Effects of Berberine on Hepatocarcinoma Cell Lines

YIP, Ka Yan

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Thesis Committee

Professor Au Wing Ngor, Shannon (Chairman)

Professor Ho Wing Shing (Thesis Supervisor)

Professor Lau Kwok Fai (Committee Member)

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Abstract

Berberine, an isoquinoline alkaloid, is an active component from a Chinese medicinal herb *Coptis chinensis* Franch. It is extensively used in traditional Chinese prescriptions. Recent studies demonstrated that berberine exerted anti-cancer activities. However, details of its biologic activity are lacking.

To understand the pharmacological actions of berberine, two hepatocarcinoma cell lines, Huh7 and JHH4, and normal liver cell line WRL68 were used in this study. The results indicate that berberine significantly reduced cell viabilities in hepatocarcinoma cell lines, while it exerted minimal inhibitory effect in normal liver cells.

The experimental findings show that apoptotic effects of berberine in hepatocarcinoma cell lines were due to its ability to activate caspases. Caspase activation led to a decrease in the protein expression of full-length Bid in cytosol, where it was likely to be truncated and translocated to mitochondria, resulting in apoptosis. Furthermore, the results indicate that berberine triggered G_1/S cell cycle arrest in a dose-dependent manner. The present study provides experimental evidences that berberine possess anti-cancer activities in human hepatocellular carcinoma, and enables us to understand the apoptotic pathways induced by berberine.

論文摘要

黃連為常用中草藥，味苦性寒，能清熱燥濕、瀉火解毒。黃連素乃從其根部萃取。近年有研究指出黃連素能抗癌，惟其生物活性細節仍然缺乏。

本研究旨在理解黃連素的藥理作用。實驗結果證明黃連素顯著抑制肝癌細胞 Huh7 及 JHH4，及輕微抑制正常肝臟細胞 WRL68。

研究證實，黃連素在肝癌細胞的凋亡作用肇因於它能激活硫胱氨酸蛋白酶 (Caspases)，從而減少細胞質內 full-length Bid 蛋白的表達。此蛋白有可能被截斷，易位到線粒體，繼而導致細胞凋亡。此外，研究結果表明黃連素觸發 G_1/ S 細胞週期停滯，並呈劑量依賴性。

總括而言，本研究提供了實驗證據，確認黃連素誘導細胞凋亡的途徑，證實黃連素有效抑制肝癌細胞生長，並指出黃連素具潛在藥用價值。

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List of Abbreviations

AF/ AFB	Aflatoxin/ Aflatoxin B
Apaf1	Apoptotic protease-activating factor
BP	Benzo[a]pyrene
CAD	Caspase-activated DNase
CAM	Complementary and Alternative Medicine
CARD	Caspase Recruitment Domain
DED	Death Effector Domain
DFF	DNA fragmentation factor
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FADD	Fas-associated death domain
FBS	Fetal Bovine Serum
G_1 phase	Gap phase 1 in cell cycle
G_2 phase	Gap phase 2 in cell cycle
HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
IAP	Inhibitor of apoptosis
IC_{50}	Half maximal inhibitory concentration
M phase	Mitosis phase in cell cycle
MEM	Minimum Essential Media
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
PAH	Polycyclic Aromatic Hydrocarbon
PARP	Poly (ADP-ribose) Polymerase
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PS	Penicillin Streptomycin antibiotic solution
RPMI	Roswell Park Memorial Institute media
S phase	DNA synthesis phase in cell cycle
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TCM	Traditional Chinese Medicine
VC	Vinyl Chloride

Chapter 1 Introduction

1.1 Hepatocellular carcinoma

1.1.1 Overview

Liver cancer is the fifth most popular cancer worldwide and the second leading cause of cancer death in men (Jemal et al., 2011). In women, it is the seventh most frequently diagnosed cancer and the sixth most common cause of cancer death.

There were 694,000 deaths from liver cancer in 2008 (Ferlay et al., 2010). The overall ratio of mortality to incidence for liver cancer was 0.93. Due to its high fatality, liver cancer is the third most common causes of death from cancer worldwide (Ferlay et al., 2010). Occurrence rate of liver cancer in males was twice as high as in females worldwide. Among primary liver cancers occurring worldwide, hepatocellular carcinoma (HCC) represents the major histological type, which accounts for 70% to 85% of cases (Perz, Armstrong, Farrington, Hutin, & Bell, 2006).

Half of the liver cancer cases and deaths were estimated to occur in China for both men and women in 2008 (Ferlay et al., 2010). The high HCC rates in China, other parts of Asia, and sub-Saharan Africa largely reflect the elevated prevalence of chronic hepatitis B virus (HBV) infection, with over 8% of the populations in these regions chronically infected with the virus (Schottenfeld, Fraumeni, Colditz, Samet, &

Whittemore, 2006). HBV infection accounts for about 60% of total liver cancer in developing countries and for about 23% of cases in developed countries (Parkin, 2006). Hepatitis C virus (HCV) infection accounts for 33% of total liver cancer in developing countries, and for 20% in developed countries. Interaction of aflatoxin B (AFB) exposure with chronic HBV infection is known to increase liver cancer (Ming et al., 2002; Parkin, 2006). However, the contribution of aflatoxin B1 (AFB1) exposure to liver cancer cases in parts of Asia, where the exposure is prevalent, is not yet known (International Agency for Research on Cancer [IARC], 2002).

In the United States and other low-risk Western countries, alcohol-associated cirrhosis and possibly non-alcoholic fatty liver disease related to obesity, are thought to be the leading causes of liver cancer (El-Serag, 2007).

Liver cancer incidences are ascending in the United States and Central Europe. Possible leading causes are obesity epidemic and the increase in HCV infection through continued transmission by injection drug users (Altekruse, McGlynn, & Reichman, 2009; Bosetti et al., 2008; El-Serag, 2007). Contrary to the trend in the low-risk areas, possibly due to the HBV vaccine, incidence rates of liver cancer in some historically high-risk areas decreased (Bosetti et al., 2008). In Taiwan, the universal infant hepatitis vaccination programs reduced liver cancer incidences by nearly two-thirds in children and young adults (Chang et al., 2009).

As of 2009, a total of 177 countries (91%) had introduced the HBV vaccine into their national infant immunization schedules (World Health Organization [WHO], 2011). No vaccine is available for HCV. Preventive strategies against HCV include screening of donor's blood for antibodies to HCV, instituting adequate infection control practices during medical procedures including the use of oral delivery medicines where possible, and needle exchange programs among injection drug users (Jemal et al., 2011). Crop substitution and improved grain storage practices have been shown to reduce AFB contamination in sub-Saharan Africa (Turner et al., 2005).

1.1.2 Risk factors

HBV or HCV infection, alcohol consumption, and diabetes mellitus alone were found to be significant risk factors for HCC development in a recent study in the United States (Hassan et al., 2002). The study also reported that synergy existed between alcohol consumption and chronic HBV or HCV infection and diabetes mellitus. Moreover, exposure to chemicals such as AF is also an independent risk factor for HCC development. In addition, androgens have been known to be associated with an increased incidence of liver neoplasm, and their hepatic effect is receptor-mediated. However, the pathogenesis of HCC is still poorly understood.

HBV and HCV infection

Chronic HBV infection is the most prevalent cause of HCC worldwide. HBV infection accounts for 60% of total liver cancer in developing countries, and for about 23% of cases in developed countries (Parkin, 2006). HCV infection accounts for 33% of total liver cancer in developing countries, and for 20% in developed countries. Epidemiologic studies have established a close association between HCC occurrence and chronic HBV infection (Kew & Popper, 1984). A recent study reported that chronic HBV and HCV infection increased the risk of HCC approximately 13-fold and 15-fold respectively (Hassan et al., 2002). In addition, the presence of cirrhosis seems to play a central role in both HBV and HCV, as generally 60% to 80% of HCC cases are associated with the disease (Bosch & Munoz, 1991). Another recent study found that 69% of HCV-infected HCC patients and 35% of HBV-infected HCC patients had cirrhosis (Hassan et al., 2002). Even though some HBV- or HCV-infected HCC patients did not develop cirrhosis, a direct carcinogenic role has been suggested for HBV and HCV. Integration of the HBV genome into cellular DNA was found in most HBV-induced HCC patients (Kew, 1996). The HCV core protein was implicated to have a direct carcinogenic role in HCC development (Moriya et al., 1998; Ray, Meyer, & Ray, 1996). Both viruses may directly induce HCC by contributing to histopathologic changes in the liver (Hassan et al., 2002).

In fact, HBV virus may be the primary carcinogen in nearly 80% of HCC patients worldwide (Oberfield, Steele, Gollan, & Sherman, 1989). A prospective study in Taiwan showed that the relative risk of HCC developing in a chronic HBV carrier was more than 200, with a lifetime risk in humans of approximately 50% (Beasley & Hwang, 1984). The mechanism by which chronic HBV infection leads to liver cancer remains unclear, but apparently it is related to the integration of hepatitis B viral DNA into the host genome (Dejean, Bougueleret, Grzeschik, & Tiollais, 1986).

Cirrhosis

Between 30-70% of HCC patients also have cirrhosis. The risk of liver cancer development varies according to the type of cirrhosis present (Melia, Wilkinson, Portmann, Johnson, & Williams, 1984). Primary liver cancers will eventually develop in 2-3% of patients with alcoholic cirrhosis, compared with 10% of patients with the macronodular, posthepatic form of cirrhosis (Oberfield et al., 1989). The risk of HCC development is about 40 times greater in patients with cirrhosis than individuals whose liver functions normally. The risk is higher in men than in women with cirrhosis, with the male-to-female ratio of about 2:1. Cirrhosis associated with α,-antitrypsin deficiency and other inborn errors of metabolism, for example, tyrosinemia, leads to an increased incidence of HCC (Oberfield et al., 1989). Rare cases were reported for patients with HCC complicating Wilson's disease (Cheng,

Govindarajan, & Redeker, 1992). Liver cancer in patients with cirrhosis associated with Wilson's disease was relatively absent (Oberfield et al., 1989). However, HCC occurs in 10-22% of cirrhotic patients with hemochromatosis (Oberfield et.al, 1989).

Exposure to chemicals

Aflatoxin

AFs are carcinogenic in several animal species but their potency varies, among which AFB1 is the most potent hepatotoxin (C. Chen & D. Chen, 2002). AFB1 is a human hepatocarcinogen, and is also a liver carcinogen when fed to rodents (Wogan & Newberne, 1967; Wogan, 1992). AFB1 is the secondary metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which grow in tropical and subtropical regions of the world. AFB1 contaminates foods such as corn, rice and peanuts which are stored under tropical conditions (Wogan, 1976). AFB1 metabolism produces the active form of AFB1, AFB1-8, 9-epoxide, which is highly mutagenic and carcinogenic for the liver in rats and other experimental animals, with mutagenicity correlating with carcinogenicity (Newberne & Wogan, 1968; Swenson, E. Miller, & J. Miller, 1974). AFB1 is implicated by epidemiological studies as a causative factor for HCC in humans (Ross et al., 1992). The clinical appearance of HCC is the result of a series of changes at cellular and molecular level. Evidences have shown that DNA damage by environmental chemical carcinogens is critical in this process (Zhang,

2010).

AFB1 forms AFB1-DNA adducts by covalently binding to guanine and cytosine residues of DNA both *in vivo* and *in vitro* (Croy, Essigmann, Reinhold, & Wogan, 1978; Yu, Bender, & Hutchcroft, 1994). It also forms RNA and protein adducts, which impairs DNA, RNA and ultimately protein synthesis (Santella, Chen, Zhang, Yu, & Wang, 1998; Meneghini & Schumacher, 1977; Amstad & Cerutti, 1983). AFB1-DNA adducts were detected using immunohistochemical assay in smeared HCC tissues and HCC sections (Chen, Zhang, Lu, & Santella, 1992; Zhang et al., 1991; Lunn et al.; 1997). The presence of AFB1-DNA adducts can lead to genetic alternations in loci involved in HCC development (Zhang, 2010). The recent epidemiologic studies suggested an additive effect of AFB1 and HBV infection on HCC risk (Wu et al., 2007). The highly aberrant patterns of genetic alternations detected in different areas suggested that AFB1 has genotoxic effects (Zhang, 2010). HBV infection and high AFB1 exposure may have combined effects which could promote HCC development (Lunn et al., 1997; Wong et al., 2000).

Molecular mechanisms underlying the carcinogenic effects of AFB1 have been investigated in rodents. HCC induced by AFB1 in Fischer 344 rats showed activating mutations in codon 12 of *K-ras* (McMaho, Davis, Huber, Kim, & Wogan, 1990). However, the incidence of point mutation of *K-ras* and *N-ras* oncogenes in human

HCC was low (Tsuda et al., 1989). AFB1 interfered with cell cycle regulation in an *in vitro* study (Ricordy, Gensabella, Cacci, & Augusti-Tocco, 2002). AFB1 also induced mitotic recombination (Stettler & Sengstag, 2001), and minisatellite rearrangements (Kaplanski, Chisari, & Wild, 1997). Therefore, AFB1 may contribute to genetic alternations in HCC by alternative mechanisms, which are mitotic recombination and genetic instability (Wild & Turner, 2002).

Vinyl chloride

Vinyl chloride (VC) is a major industrial chemical, a wide-spread environmental contaminant, and a known animal and human carcinogen (IARC, 1974). VC is a colorless toxic gas extensively used in the plastic industry. VC is absorbed after respiratory exposure, and is activated primarily in hepatocytes by the enzyme cytochrome P450 (CYP2E1) (Zhang, 2010). Metabolites of VC can react with DNA bases to form DNA adducts (Guengerich, 1992). VC induces several DNA adducts after metabolic activation, and different studies have shown that these DNA adducts are responsible for specific mutations (Barbin et al., 1997). Barbin et al. (1997) also suggested that VC is a multi-potential carcinogen in animals. The p53 mutation pattern in HCC in workers exposed to VC included point mutations in codons 175, 245, 248, 273 and 282, however, it remains unclear whether these genetic alternations were directly associated with VC exposure (Weihrauch, Lehnert, Köckerling,

Wittekind, & Tannapfel, 2000). No direct evidence has shown that genetic polymorphisms of metabolizing enzymes are correlated with HCC development due to VC exposure (Zhang, 2010).

Polycyclic aromatic hydrocarbons

Cigarette smoking is associated with a significantly increased HCC risk in some Asian countries (Chen et al., 1996; Tu, Gao, Zhang, & Gu, 1985; Goodman et al., 1995). Tobacco smoke consists of chemical carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) and aromatic amines. Benzo[a]pyrene (BP) and N-nitrosamines are examples of PAHs which can be found in Tobacco smoke. PAHs are produced during combustion of all organic materials, and are ubiquitous in the environment. Therefore, PAHs are also found in polluted air, smoked and charbroiled foods, contaminating fats and grains (Phillips, 1999). PAHs, especially BP, are known animal and human carcinogens (IARC, 1983).

PAH-DNA adducts were detected in HCC tissue samples in human (Chen et al., 2002; Zhang et al., 2006). Levels of PAH-DNA adducts in liver tissues and the combination of their levels with some susceptibility factors including HBV infection and AFB1 exposure, were found to be in association with HCC (Chen et al., 2002). A recent study reported that PAH-albumin adducts were associated with increased HCC risk, especially among those with high exposure to AFB1, and environmental PAH

exposure may enhance the hepatic carcinogenic potential of HBV infection (Chen et al., 2002).

Alcohol consumption

Alcohol consumption is an independent risk factor for HCC. Ethanol is a hepatotoxin. It is an important chemical solvent and promotes the absorption of ingested toxins (Chen, Yu, & Liaw, 1997). Alcohol consumption is the common cause of cirrhosis, a primary clinical predictor of HCC, in the US and European countries. HCC development is correlated with the magnitude of alcohol consumption (Austin et al., 1986; Tagger et al., 1999; Hassan et al., 2002). Previous studies have suggested that heavy alcohol consumption could induce direct hepatic cellular injury and toxicity, which could lead to liver fibrosis and cirrhosis (Batey, Burns, Benson, & Byth, 1992). Moreover, microsomal enzymes in liver, which oxidize and metabolize ethanol, may increase conversion of procarcinogens to carcinogens (Lieber, Seitz, Garro, & Worner, 1979).

Ethanol damages liver by oxidative stress. Alcoholic hepatitis shows increased levels of isoprostanes, which are markers of oxidative damage (McClain et al., 2002). Acetaldehyde and oxygen-free radicals are generated during ethanol metabolism, which are highly associated with the development of alcohol-related liver diseases through oxidative stress (Lieber, 1990). Oxidative stress may induce liver cell damage

directly by initiating peroxidation of membrane lipids. Moreover, oxidative stress may lead to the accumulation of oncogenic mutations (Zhang, 2010). Oxidative damage may also accelerate telomere shortening, which correlates with the development of liver cirrhosis, chromosomal instability and HCC (Kurz et al., 2004).

However, the mechanism through which heavy alcohol consumption promotes the development of HCC is not well known (Hassan et al., 2002).

Diabetes mellitus

Studies have shown that diabetes mellitus is significantly related to the risk of HCC development (Yu, Tong, Govindarajan, & Henderson, 1991; La Vecchia, Negri, Decarli, & Franceschi, 1997; Lagiou et al., 2000; Hassan et al., 2002). Diabetes mellitus promotes the development of HCC through an unknown mechanism. However, previous studies suggested oxidative stress was associated with the pathogenesis and complications of diabetes mellitus as a result of hyperglycemia (Rosen et al., 2001; Kar & Chakraborti, 1999; Ford & Cogswell, 1999; Oberley, 1988; Gillery, Monboisse, Maquart, & Borel, 1989). An increased blood glucose level in diabetes mellitus patients may stimulate glycosylation of proteins, which include hemoglobin, leading to an increase in the release of iron from hemoglobin, and further production of free radicals, which cause oxidative stress (Kar & Chakraborti, 1999; Ford & Cogswell, 1999). An early study reported a high concentration of serum

ferritin in diabetes mellitus patients, which reflected increased body iron stores in patients (Ford & Cogswell, 1999). Hassan et al. (2002) suggested that iron is a powerful pro-oxidant and oxidative stress is increased in impaired glucose tolerance states. Alcohol-induced oxidative stress may increase the susceptibility of patients having diabetes mellitus to cirrhosis, DNA damage, and HCC development (Hassan et al., 2002). Alcohol-induced oxidative stress may also increase the susceptibility of diabetes mellitus development (Hassan et al., 2002), which may explain early onset of diabetes is related to HCC development (Yu et al., 1991; La Vecchia et al., 1997).

1.1.3 Treatment of HCC

Phases of HCC

The natural history of HCC can be divided into three distinct phases, which are molecular phase, preclinical phase, and clinical phase (Trevisani, Cantarini, Wands, & Bernardi, 2008). The molecular phase includes the sequential genomic alternations leading to cell transformation (Trevisani et al., 2008). The preclinical phase covers an initial period, in which the tumor is too small to be detected by imaging techniques (Trevisani et al., 2008). The second period in preclinical phase is the diagnostic phase, during which the tumor is detectable but is still asymptomatic (Trevisani et al., 2008). Finally, the clinical phase starts with the occurrence of symptoms caused by the tumor

burdens (Trevisani et al., 2008). HCC usually becomes symptomatic when it reaches 4.5-8 cm in patients suffered from chronic liver disease (Trevisani et al., 2008).

