, 2008]. The rate of HCC is annually increasing worldwide between 3% and 9%, and the incidence of HCC is particularly higher in Southeast Asia and sub-Saharan Africa due to the higher frequency of chronic viral hepatitis [Rampone *et al.*, 2009]. Generally, HCC is associated with dietary aflatoxin B1 intake or heavy alcohol consumption, and alternative causes of hepatic cirrhosis with a persistent hepatitis B virus or C virus infection, which is a 3.2 kb, partially dsDNA, non-cytopathic virus, and the most important etiologic factor for malignant HCC [Anthony *et al.*, 2001]. Until now, although surgical resection, orthotropic liver transplantation and radiofrequency ablation have shown excellent results in the treatment of early stage liver cancer, there is no curative therapy for patients with advanced HCC [Ferlay *et al.*, 2008]. Therefore, HCC remains a serious global problem, and more effective prevention, diagnosis and treatment strategies are urgently needed.

Apoptosis is linked to cell cycle arrest, and the blockade of the cell cycle is regarded as an effective intrigue for eliminating cancer cells. In recent years, many chemotherapeutic agents have been shown to impart anti-proliferative effects via arrest of cell division at certain checkpoints in the cell cycle. The concept of cell cycle-mediated apoptosis has gained increasing attention as this pathway may provide minimal opportunity for acquired drug resistance, decreased mutagenesis and reduced toxicity [Sandal *et al.*, 2002; Kögel *et al.*, 2010]. These observations suggest new approaches could alter uncontrolled cancer cell growth by modulating

cell cycle regulators causing cell cycle arrest and could be useful in prevention and/or intervention in human cancer [Sandal *et al.*, 2002].

The present study was conducted to elucidate the underlying mechanisms of the extract of *C. amuricus*-induced antiproliferation, cell cycle arrest, and apoptosis in HCC Hep3B cells *in vitro*, so as to supply scientific rationales for using *C. amuricus* as a new promising chemopreventive and/or chemotherapeutic agent against liver cancer.

2.3. Materials and methods

2.3.1. Cell culture and reagents

Hep3B (hepatocellular carcinoma), A549 (human lung adenocarcinoma) and HaCaT (non-cancerous human keratinocyte) cells were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (for Hep3B and HaCaT), RPMI-1640 (for A549) (HyClone Laboratories, Logan, UT, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1% penicillin-streptomycin (PAA Laboratories GmbH, Australia) at 37˚C and 5% CO_2. The methyl alcohol extract from the whole plant of *C. amuricus* (distribution number: 010-032) was obtained from the Korea Plant Extract Bank (KPEB, Cheongju, Korea) with the purity of ≥99.9%, HPLC.

2.3.2. Cell viability assay

Exponential phases of Hep3B, A549 and HaCaT cells ($1x10^4$ cells/well) were seeded on 96-well plates (SPL Lifesciences, Gyeonggi, Korea) in triplicate. Following overnight incubation, cells were treated with various concentrations (50, 100, 150, and 200 μg/ml) of an extracted fraction of *C. amuricus* and incubated for 24 h. After the treatment, 10 μl of EZ-Cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Seoul, Korea) was added to each well and incubated for an additional 3 h. The absorbance of the reaction was measured using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 460 nm and cell viability was calculated. The cytotoxic activity of the extract was expressed as an IC_{50} value, which is the concentration of the extract that caused 50% cell death. The extract of *C. amuricus* with an IC_{50} value ≤150 μg/ml was considered active on Hep3B cells. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used to dilute the extract and the final concentration of DMSO in each well was not in

excess of 0.05% (v/v). No adverse effect due to the presence of DMSO was observed.

2.3.3. Treatment of Z-VAD-fmk

Hep3B cells were divided into four groups to compare caspase-dependent and caspase-independent cell death as follows: non-treated, Z-VAD-fmk (pan caspase inhibitor, Sigma-Aldrich), *C. amuricus*, and *C. amuricus* with Z-VAD-fmk groups. After 24 h, the cells were transferred to a fresh medium containing no agent (control group), Z-VAD-fmk (50 μM Z-VAD-fmk), *C. amuricus* (150 μg/ml *C. amuricus*), or a combination (150 μg/ml *C. amuricus* and 50 μM Z-VAD-fmk). After treatment for 24 h, 10 μl of WST-1 solution was added to each well, further incubated for 3 h, and then cell viability was measured at 460 nm using an ELISA reader.

2.3.4. DAPI staining

Hep3B cells were treated with the concentration of 100, 150 and 200 μg/ml of the *C. amuricus* extract for 24 h. The cells were rinsed once with phosphate-buffered saline (PBS) buffer (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium dihydrogen phosphate) and stained by addition of 1 μg/ml DAPI solution (Roche Applied Science, Indianapolis, IN, USA) in methanol (Sigma-Aldrich). After incubation in the dark at 37˚C for 20 min, cells were washed twice with PBS buffer, and then fixed with 4% formaldehyde (Junsei, Tokyo, Japan) for 15 min. The nuclear morphology of the cells was observed under a Laser Scanning Confocal Microscope (Carl Zeiss LSM 700; Carl Zeiss, Oberkochen, Germany).

2.3.5. DNA fragmentation

For detecting genomic DNA fragmentation, the treated Hep3B cells were washed with ice-cold PBS buffer and harvested. The collected cells were handled

by following the protocol of DNeasy® Blood and Tissue kit (Qiagen GmbH, Hilden, Germany). The isolated DNA was separated in a 1.5% agarose gel (Life Technologies Inc., Grand Island, NY, USA) and visualized by ethidium bromide staining (Sigma-Aldrich) under a UV transilluminator (Vilber Lourmat, Marne-la-Vallee, France).

2.3.6. Cell cycle analysis

Briefly, Hep3B cells were harvested by trypsinization and fixed with 70% ethanol overnight at 4˚C. Then, the cells were resuspended in PBS buffer containing 0.2 mg/ml RNase and incubated for 1 h at 37˚C. The cells were stained with 40 μg/ml propidium iodide (Sigma-Aldrich) at room temperature for 30 min in the dark. The cell cycle was analyzed based on DNA contents using a flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

2.3.7. Protein extraction and western blot analysis

Hep3B cells were washed once with PBS buffer and then lysed by the addition of lysis buffer [(50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, prostatin A)] (Intron Biotechnology, Gyeonggi, Korea). After 30 min on ice, lysates were collected and clarified by centrifugation at 14,000 rpm for 20 min at 4˚C. Cytosolic fractions were prepared using NE-PER® Nuclear and Cytoplasmic Extraction reagents (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's protocol. Aliquots of whole cell lysates or cytosolic fractions were subjected to 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Pall Corp., Pensacola, FL, USA). The membranes were blocked with 5% skim milk in PBST (PBS buffer and 0.5% Tween-20). After blocking non-specific sites, the membranes were probed with primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) and then washed in PBST three times, followed by incubation for 1 h with horseradish peroxidase-conjugated

anti-rabbit IgG or anti-mouse IgG as second antibodies (Cell Signaling Technology). The blots were then washed in PBST and visualized by an enhanced chemiluminescent (ECL) detection solution (Pierce, Rockford, IL, USA). Anti-Bim were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other antibodies were purchased from Cell Signaling Technology.

2.4. Results

2.4.1. Effects of *C. amuricus* on the cell growth

In order to understand the cytotoxicity of the extract, Hep3B, A549 and HaCaT cells were exposed to the methanol extract of *C. amuricus* by cell viability assay. As shown in Figure 2.1A, the *C. amuricus* extract did not have significant effects on either A549 or HaCaT cell viability while the extract (over 100 μg/ml) caused remarkable growth inhibition and a marked decrease in cell viability only in Hep3B cells. Cell death reached 50% with 150 μg/ml (IC_{50}) and 63% with 200 μg/ml of the *C. amuricus* treatment for 24 h, respectively, and those concentrations were used in subsequent experiments to investigate the mechanism of cell death. Additionally, morphological changes of Hep3B cells treated with or without the extract of *C. amuricus* for 24 h were visualized under the inverted microscope (magnification, x100). Compared with the non-treated cells, most of the *C. amuricus*-treated Hep3B cells (100-200 μg/ml of the extract) did exhibit morphological features of apoptosis, such as cell membrane blebbing, cell shrinkage, increased cytoplasm granules and detachment from culture plates (Figure 2.1B). The data initially indicate that the extract of *C. amuricus* induces selective cytotoxicity in HCC Hep3B cells.

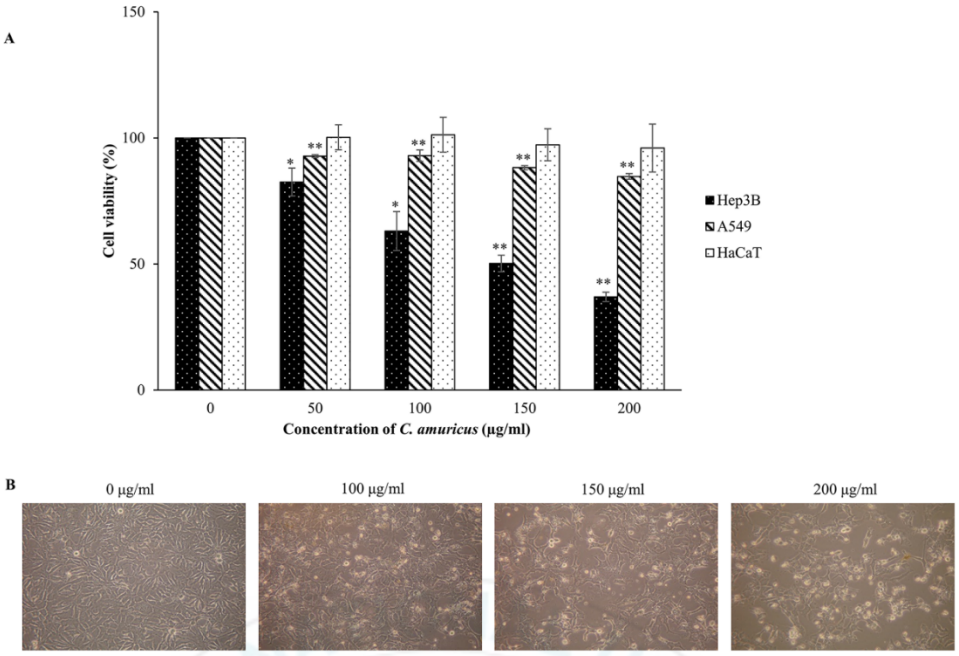

Figure 2.1. Effects of *C. amuricus* on the cell viability of Hep3B cells. (A) Cytotoxicity of the *C. amuricus* extract was determined using the cell viability assay. Hep3B, A549 and HaCaT cells were treated with 50-200 μg/ml of the extract for 24 h. Bars represent the mean ± SD of three experiments. **P*<0.05; ***P*<0.01. (B) Morphological features of apoptosis in Hep3B cells after the *C. amuricus* exposure. Hep3B cells were incubated with the extract for the indicated concentrations and visualized by the inverted microscope (magnification x100).

2.4.2. Effects of *C. amuricus* on the induction of apoptosis

Further experiments were then carried out to determine whether the extract of *C. amuricus* inhibits the proliferation of Hep3B cells through the induction of apoptosis. Morphological analysis following DAPI staining was examined to analyze the phenotypic changes in cell nucleus. The classical hallmark of apoptotic cells with chromatin condensation and apoptotic bodies, was observed in Hep3B cells treated with the *C. amuricus* extract in a dose-dependent manner, indicating that the extract of *C. amuricus*-stimulated Hep3B cell death was a typical apoptotic cell death (Figure 2.2A). More evidence in support of apoptosis was performed by DNA fragmentation assay. The biochemical hallmark of apoptotic cell death, in which cleavage of chromosomal DNA at internucleosomal fragments or multiples at ~180 bp [Nicholson and Thornberry, 1997], results in a typical DNA electrophoresis ladder, was visible during incubation with the *C. amuricus* extract (Figure 2.2B). On the basis of the above data, the profile for *C. amuricus*-caused apoptosis closely correlated with its growth suppressive effect. Thus, these phenomena demonstrate that the extract of *C. amuricus* may be considered as an inducer of apoptosis in Hep3B cells.

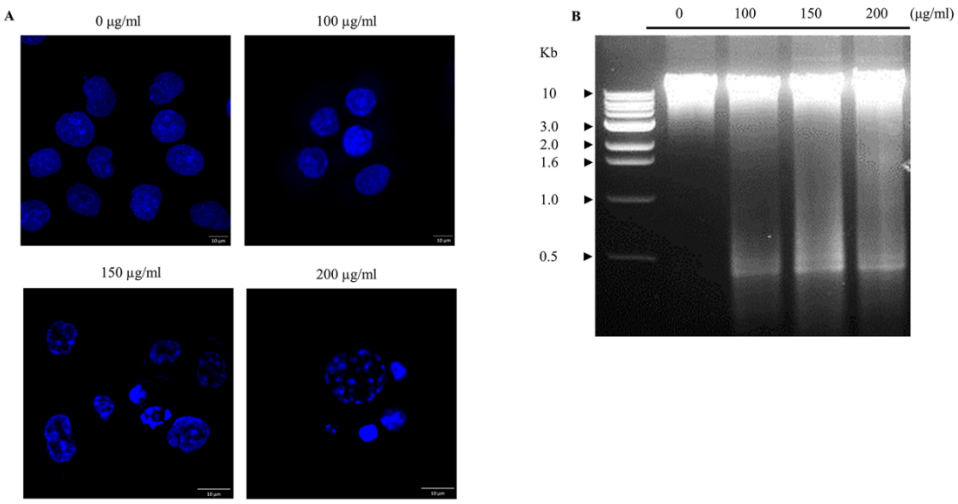

Figure 2.2. Effects of *C. amuricus* on the induction of apoptosis of Hep3B cells. Cells were treated with the extract of *C. amuricus* for the indicated concentrations for 24 h. (A) DAPI staining: the cells containing condensed chromatin or exhibiting fragmented nuclei were identified as apoptotic bodies (magnification x1,000). (B) Genomic DNA fragmentation: the isolated DNA from the treated cells was analyzed on a 1.5% agarose gel with 10 kb DNA ladder.

2.4.3. Effects of *C. amuricus* on the cell cycle distribution

In order to estimate the inhibitory effect of *C. amuricus* on Hep3B cell growth, Hep3B cells were exposed to escalating concentrations of the *C. amuricus* extract and subjected to cell cycle analysis. It could be seen that a 24 h treatment of the C. amuricus extract to Hep3B cells caused a definite rise in sub-G1 fractions in a dose-dependent manner. The relative percentages of cells staying at the sub-G1 phase were gradually increased from 2.43% in the non-treated cells to 3.36%, 8.34% and 29.84% in Hep3B cells treated with 100, 150 and 200 μg/ml of the *C. amuricus* extract, respectively (Figure 2.3A). As the elevated accumulation of sub-G1 cells exhibits the presence of apoptotic cells, the extract of *C. amuricus*-promoted cell death was concomitant with the growth inhibitory effect. To dissect the biochemical events controlling the transition of cell cycle phases, G1-related proteins subsequently were examined by western blot analysis (Figure 2.3B). Downregulation of cdc25A, cyclin D1 and cyclin E, CDK4 and 2, and upregulation of $p21^{CIP1/WAF1}$, $p27^{KIPI}$ and $p16^{INK4a}$ were noted in the Hep3B cells following *C. amuricus* treatment for 24 h. Consistently, the expression of the E2F-1 and phospho-Rb were lower in the addition of *C. amuricus*. The experimental findings imply that the methanol extract of *C. amuricus* blocks Hep3B cell cycle progression at the sub-G1 phase.

