Anti-Proliferative and Differentiation-Inducing Effects of Glycyrrhizin and 18β-Glycyrrhetinic Acid on Neuroblastoma Cells *In Vitro*

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ABBREVIATIONS

A_{540}	Absorbance at 540 nm
ADI	Acceptable Daily Intake
ALT	Alanine Transaminase
ANH	Atrial Natriuretic Hormone
APS	Ammonium Persulfate
AST	Aspartate Transaminase
ATCC	American Type Culture Collection
ATRA	All-*Trans* Retinoic Acid
bp	Base Pair
BSA	Bovine Serum Albumin
cAMP	Cyclic 3',5'-Adenosine Monophosphate
CCG	Children's Cancer Group
CDK	Cyclin-Dependent Kinase
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curie(s)
CM	Complete Medium
cpm	Counts Per Minute
CT	Computer Tomography
Ctl	Control
db-cAMP	Dibutyryl-Cyclic AMP
DEPC	Diethyl Pyrocarbonate
DMBA	7,12-Dimethylbenz[α]anthracene
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	2'-Deoxyribonucleoside 5'-Triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine Triphosphate
ECL	Enhanced Chemiluminescence
EDTA	Ethyl-diamine-tetra-acetate
EFS	Event-Free Survival
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GA	Glycyrrhetinic Acid

GAMG	Glycyrrhetinic Acid Monoglucuronide
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase (EC 1.2.1.12)
GL	Glycyrrhizin
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GRAS	Generally Recognized As Safe
HAV	Hepatitis A Virus
HBsAg	Hepatitis B Surface Antigen
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric Acid
HCMV	Human Cytomegalovirus
HEPES	N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid
HI-FCS	Heat Inactivated Fetal Calf Serum
HIV	Human Immunodeficiency Virus
hr	Hour
HSV	Herpes Simplex Virus
^{3}H-TdR	Tritiated Thymidine
HVA	Homovanillic Acid
IC_{50}	50% Inhibitory Concentration
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
INPC	International Neuroblastoma Pathology Classification
INSS	International Neuroblastoma Staging System
JEV	Japanese Encephalitis Virus
kb	Kilo-Base
kDa	Kilo-Daltons
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MAP	Microtubule-Associated Protein
2-ME	2-Mercaptoethanol
MEM	Minimum Essential Medium
MIBG	Metaiodobenzylguanidine

mg	Milligram
ml	Milliliter
mM	Millimolar
μg	Microgram
μl	Microliter
μM	Micromolar
M-MLV	Moloney Murine Leukemia Virus
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NaOH	Sodium Hydroxide
NCAM	Neuronal Cell Adhesion Molecule
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
NSE	Neuron-Specific Enolase
p	P Value
PBS	Phosphate Buffered Saline
PDA	Phorbol 12,13-Diacetate
PI	Propidium Iodide
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA/TPA	Phorbol 12-Myristate 13-Acetate/ 12-*O*-Tetradecanoyl-Phorbol 13-Acetate
POG	Pediatric Oncology Group
PSN	Penicillin-Streptomycin-Neomycin
PVDF	Polyvinylidene Difluoride
RA	Retinoic Acid
RNA	Ribonucleic Acid
RNase A	Ribonuclease A
rpm	Revolutions Per Minute
RPMI 1640	Roswell Park Memorial Institute Tissue Culture Medium 1640
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SARS-CV	Severe Acute Respiratory Syndrome-associated Coronavirus
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
S.E.	Standard Error

SFSV	Sandfly Fever Sicilian Virus
SIF	Small Intensely Fluorescent
SNMC	Stronger Neo-Minophagen C
SNS	Sympathetic Nervous System
TBE	Tris-Borate-EDTA
TEMED	N,N,N',N'-Tetra-methylethylenediamine
TNF	Tumor Necrosis Factor
TNM	Tumor-Node-Metastases
Tris	Tris[hydroxylmethyl]-amino-methane
UICC	Union International Contra le Cancer
UV	Ultraviolet
VMA	Vanillylmandelic Acid
v/v	Volume/Volume
VZV	Varicella-Zoster Virus
w/v	Weight/Volume

ABSTRACT

Licorice (*Radix Glycyrrhizae*) is one of the most widely used medicinal herbs since ancient time. Glycyrrhizin, a pentacyclic triterpenoid, is the major active constituent of licorice. Glycyrrhizin has been shown to be metabolized to its aglycone form, 18β-glycyrrhetinic acid, after oral administration. Although the importance of glycyrrhizin and 18β-glycyrrhetinic acid is becoming more prominent when their hepatoprotective, anti-inflammatory, anti-viral, immunomodulatory and cancer chemopreventive effects are unraveled, yet their direct anti-tumor activity and therapeutic potentials have not been fully explored.

In the present study, the growth-inhibitory and differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells were examined *in vitro*. Our results showed that both glycyrrhizin and 18β-glycyrrhetinic acid exhibited anti-proliferative activity on murine (Neuro-2a, BU-1 clone) and human (SK-N-DZ and SH-SY5Y) neuroblastoma cell lines in a dose-dependent manner. Kinetic studies indicated that glycyrrhizin and 18β-glycyrrhetinic acid also inhibited the proliferation of BU-1 cells time-dependently, with optimal inhibitory effect seen at 24 – 48 hours. Moreover, 18β-glycyrrhetinic acid was found to be more potent than glycyrrhizin as the former exerted its anti-proliferative activity at micromolar concentrations whereas the latter was effective at millimolar ranges. In addition, the reversibility of the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid was dependent on the drug concentration and duration of drug exposure. Interestingly, the growth-inhibitory effect of glycyrrhizin and 18β-glycyrrhetinic acid

on BU-1 cells could not be attributed directly to their cytotoxic and apoptosis-inducing effects as the percentage of viability was found to be over 90% and no DNA fragmentation could be detected after drug treatment.

On the other hand, glycyrrhizin and 18β-glycyrrhetinic acid were found to be capable of inducing neuronal differentiation of the murine neuroblastoma BU-1 cells. Morphological and biochemical changes characterized by neurite outgrowth, increase in cell size and increased expression of the neuronal differentiation markers MAP2 and neurofilament-200 were observed in drug-treated cells. Moreover, the differentiation induced by 18β-glycyrrhetinic acid in BU-1 cells was accompanied with a down-regulation of N-myc gene expression and an up-regulation in the expression of Bcl-2, NF-κB/p50 and c-fos genes.

As shown by the cell cycle analysis, the anti-proliferative activity of glycyrrhizin and 18β-glycyrrhetinic acid is probably mediated through the G_2/M phase arrest. The cell cycle arrest of the BU-1 cells induced by 18β-glycyrrhetinic acid may be brought about by the down-regulation of cell cycle regulatory proteins including cyclin A, cyclin B and cdc2. On the other hand, it was found that combinations of glycyrrhizin and 18β-glycyrrhetinic acid or all-*trans* retinoic acid and 18β-glycyrrhetinic acid had resulted in synergism on the growth inhibition and differentiation of BU-1 cells. Although 18β-glycyrrhetinic acid failed to alter the mRNA expression of protein kinase C (PKC) isoforms, addition of PKC inhibitors effectively antagonized the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells. These results suggest that the differentiation-inducing effect of 18β-glycyrrhetinic acid may be PKC-dependent. On the contrary, the

differentiation-inducing effect of 18β-glycyrrhetinic acid appears to be independent of protein kinase A (PKA) as the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells could not be modulated by PKA inhibitors although the differentiation of BU-1 cells could be augmented by cAMP elevating agent or cAMP analogs.

It is hoped that the above findings could provide better insights into the direct anti-tumor effects of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells *in vitro*. Nevertheless, more thorough understanding of the molecular mechanisms and the signaling pathways by which glycyrrhizin and 18β-glycyrrhetinic acid could exert their actions on neuroblastoma cells are required before these natural products can be considered as therapeutic agents in neuroblastoma treatment.

撮要

甘草是自遠古時代已被廣泛應用的藥草。甘草的主要成份為甘草甜素，此乃三萜類糖苷。有資料報導口服後的甘草甜素會被代謝轉化為其糖苷配基，稱之為18β-甘草次酸。縱使甘草甜素及18β-甘草次酸具有保護肝臟、抗炎、抗病毒、調節機體的免疫功能及防癌等多種作用，但直至現時為止，它們的直接抗癌活性及治病的潛力仍未被完全勘探。

在本研究計劃中，我們會通過體外實驗觀察甘草甜素及 18β-甘草次酸對神經母細胞瘤細胞的抑制增殖及誘導細胞分化效能。實驗結果證明，甘草甜素及18β-甘草次酸均能有效地抑制鼠科(Neuro-2a, BU-1 純系) 及人類(SK-N-DZ 及SH-SY5Y) 等神經母細胞瘤細胞株的增殖，且隨其濃度的增加，抑制效果越顯著。甘草甜素及18β-甘草次酸抑制 BU-1 細胞增殖的能力更是視乎作用時間之長短，其抑制細胞增殖之能力在二十四至四十八小時的作用時間最為顯著。再者，18β-甘草次酸抑制神經母細胞瘤細胞增殖的能力遠勝於甘草甜素，因前者可在百萬分之一摩爾的濃度發揮其抑制細胞增殖的效能，但後者只能在千分之一摩爾的濃度發揮其作用。此外，甘草甜素及 18β-甘草次酸抑制細胞增殖的可逆性需視乎藥物的濃度及細胞接觸藥物的時間。我們有趣地發現了甘草甜素及 18β-甘草次酸抑制神經母細胞瘤細胞增殖的能力並非基於它們對癌細胞的直接毒性及細胞凋亡之誘導，因為實驗結果顯示多於百分之九十經藥物作用的癌細胞仍然存活及經藥物作用後，癌細胞核內的脫氧核糖核酸並無斷裂。

另一方面，研究發現甘草甜素及 18β-甘草次酸均能誘導鼠科神經母細胞瘤 BU-1 細胞進行細胞分化。經過甘草甜素及 18β-甘草次酸作用的 BU-1 細胞在形態及生物化學上均擁有已分化細胞的特性，例如：細胞突起發達、細胞增大、增加神經元分化標記如：微管締合蛋白 2 (MAP2)及神經微絲-200 (neurofilament-200)的產生。再者，隨著 18β-甘草次酸誘導的細胞分化而來的轉變是 N-myc 基因之表達被下調及 Bcl-2、NF-κB/p50 和 c-fos 之基因表達被上調。

根據細胞週期分析結果，甘草甜素及 18β-甘草次酸之抑制癌細胞增殖的機制可能是通過促使癌細胞週期停留在 G_2/M 期來實現的。18β-甘草次酸誘發的細胞週期阻礙亦可能是透過細胞週期控制蛋白包括細胞週期蛋白(cyclin A, cyclin B)及細胞週期蛋白依賴性激酶(cdc2)表達的下調來實現。另一方面，我們發現甘草甜素聯合 18β-甘草次酸或全反維生素 A 酸(ATRA)聯合 18β-甘草次酸皆能對 BU-1 細胞的增殖抑制及細胞分化產生增效的現象。此外，雖然研究發現 18β-甘草次酸未能影響 BU-1 細胞的 PKC 同種型酶 mRNA 的表達，但 PKC 抑制劑能有效地對抗 18β-甘草次酸誘發的 BU-1 細胞分化。從這些實驗結果看來，18β-甘草次酸誘發的細胞分化是需要依賴 PKC 來進行。相反，這細胞分化的誘導似乎並非依賴 PKA，因為雖然 cAMP 提升劑或 cAMP 類似物能增強 BU-1 細胞的分化，但 PKA 抑制劑對 18β-甘草次酸誘發的 BU-1 細胞分化未能造成影響。

我們希望上述的研究結果能增加甘草甜素及 18β-甘草次酸在體外實驗的直接抗癌效能之了解。雖然如此，如要將甘草甜素及 18β-甘草次酸應用在神經母細胞瘤之治療上，我們必須對甘草甜素及 18β-甘草次酸在神經母細胞瘤細胞的

分子作用機制和訊號之傳遞作出更深入的理解。

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Neuroblastoma -- An Overview

1.1.1 Epidemiology of Neuroblastoma

Neuroblastoma is the most common extracranial childhood solid tumor that accounts for 8 to 10% of all childhood cancers (Gurney *et al.*, 1997; Brodeur & Maris, 2002). It accounts for about 50% cancer cases in children younger than two years old, 75% under age 4, 90% under age 10 and the mean age at diagnosis for neuroblastoma patients is about 22 months (Lanzkowsky, 2000; Cullen, 2003). Infants who are diagnosed under the age of one tend to have localized, good-prognosis tumor while children who are diagnosed over the age of one have poor outcome and usually have metastatic disease (Caron & Pearson, 1998). Approximately 50% of the infant patients and 70% of the older patients have disseminated disease at diagnosis (Reynolds & Seeger, 2001).

Neuroblastoma usually occurs sporadically although 1 – 2% was reported to be familial cases (Brodeur & Maris, 2002). The etiology of neuroblastoma is unknown. Embryologically, neuroblastoma arises from primordial neural crest cells that normally give rise to sympathetic ganglia and adrenal medulla (Lanzkowsky, 2000). The primary tumor can arise at any point where there is tissue of the sympathetic nervous system and the majority of primary tumors arise in the adrenal glands. In general, the tumors exhibit three distinct clinical behaviors: (1) spontaneous regression, (2) differentiation into a benign form, and (3) life-threatening progression. The incidence of spontaneous regression in neuroblastoma is between 10- and 100-fold greater than that for other human cancers (Westermann & Schwab, 2002). Moreover, a spectrum of maturation, differentiation, and clinical behavior are

reflected by three distinct histopathologic patterns: neuroblastoma, ganglioneuroblastoma and ganglioneuroma (Cullen, 2003). Most neuroblastomas are undifferentiated tumors consisting of small, round cells called neuroblasts. Some tumors that show partial histological differentiation are called ganglioneuroblastoma. Tumors with the most differentiated phenotype are called ganglioneuroma which consists of clusters of mature neurons surrounded by a dense stroma of Schwann cells (Brodeur, 2003).

1.1.2 Clinical Presentations of Neuroblastoma

Since neuroblastoma can arise from any site along the sympathetic system chain, the locations of primary tumors at the time of diagnosis are varied. About 25% of the primary tumors are found in the neck or the thorax, 70% in the abdomen and 5% in the pelvis (Caron & Pearson, 1998). Moreover, the frequency of adrenal tumors is slightly higher in children compared to infants and the latter have more thoracic and cervical primary tumors (Brodeur & Maris, 2002). The clinical manifestations of neuroblastoma are very diverse, depending on the site of the primary tumor and whether there is metastatic disease. Upper thoracic tumors can give rise to respiratory distress and infections, dysphagia and circulatory problems while lower thoracic tumors seldom produce symptoms (Voute *et al.*, 1992). Among the children with abdominal tumors, the predominant clinical manifestation is an abdominal mass, but anorexia, vomiting, abdominal pain, distension and weight loss are also common symptoms (Lanzkowsky, 2000; Haase & LaQuaglia, 2003). Pelvic tumors may be associated with chronic constipation or urinary tract obstruction with or without infection. In children with paraspinal neuroblastoma, the tumor can grow through

the intervertebral foramina and cause extradural spinal cord compression (Haase & LaQuaglia, 2003). Compression of the spinal cord leads to localized back pain and tenderness, immobility, weakness of lower extremities, scoliosis, bladder and anal sphincter dysfunction etc. (Voute *et al.*, 1992; Lanzkowsky, 2000). In infants, compression may cause a change in the frequency of voiding and urinary tract infections. Moreover, the most common motor manifestation in older children is disturbance of gait, starting with a limp and sometimes progressing to paraplegia (Voute *et al.*, 1992). Massive involvement of the liver in metastatic disease is particularly frequent in infants (stage 4S) (Brodeur & Maris, 2002). Rapidly enlarging abdominal tumors or massive hepatomegaly in infants may cause respiratory distress due to the compression of the diaphragm (Cullen, 2003). Bone, lymph nodes, and bone marrow are the commonest metastatic sites. Metastases to the bone manifest as painful lesions which produce an irritable and unwell child. Bone marrow involvement generally presents with anemia and later thrombocytopenia (Caron & Pearson, 1998). Orbital metastases may cause proptosis or orbital ecchymoses, causing these patients to appear as if they have "raccoon eyes" (Haase & LaQuaglia, 2003).

1.1.3 Diagnosis of Neuroblastoma

An unequivocal pathologic diagnosis of neuroblastoma requires histologic diagnosis by light microscopic examination of tumor tissue along with appropriate immunohistology. A bone marrow biopsy is done to evaluate metastatic involvement. The diagnosis may be established by a bone marrow aspiration biopsy

containing definite tumor cells in association with elevation of the serum or urine catecholamine levels (Brodeur & Maris, 2002; Haase & LaQuaglia, 2003). Urinary levels of vanillylmandelic acid (VMA) and homovanillic acid (HVA) are the most useful catecholamine metabolites to support the diagnosis. Catecholamine metabolites are abnormally elevated in the urine of nearly all patients (90 - 95%) with neuroblastoma (Brodeur & Maris, 2002). Analysis of urine for catecholamine metabolites (VMA and HVA) not only aids in the diagnosis but also in monitoring tumor response (Reynolds & Seeger, 2001).

Complete assessment of the primary tumor and extent of disease requires specific imaging studies. The conventional diagnostic imaging modalities include plain radiographs, bone scintigraphy, ultrasound, computer tomography (CT) scanning and magnetic resonance imaging (MRI). Plain radiography may show fine calcifications with a cervical, thoracic, or abdominal mass and may detect lytic lesions with cortical bone (Haase & LaQuaglia, 2003). Bone scintigraphy can be useful to detect cortical bone metastases (Brodeur & Maris, 2002). Ultrasound and CT provide more information about abdominal disease, including location of the tumor, lymph node enlargement and live metastases, while thoracic CT or MRI are useful in evaluating the extent of disease above the diaphragm. Moreover, CT or MRI of the head can further define metastases to the skull, orbits, mandible or brain (Brodeur & Maris, 2002). ^{131}I-Metaiodobenzylguanidine (MIBG) scanning is a specific and sensitive method of scintigraphic imaging for primary, metastatic or recurrent tumor and is better for assessing bone and soft tissue involvement since MIBG is taken up by catecholaminergic cells including most neuroblastomas (Brodeur & Maris, 2002; Cullen, 2003; Haase & LaQuaglia, 2003). However, about 15 – 20% of

neuroblastomas do not take up the tracer at all. At present, CT and MRI, that have excellent resolution and allow for three-dimensional reconstruction of tumors to assess volumes, are the most common imaging techniques used to detect and delineate neuroblastoma (Haase & LaQuaglia, 2003).

1.1.4 Staging of Neuroblastoma

Staging of the disease plays an important role in the evaluation of treatment results. This provides information relevant to prognosis and is useful in the selection of treatment. Four major staging systems have been developed for neuroblastoma. Before 1988, neuroblastoma was staged according to three different systems: (1) Evans classification, adopted by the Children's Cancer Group (CCG), (2) staging system developed at St. Jude Children's Research Hospital and used by the Pediatric Oncology Group (POG), and (3) the tumor-node-metastases (TNM) staging system formulated by the Union International Contra le Cancer (UICC). The oldest Evans system employs the initial distribution of disease without reference to respectability of the primary tumor. The POG system is based on a combination of tumor spread and degree of tumor resection. The TNM staging system consists of both clinical and surgicopathologic components (Caron & Pearson, 1998; Reynolds & Seeger, 2001; Haase & LaQuaglia, 2003). However, the existence of these systems raises the problems in comparing the results of therapy among various regions. This problem was solved by the emergence of International Neuroblastoma Staging System (INSS) that is being adopted worldwide. This system presents a standardized scheme for incorporating issues related to initial disease distribution, tumor resectability and

lymph node status (Reynolds & Seeger, 2001).

1.1.5 Prognostic Considerations of Neuroblastoma

Besides the stage of the disease, other clinical variables such as age of the patient at diagnosis, site of primary tumor, and biological variables such as tumor histopathology, serum markers and genetic markers have important implications on prognosis of the disease.

1.1.5.1 Age

Although the age of the patient does not seem to affect the prognosis of low-stage patients, the age of the patient at diagnosis is an important prognostic variable in the higher stages. A recent CCG study showed that the 4-year event-free survival (EFS) probability for patients with stage 3 neuroblastoma ranged from 98% for infants to 75% for patients older than two years at diagnosis (Brodeur & Maris, 2002). The outcome of infants who are younger than one year of age is substantially better than older patients with the same stage of disease, particularly those with more advanced stages of disease (Brodeur, 2003).

1.1.5.2 Primary Tumor Site

Site of primary tumor has also been proposed to be prognostically important. Thoracic neuroblastoma has a more favorable outcome than does abdominal disease

and the adrenal tumor was reported to be associated with a more aggressive clinical course than tumors originating at other sites. Moreover, patients with pelvic tumors may have a better prognosis than that of children with nonpelvic lesions of similar age and initial stage of disease (Brodeur & Maris, 2002; Haase & LaQuaglia, 2003).

1.1.5.3 Tumor Histopathology

Tumor histology plays a role in the prognosis of the disease. A variety of histopathologic classification schemes have been used as prognostic indicators. Most of these classification schemes focus on the degree of differentiation of tumor. The more detailed analysis of histology devised by Shimada takes into consideration the amount of Schwann cells in the stroma, mitotic figures and degeneration of nuclei (Brodeur, 2003). The Shimada classification divides tumors into stroma-rich and stroma-poor groups, with the stroma-rich group considered favorable (>90% survival) unless nodular elements of mature cells are present (18% survival). Stroma-poor tumors are generally classified as unfavorable (<5% survival); however, if the patient is younger than 18 months and has a low mitotic-karyorrhexis index (MKI) or is 18 – 60 months, has a low mitotic-karyorrhexis index, and at least 5% of cells show evidence of differentiation, good survival (84%) is predicted (Reynolds & Seeger, 2001). The International Neuroblastoma Pathology Classification (INPC) that based on the Schimada classification has become the international standard for histopathological classification, particularly as a prognostic variable (Table 1.1) (Shimada *et al.*, 1999).

Table 1.1: International Neuroblastoma Pathology Classification

International Neuroblastoma Pathology Classification		**Original Shimada classification**	**Prognostic group**
Neuroblastoma	(Schwannian stroma-poor)	Stroma-poor	
Favorable		Favorable	Favorable
<1.5 yrs	Poorly differentiated or differentiating & low or intermediate MKI tumor		
1.5-5 yrs	Differentiating & low MKI tumor		
Unfavorable		Unfavorable	Unfavorable
<1.5 yrs	(a) undifferentiated tumor (b) high MKI tumor		
1.5-5 yrs	(a) undifferentiated or poorly differentiated tumor (b) intermediate or high MKI tumor		
≥5 yrs	All tumors		
Ganglioneuroblastoma, intermixed	(Schwannian stroma-rich)	Stroma-rich intermixed (favorable)	Favorable*
Ganglioneuroblastoma, nodular	(composite Schwannian stroma-rich/stroma-dominate and stroma-poor)	Stroma-rich nodular (unfavorable)	Unfavorable*
Ganglioneuroma	(Schwannian stroma-dominant)		
Maturing		Well differentiated (favorable)	Favorable*
Mature		Ganglioneuroma	

MKI: mitosis-karyorrhexis index.
* Prognostic grouping for these tumor categories is not related to patient's age.

1.1.5.4 Serum Markers

Serum markers that had been used in the past to predict outcome or follow disease activity include neuron-specific enolase (NSE), ferritin, lactate dehydrogenase (LDH) and disialoganglioside G_{D2}. NSE is a cytoplasmic protein that is associated with neural cells and survival is substantially worse for patients with advanced disease and high NSE levels (>100 ng/ml). Ferritin produced by neuroblastoma cells can lead to elevations of serum ferritin to above-normal levels. Patients with increased ferritin levels have a much worse prognosis. Elevated ferritin levels (>140 ng/ml) are associated with poorer outcome in stage 4 patients (8% survival) than are normal levels (21% survival). Despite not specific to neuroblastoma, serum LDH levels have been used as a prognostic marker for neuroblastoma, and they might reflect rapid cellular turnover or large tumor burden. The disialoganglioside G_{D2} is found on the surface of most neuroblastomas. Increased levels have been found in the plasma of neuroblastoma patients and gangliosides that are shed by tumor cells might be important in accelerating tumor progression (Voute *et al.*, 1992; Reynolds & Seeger, 2001; Brodeur & Maris, 2002; Brodeur, 2003; Cullen, 2003).

1.1.5.5 Genetic Markers

Various genetic changes in neuroblastoma cells have been proposed as useful prognostic markers. These include changes in the DNA content or modal karyotype, gain or loss of genetic materials and changes in the gene expression. In children younger than two years old, tumors with an increased DNA content (DNA index > 1) exhibit a favorable response to chemotherapy. In contrast, children with diploid

tumors (DNA index = 1) have advanced-stage disease, and respond poorly to chemotherapy (Look *et al.*, 1991). Genomic amplification of N-myc is generally associated with the aggressiveness of neuroblastomas (Look *et al.*, 1991; Katzenstein *et al.*, 1998). Amplification of N-myc was reported to be associated with the worst prognosis and the estimated progression-free survival at 18 months was 70%, 30%, and 5% for patients whose tumors had 1, 3 to 10, or more than 10 N-myc copies, respectively (Seeger *et al.*, 1985). Moreover, recent study showed that infants with stage IV neuroblastoma whose tumors were without N-myc amplification had a 93% 3-year event-free survival (EFS), whereas those with amplified N-myc had a markedly reduced 3-year EFS (10%) (Schmidt *et al.*, 2000). Deletion of chromosome 1 is commonly found in patients with advanced stages of disease and 1p allelic loss is highly associated with N-myc amplification (Brodeur, 2003). Moreover, recent study indicates that allelic loss at 1p36 is an independent predictor of decreased event-free survival but not overall survival in neuroblastoma patients (Maris *et al.*, 2000). Gain of chromosome segment 17q is an important prognostic factor in children with neuroblastoma. This gain is common in advanced tumors, in tumors from children aged over one year and is strongly associated with deletion of 1p and amplification of N-myc. Among the patients with this abnormality, 5-year overall survival was 30.6%, as compared with 86% among those with normal 17q status (Bown *et al.*, 1999). On the other hand, a high incidence of 11q deletion was observed in stage 4 neuroblastoma without N-myc amplification (59%) whereas 11q loss was only observed in 15% of neuroblastomas with N-myc amplification (Plantaz *et al.*, 2001). Furthermore, 11q loss of heterozygosity (LOH) was not only inversely correlated with N-myc amplification but also positively associated with advanced stage disease, unfavorable histopathology, and decreased overall survival

probability in patients lacking N-myc amplification (Guo *et al.*, 1999; Maris *et al.*, 2001). Therefore, loss of 11q might be a useful outcome predictor in clinically high-risk patients without N-myc amplification. Altered expressions of genes have also been associated with clinical behavior in neuroblastoma. Expression of the high-affinity nerve growth factor receptor (TrkA) gene is inversely correlated with N-myc amplification and high levels of its expression are strongly associated with low-stage tumors and a favorable outcome (Nakagawara *et al.*, 1992; Suzuki *et al.*, 1993; Nakagawara *et al.*, 1993; Borrello *et al.*, 1993; Tanaka *et al.*, 1995; Tanaka *et al.*, 1998).

1.1.6 Standard Treatment Modalities for Neuroblastoma

The conventional treatment modalities in the management of neuroblastoma include surgery, chemotherapy and radiotherapy. However, the use of each is determined by the anticipated clinical behavior of the tumor in individual cases regarding the age of patients at diagnosis, tumor location, stage of disease and biologic features of the tumors.

1.1.6.1 Surgery

Surgical removal of the tumor is the most effective treatment for neuroblastoma. The majority of localized tumors can be managed with surgery alone as primary therapy, even if complete gross resection is not feasible. A recent study conducted by the Children's Cancer Group showed that surgery alone is sufficient initial therapy

for almost all patients with Evans stage I and stage II disease, with overall survival (OS) of 99% for stage I and 98% for stage II patients, respectively (Perez *et al.*, 2000). However, multimodal therapy (such as chemotherapy and radiation) should be reserved for those who develop progressive disease. Due to the difficulty in removing some stage 3 and many stage 4 tumors, it has become a common practice to delay attempts at definitive surgical removal of the primary tumor until after several courses of chemotherapy (Reynolds & Seeger, 2001). Complications generally are lower for delayed or second-look procedures, after tumor shrinkage by chemotherapy (Brodeur & Maris, 2002). It has been suggested that delayed surgery on the primary tumor should be done between the second to fourth cycle of a dose-intense regimen since neuroblastoma tumor volumes were shown to regress according to first-order kinetics and reach a plateau after the second to fourth cycle of chemotherapy in most patients (Haase & LaQuaglia, 2003). Moreover, surgery also plays a pivotal role for diagnosis. The goals of primary surgical procedures, performed before any therapy, are to provide tissue for biologic studies and to stage the tumor surgically (Brodeur & Maris, 2002).

1.1.6.2 Chemotherapy

Chemotherapy is the predominant modality for patients with intermediate- or high-risk disease. A number of drugs have been shown to be active against neuroblastoma as single agents and in various combinations. These include cyclophosphamide, ifosfamide, cisplatin, carboplatin, iproplatin, doxorubicin, teniposide, etoposide, L-phenylalanine mustard, vincristine, dacarbazine, and topotecan (Reynolds & Seeger, 2001). The commonly used agents are

cyclophosphamide, ifosfamide, vincristine, doxorubicin, teniposide, etoposide, cisplatin and carboplatin. Induction therapy attempts to deplete tumor cells rapidly, arrest tumor growth and prevent or impede tumor spread. A consolidation phase is given to eradicate residual tumor (Cullen, 2003). Multiagent regimens can be used for stage 3 patients who may not undergo consolidation but require aggressive cytoreductive chemotherapy before undergoing delayed surgery. Aggressive multiagent chemotherapy is used for high-risk patients but the chances of long-term survival for such patients treated with chemotherapy are not high. However, the probability of long-term survival can be improved for many high-risk patients by using consolidation with myeloablative chemoradiotherapy and autologous hematopoietic stem cell transplantation (Reynolds & Seeger, 2001).

1.1.6.3 Radiotherapy

Neuroblastoma is considered to be a radiosensitive tumor. Although radiotherapy is used infrequently, it may help to control the regional tumors of stage 3 patients over one year of age (Cullen, 2003). In advanced neuroblastoma, local relapse may be decreased by radiotherapy given to the primary site in combination with chemotherapy (Haase & LaQuaglia, 2003). Radiotherapy may also be used for stage 4 patients as an adjunct to myeloablative therapy prior to bone marrow transplantation (Cullen, 2003). When treatment with chemotherapy is ineffective, low-dose hepatic irradiation could be helpful in infants with stage 4S disease who develop respiratory distress secondary to hepatomegaly. Moreover, palliative radiotherapy may be useful to diminish bone pain in children with bone metastases (Brodeur & Maris, 2002; Haase & LaQuaglia, 2003).

1.1.7 Differentiation of Neuroblastoma *In Vivo* and *In Vitro*

The neural crest is an embryonic structure that is formed at the end of the third and beginning of the fourth week of human development. The neural crest cells migrate to many specific regions to form various structures, including the sympathetic nervous system (SNS). Neural crest cells are initially pluripotent but as they migrate from the neural tube, their pluripotency is gradually lost and a variety of structures are formed, depending on the inductive environment they encounter. Some ventrally migrating neural crest cells develop into the sympathoadrenal progenitor cells which subsequently differentiate into the three major adrenergic cell types (ganglionic cells, small intensely fluorescent (SIF) cells and chromaffin cells) constituting the SNS (Pahlman & Hedborg, 2000).

Neuroblastomas are tumors that arise from the neural crest-derived multipotent peripheral neuroblasts that are blocked in their differentiation (Pahlman & Hedborg, 2000). Despite its malignancy, neuroblastoma has the highest rate of spontaneous regression of any human tumor and maturation into a benign tumor known as ganglioneuroma can be observed in some patients (Westermann & Schwab, 2002; Brodeur, 2003). These clinical observations have stimulated studies of neuroblastoma differentiation *in vitro*. It has been reported that human neuroblastoma cell lines can differentiate along several pathways into cells with morphologically distinct phenotypes, with characteristics similar to the neuronal cells or to the immature Schwannian, glial or melanocytic cells (Abemayor & Sidell, 1989). Induced differentiation has been documented in neuroblastoma cell lines treated with a variety of agents including retinoic acid (RA), nerve growth factor, phorbol ester,

interferon-γ, and cAMP elevating agents (Sidell, 1982; Pavelic & Spaventi, 1987; Wu *et al.*, 1998; Guzhova *et al.*, 2001). These *in vitro* studies suggest that agents that induce differentiation of the neuroblastoma cells can reverse the malignant state or suppress the tumorigenic phenotype. Among these differentiation inducers, retinoic acid appears to be quite potent and its effectiveness as a therapeutic agent has been evaluated in clinical trials. The exposure of some neuroblastoma cells to nanomolar to micromolar concentrations of all-*trans* retinoic acid (ATRA), 13-*cis* or 9-*cis* RA induces dramatic morphological and biochemical changes including neurite extension, increases in the biosynthesis of neurospecific enzymes (such as acetylcholinesterase), neurotransmitters (such as catecholamines) and up-regulation of the 180 kDa neuronal cell adhesion molecule (NCAM) isoform expression (Raschella *et al.*, 2000). More recently, neuroblastoma differentiation-inducing property has been demonstrated in some natural products. Euxanthone isolated from the medicinal plant *Polygala caudata* was shown to be able to inhibit the growth and trigger differentiation in the murine neuroblastoma Neuro-2a, BU-1 cells (Mak *et al.*, 2000). On the other hand, pyridoacridine alkaloids isolated from the Indonesian marine sponge *Biemna fortis* was found to be capable of inducing differentiation in the Neuro-2a cells and arresting the cell cycle at the G_2/M phase (Aoki *et al.*, 2003). It seems that natural products are rich sources of bioactive substances that can be used as a differentiation inducer in neuroblastoma.

1.1.8 Differentiation Therapy of Neuroblastoma

Although the conventional modalities will continue to be important in the treatment of neuroblastoma, biologically based therapies will be used increasingly in the treatment of neuroblastoma. Retinoic acid derivatives have been shown to induce differentiation and slow the growth of neuroblastoma cells in culture (Sidell, 1982; Reynolds *et al.*, 1994; Lovat *et al.*, 1994; Han *et al.*, 1995). The finding that treatment of high-risk neuroblastoma patients with 13-*cis*-retinoic acid after bone marrow transplantation or after continuation chemotherapy significantly improved the event-free survival of patients provides a promising prospect of differentiation therapy of neuroblastoma, especially for the minimal residual disease (Matthay *et al.*, 1999). Induction of differentiation would seem to be a promising approach for the management of neuroblastoma in the future. It is hoped that more effective and less toxic differentiation-inducing agents could be found.

1.2 Glycyrrhizin — the Major Active Component of Licorice

1.2.1 Chemistry of Licorice

Licorice (Figure 1.1) has a long history and was used by the ancient Chinese, Egyptians and Greeks. The generic name for licorice is *Glycyrrhiza.* This name is derived from the Greek words meaning "sweet root" and the Chinese name "gancao" also means "sweet grass". This genus consists of 30 species and many of them are found in China. In traditional Chinese medicine, Radix glycyrrhizae is defined as *Glycyrrhiza uralensis, G. glabra* or *G. inflata* (Mills & Bone, 2000; Wang & Nixon, 2001). The licorice with which we are familiar in the Western part of the world comes from the plant *Glycyrrhiza glabra.* This plant is indigenous to Greece, Turkey, Spain, Iraq, Caucasian and Transcaspian Russia, and northern China (Davis & Morris, 1991).

Licorice is one of the oldest and most frequently used drugs in traditional Chinese medicine. It possesses various pharmaceutical functions, such as detoxification, anti-ulcer, anti-inflammation, anti-viral, anti-atherogenic and anti-carcinogenic functions etc. It is recommended for its life-enhancing properties, for improving health, for cure of injury or swelling, and for its detoxification effects (Wang & Nixon, 2001). In traditional Chinese medicine, licorice is primarily used as a tonic, an antipyretic, an antidote, a demulcent to the lungs, an expectorant, an analgesic to soothe sore throats and coughs, to treat asthma, and to alleviate toxic abscesses and acute abdominal pains (Davis & Morris, 1991). In Western countries, licorice products are used as flavoring and sweetening agents for tobaccos, chewing gums, candies, toothpaste, and beverages (Wang & Nixon, 2001).

Licorice root (Figure 1.2) contains a variety of compounds including essential oils, alkaloids, polysaccharides, polyamines, triterpenes, and flavonoids. Glycyrrhizin, also known as glycyrrhizic acid or glycyrrhizinic acid, is a major component of roots and rhizomes of licorice. It is a triterpenoid saponin that is composed of a molecule of glycyrrhetinic acid and two molecules of glucuronic acid. The intense sweetness of licorice resides mainly in glycyrrhizin (present in form of potassium and calcium salts). Glycyrrhizin is approximately between 50 and 170 times as sweet as sucrose. The content of glycyrrhizin in the licorice root depends on the source and the method of assay and varies from 6 - 14%. 18β-glycyrrhetinic acid, the aglycone of glycyrrhizin, is also present in the root of licorice (0.5% - 0.9%). Moreover, glycyrrhizin is hydrolyzed by glucuronidase to its aglycone which exists in 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid stereoisomeric forms (Huang, 1999; Mills & Bone, 2000; Wang & Nixon, 2001). The metabolism of glycyrrhizin will be discussed in a later section. The structures of glycyrrhizin and 18β-glycyrrhetinic acid are shown in Figure 1.3.

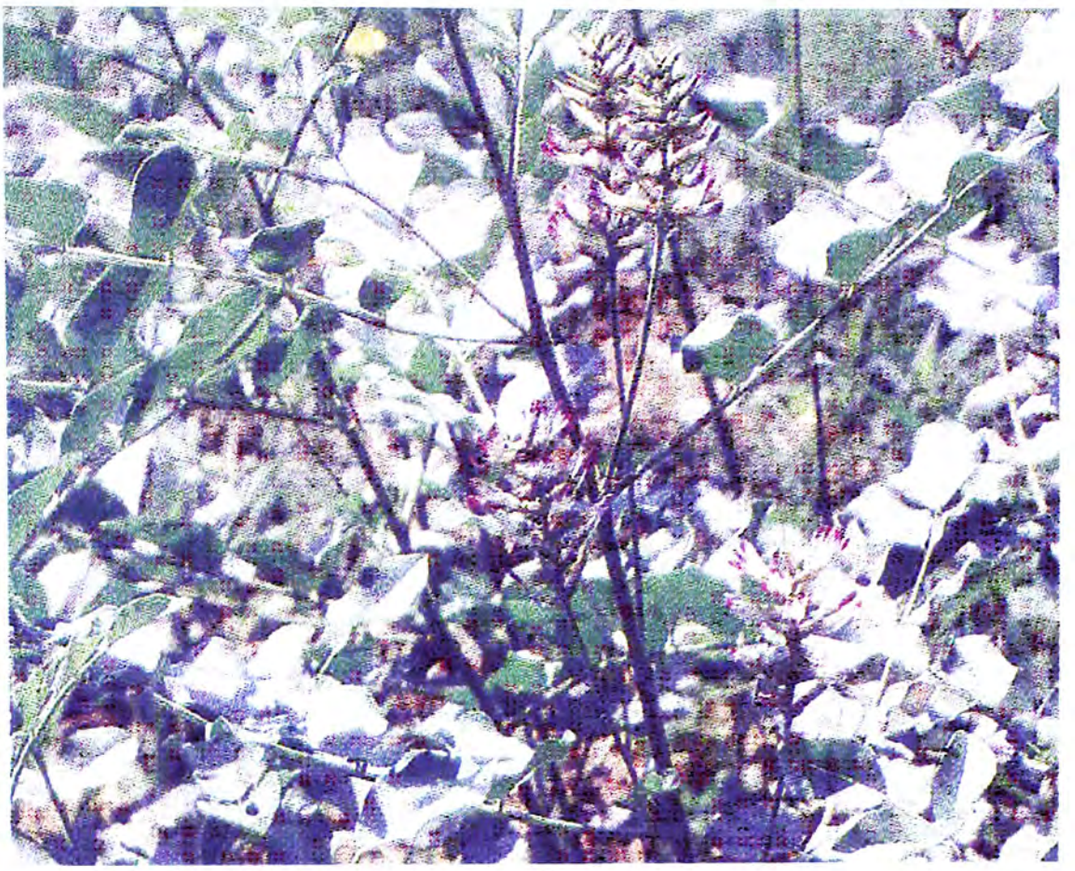
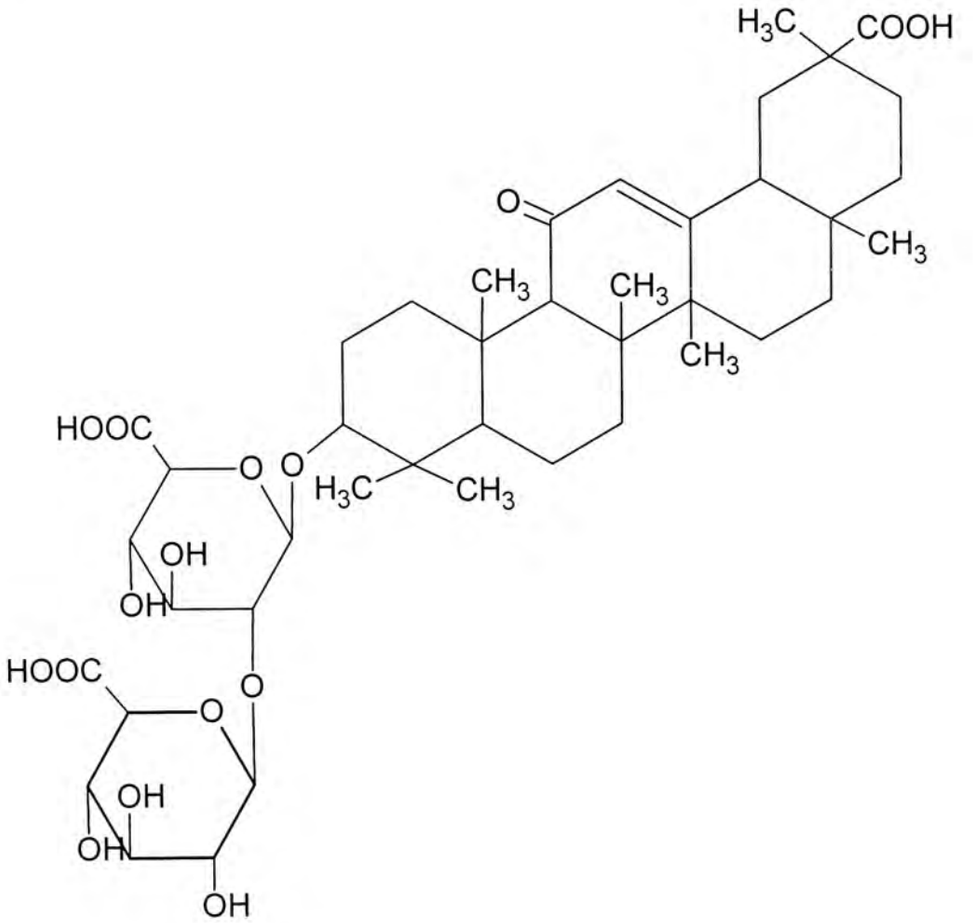


Figure 1.1: Licorice. Adapted from (Yang & Liu, 2001)

Figure 1.2: Rhizome of licorice. Adapted from (Zhongguo yi yao za zhi she, 1995)

A



B

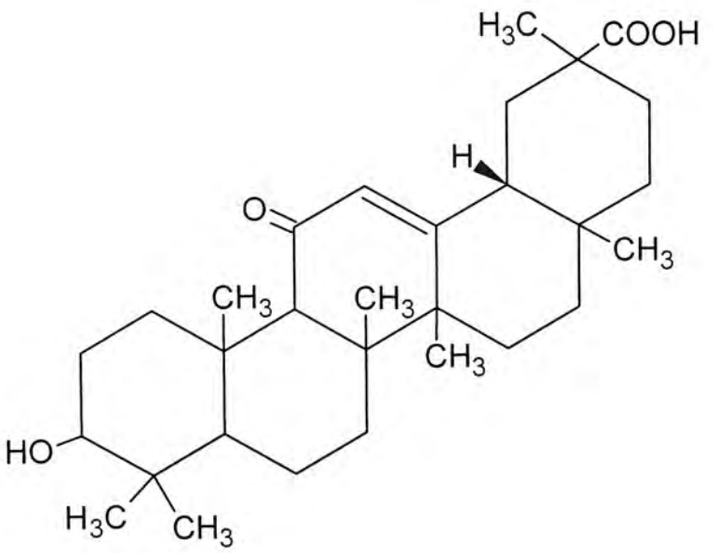


Figure 1.3: The chemical structures of (A) glycyrrhizin and (B) 18β-glycyrrhetinic acid.