Treatment options

Screening for primary liver cancer is difficult because the tumor seldom gives rise to early symptoms (Oberfield et al., 1989). Current treatment options are limited and does not provide effective cure for HCC.

Surgery

The criteria for liver resection are stringent. Patients should have good liver function, and they should not have obvious metastasis. These patients need surgical exploration to determine the location, extent and the resectability of the tumor (Oberfield et al., 1989). However, only a minority of these patients will be suitable for surgical excision due to other limitations. For example, if a patient suffers from cirrhosis, he or she will not be able to undergo liver resection, since cirrhosis impacts the regeneration of liver. Oberfield et al. (1989) suggested the criteria for resectability of a HCC are a solitary lesion limited to one lobe of the liver, and absence of cirrhosis, jaundice, or ascites. Surgical therapy offers the greatest likelihood of cure with a 10-year actual disease-specific survival rate of 17-25% at present (Tomlinson et al., 2007; Fong, Fortner, Sun, Brennan, & Blumgart, 1999). Despite complete resection, more than 75% of patients develop recurrence (Govindarajan et al., 2011). Achieving a complete

surgical resection is often technically difficult and sometimes impossible.

Radiotherapy

Radiotherapy is believed not very useful for HCC patients because normal liver tolerance is restricted to 2,500 to 3,000 cGy (Oberfield et al., 1989). Moreover, conventional photon radiotherapy for HCC may induce risk of hepatic toxicity (Komatsu et al., 2010).

Chemotherapy

Combination chemotherapy using 5-fluorouracil, cisplatin, and mitoxantrone (FMP) could achieve a response rate of more than 20% in HCC patients, however, the beneficial effect was compromised by formidable side effects (Yeh et al., 2011).

1.2 Berberine - a compound derived from Traditional Chinese Medicine

1.2.1 Traditional Chinese Medicine

Conventional HCC therapies include surgery, chemotherapy and radiotherapy, as discussed above. However, these therapies have limitations and negative impacts. First, most cancer cases are diagnosed at the late stage of development and patients are unable to undergo surgery. Second, the recurrence of cancer is common in patients who have had a resection, while the post-operative survival rate for most cancers is less than 5 years. Third, malicious side effects are observed for chemotherapy and radiotherapy against cancer (National Cancer Institute, 2008). For cancers which are relatively resistant to chemotherapy or radiotherapy, these treatments only have minimal effect at improving patients' survival (Qi et al., 2010). Despite the unsatisfactory treatment outcome of current therapies, the use of complementary and alternative medicine (CAM) has become more popular among cancer patients in Western countries, with a prevalence of 80% (Xu, Towers, Li, & Collet, 2006; Cui, Wang, Kokudo, Fang, & Tang, 2010). Traditional Chinese Medicine (TCM) has been used in China, Japan, and other Asian countries for hundreds of years. These medicines are widely accepted as current forms of CAM in cancer treatment in the United States and Europe (Wong, C.Sagar, & S.Sagar, 2001; Gai et al., 2008).

1.2.2 Berberine

Berberine is an isoquinoline alkaloid from the Chinese medicinal herb *Coptis chinensis*. It is found in the roots, rhizome and stem bark of other important medicinal plants, such as *Berberis vulgaris* (barberry), *Berberis aquifolium* (Oregon grape), and *Berberis aristata* (tree turmeric) (Singh, Vaid, Katiyar, Sharma, & Katiyar, 2011). Berberine is extensively used in traditional Chinese prescriptions due to its anti-protozoal, anti-hypertensive (Bova, Padrini, Goldman, Berman, & Cargnelli, 1992), anti-bacterial (Amin, Subbaiah, & Abbasi, 1969), anti-inflammation (Akhter, Sabir, & Bhide, 1977), anti-cholinergic (Tsai & Ochillo, 1991), and anti-arrhythmic properties (Wang & Zheng, 1997). Previous studies reported that berberine inhibited COX-2 transcriptional activity in human colon cancer cells (Lin et al., 1999; Fukuda et al., 1999b). Berberine inhibited inflammation in human hepatoma cells (Fukuda et al., 1999a), however, details of its biologic activity are lacking.

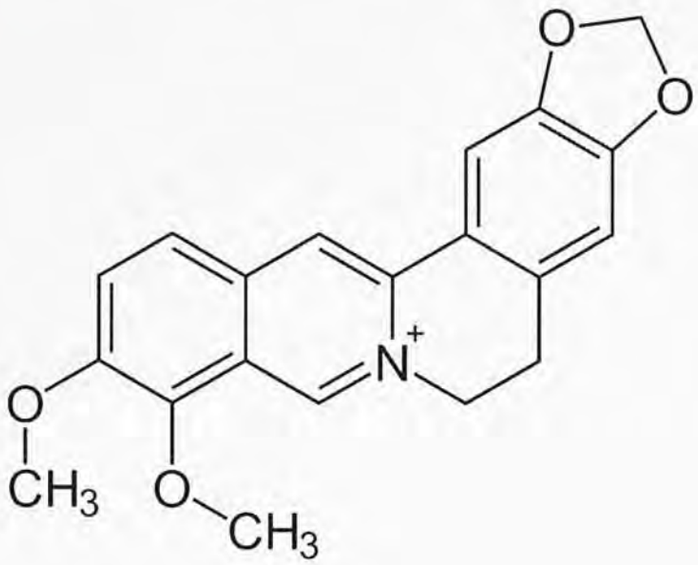


Figure 1 Structure of berberine

Berberine has a molecular formula of $C_{20}H_{18}NO_4^+$, and the molar mass of 336.361 g/mol.

1.3 Cell cycle

1.3.1 An Overview of cell cycle

The eukaryotic cell cycle comprises four phases in proliferating cells: Gap phase 1 (G_1); DNA synthesis phase (S); Gap phase 2 (G_2), and mitosis phase (M). The transition which occurs at the restriction point (R) in G_1 commits the cell to the proliferative cycle. If conditions which signal this transition are absent, the cell exits the cell cycle and enters G_0, a resting phase when the cell has stopped dividing (Cooper & Hausman, 2009). G_2 is the phase when the cell prepares for division. M phase consists of prophase, metaphase, anaphase, and telophase. The chromosomes separate while the cell divides during M phase.

1.3.2 Cell cycle and carcinogenesis

Dysregulation of the cell cycle is one of the hallmarks of carcinogenesis (Schwartz & Shah, 2005). Cell cycle protects the cell from DNA damage, while the cell cycle arrest is a survival mechanism in which the tumor cell can repair damaged DNA (Schwartz & Shah, 2005). Cell cycle is controlled at different checkpoints. When cells suffer extracellular or intracellular stress or both, the cell cycle checkpoints, especially G_1/S and G_2/M checkpoints, are activated (Wang et al., 2009). Checkpoints are a regulatory mechanism which ensures genomic integrity and prevents the propagation of

transformed cells (Hartwell & Kastan, 1994). G_1 arrest prevents aberrant replication of damaged DNA, while G_2 arrest allows cells to avoid segregation of defective chromosomes (Arima et al., 2004). Leading causes of defective cell cycle checkpoints include gene mutations, chromosome damage, and aneuploidy, and these defects may lead to tumor formation (Hartwell & Kastan, 1994; Paulovich et al., 1997).

G_1 arrest after DNA damage is primarily induced by stabilization of the p53 tumor suppressor protein (Lakin & Jackson, 1999). Responsive to DNA damage, p53 is activated and post-translationally modified (Arima et al., 2004). p53 is able to serve as a transcription factor which binds DNA sequence-specifically in response to cellular stress, and triggers the expression of genes which contain a p53 binding-site element in their promoter or introns (Bourdon et al. 1997 ; El-Deiry et al., 1992 ; Funk et al., 1992). The cdk inhibitor $p21^{Cip1/WAF1}$ was known to be a p53-responsive gene, which is an important player in p53-induced G_1 arrest (Arima et al., 2004). The inhibitory activities of $p21^{Cip1/WAF1}$ do not attribute only to inhibition of cdk activity, but also other cellular activities, such as interaction with proliferating cell nuclear antigen (PCNA) to inhibit DNA replication (Chen, Smith, O'Connor, & Fornace, 1995; Luo, Hurwitz, & Massague, 1995; Sherr & Roberts, 1995; Waga, Hannon, Beach, & Stillman, 1994). Thus, studies of the cell cycle may yield useful information on how cancer cells are regulated at the cellular level.

1.4 Molecular mechanism of apoptosis

1.4.1 Overview of apoptosis

The term apoptosis was proposed by Kerr, Wyllie, and Currie in 1972. Apoptosis describes a morphology which is a distinctive type of cell death, and is associated with normal physiology (Henkart, 1999). Apoptosis, or programmed cell death, is different from necrosis, which happens when cells are acutely injured (Henkart, 1999). Apoptosis occurs when nuclear chromatin condensation, cytoplasmic shrinkage, dilated endoplasmic reticulum and membrane blebbing happen (Henkart, 1999). Apoptosis can be induced by different types of stimuli, including cell surface receptors like FAS, the mitochondrial response to stress, and factors released from cytotoxic T cells. For example, DNA damage may lead to apoptotic death via a p53-dependent pathway (Henkart, 1999).

Intrinsic and extrinsic apoptotic pathways

Two major pathways for activating caspases have been reported (Cory & Adams, 2002). They are the intrinsic and extrinsic pathways.

The extrinsic pathway, Fas, consists of several protein members, which include the death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain (FADD), and caspases 8 and 10 (Ghobrial, Witzig, & Adjei, 2005). Caspases 8 and 10 lead to apoptosis by activating the downstream

caspases (Ghobrial et al., 2005). This pathway is induced when 'death receptors' on the plasma membrane recruit caspase-8 through the adaptor protein FADD (Ashkenazi, 2002).

The intrinsic pathway is regulated by the Bcl-2 family proteins (Cory & Adams, 2002). Programmed cell death through this pathway results in the caspase-9 activation on a scaffold which is formed by apoptotic protease-activating factor (Apaf1) (Li et al., 1997). Activation of caspase-9 occurs after Apaf1 has interacted with cytochrome c, which is released from damaged mitochondria (Li et al., 1997).

Final pathway

Both the intrinsic and extrinsic pathways lead to a common final pathway. The activation of caspases is the final pathway which leads to the execution of the death signal (Ghobrial et al., 2005). Caspase-3 is the common target of both the intrinsic and extrinsic pathways. Caspase-3 cleaves the inhibitor of the caspase-activated deoxyribonuclease, and it becomes active after cleavage, resulting in nuclear apoptosis (Ghobrial et al., 2005). Caspase-9 is the upstream caspase which converges to caspase-3 in the intrinsic pathway, while caspase-8 is the upstream caspase which converges to caspase-3 in the extrinsic pathway (Mancini et al., 1998; Thornberry & Lazebnik, 1998). The downstream caspases interfere with normal cell functions by triggering cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins,

inhibitory subunits of endonucleaes (CIDE family), and ultimately, destroy "housekeeping" cellular functions (Mancini et al.,1998; Thornberry & Lazebnik, 1998). Moreover, caspases affect cytoskeletal structure, regulation of cell cycle, and signaling pathways. It may lead to morphological signs of apoptosis, for example, DNA condensation, DNA fragmentation, and membrane blebbing.

1.4.2 Caspases cascade

The activation of caspases is one of the biochemical hallmark events of apoptosis (Thornberry & Lazebnik, 1998). Caspases inactivate proteins which prevent living cells from programmed cell death. Moreover, caspases lead to apoptosis by directly disassembling cell structures. Caspases cleave lamins at a single site during apoptosis. The cleavage may result in lamina collapse and subsequent chromatin condensation. Caspases mediate programmed cell death through an intricate mechanism. In most cells, caspases are found to be in inactive proenzyme form (Henkart, 1999). The inactive proenzymes require activation prior to execution of their enzyme activity.

Activation of effector caspases

Various biochemical evidences substantiate a cascade model for the activation of effector caspases (Thornberry & Lazebnik, 1998). The activation of an initiator caspase requires a pro-apoptotic signal (Thornberry & Lazebnik, 1998). When

activated initiator caspase activates effector caspases, cellular disassembly occurs. Initiator caspases mediate cell death by different signaling induced by different stimuli. For example, caspase-8 leads to apoptosis by binding to FADD (Ashkenazi & Dixit, 1998). Alternatively, caspase-9 is associated with programmed cell death induced by cytotoxic drugs (Filomenko et al., 2006).

Activation of initiator caspases

Biochemical evidences suggest that initiator caspases could bind to specific cofactors for activation, which is a commonly observed mechanism in proteases (Thornberry & Lazebnik, 1998). Binding of initiator caspases to specific cofactors is induced by a pro-apoptotic signal. This binding is mediated through distinct structural motifs, which is within both the caspase prodomain and its corresponding cofactor. For example, procaspase-8 needs to associate with its cofactor FADD through the death effector domain (DED) for activation (Boldin, Goncharov, Goltseve, & Wallach, 1996; Muzio et al., 1996), while activation of procaspase-9 requires association with its cofactor APAF-1 through the caspase recruitment domain (CARD) to form a complex (Li et al., 1997). Furthermore, caspase-9 also requires cytochrome c and deoxyadenosine triphosphate for activation, which indicates multiple cofactors may be required for caspase activation (Thornberry & Lazebnik, 1998).

1.4.3 Bcl-2 family

Bcl-2 family proteins regulate caspase activation; pro-apoptotic and anti-apoptotic protein members in the family determine the fate of the cell (Cory & Adams, 2002). Bcl-2 family includes pro-apoptotic proteins such as Bax, Bak, Bad, Bid and etc., and anti-apoptotic proteins such as Bcl-2, Bcl-X_L and etc (Reed, 1994). Anti-apoptotic proteins repress apoptosis by blocking the release of cytochrome-c, while pro-apoptotic proteins promote the release of cytochrome-c (Reed, 1997). Pro-apoptotic proteins are modified post-translationally after receiving a death signal (Scorrano & Korsmeyer, 2003). These modifications include dephosphorylation and cleavage, which result in activation and translocation of these proteins, resulting in apoptosis (Scorrano & Korsmeyer, 2003). To exert the intrinsic pro-apoptotic activity of BH3-only molecules, they need multi-domain BH3 proteins, such as Bax and Bak (Reed, 1994; Scorrano & Korsmeyer, 2003). The outer mitochondrial membrane becomes permeable upon apoptotic stimuli, resulting in the release of cytochrome-c and second mitochondria-derived caspase activator, which is also known as direct IAP-binding protein with low pI (Ghobrial et al., 2005). Once cytochrome-c is released in the cytosol and reacts with Apaf-1, it will lead to the activation of procaspase-9 (Reed, 1997). The active caspase-9 cleaves procaspase-3, which subsequently activates the rest of the caspase cascade, resulting in apoptosis (Reed,

1997). Activated caspases lead to nuclear lamin cleavage, and nucleus breakdown through caspase-3 (Reed, 1997).

1.5 Apoptosis as a target of cancer therapy

Since apoptosis is often defective in cancer, this could limit conventional therapy (Cory & Adams, 2002). A better understanding of apoptotic pathways is warranted to develop new and more effective therapeutic approaches (Cory & Adams, 2002).

Several agents could directly or indirectly inhibit apoptosis-related proteins (Tamm, Schriever, & Dörken, 2001). These agents target apoptosis pathways or the regulators of apoptosis. For example, caspases activators, which target on caspases, are active in the common final death pathway (Ghobrial et. al, 2005). Moreover, the activation of pro-apoptotic protein, Bax, can be induced by gene therapy with the utilization of Bax vectors. Bcl-Xs gene therapy showed promising effects in inducing tumor regression (Ferreira, Epping, Kruyt, & Giaccone, 2002).

The study of the intrinsic and extrinsic apoptotic pathways and other signaling modulators, such as the p53 showed that there were many effective novel agents on the pathways (Ghobrial et. al, 2005). These agents can function individually or in combination with conventional cytotoxic therapy or radiotherapy. Further research on different apoptotic signaling pathways in HCC can help the discovery of novel targeted agents, which affect the specific molecular defects of the tumor.

1.6 Aims of study

To evaluate the potential role of berberine as an anti-cancer agent for hepatocellular carcinoma by *in vitro* study of the compound in different cancer cell lines in terms of cell viabilities, gene expression, protein expression and cell cycle distribution, and to understand the apoptotic pathways induced by berberine.

Chapter 2 Materials and Methods

2.1 Cell culture and treatment

2.1.1 Cell lines

Huh7

Huh7 is a well differentiated human hepatocellular carcinoma cell line. It was purchased from JCRB and was derived from a 57-year-old male. The ethnicity of the origin is Japanese. Cells were grown in Roswell Park Memorial Institute media (RPMI) 1640 with 10% of fetal bovine serum.

JHH4

JHH4 is a human hepatocellular carcinoma cell line. It was purchased from JCRB and was derived from a 51-year-old male. The ethnicity of the origin is Japanese. Cells were grown in Minimum Essential Media (MEM) with 10% of fetal bovine serum.

WRL68

WRL68 is a human liver embryonic cell line purchased from ATCC. Cells were grown in RPMI 1640 with 10% of fetal bovine serum. WRL68 cells have a morphologic structure similar to hepatocytes and hepatic primary cultures (Gutierrez-Ruiz et al. 1994). WRL68 is often used as a model for studying normal liver functions.

2.1.2 Berberine

Berberine chloride was ordered from Sigma-Aldrich China, Inc. It was yellow powder.

2.1.3 Chemicals and reagents

Dulbecco's Phosphate Buffered Saline (DPBS), Fetal Bovine Serum (FBS), Penicillin Streptomycin antibiotic solution (PS), Roswell Park Memorial Institute (RPMI) 1640 media, and Trypsin-EDTA (1X) were purchased from Invitrogen. Minimal Essential Media (MEM) were purchased from ATCC. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and sodium bicarbonate ($NaHCO_3$) were from Sigma chemicals. St. Louis, USA. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific, USA. The filter millex-GP (0.22μm) was ordered from Millpore, Ma., USA. 75 cm^2 tissue culture flasks were ordered from Corning, USA. 96-well microplates were purchased from Iwaki. 100mm culture dishes were purchased from Greiner Bio-one, Germany.

2.1.4 Preparation of solutions

Berberine stock solution (10 mM)

Berberine stock solution was prepared by dissolving 0.0375g dry berberine chloride in 10 ml of Milli-Q water.

DPBS

Dulbecco's Phosphate Buffered Saline (DPBS) was ordered from Invitrogen and was stored at 4°C for cell culture use.

MEM

Liquid MEM was ordered from ATCC. Each liquid bottle contained 500 ml plain MEM. Plain MEM (50 ml) was drawn out before use. Fetal bovine serum (50ml) and PS antibiotic mixture (5ml) were added to the liquid MEM. The completed medium was stored at 4°C.

MTT solution

MTT (5 mg) was dissolved in 1 ml of DPBS to give a final concentration of 5 mg/ml. MTT solution was freshly prepared before use, and was wrapped by aluminum foil to be protected from light.

RPMI

Liquid RPMI 1640 medium was ordered from Invitrogen. Each liquid bottle contained 500 ml plain RPMI 1640 medium. Plain RPMI medium (50 ml) was drawn out before use. Fetal bovine serum (50 ml) and PS antibiotic solution (5 ml) were added to the liquid RPMI. The completed medium was stored at 4°C.