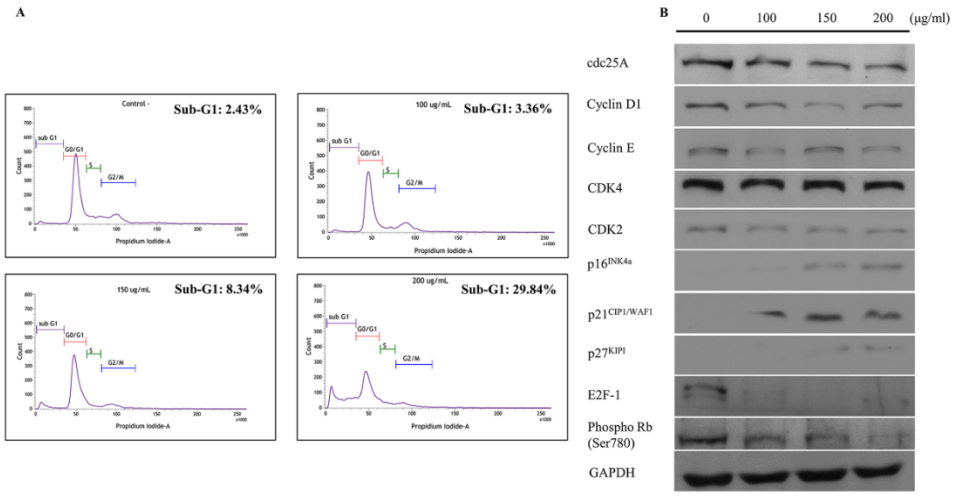

Figure 2.3. Effects of *C. amuricus* on the cell cycle progression of Hep3B cells. (A) Following the treatment with indicated concentrations of the *C. amuricus* extract for 24 h, the ratio (%) of cell distribution in the indicated stages was analyzed by flow cytometry analysis and compared to non-treated cells. (B) The expression of proteins related to cell cycle was detected by western blot analysis, and GAPDH was used as the loading control. Images show chemiluminescent detection of the blots, which are representative of three independent experiments.

2.4.4. Effects of *C. amuricus* on the expression of apoptosis-related proteins

The possible molecular mechanisms underlying *C. amuricus*-induced apoptosis in Hep3B cells were investigated. The effects of *C. amuricus* on the protein expression of Fas-associated death domain (FADD) and phospho-FADD, cleaved caspase-8, and truncated Bid (tBid, the active form of Bid) in Hep3B cells were explored. Western blot analysis revealed that *C. amuricus* caused dose-dependent increments in the levels of FADD and phospho-FADD, cleaved caspase-8 and tBid (Figure 2.4A).

The effects of *C. amuricus* on the mitochondria-mediated death pathway regulated by several members of anti- and pro-apoptotic Bcl-2 family, cytosolic cytochrome *c* and apoptotic protease activating factor-1 (Apaf-1) were next examined (Figure 2.4B). The expression of Bcl-2 was clearly decreased, by way of contrast, the protein levels of Bak, Bax and Bim were notably extended after treatment of Hep3B cells with the *C. amuricus* extract. Also, a dose-dependent advance in the expression of cytosolic cytochrome *c* and Apaf-1 was markedly detected in *C. amuricus*-treated Hep3B cells.

As a first step in identifying whether caspases were involved in the extract of *C. amuricus*-induced apoptosis, Hep3B cells were treated with the caspase inhibitor Z-VAD-fmk. As shown in the results, more cells were alive when treated with Z-VAD-fmk (114%) and the *C. amuricus* extract plus Z-VAD-fmk (59%) than treated with the *C. amuricus* extract alone (48.1%) (Figure 2.4C), suggesting that the extract of *C. amuricus* is able to affect the activity of caspases. In addition, results of western blot analysis presented the expression of multiple apoptotic proteins, including cleaved caspase-9, -3, -7 and -6 were distinctly higher in response to the extract of C. amuricus treatment in a dose-dependent manner. The activation of caspases often lead to the proteolytic cleaved of target proteins poly(ADP ribose) polymerase (PARP), lamins and DNA fragmentation factor

DFF45. Under these conditions, cleaved PARP and cleaved lamin A/C protein were expanded gently, while DFF45 was reduced effectively by the treatment with increasing concentrations of the extract of *C. amuricus* (Figure 2.4D).

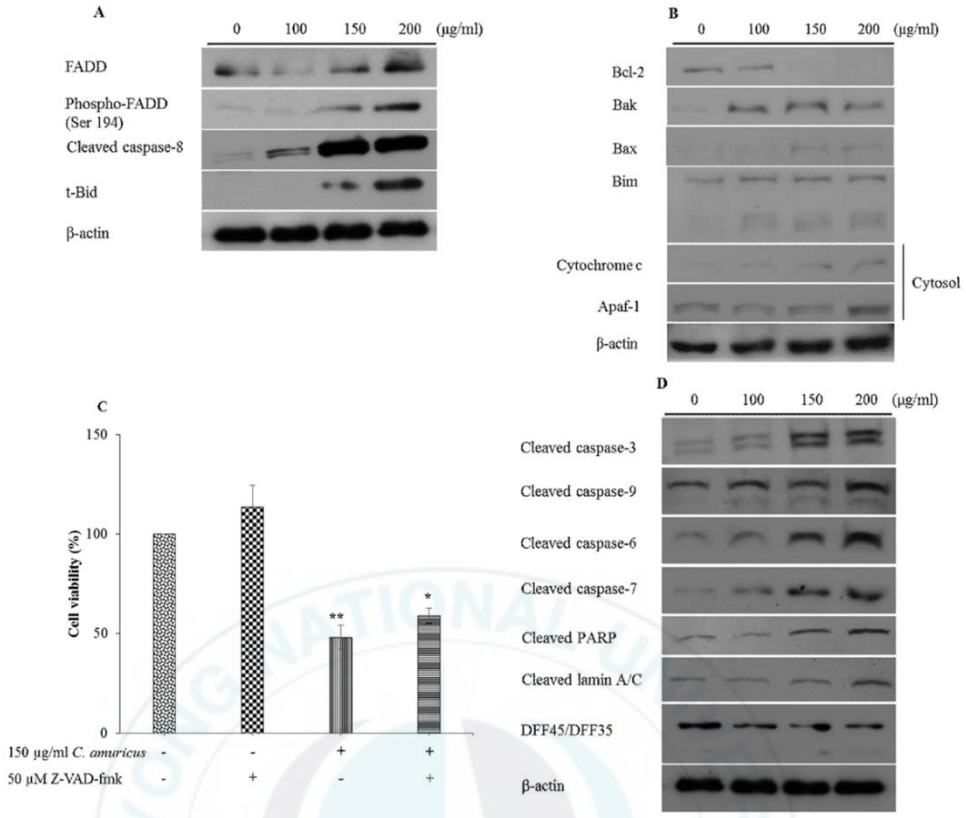

Figure 2.4. Effects of *C. amuricus* on the expression of apoptosis-related proteins. The expression of proteins related with extrinsic apoptotic pathway (A), mitochondrial membrane (B) and cleaved form of caspases (D) were detected by western blot analysis in Hep3B cells treated with various concentrations of the *C. amuricus* extract for 24 h, and β-actin was used as the loading control. Images show chemiluminescent detection of the blots, which are representative of three independent experiments. (C) Caspase activity assay: the cells were treated with 150 μg/ml of the *C. amuricus* extract for 24 h after pre-incubation with Z-VAD-fmk, a caspase inhibitor, for 1 h. Bars represent the mean ± SD of three experiments. **P*<0.05; ***P*<0.01.

2.5. Discussion

The anticancer activities of natural herbal medicine were thoroughly investigated long ago. Also, their crucial effects on the pathophysiology associated with cancers have recently received special attention, due to many epidemiological reports proposing several beneficial effects of herbal extracts significantly reduce the risk of many cancers with few side-effects. Although numerous *Cyperus* species are popularly used in folk medicine and pharmacies as antioxidants and anti-inflammatories, scientific evidence of their effects is essentially required. Therefore, the potential of *C. amuricus* as an anticancer preparation was elucidated in the present study.

The imbalance between cell proliferation and death is considered to be an important event in cancer progression. Among the effects of antitumor reagents, apoptosis and growth inhibition are the most common responses on cancer cells [Ayed-Boussema *et al.*, 2008]. The study demonstrated that the extract of *C. amuricus* was significantly toxic towards Hep3B cells, while essentially non-toxic to A549 and HaCaT cells (Figure 2.1A). Furthermore, apoptosis is one of the most prevalent pathways through which chemopreventive and/or chemotherapeutic strategies can inhibit the overall growth of cancer cells. Apoptosis involves specific morphological and biochemical changes such as cell shrinkage, chromatin condensation, membrane blebbing, more floating and DNA fragmentation [Nicholson and Thornberry, 1997]. Hence, induction of apoptosis is recognized as a useful indicator for cancer treatment and prevention. Particularly, after 24 h of treatment with *C. amuricus* extract, marked morphological changes, including the obvious destruction of the monolayer, shrinkage and extensive detachment of cells (Figure 2.1B), the increased nuclear chromatin condensation in DAPI staining (Figure 2.2A) as well as evident DNA fragmentations (Figure 2.2B) were observed, suggesting that *C. amuricus*-induced cell death involves an apoptotic mechanism.

Cancer cells lack normal growth controls, exhibit loss of cell cycle control, have unlimited reproductive potential and have growth-signal self-sufficiency [Hartwell and Kastan, 1994]. Cell cycle machinery depends on the coordinated activity of protein kinase complexes, each consisting of a cyclin-dependent kinases (CDKs) and cyclins, which act consecutively in G1 to initiate S phase and in G2 to initiate mitosis [Morgan *et al.*, 1997]. Progression through G1 phase requires the activities of cyclin D-dependent CDK4 or 6, followed by activation of the cyclin E- and cyclin A-dependent kinase CDK2. The cyclin-CDK complex formed during G1-phase catalyzes phosphorylation of the dominant inhibitor of G1/S-cell cycle progression, the retinoblastoma Rb family of tumor suppressor proteins, thereby blocking their inhibitory activity allowing the cell to progress into S phase. The Rb family proteins bind to members of the E2F transcription factor family and block the E2F-dependent transcription of genes controlling the G1 to S phase transition and subsequent DNA synthesis. The phosphorylation of Rb disrupts its association with E2F, thereby reducing the suppression of E2F target genes and activating the transcription outside of G1/S [Knudsen *et al.*, 2008]. It is also known that these cyclin-CDK complexes often bind to CDK inhibitors (CDKIs), to conduct pivotal functions in cell cycle regulation via the coordination of internal and external signals that inhibit cell cycle progression at critical checkpoints. One group is the INK4 (inhibitors of CDK4) family, which has four members $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, all of which share the ability to control the G1/S transition. The second group of CDKIs includes $p21^{CIP1/WAF1}$ and $p27^{KIPI}$ [Li *et al.*, 2006]. In the present study, cell cycle analysis exhibited that the extract of *C. amuricus* has the ability to induce sub-G1 phase arrest in Hep3B cells (Figure 2.3A). Another interesting finding is that the concomitant occurrence of apoptosis after *C. amuricus* treatment is mediated with cell cycle arrest in the sub-G1 phase. The increase in sub-G1 phase of *C. amuricus*-treated Hep3B cells may be due to the induction of S phase arrest. Western blot analysis further examined proteins

associated with the cell cycle. *C. amuricus* drastically decreased the levels of these protein kinase complexes cdc25A, cyclin D1 and cyclin E, CDK4 and 2 and increased the levels of $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIPI}$ proteins in Hep3B cells (Figure 2.3B). *C. amuricus* also induced a reduction in Rb phosphorylation, then resulted in a contraction of the E2F-1 protein (Figure 2.3B). The data indicated that *C. amuricus* elicits cell cycle arrest at sub-G1 phases in Hep3B cells through the induction of CDKIs and the inhibition of cyclins and CDKs.

Apoptotic pathways include the extrinsic death receptor- and intrinsic mitochondria-mediated pathways [Jin and El-Deiry, 2005]. The extrinsic signaling pathway is related to the membrane death receptors that belong to the tumor necrosis factor (TNF) receptor gene superfamily. To date, the fatty acid synthase ligand/receptor (FasL/FasR) and TNF-α/TNFR1 models are the best characterized ones. The ligation of FasL to FasR results in sequential recruitment of adaptor molecular FADD and a FADD associated procaspase-8 to the death receptor to form a death-inducing signaling complex (DISC), for execution of cell death [Wang *et al.*, 2013]. Advanced phospho-FADD stimulates binding of caspase-8 to FADD, leading to cleavage of procaspase-8, with the consequent generation of active caspase-8. Active caspase-8 successively amplifies the apoptotic signal through either direct activation of downstream executioner caspase-3 or cleavage of Bid [Jin and El-Deiry, 2005]. Bid, a BH3-only pro-apoptotic Bcl-2 family protein, is generally considered as a molecular linker bridging between the death receptor pathway and the mitochondria pathway [Breckenridge and Xue, 2004]. Uncleaved Bid is predominantly localized in the cytosol as an inactive precursor. Upon cleavage by caspase-8, the activated form of Bid (tBid) migrates to the mitochondria, where it enhances the permeability of the mitochondrial membrane, thus conveys extrinsic apoptotic signals from the cytoplasm to the mitochondria [Jin and El-Deiry, 2005]. In this study, *C. amuricus* may trigger caspase-8 through FADD, and then the activated caspase-8 cleaved Bid into tBid (Figure 2.4A),

which was transferred to the mitochondria where it may contribute to the *C. amuricus*-induced activation of the intrinsic apoptotic pathway. Therefore, *C. amuricus* medicated cleavage of Bid in Hep3B cells may be an important event for cross-talk between intrinsic and extrinsic pathways.

The tBid translocates to the mitochondria where it acts with the pro-apoptotic proteins Bax and Bak to initiate the release of pro-apoptotic factors from the mitochondria. The action of Bid and Bax is counteracted by the anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xL) which can inhibit mitochondrial pro-apoptotic events. The Bcl-2 family of proteins, located on the outer mitochondrial membrane, is vital in apoptotic modulation by participating in the formation of the pores and altering the mitochondrial permeability [Portt *et al.*, 2011]. The balance between pro-apoptotic (Bid, Bad, Bim, Bax, Bak and Noxa) and anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) proteins is critical for determining the release into the cytoplasm of many apoptogenic factors (cytochrome *c*), to influence cell survival [Leibowitz and Yu, 2010]. Cytochrome *c* is a soluble protein located in the mitochondrial intermembrane space, which functions as an electron carrier of the mitochondrial respiration chain between complexes III and IV [Abu-Qare and Abou-Donia, 2001]. Once mitochondria senses the cell death stimuli, cytochrome *c* from the mitochondria can be released into cytosol and binds to Apaf-1, forming of the apoptosome-deoxyadenosine triphosphate-dependent complex, which activates caspase-9, and then drives the downstream caspase cascade (caspase-3) and cell death mechanisms. Release of cytochrome *c* to the cytoplasm is a key step in the initiation of mitochondrial-dependent apoptosis [Abu-Qare and Abou-Donia, 2001; Green *et al.*, 2005]. Consistent with these findings, *C. amuricus* downregulated the levels of the anti-apoptotic proteins Bcl-2 and upregulated the expression of the pro-apoptotic proteins Bak, Bax, and Bim in Hep3B cells in a dose-dependent manner. *C. amuricus* treatment in different doses also promoted the release of cytochrome *c* and Apaf-1 into the cytosol, which in turn cleaved and activated

caspase-9 (Figure 2.4B). Collectively, *C. amuricus*-induced apoptosis of Hep3B cells occurs via the intrinsic mitochondria-mediated pathway, by regulating the expression of the Bcl-2 family proteins, by enhancing the expression of cytosolic cytochrome *c*, Apaf-1, as well as triggering the activation of caspase-9 and eventually leading to apoptosis.