1.2.2 Metabolism of Glycyrrhizin

The metabolism of glycyrrhizin was believed to take place in the liver and the large intestine. After oral administration of glycyrrhizin, it is almost completely hydrolyzed to glycyrrhetinic acid by specialized β-glucuronidase of the intestinal bacteria. Glycyrrhetinic acid formed will be transformed partly into 3α-hydroxyglycyrrhetinic acid via a metabolic intermediate 3-oxo-glycyrrhetinic acid by the intestinal bacteria. The glycyrrhetinic acid is then absorbed in the intestine, entered the systemic circulation and delivered to the liver where it is conjugated to glucuronide or sulphate prior to biliary excretion. These glycyrrhetinic acid metabolites are subsequently excreted into the bile. After outflow of the bile into the duodenum, the conjugates are hydrolyzed to glycyrrhetinic acid again by the commensal bacteria and the glycyrrhetinic acid is subsequently reabsorbed. This enterohepatic cycling of glycyrrhetinic acid is the cause of delayed terminal plasma clearance of glycyrrhetinic acid (Gunnarsdottir & Johannesson, 1997; Mills & Bone, 2000; Ploeger *et al.*, 2001; Okamura *et al.*, 2003).

After intravenous administration of glycyrrhizin, it is metabolized in the liver by lysosomal β-glucuronidase to glycyrrhetinic acid monoglucuronide (GAMG) via the removal of one glucuronic acid. GAMG is then excreted into bile and converted to glycyrrhetinic acid by intestinal bacteria via the removal of another glucuronic acid. The glycyrrhetinic acid formed is then absorbed as described previously. Therefore, after intravenous administration of glycyrrhizin, both glycyrrhizin and glycyrrhetinic acid could be found in the plasma (Akao *et al.*, 1991; Akao, 1998; Kim *et al.*, 1999; Shibata, 2000).

1.2.3 Pharmacological Effects of Glycyrrhizin and 18β-Glycyrrhetinic Acid

There have been a variety of studies on the pharmacological effects of licorice. The pharmacological activities of licorice are mainly, if not all, due to the main saponin, glycyrrhizin and its aglycone, glycyrrhetinic acid. Therefore, much of the focuses have been placed on glycyrrhizin and glycyrrhetinic acid. Although glycyrrhetinic acid lacks sweetness, it shares many diverse pharmacological effects with glycyrrhizin.

1.2.3.1 Anti-inflammatory Effect

The anti-inflammatory effect of glycyrrhizin and 18β-glycyrrhetinic acid has been demonstrated in a number of animal models (Mills & Bone, 2000). An earlier animal study demonstrated that oral administration of 18β-glycyrrhetinic acid to rats resulted in inhibition of carrageenan-induced inflammation (Capasso *et al.*, 1983). Another study demonstrated that glycyrrhetinic acid was effective in inhibiting TPA-induced mouse ear edema (Inoue *et al.*, 1989). The mode of actions of glycyrrhizin and 18β-glycyrrhetinic acid is still unclear yet. However, several possible mechanisms behind the anti-inflammatory activity have been proposed. Due to the observation that glycyrrhizin inhibited prostaglandin E2 production by activated rat peritoneal macrophages, it had been proposed that the anti-inflammatory activity of glycyrrhizin depends in part on its inhibitory effect on the formation of prostaglandin E2 (Ohuchi *et al.*, 1981). Another study showed that glycyrrhizin decreased neutrophils-generated reactive oxygen species in a dose-dependent manner, suggesting that glycyrrhizin exerts anti-inflammatory activity by inhibiting the

generation of reactive oxygen species by neutrophils (Akamatsu *et al.*, 1991). Due to the structural resemblance between 18β-glycyrrhetinic acid and corticosteroids, an action mechanism of 18β-glycyrrhetinic acid similar to that of glucocorticoids has been raised. However, a recent report showed that 18β-glycyrrhetinic acid is a potent inhibitor of the classical complement pathway and it acts at the level of complement component C2 but naturally occurring steroids, such as hydrocortisone and cortisone do not have anti-complementary activity. Therefore, the anti-complementary activity of 18β-glycyrrhetinic acid is likely to contribute to its anti-inflammatory effect (Kroes *et al.*, 1997). Most recently, the complement C3 was found to be a glycyrrhizin (GL)-binding protein and the glycyrrhetinic acid- and GL-binding domains were located to C3a. Moreover, the phosphorylation of C3α (p115) by casein kinase 2 (CK-2) was completely inhibited by glycyrrhizin and glycyrrhetinic acid. Therefore, the glycyrrhizin- and glycyrrhetinic acid-induced inhibitions of the physiological activities of C3a and C3α may partly explain their anti-inflammatory effect *in vivo* since complement plays an important role in many acute inflammatory processes (Kawakami *et al.*, 2003).

1.2.3.2 Hepatoprotective Effect

In Japan, glycyrrhizin preparation under the name of Stronger Neo-Minophagen C (SNMC) has been used clinically for decades as an anti-allergic and anti-hepatitis agent. SNMC is an intravenous drug containing glycyrrhizin as a principal ingredient along with glycine and L-cysteine. Although no anti-viral effect has been attributed to SNMC in the treatment of chronic viral hepatitis, SNMC can significantly decrease patient's serum levels of alanine transaminase (ALT) (Ito *et al.*,

1997; van Rossum *et al.*, 1999; Miyake *et al.*, 2002). Moreover, long-term administration of SNMC for chronic hepatitis C was reported to be effective in the reduction of hepatocellular carcinoma development (Arase *et al.*, 1997). Study in rats also showed that both glycyrrhizin and 18β-glycyrrhetinic acid had a hepatoprotective activity on carbon tetrachloride (CCl_4)-induced hepatotoxicity of rats. The serum aspartate transaminase (AST) and ALT levels of rats treated with glycyrrhizin and 18β-glycyrrhetinic acid orally and intraperitoneally, respectively were significantly lower than those of the CCl_4-treated control group (Shim *et al.*, 2000). Although various pharmacological actions of glycyrrhizin such as anti-inflammatory activity, interferon-γ-inducing activity and corticosteroid-enhancing activity are considered as mechanisms by which glycyrrhizin lowers the transaminase levels, other mechanisms have been proposed for the hepatoprotective effect of glycyrrhizin and 18β-glycyrrhetinic acid. The protective function has recently been explained by the inhibitory effects of glycyrrhizin on immune-mediated cytotoxicity against hepatocytes and on nuclear factor (NF)-kappa B activity in the murine liver injury induced by CCl_4-ethanol (Yoshikawa *et al.*, 1997; Wang *et al.*, 1998). Furthermore, the finding that glycyrrhizin can inhibit the TNF-α-induced apoptosis in human hepatoblastoma HepG2 cells has raised the possibility that glycyrrhizin may inhibit the hepatocyte apoptotic cell death mediated by TNF-α receptor 1 that has been believed to be involved in the pathogenesis of chronic viral hepatitis (Yoshikawa *et al.*, 1999). More recent finding suggests that glycyrrhizin and 18β-glycyrrhetinic acid may improve liver failure by stimulating liver regeneration in acute or chronic hepatitis since the growth-promoting effects of glycyrrhizin and 18β-glycyrrhetinic acid on adult rat hepatocytes via epidermal growth factor (EGF) receptors have been demonstrated *in vitro* (Kimura *et al.*, 2001).

1.2.3.3 Anti-carcinogenic and Anti-tumor Effects

During the past few decades, animal and cell culture studies indicated that glycyrrhizin and glycyrrhetinic acid have inhibitory activities against certain tumors. An earlier animal study showed that glycyrrhetinic acid inhibited the tumor promoting activity of both TPA and teleocidin on skin tumor formation in ICR mice initiated with 7,12-dimethylbenz[α]anthracene (DMBA). The percentage of tumor-bearing mice of the group treated with DMBA plus TPA was 97% at week 20, whereas that of the group treated with DMBA plus TPA and glycyrrhetinic acid was 40%. The percentage of tumor-bearing mice in the group treated with DMBA and teleocidin was 88% at week 18, whereas that of the group treated with DMBA plus teleocidin and glycyrrhetinic acid was 6% (Nishino *et al.*, 1986). In another animal study, chronic oral feeding of 0.05% glycyrrhizin in drinking water to Sencar mice resulted in substantial protection against DMBA-initiated and TPA-promoted skin tumorigenesis. Oral feeding of glycyrrhizin in drinking water also resulted in inhibition in the binding of topically applied [^{3}H]benzo[α]pryene and [^{3}H]DMBA to epidermal DNA and the authors raised the possibility that the anti-tumor-initiating activity of glycyrrhizin may be due to the involvement of glycyrrhizin as an inhibitor of the carcinogen metabolism and DNA adduct formation that finally lead to carcinogenesis (Agarwal *et al.*, 1991). The same group also demonstrated that glycyrrhetinic acid inhibited the mutagenicity of benzo[α]pyrene, 2-aminofluorene and aflatoxin B1 in *Salmonella typhimurium* strain TA98 ad TA100. Moreover, both 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid inhibited the DMBA-induced skin tumor-initiating activity but 18β-glycyrrhetinic acid was shown to be more effective than 18α-glycyrrhetinic acid as an anti-skin tumor initiating agent. However,

18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid showed comparable effect as inhibitors of TPA-induced skin tumor promotion in mice (Wang *et al.*, 1991). Besides the skin tumor, animal study also showed that long-term intramuscular treatment of mice with glycyrrhizin significantly decreased the occurrence of hepatocellular carcinoma in diethylnitrosamine-treated mice (Shiota *et al.*, 1999). Furthermore, intraperitoneal administration of glycyrrhizin (10 mg/kg) was shown to be able to inhibit the incidence of pulmonary metastases of B16 melanoma in mice inoculated with B16F10 melanoma cells. Similarly, 84% of metastases were inhibited in those mice after they were adoptively transferred with splenic $CD4^+$ T cells from mice treated with glycyrrhizin but not with normal $CD4^+$ T cells. Since tumor-associated T helper type 2 cells (Th2) play a role in the pulmonary metastasis of B16 melanoma cells, the authors proposed that glycyrrhizin inhibited pulmonary metastasis in melanoma inoculated mice by inducing antagonistic cells for Th2 cells (Kobayashi *et al.*, 2002).

The tumor suppression activity of glycyrrhizin and glycyrrhetinic acid could also be demonstrated by *in vitro* studies. An earlier study showed that both glycyrrhizin and glycyrrhetinic acid inhibited the growth of B16 melanoma cells and stimulated the melanogenesis and the growth inhibition of glycyrrhetinic acid was correlated with the inhibition of cell cycle progression from G_1 to S phase (Abe *et al.*, 1987). Another *in vitro* study also demonstrated that glycyrrhizin inhibited the proliferation of the mice submandibular gland fibrosarcoma cell line by blocking the transfer of cells from G_1 to S phase (Ge *et al.*, 1998). One study showed that glycyrrhizin, 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid displayed differential growth inhibitory effects on lymphoma and leukemia cell lines (Malagoli *et al.*, 1998). A

more recent study reported that glycyrrhizin and 18β-glycyrrhetinic acid exhibited cytotoxicity against a number of tumor cell lines including SNU C4, SNU-1, P-388, L-1210, HepG2 and A549 (Kim *et al.*, 2000). In addition, previous study in our laboratory showed that glycyrrhizin and 18β-glycyrrhetinic acid inhibited the growth of human and murine leukemia cell lines by inducing apoptosis and/or differentiation in leukemia cells (Tsang, 2001).

1.2.3.4 Anti-viral Effect

Direct anti-viral effect of glycyrrhizin has been demonstrated by *in vitro* studies. An earlier study reported that 8 mM of glycyrrhizin inhibited both the growth and cytopathic effects of vaccinia, herpes simplex type 1, Newcastle disease and vesicular stomatitis viruses (Pompei *et al.*, 1979). Apart from inhibiting the growth of several viruses, glycyrrhizin also produces irreversible inactivation of herpes simplex virus type 1 (Pompei *et al.*, 1979; Pompei *et al.*, 1980). Glycyrrhizin also possesses effective anti-viral properties against the varicella-zoster virus (VZV) (Baba & Shigeta, 1987), human immunodeficiency virus (HIV) (Ito *et al.*, 1987; Ito *et al.*, 1988; Sasaki *et al.*, 2002), hepatitis A virus (HAV) (Crance *et al.*, 1990; Crance *et al.*, 1994), human cytomegalovirus (HCMV) (Numazaki *et al.*, 1994), Japanese encephalitis virus (JEV) (Badam, 1997), sandfly fever Sicilian virus (SFSV) (Crance *et al.*, 1997) and flaviviruses (Crance *et al.*, 2003). More recently, glycyrrhizin has been shown to be more effective than several other anti-viral drugs in inhibiting the replication of the severe acute respiratory syndrome-associated coronavirus (SARS-CV) in Vero cells (Cinatl *et al.*, 2003). In addition to the inhibition of SARS-CV replication, glycyrrhizin also prevented the adsorption and penetration of the virus into Vero cells.

The mechanisms by which glycyrrhizin can exhibit anti-viral activity against different viruses remain unclear. It has been suggested that direct binding of glycyrrhizin to the vesicular stomatitis virus causes the direct inactivation of virus-associated kinase and the reduction of the viral infectivity (Ohtsuki & Iahida, 1988). Crance *et al.* (1994) also reported that glycyrrhizin inhibited the early stage of HAV replication and the HAV penetration of the plasma membrane. Moreover, the abilities of glycyrrhizin to suppress the secretion of hepatitis B surface antigen (HBsAg), to interfere with the intracellular transport of HBsAg at the trans-Golgi area and to suppress sialylation of HBsAg have been demonstrated by *in vitro* studies (Takahara *et al.*, 1994; Sato *et al.*, 1996). In the same study, glycyrrhetinic acid was found to be more effective than glycyrrhizin in suppressing the secretion of HBsAg (Sato *et al.*, 1996). Glycyrrhizin might also inhibit HIV-1 *in vivo* as suggested by one human clinical study that intravenous administration of glycyrrhizin to three hemophiliacs with acquired immune deficiency syndrome (AIDS) reduced the viral antigens levels in these patients (Hattori *et al.*, 1989). It is believed that glycyrrhizin may exert its anti-HIV-1 activity by inhibiting the virus replication and the virus-cell binding (Ito *et al.*, 1988).

On the other hand, two recent animal studies have demonstrated that the anti-viral effect of glycyrrhizin may be indirect. One animal study showed that when thermally injured mice were treated intraperitoneally with glycyrrhizin, the resistance of these mice to herpes simplex virus type 1 (HSV-1) was improved to levels observed in normal mice. In addition, adoptive transfer of splenic mononuclear cells from normal mice treated with glycyrrhizin to thermally injured mice (recipients) resulted in improved resistance of recipients to HSV infection

(Utsunomiya *et al.*, 1995). Utsunomiya *et al.* (1997) also reported that when splenic T cells from glycyrrhizin-treated mice were adoptively transferred to mice exposed to influenza virus, 100% of the recipients survived, compared to 0% survival for recipient mice inoculated with naive T cells or splenic B cells and macrophages from glycyrrhizin-treated mice. Moreover, the anti-viral activity of glycyrrhizin on influenza virus infection in mice was abolished when it was administered to infected mice in combination with anti-gamma interferon (anti-IFN-γ) monoclonal antibody, suggesting that glycyrrhizin may protect mice exposed to a lethal amount of influenza virus through the stimulation of interferon-gamma (IFN-γ) production by T cells (Utsunomiya *et al.*, 1997). Therefore, these *in vitro* and *in vivo* studies suggest that the anti-viral effect of glycyrrhizin may exert through its direct inhibitory action on viral replication and function, and indirectly by stimulating the host immune system.

1.2.3.5 Immunomodulatory Effect

Various immunomodulatory effects of glycyrrhizin and glycyrrhetinic acid have been reported. An earlier report showed that intravenous administration of glycyrrhizin or glycyrrhetinic acid induced IFN-γ production in mice (Abe *et al.*, 1982). However, another report showed that lymphocyte-macrophage culture treated with 10 – 100 μg/ml of glycyrrhizin alone did not produce interferon whereas similar glycyrrhizin treatment significantly enhanced the IFN-γ production in response to concanavalin A and surface antigen of hepatitis B virus (Shinada *et al.*, 1986). Besides, Zhang *et al.* (1993) reported that glycyrrhizin promotes receptor-mediated T-cell proliferation through the augmentation of interleukin-2 (IL-2) production and IL-2 receptor (IL-2R) expression and the primary site of action for the augmented

IL-2 production is after the activation of immediate early genes. In addition, previous studies demonstrated that nitric oxide (NO) production was enhanced in lipopolysaccharide (LPS)- or IFN-γ-activated macrophages isolated from glycyrrhizin-treated mice although glycyrrhizin alone did not induce NO from resting macrophages (Kondo & Takano, 1994; Yi *et al.*, 1996). On the contrary, 18β-glycyrrhetinic acid significantly elicited a dose-dependent increase in NO production in the absence of any stimulator and the increased production of NO was mediated by the up-regulation of inducible nitric oxide synthase (iNOS) expression via NF-κB transactivation in macrophages (Jeong & Kim, 2002). Based on the relationship between NO production and the anti-viral and anti-tumor function of macrophages, it has been suggested that the anti-viral and anti-tumor effects of glycyrrhizin and 18β-glycyrrhetinic acid *in vivo* might be mediated in part through the activation of NO production (Kondo & Takano, 1994; Jeong & Kim, 2002). Furthermore, a recent study showed that glycyrrhizin enhanced both IL-12 messenger RNA accumulation and protein secretion by peritoneal macrophages in response to LPS, and the priming effect of glycyrrhizin on IL-12 production did not depend on IFN-γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Dai *et al.*, 2001). Therefore, glycyrrhizin and 18β-glycyrrhetinic acid seem to have an important potential in activating certain immune functions.

1.2.3.6 Mineralocorticoid Effect

It has been observed that long-term administration of licorice or glycyrrhizin in humans may cause pseudohyperaldosteronism. Based on the structural similarities between glycyrrhetinic acid and the corticosteroid hormones, the mineralocorticoid

effect of glycyrrhizin had been proposed to be mediated by the direct binding of glycyrrhetinic acid to the mineralocorticoid receptor. However, the affinity of glycyrrhetinic acid for the mineralocorticoid receptor was found to be about 3,000 and 10,000 times lower than that of aldosterone itself in human mononuclear leucocytes and in renal tissue of rats, respectively. Moreover, licorice does not exert its mineralocorticoid activity in adrenalectomised animals. These observations indicate that direct binding of glycyrrhetinic acid to mineralocorticoid receptors is unlikely the predominant mode of action (Armanini *et al.*, 1983; Stewart *et al.*, 1987; Armanini *et al.*, 1989). The mineralocorticoid effect is however, mainly due to the inhibition of 11β-hydroxysteroid dehydrogenase activity by glycyrrhetinic acid (Stewart *et al.*, 1987; Monder *et al.*, 1989). The mineralocorticoid receptors have equal affinity for the aldosterone and for the glucocorticoids (cortisol and corticosterone), which circulate at much higher concentrations than aldosterone. In mineralocorticoid target tissues (kidney, parotid and colon), the mineralocorticoid receptors are selective for aldosterone because of the presence of 11β-hydroxysteroid dehydrogenase which converts cortisol and corticosterone to their 11-keto analogs (cortisone and 11β-dehydrocorticosterone) that cannot bind to mineralocorticoid receptors (Funder *et al.*, 1988). Inhibition of 11β-hydroxysteroid dehydrogenase by 18β-glycyrrhetinic acid thus leads to increased cortisol levels and exerts the mineralocorticoid effect by the binding of cortisol to the mineralocorticoid receptors. In addition, a more recent study showed that chronic high dose of glycyrrhizin (120 mg/kg of glycyrrhizin twice a day for 2 weeks) given to rats not only inhibited the 11β-hydroxysteroid dehydrogenase activity but also suppressed the mRNA and protein expression of 11β-hydroxysteroid dehydrogenase (Tanahashi *et al.*, 2002).

1.2.4 Pharmacokinetics of Glycyrrhizin and Glycyrrhetinic Acid

When 80 mg glycyrrhizin was injected into normal subjects, the maximum level of glycyrrhizin in serum was found to be 10 - 15 μg/ml, with a half-life of about 5 hours. Glycyrrhetinic acid appeared after 6 hours with a maximum level of about 150 ng/ml at 15 hours. However, when normal subjects were given 100 mg glycyrrhizin orally, a glycyrrhetinic acid level of about 50 - 500 ng/ml (100 – 1000 nM) was detected in serum. The maximum level of glycyrrhetinic acid in serum was detected between 2 and 25 hours in different individuals (Stormer *et al.*, 1993). Another study reported that glycyrrhetinic acid (< 200 ng/ml) but not glycyrrhizin appeared in plasma of healthy volunteers after oral administration of 100 mg glycyrrhizin and the appearance of glycyrrhetinic acid in the blood after oral administration of glycyrrhizin was due to the hydrolysis of glycyrrhizin by the intestinal bacteria. Moreover, the same study showed that the plasma clearance of glycyrrhizin was not dose-dependent for intravenous doses of 40 - 120 mg. For the intravenous administration of glycyrrhizin (40, 80 and 120 mg), the terminal half-life of glycyrrhizin was 2.7 – 4.8 hours (Yamamura *et al.*, 1992). Another pharmacokinetic study in 10 healthy young volunteers reported that a maximum serum level of 18β-glycyrrhetinic acid (6.3 μg/ml) was reached 2 - 4 hours after ingestion of 500 mg 18β-glycyrrhetinic acid. Moreover, seven subjects still had 18β-glycyrrhetinic acid with a mean level of 0.33 μg/ml 24 hours after ingestion (Heilmann *et al.*, 1997). As stated in the literatures, the pharmacokinetics of glycyrrhizin and glycyrrhetinic acid are characterized by a biphasic elimination from the central compartment with a dose-dependent second elimination phase. After oral dose of 1,000 mg and 1,500 mg of 18β-glycyrrhetinic acid, the mean half-life of the

second elimination phase was 11.5 ± 1.2 hours and 38.7 ± 10.5 hours, respectively (Krahenbuhl *et al.*, 1994a). The peak plasma concentration increased with increasing 18β-glycyrrhetinic acid doses. The major part of both glycyrrhizin and glycyrrhetinic acid is eliminated via the bile and the urinary elimination of glycyrrhizin and glycyrrhetinic acid over 24 hours was less than 1% of the dose administered. Glycyrrhizin can be eliminated unmetabolized while glycyrrhetinic acid is conjugated to glucuronide or sulfate prior to biliary excretion. Orally administered glycyrrhizin is almost completely hydrolyzed by intestinal bacteria and reaches the systemic circulation as glycyrrhetinic acid (Krahenbuhl *et al.*, 1994a; Krahenbuhl *et al.*, 1994b). Liver function has been shown to influence the plasma clearance of glycyrrhizin after chronic intravenous administration to patients with acute hepatitis and liver cirrhosis. The elimination half-life for glycyrrhizin in the hepatitis (2.7 – 7.6 hours) and the cirrhosis (6.2 – 40.1 hours) groups was about twice and eight times longer than that in normal subjects, respectively (Yamamura *et al.*, 1995).

1.2.5 Health Hazards of Glycyrrhizin and Glycyrrhetinic Acid

Since glycyrrhizin is converted to glycyrrhetinic acid in the intestine and absorbed as glycyrrhetinic acid, the main hazards of licorice or glycyrrhizin administration are due to the mineralocorticoid effects of glycyrrhetinic acid. Chronic administration of licorice or glycyrrhizin in humans may cause pseudohyperaldosteronism. This syndrome is characterized by sodium retention, potassium loss, low aldosterone level, depression of the rennin-angiotensin system

and increased levels of the atrial natriuretic hormone (ANH). A number of clinical signs such as edema, hypertension, headache and symptoms of hypokalemia were observed (Stormer *et al.*, 1993; Huang, 1999; Shibata, 2000). There is apparently a great individual variation in the susceptibility to glycyrrhizin. Most individuals who consume 400 mg glycyrrhizin daily experience adverse effects. In the most sensitive individuals, a regular daily intake of no more than about 100 mg glycyrrhizin is enough to produce the adverse effects (Stormer *et al.*, 1993). Moreover, people with hypertension are more sensitive to the inhibition of 11β-hydroxysteroid dehydrogenase by licorice and the inhibition of 11β-hydroxysteroid dehydrogenase causes more pronounced clinical symptoms in women than in men (Sigurjonsdottir *et al.*, 2003). In a recent study, a no-effect level of 2 mg glycyrrhizin/kg body weight is proposed. The acceptable daily intake (ADI) of 0.2 mg/kg body weight proposed lies below the quantity advised by the Dutch Nutrition Council of 200 mg/day and the relatively mild acute toxicity of glycyrrhizin may account for the large difference between the no-effect level, the doses of case reports and the quantity advised by the Dutch Nutrition Council (van Gelderen *et al.*, 2000). In addition, intravenous administration of glycyrrhizin up to 240 mg, thrice weekly for 4 weeks to patients with chronic hepatitis C appears to be safe and is well tolerated (van Rossum *et al.*, 1999). A more recent clinical study showed that intravenous administration of 200 mg glycyrrhizin (100 ml of SNMC) to patients with chronic hepatitis C six times per week, for 4 weeks only produced minor reversible symptoms of pseudoaldosteronism (van Rossum *et al.*, 2001). In fact, licorice was approved by the German Commission E to treat peptic ulcer at relatively high doses (200 – 600 mg glycyrrhizin daily) but for treatment period not exceeding 6 weeks. In USA, licorice is used as a food additive and is designated as "Generally Recognized as Safe"

(GRAS). In Japan, daily administration of glycyrrhizin is also a standard practice to treat chronic hepatitis (Newton & Cupp, 2000; Shibata, 2000).

1.3 Aims and Scopes of This Study

As stated in Section 1.2.3, a number of reports showed that glycyrrhizin and 18β-glycyrrhetinic acid possess many diverse pharmacological properties. However, there are very few studies on the action mechanisms of the direct anti-tumor activity of these two natural products on the cancer cells. In addition, to our knowledge, there has been no report demonstrating the effects of glycyrrhizin and 18β-glycyrrhetinic acid on the growth and differentiation of neuroblastoma cells. Thus, it is of great interest to study the direct anti-tumor activity of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells and to further elucidate the cellular and molecular mechanisms of the anti-tumor activity of these two compounds.

In my study, the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on one murine and two human neuroblastoma cell lines will be first examined by the tritiated thymidine (^{3}H-TdR) incorporation assay. In addition, the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on a well characterized and subcloned murine neuroblastoma Neuro-2a BU-1 cell line will also be assessed by the neutral red assay, clonogenic assay and limiting dilution assay. The kinetics and reversibility of glycyrrhizin and 18β-glycyrrhetinic acid with respect to their anti-proliferative effect on BU-1 cells will be determined by the ^{3}H-TdR incorporation assay. Moreover, the cytotoxicity of glycyrrhizin and 18β-glycyrrhetinic acid on the murine neuroblastoma BU-1 cells will be assessed by the trypan blue exclusion test. Furthermore, the ability of glycyrrhizin and 18β-glycyrrhetinic acid to induce apoptosis in the neuroblastoma BU-1 cells will be

measured by the DNA fragmentation assay and Hoechst 33342 staining.

Since there has been an increasing interest in studying the differentiation therapy of neuroblastoma, therefore, the ability of glycyrrhizin and 18β-glycyrrhetinic acid to trigger neuronal cell differentiation will also be investigated in my present study. The differentiation-inducing capacity of glycyrrhizin and 18β-glycyrrhetinic acid on the BU-1 cells will be assessed by the morphological, biochemical as well as molecular studies. Morphological changes of glycyrrhizin- or 18β-glycyrrhetinic acid-treated BU-1 cells will be examined by microscopic observation after cell staining. The biochemical changes of drug-treated BU-1 cells will be assessed by examining the expression of neuronal cell differentiation marker proteins by immunocytochemisty. The differentiation-related gene expression in BU-1 cells will also be examined at the molecular level by the RT-PCR technique.

To provide better insights into the mechanisms of the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on the BU-1 cells, the cell cycle kinetics of drug-treated BU-1 cells will be analyzed by flow cytometry. In addition, the effects of 18β-glycyrrhetinic acid on the expression of cell cycle regulatory genes and proteins will be further studied by the RT-PCR technique and Western blot, respectively. Furthermore, attempts will be made to investigate whether the combination of glycyrrhizin, 18β-glycyrrhetinic acid and all-*trans* retinoic acid will have additive or synergistic effect on the growth inhibition and differentiation of BU-1 cells. Finally, the possible involvements of protein kinase C (PKC) and protein kinase A (PKA) on the 18β-glycyrrhetinic acid-induced anti-proliferation and differentiation of BU-1 cells will be elucidated using the RT-PCR technique and by

the combined use of activators or inhibitors of PKC and PKA with 18β-glycyrrhetinic acid.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

1) Neuro-2a BU-1 (BU-1)

BU-1 is a subclone of the murine neuroblastoma Neuro-2a cell line which was established from a spontaneous tumor of a strain A albino mouse (Klebe & Ruddle, 1969). BU-1 cells were derived from the Neuro-2a cells (ATCC CCL-131) by a limiting dilution method as described previously (Mak *et al.,* 2000) and was a kind gift of Dr. N.K. Mak (Department of Biology, the Hong Kong Baptist University).

2) SK-N-DZ

SK-N-DZ (ATCC CRL-2149) purchased from the American Type Culture Collection (ATCC), USA, is a human neuroblastoma cell line which was derived in 1978 from a bone marrow metastasis from a child with poorly differentiated embryonal neuroblastoma.

3) SH-SY5Y

SH-SY5Y (ATCC CRL-2266) purchased from ATCC is a thrice cloned (SK-N-SH->SH-SY->SH-SY5->SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH which was established in 1970 from a metastatic bone tumor (Biedler *et al.*, 1978).

2.1.2 Cell Culture Media, Buffers and Other Reagents

1) Cell Culture Media

(a) RPMI 1640 Medium (Powder Form)

The powder form of RPMI 1640 medium (Gibco BRL Life Technologies, Inc.),

supplemented with 25 mM N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES) and 2 mM L-glutamine was used for the preparation of culture medium that was used for sub-culturing of BU-1 cells. The powder was dissolved in double distilled water and buffered with 2 g sodium bicarbonate ($NaHCO_3$) (Sigma Chemical Co.) per liter. The pH value of the medium was adjusted to 7.2 by 1 M HCl or NaOH (Sigma Chemical Co.). Thereafter, the medium was sterilized by filtration through a 0.22 μm Millipore filter and stored at 4°C until use.

(b) Dulbecco's Modified Eagle's Medium (DMEM Powder Form)

The powder form of DMEM medium (Gibco BRL Life Technologies, Inc.), supplemented with 4,500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, and 4 mg/L pyridoxine HCl was used for the preparation of culture medium that was used for sub-culturing of SK-N-DZ cells. The powder was dissolved in double distilled water and buffered with 1.5 g sodium bicarbonate ($NaHCO_3$) (Sigma Chemical Co.) per liter. The pH value of the medium was adjusted to 7.2 by 1 M HCl or NaOH (Sigma Chemical Co.) and the medium was then sterilized by filtration through a 0.22 μm Millipore filter and stored at 4°C until use.

(c) Minimum Essential Medium (MEM Powder Form)

The powder form of MEM medium (Gibco BRL Life Technologies, Inc.), supplemented with Earle's salts, 2 mM L-glutamine, and non-essential amino acids, was used for the preparation of culture medium which was used for sub-culturing of SH-SY5Y cells. The powder was dissolved in double distilled water and buffered with 2.2 g sodium bicarbonate ($NaHCO_3$) (Sigma Chemical Co.) per liter. The pH value of the medium was adjusted to 7.2 by 1 M HCl or NaOH (Sigma Chemical Co.)

and the medium was then sterilized by filtration through a 0.22 μm Millipore filter and stored at 4°C until use.

2) Serum Supplements

Fetal calf serum (FCS) (Gibco BRL Life Technologies, Inc.) was stored as 50 ml and 10 ml aliquots in sterile universal bottles. Heat-inactivated fetal calf serum (HI-FCS) was prepared by heating the FCS at 56°C for 30 minutes. The aliquots were then kept frozen at -20°C until use.

3) Antibiotic Mixture Solution (100x)

Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture 100x stock solution containing 5,000 μg/ml penicillin G (sodium salt), 5,000 μg/ml streptomycin sulfate and 10,000 μg/ml neomycin sulfate in 0.85% saline was purchased from Gibco BRL Life Technologies, Inc. It was stored at -20°C as 5 ml aliquots until use. 100x PSN was added to culture medium to give 1% antibiotics to prevent any possible contamination of cell culture.

4) Complete Medium

RPMI 1640, DMEM or MEM medium supplemented with 1% antibiotics was called "Plain Medium". Complete medium (CM) was prepared by supplementing the plain medium with 10% FCS or HI-FCS. CM supplemented with 10% FCS was used for sub-culturing murine and human neuroblastoma cell lines while CM supplemented with 10% HI-FCS was used for assay.

5) Dulbecco's Phosphate-Buffered Saline (PBS)

PBS (Gibco BRL Life Technologies, Inc.) was prepared by stirring the powdered salt (8 g sodium chloride, 0.2 g potassium chloride, 0.2 g monobasic potassium phosphate and 1.15 g dibasic sodium phosphate) in one liter of double distilled water until dissolved. The pH of the solution was adjusted to 7.4 by 1 M HCl or NaOH. It was then sterilized by autoclaving at 121°C for 20 minutes.

6) Dye Solutions

(a) Hemacolor Staining Solutions

Hemacolor staining solutions (Diagnostica Merck) which consist of three different solutions were used to stain cells. Hemacolor solution 1 was a fixation solution, solution 2 was a buffered color reagent red and solution 3 was a buffered color reagent blue. All solutions were stored in light-protected, tightly closed glass bottles at room temperature.

(b) Trypan Blue Staining Solution

Trypan blue solution was purchased from Gibco BRL Life Technologies, Inc. It contains 0.4% (w/v) trypan blue dissolved in 0.85% saline.

7) Trypsin-EDTA Solution

Trypsin-EDTA solution (without Ca^{2+} and Mg^{2+}) purchased from Gibco BRL Life Technologies, Inc., contains 0.25% (w/v) trypsin and 1 mM EDTA-tetrasodium in Hanks' balanced salt solution (HBSS). It was stored at -20°C as 20 ml aliquots until use.

2.1.3 Drugs and Chemicals

1) Glycyrrhizin (GL)

Glycyrrhizic acid ammonium salt (glycyrrhizin) with an estimated purity of ~95% was purchased from Fluka Chemical Company Inc. It was freshly prepared by dissolving the powder in plain RPMI 1640 medium as 11 mM solution and then filter sterilized. HI-FCS was then added to the solution to give a 10 mM stock solution in 10% HI-FCS CM.

2) 18β-Glycyrrhetinic Acid (GA)

18β-Glycyrrhetinic acid with an estimated purity of ~97% was purchased from Aldrich Chemical Company Inc. It was dissolved in cell culture-tested, sterile dimethylsulfoxide (DMSO) as a 60 mM stock solution. The stock solution was stored in dark at -20°C until use and it was used within one month after preparation.

3) Etoposide

Etoposide with an estimated purity of >97% was purchased from Calbiochem. It was dissolved in DMSO as a 100 mM stock solution and stored in dark at -20°C.

4) All-*Trans* Retinoic Acid (ATRA)

ATRA obtained from Calbiochem was solubilized in DMSO at a concentration of 30 mM and stored in dark at -20°C until use.

5) Protein Kinase C Modulators

Protein kinase C (PKC) activators phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-diacetate (PDA) were purchased from Sigma Chemical Co. They

were dissolved in DMSO and absolute ethanol respectively as a stock solution of 1 mg/ml and stored at -20°C until use. The PKC inhibitors staurosporine and calphostin C were dissolved in absolute ethanol at a concentration of 100 μM whereas Gö 6976 was first dissolved in DMSO and further diluted in plain medium as a 10 μM stock solution and stored in dark. All PKC inhibitors were purchased from Calbiochem and all stock solutions were stored at –20°C until use.

6) Protein Kinase A Modulators

Protein kinase A (PKA) activators adenosine 3',5'-cyclic phosphorothioate-Sp (Sp-isomer) and dibutyryl-cAMP, sodium salt (db-cAMP) were dissolved in sterilized double distilled water as 20 mM and 100 mM stock solution respectively while forskolin was dissolved in DMSO at a concentration of 10 mM. The PKA inhibitors 14-22 amide and adenosine 3',5'-cyclic phosphorothiolate-Rp (Rp-isomer) were dissolved in sterilized double distilled water as 1 mM and 20 mM stock solution, respectively while H-89 and KT5720 were dissolved in DMSO as 5 mM and 500 μM stock solution respectively and KT5720 was protected from light. All PKA activators and inhibitors were purchased from Calbiochem and the stock solutions were stored at –20°C until use.

2.1.4 Reagents for ^{3}H-Thymidine Incorporation Assay

1) [methyl-^{3}H] Thymidine (^{3}H-TdR)

The stock solution (Amersham Life Science Ltd.) with specific activity of 2 Ci/mmol was kept as 500 μl aliquots at 4°C. It was freshly diluted with complete medium to make up the 25 μCi/ml working solution before cell labeling. In cell labeling experiment, 20 μl working solution was added to each well of the 96-well

flat-bottomed microtiter plate.

2) Liquid Scintillation Cocktail

The OptiPhase 'HiSafe'2 liquid scintillation cocktail was purchased from Perkin-Elmer Co. The cocktail was stored in dark at room temperature.

2.1.5 Reagents for Neutral Red Assay

1) 0.5% Neutral Red Solution

Neutral red solution (0.5%, w/v) was prepared by dissolving 0.5 g neutral red powder (Sigma Chemical Co.) in 100 ml normal saline. The neutral red solution was then filtered and stored at 4°C.