2.1.5 Procedures

Seeding cells into culture flask

Cells were seeded into culture flasks inside culture hood. Cells stored in liquid nitrogen stock were thawed in the 37°C incubation. Cells were transferred to a new falcon, and were re-suspended with additional fresh completed medium (5mL) and were centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded, and the cell pellet was washed with 1 ml of DPBS, and was centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded and 1 ml of the completed medium was added to re-suspend cells. After re-suspension, cells were transferred to a new 75 cm^2 culture flask with 12 ml of completed medium. The flask was stored in a 37°C incubator supplied with 5% carbon dioxide.

Subculture of cells

Subculture was done inside culture hood. The growth medium was discarded and warm DPBS was used to rinse the flask twice. Trypsin-EDTA (1 ml) was added to the flask and incubated at 37°C for 3 minutes. With appropriate tapping, cells were completely detached from the bottom surface of the flask. The completed medium (5 ml) was added to stop the activity of trypsin. Cells were transferred to a new falcon and were centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded and the cell pellet was re-suspended in 1 ml completed medium. After re-suspension, 2 µl

of cells were mixed with 18 µl of trypan blue and placed onto a hemocytometer. The cell count was performed with the use of a microscope. Cells were seeded in a new 75 cm^2 culture flask with 12 ml of fresh completed medium at the concentration of 1 x 10^6. The culture flask was stored in a 37°C incubator supplied with 5% carbon dioxide.

Cell viability assay

Huh7, JHH4 and WRL68 cells were seeded onto 96-well plate at the concentration of 1 x 10^6 overnight. Cells were treated with different concentration of berberine for 24, 48, and 72 hours respectively. MTT solution (20 µL) was added to each well of cells in the 96-well plate, and the plate was kept at 37°C with 5% carbon dioxide inside the incubator for 4 hours. After incubation, the medium was discarded and the plate was dried. DMSO (200 µL) was added to dissolve the purple formazan formed. The plate was shaken for 3 minutes, after which the optical density was measured at 540 nm. Cell viability towards berberine was calculated by comparing the absorbance of wells of cells treated with different berberine concentration with the control. The concentration of berberine which reduced the cell viability by 50% (IC_{50}) was recorded.

2.2 Apoptosis detection by FITC Annexin V and PI co-staining

2.2.1 Chemicals and reagents

FITC Annexin V Apoptosis Detection Kit I was ordered from BD Pharmingen™. The kit consisted of 10X Annexin V Binding Buffer, FITC Annexin V and Propidium Iodide Staining Solution. The reagents were stored undiluted at 4°C and protected from exposure to light.

Dulbecco's Phosphate Buffered Saline (DPBS) was purchased from Invitrogen.

2.2.2 Procedures

Cells were harvested according to manufacturer's protocol

Huh7 or JHH4 cells were grown to 60-80% confluence before treating with berberine. Cells were treated with different concentration of berberine for 24, 48, and 72 hours respectively. After berberine treatment, cells were harvested with trypsin. After washing with DPBS, cells were stained as recommended by BD Pharmingen™ FITC Annexin V Staining Protocol to measure apoptosis. The stained cells were analyzed using a FACSCanto instrument. Data were analyzed with the software FlowJo 7.6.1.

Modifications of the protocol

Cells were treated according to the manufacturer's protocol with the modifications described below:

1. FITC Annexin V was diluted with DPBS in the ratio of 1:3 prior to use. The reagent was freshly prepared. 5 µl was used per test.
2. Propidium Iodide (PI) was diluted with DPBS in the ratio of 1:39 prior to use. The reagent was freshly prepared. An aliquot (5 µl) was used for each test.
3. Annexin V Binding Buffer (10X concentrate) was diluted with DPBS instead of distilled water. One part of the 10X Annexin V Binding Buffer was diluted to 9 parts of DPBS.

Controls for setting up flow cytometry

Controls below were used to set up compensation and quadrants for flow cytometry

1. Unstained Huh7 and JHH4 cells.
2. Huh7 and JHH4 cells stained with FITC Annexin V only.
3. Huh7 and JHH4 cells stained with PI only.

2.3 Gene expression in Berberine-induced apoptotic cells

2.3.1 Chemicals and Reagents

Human Apoptosis RT² Profiler PCR Array (PAHS-012) 96-well Plate and RT^2 SYBR Green / ROX qPCR Master Mix were ordered from SABiosciences. RNase away was ordered from Invitrogen. RNase-free water was from USB, Cleveland, USA. RNeasy Mini Kit was ordered from Qiagen. Real-time PCR primers were ordered from Sigma-Aldrich. RT^2 qPCR Primer Assay- SYBR ® Green Human ACTB, RT^2 qPCR Primer Assay- SYBR ® Green Human CIDEA, and RT^2 qPCR Primer Assay- SYBR ® Green Human HRK were ordered from SABiosciences. Transcriptor First Strand cDNA Synthesis Kit was ordered from Roche Diagnostics.

2.3.2 Procedures

Cell treatments

Huh7 or JHH4 cells were grown to 60-80% confluence prior to berberine treatment. Cells were treated with berberine for 72 hours. After incubation, cells were harvested with trypsin and centrifuged at 1000 rpm for 3 minutes.

Total RNA extraction

Total RNA was isolated using RNeasy Mini kit (Qiagen) according to manufacturer's

protocol. RNA concentration was determined using Nano Drop spectrophotometer (Thermo Fisher Scientific).

cDNA synthesis

First-strand cDNA was synthesized using 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to manufacturer's protocol. Anchored-oligo$(dT)_{18}$ Primer was used in the template-master mix.

PCR Array

Experimental cocktail was prepared as recommended by 'RT^2 Profiler™ PCR Array System Pathway-Focused Gene Expression Profiling Using Real-Time PCR' Protocol. The cycling profile was as below:

Cycles	Duration	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	1 minute	60°C

Real-time quantitations were performed using the Applied Biosystems 7500 Real-Time PCR Systems. Ct values were calculated using the ABI system software.

Real-time PCR

All Real-time PCR reactions were performed in a 10 µl mixture containing 0.4 µl of cDNA, 5 µl of RT^2 SYBR Green / ROX qPCR Master Mix, 4 µl of RNase-free water and 0.4 µl of each primer. The cycling profile was as below:

Cycles	Duration	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	1 minute	60°C

Real-time quantitations were performed using the Applied Biosystems 7500 Real-Time PCR Systems. Ct values were calculated using the ABI system software.

2.4 Protein expression in Berberine-induced apoptotic cells

2.4.1 Chemicals and Reagents

Acrylamide, ammonium persulfate, bis-acrylamide, glycerol, glycine, hydrochloric acid (HCl), potassium chloride, sodium chloride, Rodeo™ ECL Western Blotting Reagents, TEMED and tris base were from USB, Cleveland, USA. Bromophenol blue, calcium chloride, sodium dodecyl sulfate (SDS), sodium azide, sodium vanadate (V), Tween-20 and β-mercaptoethanol were from Sigma chemicals, St. Louis, USA. BSA standard and DC Protein Assay were from Bio-rad. Methanol was from BHD. Non-fat milk powder was from Nestle. Primary antibodies used were listed below: GAPDH was ordered from Applied Biosystems. Bid was from BD Biosciences. Caspase-7, caspase-9, cleaved PARP, p53, and PARP were from cell signaling. Bcl-2, MDM2 and PCNA were from Santa Cruz. As for secondary antibodies, goat anti-mouse IgG (H+L) - HRP (Zymed®) was from Invitrogen, while anti-rabbit IgG, HRP-linked antibody was from Cell Signaling. PVDF membrane was from Pall. Rodeo™ ECL Western Blotting Reagents were from USB.

2.4.2 Preparation of solution

The solutions used in protein expression studies were prepared as described below:

1.5M Tris-HCl (pH 6.8)	Tris base:	18.171g/ 100 ml
	pH was adjusted to 6.8 with HCl.	
10% Ammonium persulfate*	Ammonium persulfate:	0.1g/ ml
10% SDS	SDS:	1g/ ml
1M Tris-HCl (pH 8.8)	Tris base:	12.114g/ 100ml
	pH was adjusted to 8.8 with HCl.	
10X SDS running buffer	Tris Base:	30.2g/ L
	Glycine:	188g/ L
	10% SDS:	100ml/ L
10X Tris-buffered saline (TBS)	Sodium chloride:	88g/ L
	Potassium chloride:	2g/ L
	Tris base:	3g/ L
1X Tris-buffered saline/ Tween	10X TBS:	100 ml/ L
(TBST)	Tween-20:	1 ml/ L
2X SDS sample buffer	1M Tris (pH 6.8):	10ml/ 100ml
	10% SDS:	40ml/ 100ml
	Bromophenol blue:	0.2%

	Glycerol:	20ml/ 100ml
	β-mercaptoethanol:	15µl/ 1ml
30% Acrylamide/ Bis*	Acrylamide:	29g/ 100ml
	Bis-acrylamide:	1g/ 100ml
Blocking solution (5%)	Non-fat milk powder:	0.2g/ 4ml
	Sodium azide:	0.05%
BSA standard (4mg/ml)**	BSA standard:	4mg/ 1ml whole cell lysis buffer
Resolving gel 12%	Milli-Q water:	1.28 ml
	1.5M Tris (pH 8.8):	1.04 ml
	30% Acrylamide/ Bis:	1.6 ml
	10% SDS:	40 µl
	10% Ammonium persulfate:	40 µl
	TEMED:	5 µl
Stacking gel 4%	Milli-Q water:	1.48 ml
	0.5M Tris (pH 6.8):	1.25 ml
	30% Acrylamide/ Bis:	0.335 ml
	10% SDS:	25 µl
	10% Ammonium persulfate:	12.5 µl
	TEMED:	5 µl

Transfer buffer	Methanol:	200ml/ L
	Glycine:	2.93g/ L
	Tris:	5.82g/ L
	10% SDS:	3.75ml/ L
Whole cell protein lysis buffer	SDS:	1%
	Sodium vanadate (V):	1mM
	Tris:	10mM
	Calcium chloride:	5mM
	pH was adjusted to 7.4 with HCl.	

Remark:

Solutions marked with * were stored at 4°C.

Solutions marked with ** were stored at -20°C.

2.4.3 Procedures

Cell treatment

Huh7 and JHH4 cells were seeded onto 100mm culture dish at the concentration of 1 x 10^6 overnight. Cells were treated with different concentration for 72 hours respectively. After berberine treatment, cells were harvested with trypsin and were centrifuged at 1000 rpm for 3 minutes.

Western Blot Analysis

Cells were grown to 60-80% confluence in 100mm dishes followed by 72-hour treatment of different concentration of berberine. Whole cell lysates were extracted using whole cell lysis buffer. The protein concentration was determined by the DC Protein Assay (Bio-Rad) using BSA as the standard. 30μg of protein was separated by SDS-PAGE and transferred for an hour onto a PVDF membrane (Pall). The blots were blocked with blocking solution (5%) for an hour, and were probed with a specific primary antibody with 5% non-fat milk powder in TBST at 4°C overnight. After washing with TBST, the blots were treated with the specific HRP-linked secondary antibody for an hour and were washed several times. Proteins were detected by Rodeo™ ECL Western Blotting Reagents (USB, Cleveland, USA) according to manufacturer's protocol.

2.5 Caspase cascade studies in berberine-induced apoptosis

2.5.1 Chemicals and reagents

Apo-ONE® Homogeneous Caspase-3/7 Assay was purchased from Promega. The assay consisted of 100μl Caspase Substrate Z-DEVD-R110 (100X) and 10ml Apo-ONE® Homogeneous Caspase-3/7 Buffer.

2.5.2 Procedures

Huh7 or JHH4 cells were seeded onto 96-well plate at the concentration of 1 x 10^6 overnight. Cells were treated with different concentration of berberine for 72 hours respectively. After berberine treatment, Apo-ONE® Homogeneous Caspase-3/7 assay was performed as recommended by 'Promega Technical Bullentin- Apo-ONE® Homogeneous Caspase-3/7 Assay Instructions for use of products G7790, G7791 AND G7792'. The plate was incubated and shaken at room temperature for 2 hours. The fluorescence of each well was measured (excitation wavelength: 485nm; emission wavelength: 520nm).

2.6 Cell cycle study in berberine-induced apoptotic cells

2.6.1 Chemicals and Reagents

DPBS was purchased from Invitrogen. Ethanol was purchased from BDH. Propidium Iodide (PI) and Ribonuclease A from bovine pancreas (RNase A) were ordered from Sigma chemicals. St. Louis, USA. 5ml polystyrene round-bottom tubes were ordered from BD Falcon.

2.6.2 Preparation of solutions

PI of 40 μg/ml was prepared with 100 μg/ml RNase A and was stored at -20°C in dark. 70% (w/v) Ethanol was stored at -20°C.

2.6.3 Procedures

Cell treatments

Huh7 or JHH4 Cells were grown to 60-80% confluence on 100mm culture dishes. Different concentration of berberine was added to the cells for 24, 48 and 72 hours.

Fixing of cells

Cells were harvested with trypsin after berberine treatment. Cells were washed with cold DPBS twice and were centrifuged at 300 x g for 5 minutes. Next, cells were re-suspended in cold 70% (w/v) ethanol at a concentration of 1 x 10^6 cells per ml.

Cells were suspended in ethanol at -20°C for 24 hours. After 24 hours, cells were centrifuged at 300 x g for 5 minutes. Ethanol was discarded and cell pellets were washed by cold DPBS.

Staining of cells

After the cold DPBS wash, cells were re-suspended with 400 µl of PI solution and transferred to a 5ml polystyrene round-bottom tube. The tube was incubated for 15 minutes at 25°C in dark. Tubes were analyzed by BD FACSCanto flow cytometer within 1 hour.

Data analysis

FACSDiva software was used to operate BD FACS Canto Flow Cytometer. Experimental data were captured and were analyzed by FlowJo 7.6.1. The percentage of populations in G_1, G_2 and S phases of cell cycle was recorded. The experiment was performed three times. The ratio of cells in the G_0/G_1, intra-S, and G_2/M phases were expressed as mean ± SD.

Chapter 3 Results

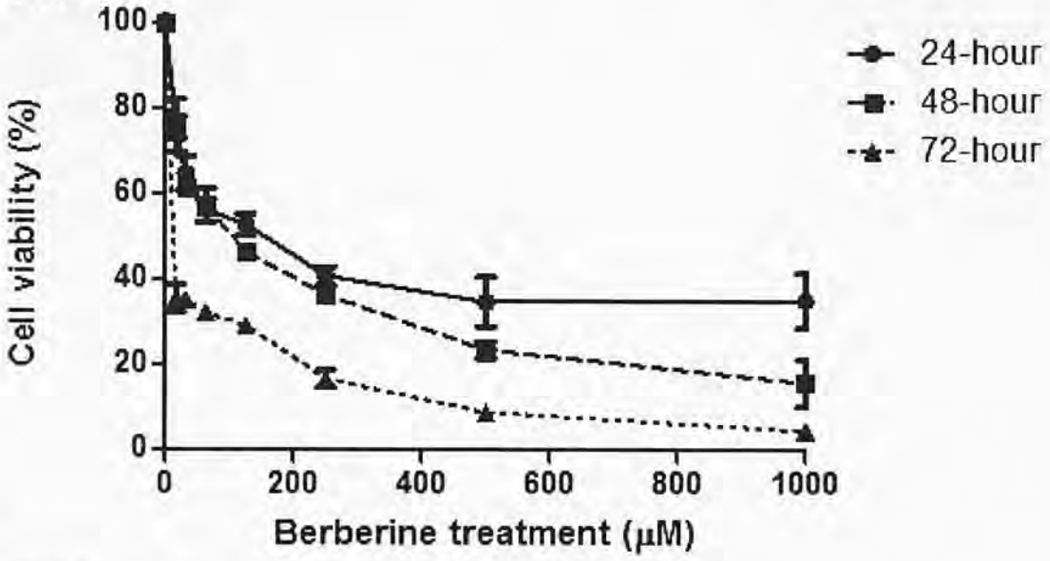
3.1 Berberine induces apoptosis in hepatocellular cells

Effects of berberine on hepatocellular carcinoma (HCC) cell lines were evaluated by the MTT assay. HCC cell lines used for the study were Huh7 and JHH4, while normal liver cell line WRL68 was used as a control experiment. The results indicate that berberine showed inhibitory effect towards the growth of HCC cells.

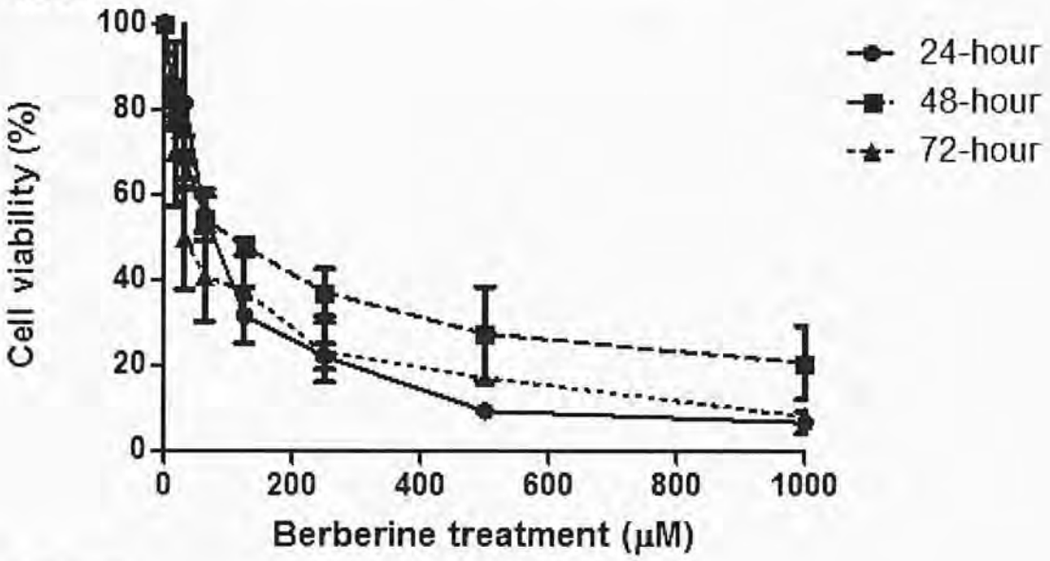
Apoptosis in HCC cell lines were detected by BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. FITC Annexin V is often used together with propidium iodide (PI) to allow the identification of early apoptotic cells, which yield PI negative and FITC Annexin V positive signals (Koopman G et al., 1994).

The treatment concentration in FITC Annexin V-PI co-staining for Huh7 cells was 0, 5, 10, and 20 μM. $IC_{50,\ 72h}$ (half maximal inhibitory concentration at 72 hours) of berberine in Huh7 was 10 μM. An intermediate dose of 5 μM, and a higher dose of 20 μM, was used for comparing effects of berberine in Huh7 cells at different concentration. For JHH4, the treatment concentration was 0, 15, 30, and 60 μM. $IC_{50,\ 72h}$ of berberine in JHH4 was 30 μM. An intermediate dose of 15 μM, and a higher dose of 60 μM, was used for comparing effects of berberine in JHH4 cells at different concentration. The results demonstrate that berberine caused apoptosis in HCC cells.