As a downstream product of cytochrome *c*, caspases are crucial mediators of the principal factors found in apoptotic cells. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many cellular substrates, most notably PARP. PARP is an enzyme involved in DNA repair, genome surveillance, and genomic integrity maintenance in response to environmental stress; thus, cleaved PARP is regarded as another hallmark of apoptosis [Jin and El-Deiry, 2005]. The cleavage of PARP induces separation of DNA-binding motifs in NH2 terminal region of catalytic domains from 116- to 89- and 24 kDa, respectively, during drug-induced apoptosis in a variety of cells [Boulares *et al.*, 1999]. In the present study, *C. amuricus*-induced cell death distinctly reduced when Hep3B cells were pre-treated with caspase inhibitor Z-VAD-fmk, showing that the activation of caspases is one of the principal mechanisms by which *C. amuricus* extract induces apoptosis (Figure 2.4C). The rise of the active (cleaved) forms of caspase-9, caspase-3, caspase-7 and caspase-6 and cleaved PARP further validated in *C. amuricus*-treated Hep3B cells in a dose-dependent manner. In addition, both the caspase-3 activation and the subsequent PARP cleavage decreased the expression of DFF45 and increased the expression of lamin A/C after treatment of *C. amuricus* (Figure 2.4D). The DFF45 protein is one of the cleavage targets of caspase-3. Activation of caspase-3 triggers the cleavage of DFF45 (or its isoform DFF35), inactivates its inhibitory function on DFF40 and causes nuclear DNA degradation by DFF40, leading to cell death in *C. amuricus* exposure at different doses [Ozoren and El-Deiry, 2003]. Another marker cleaved by caspase-6 by activating caspase-3 is lamin A/C, functioning in cell cycle control,

DNA replication and nuclear membrane structure and chromatin organization. During apoptosis, lamin A/C is specifically cleaved into large (41-50 kDa) and small (28 kDa) fragments [Lazebnik *et al.*, 1995], and the cleavage of lamin A/C results in nuclear dysregulation and cell death. Results indicated that *C. amuricus* activates caspase-3 and it cleaves specific target proteins committing Hep3B cells to apoptosis. The activation of caspases principally amplifies apoptotic signaling via two distinct pathways: the extrinsic-death receptor pathway and the intrinsic mitochondrial pathway. In the present study, the activation of the intrinsic mitochondrial pathway is involved in the permeabilization of the outer mitochondrial membrane with subsequent releases of pro-apoptotic factors, including cytochrome *c*, into the cytosol. Cytosolic cytochrome *c* alters the conformation of the cytosolic protein Apaf-1, whereas this protein oligomerizes with inactive procaspase-9, resulting in the activation of this enzyme. Additionally, the cleavage and activation of the initiator caspase-8 and -9 occurred after *C. amuricus* exposure. This, in turn, leads to the activation of executioner caspase-3, -6, and -7, ending with the cleavage of several intracellular polypeptides (PARP-1, DFF45, and lamin A/C) as well as DNA fragmentation, which provokes the induction of apoptosis [Ozoren and El-Deiry, 2003].

In conclusion, a wide range of anticancer effects of *C. amuricus* methanol extract such as cell cycle arrest and apoptosis on Hep3B cells, a representative p53-null HCC that contains copies of hepatitis B virus (HBV) genomes in their chromosomes and actively secrets HBsAg [Twist *et al.*, 1981], is illustrated in Figure 2.5. This study, therefore, extends the understanding on the molecular mechanisms underlying the diverse anticancer activities of *C. amuricus* on human liver cancer. However, further studies in animal models are needed to validate the usefulness of this strategy *in vivo*. It could be informative to elucidate the precise mechanism and biological efficacy of this medicinal herb on biological cellular response in other cancer types to chemo-sensitization.

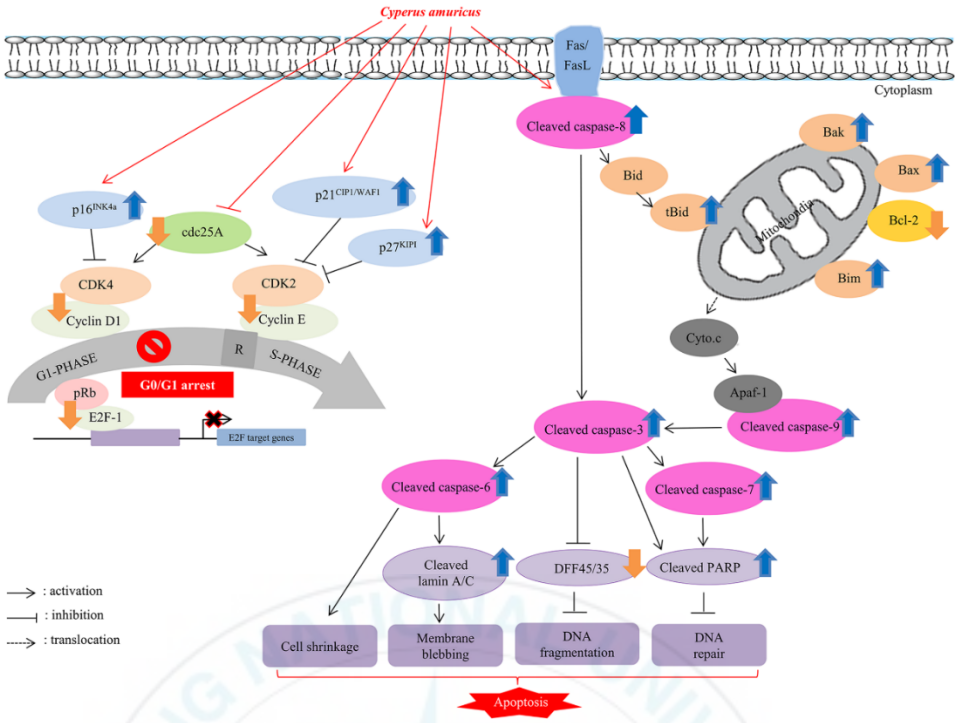

Figure 2.5. Proposed pathways for the effects of *C. amuricus* on the cell cycle arrest and apoptosis in HCC Hep3B cells.

2.6. References

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Chapter 3

Induction of G1 arrest and mitochondrial-mediated apoptosis through endoplasmic reticulum stress in Hep3B cells by *Cyperus amuricus*

3.1. Abstract

The present study investigated the effects of *C. amuricus* on the ER stress-induced cell death and G1 cell cycle arrest in human hepatocellular carcinoma (HCC) Hep3B cells. *C. amuricus* strongly exhibited cytotoxicity against Hep3B cells, but not against HEK293 cells. *C. amuricus* affected the phosphorylation levels of endoplasmic reticulum (ER) sensors and increased the expression of GRP78/BiP and CHOP, resulting in the accumulation of unfolded proteins in the ER triggered the unfolded protein response (UPR). These changes were accompanied by the elevation of intracellular Ca^{2+} concentrations, which could contribute to ER stress-induced apoptosis in *C. amuricus*-treated Hep3B cells. *C. amuricus* also coordinated the stimulation of ER chaperones, which elicited G1 cell cycle arrest through the induction of CDKIs and the inhibition of cyclins and CDKs. Furthermore, *C. amuricus* triggered apoptosis via the activation of mitochondrial-dependent pathway in Hep3B cells. Taken together, these data suggest that *C. amuricus* as a potential apoptosis-inducing agent for Hep3B cells, via the G1 arrest, ER stress and mitochondrial mediated apoptotic pathways.

3.2. Introduction

Primary liver cancer, especially hepatocellular carcinoma (HCC), represents one of the most common malignancies worldwide and its curative therapies still remain limited, mostly in patients with advanced HCC [Bosch *et al.*, 2004; Stewart and Wild, 2014]. A multitude of different signaling pathways is altered in liver cancer cells and dysregulation of the balance between cell proliferation and cell death contributes to hepatocarcinogenesis [Villanueva and Llovet, 2011]. Therefore, HCC continues to be a serious global problem, and identifying new agents with efficient pharmacological activities would promote the development of novel therapeutic target in HCC.

Apoptosis, programmed cell death, is a cell suicide mechanism that enables organisms to control cell number, eliminates excessive cells that threaten survival and hence is pivotal to the preclusion of tumor development. The process of apoptosis is generally regulated by two major signaling pathways [Jin and El-Deiry, 2005]. One is the death receptor-dependent extrinsic pathway, in which the ligation of death receptors by death ligands is followed by the recruitment of adaptor molecules and the activation of caspase-8 or -10 [Thorburn *et al.*, 2004]. The other is the mitochondrial-dependent intrinsic pathway, in which pro-apoptotic signals provoke the release of cytochrome *c* from the mitochondria into the cytosol, forming a complex with Apaf-1, procaspase-9 and dATP, known as apoptosome. Apoptosome formation subsequently inflames the activation of executioner caspase-3, -6 and -7, which in turn stimulates a series of apoptotic events [Breckenridge DG and Xue, 2004]. Both pathways continuously collect information on various aspects of signal transduction cascades and cellular metabolism, operate this information, and eventually decide on the fate of cells [Budihardjo *et al.*, 1999].

Nowadays, there has been enlarging awareness regarding the role of ER in the homeostasis of the cell. ER is an essential compartment for protein synthesis and maturation [Boyce and Yuan, 2006]. When ER function is disturbed, the unfolded protein response (UPR) can be activated, and the associated ER stress is known to be the basis of many cellular aggressions, namely apoptosis through a variety of mechanisms, including redox imbalance, alteration in Ca^{2+} levels and the activation of the Bcl-2 family proteins [Puthalakath *et al.*, 2007]. The UPR coordinates the induction of ER chaperones, which decreases protein synthesis and results in the growth arrest in G1 phase of cell cycle. A number of scientists have reported that ER stress triggers G1 cell cycle arrest in several cancer cells [Brewer *et al.*, 1999; Hamanaka *et al.*, 2005]. However, the molecular mechanism underlying UPR-induced G1 cell cycle arrest is largely unknown. Additionally, in the branch of UPR, CCAAT enhancer-binding protein (C/EBP) homologous protein (CHOP) is upregulated, and for this reason, it is a widely used marker of ER-stress [Yamaguchi and Wang, 2004; Hetz and Glimcher, 2009]. Furthermore, an increment of CHOP has been related to pro-apoptotic effects in diverse cancer cell lines, an effect attributed to CHOP-mediated repression of Bcl-2 gene family. The activation of these elements can promote the expression of cell death-related genes and/or modulate the activation of ER stress-sensible caspases, like caspase-12 or -4 [Malhi and Kaufman, 2011; Tabas and Ron, 2011]. Practically, the complete mechanisms promoting ER stress-induced cell death are still unclear.

Here, the present study was conducted to elucidate the ability of the *C. amuricus* to activate ER stress-induced cell death in HCC Hep3B cells, supporting a promising anticancer drug for liver cancer.

3.3. Materials and methods

3.3.1. Cell culture and reagents

Hep3B (hepatocellular carcinoma) and HEK293 (human embryonic kidney) cells were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (for Hep3B) or Minimum essential medium (MEM) (for HEK293) (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1% penicillin-streptomycin (PAA Laboratories GmbH, Australia) at 37˚C and 5% CO_2. The methyl alcohol extract from the whole plant of *C. amuricus* (distribution number: 010-032) was obtained from the Korea Plant Extract Bank (KPEB, Cheongju, Korea) with the purity of ≥99.9%, HPLC.

3.3.2. Cell viability assay

Exponential phases of Hep3B and HEK293 cells ($1x10^4$ cells/well) were seeded on 96-well plates (SPL Lifesciences, Gyeonggi, Korea) in triplicate. Following overnight incubation, cells were treated with various concentrations (50, 100, 150 and 200 μg/ml) of *C. amuricus* and incubated for 24 h. After the treatment, 10 μl of EZ-Cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Seoul, Korea) was added to each well and incubated for an additional 3 h. The absorbance of the reaction was measured using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 460 nm and cell viability was calculated. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used to dilute the *C. amuricus* extract and the final concentration of DMSO in each well was not in excess of 0.05% (v/v). No adverse effect due to the presence of DMSO was observed.

3.3.3. DAPI staining

Hep3B cells treated with the indicated concentrations of *C. amuricus* were rinsed with phosphate-buffered saline (PBS) buffer (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium dihydrogen phosphate) and stained by addition of 1 μg/ml DAPI solution (Roche Applied Science, Indianapolis, IN, USA) in methanol (Sigma-Aldrich). After incubation in the dark at 37˚C for 20 min, cells were washed with PBS buffer, and then fixed with 4% formaldehyde (Junsei, Tokyo, Japan) for 15 min. The nuclear morphology of the cells was observed under a Laser Scanning Confocal Microscope (Carl Zeiss LSM 700; Carl Zeiss, Oberkochen, Germany).

3.3.4. Fluo3-AM calcium assay

In order to detect the change of intracellular Ca^{2+} concentration, Hep3B cells on cover-glass bottom dish were incubated with 150 μg/ml *C. amuricus*, and then stained with 1.5 μM of Fluo3-AM (Invitrogen, Grand Island, NY, USA) for 30 min at 37˚C. The cells on the slides were washed twice with PBS and mounted in Prolong Gold Antifade Reagent (Invitrogen) followed by observation under a Laser Scanning Confocal Microscope (Carl Zeiss LSM 700; Carl Zeiss, Oberkochen, Germany).

3.3.5. Immunofluorescence staining

Hep3B cells on cover-glass bottom dish were incubated with 150 μg/ml *C. amuricus* for 24 h. After the treatment, Hep3B cells were fixed with 4% formaldehyde for 15 min at room temperature and blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS at room temperature. Fixed and blocked cells were incubated with the primary antibodies ($p21^{CIP1/WAF1}$ or caspase-12) for overnight at 4˚C and then incubated with Alexa488-conjugated goat anti-rabbit immunoglobulin IgG (Cell Signaling Technology, Inc., Danvers, MA, USA). After

being washed with PBS, cells were stained with DAPI and examined under a Laser Scanning Confocal Microscope (Carl Zeiss LSM 700; Carl Zeiss, Oberkochen, Germany).

3.3.6. Cell cycle analysis

Briefly, Hep3B cells were harvested by trypsinization and fixed with 70% ethanol overnight at 4˚C. Then, the cells were resuspended in PBS buffer containing 0.2 mg/ml RNase and incubated for 1 h at 37˚C. The cells were stained with 40 μg/ml propidium iodide (Sigma-Aldrich) at room temperature for 30 min in the dark. The cell cycle was analyzed based on DNA contents using a flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

3.3.7. Protein extraction and western blot analysis

Hep3B cells were washed with PBS buffer and then lysed by the addition of lysis buffer [(50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, prostatin A)] (Intron Biotechnology, Gyeonggi, Korea). After 30 min on ice, lysates were collected and clarified by centrifugation at 14,000 rpm for 20 min at 4˚C. Aliquots of whole cell lysates were subjected to 10% SDS-PAGE and then transferred to nitrocellulose membrane (Pall Corp., Pensacola, FL, USA). The membranes were blocked with 5% skim milk in PBST (PBS buffer and 0.5% Tween-20). After blocking non-specific sites, the membranes were probed with primary antibodies (Cell Signaling Technology) and then washed in PBST three times, followed by incubation for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as second antibodies (Cell Signaling Technology). The blots were then washed in PBST and visualized by an enhanced chemiluminescent (ECL) detection solution (Pierce, Rockford, IL, USA).