2) Normal Saline

Normal saline was prepared by dissolving 1.8 g sodium chloride (Sigma Chemical Co.) in 200 ml of double distilled water and sterilized by autoclaving at 121°C for 20 minutes.

3) 1% Sodium Dodecyl Sulfate (SDS) Solution

SDS solution (1%, w/v) was prepared by dissolving 1 g SDS (Sigma Chemical Co.) in 100 ml double distilled water and kept at room temperature.

2.1.6 Reagents for Clonogenic Assay

1) Citrate Solution

Citrate solution containing 18 mM citric acid, 9 mM sodium citrate and 12 mM sodium chloride was prepared and stored at 4°C until use.

2) Citrate-Acetone-Formaldehyde (CAF) Solution

CAF solution was prepared by mixing 25 ml citrate solution, 65 ml acetone and 8 ml 37% formaldehyde together and stored at 4°C before use.

3) Hematoxylin Solution

Hematoxylin solution containing 6 g/L hematoxylin, 52.8 g/L aluminum sulfate, and 0.6 g/L sodium iodate was purchased from Sigma Chemical Co.

2.1.7 Reagents and Buffers for Immunocytochemistry

1) 4% Paraformaldehyde

Freshly prepared 4% (w/v) paraformaldehyde solution was used as the fixative. Briefly, 2 g paraformaldehyde (Sigma Chemical Co.) was first dissolved in 25 ml of double distilled water by heating to 60°C with stirring in a fume hood for one hour. A few drops of 1 M NaOH were added to help dissolving until the solution became clear. After cooling to room temperature, 25 ml 2X PBS was added to form 4% paraformaldehyde in 1X PBS and stored in dark at 4°C until use.

2) 0.1% Triton X-100

Triton X-100 solution (0.1%, w/v) was prepared by diluting one part of Triton X-100 solution (Sigma Chemical Co.) with 999 parts of PBS. It was used as a cell permeabilization agent.

3) 1% BSA

BSA solution (1%, w/v) was prepared by dissolving 1 g of bovine serum albumin (Sigma Chemical Co.) in 100 ml PBS and it was used as a blocking agent.

4) Normal Goat Serum

Lyophilized normal goat serum was purchased from Zymed Laboratories, Inc. The lyophilized normal goat serum was reconstituted with 10 ml of double distilled water to give 10 ml of whole goat serum with 0.05% sodium azide. Reconstituted normal goat serum was stored at –20°C until use. Ten percent normal goat serum was prepared by diluting 1 part of the reconstituted normal goat serum with 9 parts of PBS. It was used as a diluting agent for secondary antibodies.

5) Primary Antibodies

(a) Rabbit Anti-MAP2 Polyclonal Antibody

It is specific for microtubule-associated protein 2 (MAP2) and was purchased from Chemicon International, Inc. The antibody recognizes all types of MAP2 isoforms (MAP2A, MAP2B, MAP2C and MAP2D) and it shows greatest immunoreactivity with MAP2A and MAP2B. The antibody reacts with MAP2 of human, rat, and mouse origin. Undiluted aliquots of antibody were kept at –20°C until use.

(b) Mouse Anti-Neurofilament 200 Monoclonal Antibody

It was purchased from Sigma Chemical Co. and reacts with an epitope in the tail domain of neurofilament 200 (also referred to as the H-subunit) that is present on both the phosphorylated and non-phosphorylated forms of this polypeptide. It shows broad species cross-reactivity by recognizing neurofilaments of human, monkey, pig, rabbit, hamster, rat and mouse. Undiluted aliquots of antibody were kept at –20°C until use.

6) Secondary Antibodies

FITC Goat Anti-Rabbit IgG (H+L) Conjugate (ZyMax^{TM} Grade) and FITC Goat Anti-Mouse IgG (H+L) Conjugate (ZyMax^{TM} Grade) were purchased from Zymed Laboratories, Inc. The undiluted antibodies were stored in dark at 4°C until use. The secondary antibodies were diluted in 10% normal goat serum before use.

2.1.8 Reagents for DNA Extraction

1) IGEPAL CA-630 Lysis Buffer

The buffer was prepared in 50 mM Tris [hydroxylmethyl] amino methane (Tris)-HCl, pH 7.5 with 3% non-ionic detergent IGEPAL CA-630 (Sigma Chemical Co.) and 20 mM EDTA. It was kept at room temperature.

2) Proteinase K

It was purchased from Boehringer Mannheim. It is a highly active subtilisin type of protease. It was purified from the mold *Tritirachium album* Limber and the stock solution was prepared by dissolving it in autoclaved double distilled water to a concentration of 20 mg/ml and stored as 500 µl aliquots at –20°C until use.

3) RNase A

The pancreatic RNase (Boehringer Mannheim) was dissolved at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. The stock solution was stored as 500 µl aliquots at –20°C until use.

4) Sodium Acetate Solution (NaOAc)

Sodium acetate solution (3 M) was prepared by dissolving 24.61 g sodium

acetate (Sigma Chemical Co.) in 100 ml double distilled water. It was then sterilized by autoclaving at 121°C for 20 minutes. The solution was kept at room temperature.

5) $T_{10}E_{0.1}$ Buffer

The $T_{10}E_{0.1}$ buffer contains 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA in double distilled water. The solution was kept at room temperature.

2.1.9 Reagent for DNA Staining

The membrane-permeable nucleic acid stain Hoechst 33342 dye of 10 mg/ml was purchased from Molecular Probes Inc. (USA) and stored in dark at 4°C. It is a nuclear counterstain that emits blue fluorescence when bound to double stranded DNA.

2.1.10 Reagents and Buffers for Flow Cytometry

1) Propidium Iodide (PI) DNA Staining Buffer

The PI DNA staining buffer containing 400 μg/ml ribonuclease A (RNase A) (Boehringer Mannheim), 50 μg/ml propidium iodide (Boehringer Mannheim), 10 mM EDTA, pH 7.4 (Sigma Chemical Co.), 0.1% trisodium citric acid (Sigma Chemical Co.) and 0.1% Triton X-100 (Sigma Chemical Co.) was freshly prepared in PBS and kept in dark at 4°C until use.

2) FACS Flow Shealth Fluid

The shealth fluid is a ready-to-use balanced electrolyte solution containing sodium chloride, potassium chloride, disodium EDTA, sodium fluoride and anti-microbial agent. It was purchased from the Becton Dickinson International and

stored at room temperature.

2.1.11 Reagents for Total RNA Isolation

1) DEPC-treated Double Distilled Water

Diethyl pyrocarbonate (DEPC) (Sigma Chemical Co.) was added to double distilled water at 1:1,000 ratio to give 0.1% (v/v) DEPC water. Thereafter, it was shaken vigorously to disperse the DEPC. After standing overnight, the solution was autoclaved at 121°C for 20 minutes to destroy the remaining DEPC.

2) Trizol Reagent

Trizol reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, was purchased from Gibco BRL Life Technologies, Inc. It is a ready-to-use reagent for the isolation of total RNA from cells and tissues. It was stored in dark at 4°C until use.

2.1.12 Reagents and Buffers for RT-PCR

1) Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)

The M-MLV reverse transcriptase (Gibco BRL Life Technologies, Inc.) was isolated from *E. coli* expressing a portion of the *pol* gene of M-MLV on a plasmid. This enzyme was stored at –20°C in a buffer containing 20 mM Tris-HCl pH 7.5, 1 mM dithiothreitol (DTT), 0.01% (v/v) NP-40, 0.1 mM EDTA, 100 mM NaCl and 50% (v/v) glycerol. One unit of its activity was defined as the amount of enzyme that would incorporate 1 nmole of deoxythymidine triphosphate (dTTP) into acid-precipitable material in 10 minutes at 37°C using poly(A)•oligo$(dT)_{25}$ as template and primer respectively.

2) First Strand Buffer (5X)

The first strand buffer (Gibco BRL Life Technologies, Inc.) supplied as a 5X solution of 250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$ was stored at –20°C until use.

3) 0.1 M Dithiothreitol (DTT)

DTT (0.1 M) purchased from Gibco BRL Life Technologies, Inc. was stored at –20°C until use.

4) $T_{10}E_{0.1}$ Buffer

The $T_{10}E_{0.1}$ buffer contains 10 mM Tris-HCl pH 8 and 0.1 mM EDTA in DEPC-treated distilled water. All chemicals were purchased from Sigma Chemical Co.

5) $RN_{ASE}OUT^{TM}$ Recombinant Ribonuclease Inhibitor

$RN_{ASE}OUT^{TM}$ recombinant ribonuclease inhibitor (Gibco BRL Life Technologies, Inc.) was affinity purified from a recombinant strain of *E. coli* expressing a cloned porcine liver gene. This inhibitor has a very high binding affinity for pancreatic-type ribonucleases such as RNase A and is active against RNase A, RNase B and RNase C. It has a concentration of 40 units/μl but requires a minimum of 1 mM DTT to maintain activity. It was stored at –20°C in a buffer which contains 20 mM Tris-HCl (pH 8), 50 mM KCl, 0.5 mM EDTA, 8 mM DTT and 50% (v/v) glycerol. One unit was defined as the amount of inhibitor required to inhibit by 50% the activity of 5 ng of RNase A as determined by the inhibition of hydrolysis of cytidine 2', 3'-cyclic monophosphate by RNase A.

6) Oligo(dT)$_{12-18}$, Sodium Salt (pd(T)$_{12-18}$ 5'-PO$_4$, sodium salt)

Oligo(dT)$_{12-18}$ purchased from Pharmacia Biotech was prepared as a stock solution of 1 μg/μl in $T_{10}E_{0.1}$ buffer and stored at –20°C.

7) Ultrapure dNTP Set, 2'-Deoxynucleoside 5'-Triphosphate, Sodium Salt

The deoxynucleoside triphosphate (dNTP) set was purchased from Pharmacia Biotech, with each nucleotide supplied as 100 mM solution in double distilled water (pH 7.5). A stock solution with 10 mM of each dNTP was prepared in 1 mM Tris-HCl (pH 7.5) and was stored at –20°C.

8) Thermoprime ^{plus} DNA Polymerase

Thermoprime ^{plus} DNA Polymerase (Advanced Biotechnologies Ltd.) stored at –20°C is a thermostable DNA polymerase isolated from Thermophilic bacteria. Thermoprime ^{plus} DNA Polymerase is a single polypeptide of approximately 94 kDa which has 5' to 3' polymerization-dependent exonuclease replacement activity, but lacks the 3' to 5' exonuclease activity. One unit of enzyme was defined as the amount that would incorporate 10 nmoles of dNTPs into acid insoluble material in 30 minutes at 47°C.

9) Reaction Buffer IV (10X)

The reaction buffer (Advanced Biotechnologies Ltd.) supplied as a 10X solution of 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl (pH 9) and 0.1% (w/v) Tween was stored at –20°C until use.

10) 25 mM $MgCl_2$ solution

$MgCl_2$ solution (25 mM) purchased from Advanced Biotechnologies Ltd. was stored at –20°C until use.

11) Primer Pairs

The oligonucleotide primers were designed on the basis of the published sequences of cloned cDNA. They were designed to prime on the sense and antisense sequences of the corresponding cDNA respectively. The PCR primer sets for PCR amplifications of specific cDNA were synthesized by Gibco BRL Life Technologies, Inc. and their sequences and predicted sizes of the PCR products are shown in Table 2.1. The lyophilized primer pairs were reconstituted in DEPC-treated double distilled water to a working concentration of 2.5 μM. The primers were stored as 500 μl aliquots at –20°C. They have the annealing temperature of 52-61°C.

Table 2.1: Primers used in RT-PCR and the predicted sizes of the PCR products.

cDNA amplified	Sequence (5' to 3')	Predicted size of the amplified PCR product (bp)
N-myc	Sense strand: CGA ATT GGG CTA CGG AGA TGC T Antisense strand: TTG TGC TGC TGA TGG ATG GG	763
c-myc	Sense strand: TTT GGG GAC AGT GTT CTC TGC CT Antisense strand: GTT CTT GAT GAA GGT CTC GTC GTC	501
c-fos	Sense strand: CCA GTC AAG AGC ATC AGC AA Antisense strand: AAG TAG TGC AGC CCG GAG TA	247
c-jun	Sense strand: GCA TGA GGA ACC GCA TTG CCG CCT CCA AGT Antisense strand: CGC AAA GTC TGC CGG CCA ATA GGC CGC T	459

NF-κB/p50	Sense strand: AAA GGT TAT CGT TCA GTT Antisense strand: TTG TAG ATA GGC AAG GTC	250
Bcl-2	Sense strand: AGC TGC ACC TGA CGC CCT T Antisense strand: CAG CCA GGA GAA ATC AAA CAG AGG	293
Cyclin A	Sense strand: TAC TTC CTG CAC CTG CAG CCT Antisense strand: AGC ATG GAC TCC GAG CGA CT	399
Cyclin B	Sense strand: CCA TTA TTG ATC GGT TCA TGC AGA Antisense strand: ATG TGT ACA GTT CAG CTG TGC CAG	584
Cdc 2	Sense strand: CTC GGC TCG TTA CTC CAC TC Antisense strand: CCA TTT TGC CAG AGA TTC GT	311
PKC-α	Sense strand: TGA ATC CTC AGT GGA ATG AGT Antisense strand: GGT TGC TTT CTG TCT TCT GAA	324
PKC-δ	Sense strand: GAT CGC TGA CTT TGG GAT GT Antisense strand: GGG GAT AGT GTG GTG TGT CC	242
PKC-ε	Sense strand: CAT CGA TCT CTC GGG ATC ATC G Antisense strand: CGG TTG TCA AAT GAC AAG GCC	732
PKC-ζ	Sense strand: CGA TGG GGT GGA TGG GAT CAA AA Antisense strand: GTG TTC ATG TCA GGG TTG TCC G	681
PKC-λ	Sense strand: CGT TGG GAG CTC TGA CAA TC Antisense strand: ACC TGC TTT TGC TCC ATC ATG	240
GAPDH	Sense strand: AAT GGT GAA GGT CGG TGT GAA C Antisense strand: GAA GAT GGT GAT GGG CTT CC	226

2.1.13 Reagents and Buffers for Gel Electrophoresis

1) Tris-Borate-EDTA (TBE) Electrophoresis Buffer (5X)

The 5X TBE electrophoresis buffer stock was prepared by dissolving 54 g Tris, 27.5 g boric acid and 20 ml 0.5 M EDTA in 1 liter double distilled water. The pH of the buffer was adjusted to 8. The working TBE buffer (0.5X) was prepared by diluting one part of the 5X stock TBE buffer with nine parts of double distilled water. Both of the working and stock solutions were kept at 4°C.

2) Agarose Gel

1% (w/v) and 2% (w/v) agarose gels were prepared by dissolving 10 g and 20 g agarose (USB) respectively in 1 liter 0.5X TBE buffer by heating at 70°C with constant stirring on a hot plate. The solidified agarose gel was heated and melted in a microwave oven before use. The 1% agarose gel was used for RNA gel electrophoresis while the 2% agarose gel was used for DNA and PCR products gel electrophoresis.

3) Gel Loading Solution (5X)

The gel loading solution (Sigma Chemical Co.) was a 5X solution containing 0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1 M EDTA (pH 8.0) and 0.5% (w/v) sodium dodecyl sulfate (SDS). One volume of the solution was mixed with four volumes of sample before loading into agarose gel. The solution was used for non-denaturing agarose gel electrophoresis of nucleic acids. It was stored at room temperature.

4) Ethidium Bromide (EtBr)

A stock solution (10 mg/ml) of ethidium bromide was prepared by dissolving the EtBr (Sigma Chemical Co.) in double distilled water. The working solution was prepared by diluting the stock solution by 1,000 fold. Both the stock and working solution were kept in dark at room temperature.

2.1.14 Reagents and Buffers for Western Blot Analysis

1) Cell Lysis Buffer

The cell lysis buffer contains 1% (v/v) IGEPAL-CA 630 (Sigma Chemical Co.), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) and one tablet of complete protease inhibitor cocktail (Complete; Roche Molecular Biochemicals) in 50 ml double distilled water. Each tablet of complete protease inhibitor cocktail when dissolved in 50 ml lysis buffer yielded a mixture of several protease inhibitors with a broad spectrum of activity on serine, cysteine and metalloproteases and calpains inhibitors. The cell lysis buffer was kept at 4°C until use.

2) 30% (w/v) Acrylamide / Bis Solution, 37.5:1

The ready-to-use 500 ml acrylamide solution containing 30% (w/v) of acrylamide (146.1 g) and N,N'-methylene-bis-acrylamide (3.9 g) for a total monomer to crosslinker ratio of 37.5:1 was purchased from Bio-Rad Laboratories. It was light-protected and stored at 4°C until use.

3) Lower Buffer for Separating Gel

It is a 1.5 M Tris-HCl buffer adjusted to pH 8.8 (Sigma Chemical Co.) and kept at 4°C.

4) 10% Sodium Dodecyl Sulfate (SDS) Solution

SDS solution (10%, w/v) was prepared by dissolving 10 g SDS (Sigma Chemical Co.) in 100 ml double distilled water and kept at room temperature.

5) 10% Ammonium Persulfate (APS)

Ammonium persulfate (10%, w/v) was prepared by dissolving 0.5 g APS (Bio-Rad Laboratories) in 5 ml double distilled water. The solution was kept as 500 μl aliquots at -20°C until use.

6) N,N,N',N'-Tetra-methylethylenediamine (TEMED)

TEMED purchased from Bio-Rad Laboratories was the last to add to initialize the polymerization of the SDS-polyacrylamide gel. It was stored at 4°C until use.

7) Upper Buffer for Stacking Gel

It is a 0.5 M Tris-HCl buffer adjusted to pH 6.8 (Sigma Chemical Co.) and was kept at 4°C.

8) 2X SDS Gel Loading Buffer

2X SDS gel loading buffer containing 2-mercaptoethanol and bromophenol blue was prepared by mixing 0.2 ml 0.05% (w/v) bromophenol blue, 1 ml upper buffer, 1 ml glycerol, 1 ml 10% (w/v) SDS, 4.7 ml double distilled water and 0.1 ml 2-mercaptoethanol. All reagents were purchased from Sigma Chemical Co. The buffer was protected from light and stored at 4°C until use.

9) Kaleidoscope Prestained Standards

The Kaleidoscope prestained standards (Bio-Rad Laboratories) consist of seven uniquely colored proteins including myosin (199,000 Da), β-galactosidase (128,000 Da), bovine serum albumin (85,000 Da), carbonic anhydrase (41,700 Da), soybean trypsin inhibitor (32,100 Da), lysozyme (18,300 Da) and aprotinin (7,500 Da). The standard colored proteins prepared in 33% (v/v) glycerol, 3% SDS, 10 mM Tris (pH 7), 10 mM DTT, 2 mM EDTA, 0.01% NaN_3 were stored at -20°C until use.

10) Tris-Glycine-SDS Electrophoresis Buffer (10X)

Tris-glycine-SDS electrophoresis buffer 10X concentrate contains 0.25 M Tris-HCl, pH 8.6, 1.92 M glycine and 1% SDS in double distilled water. It was filtered by filtration through a 0.2 μm Millipore filter and was stored at 4°C. The 10X concentrate was freshly diluted to 1X working buffer solution for SDS-PAGE.

11) Coomassie Blue Staining and Destaining Solution

The staining solution was prepared by mixing one volume of 0.05% Coomassie blue (Bio-Rad Laboratories) in one volume of acetic acid, three volumes of methanol and ten volumes of double distilled water. For the destaining solution, it was prepared by mixing one volume of acetic acid, three volumes of methanol and ten volumes of double distilled water. The Coomassie blue staining and destaining solutions were used to stain and destain the SDS-polyacrylamide gel and the PVDF Western blotting membrane.

12) Tris-Glycine Buffer (10X)

Tris-glycine buffer 10X concentrate contains 0.25 M Tris-HCl, pH 7.5 and 1.92

M glycine in double distilled water. It was filtered by filtration through a 0.22 μm Millipore filter and stored at 4°C.

13) Tris-Glycine-Methanol Transfer Buffer (1X)

The transfer buffer was prepared by mixing 100 ml methanol with 50 ml 10X Tris-glycine buffer and 350 ml double distilled water. The buffer solution was stored at 4°C.

14) PVDF Western Blotting Membranes

The microporous polyvinylidene difluoride (PVDF) membrane (Roche Applied Science) with pore size of 0.45 μm was stored at room temperature.

15) Washing Buffer

The washing buffer contains 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% (v/v) Tween 20 (Sigma Chemical Co.). The solution was kept at 4°C.

16) 5% Skimmed Milk Solution (Blocking Solution)

It was prepared by dissolving Nestle's skimmed milk powder in washing buffer at a concentration of 5% (w/v).

17) Primary Antibodies

(a) Mouse Anti-β-Actin Monoclonal Antibody

It is a mouse monoclonal antibody (Sigma Chemical Co.) which recognizes an epitope located on the N-terminal end of the β-isoform of actin. It is provided as ascities fluid containing 0.1% sodium azide as a preservative. The antibody cross

reacts with β-actin in human, bovine, sheep, pig, rabbit, cat, dog, mouse, rat, guinea pig, chicken, carp, leech, and fruit fly tissues. The antibody was stored as small aliquots at -20°C.

(b) Rabbit Anti-Cyclin A Polyclonal Antibody

It is an affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) raised against a recombinant protein corresponding to amino acids 1 - 432 representing full length cyclin A of human origin. Each vial contains 200 μg IgG in 1 ml PBS supplemented with 0.1% sodium azide and 0.2% gelatin. It reacts with cyclin A p60 and cyclin A1 of mouse, rat and human origin and was stored at 4°C.

(c) Rabbit Anti-Cyclin B1 Polyclonal Antibody

It is an affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) raised against a recombinant protein corresponding to amino acids 1 - 433 representing full length cyclin B1 of human origin. Each vial contains 200 μg IgG in 1 ml PBS supplemented with 0.1% sodium azide and 0.2% gelatin. It reacts with cyclin B1 of mouse, rat and human origin and was stored at 4°C.

(d) Rabbit Anti-Cdc2 Polyclonal Antibody

It is an affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) raised against a peptide mapping at the carboxy terminus of Cdc2 p34 of human origin. Each vial contains 200 μg IgG in 1 ml of PBS supplemented with 0.1% sodium azide and 0.2% gelatin. The antibody reacts with Cdc2 p34 of mouse, rat and human origin and is non cross-reactive with other cyclin dependent kinases. It

was stored at 4°C.

18) Secondary Antibodies

Sheep anti-mouse IgG, peroxidase-linked species-specific whole antibody and donkey anti-rabbit IgG, peroxidase-linked species-specific whole antibody were purchased from Amersham Pharmacia Biotech. Both of them had a concentration of 1 mg/ml and were stored at 4°C.

19) RPN 2106 ECL™ Western Blotting Detection Reagents

The Western blotting detection reagents (Amersham Pharmacia Biotech.) which contain detection reagent 1 (250 ml) and detection reagent 2 (250 ml) were stored at 4°C. Equal volume of detection reagent 1 and 2 was mixed before use and a final volume of 0.125 ml/cm^2 membrane was required.

20) X-ray film (Fuji, Japan)

The Fuji X-ray film was stored in dark at 4 °C.

2.2 Methods

2.2.1 Cell Culture Methodology

The murine neuroblastoma Neuro-2a BU-1 cells were maintained in RPMI 1640 medium containing 2 mM glutamine and 2 g/L sodium bicarbonate supplemented with 10% FCS and 1% PSN antibiotics. For the human neuroblastoma SK-N-DZ cells, they were maintained in DMEM supplemented with 10% FCS and 1% PSN antibiotics and the SH-SY5Y cells were maintained in MEM supplemented with 10% FCS and 1% antibiotics. All the cell lines were maintained in tissue culture flasks under a humidifying atmosphere containing 5% CO_2 / 95% humidified air at 37°C. The cell lines were subcultured every 2 - 3 days or twice weekly depending on their doubling times. Cells of 5 - 25 passages growing exponentially were used to perform all experiments and long-term storage of cell lines was done by cryo-preservation in liquid nitrogen.

For the subculture procedure, growth medium was first decanted, and the adherent cells were rinsed once with warm PBS before mild trypsinization with 0.5 ml (for 25 cm^2 culture flask) or 1 ml (for 75 cm^2 culture flask) of trypsin-EDTA solution at 37°C for 2 minutes. Then 10 ml of complete medium (CM) was added to stop the trypsinization process when all the cells were detached from the culture flask. Cells were then collected by centrifugation. After resuspension in CM, appropriate aliquots of cell suspension were added to new culture flasks with complete medium.

2.2.2 Determination of Cell Proliferation

1) ^{3}H-thymidine (^{3}H-TdR) Incorporation Assay

The anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on the

neuroblastoma cells was assessed by ^{3}H-TdR incorporation. Briefly, cells with a specified cell number (in 100 μl CM) were seeded in quadruplicates in wells of 96-well flat-bottomed microtiter plates at 37°C overnight inside a humidified 5% CO_2 incubator, after which CM or different concentrations of drug (100 μl) was added and further incubated for various time periods as indicated in the text. During this period, cells were pulsed with 0.5 μCi ^{3}H-TdR per well for the last 6 - 10 hours (6 hours for BU-1 cells and 10 hours for SK-N-DZ and SH-SY5Y cells) of incubation. The cells were then frozen in a -20°C freezer overnight, thawed at room temperature and harvested onto glass fiber filter with a cell harvester (Skatron). The filters were transferred to vials containing 0.5 ml scintillation fluid and the amount of ^{3}H-TdR incorporated into DNA was determined by liquid scintillation counting. The radioactivity of each filter was measured by the Beckman LS6000 liquid scintillation counter. The results were expressed as the percentage inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control (Leung *et al.*, 1994). The percentage inhibition of ^{3}H-TdR incorporation was calculated as follows:

% inhibition = [(cpm of control – cpm of test sample) / cpm of control] x 100%

2) Neutral Red Assay

The neutral red assay was also used as a non-radioactive method for the measurement of cellular proliferation (Borenfreund *et al.*, 1990). In brief, BU-1 cells (7,500 cells/100μl) in quadruplicates were seeded in wells of 96-well flat-bottomed microtiter plates at 37°C overnight inside a humidified 5% CO_2 incubator, after which CM or different concentrations of drug (100 μl) was added and further incubated for 48 hours. At the end of the incubation period, the medium was discarded and the cells were washed with 150 μl pre-warmed normal saline.

Thereafter, cells were incubated with 50 µl 0.5% neutral red solution at 37 °C for one hour to allow the uptake of the neutral red. After incubation, the neutral red solution was removed and the cells were washed six times with pre-warmed normal saline. After washing, the intracellular neutral red absorbed by the cells was solubilized with 100 µl of 1% (w/v) SDS on a shaker platform for 2 hours and the absorbance at 540 nm was measured spectrophotometrically with a microplate reader (Bio-Rad, Model 3550) using 1% SDS as blank. The result expressed as percentage decrease in absorbance measured at 540 nm was calculated as follows:

% decrease in absorbance measured at 540 nm

= [(A_{540} of control – A_{540} of test sample) / A_{540} of control] x 100%

2.2.3 Determination of Cell Viability by Trypan Blue Exclusion Test

Trypan blue exclusion test was used to determine the number of viable cells in cultures (McGahon *et al.*, 1995). BU-1 cells (7.5 x 10^4 cells/ml) were cultured in 6-well plates overnight for cell attachment. Different concentrations of drugs or CM were then added and further incubated for 48 hours at 37°C inside a humidified 5% CO_2 incubator. Cells were then trypsinized and collected by centrifugation. After resuspension in PBS, cells were mixed with an equal volume (10 µl) of 0.4% trypan blue staining solution. The viable cells were visible as clear cell bodies while the non-viable cells were stained blue. The number of viable and non-viable cells was counted using a hemacytometer. The results were expressed as the mean percentage of cell viability ± S.E. of triplicate cultures. The percentage viability was calculated as follows:

% cell viability = (no. of unstained cells / total no. of cells counted) x 100%

2.2.4 Limiting Dilution Assay

Limiting dilution assay was used to determine the proliferating potential of cells after drug treatment. Briefly, BU-1 cells (2.5×10^4 cells/ml) were cultured overnight and incubated with 3.5 mM glycyrrhizin or 80 μM 18β-glycyrrhetinic acid in the wells of a 6-well plate for 4 days at 37°C. Thereafter, the cells were washed and harvested by mild trypsinization. Various concentrations of viable cells in a total volume of 200 μl were seeded into the wells of 96-well plates, with 48 replicates for each cell concentration. The cells were allowed to incubate for 7 days at 37°C in a humidified 5% CO_2 incubator. Each well was scored by microscopic observation as positive or negative growth based on the presence or absence of cell growth. The frequency of proliferating neuroblastoma cells was determined by analysis of the number of cells plated per well and the percentage of non-proliferating cultures using the limiting dilution analysis method based on Poisson distribution. In brief, the frequency is estimated by the cell concentration in which 37% of the wells are negative for growth (Hay & Westwood, 2002).

2.2.5 Clonogenic Assay

The clonogenic assay was carried out on 24-well flat-bottomed plates (Nunc Co.) as described by Kobayashi *et al.* (1989) with minor modifications. Briefly, 0.4 ml semi-solid agar cell culture containing 0.33% Bacto agar (Difco, Detroit, MI), RPMI medium with 1% PSN and 20% FCS, 2 mM glutamine and 250 untreated BU-1 cells was set up in each well. When the agar culture was solidified, 20 μl of appropriately diluted DMSO or 18β-glycyrrhetinic acid was added and slowly diffused into the agar. The plate was then put into the 37°C 5% CO_2 incubator. After 9 day incubation, the agar disc was rinsed out by cold PBS and dried on microscopic slides. The disc was

then fixed with citrate-acetone-formaldehyde solution. Cell colonies formed were stained with hematoxylin solution and the number of colonies with at least 50 cells was counted using a dissecting microscope.

2.2.6 Measurement of Apoptosis by DNA Fragmentation Analysis

DNA fragmentation is one of the characteristics of apoptosis. DNA fragments were isolated from the cells by the method of Herrmann *et al.* (1994). Briefly, 10^6 BU-1 cells (1 x 10^5 cells/ml) were seeded in a 10-cm culture dish overnight and then incubated with appropriate concentrations of drugs at 37°C for different periods of time and the DMSO-treated cells were acted as a control. Cells were harvested by mild trypsinization, washed with PBS and pelleted by centrifugation. The cell pellets were then lysed in 200 µl of IGEPAL CA-630 lysis buffer for 10 minutes at 37°C. After centrifugation at 7,500 rpm for 5 minutes, the supernatants containing the DNA were collected to a new microcentrifuge tube. Fifty microliter of 5% SDS was added to the supernatant and the mixture was incubated with 0.4 µg/µl RNase A at 56°C for 1.5 hours to remove the cellular RNA. Thereafter, proteinase K (at a final concentration of 1.5 µg/µl) was added and the mixture was incubated for 1.5 hours at 56°C to remove the proteins. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. After centrifugation at 14,000 rpm for 30 minutes, the DNA pellets were washed with 70% ethanol followed by absolute ethanol. The DNA pellets were then air dried for about 10 minutes. The dried pellets were resuspended in 20 µl $T_{10}E_{0.1}$ buffer and incubated at 65°C for 5 minutes. Finally, the resuspended DNA was mixed with 5X gel loading solution and subjected to 2% agarose gel electrophoresis.

2.2.7 Assessment of Apoptosis by Hoechst 33342 Staining

The membrane-permeable Hoechst 33342 fluorescent dye has been extensively used for staining the nuclei of living cells. The Hoechst 33342 dye binds to the minor groove of DNA at AT-rich sequences and is commonly used to distinguish the compact chromatin of apoptotic nuclei. The apoptotic cells with condensed chromatin or dispersed and fragmented nuclei can be easily observed after Hoechst 33342 staining (Huschtscha *et al.*, 1995). Briefly, BU-1 cells (40,000 cells/well) were grown on sterile microscopic cover glasses (Fisher Scientific) in the wells of a 6-well plate in a total volume of 2 ml per well. After overnight incubation, control solvent, glycyrrhizin, 18β-glycyrrhetinic acid or etoposide was added and incubated at 37°C for 48 hours. After drug treatment, the culture medium was aspirated, and the cells were washed with PBS and fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature. After washing with PBS, the cells were incubated with Hoechst 33342 (20 μg/ml) in PBS for 15 minutes at room temperature. After washing with PBS, the stained cells were observed under a fluorescence microscope (Zeiss Axioskop) using (G 365, FT 395, LP 420) filter set and 20 x Plan-NEOFLUAR objective. Nuclei were identified as normal, fragmented or condensed. Cells with fragmented or condensed nuclei were classified as apoptotic.

2.2.8 Cell Morphological Study

Morphological study of the effects of glycyrrhizin and 18β-glycyrrhetinic acid on the neuronal differentiation of the neuroblastoma cells was carried out as described by Mak *et al.* (2001). BU-1 cells (5,000 cells/well) were grown on sterile thermanox plastic 13 mm coverslips (Nalge Nunc International) placed in the wells of a 24-well plate in a total volume of 0.25 ml per well overnight. Cells were then incubated with

different concentrations of drugs in a final volume of 0.5 ml at 37°C for 3 - 4 days. After washing with PBS, the cells were stained with each Hemacolor staining solutions for 15 seconds. After destaining with distilled water, the coverslips were air dried and mounted onto glass slides with nail tonish. The morphology of the cells was observed under the Zeiss Axioskop microscope and photographs were taken at 200x magnification. Differentiation of BU-1 cells was characterized by the enlargement in cell size and elongation in neurite length.

2.2.9 Immunocytochemistry

BU-1 cells (20,000 cells/well) were grown on sterile microscopic cover glasses (Fisher Scientific) in the wells of a 6-well plate in a total volume of 1 ml per well. After overnight incubation, control solvent, glycyrrhizin or 18β-glycyrrhetinic acid was added and incubated at 37°C for an additional 4 days. After drug treatment, the culture medium was aspirated, and the cells were washed with PBS and fixed with 4% freshly made paraformaldehyde in PBS for 15 minutes at room temperature. After washing twice with PBS, cells were permeabilized by treatment with PBS containing 0.1 % Triton X-100 for 30 minutes at room temperature. After a brief wash with PBS, nonspecific binding sites were blocked with 1% BSA in PBS for 3 hours. Cells were then washed with PBS and incubated overnight at 4°C with appropriately diluted rabbit anti-MAP2 (1:500) polyclonal antibody or mouse anti-Neurofilament 200 (1:200) monoclonal antibody in PBS. Samples incubated without primary antibody served as negative controls. Following incubation with the primary antibody, cultures were extensively washed and then incubated with the appropriately diluted FITC-conjugated goat anti-rabbit IgG (1:100) or FITC-conjugated goat anti-mouse IgG (1:100) secondary antibodies in 10% goat serum for 1 hour at room temperature.

After the washing and drying procedures, the microscopic cover glasses with immunostained cells were mounted onto the glass slide with 50% glycerol as mounting medium, examined and photographed under a laser confocal microscope. A laser scanning confocal microscope (Leica Model TCS-NT) with epifluorescence and a phase contrast system was used. An argon/krypton laser line with a wavelength of 488 nm was used for excitation, and a predefined filter setting for FITC was used for emission. The oil immersion objective with magnification of 40 times (UV 40x1.25NA oil PL APO 1.25-0.75) was used for image capturing. The images were analyzed by the PowerScan software LEICA TCS NT 1.6.582.

2.2.10 Flow Cytometric Analysis of Cell Cycle Profile

The cell cycle profile of drug-treated BU-1 cells was analyzed by flow cytometry. Briefly, BU-1 cells (2 x 10^5 cells) were seeded at a density of 10^5 cells/ml in the wells of 6-well plates in RPMI medium supplemented with 2% HI-FCS for synchronization. After serum starvation overnight, the culture medium was replaced with RPMI medium containing 10% HI-FCS. Glycyrrhizin or 18β-glycyrrhetinic acid was added and incubated for 1 - 3 days at 37°C. After drug treatment, cells were trypsinized, washed with PBS and pelleted by centrifugation at 2,000 rpm for 3 minutes. Cells were then fixed with 70% ethanol (10^6 cells/ml) at 4°C for 30 minutes. For staining, fixed cells were centrifuged at 2,000 rpm for 10 minutes and washed with PBS to remove the ethanol. After removal of ethanol, cells were resuspended in 1 ml freshly prepared DNA staining solution containing 50 μg/ml propidium iodide (PI) and 400 μg/ml RNase A and incubated for 1.5 hours in dark at room temperature. Stained cells were analyzed for fluorescence intensity with a fluorescence-activated cell sorter (Becton Dickinson FACSort) equipped with an

argon laser emitting at 488 nm, using the CellQuest software. A minimum of 10,000 events were acquired for each determination. The percentages of cells in G_0/G_1, S and G_2/M cell cycle phases were calculated by the ModFit program (Becton Dickinson).

2.2.11 Gene Expression Study

1) Isolation of Total Cellular RNA

Briefly, 10^6 BU-1 cells (10^5 cells/ml) were cultured in a 10-cm culture dish at 37°C in a humidified 5% CO_2 incubator. After overnight incubation, the cells were exposed to appropriate concentrations of 18β-glycyrrhetinic acid for different periods of time as indicated in the text. The cells were then collected by mild trypsinization and centrifugation at 2,000 rpm for 3 minutes at 4°C. The cell pellets were then lysed by 1 ml TRIZOL reagent containing guanidine thiocyanate with vigorous shaking. The cell lysate solutions were incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. RNA was then extracted by adding 200 μl chloroform (BDH), followed by shaking vigorously for 15 seconds. After incubation at room temperature for 3 minutes, the mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. Thereafter, the upper aqueous phase containing RNA was collected (~400 μl) and transferred to another new microcentrifuge tube. RNA was then precipitated by mixing with 500 μl isopropanol (Merck) and standing at –20°C overnight. After centrifugation at 12,000 x g at 4°C for 10 minutes, the supernatants were discarded and the RNA pellets were washed once with 1 ml ice-cold 75% ethanol. After centrifugation at 7,500 x g at 4°C for 5 minutes, the supernatants were removed by aspiration and the RNA pellets were air-dried for about 5 minutes. The RNA pellets were redissolved in 30 μl

DEPC-treated water and stored at -70°C until use. The RNA concentration was quantified by spectrophotometry at 260 nm. The purity was estimated by the ratio of A_{260}/A_{280} and was found to be between 1.6 and 1.8. Small aliquots of RNA with a working concentration of 0.5 μg/μl were prepared with DEPC-treated water and stored at –70°C. The integrity of the isolated RNA was checked by gel electrophoresis on 1% agarose gel and was found to be intact.

2) Reverse Transcription (RT)

The reverse-transcription polymerase chain reaction (RT-PCR) was used to study the differentiation and growth-related gene expression. One microgram of the total RNA was reverse transcribed in a 20 μl reaction mixture containing 40 units of $RN_{ASE}OUT^{TM}$ recombinant ribonuclease inhibitor, 1X M-MLV first strand buffer, 0.5 mM of each dNTP, 10 mM DTT, 0.1 μg oligo$(dT)_{12\text{-}18}$ and 200 units of M-MLV reverse transcriptase. The reaction mixture was incubated at 37°C for 1 hour, followed by 99°C for 5 minutes to inactivate the reverse transcriptase and to completely denature the template, and then cooled to 4 °C. The resulting RT sample was stored at -20°C until use.

3) Polymerase Chain Reaction (PCR)

PCR was performed in a 25 μl reaction mixture containing cDNA equivalent to 0.1 μg of total RNA, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 unit of Thermoprime^{Plus} DNA polymerase, and 0.2 μM of both sense and antisense oligonucleotide primers by the GeneAmp PCR System 9700 programmable thermal controller (Perkin-Elmer Co.). Cycling conditions comprised an initial denaturation of 5 minutes at 94°C, followed by 20 - 30 thermal cycles with denaturation at 94°C for

30 seconds, annealing at 52-61°C for 1 minute to 1 minute and 15 seconds and elongation at 72°C for 1 minute. A final extension of 5 minutes at 72°C was performed to complete the reaction. The annealing temperature and the number of PCR cycles were varied and optimized empirically for each primer set to allow the detection of the amplified products.

4) Agarose Gel Electrophoresis

The PCR products were analyzed by 2% agarose gel electrophoresis. Briefly, 5 μl aliquot of PCR product was mixed with 1 μl of 5X gel loading buffer. The mixtures were electrophoresed through a 2% agarose gel in 0.5X TBE electrophoresis buffer at a constant voltage of 100 volts for 1.5 - 2 hours. Five hundred nanogram of 100 bp DNA ladder (Gibco BRL Life Technologies, Inc.) was loaded as a size marker. After electrophoresis, the gel was stained with 10 μg/ml ethidium bromide solution for 5 minutes and destained with distilled water for 10 minutes. The stained gel was visualized and photographed by the Bio-Rad Gel Doc 2000 under UV illumination.

5) Quantification of RT-PCR Product Band Intensity

The gel photographs were analyzed in an objective manner by ImageQuant software (Molecular Dynamics) on a densitometry system. The relative intensity of each band of the RT-RCR product was normalized after dividing by the relative intensity of the corresponding band of GAPDH, and the relative intensity of each GAPDH band in the experimental group was expressed as the fraction of the control GAPDH band which was taken as unity.