A: Huh7



B: JHH4



C: WRL68

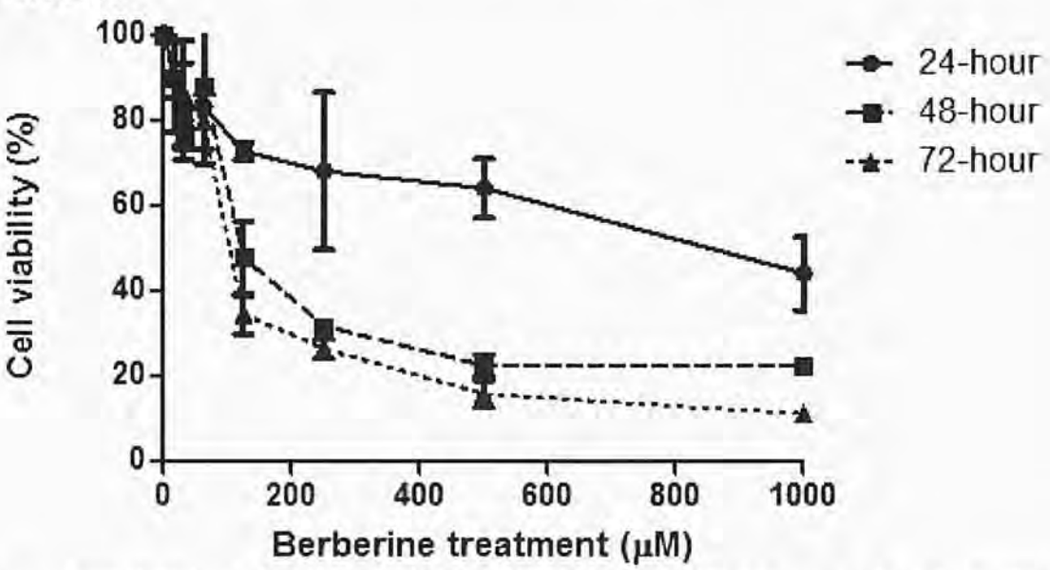


Figure 2 Viabilities of (A) Huh7, (B) JHH4, and (C) WRL68 treated with berberine for 24, 48 and 72 hours

Evaluation of the cell viability of Huh7, JHH4 and WRL68 cells treated with berberine for 24, 48 and 72 hours

Huh7 cells (Figure 2A), JHH4 cells (Figure 2B) and WRL68 (Figure 2C) cells were incubated with 0-1000 μM berberine for 24, 48 and 72 hours respectively. The percentage of viable cells was determined using the MTT assay as mentioned in Materials and Methods. Control cells were treated with vehicle only. The above experiments were performed in triplicate and the data represent the means ± SD. The IC_{50} for each cell line at each treatment length was recorded in the tables below.

Huh7 (Figure 2A):

Time (hour)	24	48	72
IC_{50} (μM)	150	100	10

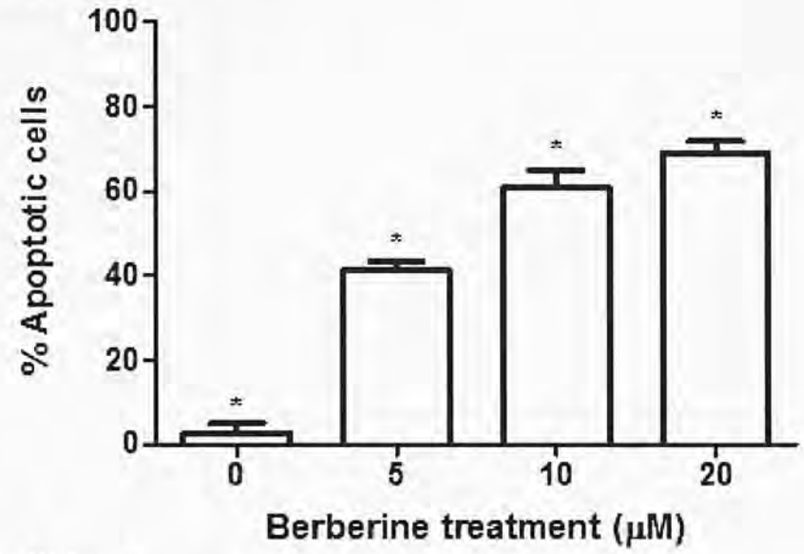
JHH4 (Figure 2B):

Time (hour)	24	48	72
IC_{50} (μM)	105	60	30

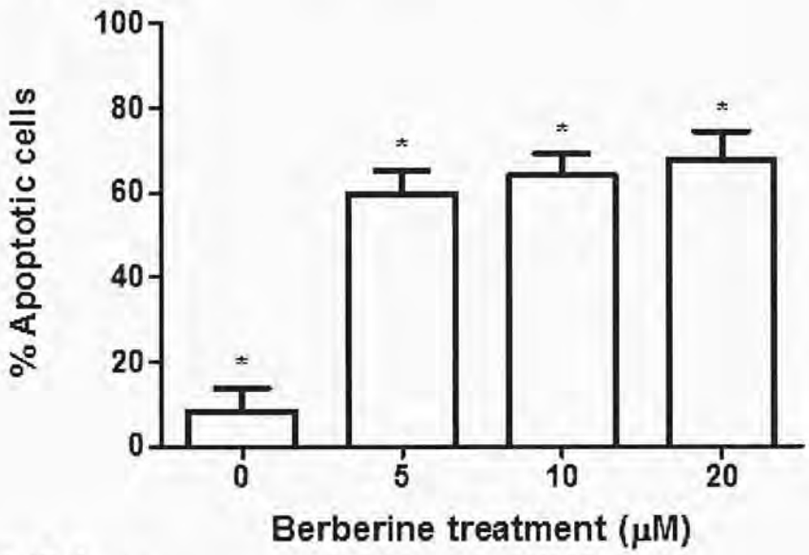
WRL68 (Figure 2C):

Time (hour)	24	48	72
IC_{50} (μM)	1000	120	100

A: 24-hour



B: 48-hour



C: 72-hour

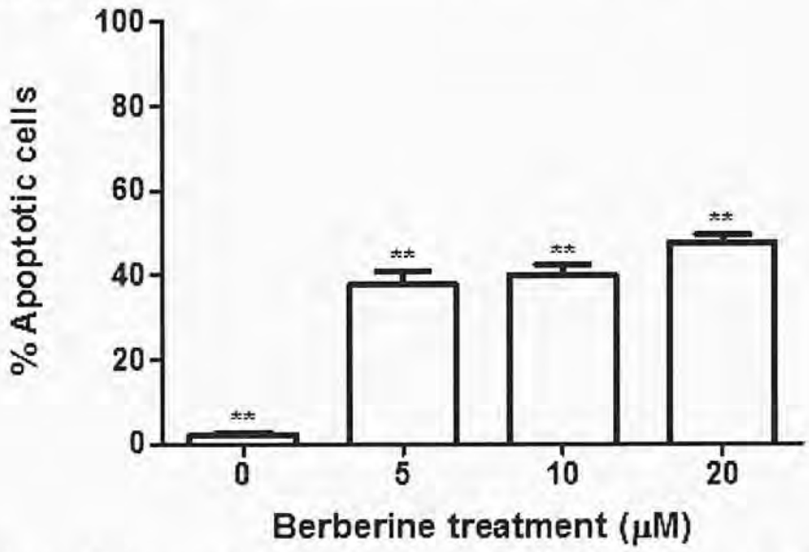


Figure 3 Apoptotic cells after (A) 24-hour, (B) 48-hour, and (C) 72-hour berberine treatment in Huh7 cell line

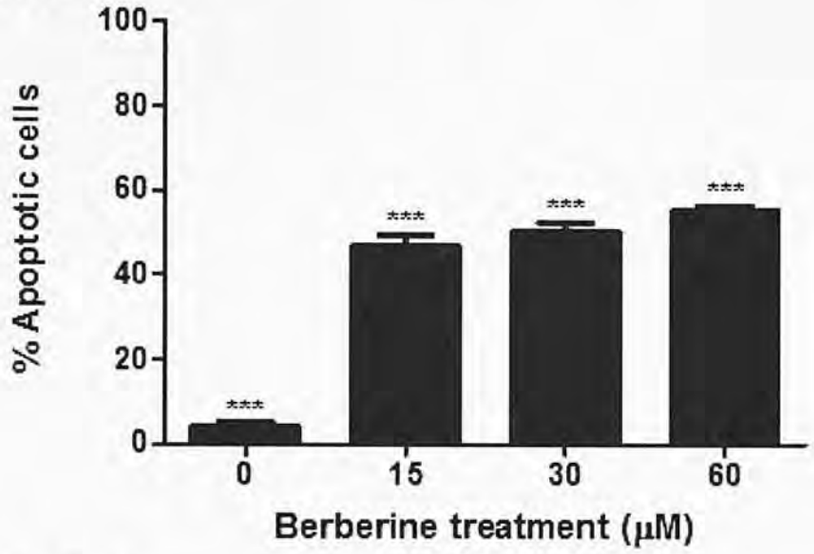
Berberine leads to apoptosis in Huh7 cells

BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I was used to detect apoptosis in Huh7 cells. The experiment was done according to manufacturer's protocol with modifications (Chapter 2.2).

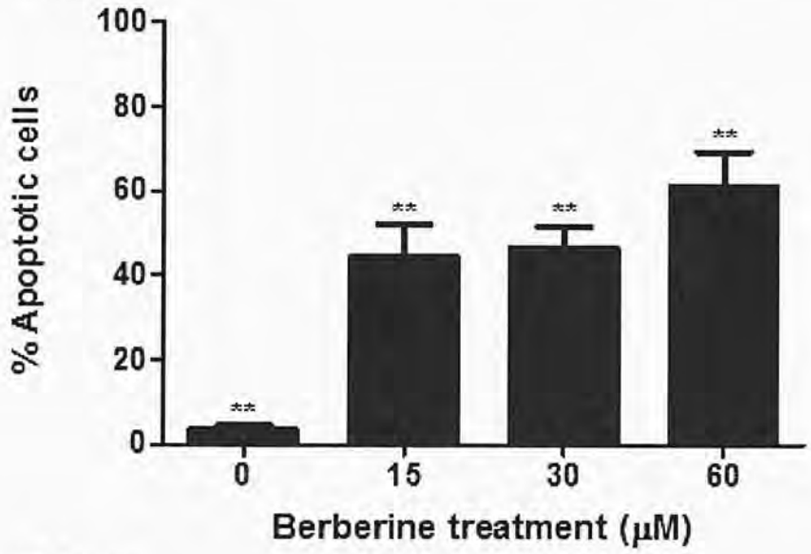
Huh7 cells were seeded into 6-well plates. Cells were treated with Berberine at 0, 5, 10 and 20 μM for 24, 48, and 72 hours respectively. The results with triplicate experiments (* $P < 0.05$; ** $P < 0.01$) were shown in Figure 3.

For Huh7 cells treated with berberine for 24 hours (Figure 3A), the percentage of apoptotic cells increased along with the increase in berberine concentration. For Huh7 cells treated with berberine for 48 hours and 72 hours (Figure 3B and Figure 3C), the treated cells showed a significant increase in the percentage of apoptotic cells compared with the untreated cells.

A: 24-hour



B: 48-hour



C: 72-hour

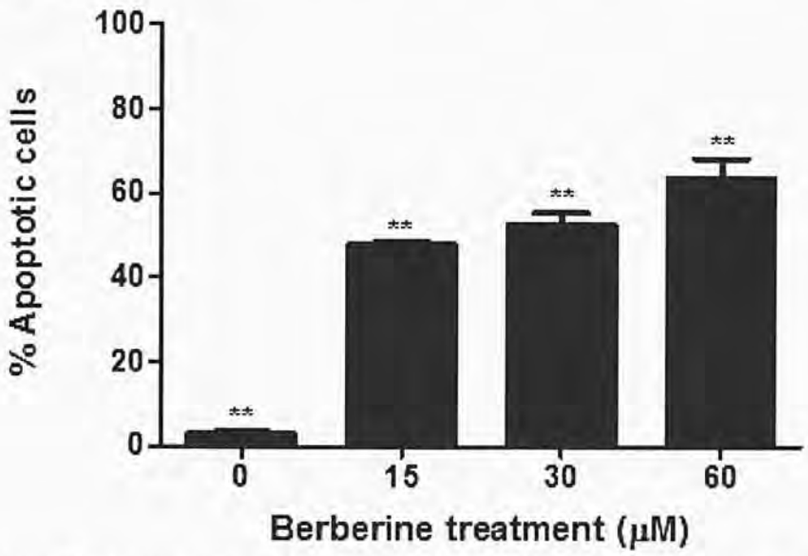


Figure 4 Apoptotic cells after (A) 24-hour, (B) 48-hour, and (C) 72-hour berberine treatment in JHH4 cell line

Berberine leads to apoptosis in JHH4 cells

Cells were treated with Berberine at 0, 15, 30 and 60 μM for 24, 48, and 72 hours respectively. The results with triplicate experiments (** $P < 0.01$; *** $P < 0.001$) were shown in Figure 4.

For 24-hour (Figure 4A), 48-hour (Figure 4B), and 72-hour (Figure 4C) berberine treatment, the treated cells showed a significant increase in the percentage of apoptotic cells compared with the untreated cells.

3.2 Gene expression in Berberine-induced apoptotic cells

Gene expression in HCC cell lines were studied by PCR array and real-time PCR. PCR array was used to profile the gene expression in Huh7 cells. Real-time PCR was used as a quantitative experiment to study the gene expression in berberine-treated HCC cells.

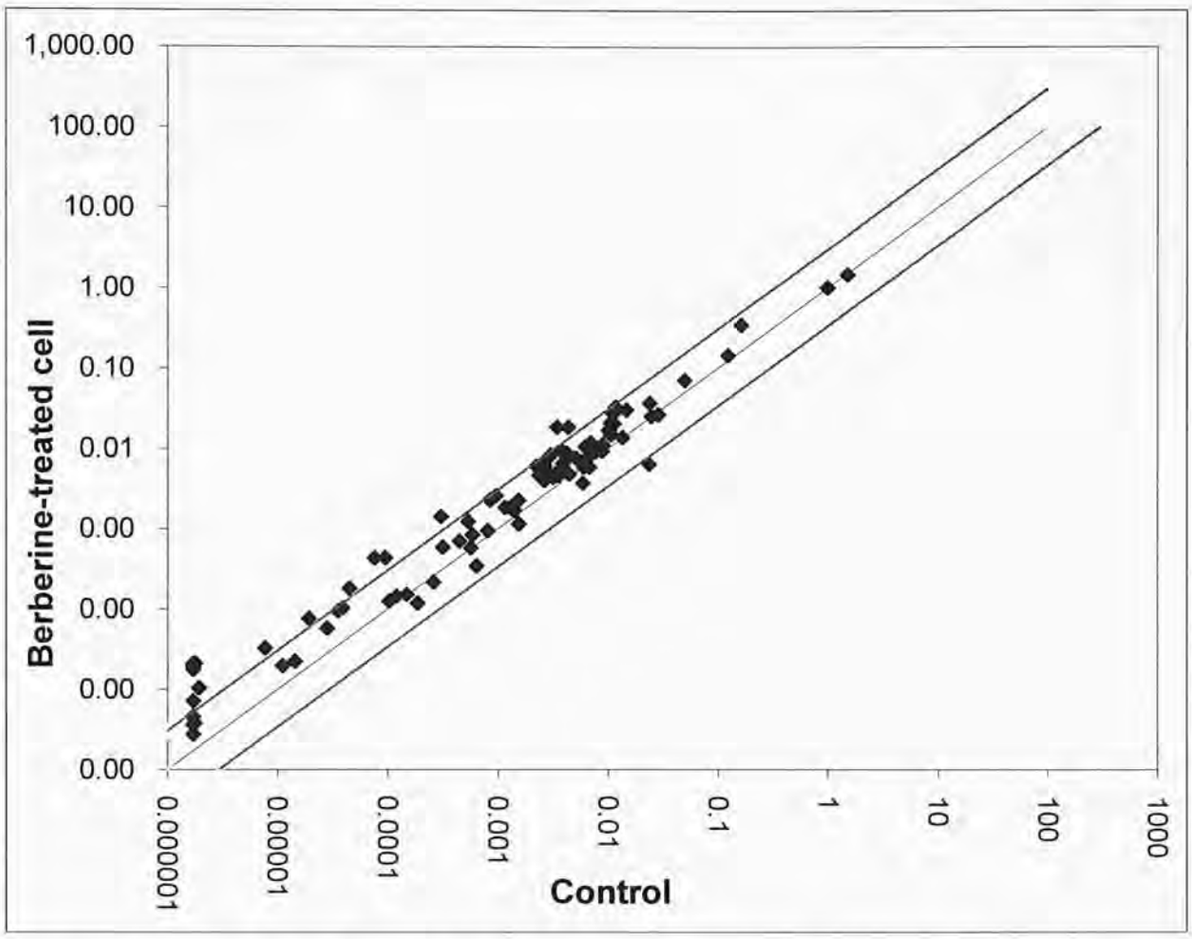


Figure 5 Apoptosis PCR Array profiled Up- or Down- Regulated Genes in Huh7 cells upon 72-hour berberine treatment

Apoptosis PCR Array profiled apoptotic genes in various apoptotic signaling pathways. Experimental Huh7 cells were treated with 10 μM berberine for 72 hours, and control Huh7 cells were treated with vehicle for 72 hours. Total RNA from untreated Huh7 cell and berberine-treated Huh7 cell were characterized. cDNA was synthesized from the total RNA as mentioned in Chapter 2.3.2. The relative expression levels for each gene in both samples were plotted against each other in the Scatter Plot using the program provided by the manufacturer. The middle line indicates fold changes ((2^(-Δ Ct)) of 1. The left and right lines indicate the fold-change in gene expression threshold, which was defined at 3-fold. Outliers of the

left line were genes up-regulated by at least three-fold, which included BCL2, BCL2L1, CASP14, CD40, CIDEA, FASLG, GADD45A, HRK, LTA, TNFRSF10A, TNFRSF10B, CD27, TNFRSF9, and CD70. The outliner of the right line was BCLAF1, which was down-regulated by at least three-fold.