3.4. Results

3.4.1. Effects of *C. amuricus* on Hep3B cell viability

In order to understand the cytotoxicity, Hep3B and HEK293 cells were exposed to *C. amuricus* by cell viability assay. As shown in Figure 3.1A, the *C. amuricus* extract had no cytotoxicity on HEK293 cells, while the extract (over 100 μg/ml) caused remarkable decrease in cell viability in Hep3B cells. Treatment with 150 μg/ml and 200 μg/ml of *C. amuricus* for 24 h dramatically reduced the numbers of viable Hep3B cells by 48.53% and 35.63%, respectively, when compared to that of the non-treated cells. The extract of *C. amuricus* with an IC_{50} value ≤150 μg/ml was considered active on Hep3B cells, hence this concentration was used for following investigations. An extensive distension of the ER and typical apoptotic morphology also correlated to a 24 h exposure of 150 and 200 μg/ml *C. amuricus* to Hep3B cells, with condensation and margination of nuclear chromatins using DAPI staining (Figure 3.1B). The data initially indicate that the diminished viability in *C. amuricus*-treated Hep3B cells is for apoptosis induction.

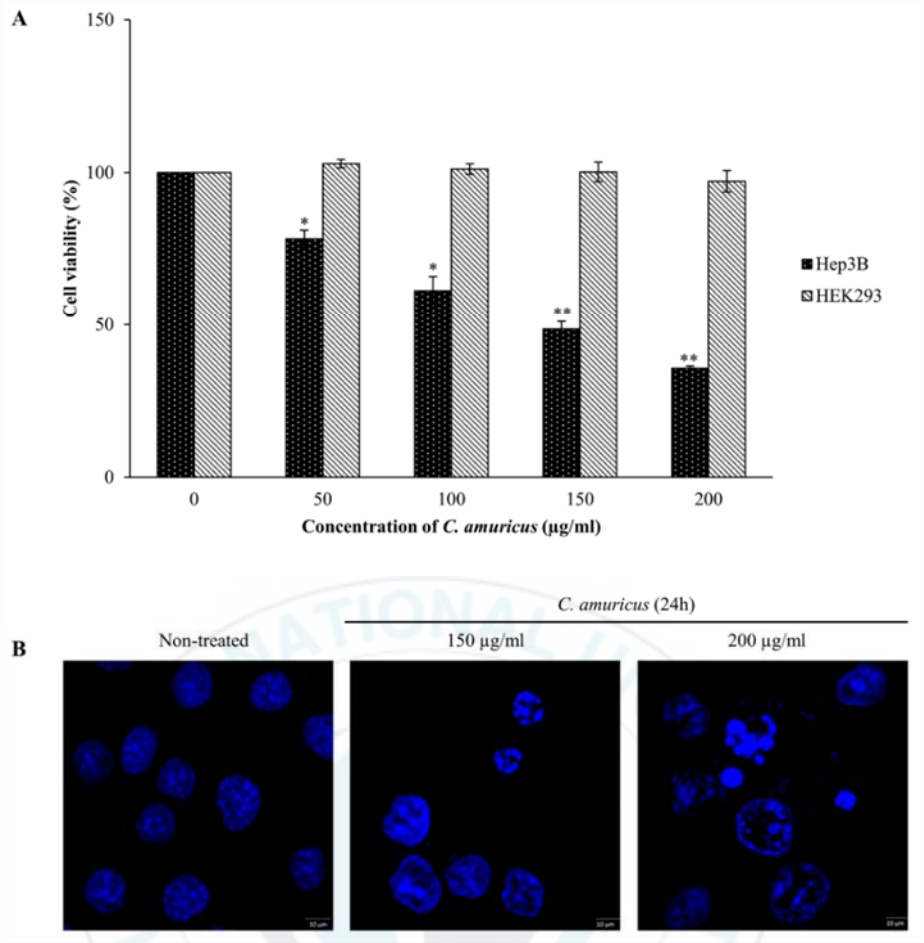

Figure 3.1. Effects of *C. amuricus* on the cell viability of Hep3B cells. (A) Cytotoxicity of *C. amuricus* was determined using the cell viability assay. Hep3B, HEK293 cells were treated with 50-200 μg/ml of *C. amuricus* for 24 h. Bars represent the mean ± SD of three experiments. **P*<0.05; ***P*<0.01. (B) Morphological features of apoptosis in Hep3B cells after the *C. amuricus* exposure were observed using DAPI staining. Hep3B cells were incubated with *C. amuricus* for the indicated concentrations and visualized by the inverted microscope (magnification x1,000).

3.4.2. Effects of *C. amuricus* on the cell cycle progression

The inhibitory effect of *C. amuricus* on Hep3B cell cycle distribution was next estimated using flow cytometry. It could be seen that treatment of the *C. amuricus* extract to Hep3B cells posed a definite rise in sub-G1 fractions in a time-dependent manner. The relative percentages of sub-G1 phase cells was progressively increased from 4.93% in the non-treated cells to 8.07%, 8.45% and 22.22% in the 150 μg/ml *C. amuricus*-treated Hep3B cells for 6, 12, and 24 h, respectively (Figure 3.2A). To dissect the biochemical events controlling the transition of cell cycle phases, G1-related proteins were examined by western blot analysis (Figure 3.2B). Elevated levels of $p21^{CIP1/WAF1}$, $p27^{KIPI}$ and $p16^{INK4a}$ and depressed levels of cyclin D1 and cyclin E, CDK4 and 2 were detected in Hep3B cells in response to 150 μg/ml *C. amuricus* after 6 h and longer exposition. Concomitantly, time-dependent diminution of E2F-1 and phospho-Rb expressions were appeared in the addition of *C. amuricus*. Furthermore, *C. amuricus*-treated Hep3B cells had significantly increasing immunofluorescence of $p21^{CIP1/WAF1}$ expression (green) nearby the nucleus in the cytoplasm although there is a very few notice in the non-treated cells (Figure 3.2C). The experimental findings imply that *C. amuricus* blocks Hep3B cell cycle progression at the sub-G1 phase.

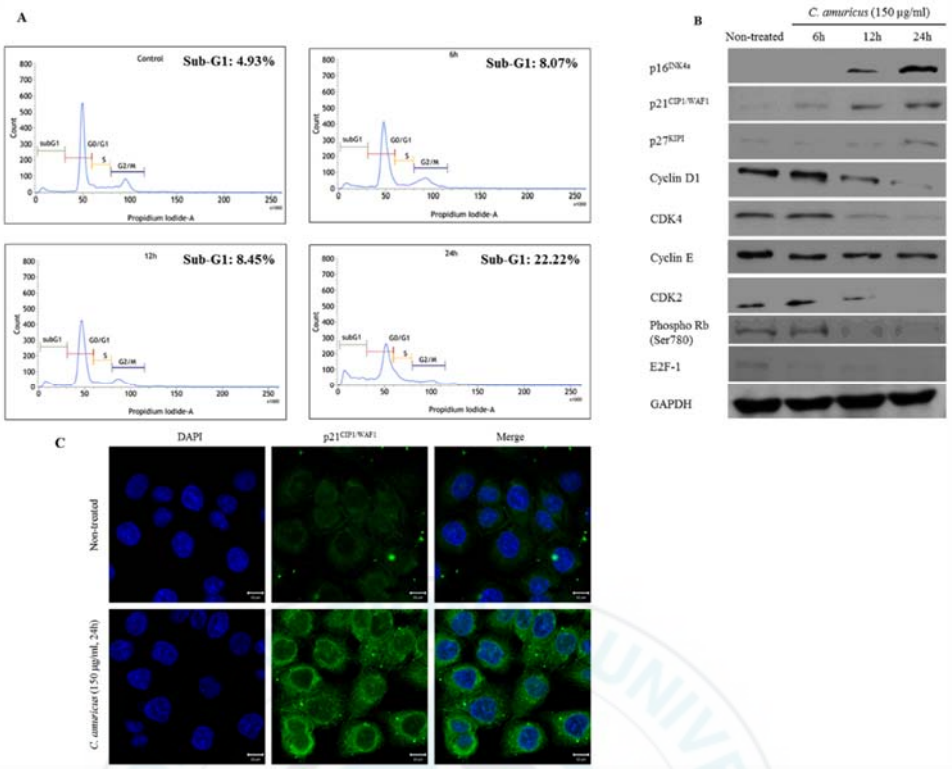

Figure 3.2. Effects of *C. amuricus* on the cell cycle progression of Hep3B cells. (A) Following the treatment with 150 μg/ml *C. amuricus* for the indicated times, the ratio (%) of cell distribution in the indicated stages was analyzed by flow cytometry analysis and compared to the non-treated cells. (B) The expression of proteins related to cell cycle was detected by western blot analysis, and GAPDH was used as the loading control. Images show chemiluminescent detection of the blots, which are representative of three independent experiments. (C) Immunofluorescence microscopy images of $p21^{CIP1/WAF1}$ (green) with DAPI (nuclei; blue) were detected by fluorescence microscopy (magnification x1,000).

3.4.3. Effects of *C. amuricus* on the expression of apoptosis-related proteins

The effects of *C. amuricus* on the mitochondria-mediated death pathway regulated by several members of anti- and pro-apoptotic Bcl-2 family and cytochrome *c* were carried out. The time course results indicated that the expressions of Bax and cytosolic cytochrome *c* were greatly increased, by contrast, the level of Bcl-2 was clearly decreased in *C. amuricus*-treated Hep3B cells from 6 h to 24 h (Figure 3.3A).

To further validate whether *C. amuricus* stimulates apoptosis associated with the caspase family proteins, western blot analysis presented the expressions of cleaved caspase-9 and -3 were distinctly higher in response to 150 μg/ml *C. amuricus* treatment at each of the respective time points. Under these conditions, cleaved poly(ADP ribose) polymerase (PARP) gently enlarged at indicated time points of *C. amuricus* treatment (Figure 3.3B).

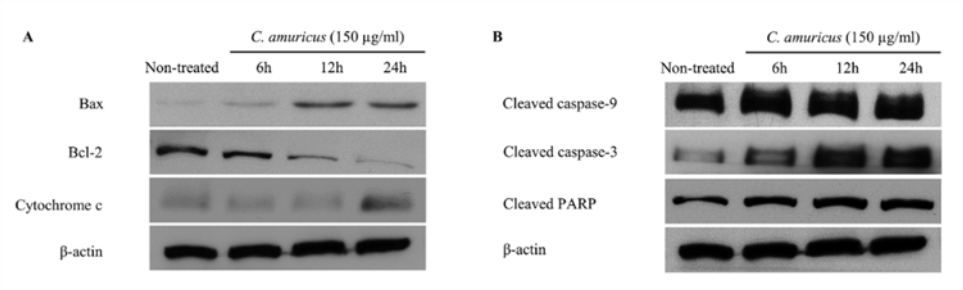

Figure 3.3. Effects of *C. amuricus* on the expression of apoptosis-related proteins. The expression of proteins related with mitochondrial membrane (A) and cleaved form of caspases (B) were detected by western blot analysis in Hep3B cells treated with 150 μg/ml *C. amuricus* for the indicated times, and β-actin was used as the loading control. Images show chemiluminescent detection of the blots, which are representative of three independent experiments.

3.4.4. Effects of *C. amuricus* on the ER stress regulatory proteins

Altered Ca^{2+} homeostasis can disorder numerous cellular responses, basically ER stress and accumulation of misfolded proteins [Orrenius *et al.*, 2003]. Accordingly, Fluo-3 AM calcium staining was performed to assess whether *C. amuricus* turn on an expanded cytosolic Ca^{2+} levels and whether the ER is the source of Ca^{2+} release in Hep3B cells. The green fluorescence representing cytosolic Ca^{2+} was visible at 6 h and the signal was increased until 24 h after *C. amuricus* stimulation (Figure 3.4A).

The levels of ER stress-associated chaperone proteins to determine the UPR activation were measured. Western blot analysis revealed that the expressions of Bip, IRE1α, ATF6 cleavage and calpain were obviously extended at 6 h to 24 h, contrastingly, the levels of phospho-PERK and phospho-eIF2α were gradually attenuated after 12-24 h of *C. amuricus* treatment. Notably, two ER stress-associated pro-apoptotic markers, caspase-12 and CHOP, were intensive based on the treatment time with 150 μg/ml of *C. amuricus* (Figure 3.4B). To confirm the prediction of ER stress, the immunofluorescence of caspase-12, a protein located on the ER membrane, was conducted. In view of Figure 3.4C, ER membrane staining displayed the dilation of ER, indicating that cytoplasmic vacuoles amplified by *C. amuricus* might be dilated cisternae of ER. These results suggest that *C. amuricus*-induced ER stress may initiate programmed cell death in Hep3B cells.

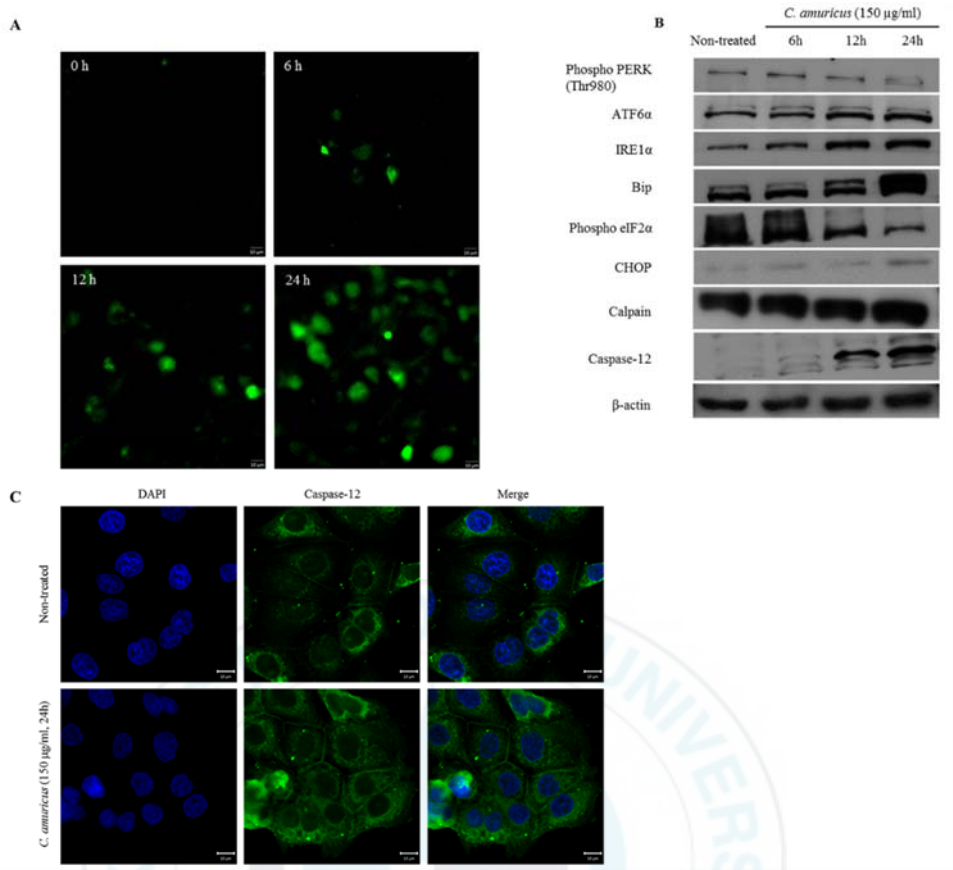

Figure 3.4. Effects of *C. amuricus* on the ER stress-regulated proteins. (A) Intracellular Ca^{2+} mobilization by *C. amuricus* was detected using Fluo-3 AM under fluorescence microscopy (magnification x400). (B) Induction of ER stress mediators in *C. amuricus*-treated Hep3B cells were detected by western blot analysis in a time-dependent manner, and β-actin was used as the loading control. Images show chemiluminescent detection of the blots, which are representative of three independent experiments. (C) Immunofluorescence microscopy images of caspase-12 (green) with DAPI (nuclei; blue) were detected by fluorescence microscopy (magnification x1,000).