2.2.12 Protein Expression Study

1) Treatment of Cells and Protein Extraction

In brief, 1.5 x 10^6 BU-1 cells (1.5 x 10^5 cells/ml) were cultured in a 10-cm culture dish overnight. Cells were then either treated with DMSO as a control or with different concentrations of 18β-glycyrrhetinic acid for different periods of time as indicated in the text at 37°C. The cells were harvested by mild trypsinization, washed with cold PBS and collected by centrifugation at 2,000 rpm for 3 minutes at 4°C. Cell pellets were then resuspended and vortexed vigorously with lysis buffer and stand on ice for at least 30 minutes. The cell lysates were clarified by centrifugation at 14,000 rpm for 5 minutes at 4°C, and the resulting supernatants were collected and stored at -20°C until further assay.

2) Quantification of Proteins

The protein content of the cell lysate was estimated by the Bio-Rad protein assay with bovine serum albumin (BSA) as standard. BSA solutions at 2 μg/ml, 4 μg/ml, 6 μg/ml, 8 μg/ml, and 10 μg/ml were prepared with double distilled water in duplicate with 2 mg/ml BSA stock solution. Two microliters protein samples were diluted with 1,598 μl double distilled water. Diluted BSA standards or protein samples (800 μl) were mixed with 200 μl of Bio-Rad protein assay dye reagent concentrate. The reaction mixtures were allowed to stand at room temperature for 5 minutes before the absorbance at 595 nm was recorded using the dye reagent as a blank. The concentrations of the protein samples were determined spectrophotometrically by BioPhotometer (Eppendorf) and an equal amount of the protein (25 μg) was subjected to SDS-PAGE after equalizing the amount of protein loaded per lane.

3) Western Blot Analysis

SDS-PAGE was carried out to separate the proteins according to the molecular weights before Western blotting. The percentage of acrylamide added in the running gel varied according to the molecular weight of the target protein being separated. Table 2.2 shows the composition of the chemical reagents needed for various percentages of SDS-polyacrylamide gel. Samples containing the same amount of protein (25 μg) were mixed with an equal volume of 2X SDS gel loading buffer and boiled for 10 minutes before loading onto the SDS-polyacrylamide gel with a 5% stacking gel. Eight microliters of Kaleidoscope prestained standards was loaded as molecular weight marker and the gel was run under constant voltage of 100 volts for 2 to 3 hours.

Table 2.2: Composition of the SDS-polyacrylamide gel.

Reagents	5 % Stacking Gel (3 ml)	Separating Gel (5ml)		
		7.5%	10%	12%
Distilled water	1.65 ml	2.4 ml	2 ml	1.65 ml
Lower Buffer	-	1.25 ml	1.25 ml	1.25 ml
Upper Buffer	0.75 ml	-	-	-
30% Acrylamide Stock	0.5 ml	1.25 ml	1.665 ml	2 ml
10% SDS	30 μl	25 μl	25 μl	25 μl
10% APS	40 μl	75 μl	75 μl	75 μl
TEMED	3 μl	2 μl	2 μl	2 μl

After electrophoresis, the stacking gel was cut and removed. The gel was rinsed with distilled water for 10 minutes to remove SDS that could hinder the protein transfer. Thereafter, the gel was soaked in transfer buffer for 10 minutes. Three pieces of 3 MM Whatman chromatography papers soaked with transfer buffer were placed on the semi-dry blotting apparatus (Bio-Rad Laboratories). They were then

covered by one piece of PVDF membrane activated by soaking in 100% methanol for 2 seconds and in transfer buffer for 10 minutes. The gel was carefully placed on the membrane and three more pieces of chromatography papers soaked with transfer buffer were placed on the gel. Finally, air bubbles were excluded by rolling a glass tube on the whole pack. The proteins on the gel were then electroblotted onto the PVDF membrane at 16 volts for 35 minutes. After the transfer, the membrane with transferred proteins was washed with washing buffer and incubated with 5% skimmed milk blocking solution with constant rotation at room temperature for at least 1 hour to block the non-specific sites for probing. The membrane was then incubated with the desired primary antibody diluted at a ratio of 1:250 – 1:100,000 in 5% skimmed milk at 4°C overnight. After blotting with primary antibody, the membrane was washed for 20 minutes with three changes of washing buffer. After washing, the membrane was incubated with the corresponding appropriately diluted (1:1,000) horseradish peroxidase-linked secondary antibody in washing buffer for one hour at room temperature. Incubation was followed by four washes in washing buffer for 20 minutes. After a final round of washing, the membrane was subjected to ECL assay.

4) ECL Assay

ECL™ detection reagent 1 (0.5 ml) was freshly mixed with 0.5 ml of detection reagent 2 and poured onto the membrane. The membrane was then immersed in the reagent mixture and allowed to react for 1 minute. Excess reagent mixture was removed by holding the membrane vertically and touching the edge of the membrane against tissue paper. The membrane covered with Glad Wrap, with the protein side up, was placed in the film cassette. A sheet of X-ray film (Fuji, Japan) was then carefully placed on top of the membrane and allowed to expose in dark for times

varying from 30 seconds to 5 minutes. Finally, the film was developed and the immunoreactive bands could be visualized on the X-ray film. Semi-quantitative scanning densitometry was performed using the ImageQuant software (Molecular Dynamics). Bands for analysis were outlined and their volumes were integrated to obtain an indication of band density. The densitometric values were normalized with respect to the value obtained for a control protein (β-actin) in order to correct for any deviation in the loaded protein.

2.2.13 Statistical Analysis

Each experiment was performed at least twice and the results of only one representative experiment were presented. All data were presented as arithmetic mean ± standard error. The Student's *t*-test was used for statistical comparison of data and difference was considered to be statistically significant if $p < 0.05$.

CHAPTER 3

ANTI-PROLIFERATIVE EFFECTS OF GLYCYRRHIZIN AND 18β-GLYCYRRHETINIC ACID ON NEUROBLASTOMA CELLS

3.1 Introduction

Herbs have been used for medicinal purposes for centuries. Herbal medicine is based on the premise that plants contain natural substances that promote health and alleviate illness. A variety of herbs and herbal extracts contain different phytochemicals with a wide range of biological activities that may have therapeutic effects. Moreover, many Western drugs have their origin in a plant extract (Craig, 1999). The traditional Chinese medicine licorice (*Glycyrrhiza glabra* L.) has been widely used for the treatment of cough, bronchitis, peptic ulcer, gastritis, adrenal insufficiency and urinary tract inflammation (Mills & Bone, 2000). Recently, research interest has focused on herbs that possess anti-tumor or immune-stimulating properties that may be useful adjuncts which can help to reduce the risk of cancer. Glycyrrhizin, a major component of licorice root, is a sweet-tasting triterpenoid saponin. Glycyrrhizin and its aglycone, glycyrrhetinic acid, have been reported to possess anti-mutagenic, anti-carcinogenic, anti-tumor, anti-inflammatory, anti-viral, immunomodulatory and hepatoprotective activities (Mills & Bone, 2000; Wang & Nixon, 2001).

Earlier work had shown that glycyrrhizin and 18β-glycyrrhetinic acid could inhibit the growth of B16 melanoma cells (Abe *et al.*, 1987), SupT1 non-Hodgkin's lymphoma cells, JJhan and HSB-2 chronic myeloid leukemia cells *in vitro* (Malagoli *et al.*, 1998). More recently, glycyrrhizin and 18β-glycyrrhetinic acid were found to inhibit the growth of myeloid leukemia cells *in vitro* by inducing the apoptosis and differentiation of the leukemia cells (Tsang, 2001). These studies showed that glycyrrhizin and 18β-glycyrrhetinic acid could inhibit the growth of various types of

cancer cells *in vitro*. A recent report showed that euxanthone isolated from the medicinal plant *Polygala caudata* could inhibit the growth and induce the differentiation of neuroblastoma cells (Mak *et al.*, 2000). To our knowledge, however, there has been no report demonstrating the effect of glycyrrhizin and 18β-glycyrrhetinic acid, the major active constituents of licorice, on the growth of neuroblastoma cells. Therefore, in this chapter, attempts have been made to investigate the effects of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells and to further elucidate their anti-tumor mechanisms. Firstly, the differential anti-proliferative and cytotoxic effects of glycyrrhizin and 18β-glycyrrhetinic acid on various murine and human neuroblastoma cell lines were studied. In addition, the kinetics and reversibility of glycyrrhizin and 18β-glycyrrhetinic acid with respect to their anti-proliferative effect on a murine neuroblastoma cell line (Neuro-2a, subclone BU-1 cells) were examined. Furthermore, the ability of glycyrrhizin and 18β-glycyrrhetinic acid to induce apoptosis in BU-1 cells was also investigated.

3.2 Results

3.2.1 Differential Anti-proliferative Effect of Glycyrrhizin and 18β-Glycyrrhetinic Acid on Various Neuroblastoma Cell Lines *In Vitro*

The effect of glycyrrhizin and 18β-glycyrrhetinic acid on the proliferation of neuroblastoma cells was studied using tritiated thymidine (^{3}H-TdR) incorporation assay. The incorporation of labeled DNA precursor, ^{3}H-TdR, into the DNA of the cells is directly proportional to the extent of cell proliferation. Two human and one murine neuroblastoma cell lines with appropriate cell density were treated with various concentrations of glycyrrhizin and 18β-glycyrrhetinic acid for 48 hours as indicated in the figures and the amount of radioactivity incorporated into DNA was measured. It was found that both glycyrrhizin and 18β-glycyrrhetinic acid could inhibit the proliferation of the murine neuroblastoma BU-1 cells (Figure 3.1 & 3.4), and the human neuroblastoma SK-N-DZ (Figure 3.2 & 3.5), and SH-SY5Y (Figure 3.3 & 3.6) cells in a dose-dependent manner. The estimated IC_{50} values of glycyrrhizin and 18β-glycyrrhetinic acid at 48 hours of incubation were summarized in Table 3.1.

It is interesting to note that at lower concentrations of 18β-glycyrrhetinic acid (< 80 μM), the anti-proliferative effect on murine and human neuroblastoma cells was minimal but it was sharply increased at concentrations ≥ 80 μM (Figure 3.4 - 3.6). As indicated in Table 3.1, different neuroblastoma cell lines had differential sensitivity to the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid. By comparing the IC_{50} values at 48 hours of treatment, the SK-N-DZ and BU-1 cells

were found to be more sensitive to the anti-proliferative effect of glycyrrhizin than the SH-SY5Y cells, with an estimated IC_{50} value of about 3 mM. On the other hand, the BU-1 cells were found to be the most sensitive cells in response to 18β-glycyrrhetinic acid, with an estimated IC_{50} value of about 75 μM. The proliferation of neuroblastoma cells was completely inhibited by 18β-glycyrrhetinic acid at a concentration of 120 - 130 μM but no complete inhibition of cell proliferation was achieved at the highest concentration of glycyrrhizin (4.5 mM) tested in this study. This suggests that 18β-glycyrrhetinic acid was more potent than glycyrrhizin as it inhibited the growth of neuroblastoma cells at micromolar level while glycyrrhizin inhibited the growth of neuroblastoma cells at millimolar level.

The neutral red assay was also used as a non-radioactive method for the measurement of cellular proliferation of BU-1 cells after 48 hours of treatment with different concentrations of glycyrrhizin and 18β-glycyrrhetinic acid. The uptake, transport, and storage of neutral red dye occurs via active biological processes that require energy, as well as intact cellular and lysosomal membranes. Viable cells take up the neutral red dye and transport it to a specific cellular compartment, the lysosome. Damage to any of the systems involved in the process or a reduction in cell number due to cell death would result in decreased uptake of the neutral red dye. Therefore, the number of viable cells can be indicated by the amount of neutral red taken up by the cells. The neutral red taken up by the cells was released by 1% SDS and can be quantified by measuring the absorbance at 540 nm which reflects the number of viable cells.

The results of neutral red assay (Figure 3.7 & 3.8) showed that glycyrrhizin and

18β-glycyrrhetinic acid caused a dose-dependent reduction in the absorbance measured at 540 nm, indicating that glycyrrhizin and 18β-glycyrrhetinic acid exerted an inhibitory effect on the growth of BU-1 cells *in vitro*. Moreover, the results were similar to that of ^{3}H-TdR incorporation assay although the estimated IC_{50} values were slightly higher than that of ^{3}H-TdR incorporation assay. Due to the higher sensitivity of the ^{3}H-TdR incorporation assay, it was the method of choice for measuring the proliferation of neuroblastoma cells in all subsequent experiments.

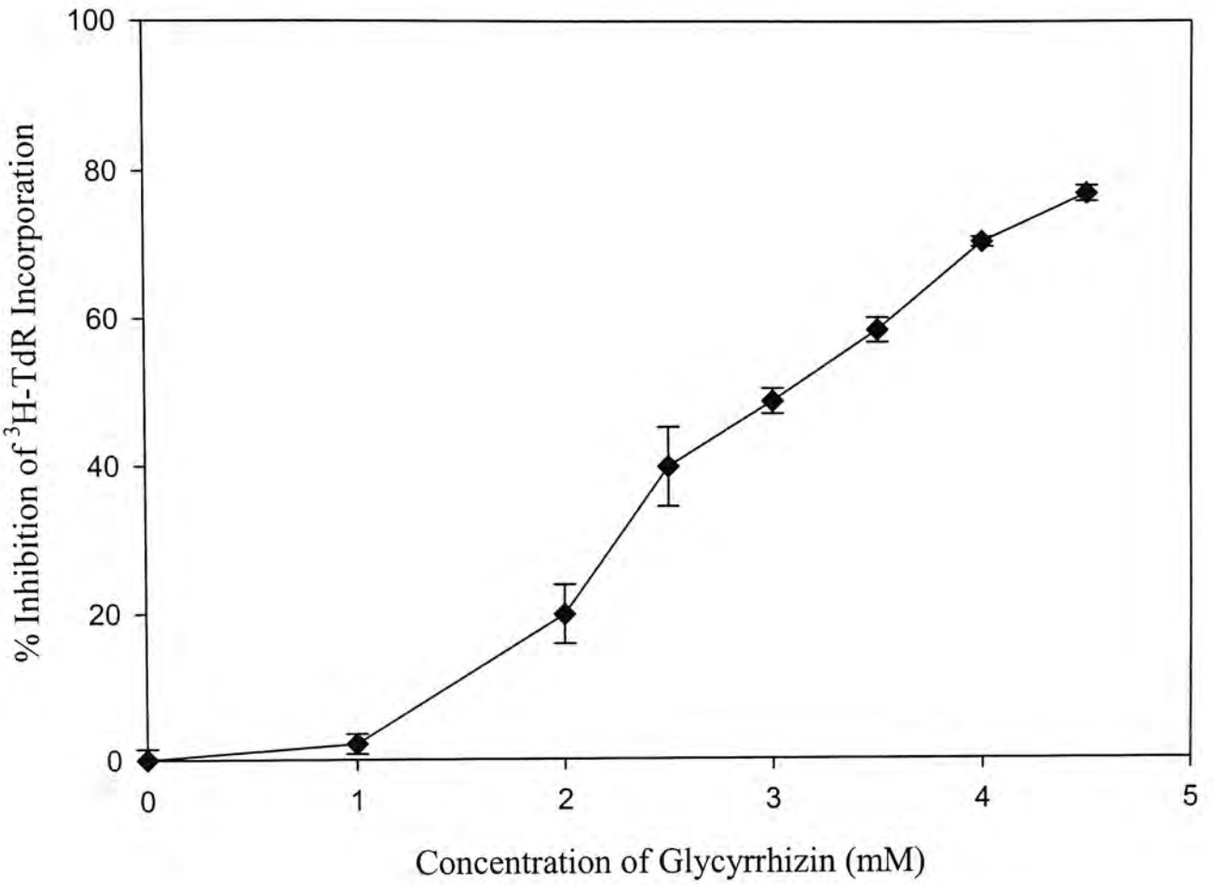


Figure 3.1: Anti-proliferative effect of glycyrrhizin on the murine neuroblastoma Neuro-2a BU-1 cells as determined by the tritiated thymidine incorporation assay. BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with different concentrations (0 - 4.5 mM) of glycyrrhizin at 37°C for 48 hours and were pulsed with 0.5 μCi of ^{3}H-TdR during the last 6 hours. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

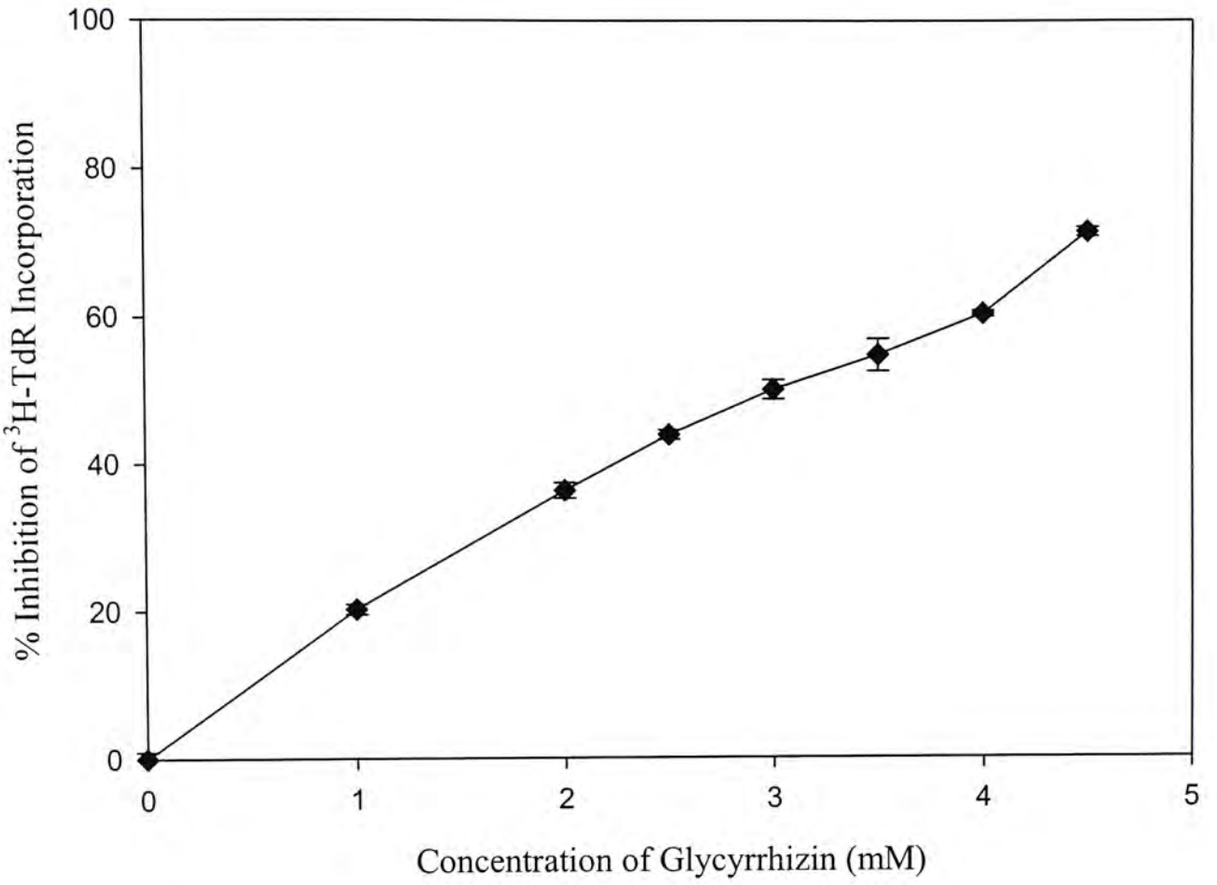


Figure 3.2: Anti-proliferative effect of glycyrrhizin on the human neuroblastoma SK-N-DZ cells as determined by the tritiated thymidine incorporation assay. SK-N-DZ cells (10^5 cells/ml) were cultured overnight and incubated with different concentrations (0 - 4.5 mM) of glycyrrhizin at 37°C for 48 hours and were pulsed with 0.5 μCi of ^{3}H-TdR during the last 10 hours. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

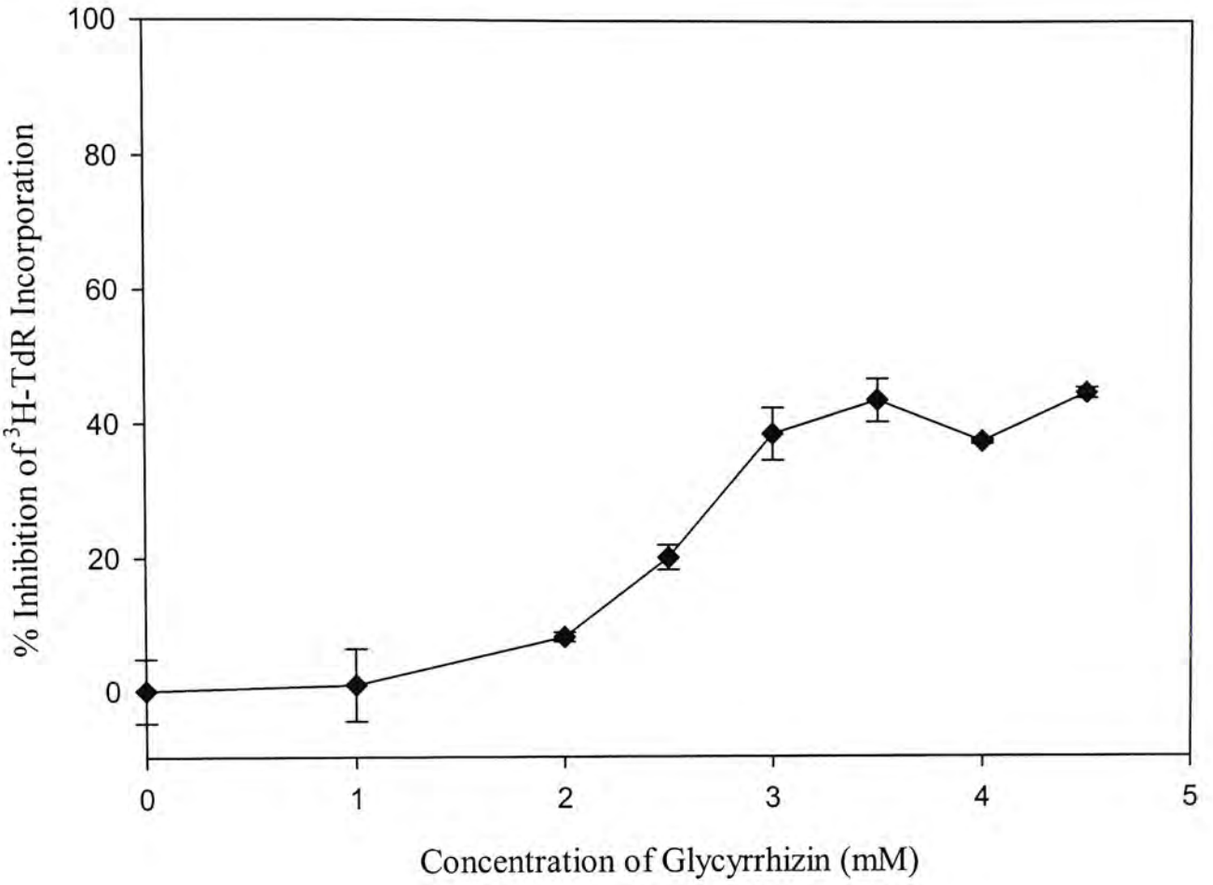


Figure 3.3: Anti-proliferative effect of glycyrrhizin on the human neuroblastoma SH-SY5Y cells as determined by the tritiated thymidine incorporation assay. SH-SY5Y cells (1.5 x 10^5 cells/ml) were cultured overnight and incubated with different concentrations (0 - 4.5 mM) of glycyrrhizin at 37°C for 48 hours and were pulsed with 0.5 μCi of ^{3}H-TdR during the last 10 hours. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

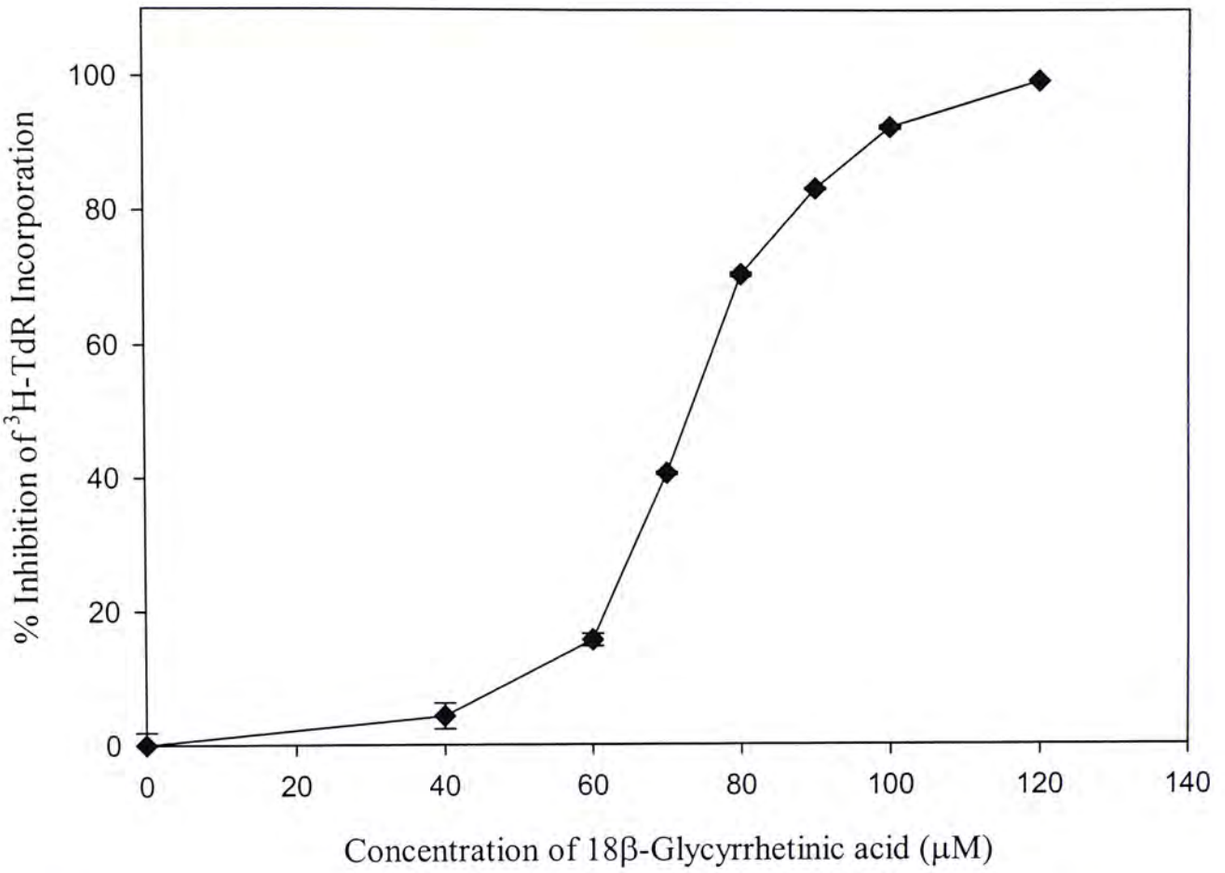


Figure 3.4: Anti-proliferative effect of 18β-glycyrrhetinic acid on the murine neuroblastoma Neuro-2a BU-1 cells as determined by the tritiated thymidine incorporation assay. BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with different concentrations (0 - 120 μM) of 18β-glycyrrhetinic acid at 37°C for 48 hours and were pulsed with 0.5 μCi of ^{3}H-TdR during the last 6 hours. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

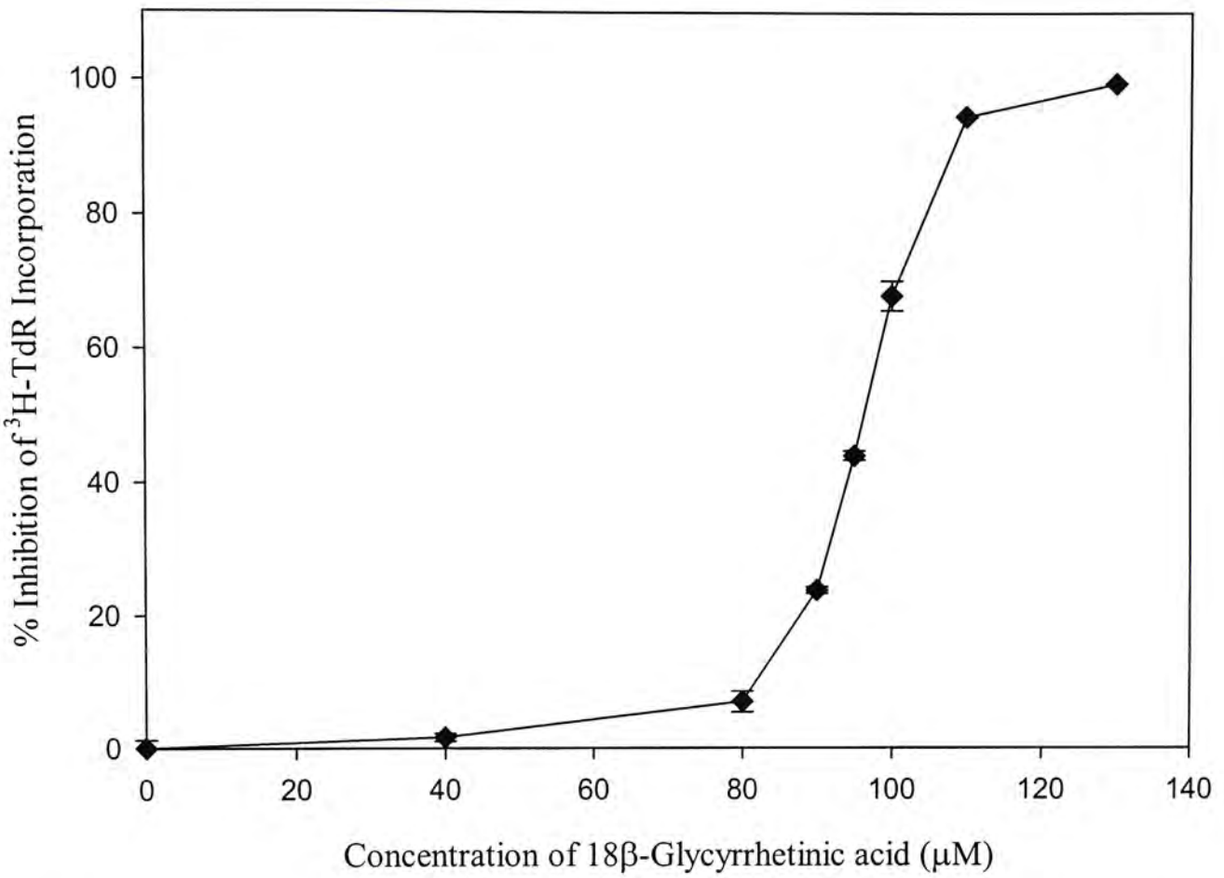


Figure 3.5: Anti-proliferative effect of 18β-glycyrrhetinic acid on the human neuroblastoma SK-N-DZ cells as determined by the tritiated thymidine incorporation assay. SK-N-DZ cells (10^5 cells/ml) were cultured overnight and incubated with different concentrations (0 - 130 μM) of 18β-glycyrrhetinic acid at 37°C for 48 hours and were pulsed with 0.5 μCi of ^{3}H-TdR during the last 10 hours. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

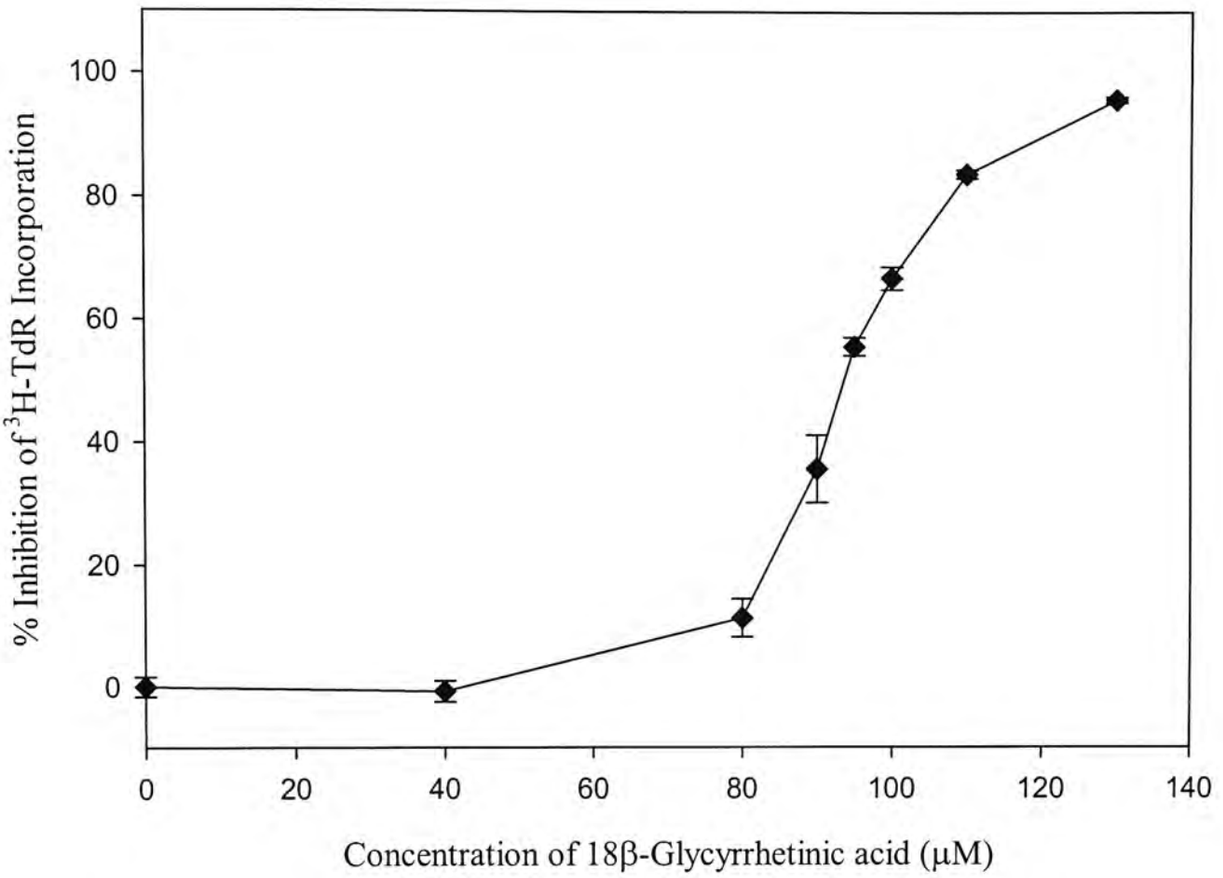


Figure 3.6: Anti-proliferative effect of 18β-glycyrrhetinic acid on the human neuroblastoma SH-SY5Y cells as determined by the tritiated thymidine incorporation assay. SH-SY5Y cells (1.5 x 10^5 cells/ml) were cultured overnight and incubated with different concentrations (0 - 130 μM) of 18β-glycyrrhetinic acid at 37°C for 48 hours and were pulsed with 0.5 μCi of ^{3}H-TdR during the last 10 hours. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

Table 3.1: The estimated IC_{50} values of glycyrrhizin and 18β-glycyrrhetinic acid on various neuroblastoma cell lines after 48 hours of treatment.

Cell Line		Estimated IC_{50} Value	
		Glycyrrhizin (mM)	18β-Glycyrrhetinic acid (μM)
Murine Neuroblastoma	Neuro-2a BU-1	3.2	75
Human Neuroblastoma	SK-N-DZ	3	97
Human Neuroblastoma	SH-SY5Y	> 4.5	93

IC_{50} value is the estimated concentration of glycyrrhizin or 18β-glycyrrhetinic acid which can cause 50% inhibition of ^{3}H-TdR incorporation under the specified experimental conditions.

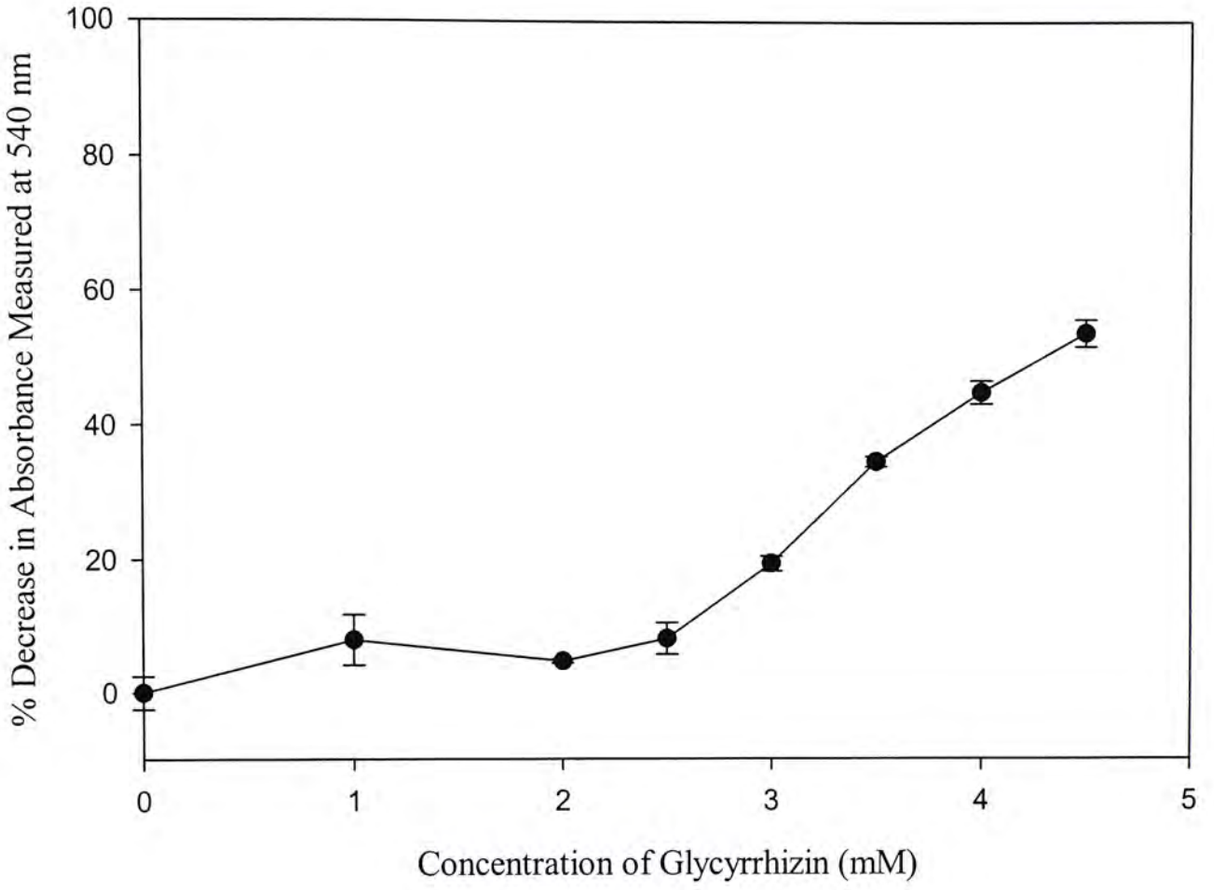


Figure 3.7: Anti-proliferative effect of glycyrrhizin on the murine neuroblastoma BU-1 cells as determined by the neutral red assay. BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with different concentrations (0 – 4.5 mM) of glycyrrhizin at 37°C for 48 hours and cell proliferation was determined by the neutral red assay as described under Materials and Methods. Results were expressed as % decrease in absorbance measured at 540 nm, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

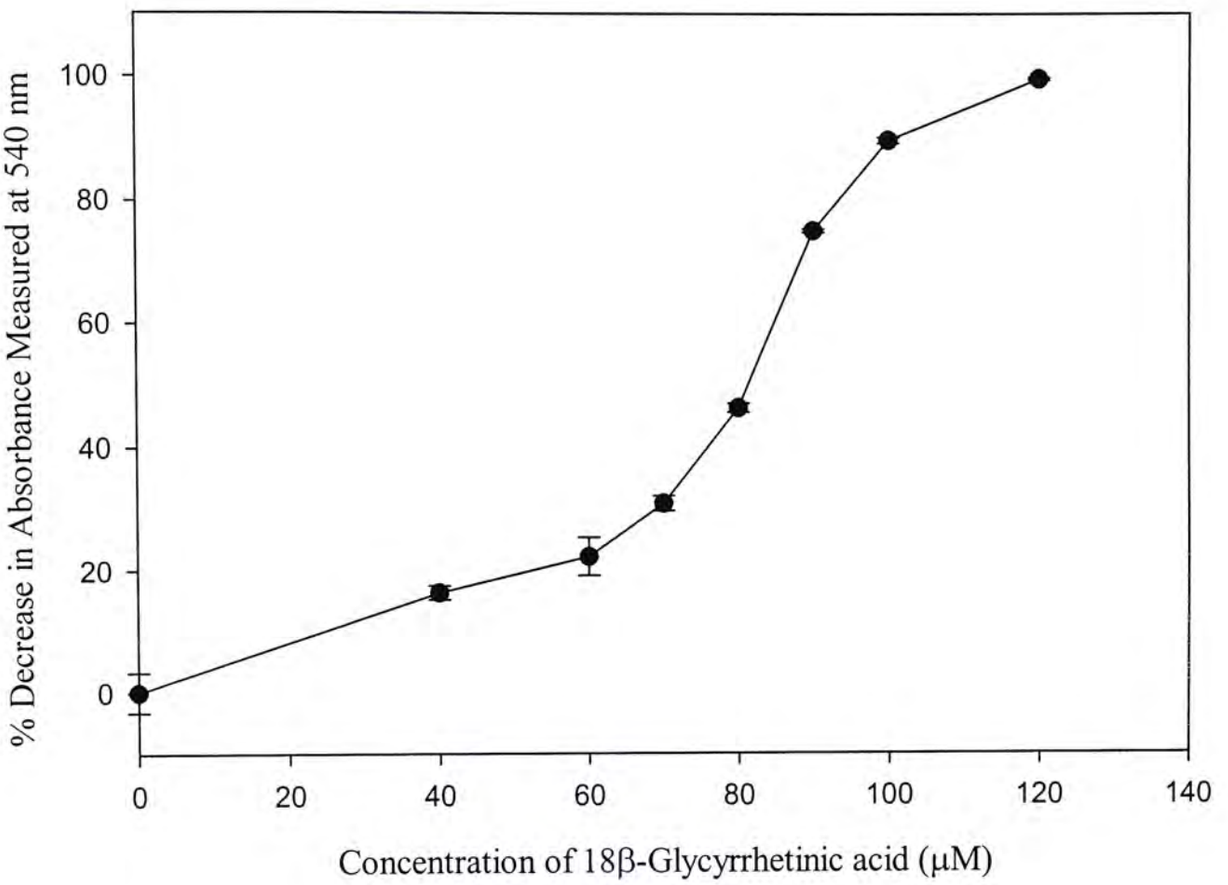


Figure 3.8: Anti-proliferative effect of 18β-glycyrrhetinic acid on the murine neuroblastoma BU-1 cells as determined by the neutral red assay. BU-1 cells (7.5×10^4 cells/ml) were cultured overnight and incubated with different concentrations (0 - 120 μM) of 18β-glycyrrhetinic acid at 37°C for 48 hours and cell proliferation was determined by the neutral red assay as described under Materials and Methods. Results were expressed as % decrease in absorbance measured at 540 nm, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

3.2.2 Effect of 18β-Glycyrrhetinic Acid on the Clonogenicity of the Murine Neuroblastoma BU-1 Cells *In Vitro*

In the clonogenic assay, the ability of a single cell to divide enough times to form a visible, countable colony is evaluated. The ability to form colonies is a measure of the reproductive integrity or the proliferative capability of a cell population and this assay is widely used to determine the effect of cytostatic/cytotoxic agents on tumor cells *in vitro*. Since 18β-glycyrrhetinic acid exhibited potent anti-proliferative effect on BU-1 cells *in vitro*, its influence on the clonogenicity of BU-1 cells *in vitro* was also examined. It was found that the colony-forming ability of 18β-glycyrrhetinic acid-treated BU-1 cells in soft agar was markedly reduced when compared with the control cells. Moreover, this effect seems to be dose-dependent with the cloning efficiency reduced from 44.13% for DMSO-treated control BU-1 cells to 22.53% and 12.53% for 80 μM and 100 μM 18β-glycyrrhetinic acid-treated cells, respectively (Table 3.2).