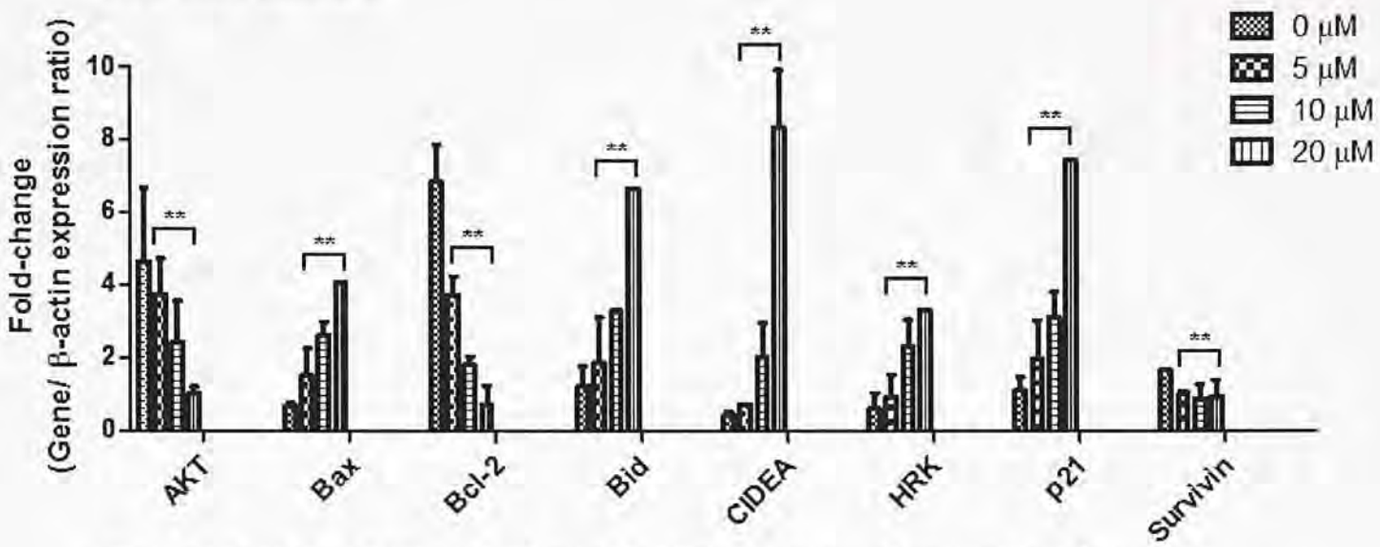


Figure 6 Real-time PCR result showed berberine regulated expression of genes in Huh7 cells in a dose-dependent manner

Total RNA from untreated Huh7 cells and berberine-treated Huh7 cells were characterized. Cells were treated with berberine at different concentration for 72 hours. After 72 hours, total RNA was extracted and cDNA was synthesized from the total RNA. Real-time PCR was done according to the procedure mentioned in Chapter 2.3.2. Bax, Bid, CIDEA, HRK, and p21 were found to be up-regulated by berberine in a dose-dependent manner, while AKT and Bcl-2 were found to be down-regulated by berberine in a dose-dependent manner. The gene expression of Survivin decreased along with the increase in berberine concentration. The data represent the mean ± SD of three individual experiments (** $P < 0.01$).

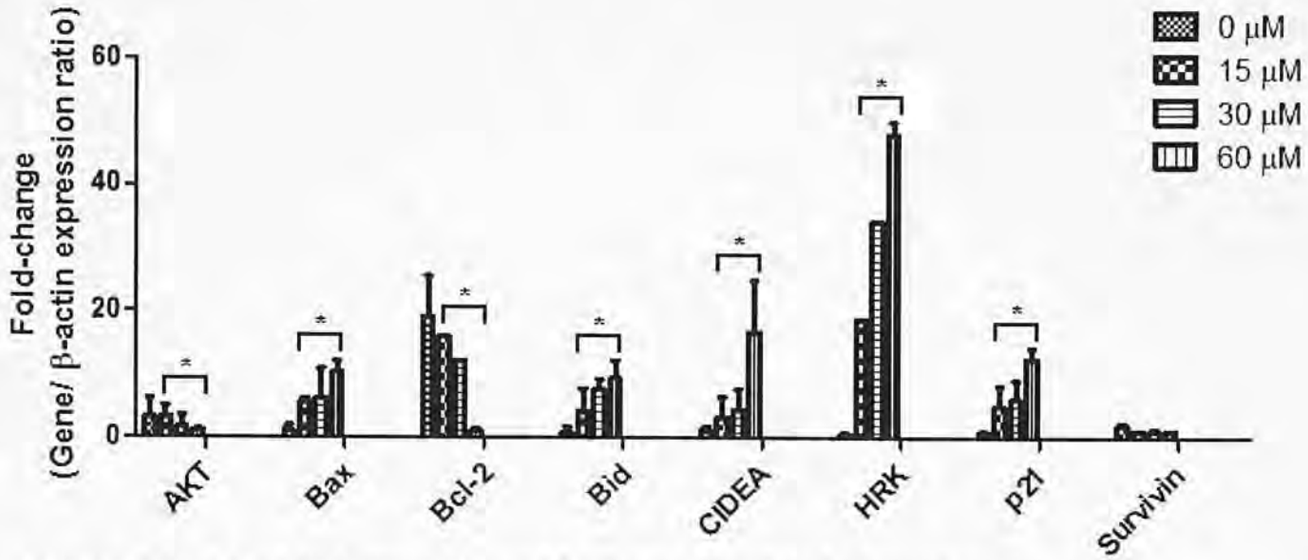


Figure 7 Real-time PCR result showed berberine regulated expression of genes in JHH4 cells in a dose-dependent manner

Total RNA from untreated JHH4 cells and berberine-treated JHH4 cells were characterized. Cells were treated with berberine at different concentration for 72 hours. After 72 hours, total RNA was extracted and cDNA was synthesized from the total RNA. Real-time PCR was done according to the procedure mentioned in Chapter 2.3.2. Bax, Bid, CIDEA, HRK, and p21 were found to be up-regulated by berberine in a dose-dependent manner, while AKT and Bcl-2 were found to be down-regulated by berberine in a dose-dependent manner. An increase in berberine concentration did not affect the gene expression of Survivin. The data represent the mean ± SD of three individual experiments (* $P < 0.05$).

3.3 Caspase cascade studies in berberine-induced apoptosis

Apo-ONE ® Homogeneous Caspase-3/7 Assay Kit was used to measure caspase3/7 activities of the HCC cells after Berberine treatment. The assay measured the non-fluorescent caspase substrate Z-DEVD-R110 that is cleaved by Caspase-3/7, producing the fluorescent Rhodamine 110. Fluorescence generated in the experiment is proportional to the amount of caspase-3/7 cleavage activity present in the cultured sample.

Protein members in caspase cascade were studied by SDS-PAGE and Western Blot Analysis. For Huh7, treatment concentration was 0, 5, 10, and 20 μM. $IC_{50,\ 72h}$ of berberine in Huh7 was 10 μM. An intermediate dose of 5 μM, and a higher dose of 20 μM, was used for comparing effects of berberine in Huh7 cells at different concentration. For JHH4, treatment concentration was 0, 15, 30, and 60 μM. $IC_{50,\ 72h}$ of berberine in JHH4 was 30 μM. An intermediate dose of 15 μM, and a higher dose of 60 μM, was used for comparing effects of berberine in JHH4 cells at different concentration.

In the Western Blot Analysis, GAPDH was used as the control protein. Intensities of bands were analyzed by Image J. Normalization of GAPDH was included in the analysis.

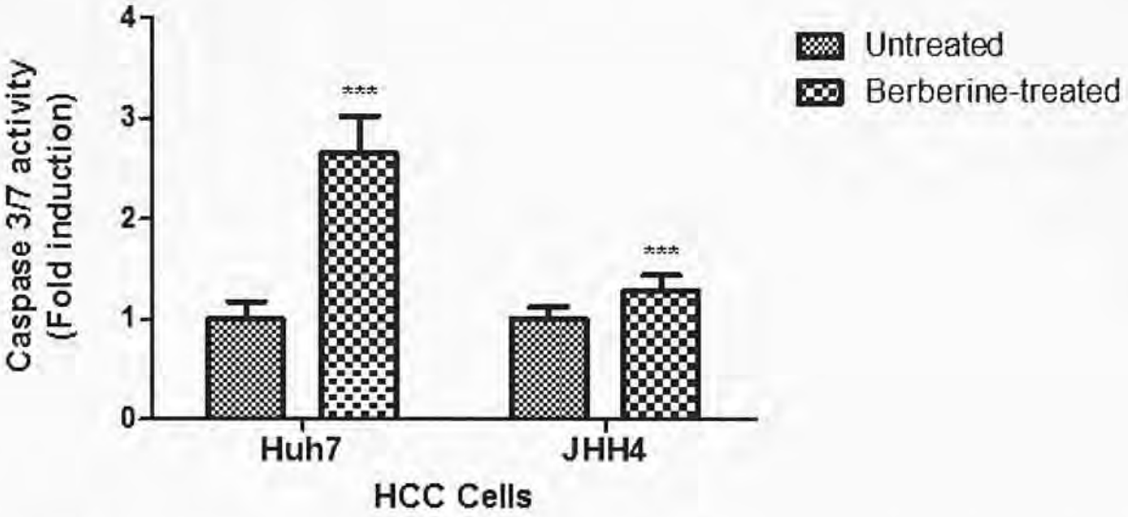


Figure 8 Caspase-3/7 activities in Huh7 and JHH4 cells
Evaluation of caspase3/7 activities of Huh7 in JHH4 cells treated with berberine for 72 hours

Apo-ONE ® Homogeneous Caspase-3/7 Assay Kit was used to measure caspase3/7 activities of the HCC cells after Berberine treatment. Huh7 or JHH4 cells were seeded into 96-well plates. Cells were either remained untreated or treated with Berberine at 30 μM for 72 hours. The treatment dose was designated as 30 μM because it was the $IC_{50,\ 72h}$ in JHH4 cells. After treatment, caspase substrate Z-DEVD-R110 was added according to the assay kit protocol. The fluorescence was measured with the excitation wavelength at 495nm, and the emission wavelength at 595nm.

The data represent the mean ± SD of three individual experiments (*** $P < 0.001$).

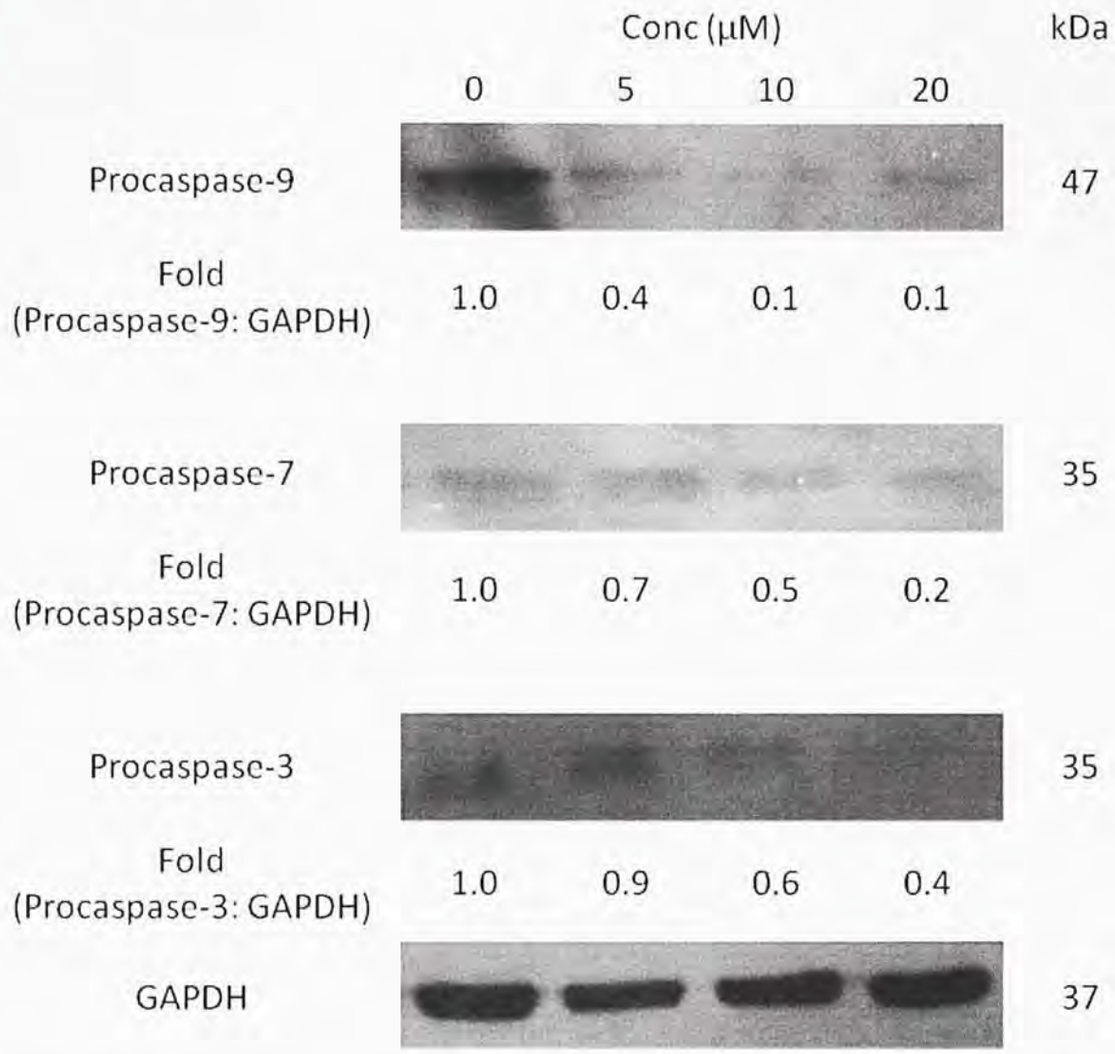


Figure 9 Western Blot Analysis of Procaspase-9, Procaspase-7, and Procaspase-3 in Huh7 cells after 72-hour berberine treatment at different concentration

GAPDH was used as the control protein. Intensities of bands were analyzed by Image J. Figure 9 showed the representative experimental results of three experiments. The results suggest the expression of procaspase-9, and its effector caspases, procaspase-7 and procaspase-3 in Huh7, was decreased along with the increase in berberine concentration.

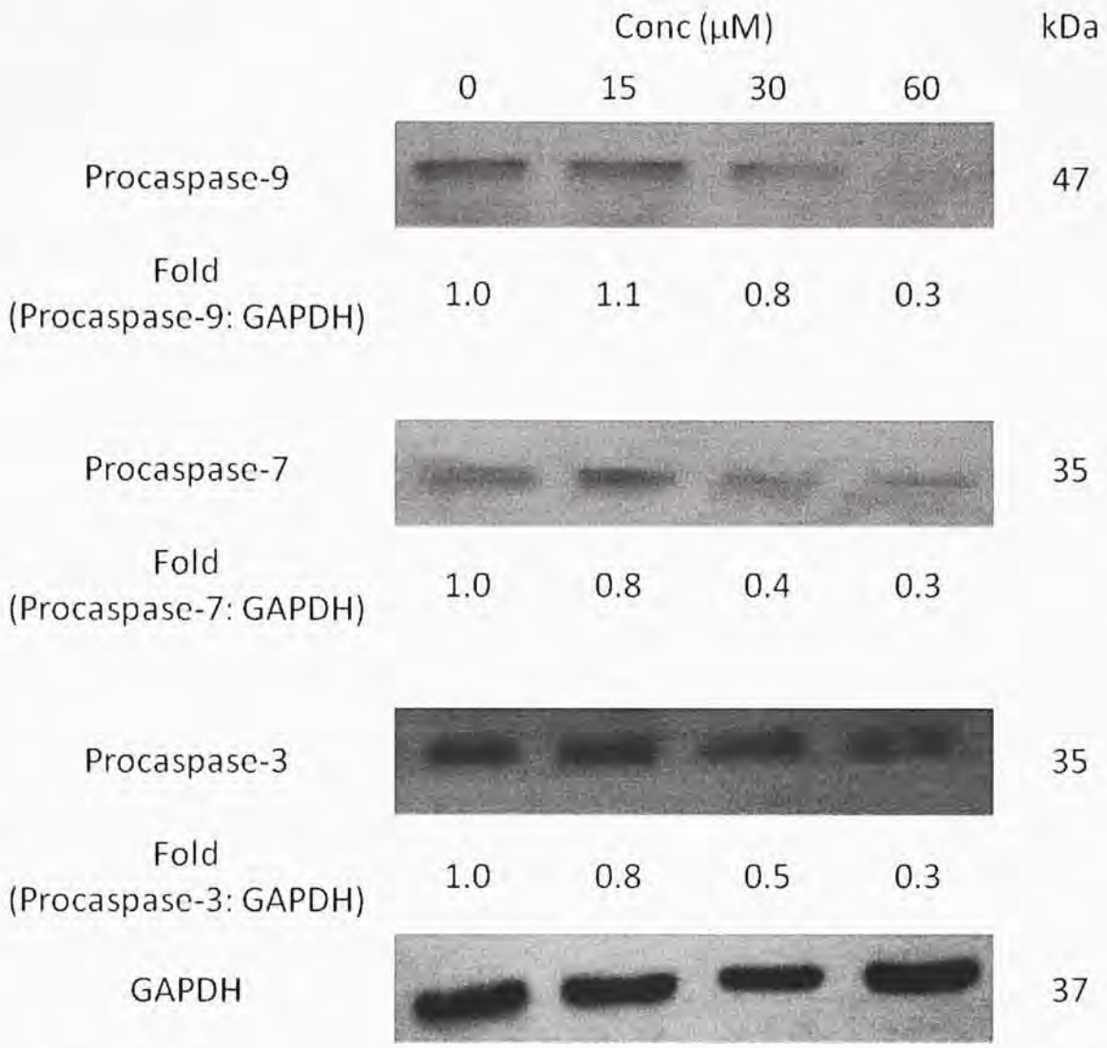


Figure 10 Western Blot Analysis of Procaspase-9, Procaspase-7, and Procaspase-3 in JHH4 cells after 72-hour treatment at different concentration

GAPDH was used as the control protein. Intensities of bands were analyzed by Image J. Figure 10 showed the representative experimental results of three experiments. The results show the expression of procaspase-9, and its effector caspases, procaspase-7 and procaspase-3 in JHH4, was decreased along with the increase in berberine concentration.

3.4 Protein expression in Berberine-induced apoptotic cells

The protein expression was studied in both Huh7 and JHH4 cells. The expression of Bcl-2 protein family members such as Bcl-2 and Bid, and other proteins including PARP and PCNA was studied by SDS-PAGE and Western Blot Analysis.