3.5. Discussion

Recent studies have pointed out that during tumorigenesis, cancer cells are often exposed to nutrient deprivation, hypoxia, oxidative stress and other metabolic dysregulations that cause the accumulation of unfolded or misfolded proteins in the ER lumen, a condition referred to as "ER stress". To cope with ER stress, cells evoke an adaptive mechanism, named UPR, to modify transcriptional and translational programs in attempt to reestablish ER homeostasis [Wang *et al.*, 2010]. However, masses of normal cells do not undergo an active "stress" response, and the UPR pathways keep on quiescent. This discrepancy between tumor and normal cells allows for the improvement of agents that target ER stress to achieve specificity in malignant cancer therapy. Clinical data favor the hypothesis that the manipulation of ER stress may improve the efficacy of chemotherapeutic agents and ER stress-mediated apoptotic pathways may provide novel approaches for the development of antitumor drugs [Ni and Lee, 2007; Liao *et al.*, 2008].

The UPR is orchestrated by three ER-transmembrane transducers: inositol requiring enzyme-1 α (IRE1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [Hetz and Glimcher, 2009; Malhi and Kaufman, 2011], which are maintained in an inactive state through association with the ER chaperone binding immunoglobulin protein (GRP78/BiP) under physiological conditions [Malhi and Kaufman, 2011]. GRP78/BiP, a member of glucose-regulated protein family (GRPs), is responsible for the folding and maturation of non-glycosylated proteins. GRP78/BiP is ubiquitously expressed at very low levels in growing cells, but it is highly expressed in response to numerous cellular stresses. Therefore, GRP78/BiP is a major target of the UPR. As massive prolonged or unresolved ER stress, the accumulation of unfolded proteins leads to GRP78/BiP dissociation and activation of the three ER stress sensors, triggering the UPR signal that aims to facilitate

proper protein folding, to prevent protein aggregate formation, and to drive misfolded proteins to the final proteasomal degradation [Lee *et al.*, 2007]. If the stress is protracted and the function of ER is severely impaired, this adaptive action switches to a pro-apoptotic response, ending with the elimination of cells unable to handle the unfolded protein aggregation through the UPR intervention [Gorman *et al.*, 2012; Tabas and Ron, 2011]. In Hep3B cells, *C. amuricus* caused the significant upregulation of GRP78/BiP.

Upon ER stress, GRP78/BiP detaches from the luminal domains of IRE1α, subsequently activates IRE1α through trans-autophosphorylation on Ser724 and catalyzes XBP-1 splicing to launch UPR for restoring ER homeostasis. Then, XBP-1s can induce the expression of several genes involved in different aspects of UPR [Tabas and Ron, 2011; Acosta-Alvear *et al.*, 2007], regulating not only the adaptive response to ER stress but also apoptosis. Moreover, at high stress signaling levels, IRE1α may contribute to membrane-associated mRNA degradation through a proceeding named regulated IRE1α-dependent decay that could first defend cells against ER stress by limiting new protein translation and further urge apoptotic mechanism in the setting of intense ER stress [Tabas and Ron, 2011]. In this experiment, IRE1α, a transmembrane protein harboring a dual enzymatic activity of protein kinase and endoribonuclease [Hetz and Glimcher, 2009], was extended after *C. amuricus* treatment in Hep3B cells (Figure 3.4B).

During UPR, PERK, an ER resident protein kinase, phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), which alleviates ER stress through attenuation of global mRNA translation by decreasing the overabundance of misfolded proteins and favoring the expression of ATF6 transcription factor [Hetz and Glimcher, 2009; Tabas and Ron, 2011]. Active ATF6 then translocates into the nucleus and regulates the ER stress response elements, including XBP-1, Bip and CHOP [Haze *et al.*, 1999]. Following *C. amuricus* exposure, PERK and eIF2α were phosphorylated while ATF6 was cleaved to the

activated form (Figure 3.4B). These signaling transduction pathways are the adaptation responses to ER stress which can suppress the protein synthesis to relieve the burden of ER and increase the transcription of ER-associated chaperone genes [Boyce and Yuan, 2006]. Continual increase of IRE1α and decrease of phospho-PERK and phospho-eIF2α might exacerbate the situation.

Among different ER-stress sensors, CHOP, a downstream component of the ER stress-mediated apoptotic pathway [Oyadomari and Mori, 2004], was slightly increased in *C. amuricus*-treated Hep3B cells (Figure 3.4B). CHOP overexpression sponsors the transcription of some BH3-only and growth arrest and DNA damage-inducible 34 genes, and represses the expression of the anti-apoptotic Bcl-2 gene, thus providing confirmation that the pro-apoptotic functions of CHOP are associated with the mitochondrial-dependent mechanisms of cell demise [Hetz *et al.*, 2012; McCullough *et al.*, 2001]. Under normal conditions, Bcl-2 forms heterodimers with the pro-apoptotic protein, Bax. Under stress conditions, Bax enters the mitochondrial outer membrane, adjusts mitochondrial permeability, and thereafter expedites cytochrome *c* release by forming pores on the outer mitochondrial membrane, to impact on cell survival. Cytochrome *c* is a soluble protein located in the mitochondrial intermembrane space, which functions as an electron carrier of the mitochondrial respiration chain between complexes III and IV. Once mitochondria senses the cell mortality stimuli, cytochrome *c* from mitochondria can be discharged into cytosol and binds to Apaf-1, forming of the apoptosome-deoxyadenosine triphosphate-dependent complex, which drives the downstream signaling (caspase-9, -3 and cleaved PARP). Release of cytochrome *c* is a key step in the initiation of mitochondrial-dependent apoptosis [Abu-Qare and Abou-Donia, 2001]. Consistent with these findings, *C. amuricus* downregulated the level of Bcl-2 and upregulated the expression of Bax, leading to upgrade expression of cytosolic cytochrome *c* in Hep3B cells (Figure 3.3A). In addition, *C. amuricus* treatment improved the cleaved forms of caspase-9, -3 and cleaved PARP

in a time-dependent manner (Figure 3.3B). Collectively, *C. amuricus*-induced apoptosis of Hep3B cells occurred via the intrinsic mitochondria-mediated pathway by regulating the expression of the Bcl-2 family proteins, enhancing the expression of cytosolic cytochrome *c*, as well as triggering the activation of caspase cascade and eventually leading to apoptosis. According to these observations above, these results implicate that ER stress-mediated BiP-phospho-eIF2α-CHOP is vital in *C. amuricus*-induced Hep3B apoptosis.

Intracellular Ca^{2+} is primarily stored in the ER, which is critical for protein folding and Ca^{2+} homeostasis [Gorlach *et al.*, 2006]. Ca^{2+} is moved from the ER into the cytoplasm as shown in Figure 3.4A, suggesting the implication of ER stress in *C. amuricus*-treated Hep3B cells. Additionally, the activation of ER stress proteins is a downstream consequence of Ca^{2+} depletion which is closely related with apoptosis [Mekahli *et al.*, 2011]. Elevated cytosolic Ca^{2+} incites apoptosis because of mitochondrial Ca^{2+}overload. The pathological overcharge of Ca^{2+} in the mitochondria triggers the opening of the mitochondrial permeability transition pores, which leads to mitochondrial dysfunction, resulting in the release of the mitochondrial apoptogenic factor, cytochrome *c* [Boitier *et al.*, 1999]. Thence, the potential conclusion could be draw the crosstalk between ER stress and apoptosis in *C. amuricus*-treated Hep3B cells. In addition to cytosolic Ca^{2+}, activation of calcium-dependent proteases such as calpain is thought to play an important role in apoptotic cell death. Earlier studies also showed that calpain can engage in tumor death, and calpain inhibitors have been used to block apoptosis [Rasheva and Domingos, 2009]. In the present study, enhanced cytosolic Ca^{2+} and calpain expression after *C. amuricus* treatment (Figure 3.4A and 3.4B) acted as convincing evidence to support the occurrence of ER stress-mediated apoptosis.

Caspase 12, a murine member of the inflammatory group of caspase family, has been described as a mediator of ER-specific apoptosis [Szegezdi *et al.*, 2003; Nakagawa and Yuan, 2000]. In particular, ER stress commences the decrement of

Ca^{2+} through the inositol 1,4,5-triphosphate receptors (IP3R) and ryanodine receptor (RyR) present on ER, afterwards activates calpain-dependent apoptosis in the cytosol, which proteolytically cleaves procaspase-12 to mature caspase-12 in the ER [Nakagawa and Yuan, 2000]. Prolonged ER stress caused by the increment of calpain together with caspase-12 (Figure 3.4B and 3.4C) encourages *C. amuricus*-induced Hep3B apoptosis through the activation of the ER resident caspase-12. Overall, the data demonstrate that the capacity of *C. amuricus* to activate the key proteins of ER stress as well as ER-associated apoptotic proteins, CHOP and caspase-12, which indicates that both effectors are possibly attributed to ER stress-induced apoptosis in *C. amuricus*-treated Hep3B cells.

After its activation at the ER, caspase-12 prompts the subsequent activation of caspase-9, further intensifying caspase-3, which simultaneously are the executioners of apoptosis [Rasheva and Domingos, 2009]. As a downstream product of caspase-12, caspase-3 is one of the principal factors found in apoptotic cells. In detail, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many cellular substrates, most notably PARP, which involved in DNA repair, genome surveillance, and genomic integrity maintenance in response to environmental stress [Jin and El-Deiry, 2005]. In agreement with these reports, the rise of the active (cleaved) forms of caspase-9, -3 and cleaved PARP ensued after *C. amuricus* exposure (Figure 3.3B). This, in turn, incites the induction of apoptosis.

Apoptosis is linked to cell cycle arrest, and the blockade of the cell cycle is regarded as an effective intrigue for eliminating cancer cells. Cell cycle machinery depends on the coordinated activity of protein kinase complexes, each consisting of a cyclin-dependent kinases (CDKs) and cyclins, which act consecutively in G1 to initiate S phase and in G2 to initiate mitosis [Morgan *et al.*, 1997]. Progression through G1 phase requires the activities of cyclin D-dependent CDK4 or 6, followed by activation of the cyclin E- and cyclin A-dependent kinase CDK2. The

cyclin-CDK complex formed during G1 phase catalyzes phosphorylation of the dominant inhibitor of G1/S cell cycle distribution, the retinoblastoma Rb family of tumor suppressor proteins, thereby blocking their inhibitory activity allowing the cell to progress into S phase. The Rb family proteins bind to members of the E2F transcription factor family and block the E2F-dependent transcription of genes controlling the G1 to S phase transition and consequent DNA synthesis. The phosphorylation of Rb disrupts its association with E2F, thereby reducing the suppression of E2F target genes and activating the transcription outside of G1/S [Knudsen *et al.*, 2008]. It is also known that these cyclin-CDK complexes often bind to CDK inhibitors (CDKIs), to conduct pivotal functions in cell cycle regulation via the coordination of internal and external signals that inhibit cell cycle at decisive checkpoints. One group is the INK4 (inhibitors of CDK4) family, which has four members $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, all of which share the ability to control the G1/S transition. The second group of CDKIs includes $p21^{CIP1/WAF1}$ and $p27^{KIPI}$ [Li *et al.*, 2006]. In the present study, cell cycle analysis exhibited that *C. amuricus* has the ability to induce sub-G1 phase arrest in Hep3B cells (Figure 3.2A). Another interesting finding is that the concomitant occurrence of apoptosis after *C. amuricus* treatment is mediated with cell cycle arrest in the sub-G1 phase. The increase in sub-G1 phase of *C. amuricus*-treated Hep3B cells may be due to the induction of S phase arrest. Western blot analysis further examined proteins associated with the cell cycle analysis. *C. amuricus* drastically decreased the levels of cyclin D1 and cyclin E, CDK4 and 2 and increased the levels of $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIPI}$ proteins in Hep3B cells (Figure 3.2B and 3.2C). *C. amuricus* also induced a reduction in Rb phosphorylation, then resulted in a contraction of the E2F-1 protein (Figure 3.2B).

Preceding studies have found that CDKIs and cyclins have influence on ER stress and cell cycle arrest. $p27^{KIPI}$ acts as a crucial negotiator of ER stress-induced G1 cell cycle arrest in melanoma cells [Han *et al.*, 2013]. $p21^{CIP1/WAF1}$ integrates the

DNA damage response with ER stress signaling, which then regulates mitochondrial death pathways during chronic genotoxic stress [Vitiello *et al.*, 2009]. Translational regulation of cyclin D1 in response to ER stress is a mechanism for checkpoint control that prevents cell cycle procession. PERK has been shown to mediate cell cycle arrest by blocking cyclin D1 translation during UPR [Brewer *et al.*, 1999; Hamanaka *et al.*, 2005]. Similarly, the present study showed that induction of members of the INK4 ($p16^{INK4a}$) or CDKIs ($p21^{CIP1/WAF1}$, $p27^{KIPI}$) families of cell cycle kinase inhibitors causes ER stress, and the accumulation of unfolded proteins in the ER triggers UPR, which is a stress signaling pathway. The UPR coordinates the induction of ER chaperones with decreased protein synthesis and growth arrest in G1 phase of the cell cycle. The data show that *C. amuricus* elicits cell cycle arrest at the sub-G1 phases in Hep3B cells mainly via transcriptional regulation of $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIPI}$.

In conclusion, a wide range of anticancer effects of *C. amuricus* on cell cycle arrest and apoptosis via mitochondrial-dependent pathways related with ER stress in Hep3B cells, are proposed in Figure 3.5. A new insight into the molecular mechanisms of *C. amuricus*-promoted Hep3B cells death is supplied as a basic step in clinical therapeutic options. It could be informative to ongoing identify the precise targets and biological efficacies of this medicinal herb on animal models.

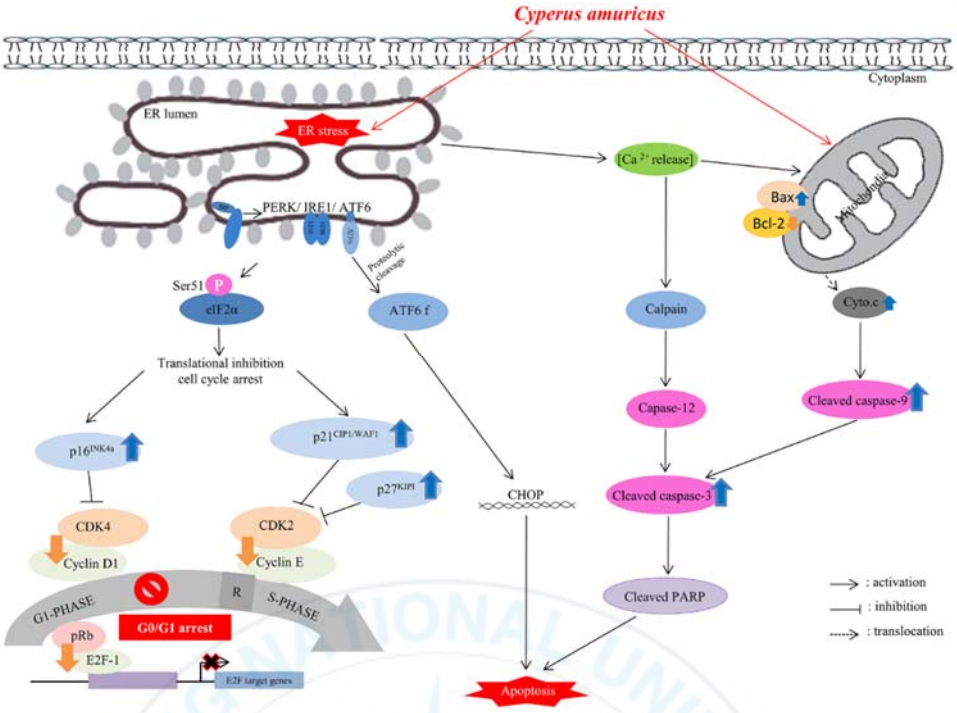

Figure 3.5. Proposed pathways for *C. amuricus*-induced apoptosis, ER stress and cell cycle arrest in HCC Hep3B cells.