Table 3.2: Effect of 18β-glycyrrhetinic acid on the colony-forming ability of BU-1 cells in soft agar.

Concentration of 18β-glycyrrhetinic acid (μM)	Number of colonies formed in soft agar	% Cloning efficiency in soft agar
DMSO control	110 ± 6.51	44.13 ± 2.60
80	56 ± 3.06 *	22.53 ± 1.22 *
100	31 ± 2.52 *	12.53 ± 1.01 *

BU-1 cells (250 cells in each well of the soft agar plate) were incubated with two different concentrations of 18β-glycyrrhetinic acid (80 and 100 μM) at 37°C for 9 days. After staining with hematoxylin, the number of colonies containing more than 50 cells was counted. The results of triplicate cultures were expressed as % cloning efficiency in soft agar, which represents the number of colonies per 100 cells initially plated in soft agar plate. * $p < 0.01$ vs. DMSO control.

3.2.3 Kinetic and Reversibility Studies of the Anti-proliferative Effect of Glycyrrhizin and 18β-Glycyrrhetinic Acid on the Neuroblastoma BU-1 Cells

Kinetic study using the well-characterized and subcloned murine neuroblastoma BU-1 cells showed that glycyrrhizin and 18β-glycyrrhetinic acid inhibited the proliferation of the neuroblastoma cells time-dependently (Figure 3.9 & 3.10) and marked inhibition of BU-1 cell proliferation was observed after 24 hours of treatment. However, for both glycyrrhizin and 18β-glycyrrhetinic acid treatment, only a slight difference in the extent of growth inhibition was found between 24 and 48 hours of incubation although maximum inhibition of cell growth was obtained after 48 hours of treatment.

To test whether the neuroblastoma cells resume proliferation after the removal of drugs, reversibility study was performed. In this study, the cells were treated either with 70 and 80 μM 18β-glycyrrhetinic acid or 3.5 and 4.5 mM glycyrrhizin and the culture medium containing the drug was removed, replaced with fresh culture medium 8 or 24 hours after drug addition, and the ^{3}H-TdR incorporation assay was performed 48 hours after the initiation of experiment. As shown in Figure 3.11, exposure of the BU-1 cells to glycyrrhizin and 18β-glycyrrhetinic acid for only 8 or 24 hours still resulted in growth inhibition although the percentage inhibition was less than that observed with 48 hours of drug treatment. Moreover, the effect of glycyrrhizin on BU-1 cells seems to be more reversible than that of 18β-glycyrrhetinic acid since the BU-1 cells were more capable of regaining proliferation after removal of glycyrrhizin and the percentage inhibition of cell growth decreased to less than 20%.

Furthermore, for both treatments with glycyrrhizin and 18β-glycyrrhetinic acid, the higher the concentration of drugs used, the greater the growth inhibitory effect was observed despite the drug was removed. Therefore, the capability of neuroblastoma cells to recover from treatment with glycyrrhizin or 18β-glycyrrhetinic acid was dependent on both drug concentration and duration of incubation.

Besides the reversibility test, limiting dilution assay was also employed to determine the proliferating potential of BU-1 cells after drug treatment. This assay system is efficient and provides an informative method to quantitatively analyze the responding cell frequencies. BU-1 cells treated with glycyrrhizin or 18β-glycyrrhetinic acid for 4 days were harvested and seeded into the wells of 96-well flat-bottomed plates with different cell densities for 7 days and finally the fraction of negative wells in log scale was plotted against each cell concentration. The frequency of proliferative cells was estimated by the cell concentration in which 37% of the wells were negative for growth.

As shown in Figure 3.12, the frequency of proliferative cells in medium control was 1 in 1.6 while treatment of BU-1 cells with 3.5 mM glycyrrhizin for 4 days reduced the frequency of proliferative cells to 1 in 6. Moreover, Figure 3.13 shows that the frequency of proliferative cells after 4 days of treatment with 80 μM 18β-glycyrrhetinic acid declined from 1 in 1.9 of solvent control to 1 in 7.9 of drug-treated cells. These results confirmed that both glycyrrhizin and 18β-glycyrrhetinic acid could exert some irreversible changes to the BU-1 cells so that the proliferating potential of drug-treated cells decreased despite the drugs were removed.

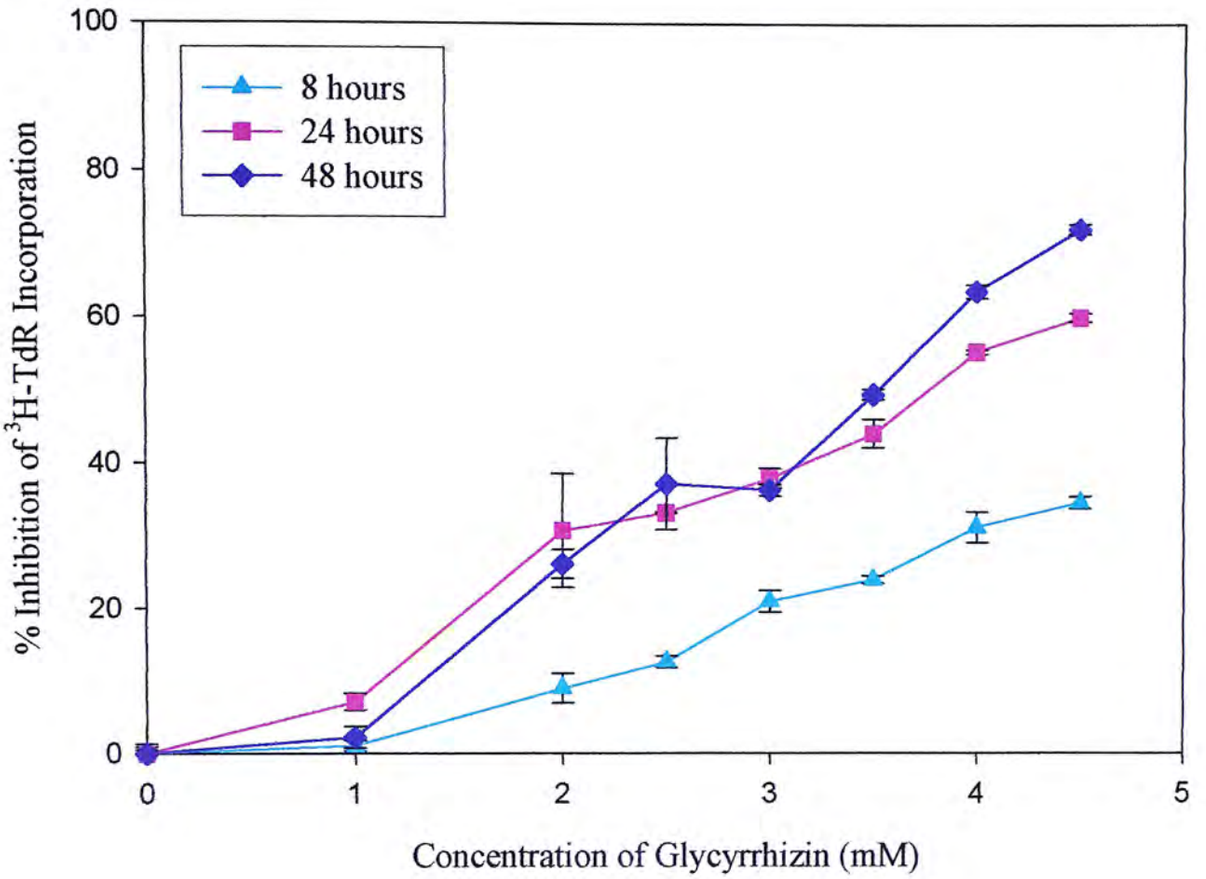


Figure 3.9: Kinetic study on the anti-proliferative effect of glycyrrhizin on the murine neuroblastoma BU-1 cells. BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with different concentrations (0 - 4.5 mM) of glycyrrhizin at 37°C for different periods of time (8, 24 or 48 hours). Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

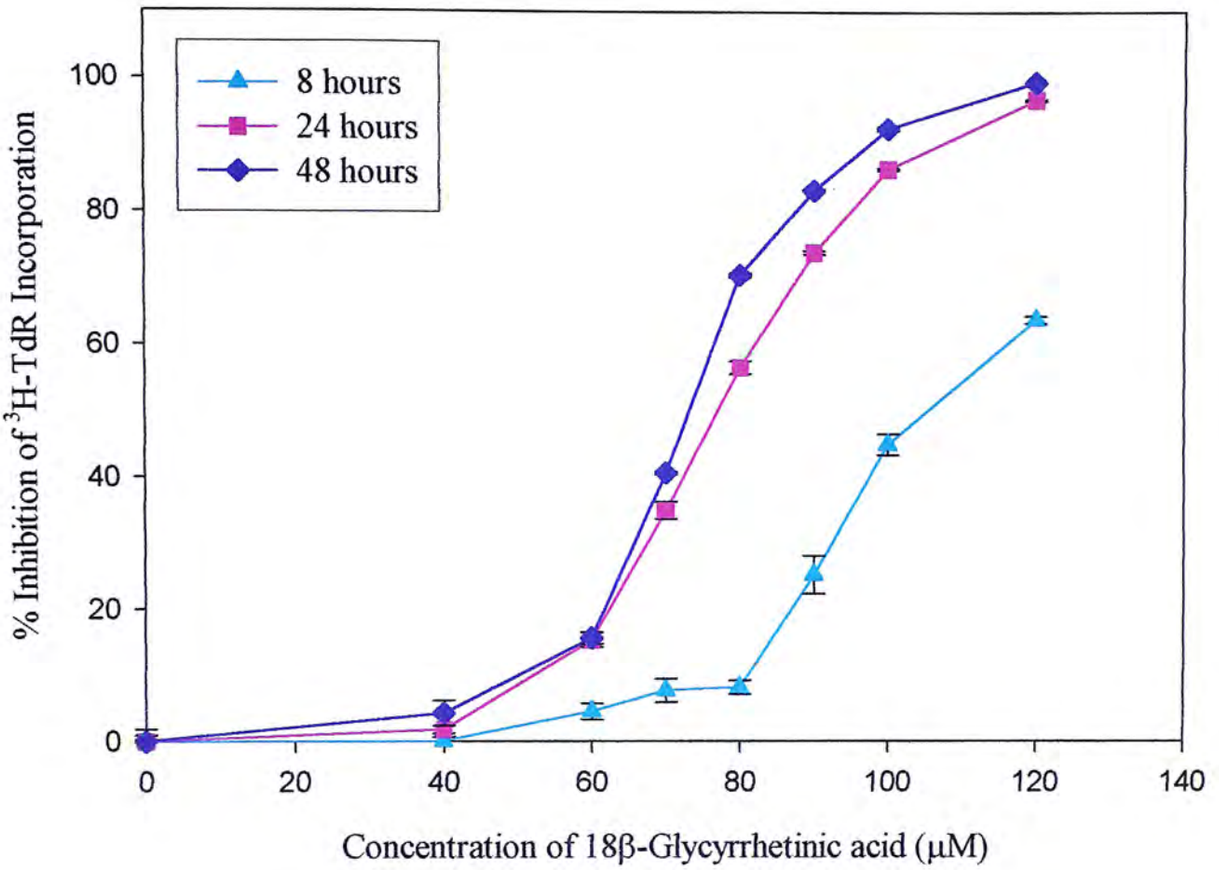


Figure 3.10: Kinetic study on the anti-proliferative effect of 18β-glycyrrhetinic acid on the murine neuroblastoma BU-1 cells. BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with different concentrations (0 – 120 μM) of 18β-glycyrrhetinic acid at 37°C for different periods of time (8, 24 or 48 hours). Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

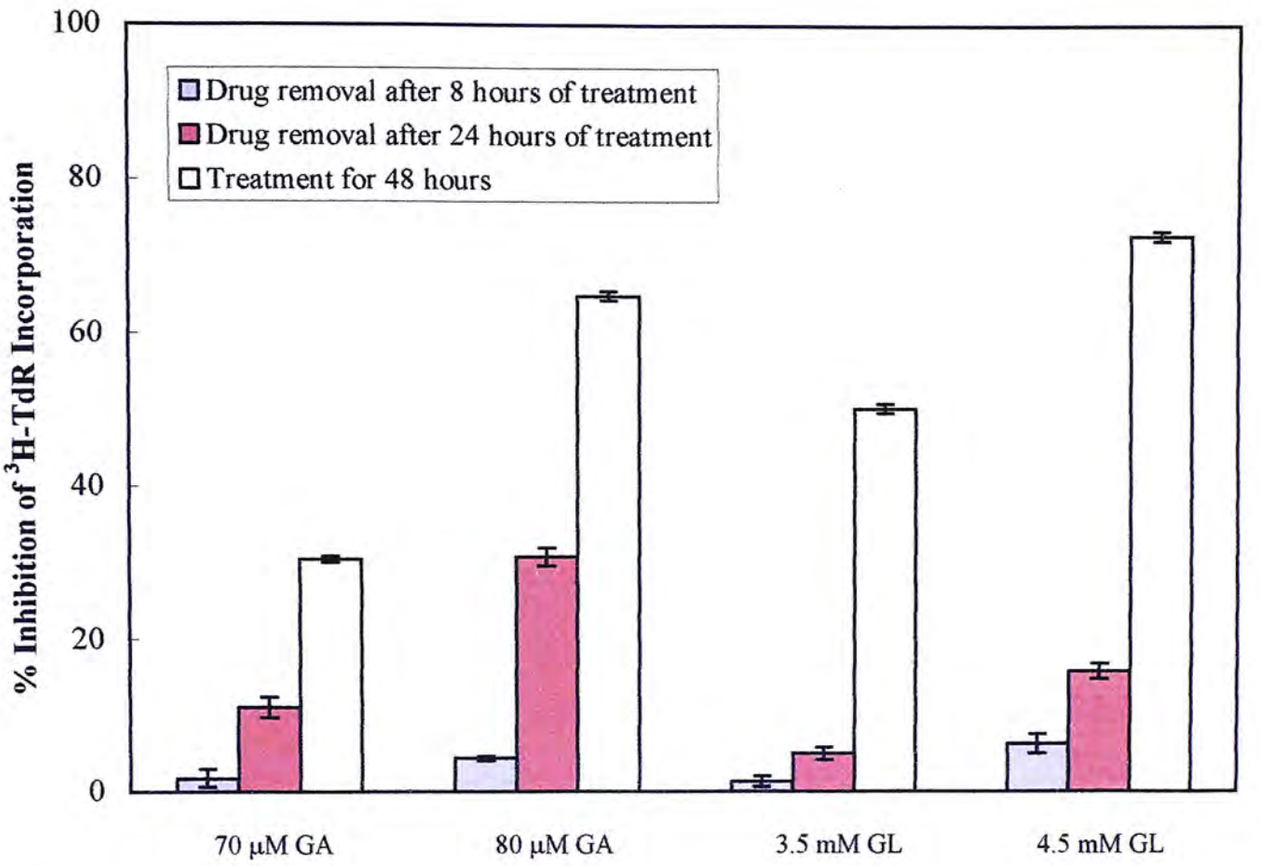


Figure 3.11: Reversibility study on the anti-proliferative effect of 18β-glycyrrhetinic acid (GA) and glycyrrhizin (GL) on the murine neuroblastoma BU-1 cells. BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with two different concentrations (70 or 80 μM) of 18β-glycyrrhetinic acid (GA) and two different concentrations (3.5 or 4.5 mM) of glycyrrhizin (GL) at 37°C. Drugs were removed and replaced by fresh culture medium at the corresponding time periods (8 or 24 hours after drug addition) and all cultures were incubated up to 48 hours after drug addition. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

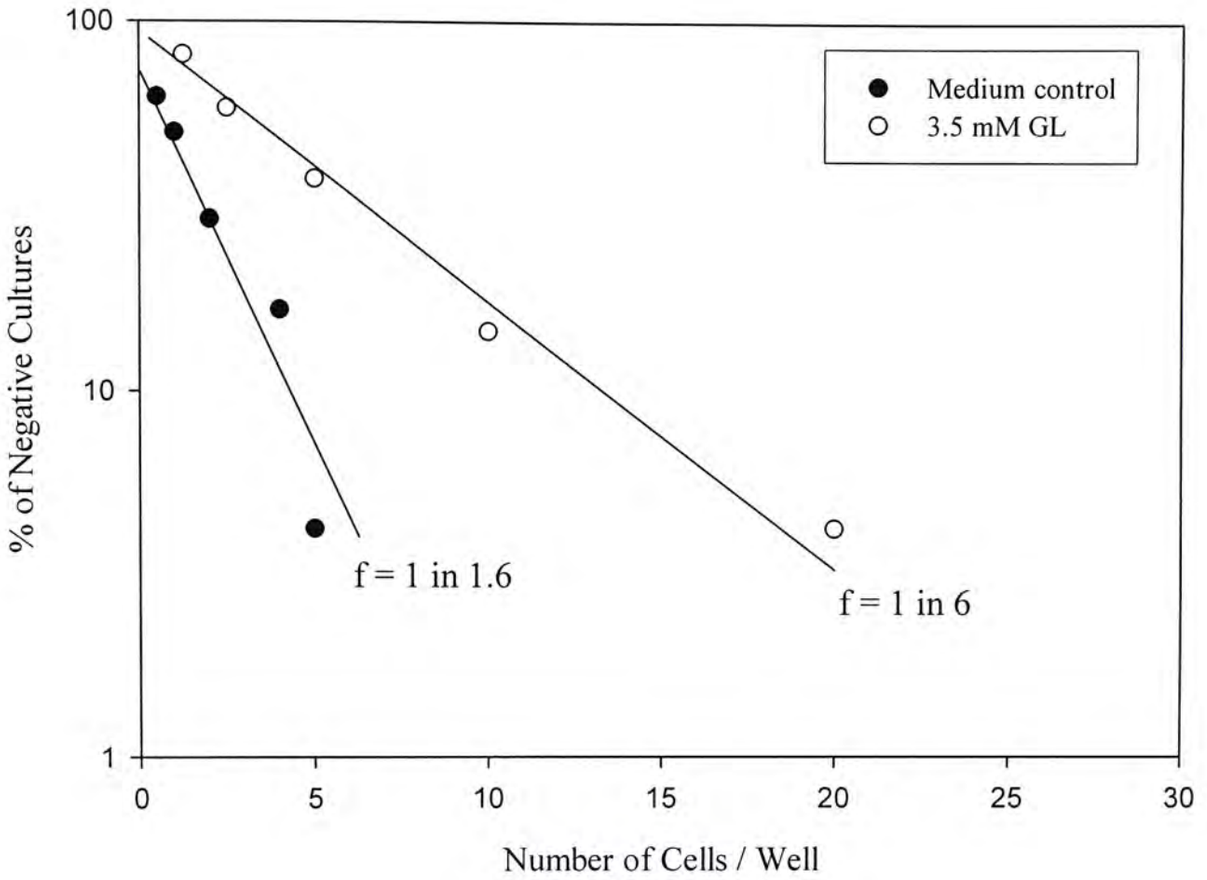


Figure 3.12: Frequency analysis of proliferative BU-1 cells after 4 days of treatment with glycyrrhizin (GL). BU-1 cells (2.5 x 10^4 cells/ml) were cultured overnight and incubated with either culture medium (solid circle) or 3.5 mM glycyrrhizin (open circle) in the wells of a 6-well plate for 4 days. The cells were then washed and various concentrations of viable cells were seeded into the wells of 96-well plates, with 48 replicates for each cell concentration. The cells were incubated for 7 days at 37°C in a humidified 5% CO_2 incubator. The frequency of proliferating neuroblastoma cells indicated in the figure was determined as stated under Materials and Methods.

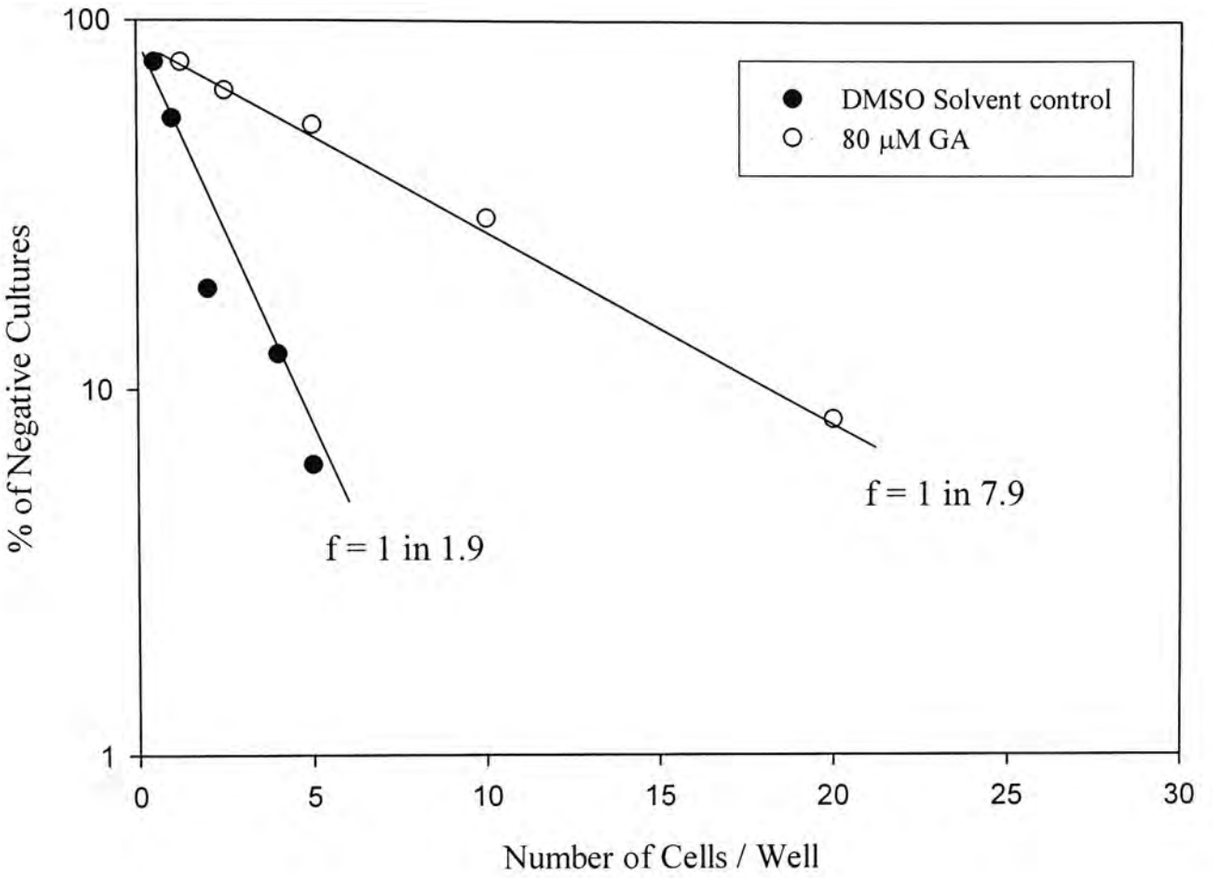


Figure 3.13: Frequency analysis of proliferative BU-1 cells after treatment with 18β-glycyrrhetinic acid (GA). BU-1 cells (2.5 x 10^4 cells/ml) were cultured overnight and incubated with either DMSO control solvent (solid circle) or 80 μM 18β-glycyrrhetinic acid (open circle) in the wells of a 6-well plate for 4 days. The cells were then washed and various concentrations of viable cells were seeded into the wells of 96-well plates, with 48 replicates for each cell concentration. The cells were incubated for 7 days at 37°C in a humidified 5% CO_2 incubator. The frequency of proliferating neuroblastoma cells indicated in the figure was determined as stated under Materials and Methods.

3.2.4 Cytotoxic Effect of Glycyrrhizin and 18β-Glycyrrhetinic Acid on the Neuroblastoma BU-1 Cells *In Vitro*

To determine whether the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid was due to their direct cytotoxicity on the treated cells, the viability of glycyrrhizin- and 18β-glycyrrhetinic acid-treated murine neuroblastoma BU-1 cells was examined by the trypan blue exclusion assay after 48 hours of treatment, an incubation time that yielded maximum inhibition of neuroblastoma cell proliferation. It was found that at all concentrations of glycyrrhizin and 18β-glycyrrhetinic acid tested, the percentage of viable cells remained over 90% after 48 hours of treatment (Table 3.3). This suggests that glycyrrhizin and 18β-glycyrrhetinic acid did not exhibit any significant direct cytotoxic activity on the neuroblastoma cells. Taken together with the findings of previous experiments, it is believed that glycyrrhizin and 18β-glycyrrhetinic acid exert a cytostatic rather than cytotoxic effect on the neuroblastoma cells.

Table 3.3: Effect of glycyrrhizin and 18β-glycyrrhetinic acid on the viability of the murine neuroblastoma BU-1 cells *in vitro*.

BU-1 cells treated with	Concentration (mM)	Cell viability (%)
Glycyrrhizin	0	95.40 ± 0.004
	1	93.79 ± 0.015
	2	95.94 ± 0.002
	3	95.03 ± 0.012
	4	96.49 ± 0.007
	4.5	96.91 ± 0.003
18β-Glycyrrhetinic acid	0	97.12 ± 0.004
	0.04	96.97 ± 0.001
	0.06	97.14 ± 0.009
	0.08	97.60 ± 0.009
	0.10	95.60 ± 0.007
	0.12	95.29 ± 0.009

BU-1 cells (7.5×10^4 cells/ml) were cultured overnight and incubated with different concentrations of glycyrrhizin (0 - 4.5 mM) or 18β-glycyrrhetinic acid (0 - 0.12 mM) at 37°C for 48 hours. Cell viability was determined by the trypan blue exclusion test, and the results were expressed as mean % of viable cells ± S.E. of triplicate cultures.

3.2.5 Inability of Glycyrrhizin and 18β-Glycyrrhetinic Acid to Induce DNA Fragmentation in the Neuroblastoma BU-1 Cells

Recent researches have shown that induction of apoptosis is one of the anti-tumor mechanisms of natural products (Okabe *et al.*, 1999; Ahmad *et al.*, 2000). Morphological changes of apoptosis include shrinkage of cells and nuclei, chromatin condensation, and fragmentation into membrane-bound apoptotic bodies (Saraste & Pulkki, 2000). Moreover, the occurrence of DNA ladder is the result of cleavage of DNA by endonucleases (Herrmann *et al.*, 1994).

In the present study, the ability of glycyrrhizin and 18β-glycyrrhetinic acid to induce apoptosis in BU-1 cells was examined by DNA fragmentation assay. As shown in Figure 3.14, at all concentrations of glycyrrhizin and 18β-glycyrrhetinic acid tested, no DNA laddering was observed after 48 hours. No DNA fragmentation can be detected even after 72 hours of treatment with glycyrrhizin and 18β-glycyrrhetinic acid (data not shown). Possible explanations for this could be due to the inability of glycyrrhizin and 18β-glycyrrhetinic acid to induce apoptosis in the BU-1 cells or due to the intrinsic property of BU-1 cells. In order to test whether BU-1 cells were intrinsically resistant to apoptosis induction or glycyrrhizin and 18β-glycyrrhetinic acid were incapable of inducing apoptosis in BU-1 cells, a well known apoptosis inducing agent, etoposide was employed in my study. The results showed that BU-1 cells responded dose-dependently to etoposide to give DNA ladders while glycyrrhizin and 18β-glycyrrhetinic acid failed to induce apoptosis (Figure 3.15).

In addition to the DNA fragmentation assay, Hoechst 33342 staining was also used to study the apoptosis-inducing capability of glycyrrhizin and 18β-glycyrrhetinic acid. In agreement with the DNA fragmentation assay, treatment with 4.5 mM glycyrrhizin and 100 μM 18β-glycyrrhetinic acid failed to induce apoptosis in BU-1 cells, and giving the appearance of round and intact nuclei after staining with Hoechst 33342 dye (Figure 3.16B & 3.16C). However, treatment of BU-1 cells with 1 μM etoposide as a positive control induced apoptosis of the cells, as evidenced by the formation of condensed chromatin and fragmented nuclei (Figure 3.16D). These results suggest that both glycyrrhizin and 18β-glycyrrhetinic acid are incapable of inducing apoptosis in BU-1 cells under the prescribed experimental conditions.

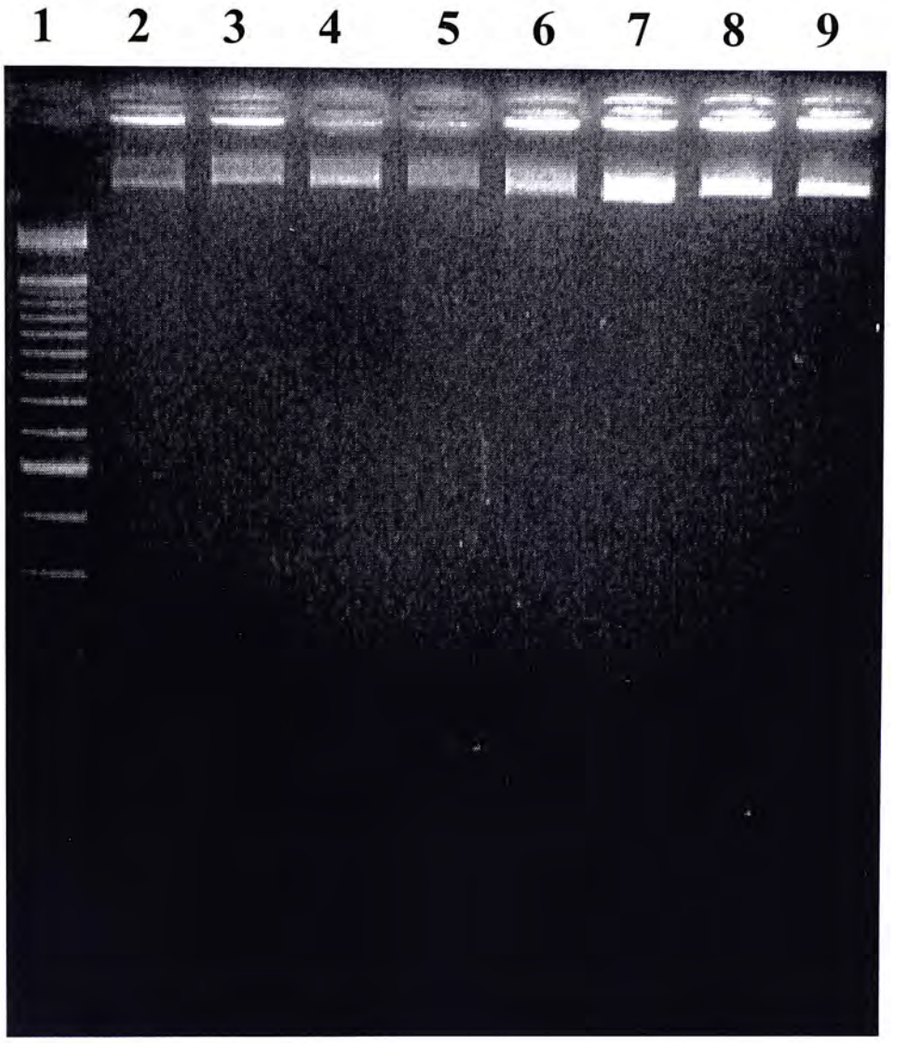


Figure 3.14: Inability of glycyrrhizin and 18β-glycyrrhetinic acid to induce apoptosis in the murine neuroblastoma BU-1 cells. BU-1 cells (10^5 cells/ml) were cultured overnight and treated with control solvent (Lanes 2 & 6), 80 - 120 μM 18β-glycyrrhetinic acid (Lanes 3 - 5) or 1.5 - 4.5 mM glycyrrhizin (Lanes 7 - 9) at 37°C for 48 hours. DNA were extracted by mild detergent IGEPAL CA-630 lysis buffer, and analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA markers

Lane 2: DMSO-treated cells

Lane 3: With 80 μM 18β-glycyrrhetinic acid

Lane 4: With 100 μM 18β-glycyrrhetinic acid

Lane 5: With 120 μM 18β-glycyrrhetinic acid

Lane 6: Untreated cells

Lane 7: With 1.5 mM glycyrrhizin

Lane 8: With 3 mM glycyrrhizin

Lane 9: With 4.5 mM glycyrrhizin

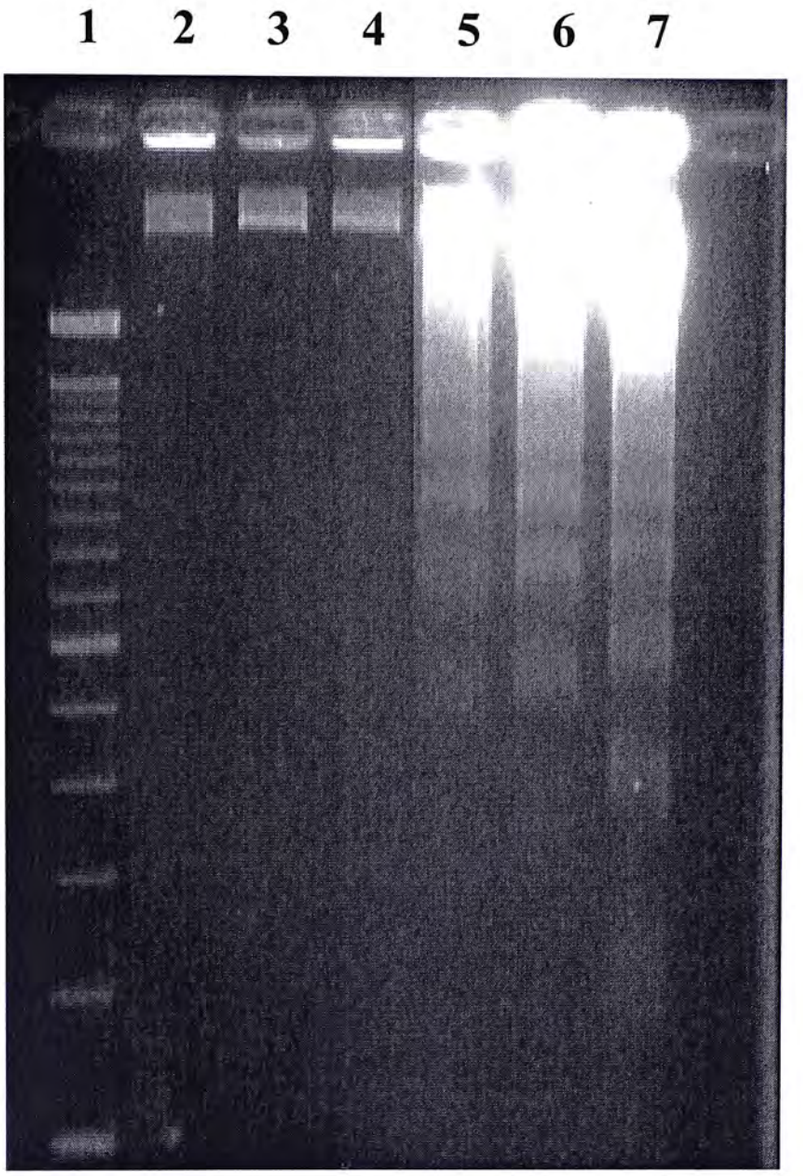


Figure 3.15: Inability of glycyrrhizin and 18β-glycyrrhetinic acid to induce apoptosis in the murine neuroblastoma BU-1 cells. BU-1 cells (10^5 cells/ml) were cultured overnight and treated with control solvent (Lane 2), 100 μM 18β-glycyrrhetinic acid (Lane 3), 4.5 mM glycyrrhizin (Lane 4) or 0.1 - 2.5 μM etoposide (Lanes 5 - 7) at 37°C for 48 hours. DNA were extracted by mild detergent IGEPAL CA-630 lysis buffer, and analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA markers

Lane 2: DMSO-treated cells

Lane 3: With 100 μM 18β-glycyrrhetinic acid

Lane 4: With 4.5 mM glycyrrhizin

Lane 5: With 0.1 μM etoposide

Lane 6: With 0.5 μM etoposide

Lane 7: With 2.5 μM etoposide

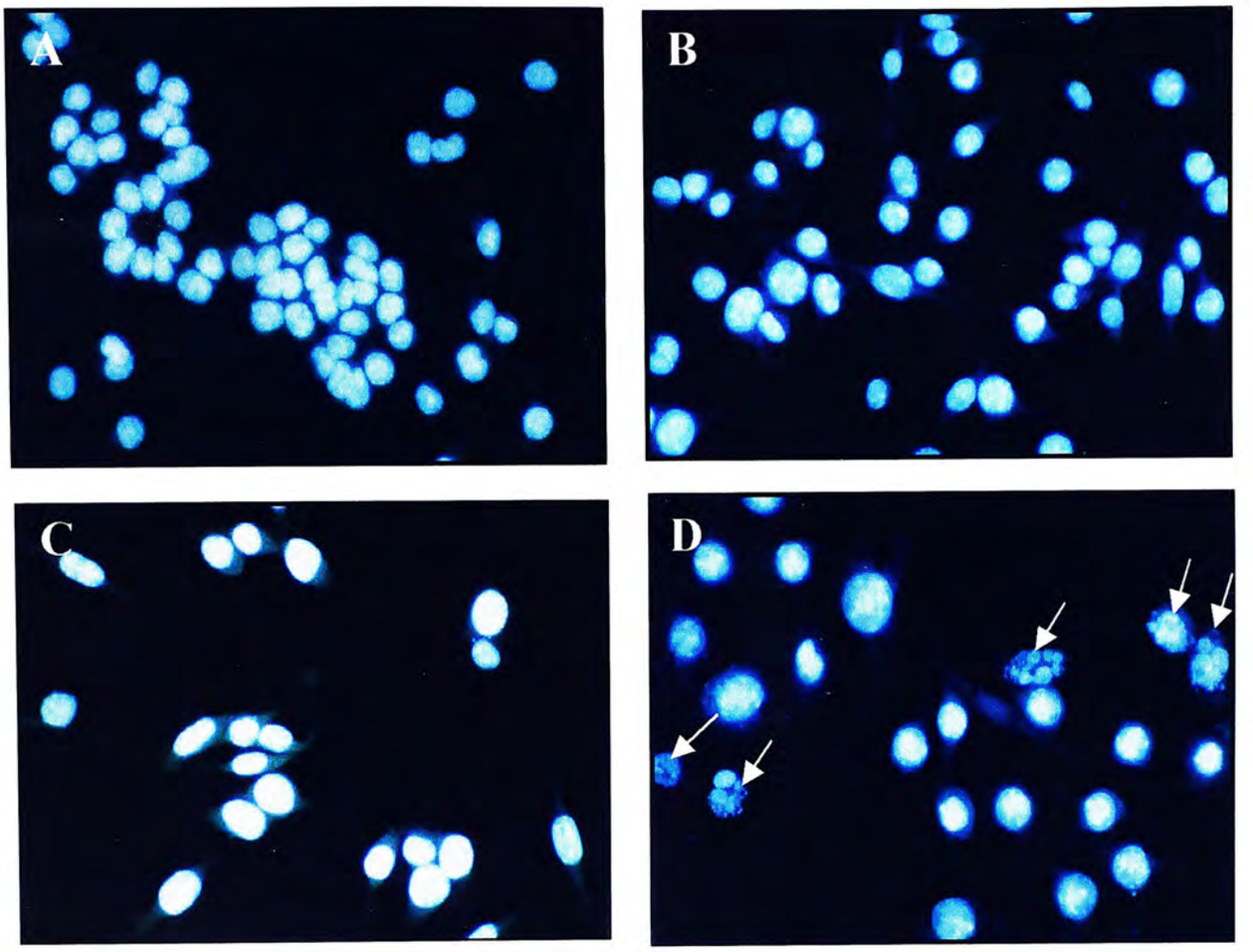


Figure 3.16: Fluorescent images of Hoechst 33342 stained BU-1 cells. BU-1 cells (40,000 cells/well) grown overnight on cover glasses in the wells of a 6-well plate were incubated with (A) control solvent (0.17% DMSO), (B) 4.5 mM glycyrrhizin, (C) 100 μM 18β-glycyrrhetinic acid or (D) 1 μM etoposide (a positive control) at 37°C for 48 hours. The cells were stained with Hoechst 33342 as described under Materials and Methods. The apoptotic cells were indicated by white arrows. (Magnification x 200)