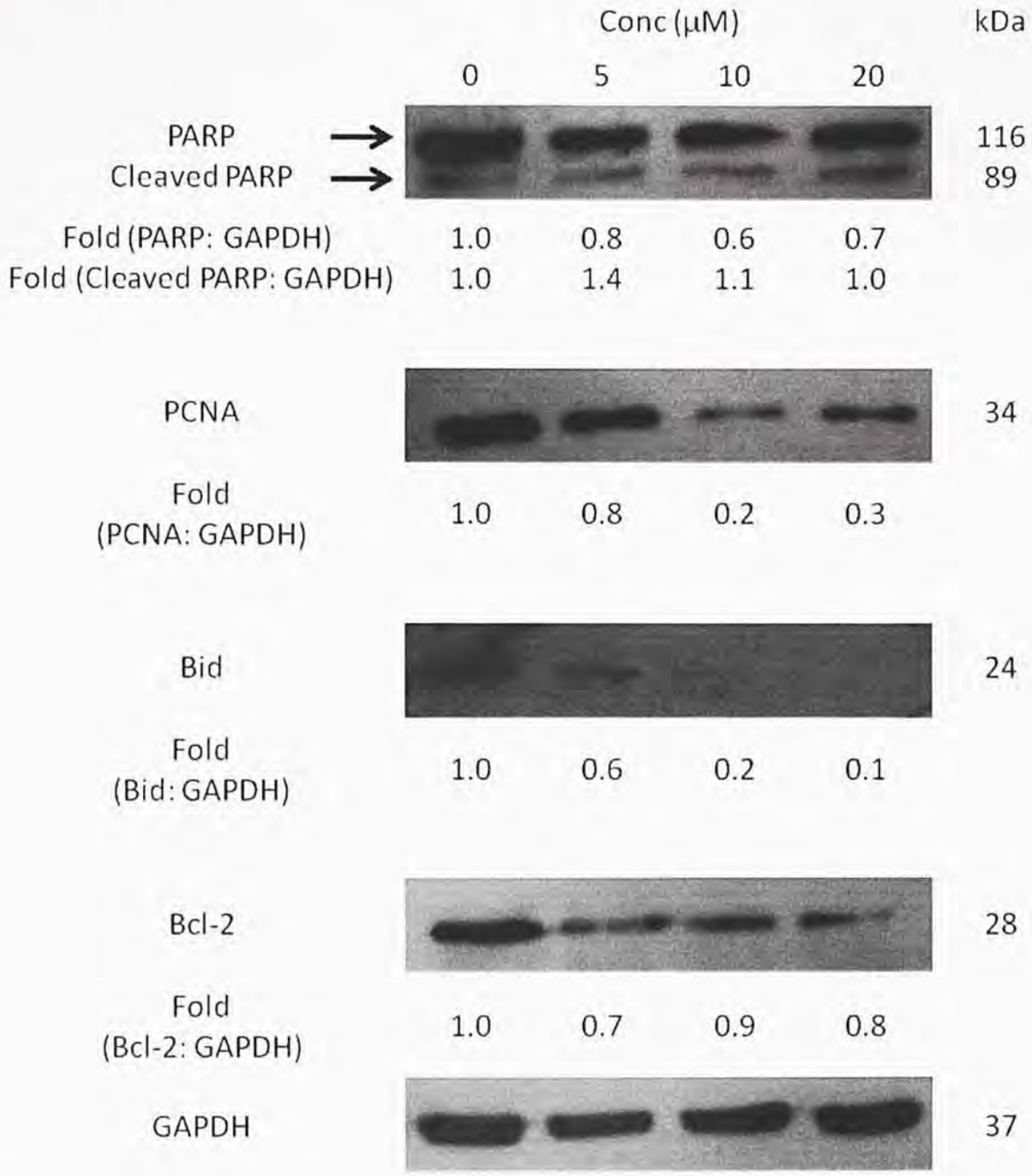


Figure 11 Western Blot Analysis of PARP, cleaved PARP, PCNA, Bid, and Bcl-2 in Huh7 cells after 72-hour berberine treatment at different concentration

GAPDH was used as the control protein. Intensities of bands were analyzed by Image J. Figure 11 showed the representative experimental results of three individual experiments. The results indicate that berberine caused cleavage of PARP, which is an important apoptosis marker, after 72-hour treatment. The expression of PCNA, Bid, and Bcl-2 in Huh7 was decreased along with the increase in berberine concentration.

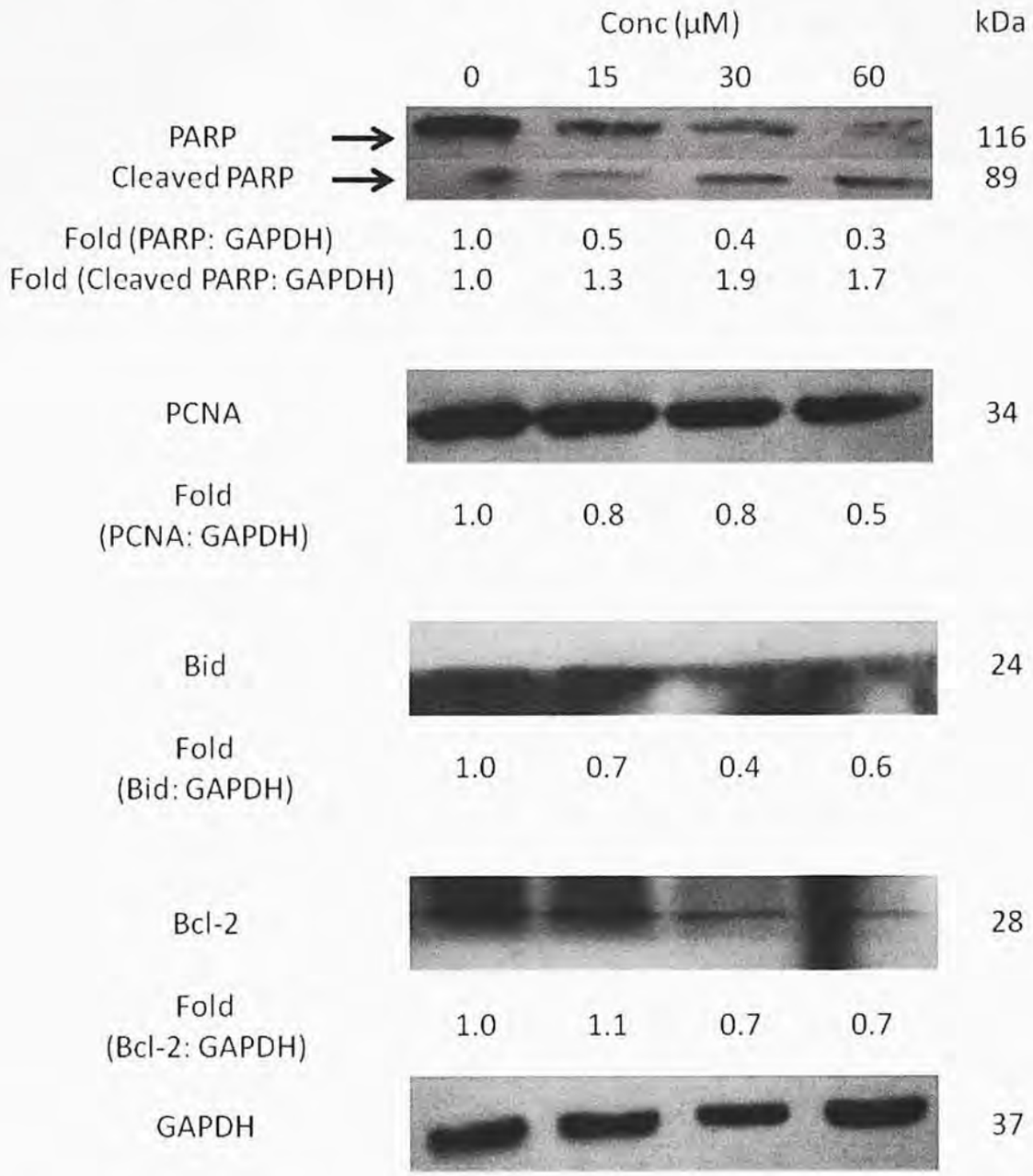


Figure 12 Western Blot Analysis of PARP, cleaved PARP, PCNA, Bid, and Bcl-2 in JHH4 cells after 72-hour berberine treatment at different concentration

GAPDH was used as the control protein. Intensities of bands were analyzed by Image J. Figure 12 showed the representative experimental results of three individual experiments. The results indicate that berberine caused cleavage of PARP, which is an important apoptosis marker, after 72-hour treatment. The expression of PCNA, Bid, and Bcl-2 in JHH4 was decreased along with the increase in berberine concentration.

3.5 Berberine causes G_1 cell cycle arrest in HCC cell lines

Flow cytometry was used to study if berberine could cause cell cycle arrest. The results show that there was an increase of G_1 cell population along with the increase in the concentration of berberine, suggesting that berberine causes G_1 cell cycle arrest in both Huh7 and JHH4 cell lines. The cell cycle was studied at three time points: 24, 48, and 72 hours, respectively.

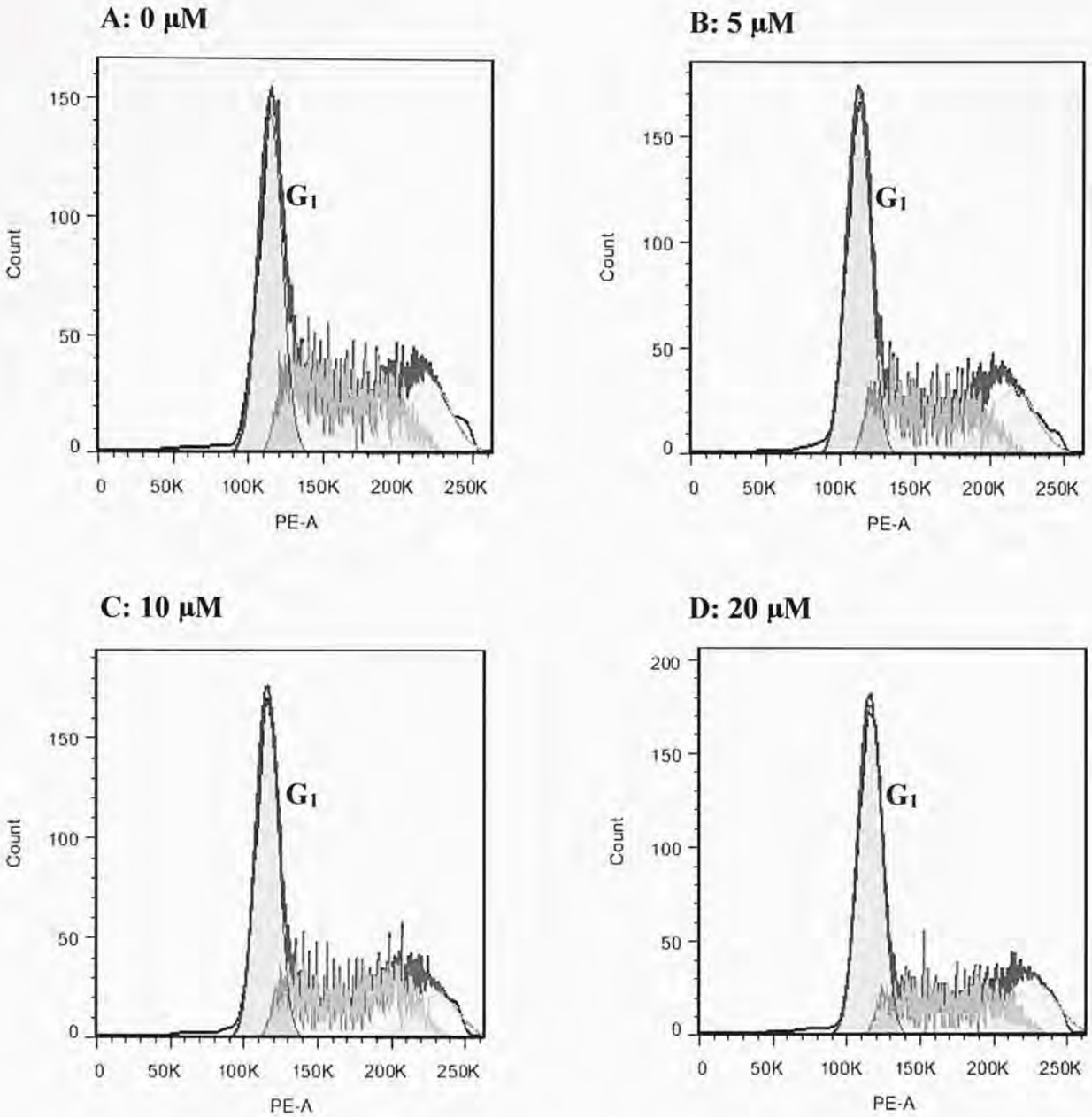


Figure 13 The cell cycle distribution of Huh-7 cells after berberine treatment for 24 hours

Huh-7 cells were treated with different concentration, (A) 0 μM, (B) 5 μM, (C) 10 μM, and (D) 20 μM. Figure 13 showed the representative experimental results of three individual experiments. The results suggest that berberine caused G_1/S cell cycle arrest in Huh-7 cells after 24-hour treatment.

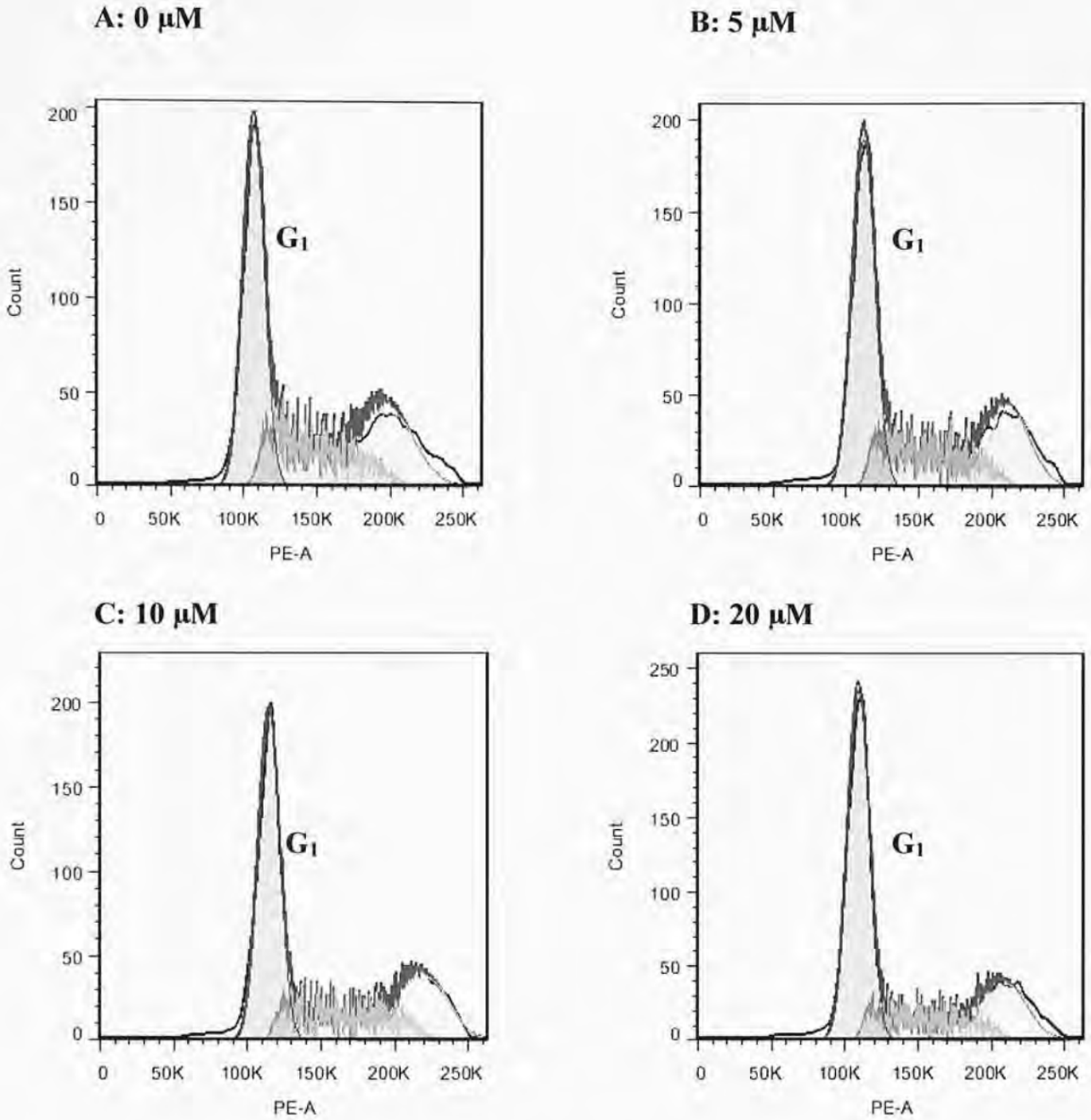


Figure 14 The cell cycle distribution of Huh-7 cells after berberine treatment for 48 hours

Huh-7 cells were treated with different concentration, (A) 0 μM, (B) 5 μM, (C) 10 μM, and (D) 20 μM. Figure 14 showed the representative experimental results of three individual experiments. The results indicate that berberine caused G_1/S cell cycle arrest in Huh-7 cells after 48-hour treatment.

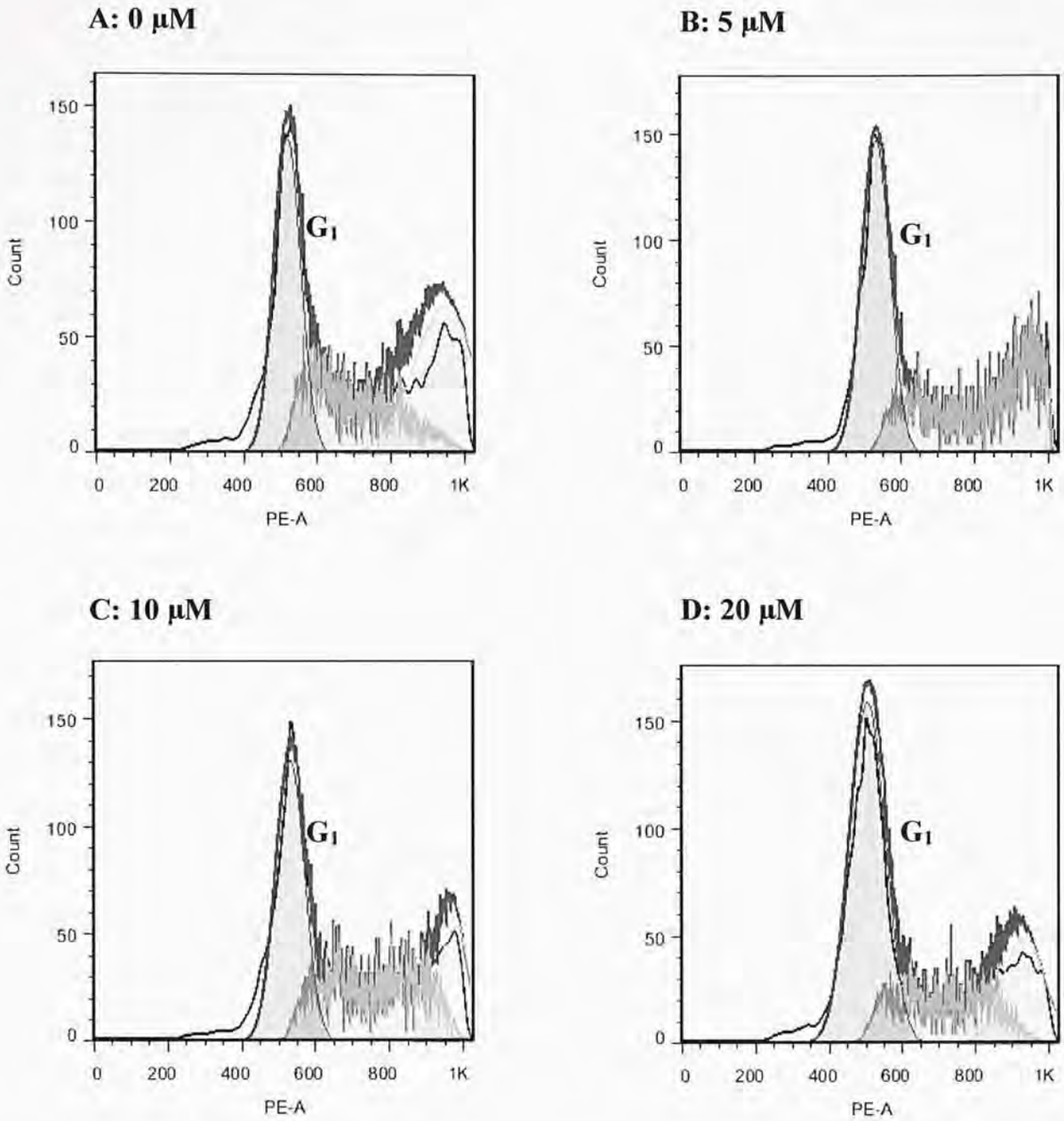


Figure 15 The cell cycle distribution of Huh-7 cells after berberine treatment for 72 hours

Huh-7 cells were treated with different concentration, (A) 0 μM, (B) 5 μM, (C) 10 μM, and (D) 20 μM. Figure 15 showed the representative experimental results of three individual experiments. The results show that berberine caused G_1/S cell cycle arrest in Huh-7 cells after 72-hour treatment.