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Chapter 4

Induction of apoptosis and autophagy in Hep3B cells by *Cyperus amuricus* via AMPK and PI3K/Akt/mTOR signaling pathways

4.1. Abstract

Recent studies have demonstrated that *C. amuricus* retains potent pharmacological efficiency in anti-lipase, antioxidant and antineoplastic capabilities. However, it remains unclear whether *C. amuricus*-induced apoptosis is associated with other mechanisms such as autophagy. Therefore, the present study was designed to elucidate the cytotoxic mechanisms of *C. amuricus* with respect to autophagy and apoptosis in Hep3B cells. The results initially found that *C. amuricus* dose-dependently caused apoptotic cell death and arrested in sub-G1 phase in Hep3B cells. In addition, during early exposure (3-12 h), *C. amuricus* triggered autophagy followed by an increased accumulation of AVO-positive cells concomitant with the conversion of microtubule-associated protein1 light chain 3 (LC3) I to LC3-II and the upregulation of Atg5-Atg12 conjugate, Atg7, Beclin-1 and DAPK3 proteins, indicating autophagosome formation. Subsequently, *C. amuricus* exposure elicited early autophagy and late apoptosis which was associated with the increment of the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and the suppression of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR). Moreover, co-treatment of the autophagosome inhibitor, 3-methyladenine (3-MA), nearly decreased the apoptosis rates and blocked *C. amuricus*-promoted intension in Atg7,

Beclin-1, LC3-II and AMPK phosphorylation, indicating the crosstalk between *C. amuricus*-induced apoptosis and autophagy. Taken together, the present study extends the mechanism behind the cell death pathway stimulated by *C. amuricus* and the development of *C. amuricus* as a prospective cancer chemotherapy for liver cancer.

4.2. Introduction

Apoptosis (type I cell death) and autophagy (type II cell death) are two primary morphologically distinctive modes of programmed cell death. Apoptosis is described by cell shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, loss of adherence to extracellular matrix, formation of apoptotic bodies and activation of proteases and externalization of phosphatidylserine [Taylor *et al.*, 2008]. Autophagy is a self-cannibalization process that is characterized by the formation of double-membrane vesicles (autophagosomes), capable of engulfing large amounts of damaged organelles and misfolded protein aggregates via the lysosomal degradation pathway. Autophagy is frequently upregulated in by a range of stimuli, such as nutrient starvation, hypoxia, metabolic and genotoxic stress to maintain energy metabolism and help cells deal with the harsh conditions until the environment is improved. In this regard, autophagy can play as a survival mechanism promoting cancer cell survival and propagation; thus, it may have a negative effect in cancer therapy and limit the therapeutic efficacy of chemotherapeutic agents [Kondo *et al.*, 2005]. However, there is evidence that excessive and sustained autophagy by various anti-cancer therapies can ultimately lead to cell death, alternative to apoptosis and potentially eliminate tumor cells [Moretti *et al.*, 2007]. Collectively, the phenotypic outcome of the interaction between these two processes is extremely complex, inferring the notion that apoptosis and autophagy can exhibit either synergistic or antagonistic effects on each other to modulate programmed cell death [Shen *et al.*, 2011]. In some circumstances, autophagy and apoptosis occur simultaneously in cancers and may be extensively interrelated by some signaling pathways. Among them, the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) and adenosine monophosphate-activated protein kinase (AMPK) pathways are major regulators of

autophagy and apoptosis [Levine and Yuan, 2005; Sridharan *et al.*, 2011]. Namely, AMPK is a principal energy-preserving intracellular enzyme activated in stress conditions that can induce autophagy by inactivation of mTOR, known as a repressor of autophagy. The interplay between AMPK-dependent energy metabolism and autophagic adaptive self-sustaining actions has prompted a growing number of investigations in AMPK-mTOR-autophagy mechanisms [Liu *et al.*, 2011]. Furthermore, the constitutively activated class I PI3K/Akt pathway inhibits both apoptosis and autophagy because it acts as a positive director of the mTOR pathway and regulates transcription factors which modulate distinct sets of genes involved in tumorigenesis , such as protein synthesis, cell cycle progression, cancer cell growth and survival. Disruption of the PI3K/Akt/mTOR pathway by anticancer drugs promotes apoptosis and autophagy [Takeuchi *et al.*, 2005]. In spite of these advances, the existence of crosstalk between apoptotic and autophagic cell death needed to be further elicited.

Consequently, the present study was carried out to explore the cytotoxic mechanisms of *C. amuricus* with respect to autophagy and apoptosis in HCC Hep3B cells.

4.3. Materials and methods

4.3.1. Cell culture and reagents

The hepatocellular carcinoma (Hep3B) cell line was purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone Laboratories, Logan, UT, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1% penicillin-streptomycin (PAA Laboratories GmbH, Australia) at 37˚C and 5% CO_2. The methyl alcohol extract from the whole plant of *C. amuricus* (distribution number: 010-032) was obtained from the Korea Plant Extract Bank (KPEB, Cheongju, Korea) with the purity of ≥99.9%, HPLC.

4.3.2. Cell viability assay

Exponential phases of Hep3B cells ($1x10^4$ cells/well) were seeded on 96-well plates (SPL Lifesciences, Gyeonggi, Korea) in triplicate. Following overnight incubation, cells were treated with various concentrations (50, 100, 150, and 200 μg/ml) of *C. amuricus* for 24 h. After the treatment, 10 μl of EZ-Cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Seoul, Korea) was added to each well and incubated for an additional 3 h. The absorbance of the reaction was measured using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 460 nm and cell viability was calculated. The cytotoxic activity of the extract was expressed as an IC_{50} value, which is the concentration of the extract that caused 50% cell death. The extract of *C. amuricus* with an IC_{50} value ≤150 μg/ml was considered active on Hep3B cells. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used to dilute the extract and the final concentration of DMSO in each well was not in excess of 0.05% (v/v). No adverse effect due to the presence of DMSO was observed.

4.3.3. Cell cycle analysis

Briefly, Hep3B cells were harvested by trypsinization and fixed with 70% ethanol overnight at 4˚C. Then, the cells were resuspended in PBS buffer containing 0.2 mg/ml RNase and incubated for 1 h at 37˚C. The cells were stained with 40 μg/ml propidium iodide (PI, Sigma-Aldrich) at room temperature for 30 min in the dark. The cell cycle was analyzed based on DNA contents using a flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

4.3.4. Annexin V-FITC staining

Hep3B cells were treated with the graded concentrations of *C. amuricus* and/or 1 mM 3-methyladenine (3-MA, Sigma-Aldrich) for 24 h. Following collection, cells were stained with annexin V-FITC and PI staining kit (BD Biosciences) according to the manufacturer's protocol. Apoptotic cells were evaluated by flow cytometry (Becton-Dickinson, Mountain View, CA, USA).

4.3.5. Quantification of acidic vesicular organelles (AVOs) by acridine orange

After exposure to the different experimental conditions, cells were stained with 1mg/ml acridine orange (AO, Sigma-Aldrich) at room temperature for 15 min in the dark, and washed with PBS, then immediately analyzed by flow cytometer. Depending on their acidity, autophagic lysosomes appeared as bright orange/red cytoplasmic vesicles, while cytoplasm and nucleolus fluoresced bright green. Quantification were measured based on the ratio of red/green fluorescence (FL3/FL1) on a flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

4.3.6. Protein extraction and western blot analysis

Hep3B cells were washed once with PBS buffer and then lysed by the addition of lysis buffer [(50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40,

1% Triton X-100, 1% Deoxycholate, 0.1% SDS and proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, prostatin A)] (Intron Biotechnology, Gyeonggi, Korea). After 30 min on ice, lysates were collected and clarified by centrifugation at 14,000 rpm for 20 min at 4˚C. Aliquots of whole cell lysates or cytosolic fractions were subjected to 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Pall Corp., Pensacola, FL, USA). The membranes were blocked with 5% skim milk in PBST (PBS buffer and 0.5% Tween-20). After blocking non-specific sites, the membranes were probed with primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) and then washed in PBST three times, followed by incubation for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as second antibodies (Cell Signaling Technology). The blots were then washed in PBST and visualized by an enhanced chemiluminescent (ECL) detection solution (Pierce, Rockford, IL, USA). All other antibodies were purchased from Cell Signaling Technology.

4.4. Results

4.4.1. Cytotoxicity and apoptosis induction

A dose-response study was conducted to investigate the cytotoxicity of *C. amuricus* using cell viability assay. *C. amuricus* obviously inhibited the numbers of viable Hep3B cells with an IC_{50} of 150 µg/ml at 24 h (Figure 4.1A), hence this concentration was used in the following experiments. From morphology observation, 100 and 150 µg/ml treatment of *C. amuricus* provoked large vacuoles inside the cells while 200 µg/ml treatment evoked massive cell rounding, shrinkage and detachment from the culture plates. Cell cycle analysis also showed a gradual rise of sub-G1 population in response to *C. amuricus* treatment (Figure 4.1B). To identify if the *C. amuricus*-induced reduction in the viability of Hep3B cells occurred via apoptosis, annexin V-FITC/PI double staining analysis was performed. The corresponding quantities of apoptosis expressed as annexin V-positive cells were markedly raised after exposed to 100 µg/ml (10.9%), 150 µg/ml (20.4%) and 200 µg/ml *C. amuricus* (36.7%) for 24 h, compared to non-treated controls (3.1%) (Figure 4.1C). Concomitantly, according to the previous studies, the upregulated Bax/Bcl-2 ratio, the advanced levels of cytoplasmic cytochrome *c* as well as the sequential activation of various caspases (cleaved of caspase-3 and cleaved PARP) were evidently detected *C. amuricus*-treated Hep3B cells [Pham *et al.*, 2016]. These experimental findings initially indicate that *C. amuricus* promotes apoptotic cell death in Hep3B cells. Thence, the effects of *C. amuricus* on other cellular responses associated with cell death to better understand its anticancer effects were next examined.

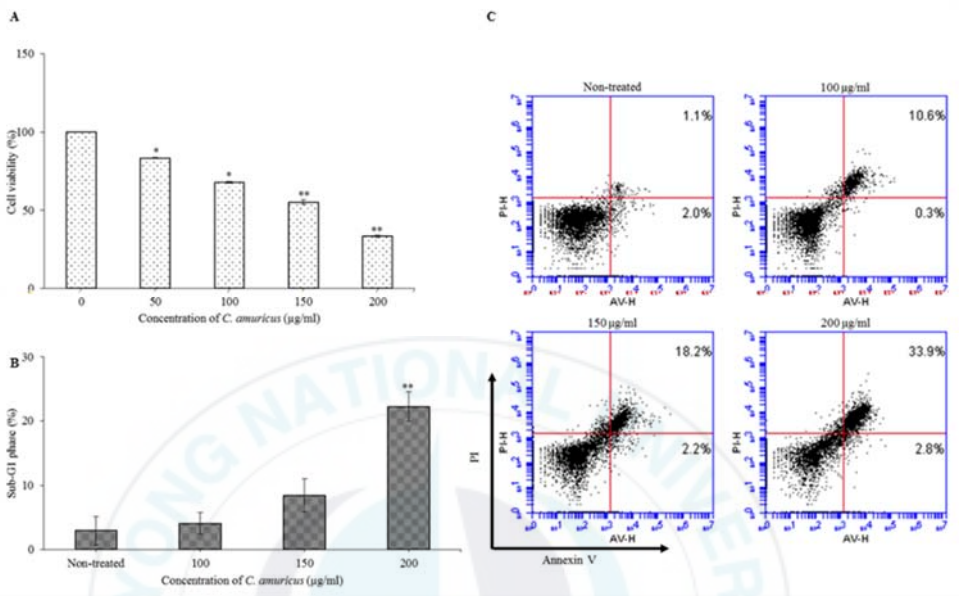

Figure 4.1. *C. amuricus* induced apoptotic cell death in Hep3B cells. The cells were treated with the indicated concentrations of *C. amuricus* for 24 h. (A) Effects of *C. amuricus* on the cell viability. (B) Effects of *C. amuricus* on the cell cycle progression. Following the *C. amuricus* treatment, quantification of the sub-G1 DNA content was determined by flow cytometry analysis. Bars represent the mean ± SD of three experiments. **P*<0.05; ***P*<0.01. (C) Effects of *C. amuricus* on the apoptosis induction. The apoptosis rates were quantified by flow cytometry analysis following annexin V-FITC and PI staining. Three independent experiments were performed.

4.4.2. Induction of autophagy

Vacuole formation, as a common morphological feature of autophagy, could be observed after early *C. amuricus* treatment in Hep3B cells (Figure 4.2A). Therefore, a series of experiments were farther performed to shed light on the autophagic profiles of *C. amuricus*. Autophagic response to *C. amuricus* treatment was monitored by quantification of AVOs, which are the hallmark of autophagy**.** A statistically significant accumulation of AVO-positive cells exposed 61.0%, 68.7% and 76.2% in the 150 μg/ml *C. amuricus*-treated Hep3B cells for 3, 6, and 12 h, respectively (Figure 4.2B). The formation of autophagosomes correlates with the increment of Beclin-1 and the conversion of microtubule-associated protein1 light chain 3 (LC3) I to LC3-II serve as reliable markers for autophagy [Suzuki *et al.*, 2001; Maiuri *et al.*, 2007; Kang *et al.*, 2011]. Western blot analysis revealed time-dependently extended Beclin-1 expression and improved LC3-I to LC3-II conversion in Hep3B cells after *C. amuricus* exposure, especially the conversion of LC3 began very early, at 3 h of *C. amuricus* treatment, and notably extended over time (Figure 4.2C). Simultaneously, *C. amuricus* greatly elevated the expression of several autophagy-related proteins including Atg5-Atg12 conjugate, Atg7 and DAPK3 in the treated condition (Figure 4.2C). The data imply that *C. amuricus* has the potential to trigger autophagy in Hep3B cells

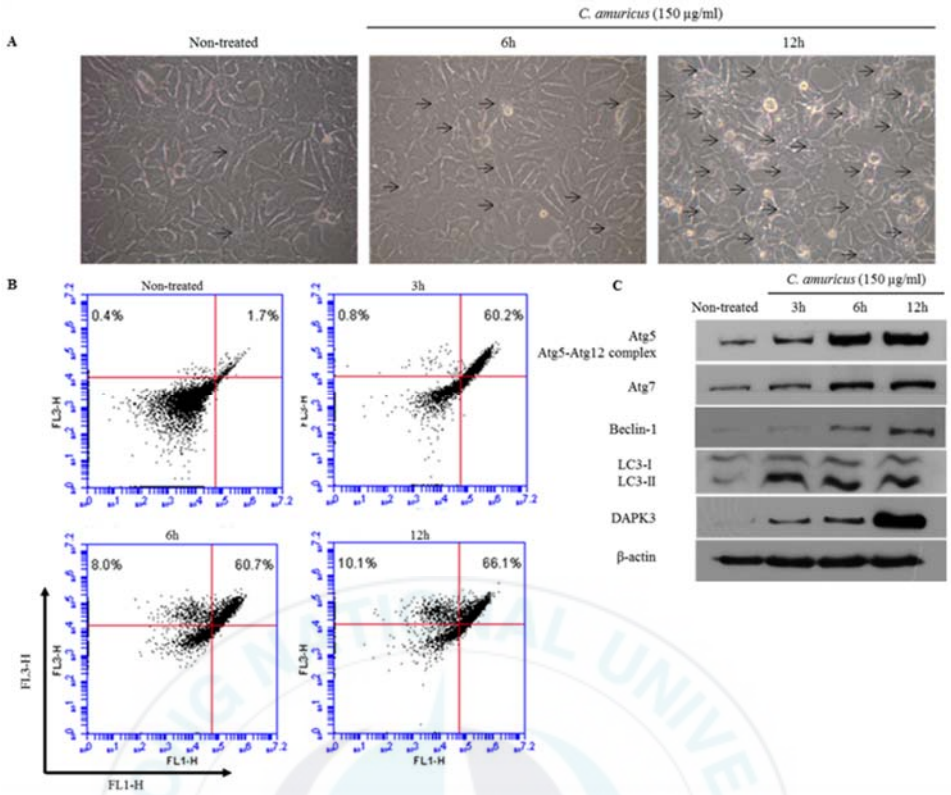

Figure 4.2. *C. amuricus* elicited autophagy in Hep3B cells. (A) The autophagic vacuoles developed by 150 μg/ml *C. amuricus* at early time points (6 h and 12 h) were counted under phase contrast microscope (magnification x200). (B) The AVO-positive cells were quantified by flow cytometry analysis following the AO staining. (C) Induction of autophagy related genes by *C. amuricus* in Hep3B cells were detected by Western blot, and β-actin was used as the loading control. Three independent experiments were performed.