3.3 Discussion

In this chapter, the effects of the glycyrrhizin and 18β-glycyrrhetinic acid on the proliferation of neuroblastoma cells were investigated, using well-characterized murine and human neuroblastoma cell lines as the cellular models. In the present study, both glycyrrhizin and 18β-glycyrrhetinic acid were found to inhibit the proliferation of murine and human neuroblastoma cells in a dose-dependent manner as determined by the ^{3}H-TdR incorporation assay and the neutral red assay. Moreover, the effect of 18β-glycyrrhetinic acid was found to be more pronounced than glycyrrhizin as it exerted its effect at micromolar concentrations. The results are in agreement with an earlier finding that the effective concentration of glycyrrhizin to inhibit the growth of B16 melanoma cells was about 20 times more than that required for glycyrrhetinic acid (Abe *et al.*, 1987). It had been suggested that the difference in the effects of glycyrrhizin and glycyrrhetinic acid on the cell membrane caused the different efficacy in cell growth inhibition (Abe *et al.*, 1987). Similarly, 18β-glycyrrhetinic acid was found to be a more potent anti-hepatotoxic agent than glycyrrhizin both *in vitro* and *in vivo*, and it had been suggested that the higher potency of 18β-glycyrrhetinic acid could be attributed to its higher adsorbability in hepatocytes (Nose *et al.*, 1994). Furthermore, it is interesting to note that the shape of the growth inhibition curve for the glycyrrhizin- and 18β-glycyrrhetinic acid-treated neuroblastoma BU-1 cells was different (growth inhibition of cells increased gradually with increasing concentration of glycyrrhizin vs. growth inhibition of cells increased sharply within a narrow range of 18β-glycyrrhetinic acid concentration). The reason for the difference in the growth

inhibition curves remains unclear, it may be due to the differential cellular uptake and/or metabolism of glycyrrhizin and 18β-glycyrrhetinic acid in neuroblastoma cells. Results of the clonogenic assay also showed that the colony-forming ability of the murine neuroblastoma BU-1 cells was reduced by the 18β-glycyrrhetinic acid treatment in a dose-dependent manner, further confirming the anti-proliferative effect of 18β-glycyrrhetinic acid on neuroblastoma cells. This assay was not performed with glycyrrhizin due to the inability of dissolving high concentration of glycyrrhizin in the soft agar culture. In addition, the kinetic study of the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on BU-1 cells showed that both glycyrrhizin and 18β-glycyrrhetinic acid could inhibit the proliferation of BU-1 cells in a time-dependent manner and maximal anti-proliferative effect could be demonstrated after 48 hours of treatment. Regarding the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on cancer cells, the data in the literature are contradictory. Abe *et al.* (1987) showed that glycyrrhizin and 18β-glycyrrhetinic acid alone was effective in inhibiting the growth of melanoma cells. More recent studies also showed that glycyrrhizin and 18β-glycyrrhetinic acid inhibited the growth of Vero and HepG2 cells with the IC_{50} values at millimolar and micromolar level, respectively (Hsiang *et al.*, 2002). In addition, 5 μM glycyrrhetinic acid alone was found to reduce the proliferation of pituitary tumor cells by 30% (Rabbitt *et al.*, 2003). In contrast, 18β-glycyrrhetinic acid alone was reported to have no effect on the proliferation of breast cancer MCF-7 and ZR-75-1 cells but it could potentiate the growth inhibitory effect of glucocorticosteroids (Hundertmark *et al.*, 1997). These discrepancies may be due to the differences in the concentration of drugs used and the sensitivity of different cell lines to the anti-proliferative effect of drugs. Glycyrrhizin and 18β-glycyrrhetinic acid had been shown to be inhibitors of

11β-hydroxysteroid dehydrogenase (Stewart *et al.*, 1987; Monder *et al.*, 1989). Elevated 11β-hydroxysteroid dehydrogenase expression was found in breast cancer and pituitary tumor cells (Hundertmark *et al.*, 1997; Koyama *et al.*, 2001; Rabbitt *et al.*, 2003) and 11β-hydroxysteroid dehydrogenase expression was also demonstrated in human prostate and colon carcinoma cell lines and endometrial cancer cells (Nath *et al.*, 1993; Reeves, 1995; Koyama & Krozowski, 2001). It had been suggested that inappropriate expression of 11β-hydroxysteroid dehydrogenase may act as a pro-proliferative and neoplastic stimulus to cell growth and thus modulation of 11β-hydroxysteroid dehydrogenase activity could alter the growth of cancer cells (Koyama & Krozowski, 2001; Rabbitt *et al.*, 2003). However, to our knowledge, there are no literatures reporting the expression of 11β-hydroxysteroid dehydrogenase in neuroblastoma cells. Whether neuroblastoma cells express 11β-hydroxysteroid dehydrogenase and whether the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells could be attributed by their inhibitory effects on 11β-hydroxysteroid dehydrogenase await further investigations.

In order to study the reversibility of the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on BU-1 cells, these two drugs were replaced by complete medium at the specific times and all cultures were incubated up to 48 hours. It was found that the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid was reversible and that the ability of neuroblastoma cells to recover from treatment with glycyrrhizin and 18β-glycyrrhetinic acid was dependent on both the concentration and duration of exposure to the drug. The higher the concentration used and the longer the incubation time, the less the ability of cells to recover from

treatment. Long time treatment of cells with glycyrrhizin and 18β-glycyrrhetinic acid seems to result in irreversible changes as supported by the findings of limiting dilution assay that the proliferating potential of drug-treated cells was markedly decreased after 4 days of treatment. Our results are consistent with the previous findings that when glycyrrhetinic acid was removed after 84 hours of treatment, the growth of B16 melanoma cells recovered slightly but the doubling time was lengthened (Abe *et al.*, 1987).

The present study showed that glycyrrhizin and 18β-glycyrrhetinic acid did not exhibit any significant cytotoxic effect on the neuroblastoma cells. Moreover, these two drugs also failed to induce apoptosis in neuroblastoma cells over a wide range of concentrations after 48 hours of incubation. These imply that the observed growth-inhibitory effect of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells could not be attributed directly to their cytotoxic and/or apoptosis-inducing effects on the neuroblastoma cells. Our results are similar to the others who found that the growth-inhibitory effect of glycyrrhetinic acid on the B16 melanoma cells was due to the cytostatic but not the cytotoxic effect (Abe *et al.*, 1987). Previous work in this laboratory had demonstrated that both glycyrrhizin and 18β-glycyrrhetinic acid were able to induce apoptosis in the human myeloid leukemia HL-60 cells and the murine myeloid leukemia WEHI-3B JCS cells (Tsang, 2001). On the contrary, one report showed that glycyrrhizin alone did not induce apoptosis in the human T cell leukemia Jurkat and Molt-4F cells (Ishiwata *et al.*, 1999). Moreover, no apoptotic effect was found in cultured splenocytes treated with glycyrrhetinic acid (Horigome *et al.*, 2001) and the present study also showed that both glycyrrhizin and 18β-glycyrrhetinic acid failed to induce apoptosis in

neuroblastoma cells, suggesting that the apoptosis-inducing effect of glycyrrhizin and 18β-glycyrrhetinic acid is cell type-specific.

In conclusion, both glycyrrhizin and 18β-glycyrrhetinic acid could exert significant anti-proliferative effect on the murine and human neuroblastoma cells. It is clear that the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on the neuroblastoma cells was cytostatic rather than cytotoxic in nature as the viability of the drug-treated BU-1 cells remained high and no apparent apoptosis could be detected. Among the three neuroblastoma cell lines tested, it was found that BU-1 cells were the most sensitive cells in response to 18β-glycyrrhetinic acid. Therefore, the BU-1 cells were chosen as a model for further mechanistic studies of the anti-tumor effect of 18β-glycyrrhetinic acid on neuroblastoma cells.

CHAPTER 4

DIFFERENTIATION-INDUCING EFFECTS OF GLYCYRRHIZIN AND 18β-GLYCYRRHETINIC ACID ON NEUROBLASTOMA CELLS

4.1 Introduction

Neuroblastoma is a childhood tumor that arises from the neural crest neuroectodermal cells (Westermann & Schwab, 2002). Despite their tumoral origin, neuroblastoma cell lines can be induced to differentiate *in vitro* by several agents including retinoic acid, nerve growth factor, phorbol ester TPA, dibutyryl cyclic AMP and phytochemicals (Ghigo *et al.*, 1998; Guzhova *et al.*, 2001; Pavelic & Spaventi, 1987; Fagerstrom *et al.*, 1996; Wu *et al.*, 1998; Wong *et al.*, 2002; Mak *et al.*, 2000). *In vitro* differentiated neuroblastoma cells have a neuronal phenotype, as judged by their morphology and the expression of biochemical and functional markers.

Glycyrrhizin and glycyrrhetinic acid had been reported to have differentiation-inducing activity (Abe *et al.*, 1987; Tsang, 2001). An earlier study showed that glycyrrhizin and glycyrrhetinic acid inhibited the growth of melanoma B16 cells as well as inducing morphological alterations and melanogenesis (Abe *et al.*, 1987). Moreover, previous study in our laboratory showed that glycyrrhizin and 18β-glycyrrhetinic acid could trigger the monocytic differentiation of the leukemia JCS cells accompanied with the enhancement of macrophage differentiation antigen and adhesion molecule expressions on the cell surfaces (Tsang, 2001).

The concept of "differentiation therapy" of cancer has gained considerable interest in recent years. This strategy is based on observations that neuroblastoma and leukemic cell lines represent immature, multipotent malignant cells that are blocked in their differentiation pathway(s), yet they can be induced to terminal differentiation and permanent cell cycle arrest *in vitro* by physiologic or

non-physiologic agents that apparently re-establish normal growth control. Recently, there has been an increasing interest in the therapeutical use of agents known to induce differentiation and growth arrest of neuroblastoma cells *in vitro*, in particular their applications for treatment of minimal residual disease (Lovat *et al.*, 1997). In chapter three, it was found that glycyrrhizin and 18β-glycyrrhetinic acid exhibited potent anti-proliferative activity on human and murine neuroblastoma cells *in vitro*. In the present study, the differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid on the murine neuroblastoma Neuro-2a BU-1 cells had been investigated. The BU-1 cell line is a recently established and well characterized model and can be induced to undergo differentiation in response to the phytochemical euxanthone (Mak *et al.*, 2000; Mak *et al.*, 2001). The differentiation-inducing effects were investigated by a number of criteria, including morphological and biochemical changes. Morphological changes were examined by microscopic observation of drug-treated cells while the biochemical changes were studied by immunocytochemistry. Moreover, the modulation of differentiation-related gene expressions by 18β-glycyrrhetinic acid was investigated by the RT-PCR technique.

4.2 Results

4.2.1 Morphological Changes in Glycyrrhizin and 18β-Glycyrrhetinic Acid-treated Neuroblastoma BU-1 Cells.

As demonstrated in the previous chapter, glycyrrhizin and 18β-glycyrrhetinic acid exhibited potent anti-proliferative activity on the murine neuroblastoma BU-1 cells, and the proliferative potential of the BU-1 cells was markedly decreased after 4 days of treatment with glycyrrhizin and 18β-glycyrrhetinic acid. It is possible that glycyrrhizin and 18β-glycyrrhetinic acid may cause terminal differentiation of the BU-1 cells, thus decreasing their proliferative potential. Therefore, the differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid were investigated. It is well known that neurite extension is a morphological expression of neuronal differentiation (Wu *et al.*, 1998) and thus morphological study was carried out in order to investigate the differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid on neuroblastoma cells. Briefly, BU-1 cells were incubated at 37°C for 3 – 4 days with 0 - 4 mM glycyrrhizin, or 0 - 100 μM 18β-glycyrrhetinic acid. The cells were then stained with Hemacolor solutions and examined for the morphological changes under the light microscope. Differentiation of BU-1 cells was characterized by the enlargement in cell size and the elongation in their neurite length.

As shown in Figure 4.1 & 4.2, differentiation could be induced in BU-1 cells after treatment with glycyrrhizin and 18β-glycyrrhetinic acid with significant, albeit morphologically diverse, neurite outgrowth. Untreated BU-1 cells grew as densely

packed, rounded and flattened cells with generally oval appearance (Figure 4.1A & 4.2A). However, cells treated with 3 and 4 mM glycyrrhizin showed a different spatial configuration with a flattened morphology and a considerably extended cell body with long and multipolar neurites around the cell body (Figure 4.1C & 4.1D). Such morphological changes were not observed in cells treated with a lower concentration of glycyrrhizin (Figure 4.1B), suggesting that the differentiation-inducing effect of glycyrrhizin was dose-dependent. On the other hand, treatment of BU-1 cells with 60 μM 18β-glycyrrhetinic acid had not resulted in any significant morphological changes (Figure 4.2B) but treatment with 80 μM 18β-glycyrrhetinic acid produced multipolar (multiple neurite bearing) star-shaped cells with several neurites projecting from the cell bodies along with cell body and nucleus enlargement (Figure 4.2C). However, these changes did not equally affect the entire cell population and some cells retained their basic spherical form. In contrast to the multipolar type of neurite growth observed in cells treated with 80 μM 18β-glycyrrhetinic acid, treatment of cells with 100 μM 18β-glycyrrhetinic acid produced thin and bipolar cells with two long neurites emanating from opposite sides of the cell body (Figure 4.2D). Treatment of cells with glycyrrhizin and 18β-glycyrrhetinic acid for 4 days resulted in similar morphological changes with slightly greater extent of differentiation than that observed after 3 days of treatment (data not shown). These results indicated that both glycyrrhizin and 18β-glycyrrhetinic acid exhibited differentiation-inducing activities on the neuroblastoma BU-1 cells.

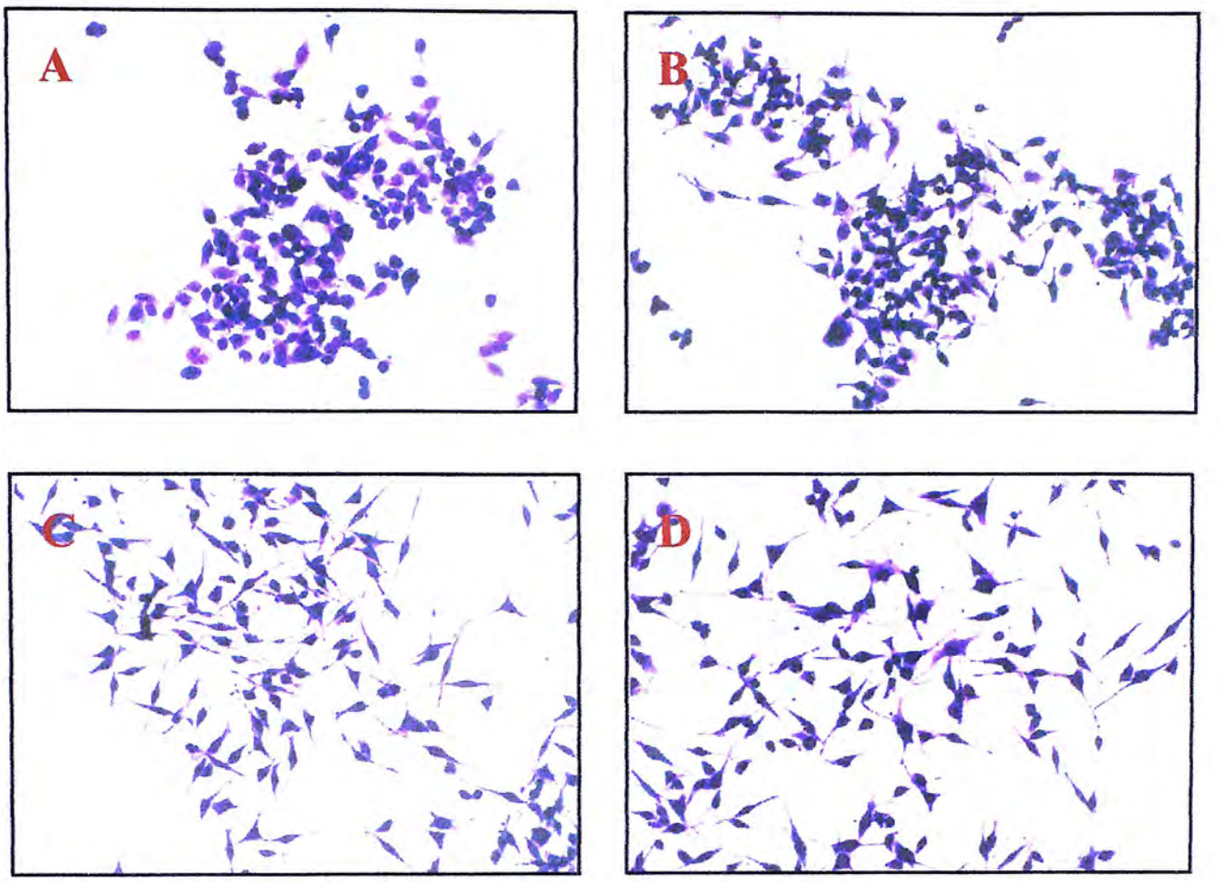


Figure 4.1: Morphological changes in glycyrrhizin-treated BU-1 cells. BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were incubated with (A) culture medium; (B) 1 mM glycyrrhizin; (C) 3 mM glycyrrhizin; or (D) 4 mM glycyrrhizin at 37°C for 3 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

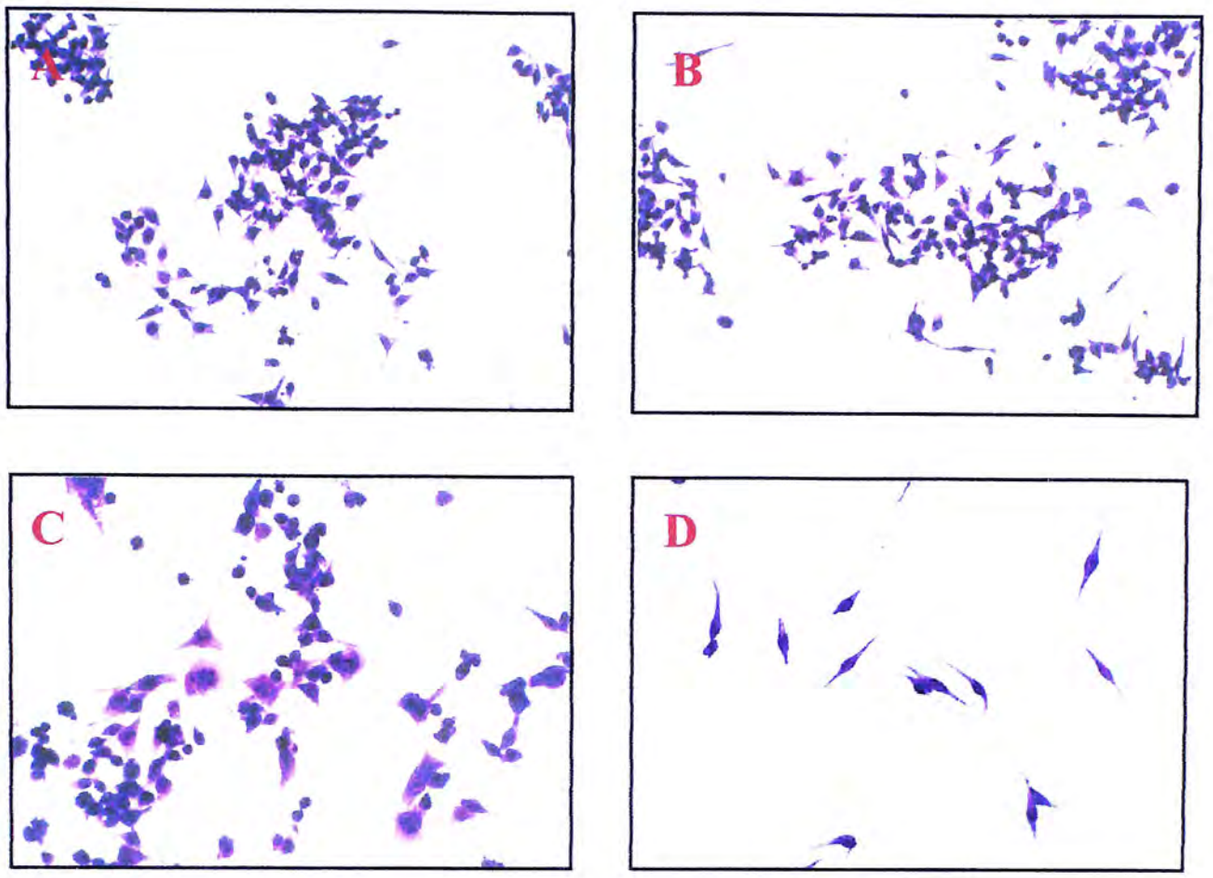


Figure 4.2: Morphological changes in 18β-glycyrrhetinic acid-treated BU-1 cells. BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were incubated with (A) control solvent (0.17% DMSO); (B) 60 μM 18β-glycyrrhetinic acid; (C) 80 μM 18β-glycyrrhetinic acid; or (D) 100 μM 18β-glycyrrhetinic acid at 37°C for 3 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

4.2.2 Immunocytochemistry of Glycyrrhizin and 18β-Glycyrrhetinic Acid-treated Neuroblastoma BU-1 Cells

Apart from morphological changes, the differentiation of the neuroblastoma BU-1 cells induced by glycyrrhizin and 18β-glycyrrhetinic acid was studied biochemically by examining the expression of neuronal differentiation markers. As well-known markers for neuronal differentiation, the expression of microtubule-associated protein 2 (MAP2) and neurofilament-200 was analyzed by immunocytochemistry after 4 days of drug treatment. Confocal microscopy was used to determine if there was any up-regulation of neuronal cell differentiation marker expression. The BU-1 cells were first incubated with 3 mM glycyrrhizin or 80 μM 18β-glycyrrhetinic acid at 37°C for 4 days and immunofluorescence study on drug-treated BU-1 cells was then performed using an antibody that specifically recognizes MAP2 and one that recognizes the neurofilament-200. MAP2 is a dendritic marker that is present in the dendrites and the perikaryon of neurons (Bernhardt & Matus, 1984; De Camilli *et al.*, 1984). It is a major component of the neuronal cytoskeleton that promotes the assembly and stabilization of microtubules and is implicated in neuronal differentiation (Przyborski & Cambray-Deakin, 1995). Neurofilament-200 is a high molecular weight neurofilament (NF-H) with an apparent molecular mass of 200 kDa (Shea *et al.*, 1988). It is a neuronal differentiation marker whose expression increases during neuronal differentiation and is localized in the mature axons (Paterno *et al.*, 1997; Wu *et al.*, 1998).

Figure 4.3 shows that MAP2 was minimally expressed in control BU-1 cells and that treatment of cells with glycyrrhizin increased the expression of MAP2 since

glycyrrhizin-treated cells showed more intense labeling of MAP2. Similarly, there was little staining of MAP2 in solvent-treated control cells but treatment of cells with 80 μM 18β-glycyrrhetinic acid resulted in increased intensity of the MAP2 immunofluorescence staining (Figure 4.4). The same phenomenon was observed in the expression of neurofilament-200, little staining of neurofilament-200 was observed in control cells while neuroblastoma cells treated with 3 mM glycyrrhizin or 80 μM 18β-glycyrrhetinic acid showed an enhanced labeling of neurofilament-200 (Figure 4.5 & 4.6). Moreover, the weak staining of MAP2 and neurofilament-200 in control cells was localized almost exclusively in the cell bodies while exposure of cells to glycyrrhizin and 18β-glycyrrhetinic acid for 4 days showed intense staining of both cell bodies and neuritic processes. The immunostaining of MAP2 and neurofilament-200 was specific since the negative controls without primary antibodies failed to show any significant increase in staining in the drug-treated cells as compared to the untreated control cells (data not shown). Taken together, these results suggest that both glycyrrhizin and 18β-glycyrrhetinic acid could induce morphological as well as biochemical differentiation of neuroblastoma cells.

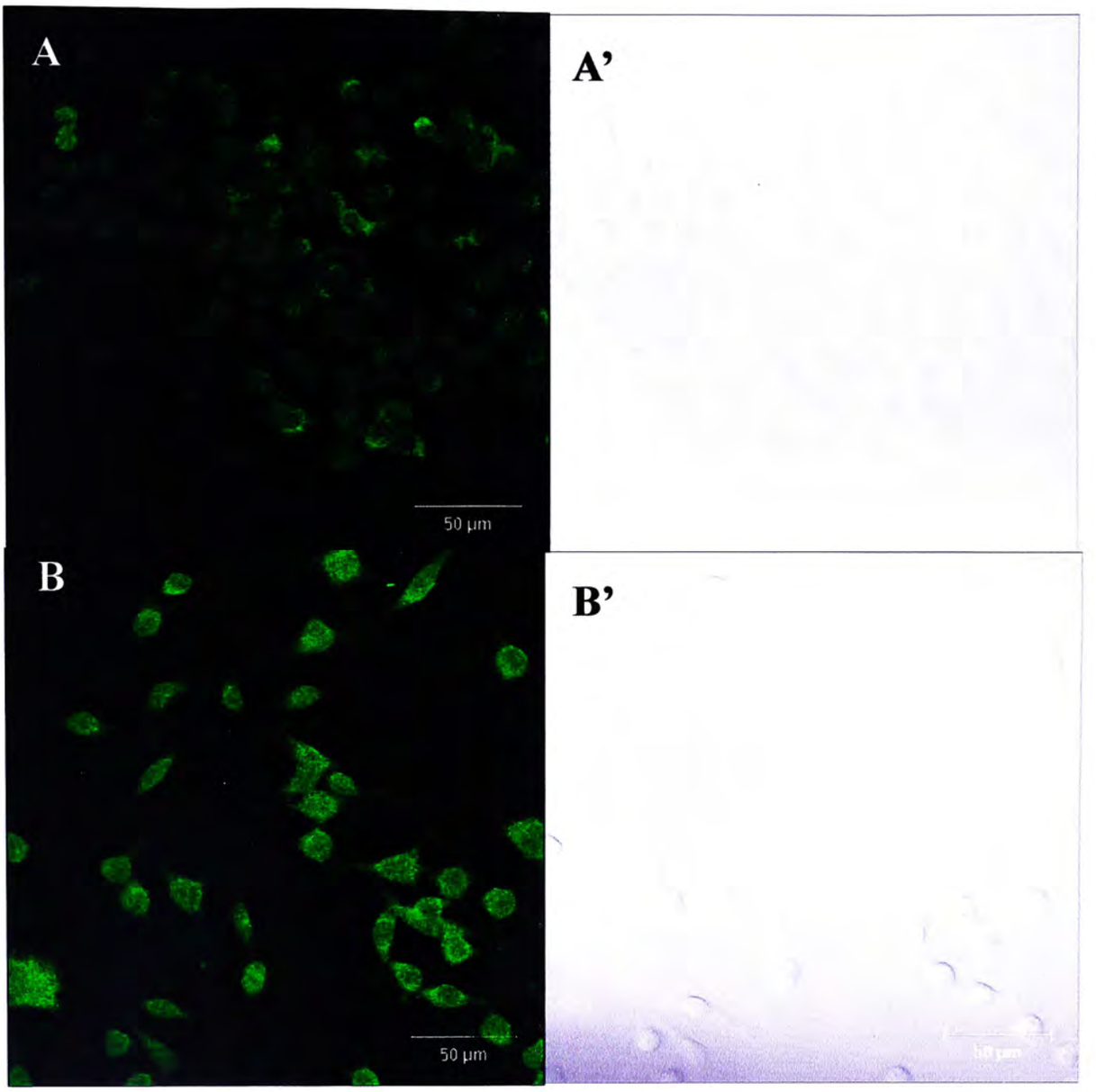


Figure 4.3: Immunocytochemical localization of MAP2 in glycyrrhizin-treated BU-1 cells. BU-1 cells (20,000 cells/well) grown overnight on cover glasses in the wells of a 6-well plate were incubated with glycyrrhizin at 37°C for 4 days. Immunocytochemistry was done as described under Materials and Methods. Confocal fluorescent images were shown in parallel with the phase-contrast images. (A-B): Fluorescent images. (A'-B'): Corresponding phase-contrast images. (A,A'): Medium control. (B,B'): 3 mM glycyrrhizin-treated cells. Scale bars represent 50 µm.

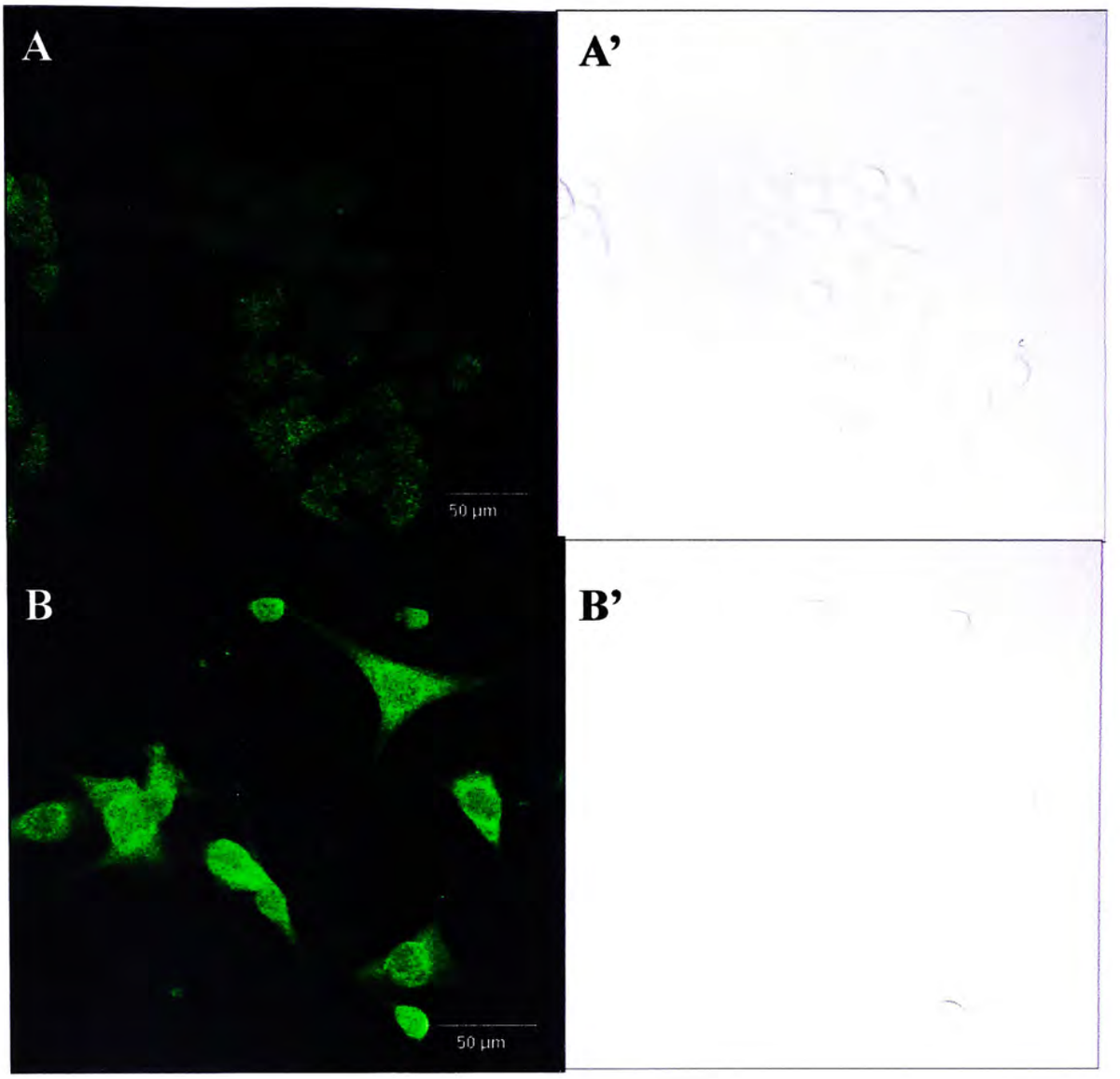


Figure 4.4: Immunocytochemical localization of MAP2 in 18β-glycyrrhetinic acid-treated BU-1 cells. BU-1 cells (20,000 cells/well) grown overnight on cover glasses in the wells of a 6-well plate were incubated with 18β-glycyrrhetinic acid at 37°C for 4 days. Immunocytochemistry was done as described under Materials and Methods. Confocal fluorescent images were shown in parallel with the phase-contrast images. (A-B): Fluorescent images. (A'-B'): Corresponding phase-contrast images. (A,A'): Solvent control (0.13% DMSO). (B,B'): 80 µM 18β-glycyrrhetinic acid-treated cells. Scale bars represent 50 µm.

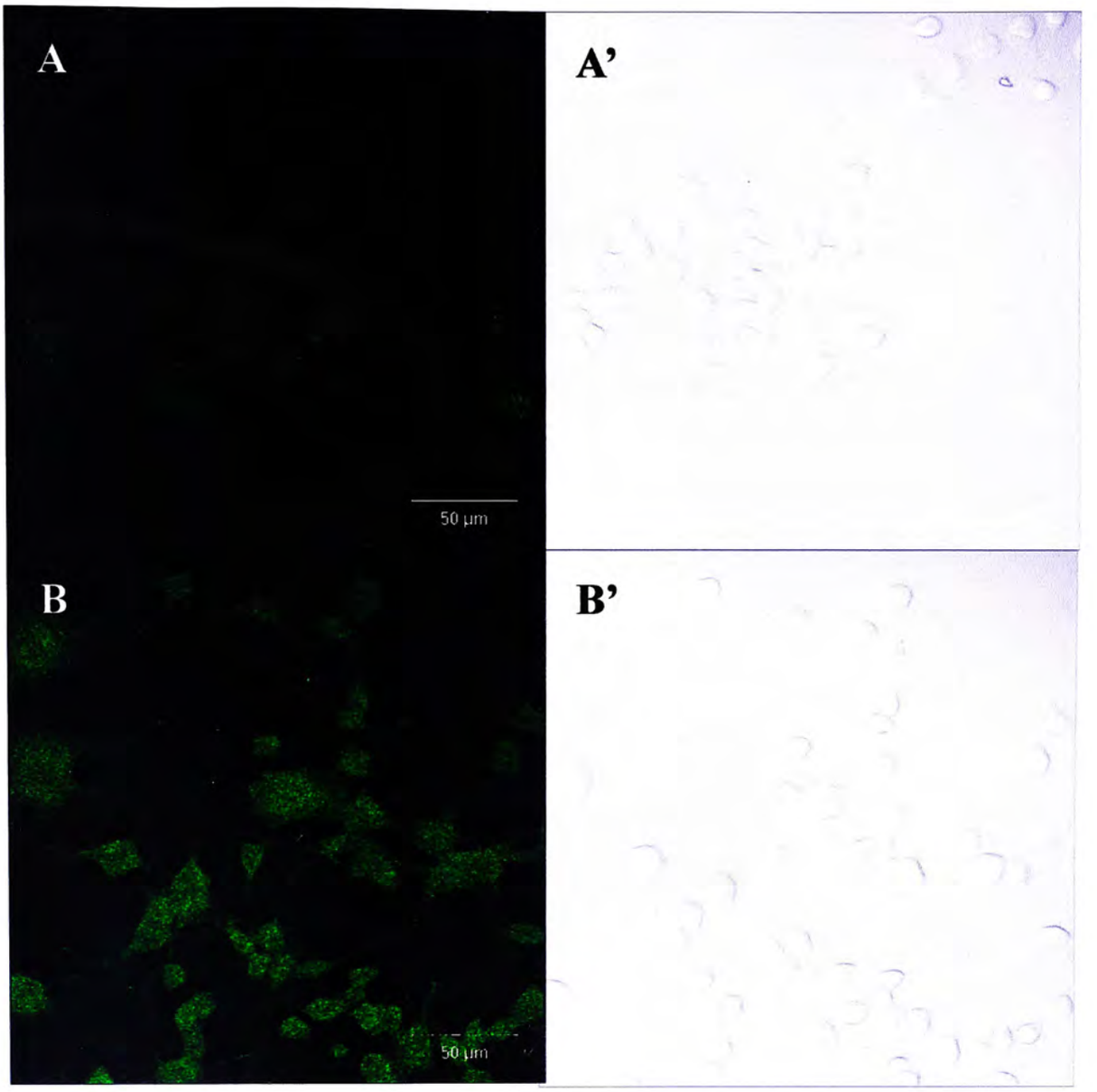


Figure 4.5: Immunocytochemical localization of neurofilament-200 in glycyrrhizin-treated BU-1 cells. BU-1 cells (20,000 cells/well) grown overnight on cover glasses in the wells of a 6-well plate were incubated with glycyrrhizin at 37°C for 4 days. Immunocytochemistry was done as described under Materials and Methods. Confocal fluorescent images were shown in parallel with the phase-contrast images. (A-B): Fluorescent images. (A'-B'): Corresponding phase-contrast images. (A,A'): Medium control. (B,B'): 3 mM glycyrrhizin-treated cells. Scale bars represent 50 µm.

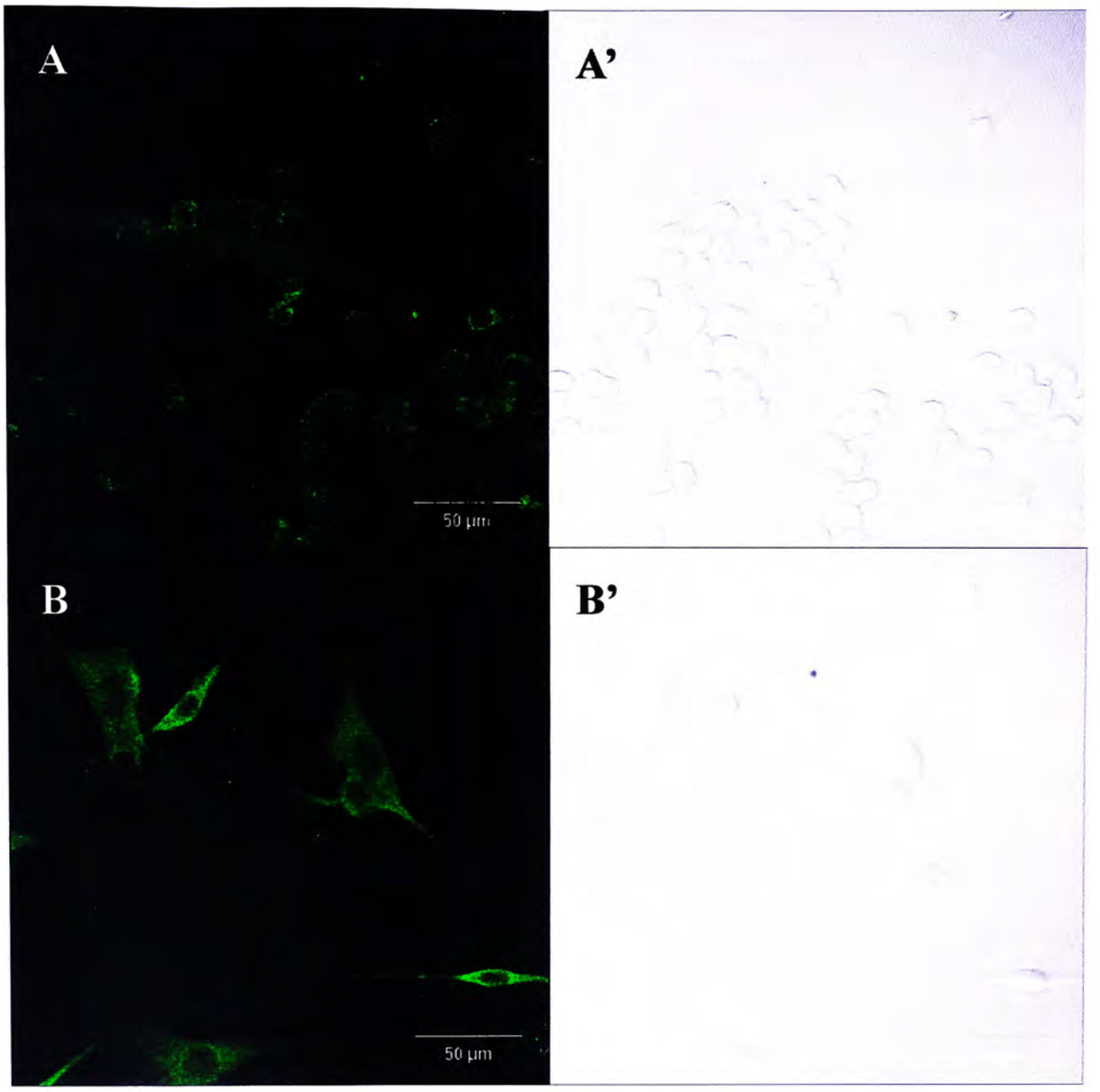


Figure 4.6: Immunocytochemical localization of neurofilament-200 in 18β-glycyrrhetinic acid-treated BU-1 cells. BU-1 cells (20,000 cells/well) grown overnight on cover glasses in the wells of a 6-well plate were incubated with 18β-glycyrrhetinic acid at 37°C for 4 days. Immunocytochemistry was done as described under Materials and Methods. Confocal fluorescent images were shown in parallel with the phase-contrast images. (A-B): Fluorescent images. (A'-B'): Corresponding phase-contrast images. (A,A'): Solvent control (0.13% DMSO). (B,B'): 80 µM 18β-glycyrrhetinic acid-treated cells. Scale bars represent 50 µm.