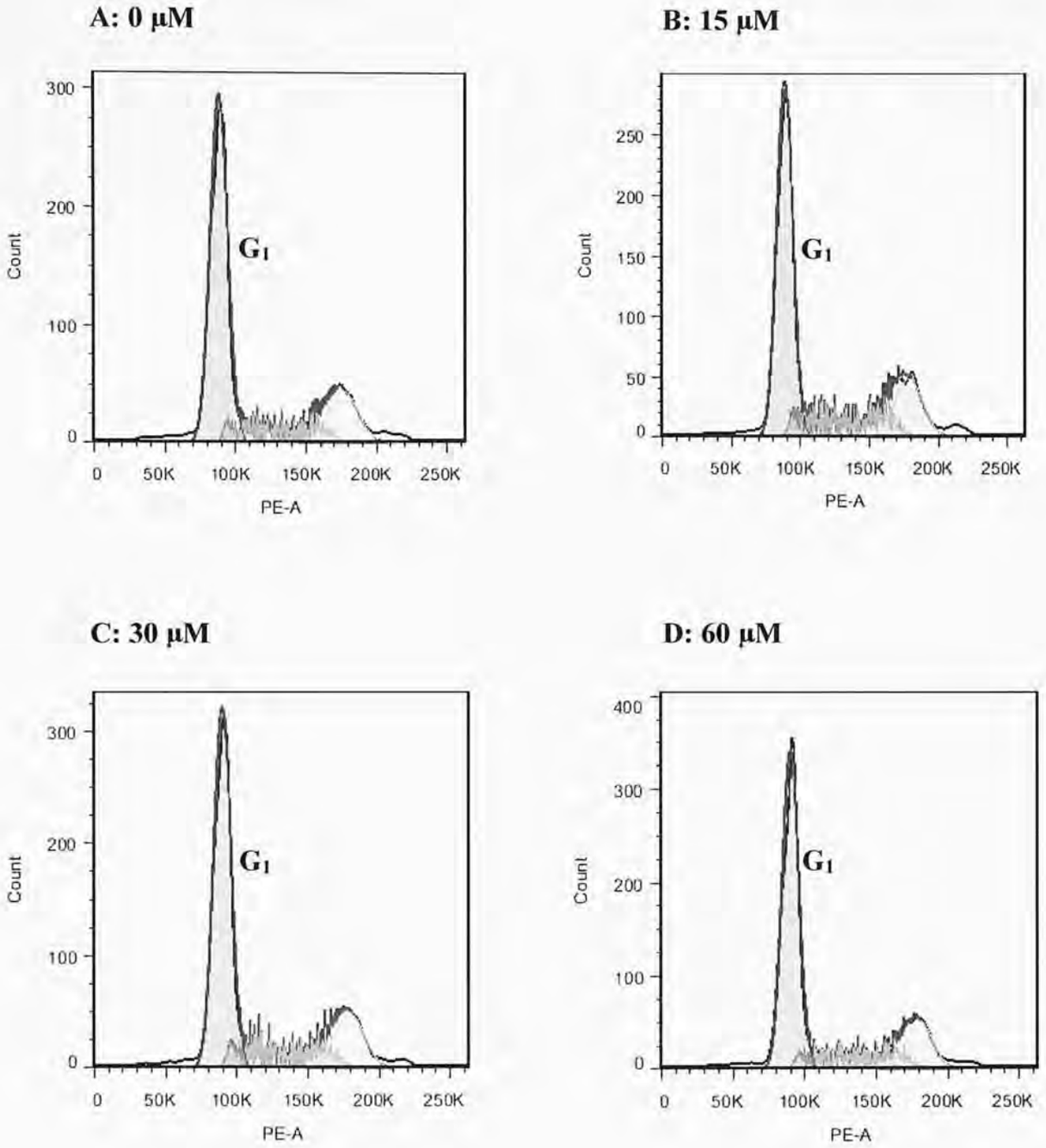


Figure 16 The cell cycle distribution of JHH-4 cells after berberine treatment for 24 hours

JHH-4 cells were treated with different concentration, (A) 0 μM, (B) 15 μM, (C) 30 μM, and (D) 60 μM. Figure 16 showed the representative experimental results of three individual experiments. The results demonstrate that berberine caused G_1/S cell cycle arrest in JHH-4 cells after 24-hour treatment.

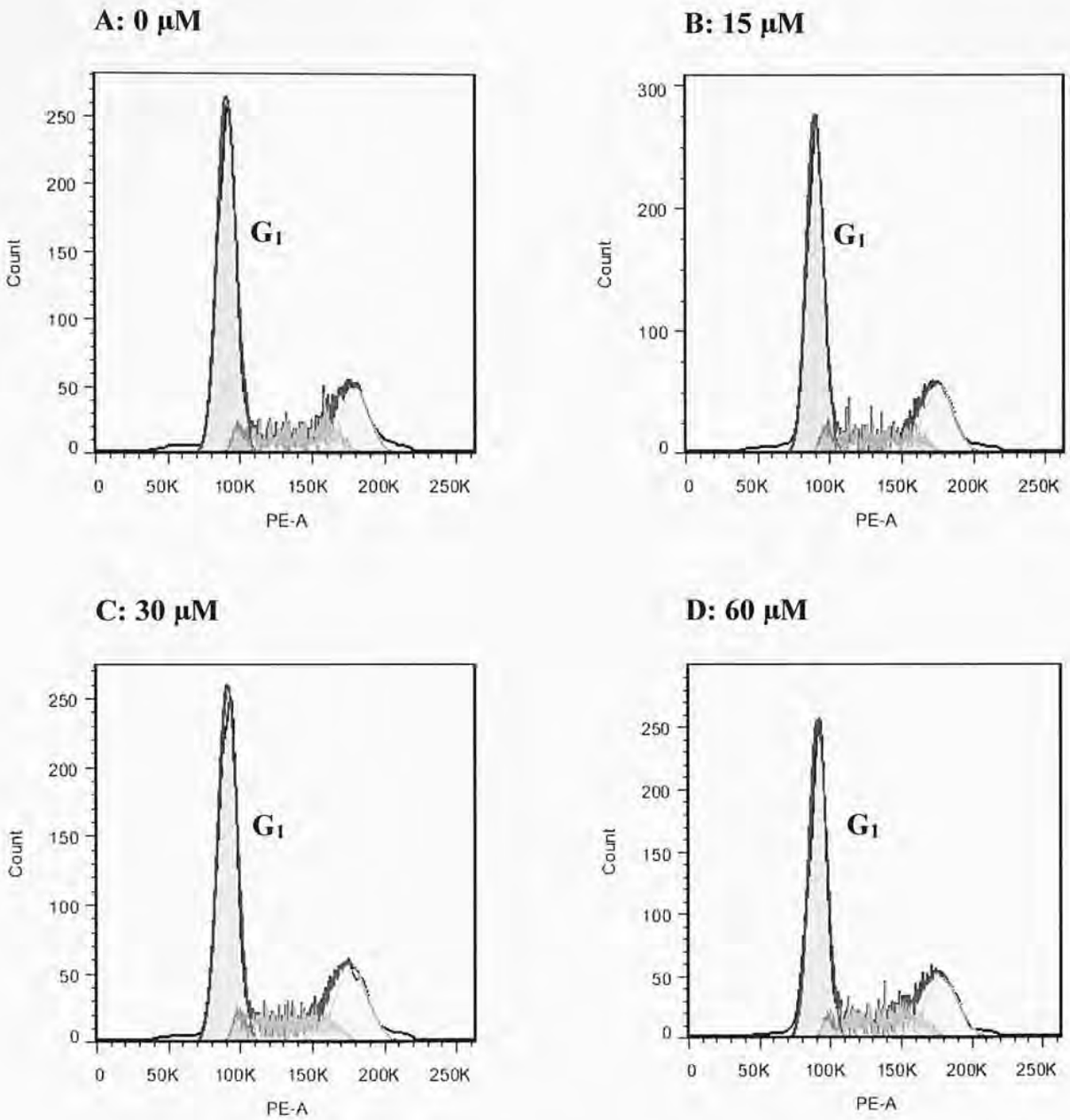


Figure 17 The cell cycle distribution of JHH-4 cells after berberine treatment for 48 hours

JHH-4 cells were treated with different concentration, (A) 0 μM, (B) 15 μM, (C) 30 μM, and (D) 60 μM. Figure 17 showed the representative experimental results of three individual experiments. The results indicate that berberine caused G_1/S cell cycle arrest in JHH-4 cells after 48-hour treatment.

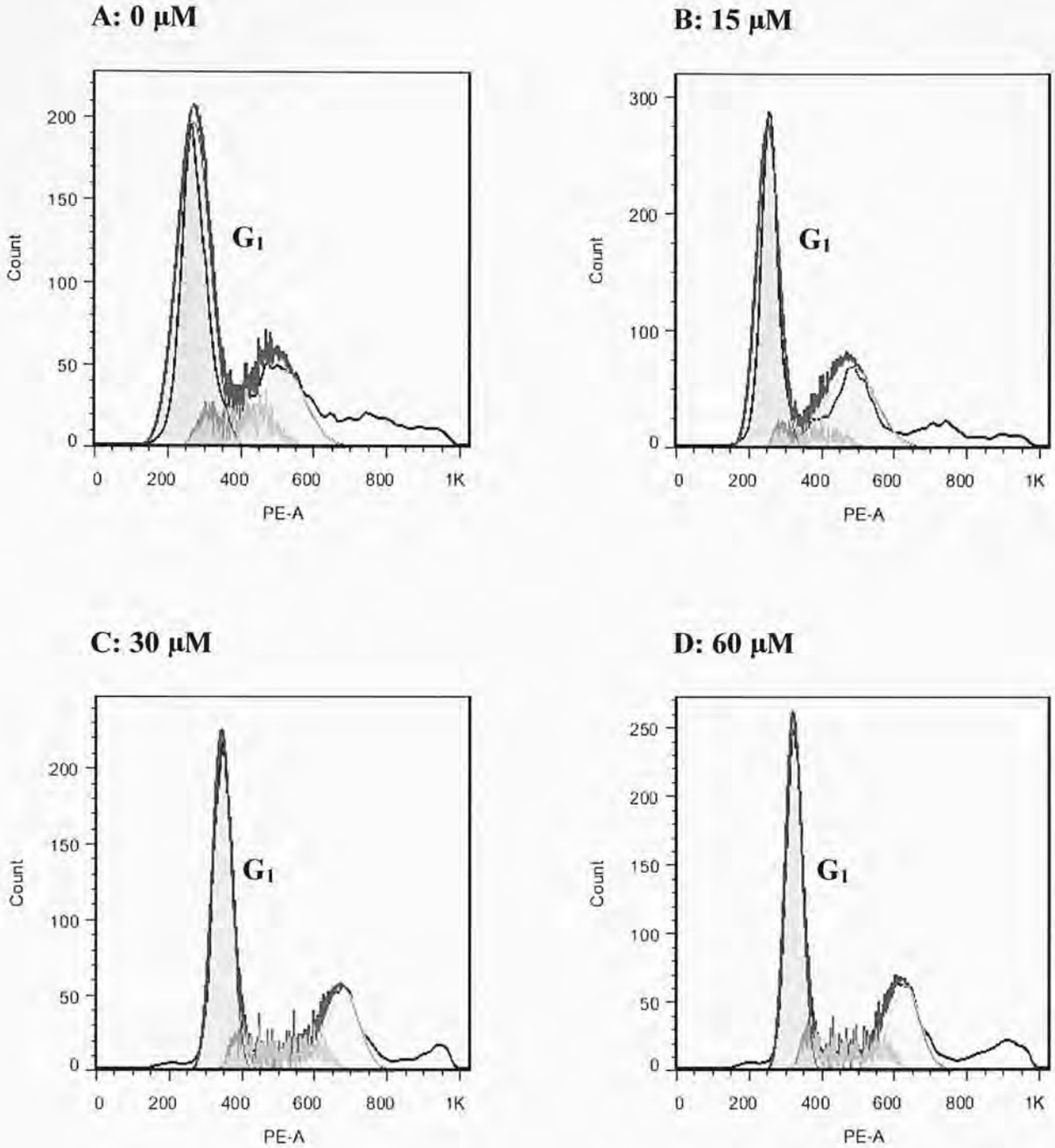
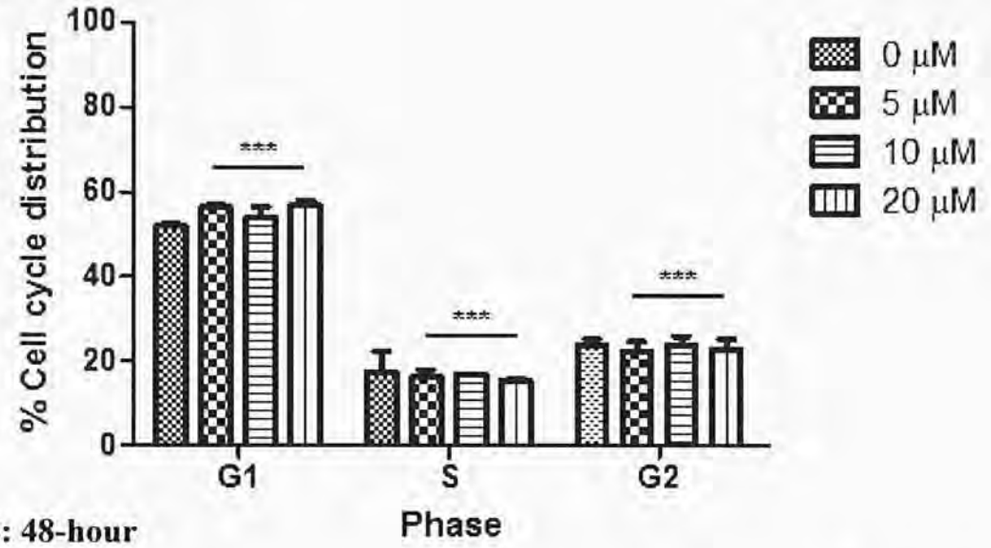


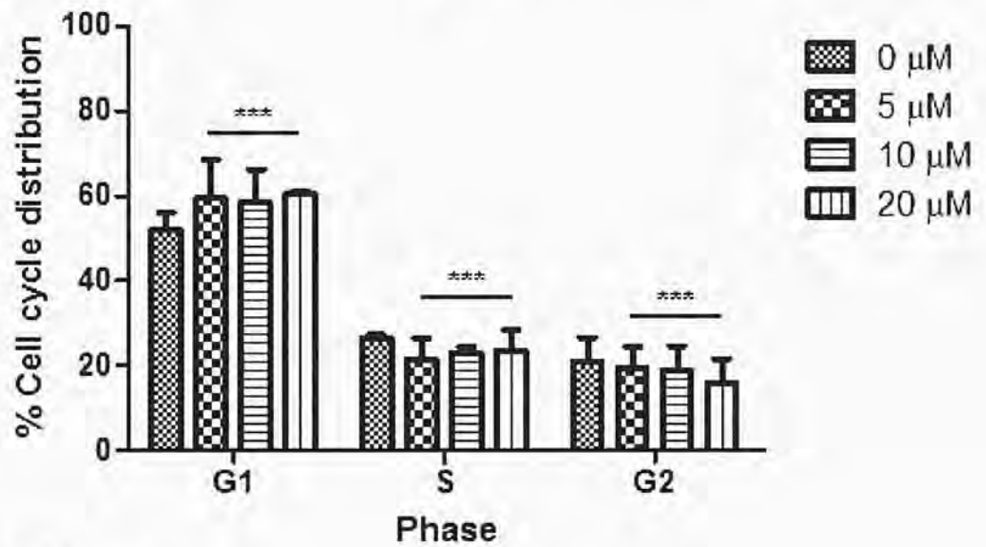
Figure 18 The cell cycle distribution of JHH-4 cells after berberine treatment for 72 hours

JHH-4 cells were treated with different concentration, (A) 0 μM, (B) 15 μM, (C) 30 μM, and (D) 60 μM. Figure 18 showed the representative experimental results of three individual experiments. The results suggest that berberine caused G_1/S cell cycle arrest in JHH-4 cells after 72-hour treatment.

A: 24-hour



B: 48-hour



C: 72-hour

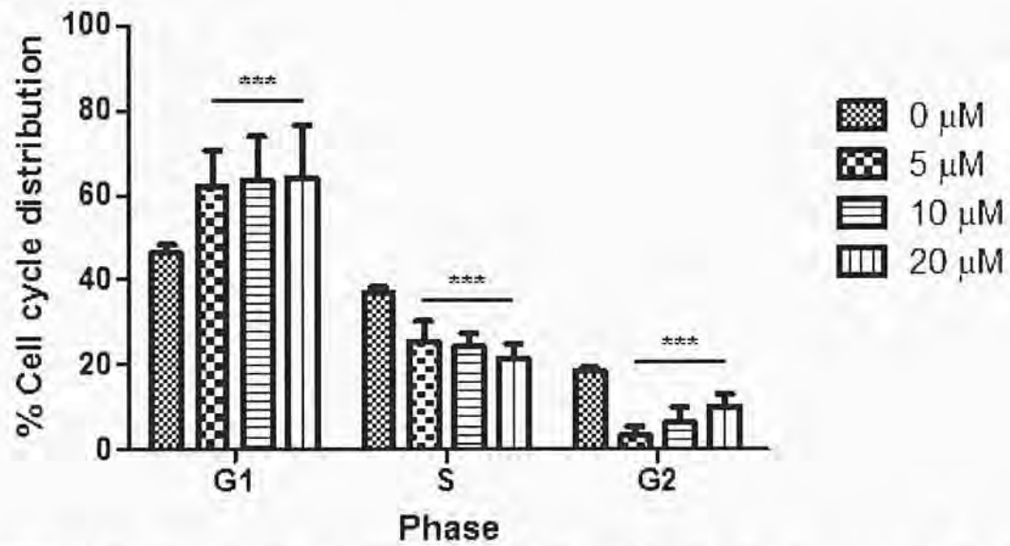


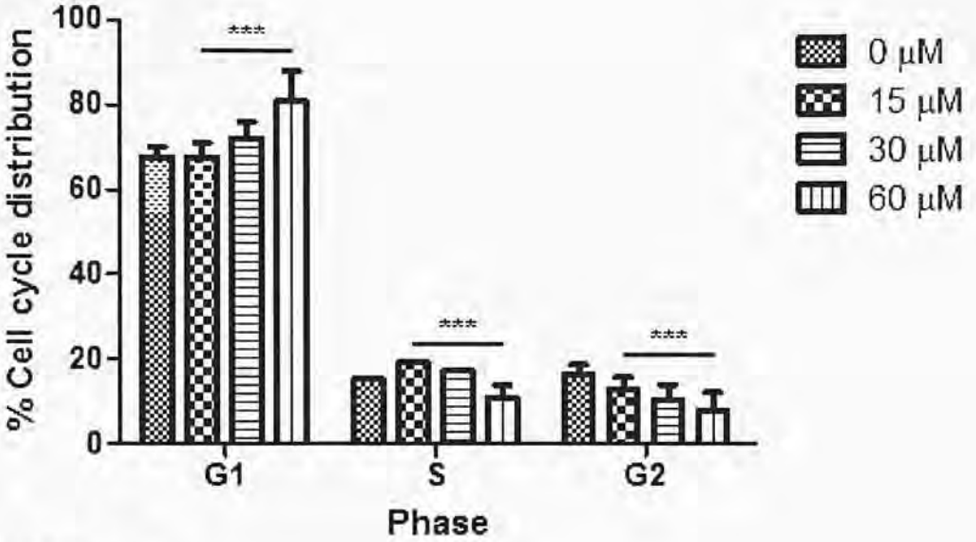
Figure 19 The cell cycle distribution (%) of Huh7 cells after treatment with berberine for (A) 24 hours, (B) 48 hours, and (C) 72 hours

Flow cytometric analysis of cell cycle in Huh7 cells after berberine treatment

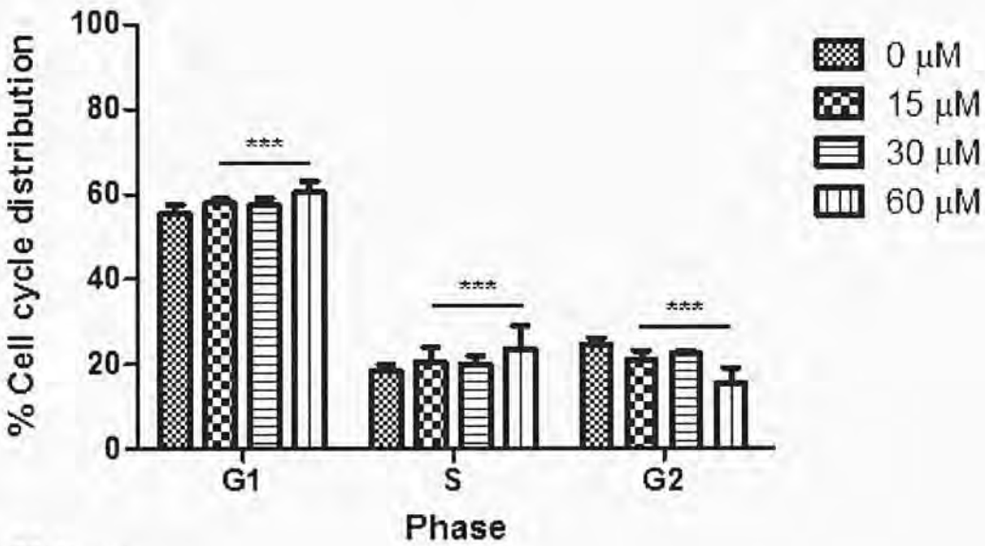
Cell cycle arrest was studied by propidium iodide (PI) staining. Huh7 cells were treated with berberine at the concentration of 0, 5, 10 and 20 μM for 72 hours, respectively. Cells were harvested after treatment, and were fixed with 70% ethanol overnight. After fixation, cells were stained with PI and were subject to analysis by flow cytometry.