4.4.3. Effects of *C. amuricus* on the AMPK and PI3K/Akt/mTOR/ p70S6K pathways in Hep3B cells

Previous studies have demonstrated that *C. amuricus* induced the intrinsic mitochondrial pathway, resulting in the dysfunction of mitochondria in Hep3B cells [Pham *et al.*, 2016]. Mitochondria is considered as the energy source of cell, which is also an important determinant for the formation of autophagosomes [Tolkovsky *et al.*, 2002; Kanki and Klionsky, 2010]. Thus, *C. amuricus* might prompt autophagy by affecting cellular dissipation of energy. In general, AMPK is a principal energy-preserving intracellular enzyme activated in stress conditions that can induce autophagy by inhibition of mTOR [Hamilton *et al.*, 2002]. PI3K/Akt/mTOR is also a well-known intracellular pathway, frequently implicated in a wide variety of cellular processes including cell proliferation, cell cycle, cellular transformation, metastasis, tumorigenesis, metabolism, autophagy and apoptosis in malignancies [Nicholson and Anderson, 2002; Altomare and Testa, 2005; Shaw and Cantley, 2006]. The effects of *C. amuricus* on the AMPK and PI3K/Akt/mTOR pathways were assessed by western blot analysis. Expectedly, the AMPK and PI3K/Akt/mTOR pathways were involved in *C. amuricus*-treated Hep3B cells, which was followed by dose-dependently enhanced the activation of AMPK and decreased the phosphorylation of both PI3K/Akt/mTOR and the substrate p70S6K (Figure 4.3).

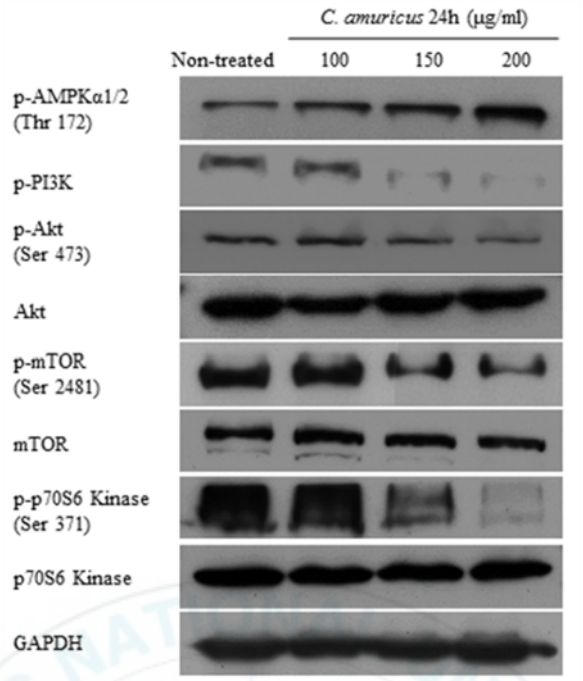

Figure 4.3. *C. amuricus* activated AMPK and inhibited PI3K/Akt/mTOR/p70S6K pathways. Whole-cell extracts were prepared and detected by western blot analysis, and GAPDH was used as the loading control. Three independent experiments were performed.

4.4.4. Effects of *C. amuricus*-induced autophagy on the cell death of Hep3B cells

Further, to elucidate underlying mechanisms responsible for *C. amuricus*-triggered autophagy in Hep3B cells, the autophagy inhibitor 3-MA was introduced. Figure 4.4A demonstrated that co-treating Hep3B cells with 3-MA inhibited *C. amuricus*-induced activation of AMPK. Interestingly, blocking AMPK activation also blocked the expression of Atg7, Beclin-1 and LC3 proteins in Hep3B cells, proposing that AMPK is a mediator of *C. amuricus*-induced autophagy in Hep3B cells. Additionally, flow cytometry analysis was employed to validate inhibition of autophagy affected cell sensitivity to *C. amuricus*. Singular 150 μg/ml *C. amuricus* treatment incited higher apoptotic rate (30.2%) in Hep3B cells compared to 3-MA co-treatment (26%) (Figure 4.4B). All of these results strongly suggest that blockage of autophagy attenuates the process of apoptotic cell death in *C. amuricus*-treated Hep3B cells and fully confirm that *C. amuricus*-induced apoptotic cell death in Hep3B cells is dependent, at least in part, on the induction of autophagy via AMPK activation.

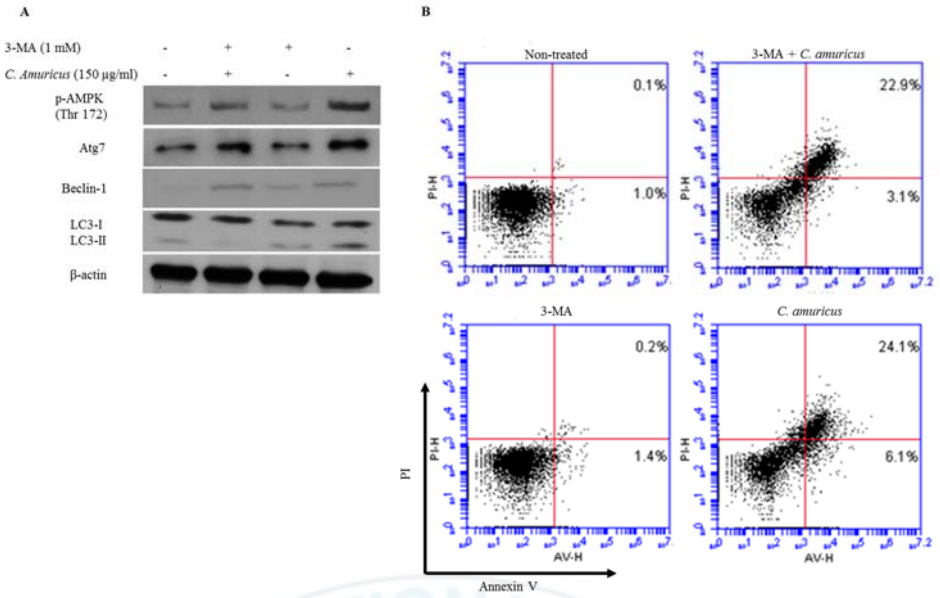

Figure 4.4. Effects of *C. amuricus*-induced autophagy on the cell death of Hep3B cells. Cells were treated with *C. amuricus* (150 μg/ml) in the presence or absence of 3-MA for 24 h. (A) Autophagy inhibitor 3-MA pretreatment attenuated *C. amuricus*-induced the phosphorylation of AMPK and the expressions of autophagic indicators. Whole-cell extracts were prepared and detected by western blot analysis, and β-actin was used as the loading control. (B) The apoptosis rates in the cells treated with *C. amuricus* alone with those in 3-MA combined with *C. amuricus* were measured by flow cytometric analysis following annexin V-FITC and PI staining. Three independent experiments were performed.

4.5. Discussion

The treatment of HCC remains an urgent challenge because of poor efficacy and severe toxicities of standard and new chemotherapy. There is an increased interest in seeking new therapies for liver cancers from medicinal herbs. Studies on the antitumor activities of medicinal herbs on HCC cells are ongoing to explore their chemopreventive drug development for liver cancer. *C. amuricus* has been found to exhibit anticancer activities *in vitro*, which are attributed to its effects on intrinsic apoptotic pathways, with a marked observation in condensed apoptotic nuclei, a sub-G1 phase arrest, mitochondrial membrane degradation, increment of Bax/Bcl-2 expression ratio, cytosolic cytochrome *c* translocation and activation of caspase-3 and -9 as well as cleavage of PARP [Pham *et al.*, 2016]. Consistent with the above results, the growth inhibitory effects of *C. amuricus* acted in a similar fashion in the present study. Exposure of Hep3B cells to escalating concentrations of *C. amuricus* for 24 h resulted in a notable decrease in cell viability and a definite rise in sub-G1 fractions as compared to that of the non-treated cells (Figure 4.1A and 4.1B). Accumulated data have expressed that many chemotherapeutic and chemopreventive agents have potential anti-proliferative effects via arresting the cell division at certain checkpoints in the cell cycle. Thus, the results strongly implicate sub-G1 cell cycle arrest by *C. amuricus* as one of the mechanisms that induced cytotoxicity in Hep3B cells. In addition, apoptosis is one of the modes of cell death and induction of apoptosis is a key characteristic of anticancer drugs as it plays an imperative role in the elimination of damaged cells and the maintenance of homeostasis. Nowadays, many of the natural chemopreventive agents, including *C. amuricus*, exert their effects via induction of apoptosis in cancer cells [Pham *et al.*, 2016]. *C. amuricus* treatment dose-dependently raised the apoptotic cells (Figure 4.1C), suggesting that *C. amuricus* suppresses proliferation of Hep3B cells by inducing apoptosis with similar phenomena described in recent literatures.

As one of the two primary programmed cell death pathways in eukaryotes, autophagy is an evolutionarily conserved catabolic process instigated by stress, involving sequestration of misfolded proteins and damaged organelles within double membraned autophagosomes which subsequently fuse with lysosomes for breakdown and recycling. In fact, autophagy has a dual-funtion as either a promoter or a suppressor of cancer cells, which makes it a promising and challenging therapeutic target in cancer treatment [Maiuri *et al.*, 2007; Eisenberg-Lerner *et al.*, 2009]. Recently, many chemotherapeutic agents have been found to arouse autophagy instead of apoptosis in different cancer cells. In the present study, one of the most interesting events in the early stage following treatment with *C. amuricus* was the cytoplasmic vacuolation identified by morphological observation and quantification of AVOs (Figure 4.2A and 4.2B). Remarkably, *C. amuricus*-treated Hep3B cells did not undergo cell death at 3-12 h, while at late time point (24 h) showed significant cell death. The results have been hypothesized that treatment with *C. amuricus* may induce autophagy at an early stage in Hep3B cells. To test this possibility, the role of *C. amuricus*-mediated autophagy in Hep3B cells were defined. As well as apoptosis, autophagy is excited by activation of the autophagy-related genes (Atgs). One of the most noticeable findings amongst the Atg proteins is the discovery of two ubiqutin-like conjugation systems. The first system leads to covalent attachment of the ubiquitin-like protein Atg5-Atg12 (together with Atg16) catalyzed by Atg7 and Atg10 [Suzuki *et al.*, 2001; Kuma *et al.*, 2002]. In the second system, LC3/Atg8 protein is converted to a membrane conjugated form by Atg4, while Atg7 and Atg3 proteins function as E1-like (ubiquitin-activating enzyme) and E2-like (ubiquitin-conjugating enzyme) enzymes, respectively [Mizushima *et al.*, 2011]. These two conjugation reactions are hierarchically essential for the construction of the double-membraned autophagosomes [Maiuri *et al.*, 2007]. On the other hands, Beclin-1/Atg6 is a subunit of the class III PI3-kinase complex, which is necessary to form

preautophagosomal structures [Suzuki *et al.*, 2001; Kang *et al.*, 2011]. As shown in Figure 4.2B and 4.2C, *C. amuricus* could induce autophagy in Hep3B cells as evidenced by the autophagosome accumulation stained with AVO, enhanced AVO-positive cells as well as the conversion of LC3-I to LC3-II. At the same time, the expression levels of specific autophagy mediators associated with the formation of autophagosome, including Atg5-Atg12 conjugate, Atg7, Beclin-1 and DAPK3, were intensive following exposure to *C. amuricus* (Figure 4.2C), further confirming the existence of autophagic mechanism in *C. amuricus*-treated Hep3B cells.

Although apoptosis and autophagy are classified separately and presented with distinct morphological features, these two types of cell death are thought to have extremely intricate interrelationships and can overlap at the level of various signaling steps. Multiple stimuli can inspire either apoptosis or autophagy, or both; and morphologic features of both cell death. Mechanisms can be observed concurrently in the same cell [Maiuri *et al.*, 2007]. Indeed, the final life-or-death destiny of cancer cells is under the influence of the interaction between autophagy and apoptosis, which, in turn, depends on the cell type and/or the type, concentration and duration of treatment of chemotherapeutic drugs [Dalby *et al.*, 2010]. Thence, understanding the crosstalk between apoptosis and autophagy may be crucial for the development of therapeutic strategies to improve the efficacy of anticancer agents. The Bcl-2-Beclin-1/Atg6 complex mainly located on the endoplasmic reticulum is possibly involved in the molecular mechanisms of both apoptosis and autophagy. Therein, Bcl-2 has been described as a central moderator of both pathways, whereas Beclin-1/Atg6 has been identified originally as Bcl-2-interacting protein, which possesses a Bcl-2 homology-3 domain (BH3) for its binding to the BH3 receptor domain of Bcl-2 or Bcl-xL. Bcl-2 and Bcl-xL then operate as critical nodes in complex networks to integrate information and make ultimate decisions on whether to initiate apoptosis. Although Bcl-2 and Bcl-xL are

well identified in the apoptotic pathway, they appear to be important factors in impeding autophagy by binding to the protein Beclin-1 and can therefore aid cells to evade cell death by autophagy [Pattingre *et al.*, 2005; Levine *et al.*, 2008; Cho *et al.*, 2009; Marquez *et al.*, 2012]. Recent discoveries have intimated that *C. amuricus* induced the intrinsic apoptotic pathway in Hep3B cells by regulating the expression of the Bcl-2 family proteins, altering the mitochondrial permeability and promoting the release of cytosolic cytochrome *c* [Pham *et al.*, 2016]. As a consequence, the phenomenon of mitochondrial depolarization in *C. amuricus*-treated Hep3B cells could activate autophagy and apoptosis [Tolkovsky *et al.*, 2002; Kanki and Klionsky, 2010], highlighting the mitochondria switches from autophagy to apoptosis caused by *C. amuricus* in this model. However, the other interplay between autophagy and apoptosis, such as caspase activation, Bcl-2 family proteins post-translational modifications and other possible upstream signals alterations stimulated by *C. amuricus* needs to be clarified by in-depth studies in future. Surprisingly, the promoted effect of apoptosis due to *C. amuricus* treatment was reversed when autophagy was blocked by 3-MA in Hep3B cells in the present study. Supression of autophagy by 3-MA not only attenuated *C. amuricus*-induced cell death but also repressed the expression of Atg7, Beclin-1 and LC3-II (Figure 4.4A and 4.4B). Besides, *C. amuricus*-stimulated autophagy took place at 3 h, which was well before the occurrence of apoptosis that took place at 12 h to 24 h after treatment. It has been documented that autophagy may perform as an enabler of apoptosis, contributing in certain morphological and cellular events (ATP, cell blebbing and DNA fragmentation) that take place in apoptotic cell death, without leading to cell death by itself **[**Eisenberg-Lerner *et al.*, 2009]. Based on the determination, the results can be reached that autophagy triggered by *C. amuricus* acts as a pro-apoptotic mechanism in Hep3B cells.