4.2.3 Effect of 18β-Glycyrrhetinic Acid on the Expression of Proto-oncogenes in Neuroblastoma BU-1 Cells

N-myc is one of the members of the myc transcription factor family (Cole & McMahon, 1999) and its overexpression has been closely associated with malignancy and poor prognosis of neuroblastoma (Taguchi *et al.*, 1997; Goodman *et al.*, 1997; George *et al.*, 2001). Previous studies have shown that N-myc expression in neuroblastoma cells is associated with high proliferative rate and is rapidly repressed by retinoic acid *in vitro* (Peverali *et al.*, 1996). In addition to its association with neuroblastoma progression, it is also a critical factor in the capacity of neuroblastoma cells to terminally differentiate in response to retinoic acid (Wada *et al.*, 1997). Decreased expression of N-myc during neuroblastoma differentiation induced by agents such as retinoic acid, interferon-γ and dibutyryl cAMP had been reported (Amatruda, III *et al.*, 1985; Thiele *et al.*, 1985; Peverali *et al.*, 1996; Wada *et al.*, 1997; Murakami *et al.*, 1991). Moreover, introduction of N-myc antisense oligomer to the human neuroblastoma cell line LAN-5 that is known to have N-myc amplification caused the induction of differentiation (Negroni *et al.*, 1991). On the other hand, transfection of N-myc gene construct into neuroblastoma SK-N-BE cells blocked the retinoic acid-induced differentiation (Peverali *et al.*, 1996). In addition, decreased N-myc expression is considered to be a molecular marker of differentiation of neuroblastoma cell lines (De, V *et al.*, 2000; Han *et al.*, 2001). In some studies, the expression of c-myc, another member of the myc family of nuclear phosphoproteins, was reported to be reduced upon differentiation induction (Hammerling *et al.*, 1987; Cohrs *et al.*, 1991). Therefore, it is intriguing to examine whether the expression of N-myc and c-myc would be modulated by 18β-glycyrrhetinic acid which could

induce differentiation in the neuroblastoma BU-1 cells.

In the present study, the modulatory effect of 18β-glycyrrhetinic acid on the expression of two proto-oncogenes N-myc and c-myc in the BU-1 cells was investigated by the technique of RT-PCR after the cells were treated with various concentrations of 18β-glycyrrhetinic acid for 8 and 24 hours. The amount of PCR product was normalized by comparison with the amplification of the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 4.7 shows that the expression of N-myc in BU-1 cells decreased to about 55% of that of the control as early as 8 hours after treatment with 100 μM and 120 μM of 18β-glycyrrhetinic acid. However, a decrease in the level of N-myc was observed in 80 μM 18β-glycyrrhetinic acid-treated BU-1 cells only after a longer period (24 hours) of treatment. The extent of decline in N-myc mRNA level (25 – 80% decrease) was more significant after 24 hours of treatment, suggesting that 18β-glycyrrhetinic acid could down-regulate the expression of N-myc both dose- and time-dependently. In contrast, no significant change could be observed in the expression level of c-myc in BU-1 cells even after 24 hours of treatment with three different concentrations of 18β-glycyrrhetinic acid.

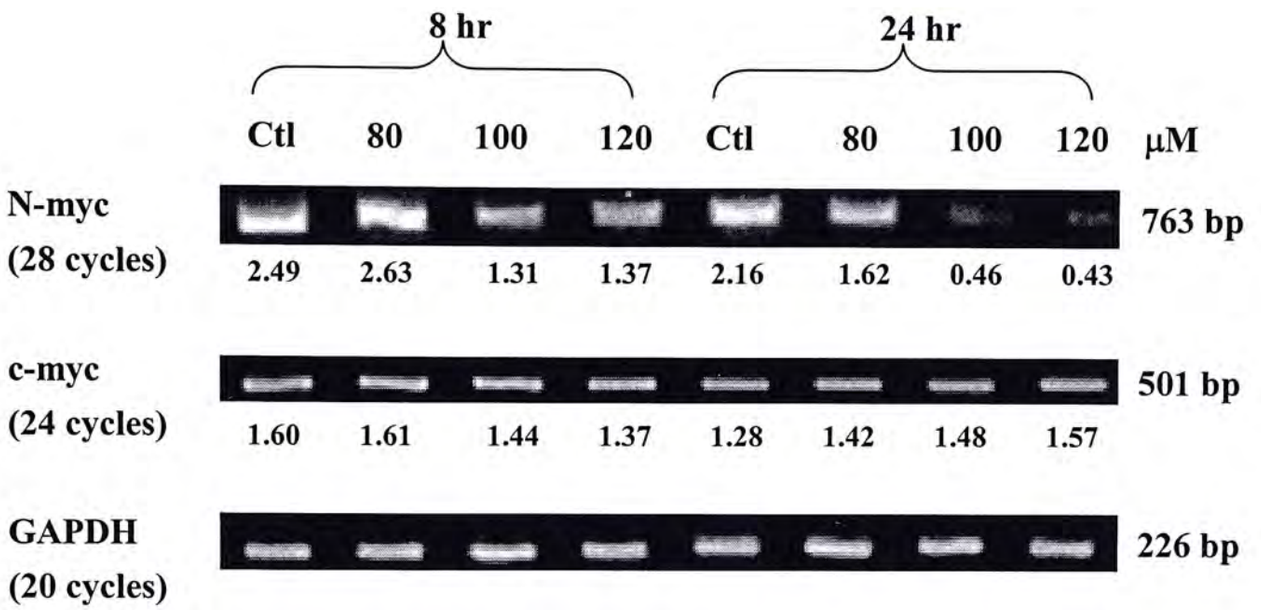


Figure 4.7: Effect of 18β-glycyrrhetinic acid on the mRNA expression of N-myc and c-myc genes in BU-1 cells. BU-1 cells (10^6) were cultured overnight and treated with either 0.2% DMSO (Ctl) or 80 - 120 μM 18β-glycyrrhetinic acid for 8 and 24 hours. Total RNA was isolated and subjected to RT-PCR as described under Materials and Methods. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide. The amounts of the PCR cDNA products were semi-quantified by the ImageQuant software (Molecular Dynamics). The value below each band represents the relative intensity after normalization with respect to GAPDH. The number of PCR cycles completed for each set of primers is indicated in the parentheses and the number on the right indicates the size of the band in base pairs (bp).

4.2.4 Effect of 18β-Glycyrrhetinic Acid on the Expression of Differentiation-Related Genes in Neuroblastoma BU-1 Cells

Besides N-myc, a number of differentiation-related genes had been identified. An earlier study reported that the c-fos mRNA expression was increased in TPA-treated SH-SY5Y cells (Hammerling *et al.*, 1987). Moreover, Sano *et al.* (1991) showed that there were a decrease in the N-myc gene expression and an increase in the c-fos gene expression during the differentiation of NB-1 cells. On the other hand, the expression of an immediate early gene, c-jun, was enhanced during differentiation of N1E-115 neuroblastoma cells in the absence of c-fos induction (de Groot & Kruijer, 1991). A recent study demonstrated that c-fes, c-fos, and RAG-1 gene activator (RGA) were significantly up-regulated during the differentiation of mouse neuroblastoma NBP_2 cells (Rudie *et al.*, 2001). Therefore, it is of interest to investigate the modulatory effect of 18β-glycyrrhetinic acid on the expression of the immediate early genes. Figure 4.8 shows that transient up-regulation of c-fos was observed after treatment of BU-1 cells with 100 μM 18β-glycyrrhetinic acid. The expression of c-fos increased as early as 30 minutes after treatment (67% increase), peaked at 1 hour (3.5 fold increase) and declined to the level slightly higher than that of the control at 8 hours of drug treatment. In contrast, the expression level of c-jun remained relatively constant throughout the time period examined.

It had been suggested that one of the potential mechanisms of N-myc action may be mediated through suppression of p50 expression, a component of the NF-κB transcription factor (van't Veer *et al.*, 1993). Moreover, transient activation of NF-κB was reported to be essential for retinoic acid- or TPA-induced differentiation

of SH-SY5Y cells (Feng & Porter, 1999). As mentioned before, N-myc expression in BU-1 cells was decreased by treatment with 18β-glycyrrhetinic acid, it is of interest to investigate whether the expression of NF-κB/p50 would be increased due to the decreased expression of N-myc. As shown in Figure 4.8, the expression level of NF-κB/p50 increased gradually from 30 minutes to 8 hours following treatment with 18β-glycyrrhetinic acid and a 64% increase was observed after 8 hours of treatment.

It has been reported that Bcl-2, in addition to its ability to protect cells against apoptosis, may also be involved in the regulation of cell differentiation, as elevated expression of the Bcl-2 protein had been found in differentiated neuroblastoma cells induced by TPA (Hanada *et al.*, 1993). Moreover, overexpression of Bcl-2 cDNA induced extensive neurite outgrowth and increased expression of neuronal marker in a human neural crest-derived tumor cell line, Paju (Zhang *et al.*, 1996). Therefore, Bcl-2 can also be considered as a biochemical marker of neuronal cell differentiation (Feng & Porter, 1999). In the present study, the expression level of Bcl-2 was examined by RT-PCR after treatment with 18β-glycyrrhetinic acid. Figure 4.8 shows that the Bcl-2 mRNA level in BU-1 cells increased gradually and a 73% increase was found after 8 hours of treatment with 18β-glycyrrhetinic acid.

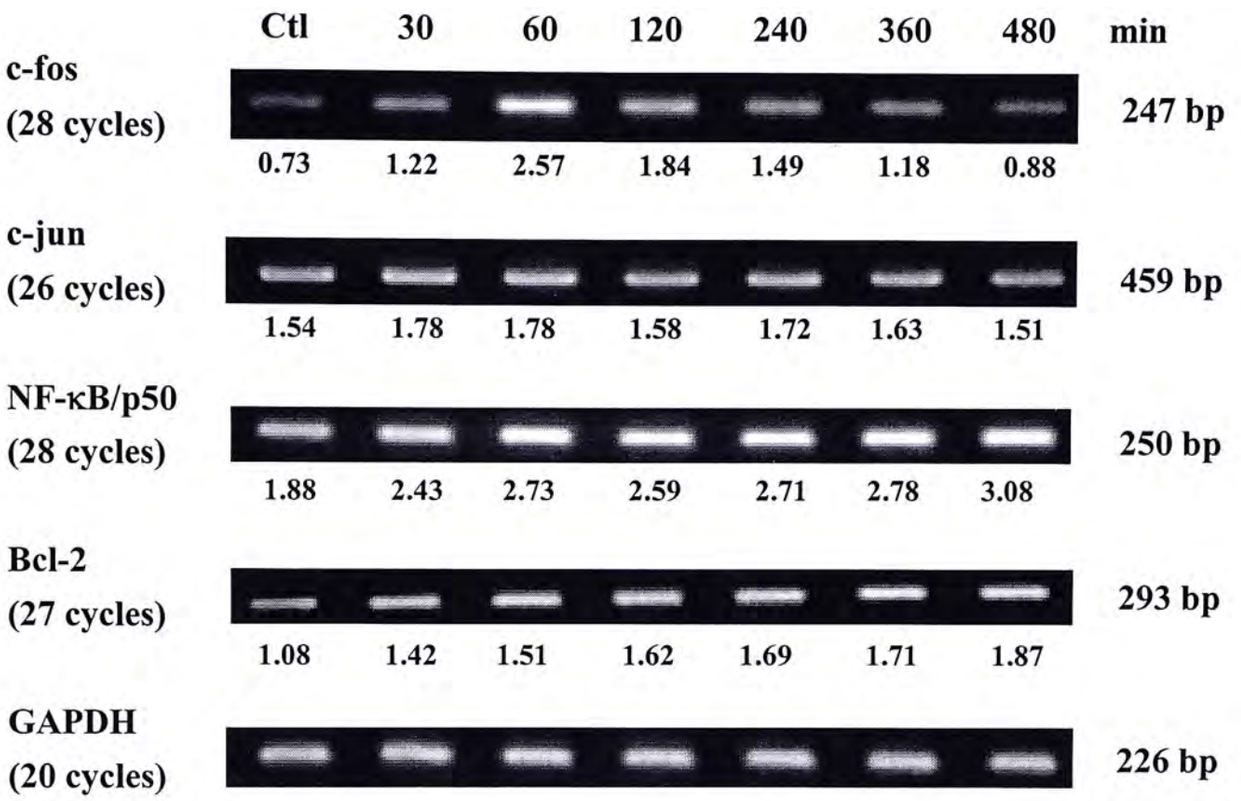


Figure 4.8: Effect of 18β-glycyrrhetinic acid on the mRNA expression of c-fos, c-jun, NF-κB/p50 and Bcl-2 genes in BU-1 cells. BU-1 cells (10^6) were cultured overnight and treated with either 0.17% DMSO (Ctl) or 100 μM 18β-glycyrrhetinic acid for various time periods (30 minutes to 8 hours). Total RNA was isolated and subjected to RT-PCR as described under Materials and Methods. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide. The amounts of the PCR cDNA products were semi-quantified by the ImageQuant software (Molecular Dynamics). The value below each band represents the relative intensity after normalization with respect to GAPDH. The number of PCR cycles completed for each set of primers is indicated in the parentheses and the number on the right indicates the size of the band in base pairs (bp).

4.3 Discussion

Neuroblastoma, a tumor of the sympathetic nervous system, is the most common solid tumor in children. In the majority of patients, neuroblastoma is metastatic at the time of diagnosis and rapid progression results in a fatal outcome. However, neuroblastoma has the highest rate of spontaneous regression among other human cancers and some tumors may undergo complete spontaneous regression even without therapy (Westermann & Schwab, 2002). A number of differentiating agents including retinoic acid (Sidell, 1982; Sidell *et al.*, 1983), interferon-γ (Watanabe *et al.*, 1989; Parodi *et al.*, 1989), dibutyryl cAMP (Rupniak *et al.*, 1984; Wong *et al.*, 2002) and phorbol esters (Pahlman *et al.*, 1981; Pahlman *et al.*, 1983) that induce neuroblastoma cell differentiation *in vitro* had been reported. Moreover, a recent study demonstrated that high-dose 13-*cis*-retinoic acid treatment after autologous bone marrow transplantation showed encouraging results for the therapy of high-risk neuroblastoma in children (Matthay *et al.*, 1999). This fact has prompted investigations into the possibility of differentiation therapy for this malignancy.

Since glycyrrhizin and 18β-glycyrrhetinic acid had been reported to induce morphological differentiation of melanoma B16 cells and myeloid leukemia JCS cells (Abe *et al.*, 1987; Tsang, 2001), it is of interest to investigate whether glycyrrhizin and 18β-glycyrrhetinic acid can induce differentiation of the neuroblastoma cells. In the present study, morphological examination showed that both glycyrrhizin and 18β-glycyrrhetinic acid could induce differentiation of BU-1 cells after 3 days of treatment. Differentiation was characterized by the increase in neurite outgrowth and cell size enlargement. However, exposure of BU-1 cells to glycyrrhizin and

18β-glycyrrhetinic acid resulted in different morphological changes. Treatment of cells with 80 μM 18β-glycyrrhetinic acid produced multipolar cells with a general increase in cell size while treatment of cells with 100 μM 18β-glycyrrhetinic acid and 3 – 4 mM glycyrrhizin resulted in extension of neurites without any changes in the size of the cells. This discrepancy in the morphological changes of drug-treated cells remains unknown and further investigation is required. However, it is not uncommon that different concentrations of drug result in different morphological changes. One report showed that treatment of Neuro-2a cells with the PKC inhibitor H7 induced unipolar neurites at lower concentrations of H7 whereas bipolar or multipolar neurites were seen at higher concentrations (Minana *et al.*, 1990).

To further characterize the effects of glycyrrhizin and 18β-glycyrrhetinic acid on the differentiation of BU-1 cells, immunocytochemistry was carried out to investigate whether the morphological differentiation was accompanied by biochemical differentiation of BU-1 cells. The neuronal phenotype of differentiated BU-1 cells was demonstrated by immunocytochemistry using specific antibodies to neuronal markers MAP2 and neurofilament-200. Microtubule-associated protein-2 (MAP2) is a prominent cytoskeletal protein in the mammalian nervous system (Kalcheva *et al.*, 1998). It is a neuron-specific MAP which is a major component of the neuronal cytoskeleton. It promotes the assembly and stabilizes the microtubule bundles, allows outgrowth of cellular processes and is implicated in neuronal differentiation (Przyborski & Cambray-Deakin, 1995). Neurofilament-200, a high molecular weight neurofilament (NF-H) with an apparent molecular mass of 200 kDa (Shea *et al.*, 1988), is localized in the mature axons and its level is increased during neuronal differentiation (Paterno *et al.*, 1997; Wu *et al.*, 1998). Using the laser confocal

scanning microscopy, the neuronal phenotype of differentiated BU-1 cells was demonstrated by increased expression of MAP2 and neurofilament-200, which are markers of neuronal cell differentiation. Therefore, in addition to induction of morphological changes, glycyrrhizin and 18β-glycyrrhetinic acid also induced the expression of biochemical markers characteristic of the neuronal differentiation. The positive staining of both markers was indicative of mixed phenotypes with dendrite-like and axon-like processes.

N-myc, like other members of the myc gene family, encodes a nuclear phosphoprotein containing a transactivation domain at the N-terminal and a C-terminal domain with regions that are required for dimerization with Max and for sequence specific DNA binding (Cole & McMahon, 1999). A close relationship between N-myc amplification and advanced stages of neuroblastoma, rapid tumor progression and poor prognosis was documented by clinical studies (George *et al.*, 2001). Moreover, N-myc expression is intimately related to an undifferentiated phenotype in neuroblastoma cells and a number of studies demonstrated that the induction of neuronal differentiation of neuroblastoma cells *in vitro* is accompanied by N-myc down-regulation (Amatruda, III *et al.*, 1985; Thiele *et al.*, 1985; Murakami *et al.*, 1991; Peverali *et al.*, 1996; Wada *et al.*, 1997). On the other hand, the expression of c-myc, another member of the myc family of nuclear phosphoproteins, was also reported to be reduced upon differentiation (Hammerling *et al.*, 1987; Cohrs *et al.*, 1991). In the present study, 18β-glycyrrhetinic acid was found to be able to induce the differentiation of BU-1 cells at micromolar concentrations, therefore, we further examined the modulatory effect of 18β-glycyrrhetinic acid on the expression of N-myc and c-myc. It has been reported that retinoic acid-induced differentiation

of SMS-KCNR neuroblastoma cells caused a reduction in the levels of N-myc mRNA within 6 hours of treatment and this change preceded the onsets of both cell-cycle changes and morphological differentiation (Thiele *et al.*, 1985). Using RT-PCR, our results showed that the mRNA level of N-myc decreased as early as 8 hours after treatment of the neuroblastoma BU-1 cells with 18β-glycyrrhetinic acid, the time at which no obvious morphological change was observed. In contrast, no significant difference between the level of c-myc in 18β-glycyrrhetinic acid-treated cells and control cells was found. Therefore, our results suggest that 18β-glycyrrhetinic acid may induce the differentiation of neuroblastoma cells by the reduction of N-myc expression and this is consistent with an earlier study showing that down-regulation of N-myc preceded the morphological differentiation of neuroblastoma cells (Thiele *et al.*, 1985). Interestingly, another study showed that treatment of SH-SY5Y cells with TPA and retinoic acid induced morphological and functional differentiation in SH-SY5Y cells and resulted in a decrease in the expression of both N-myc and c-myc genes (Hammerling *et al.*, 1987). However, in the present study, the down-regulation of N-myc cannot be ascribed to be a general effect of 18β-glycyrrhetinic acid on the myc family since no significant change in the level of c-myc was found at the times when N-myc expression was dramatically altered by 18β-glycyrrhetinic acid. It seems that N-myc and c-myc expressions are differentially regulated in 18β-glycyrrhetinic acid-treated neuroblastoma BU-1 cells. A recent study using transfection assay showed that retinoic acid and interferon-γ regulated N-myc expression through different regions of the N-myc promoter. Interferon-γ was also found to exert post-transcriptional effects on N-myc expression by shortening the half-life of N-myc mRNA (Wada *et al.*, 1997). However, the mechanism for the down-regulation of N-myc expression by 18β-glycyrrhetinic acid

awaits further investigations.

A number of studies showed that the immediate early genes, including c-fos and c-jun, were up-regulated during differentiation of neuroblastoma cells (Hammerling *et al.*, 1987; Sano *et al.*, 1991; de Groot & Kruijer, 1991; Rudie *et al.*, 2001), suggesting that these genes may be involved in the differentiation of neuroblastoma cells. In view of these findings, we had investigated whether these genes might be involved in the differentiation of BU-1 cells induced by 18β-glycyrrhetinic acid. Our results showed that c-fos mRNA was transiently up-regulated after treatment with 18β-glycyrrhetinic acid whereas the mRNA level of c-jun remained fairly constant. C-fos encodes a nuclear protein that is a member of activator protein-1 (AP-1) transcription factor. C-fos protein activates transcription of target genes via the tetradecanoyl phorbol acetate response element (TRE) when bound as a heterodimer with Jun (McBride & Nemer, 1998). It is possible that up-regulation of c-fos by 18β-glycyrrhetinic acid would increase the transcription of genes that are required for differentiation of neuroblastoma cells.

The transient activation of the NF-κB transcription factor was reported to be essential for retinoic acid- or TPA-induced differentiation of SH-SY5Y cells (Feng & Porter, 1999). Moreover, an earlier study suggested that one of the potential mechanisms of N-myc action may be mediated through suppression of p50 expression, a component of the NF-κB transcription factor (van't Veer *et al.*, 1993). As mentioned before, N-myc expression in BU-1 cells was decreased by treatment with 18β-glycyrrhetinic acid, it is of interest to investigate whether the expression of NF-κB/p50 would be increased when there was a decreased expression of N-myc in

BU-1 cells. Our results showed that the NF-κB/p50 mRNA level increased time-dependently after treatment with 18β-glycyrrhetinic acid. However, whether 18β-glycyrrhetinic acid could directly activate the NF-κB protein complex in BU-1 cells is an intriguing aspect that is worthy of future investigations.

The anti-apoptotic gene, Bcl-2, was found to be strongly up-regulated in parallel with neuronal differentiation (Hanada *et al.*, 1993; Feng & Porter, 1999). In addition, Bcl-2 appears to be required for neuronal differentiation of neural crest-derived Paju cells (Zhang *et al.*, 1996). In the present study, we also found that the expression of Bcl-2 gene was up-regulated after treatment with 18β-glycyrrhetinic acid, suggesting that there may be a close relationship between Bcl-2 expression and neuronal cell differentiation, though the precise mechanism has yet to be defined.

In conclusion, both glycyrrhizin and 18β-glycyrrhetinic acid were found to be capable of inducing the morphological and biochemical differentiation of neuroblastoma BU-1 cells. Furthermore, the differentiation-inducing effect of 18β-glycyrrhetinic acid was accompanied with a down-regulation of N-myc gene expression, and an up-regulation of Bcl-2, NF-κB/p50 and c-fos gene expression. However, the mechanisms by which 18β-glycyrrhetinic acid could alter the expression of these genes remain unclear and await further investigations.

CHAPTER 5

MECHANISTIC STUDIES ON THE ANTI-PROLIFERATIVE AND DIFFERENTIATION-INDUCING EFFECTS OF GLYCYRRHIZIN AND 18β-GLYCYRRHETINIC ACID

5.1 Introduction

In the previous two chapters, glycyrrhizin and 18β-glycyrrhetinic acid had been shown to exert anti-proliferative and differentiation-inducing effects on the neuroblastoma cells *in vitro*. However, the mechanisms by which they exerted their anti-tumor effects on the neuroblastoma cells had not been fully elucidated. It is well known that disturbance to the cell cycle may result in growth inhibition. Therefore, in the present study, the effects of glycyrrhizin and 18β-glycyrrhetinic acid on the cell cycle progression of BU-1 cells were studied by flow cytometry. In view of the fact that cell cycle progression is tightly regulated by cyclins and cyclin-dependent kinases, and progression through the cell cycle is brought about by the differential expression and activation of these cell-cycle regulatory proteins, thus, the modulatory effect of 18β-glycyrrhetinic acid on the mRNA and protein expression of the cyclins and cyclin-dependent kinases was further studied by RT-PCR and Western blot respectively.

Results in chapter three showed that the effective concentration of glycyrrhizin was relatively high for the inhibition of neuroblastoma cell proliferation, therefore, it is of great interest to find a way that could increase the efficacy of glycyrrhizin. As shown in the previous two chapters, both glycyrrhizin and 18β-glycyrrhetinic acid were found to be able to inhibit the growth and induce differentiation in the neuroblastoma BU-1 cells. In addition, glycyrrhizin is hydrolyzed to its major metabolite 18β-glycyrrhetinic acid by the bacterial β-D-glucuronidase in the intestine. Thus, it is of interest to investigate whether there are any synergistic actions between glycyrrhizin and 18β-glycyrrhetinic acid on the proliferation and differentiation of

neuroblastoma cells. All-*trans* retinoic acid (ATRA) is a well documented anti-proliferative and differentiation-inducing agent on neuroblastoma cells (Sidell, 1982; Sidell *et al.*, 1983; Thiele *et al.*, 1985). It is intriguing to investigate whether the combined treatment with 18β-glycyrrhetinic acid and ATRA would result in synergistic effect on the proliferation and differentiation of neuroblastoma cells. In the present study, the combined effect of glycyrrhizin, 18β-glycyrrhetinic acid and ATRA on the proliferation and differentiation of neuroblastoma cells was examined by the ^{3}H-TdR incorporation assay after 48 hours of treatment and by the morphological study after 4 days of treatment respectively.

Protein kinase C (PKC) is a family of serine-threonine kinases that consists of at least 10 members. Based on the structural and enzymatic properties, the PKC isoforms are divided into three groups: the conventional PKC isoforms (α, βI, βII and γ) that are Ca^{2+}-dependent and activated by both phosphatidylserine and diacylglycerol (DAG); the novel PKC isoforms (δ, ε, η and θ) that are Ca^{2+}-insensitive and regulated by DAG and phosphatidylserine; and the atypical PKC isoforms (ζ and ι/λ) that are Ca^{2+}- and phospholipid-independent (Mellor & Parker, 1998; Way *et al.*, 2000). Moreover, PKC μ takes an intermediate position between the novel and atypical PKC subgroups (Liu, 1996). PKC was suggested to play an important role in the growth, survival and differentiation of cells (Zeidman *et al.*, 1999a; Zeidman *et al.*, 1999b; Svensson *et al.*, 2000; Mak *et al.*, 2001). A number of reports had described an altered expression pattern of PKC isozymes during differentiation of neuroblastoma cells. An earlier study demonstrated that down-regulation of mRNAs for both PKC α and PKC ε was associated with the *in vitro* differentiation of Neuro-2a cells (Wada *et al.*, 1989). A more recent study

demonstrated that the differentiation of the murine neuroblastoma BU-1 cells induced by euxanthone was accompanied with induction of certain isoforms of PKC (PKC-α, -β, -δ, -λ and -ζ) (Mak *et al.*, 2001). Therefore, it is of interest to examine whether the expression of PKC isoforms would be modulated by 18β-glycyrrhetinic acid using the RT-PCR technique. In addition, one earlier report showed that concentrations of 18β-glycyrrhetinic acid below 200 μM slightly enhanced PKC activity in the presence of Ca^{2+} and phosphatidylserine while higher concentrations of 18β-glycyrrhetinic acid inhibited PKC activity with IC_{50} of approximately 450 μM (O'Brian *et al.*, 1990). Therefore, in this chapter attempts had been made to investigate whether the anti-proliferative and differentiation-inducing effects of 18β-glycyrrhetinic acid were PKC dependent or not. The dependency of PKC on the anti-proliferative and differentiation-inducing effects of 18β-glycyrrhetinic acid was elucidated by the use of PKC activators and selective PKC inhibitors in combination with 18β-glycyrrhetinic acid. Their effects on the neuroblastoma BU-1 cells were measured by the ^{3}H-TdR incorporation assay and morphological study.

In view of the fact that cyclic AMP (cAMP) analogs are able to induce differentiation of cells of neuronal origin (Wu *et al.*, 1998; Hansen *et al.*, 2000; Wong *et al.*, 2002) and that a number of cAMP actions are mediated through the activation of the PKA signaling pathways, therefore, the role of PKA in the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation in BU-1 cells was investigated by the use of PKA activators and inhibitors.

5.2 Results

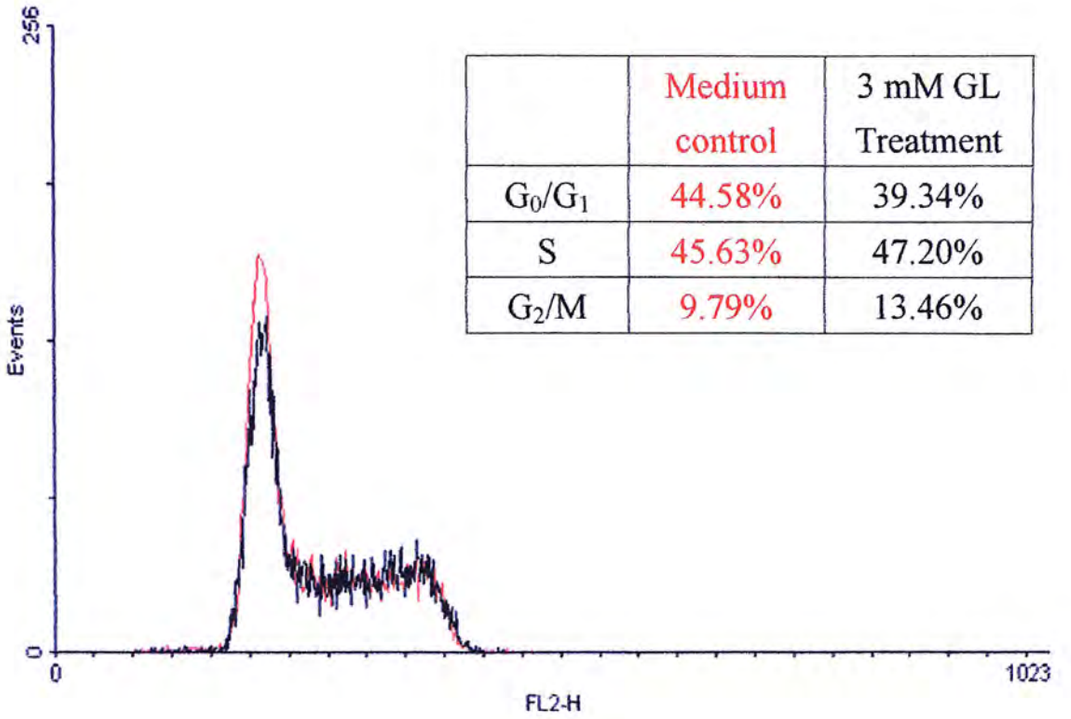
5.2.1 Effects of Glycyrrhizin and 18β-Glycyrrhetinic Acid on the Cell Cycle Kinetics of Neuroblastoma BU-1 Cells *In Vitro*

As shown in chapter three, glycyrrhizin and 18β-glycyrrhetinic acid were found to be able to exert a cytostatic effect on the neuroblastoma BU-1 cells. Thus, attempts were made to study the mechanistic actions of glycyrrhizin and 18β-glycyrrhetinic acid on the proliferation of the BU-1 cells. In order to determine whether the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid was specific to the G_0/G_1, S or G_2 /M phase of the cell cycle, the effects of glycyrrhizin and 18β-glycyrrhetinic acid on the cell cycle distribution of the neuroblastoma BU-1 cells were examined by flow cytometry. BU-1 cells were either treated with 3 and 4.5 mM of glycyrrhizin for 48 and 72 hours or treated with 80 and 100 μM of 18β-glycyrrhetinic acid for 24 and 48 hours before flow cytometric analysis.

Cell cycle analysis of BU-1 cells treated with 4.5 mM glycyrrhizin for a period of 48 and 72 hours showed accumulation of cells in the G_2/M phase with a corresponding reduction in the percentage of cells in the G_0/G_1 and S phases (Figure 5.1B & 5.2B). However, when the cells were treated with 3 mM glycyrrhizin, such changes were observed only after 72 hours (Figure 5.2A), suggesting that the effect of glycyrrhizin on the cell cycle profile of BU-1 cells was dose- and time-dependent. In the case of 18β-glycyrrhetinic acid, a more marked increase in the percentage of cells in the G_2/M phase and a decrease in the percentage of cells in the G_0/G_1 and S phases of the cell cycle were observed for the two different concentrations (80 μM

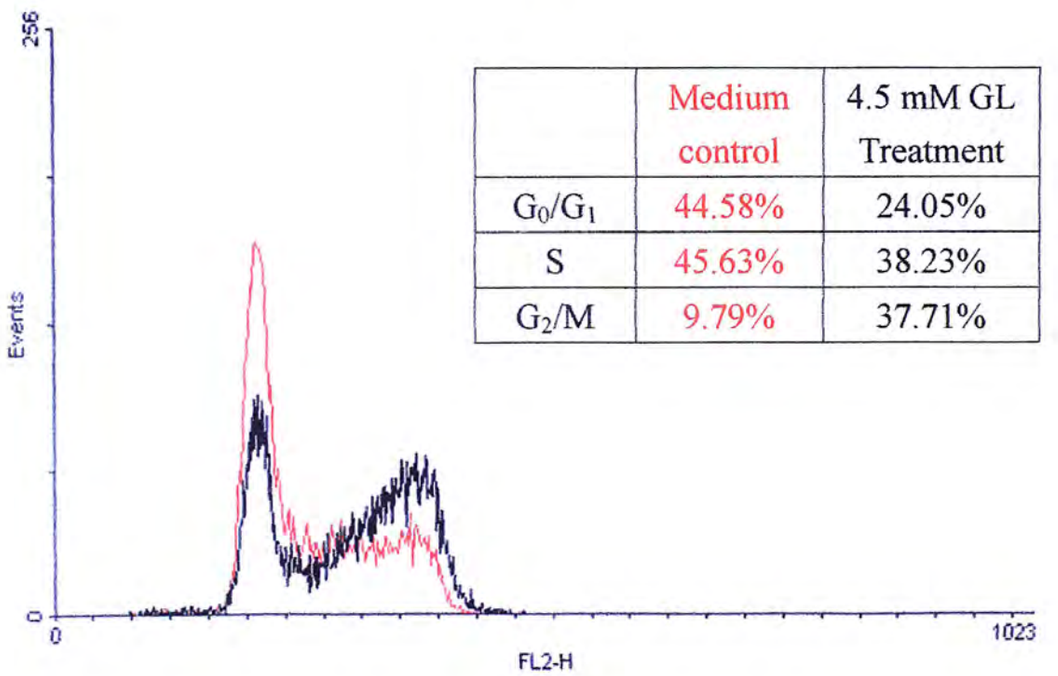
and 100 μM) of 18β-glycyrrhetinic acid used as early as after 24 hours of treatment (Figure 5.3). The effect of 18β-glycyrrhetinic acid on the cell cycle profile of BU-1 cells was even more drastic after 48 hours of treatment (Figure 5.4), suggesting that the effect of 18β-glycyrrhetinic acid on the cell cycle profile of BU-1 cells was also dose- and time-dependent. Therefore, cell cycle analysis clearly demonstrated that glycyrrhizin and 18β-glycyrrhetinic acid could suppress the proliferation of BU-1 cells by arresting the cells at the G_2/M phase.

(A)



	Medium control	3 mM GL Treatment
G_0/G_1	44.58%	39.34%
S	45.63%	47.20%
G_2/M	9.79%	13.46%

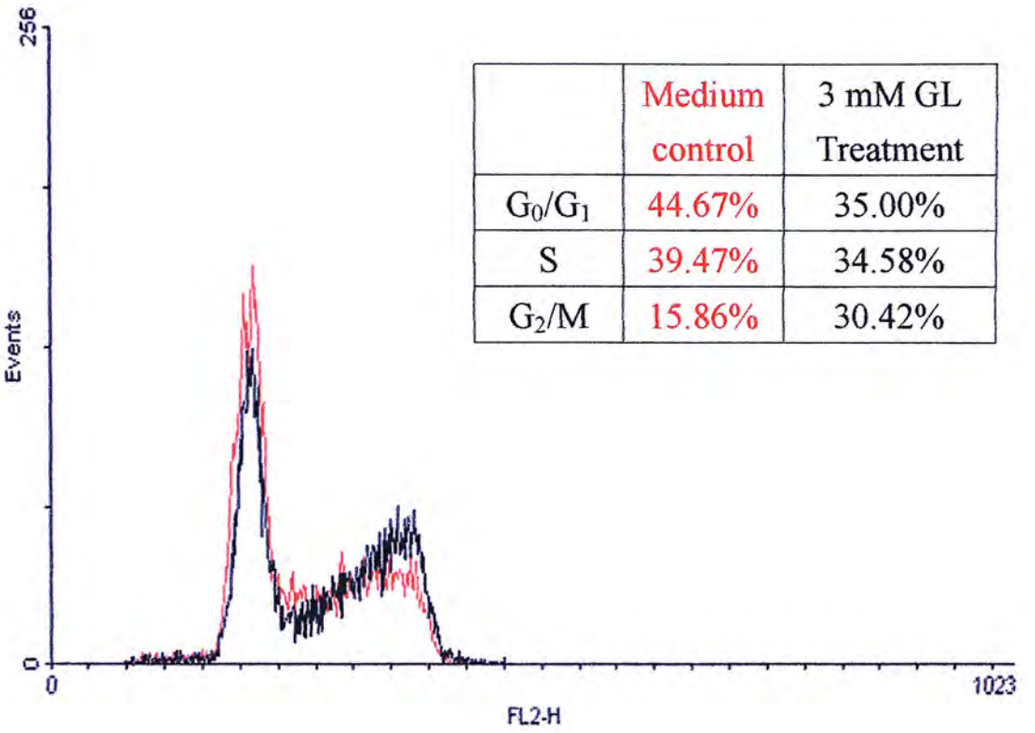
(B)



	Medium control	4.5 mM GL Treatment
G_0/G_1	44.58%	24.05%
S	45.63%	38.23%
G_2/M	9.79%	37.71%

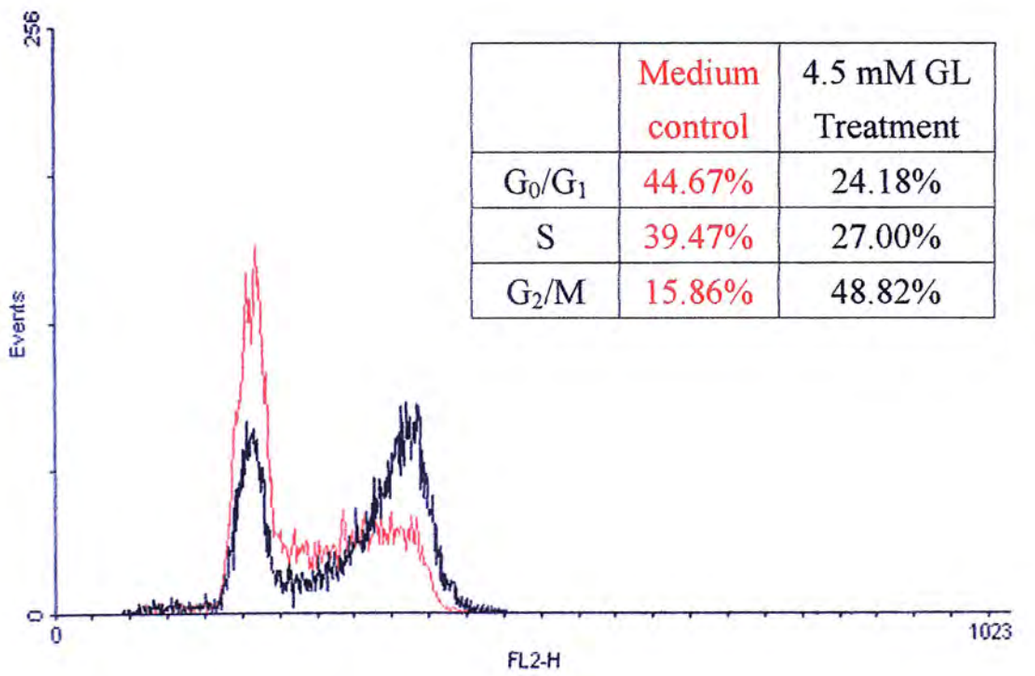
Figure 5.1: Effect of glycyrrhizin on the cell cycle profile in BU-1 cells. BU-1 cells (10^5 cells/ml) were cultured overnight and incubated with either medium (the untreated control) or with two different concentrations of glycyrrhizin (3 and 4.5 mM) at 37°C for 48 hours. 10^6 untreated and treated BU-1 cells were fixed with 70% ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model. Untreated BU-1 cells are shown in red DNA histogram and the black one indicates BU-1 cells cultured in (A) 3 mM and (B) 4.5 mM of glycyrrhizin.