Figure 19A and Figure 19B showed that there was a slight increase in percentage of cells in G_1 phase along with the increase in berberine concentration, after 24-hour and 48-hour treatment, respectively. Figure 19C demonstrated that the percentage of cells in G_1 phase in 5, 10, and 20 μM increased by 10% at least compared with that in 0 μM berberine treatment. The results in Figure 19 suggest that berberine triggered G_1 cell cycle arrest in Huh7 cells ($n = 3$; *** $P < 0.001$).

A: 24-hour



B: 48-hour



C: 72-hour

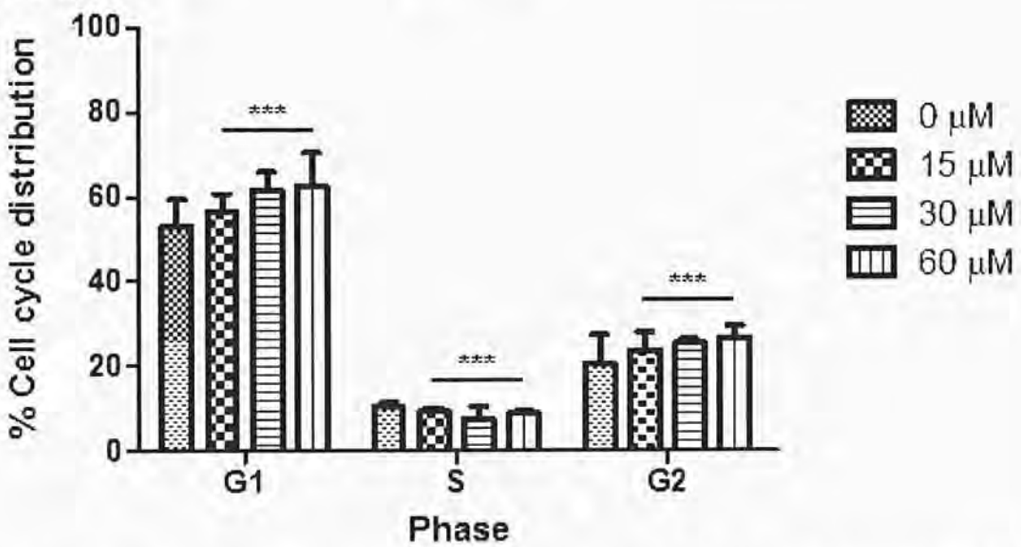


Figure 20 The cell cycle distribution (%) of JHH4 cells after treatment with berberine for (A) 24 hours, (B) 48 hours, and (C) 72 hours

Flow cytometric analysis of cell cycle in JHH4 cells after berberine treatment

Cell cycle arrest was studied by propidium iodide (PI) staining. JHH4 cells were treated with 0, 15, 30 and 60 μM berberine for 72 hours, respectively. Cells were harvested after treatment, and were fixed with 70% ethanol overnight. After fixation, cells were stained with PI and were subject to analysis by flow cytometry.

Figure 20A and Figure 20C demonstrated that as the percentage of cells in G1 phase increased along with the increase in berberine concentration after 24-hour and 72-hour treatment, respectively. Figure 20B showed there was a slight increase in percentage of cells in G_1 phase along with the increase in berberine concentration after 48-hour treatment.

The results in Figure 20 suggest that berberine triggered G_1 cell cycle arrest in JHH4 cells ($n = 3$; *** $P < 0.001$).

Chapter 4 Discussion

Berberine showed inhibitory effect towards the proliferation of HCC cells

In this study, berberine was shown to significantly inhibit proliferation of HCC cells, and reduce cell viabilities in a dose-dependent manner. Berberine did not show significant inhibitory effect on normal liver cells upon 24-hour treatment, and it exhibited inhibitory effect on normal liver cells at a lesser extent upon 48-hour and 72-hour treatment, compared with that in Huh7 and JHH4 cells. Berberine exhibited more potent effect after 72-hour treatment with berberine in both Huh7 and JHH4 cell lines. The half maximal inhibitory concentration (IC_{50}) of berberine in Huh7 after 72-hour treatment was 10 μM; while the IC_{50} of berberine in JHH4 after 72-hour treatment was 30 μM. IC_{50} of berberine in WRL68 after 72-hour treatment was 100 μM. The results suggest that HCC cell lines and normal liver cells may show different cellular response towards berberine. The fact that Huh7 and JHH4 have different IC_{50} of berberine treatment suggests their difference in cellular response towards berberine. Berberine could induce apoptotic effects on HCC cells and consequently, it causes apoptotic death in HCC cells but not in normal liver cells. The difference in inhibitory activities towards cancer cells and normal cells suggests berberine could be a good apoptotic agent in HCC cells.

While comparing berberine with traditional drugs used in chemotherapy, such as

doxorubicin, the $IC_{50, 72h}$ of doxorubicin in HepG2 was 1 μM (Al-Qubaisi et al., 2011), while the $IC_{50, 72h}$ of doxorubicin in Chang liver cell was less than 1 μM (Al-Qubaisi et al., 2011). Although doxorubicin has a very low IC_{50}, it has a low therapeutic index, and its clinical use is limited since it leads to acute and chronic toxicities (Bandyopadhyay et al., 2010). It is imperative to find an alternative agent for cancer treatment. The results in the present study suggest that berberine should be a suitable candidate since it did not produce toxic effects and it showed specific activity towards liver cancer cells, while its effects on normal liver cells were not observable at the same concentration. Considering the $IC_{50, 72h}$ of berberine in Huh7 and WRL68, it may require 10-fold increase in concentration for berberine to produce a toxic effect in normal cells.

FITC Annexin V and PI co-staining showed that berberine caused apoptosis in HCC cells

The loss of membrane integrity occurs during the latest stages of cell death either from apoptosis or necrosis (Koopman G et al., 1994). FITC Annexin V staining is succeeded by the loss of membrane integrity; therefore, FITC Annexin V staining is often used together with vital dyes such as propidium iodide (PI) (Koopman G et al., 1994). The FITC Annexin V and PI co-staining technique allows the identification of

early apoptotic cells, which yields PI negative and FITC Annexin V positive signals (Koopman G et al., 1994). FITC Annexin V and PI co-staining provides experimental evidences (Figure 3 and Figure 4) that berberine caused apoptosis in both Huh7 and JHH4 cells. For Huh7 cells, the 72-hour treatment (Figure 3C) resulted in a lower percentage of apoptotic cells compared with the 24-hour treatment (Figure 3A). The phenomenon may be explained by more cell death after 72-hour berberine treatment than that after 24-hour berberine treatment. Cells may undergo early apoptotic process after 24-hour berberine treatment, while after 72-hour treatment cells may have died via apoptosis. In this way, less percentage of apoptotic cells would be observed.

For JHH4 cells, a significant higher percentage of apoptotic cells was observed in treated cells for 24-hour (Figure 4A), 48-hour (Figure 4B), and 72-hour (Figure 4C) berberine treatment compared with the untreated cells.

Berberine regulated gene expression in HCC cell lines

In this study, PCR array was used as a preliminary screening procedure for genes that were affected by berberine significantly (Figure 5). The gene expression of Bcl-2, CIDEA and HRK were validated by Real-time PCR. The real-time PCR results corroborated with PCR Array analysis for CIDEA gene. CIDE family is known as the inhibitory subunits of endonucleaes, cleavage of which is induced by downstream

caspases (Ghobrial et al., 2005).

In real-time PCR results for both Huh7 and JHH4 cells (Figure 6 and Figure 7), Bax, Bid, CIDEA, HRK, and p21 were found to be up-regulated by berberine in a dose-dependent manner, while AKT and Bcl-2 were found to be down-regulated by berberine in a dose-dependent manner.

All BH3-only molecules need Bax, which is a multi-domain BH3 protein, to exert their intrinsic pro-apoptotic activity (Ghobrial et al., 2005). In response to apoptotic stimuli, Bax is an essential gateway to mitochondrial dysfunction required for cell death (Wei et al., 2001). An increase in the gene expression of Bax suggests an increased protein expression of Bax, which is crucial to the intrinsic apoptotic pathway. The p21 gene codes for the cyclin-dependent kinase inhibitor 1 (CDK-interacting protein 1). The p21 protein serves as a regulator of cell cycle progression at G_1 phase (Luo et al., 1995). An increase in p21 gene expression (Figure 6 and Figure 7) suggests an increased protein expression of p21 protein, the transcriptional target of the tumor suppressor protein p53 (El-Deiry et al., 1993).

AKT protein takes part in the regulation of tumor cell survival and proliferation by stabilizing p21 protein (Li, Dowbenko, & Lasky, 2002). When AKT gene expression decreased (Figure 6 and Figure 7), it suggests that less AKT protein would be expressed. A decreased AKT protein expression suggests less inhibition of p21 protein

activity, which results in cell cycle arrest at G_1 phase. Bcl-2 gene is over-expressed in certain cancers, such as folicular B-cell lymphoma, chronic lymphocytic leukemia, and B-cell non-Hodgkin lymphomas (Ghobrial et al., 2005). While Bcl-2 gene expression was found to be decreased in the present study, the result suggests that berberine may inhibit the gene expression of Bcl-2 gene in HCC cells. Bid is a well-known pro-apoptotic protein player in the Bcl-2 family, which requires proteolytical cleavage to become active (Li et al., 1998). An increased gene expression of Bid implies an increased protein expression of Bid in the cytosol, while the activity of Bid in the cytosol should be evaluated by protein expression analysis. Survivin is an anti-apoptosis gene expressed in cancer and lymphoma (Ambrosini, Adida, & Altieri, 1997). Survivin gene codes for the protein Survivin, which belongs to the inhibitor of apoptosis (IAP) family. Survivin negatively regulates apoptosis by inhibiting caspase activation. Survivin gene is a potential apoptosis-based therapeutic target. The findings from the present study demonstrated that berberine may inhibit Survivin gene expression in Huh7, while berberine did not affect the expression of Survivin gene expression in JHH4 significantly.

The real-time PCR results agreed with PCR Array analysis for HRK gene. The HRK gene encodes the protein, activator of apoptosis harakiri (Inohara, Ding, Chen, & Nunez, 1997). Early studies showed that the expression of HRK induced cell death

which was inhibited by Bcl-2 and Bcl-X(L) (Inohara et al., 1997). While berberine up-regulated the expression of HRK gene (Figure 6), more HRK protein may be expressed, which may increase apoptotic cell death.

The Bcl-2 gene expression was found to be down-regulated by berberine in real-time PCR experiments. Early studies reported that increased Bcl-2 protein expression causes resistance to chemotherapeutic drugs and radiation therapy, while decreasing Bcl-2 expression may promote apoptosis induced by anti-cancer drugs (Ghobrial et al., 2005). Furthermore, over-expression of Bcl-2 may cause accumulation of cells in the G_0 phase of cell cycle division, resulting in chemoresistance (Reed, 1997). The present study provides experimental evidences that berberine negatively regulated the protein expression of Bcl-2. The results suggest that berberine may cause apoptosis in HCC cells through the intrinsic apoptotic pathway.

Caspase3/7 activities in Huh7 and JHH4 cells

Caspase-3/7 activities in both Huh7 and JHH4 cells were evaluated by Apo-ONE ® Homogeneous Caspase-3/7 Assay Kit. The principle of the assay is that the non-fluorescent caspase substrate Z-DEVD-R110 is cleaved by Caspase-3/7 to create the fluorescent Rhodamine 110. The fluorescence generated in the experiment is proportional to the amount of caspase-3/7 cleavage activity present in the cultured

sample. The results indicate berberine triggered caspase-3/7 activities in both HCC cell lines. Among Huh7 and JHH4 cell lines, higher caspase-3/7 activities were observed in Huh7. The results suggest Huh7 and JHH4 cells may exhibit different cellular response towards berberine. The protein expression of caspase-3 and caspase-7 was further evaluated by SDS-PAGE and Western Blot Analysis.

Protein expression studies showed that berberine caused apoptosis through Caspase Cascade

In the present study, the protein expression studies were focused on caspase cascade and the Bcl-2 protein family. The results suggest that berberine induces apoptosis through the intrinsic apoptotic pathway. While the release of cytochrome c from mitochondria begins, the mitochondrial stress pathway is activated (Earnshaw, Martins, & Kaufmann, 1999). Cytochrome c interacts with Apaf-1, which activates caspase-9 (Earnshaw et al., 1999). The effector caspases, caspase-7, and caspase-3 are downstream targets of caspase-9 (Earnshaw et al., 1999). Poly (ADP-ribose) Polymerase (PARP) is a well-known downstream target of active caspase-3 (Boulares et al., 1999). While apoptosis is induced in cells, PARP can be cleaved (Boulares et al., 1999). PARP was reported to produce poly(ADP-ribosyl)ation of nuclear proteins with NAD as substrate (Boulares et al., 1999). It was shown to cause cell death by

depleting NAD and ATP of the cell, as it is activated through binding to DNA ends or strand breaks (Boulares et al., 1999). Previous studies revealed that during drug-induced apoptosis in different cells, PARP was shown to be cleaved into fragments which contain the active site as well as the DNA-binding domain of the enzyme (Boulares et al., 1999). PARP is inactivated by such cleavage as its ability to respond to DNA strand breaks is destroyed (Boulares et al., 1999). The present study enables us to confirm that berberine caused apoptosis in HCC cells through the intrinsic caspase-mediated pathway. The protein expression of full-length PARP decreased while cleaved form of which increased. Since berberine decreased the protein expression of procaspase-9, and its downstream effector caspases, procaspase-3 and procaspase-7, it may be possible that berberine cleaves caspase-9, caspase-3 and caspase-7. Cleaved caspases become active executioners of the intrinsic apoptotic pathway.

The intrinsic apoptosis pathway is also regulated by Bcl-2 protein family. The study showed that Bcl-2, an anti-apoptotic member in the Bcl-2 protein family, was down-regulated by berberine in dose-dependent fashion in both Huh7 and JHH4 cells after 72-hour treatment (Figure 11 and Figure 12). The results indicated that the expression of another pro-apoptotic member in the Bcl-2 protein family, Bid, was down-regulated after treating with berberine for 72 hours, in both Huh7 and JHH4

cells. Bid is a BH3 domain-containing pro-apoptotic Bcl-2 protein family member, which is a mediator of mitochondrial damage induced by caspase-8 (Li, Zhu, Xu, & Yuan, 1998). Full-length Bid is localized in cytosol, while truncated Bid (tBid) translocates to mitochondria (Li et al., 1998). Transduction of apoptotic signals from cytoplasmic membrane to mitochondria is mediated by tBid (Li et al., 1998). tBid was reported to induce the clustering of mitochondria around the nuclei and release of cytochrome c in a caspase-independent fashion, and then the loss of mitochondrial membrane potential, cell shrinkage, and condensation of nuclei in a caspase-dependent manner (Li et al., 1998).

The present study demonstrated that the protein expression of Bid in cytosol was decreased with increasing concentration of berberine, while the gene expression of Bid was increased with berberine treatment in a dose-dependent manner; a possible explanation is that Bid in cytosol was truncated and was translocated to mitochondria. Upon this translocation, it was likely that tBid led to the apoptotic consequence afterwards. Further investigation of the protein expression of tBid (which has a molecular size of 15 kDa) in mitochondria is recommended to gain a more complete understanding of biologic activity of berberine in the intrinsic apoptotic pathway.

The protein expression of proliferating cell nuclear antigen (PCNA) was found to be down-regulated by berberine in both Huh7 and JHH4 cells in a dose dependent

manner after 72-hour treatment. Previous studies showed that PCNA is essential for both DNA synthesis and DNA repair (Shivji, Kenny, & Wood, 1992). The experimental results imply that as the expression of PCNA was reduced by berberine, fewer cells were able to repair damaged DNA.

Berberine caused G_1/S cell cycle arrest in both Huh7 and JHH4 cells

The findings from the present study provide supportive evidences that berberine caused G_1/S cell cycle arrest in HCC cell lines (Figure 19 and Figure 20). G_1 is the first cell cycle checkpoint, which determines whether the cell should divide, delay division, or enter a resting stage, before the cells entering into S phase. The results suggest berberine may stop HCC cells from dividing, thereby halting the deregulated cell proliferation.

Conclusion

The present study has demonstrated that berberine inhibits growth of HCC cells by inducing the intrinsic apoptotic pathway, and by arresting the cell cycle at G_1/S phase. Berberine induces apoptotic events in HCC cell lines through procaspase-9, and its effector caspases, procaspase-3, and procaspase-7, which were involved in berberine-induced apoptosis. Caspase activation caused a decrease in the protein expression of full-length Bid in cytosol, where full-length Bid was likely to be

truncated and translocated to mitochondria, which consequentially resulted in apoptotic death. Our findings show that berberine possesses potent anti-cancer activities in human hepatocellular carcinoma.

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