In spite of these findings, some intriguing questions remain to be answered, such as what the key signal molecule for the switch from autophagy to initiate

apoptosis would be. Some upstream signaling molecules are common to both apoptotic and autophagic processes and the complicated interactions between them may arise as a result of crosstalk, such as the AMPK and PI3K/Akt/mTOR pathways [Levine and Yuan, 2005; Sridharan *et al.*, 2011]. AMPK serves as a key energy sensor involving in lipid metabolism, biosynthesis, protein folding and modification of various soluble and insoluble proteins in response to nutrient scarcity. When activated, AMPK stimulates energy-producing catabolic pathways including autophagy by upregulating Atg12 and LC3 conversion, while inhibiting anabolic pathways [Kroemer *et al.*, 2010]. Activated AMPK also phosphorylates and inhibits its downstream target mTOR thus allowing for the initiation of autophagy. Additionally, mTOR is recognized as a negative effector of apoptosis when inhibition of mTOR could repress Bcl-xL to promote apoptosis [Tirado *et al.*, 2005; Chen *et al.*, 2012]. Moreover, the downstream target of mTOR/p70S6K, can potently block Bad-stimulated apoptosis by phosphorylation of Bad at S136 site to disrupt Bad's binding to Bcl-xL and/or Bcl-2 [Harada *et al.*, 2001]. Otherwise, the overexpression of PI3K/Akt is implicated to a poor prognosis, tumor progression and resistance to systematic therapy in the pathogenesis of HCC. Phosphorylation of PI3K/Akt is able to significantly upregulate the expression of Bcl-2 and downregulate the expression of Bax in hepatic epithelial cells [Altomare and Testa, 2005; Zhou *et al.*, 2011]. Activation of class I PI3K also inhibits apoptosis and autophagy by activation of Akt and mTOR in numerous cancer cell lines [Shaw and Cantley, 2006]. Accordingly, inhibition of PI3K/Akt/ mTOR pathway has been proposed as a probable target for therapeutic strategy against cancer, especially in the lipogenesis inhibition and the hepatocarcinogenesis suppression. Through appropriate inhibition studies, Figure 4.3 displayed that upon *C. amuricus* treatment caused a dose-dependent downregulation of phosphorylated PI3K, Akt, mTOR and p70S6K, whereas the same treatment affected upregulation of AMPK phosphorylation in Hep3B cells. Consistently, blocking autophagic activation with

3-MA also inhibited *C. amuricus*-induced apoptosis and blocked the activation of AMPK and the expression of Atg7, Beclin-1 and LC3 in Hep3B cells (Figure 4.4), indicating that AMPK is a major mediator of *C. amuricus*-induced autophagy in Hep3B cells. Overall, the results support that *C. amuricus* causes early autophagy and late apoptosis through the activation of AMPK and inhibition of PI3K/Akt/mTOR pathways and when excessive autophagy becomes insurmountable, the programmed cell death is activated in *C. amuricus*-treated Hep3B cells.

In conclusion, the present study herein, provides a novel evidence for the potential effects of *C. amuricus* in stimulation of early autophagy and late apoptosis by activation of AMPK and inhibition of PI3K/Akt/mTOR pathways (Figure 4.5), submitting the comprehensive potency of *C. amuricus* on HCC cells *in vitro*. This opens up a new window for regulation of autophagy and apoptosis by controlling the external pathways in comparison to the mitochondria-mediated pathways that occur through an irreversible one-way process. More studies are required in the future to elucidate the associations and develop the basic therapeutic interventions for liver cancer.

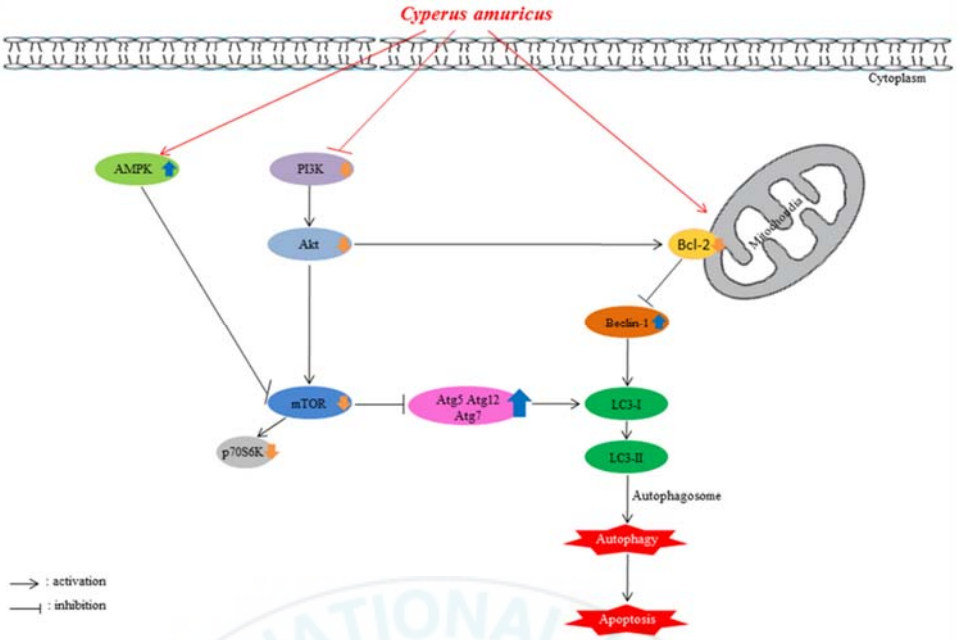

Figure 4.5. Proposed pathways for *C. amuricus*-induced autophagy and apoptosis in HCC Hep3B cells via AMPK and PI3K/Akt/mTOR pathways.

4.6. Conclusion

The purpose of this research is to examine the anticancer effects of *C. amuricus* methanol extract and to investigate the potential cell death pathway caused by *C. amuricus* in Hep3B cells.

C. amuricus obviously elicited sub-G1 arrest in Hep3B cells concomitant with the upregulation of $p21^{CIP1/WAF1}$, $p27^{KIPI}$ and $p16^{INK4a}$ proteins and the downregulation of cdc25A, cyclin D1 and cyclin E, CDK4 and 2 as well as E2F-1, phospho-Rb. Additionally, the sequential activation of various caspases (cleaved of caspase-8, -9, -3, -7, and -6, and cleaved PARP) and the changed expression of other proteins related to the apoptosis pathway were observed after *C. amuricus* exposure. An increment in the pro-apoptotic proteins (Bim, tBid, Bax and Bak) and a reduction of anti-apoptotic protein (Bcl-2) regulate Hep3B cell death by controlling the permeability of mitochondrial membranes and the release of cytochrome *c* from mitochondria into the cytosol with Apaf-1 after *C. amuricus* treatment, indicate that *C. amuricus* induces apoptosis via the activation of both extrinsic-death receptor and intrinsic-mitochondrial pathways in HCC Hep3B cells.

ER stress-induced apoptosis in *C. amuricus*-treated Hep3B cells is characterized by the disruption of Ca^{2+} homeostasis, the enhancement of GRP78/BiP and CHOP, resulting in the accumulation of UPR sensors, as well as activation of calpain and caspase-12, leading to Hep3B cell death by controlling the permeability of mitochondrial membranes.

Furthermore, *C. amuricus* induced autophagy followed by the increased cytoplasmic vacuolation and associated with the conversion of LC3-I to LC3-II and intension of Atg5-Atg12 conjugate, Atg7, Beclin-1 and DAPK3 proteins. Particularly, *C. amuricus*-induced apoptotic cell death in Hep3B cells is dependent, at least in part, on the induction of autophagy via AMPK activation and inhibition

of PI3K/Akt/mTOR pathways, submitting the mechanism behind He3B cell death pathway stimulated by *C. amuricus*.

In conclusion, all of the results elucidated the underlying mechanisms of the extract of *C. amuricus*-induced antiproliferation, cell cycle arrest, autophagy and apoptosis in HCC Hep3B cells *in vitro*, so as to supply scientific rationales for using *C. amuricus* as a new promising chemopreventive and/or chemotherapeutic agent against liver cancer.

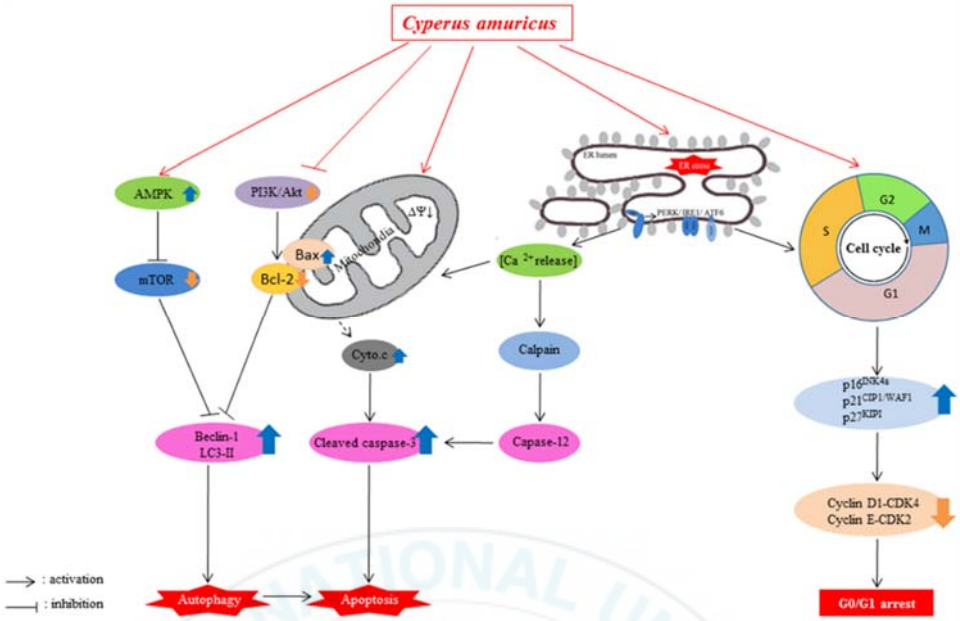

Figure 4.6. Proposal of integrated cell death signaling pathways in *C. amuricus*-treated Hep3B cells.

4.7. References

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국문요약

Cyperus amuricus (*C. amuricus*)는 Cyperaceae과에 속하며 수세기 동안 수렴제, 이뇨제, 상처 치료 및 소화관 장애 등에 널리 사용되어져 왔다. 최근 *C. amuricus*의 anti-lipase, 항산화, 항신생혈관 형성 효과 등 약리학적인 효능이 알려지고 있으나 항암 효과에 대한 분자 기전은 밝혀져 있지 않다. 그리하여 *C. amuricus*에 의한 human hepatocellular carcinoma cells (HCC) 사멸의 정확한 기전을 밝히기 위해 이 연구를 수행하였다.

*C. amuricus*는 A549와 HaCaT, HEK293 세포주에는 독성이 없었으나, Hep3B에는 상당한 세포 독성을 나타냈다. *C. amuricus*는 Hep3B에서 $p21^{CIP1/WAF1}$와 $p27^{KIPI}$, $p16^{INK4a}$의 발현 증가와 cdc25A, cyclin D1, cyclin E 및 CDK4와 2뿐만 아니라 E2F-1과 phospho-Rb의 발현 감소 수반하며 G1 세포주기 정지(cell cycle arrest)를 유도했다. 또한, *C. amuricus*는 여러 caspase (cleaved of caspase-8, 9, 3, 7 및 6과 cleaved PARP)를 활성화 시키고, anti-apoptotic Bcl-2에 대한 pro-apoptotic Bcl-2의 발현량을 증가시켜 미토콘드리아 막의 투과성 변화와 미토콘드리아로부터의 시토크롬 c의 방출을 촉발시켰다. 이러한 결과에 기초하여, *C. amuricus*는 HCC Hep3B세포에서 외인성 사멸 수용체 매개성 경로와 내인성 미토콘드리아 매개성 경로를 활성화시켜 세포의 사멸을 유도하였다.

Hep3B 세포에서 소포체 (ER) 스트레스 매개성 세포 사멸 및 G1 세포주기 정지에 대한 *C. amuricus*의 가능성을 깊이 연구하였다. *C. amuricus*는 unfolded protein response (UPR)의 활성화를 통해 ER 스트레스를 유발하여 ER 센서들의 인산화 수준의 변화, 즉 GRP78/BiP의 해리, p-PERK의 감소 및 ATF6와 IRE1α의 증가를 가져왔다. 이러한 결과는 *C. amuricus*를 처리한 Hep3B 세포에서 세포질 내 Ca^{2+} 수준의 증가와 caspase-12 및 CHOP의 활성화를 동반하여 ER 스트레스에 의한 세포 사멸을 유도하였다. 또한 *C. amuricus* 매개성 G1 세포주기 정지의 효과는 세포주기 조절 분자를 조정하는 ER chaperones의 유도에 의해 명확해졌다. 위의 결과들을 볼 때, *C. amuricus*가 Hep3B 세포에서 G1 세포주기 정지, ER stress 및 미토콘드리아 매개 내재성 경로(mitochondrial-dependent intrinsic pathways)를 통해 세포 사멸에 이르게 하는 효율적인 세포 사멸 유도물질이이다.

다음으로 *C. amuricus* 유도성 세포 사멸에서 autophagy 연관성에 대한 상세한 기전을 조사하였다. 노출 초기(3-12 시간)에 *C. amuricus*는 AVO 양성 세포의 축적 및 Atg5-Atg12 접합체와 Atg7, Beclin-1, LC3-II, DAPK3 단백질의 증가를 통해 autophagy를 유도하였다. 흥미롭게도 *C. amuricus*는 phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)을 억제하고 apoptosis의 활성화를 촉진하는 adenosine monophosphate-activated protein kinase (AMPK)의 인산화를 증가시켰다. 특히,

3-methyladenine (3-MA)의 전처리 결과는 *C. amuricus*에 의한 Atg7, Beclin-1, LC3-II 및 AMPK의 인산화의 증가를 차단하여 *C. amuricus*에 의한 apoptosis와 autophagy 사이의 crosstalk를 보여 주었다.

결론적으로, 이 연구는 HCC Hep3B 세포에서 *C. amuricus*가 세포주기 정지 및 ER 스트레스, 세포 사멸, autophagy에 미치는 영향을 보여주는 첫 연구이다. 다른 유형의 암세포에서chemo-sensitization에 대한 세포 반응에 곤한 *C. amuricus*의 정확한 메커니즘과 생물학적 효능을 밝히는 데 유익한 정보를 제공할 수 있을 것이다.

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