(A)



	Medium control	3 mM GL Treatment
G_0/G_1	44.67%	35.00%
S	39.47%	34.58%
G_2/M	15.86%	30.42%

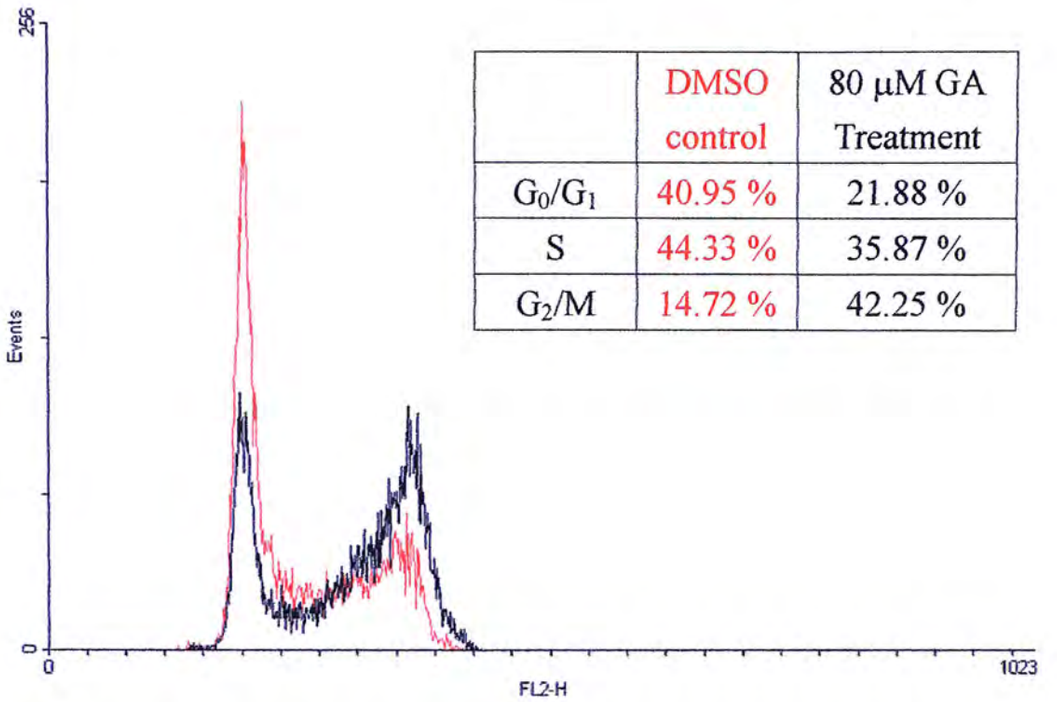
(B)



	Medium control	4.5 mM GL Treatment
G_0/G_1	44.67%	24.18%
S	39.47%	27.00%
G_2/M	15.86%	48.82%

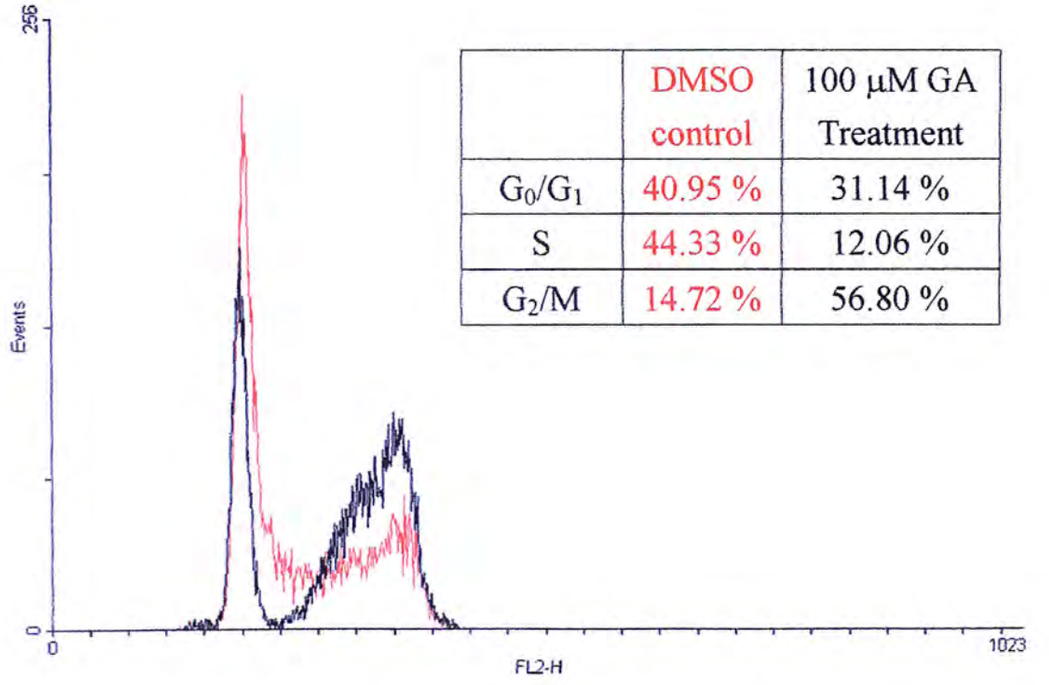
Figure 5.2: Effect of glycyrrhizin on the cell cycle profile in BU-1 cells. BU-1 cells (10^5 cells/ml) were cultured overnight and incubated with either medium (the untreated control) or with two different concentrations of glycyrrhizin (3 and 4.5 mM) at 37°C for 72 hours. 10^6 untreated and treated BU-1 cells were fixed with 70% ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model. Untreated BU-1 cells are shown in red DNA histogram and the black one indicates BU-1 cells cultured in (A) 3 mM and (B) 4.5mM of glycyrrhizin.

(A)



	DMSO control	80 μM GA Treatment
G_0/G_1	40.95 %	21.88 %
S	44.33 %	35.87 %
G_2/M	14.72 %	42.25 %

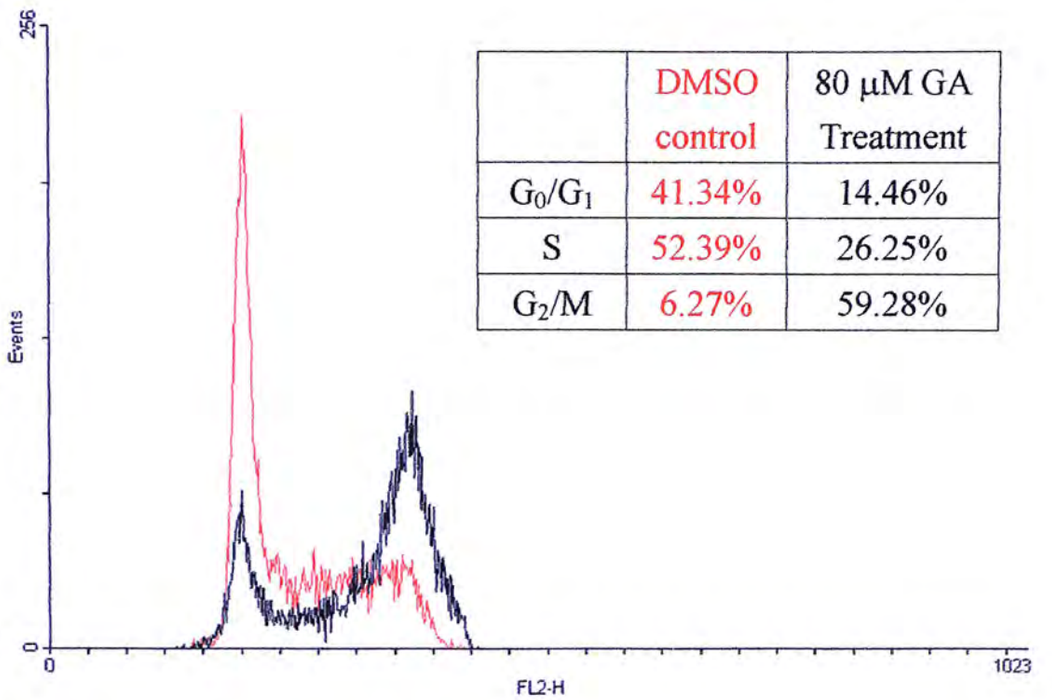
(B)



	DMSO control	100 μM GA Treatment
G_0/G_1	40.95 %	31.14 %
S	44.33 %	12.06 %
G_2/M	14.72 %	56.80 %

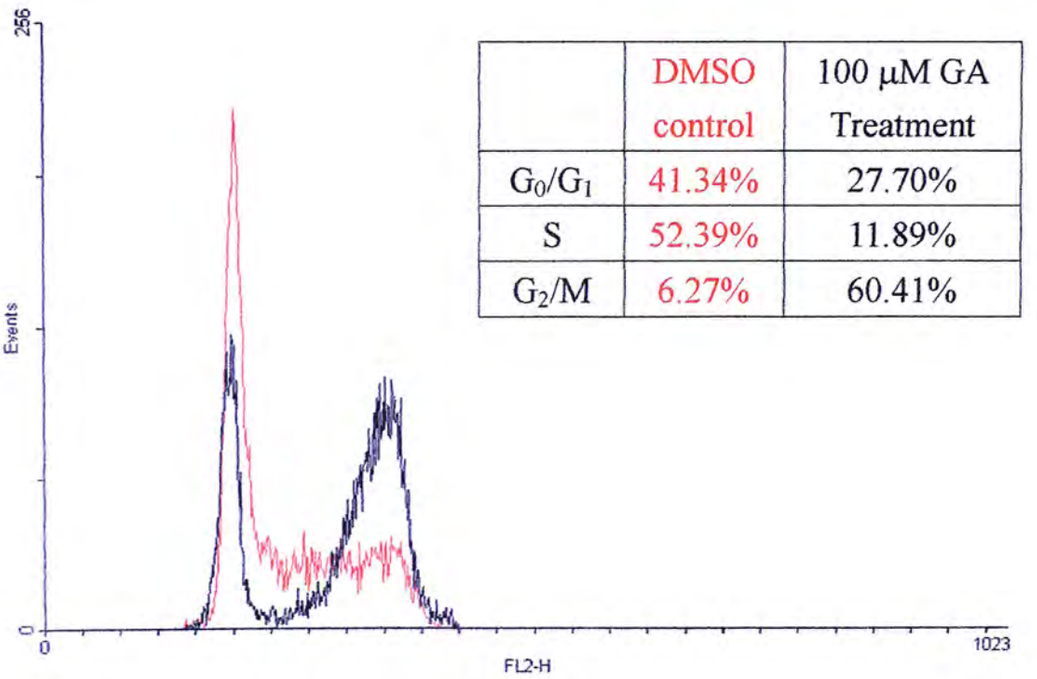
Figure 5.3: Effect of 18β-glycyrrhetinic acid (GA) on the cell cycle profile in BU-1 cells. BU-1 cells (10^5 cells/ml) were cultured overnight and incubated with either 0.17% DMSO (the solvent control) or with two different concentrations of 18β-glycyrrhetinic acid (80 and 100 μM) at 37°C for 24 hours. 10^6 treated BU-1 cells were fixed with 70% ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model. BU-1 cells cultured with the control solvent (0.17% DMSO) are shown in red DNA histogram and the black one indicates BU-1 cells cultured in (A) 80 μM and (B) 100 μM of 18β-glycyrrhetinic acid.

(A)



	DMSO control	80 μM GA Treatment
G_0/G_1	41.34%	14.46%
S	52.39%	26.25%
G_2/M	6.27%	59.28%

(B)



	DMSO control	100 μM GA Treatment
G_0/G_1	41.34%	27.70%
S	52.39%	11.89%
G_2/M	6.27%	60.41%

Figure 5.4: Effect of 18β-glycyrrhetinic acid (GA) on the cell cycle profile in BU-1 cells. BU-1 cells (10^5 cells/ml) were cultured overnight and incubated with either 0.17% DMSO (the solvent control) or with two different concentrations of 18β-glycyrrhetinic acid (80 and 100 μM) at 37°C for 48 hours. 10^6 treated BU-1 cells were fixed with 70% ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model. BU-1 cells cultured with the control solvent (0.17% DMSO) are shown in red DNA histogram and the black one indicates BU-1 cells cultured in (A) 80 μM and (B) 100 μM of 18β-glycyrrhetinic acid.

5.2.2 Modulatory Effects of 18β-Glycyrrhetinic Acid on the Expression of Cell Cycle Regulatory Genes and Proteins

Passage through the cell cycle is regulated by a family of cyclins that act as regulatory subunits for the cyclin-dependent kinases (CDK). The activity of various cyclin-CDK complexes that regulate the progression through G_1-S-G_2 phase of the cell cycle is controlled by the synthesis of the appropriate cyclins during a specific phase of the cell cycle as well as the sequential phosphorylation and dephosphorylation of the key residue of the complex, located principally on the CDK subunits (Puri *et al.*, 1999; Denhardt, 1999). As mentioned previously, 18β-glycyrrhetinic acid was shown to arrest the neuroblastoma BU-1 cells at the G_2/M phase of the cell cycle. Therefore, the effect of 18β-glycyrrhetinic acid on the expression of G_2 cyclins and cyclin-dependent kinase was examined. The modulatory effects of 18β-glycyrrhetinic acid on the mRNA and protein expression of cyclin A, cyclin B and cdc2 (CDK1) were examined by RT-PCR and Western blot, respectively. The mRNA level was examined after treatment with 80 – 120 μM 18β-glycyrrhetinic acid for 8 and 24 hours while the protein expression was examined after 24 and 48 hours of treatment.

Figure 5.5 shows that the transcription of cyclin A, cyclin B and cdc2 decreased dose-dependently as early as after 8 hours of treatment with 18β-glycyrrhetinic acid. Moreover, the reduction in the mRNA levels of cyclin A, cyclin B and cdc2 persisted after 24 hours of treatment. However, the modulatory effect of 18β-glycyrrhetinic acid on the protein levels of cyclin A, cyclin B and cdc2 appeared at a later time period. As shown in Figure 5.6, the protein level of cdc2 remained fairly constant

after 24 hours of treatment with 18β-glycyrrhetinic acid. The decline in protein level of cdc2 was observed after 48 hours in a dose-dependent manner and its level reduced to 42% after treatment with 120 μM 18β-glycyrrhetinic acid. 18β-glycyrrhetinic acid also decreased the protein expression of cyclin A and cyclin B in a dose-dependent manner and such an effect occurred as early as after 24 hours of treatment, a time period at which no significant change in the expression of cdc2 was observed. Moreover, the effect of 18β-glycyrrhetinic acid on the expression of cyclin B seems to be more drastic than that of cyclin A since the protein expression of cyclin B was almost completely suppressed by a higher concentration (120 μM) of 18β-glycyrrhetinic acid. Together with the results of cell cycle analysis, it is believed that 18β-glycyrrhetinic acid arrested the BU-1 cells at the G_2/M phase by the down-regulation of cyclin A, cyclin B and cdc2 at both transcriptional and translational levels.

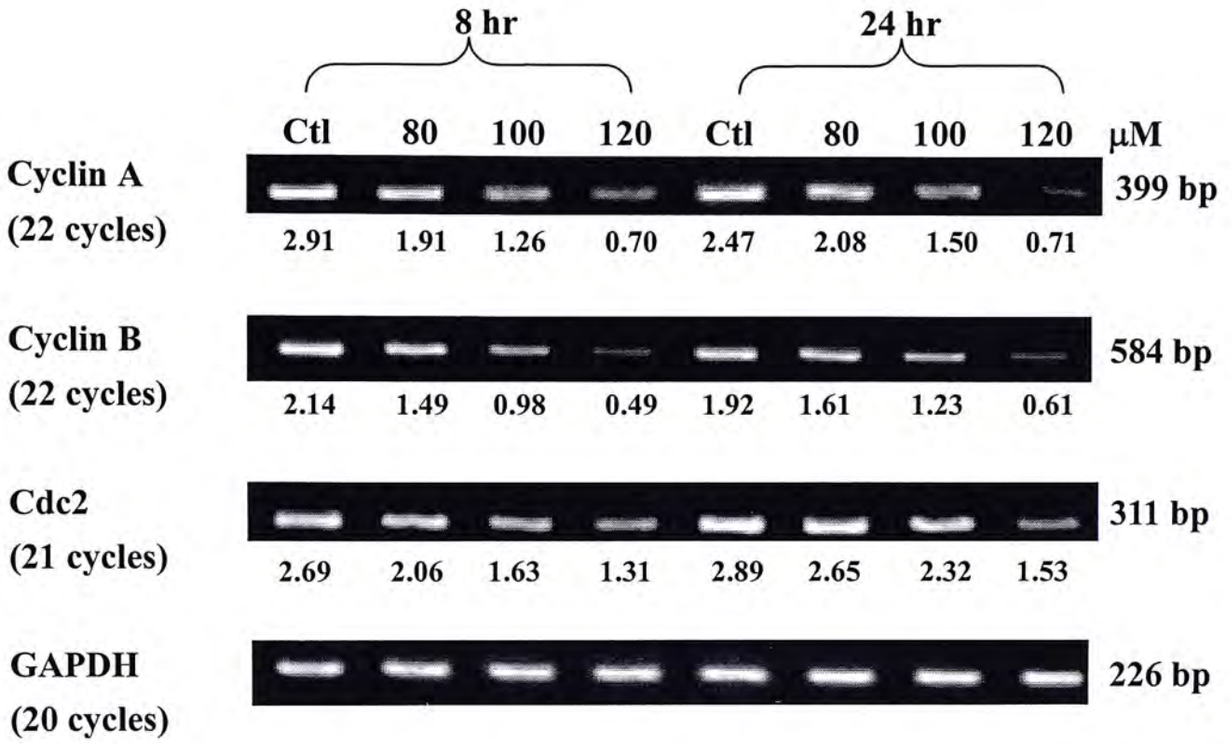


Figure 5.5: Effect of 18β-glycyrrhetinic acid on the mRNA expression of cell cycle regulatory genes in BU-1 cells. BU-1 cells (10^6) were cultured overnight and treated with either 0.2% DMSO (Ctl) or 80 - 120 μM 18β-glycyrrhetinic acid for 8 and 24 hours. Total RNA was isolated and subjected to RT-PCR as described under Materials and Methods. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide. The amounts of the PCR cDNA products were semi-quantified by the ImageQuant software (Molecular Dynamics). The value below each band represents the relative intensity after normalization with respect to GAPDH. The number of PCR cycles completed for each set of primers is indicated in the parentheses and the number on the right indicates the size of the band in base pairs (bp).

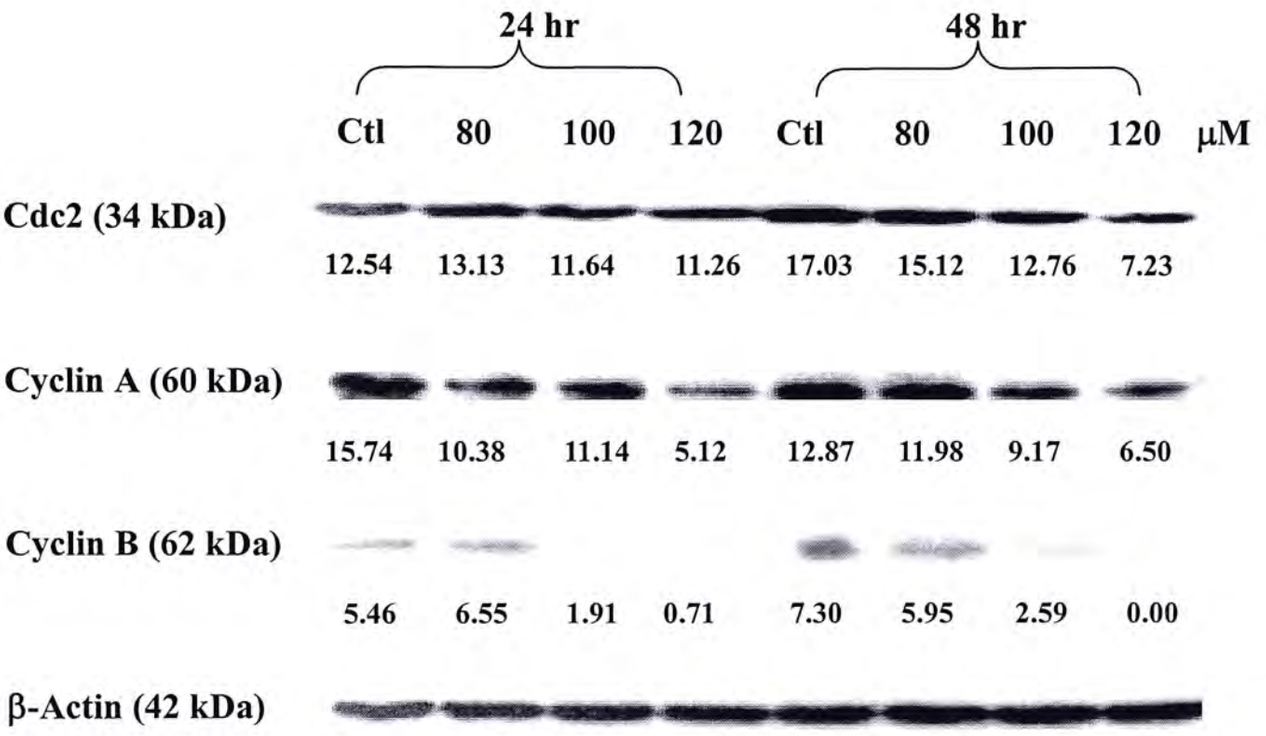


Figure 5.6: Effect of 18β-glycyrrhetinic acid on the expression of cell cycle regulatory proteins in BU-1 cells. BU-1 cells (1.5 x 10^6) were cultured overnight and treated with either 0.2% DMSO as a control (Ctl) or with 80 - 120 μM 18β-glycyrrhetinic acid for 24 and 48 hours. Proteins were extracted and subjected to Western blot as described under Materials and Methods. The extracted proteins (25 μg) were electrophoresed in a 10% polyacrylamide gel, transferred onto a PVDF membrane, exposed to anti-cdc2 (1:10,000), -cyclin A (1:1,000), -cyclin B (1:250) or -β-actin (1:100,000) antibodies, then to horseradish peroxidase conjugated secondary antibodies (1:1,000) and subjected to ECL detection. The signal generated was detected by exposing the membrane to a X-ray film. β-actin was used as a control for loading. Intensity of the signal was quantified and presented as relative units of protein expression after normalization with β-actin. The value below each band represents the relative intensity. The molecular sizes of protein bands were indicated on the left.

5.2.3 Combined Effects of Glycyrrhizin, 18β-Glycyrrhetinic Acid and All-*Trans* Retinoic Acid on the Proliferation of Neuroblastoma BU-1 Cells *In Vitro*

As shown in chapter three, glycyrrhizin exerted its effects on the proliferation and differentiation of BU-1 cells at the millimolar level which is relatively high from the pharmacological point of view. Therefore, it is of great interest to examine whether the effective concentration of glycyrrhizin could be lowered by combining with another agent that is capable of modulating the growth and differentiation of neuroblastoma cells. In the present study, the combined effects of glycyrrhizin and its major metabolite, 18β-glycyrrhetinic acid, were examined since both of them were able to inhibit the growth and induce neuronal differentiation in BU-1 cells. Moreover, the combined effects of 18β-glycyrrhetinic acid and all-*trans* retinoic acid (ATRA) on the proliferation and differentiation of BU-1 cells were also investigated.

As shown in Figure 5.7, treatment of cells with 40 μM and 60 μM 18β-glycyrrhetinic acid or 0.5 mM and 1 mM glycyrrhizin alone resulted in less than 10% inhibition of the proliferation of BU-1 cells, as measured by the ^{3}H-TdR incorporation assay. However, combined treatment of glycyrrhizin and 18β-glycyrrhetinic acid greatly enhanced the growth inhibition (≥ 70%) of BU-1 cells, suggesting that glycyrrhizin and 18β-glycyrrhetinic acid acted in synergy on the growth inhibition of BU-1 cells. Moreover, this synergistic effect occurred in a dose-dependent manner.

Similar synergistic anti-proliferative effect could also be found between 18β-glycyrrhetinic acid and ATRA. Figure 5.8 shows that combined treatment of

BU-1 cells with 18β-glycyrrhetinic acid and ATRA significantly increased the anti-proliferative effect of either compound when compared with the percentage growth inhibition of cells treated with either compound alone. Moreover, the synergistic effect was also dose-dependent. However, the extent of synergism between 18β-glycyrrhetinic acid and ATRA was less than that observed between glycyrrhizin and 18β-glycyrrhetinic acid.

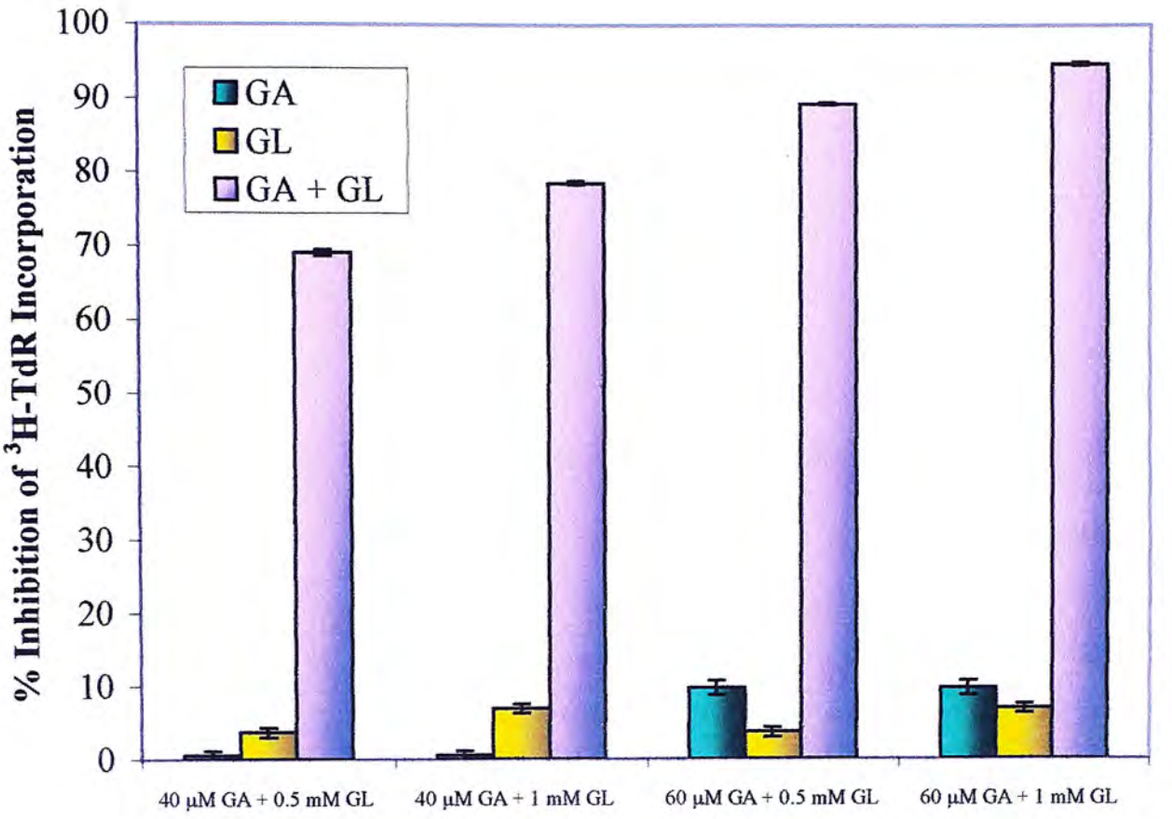


Figure 5.7: Synergistic anti-proliferative effect of glycyrrhizin (GL) and 18β-glycyrrhetinic acid (GA). BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with 40 or 60 μM of 18β-glycyrrhetinic acid (green bars), 0.5 or 1 mM of glycyrrhizin (orange bars), or combinations of glycyrrhizin and 18β-glycyrrhetinic acid (purple bars) at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

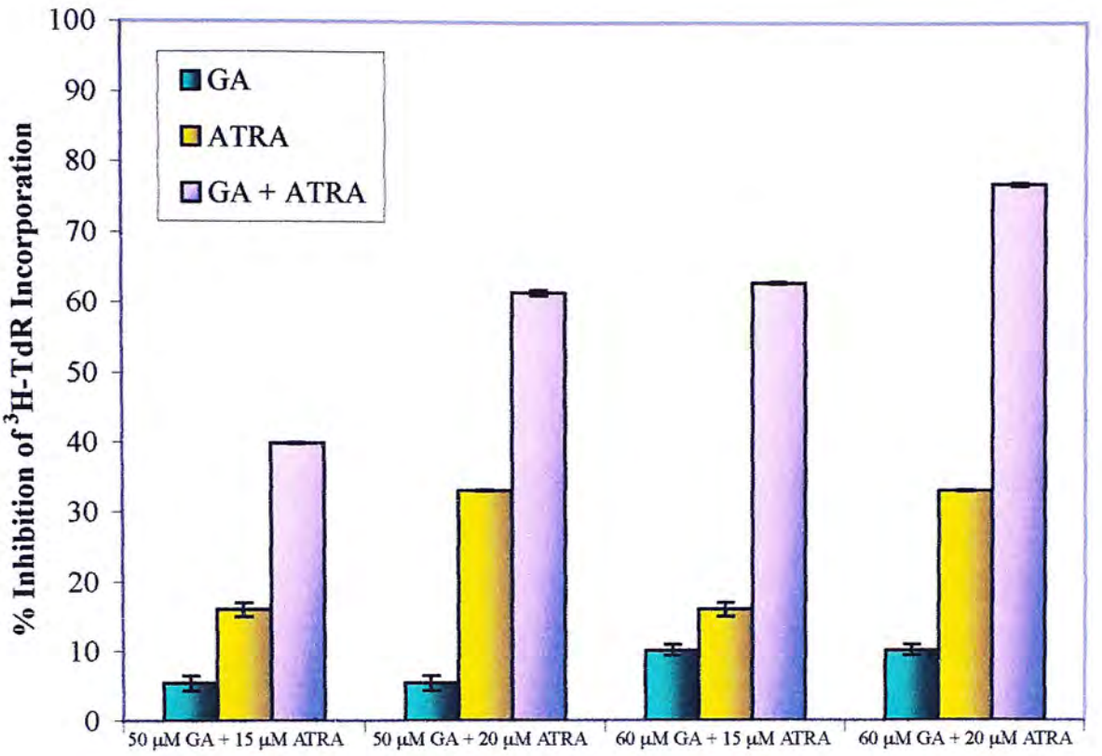


Figure 5.8: Synergistic anti-proliferative effect of 18β-glycyrrhetinic acid (GA) and all-*trans* retinoic acid (ATRA). BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated with 50 or 60 μM of 18β-glycyrrhetinic acid (green bars), 15 or 20 μM of all-*trans* retinoic acid (orange bars), or combinations of 18β-glycyrrhetinic acid and all-*trans* retinoic acid (purple bars) at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity expressed as counts per minute (cpm), was measured using a Beckman liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

5.2.4 Combined Effects of Glycyrrhizin, 18β-Glycyrrhetinic Acid and All-*Trans* Retinoic Acid on the Differentiation of Neuroblastoma BU-1 Cells *In Vitro*

In order to further investigate whether the synergistic effect observed in anti-proliferation assay could be applied to the differentiation of BU-1 cells, morphological study was performed after 4 days of treatment. As shown in Figure 5.9, treatment of BU-1 cells with 40 μM 18β-glycyrrhetinic acid or 0.5 mM glycyrrhizin did not cause any significant change in the morphology of the cells. However, combined treatment of cells with both glycyrrhizin and 18β-glycyrrhetinic acid caused the extension of neurite length and an increase in cell size of BU-1 cells characteristic of neuronal cell differentiation.

Figure 5.10 shows that treatment of BU-1 cells with 60 μM 18β-glycyrrhetinic acid did not result in any morphological changes while treatment with 20 μM ATRA resulted in differentiation of a small population of cells with neurite extension and an increase in cell size. However, combined treatment of cells with 18β-glycyrrhetinic acid and ATRA resulted in differentiation of nearly all cells with a marked increase in the neurite length.

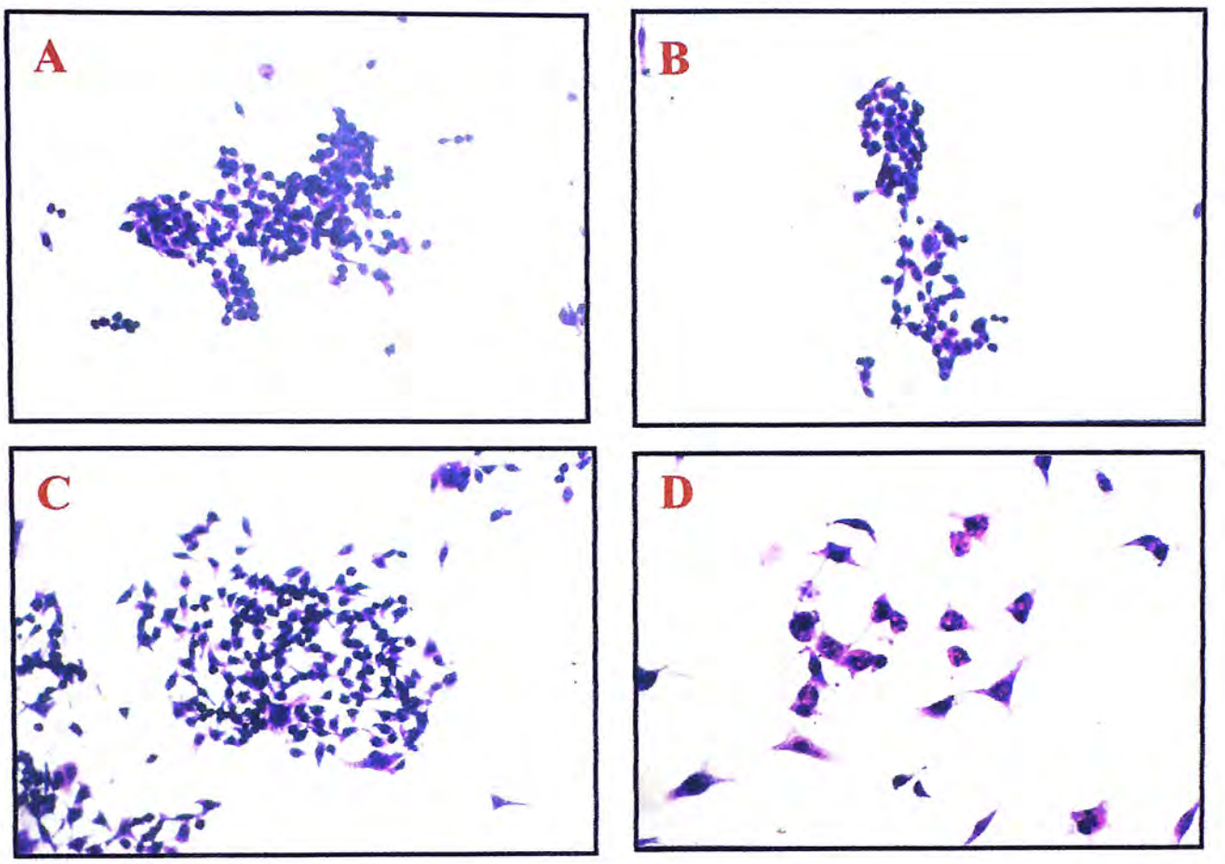


Figure 5.9: Synergistic effect of glycyrrhizin and 18β-glycyrrhetinic acid in the induction of neuronal cell differentiation in BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.07 % DMSO), (B) 40 μM 18β-glycyrrhetinic acid, (C) 0.5 mM glycyrrhizin, or (D) the combination of 40 μM 18β-glycyrrhetinic acid and 0.5 mM glycyrrhizin at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

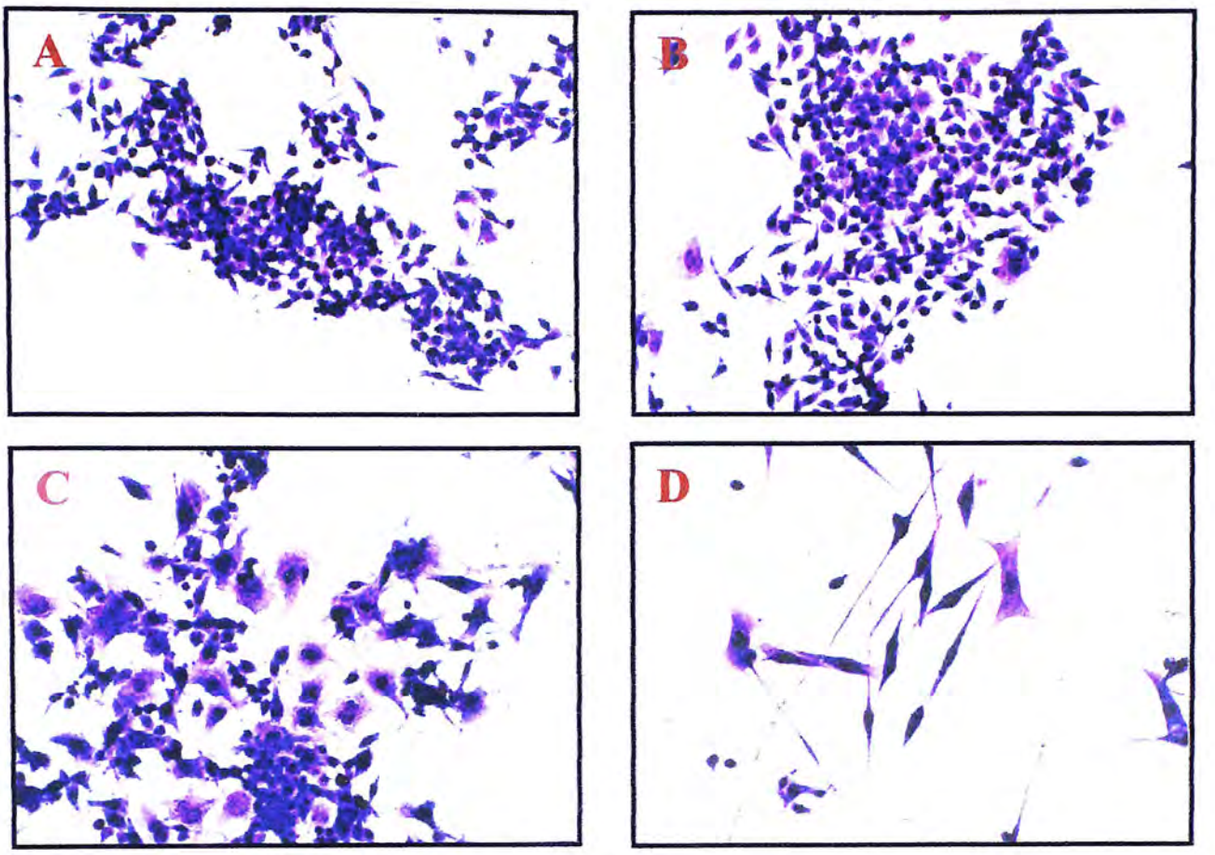


Figure 5.10: Synergistic effect of 18β-glycyrrhetinic acid and all-*trans* retinoic acid in the induction of neuronal cell differentiation in BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.1 % DMSO), (B) 60 μM 18β-glycyrrhetinic acid, (C) 20 μM all-*trans* retinoic acid, or (D) the combination of 60 μM 18β-glycyrrhetinic acid and 20 μM all-*trans* retinoic acid at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

5.2.5 Modulatory Effect of 18β-Glycyrrhetinic Acid on the Expression of PKC Isoforms in Neuroblastoma BU-1 Cells

Altered expression pattern of PKC isozymes during differentiation of neuroblastoma cells had been reported (Wada *et al.*, 1989; Mak *et al.*, 2001). Therefore, in the present study, the expression of various PKC isoforms in 18β-glycyrrhetinic acid-treated BU-1 cells was examined to see whether there are any correlations between their expressions and the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation of BU-1 cells. Previous study showed that BU-1 cells express two Ca^{2+}-dependent PKC isoforms (PKC α and PKC β), and four Ca^{2+}-independent PKC isoforms (PKC δ, PKC ε, PKC ζ and PKC λ) (Mak *et al.*, 2001). In the present study, the expression of PKC α, δ, ε, ζ and λ was examined by RT-PCR after treatment with 100 μM 18β-glycyrrhetinic acid for 30 minutes to 8 hours.

The expression profile of PKC isoforms in BU-1 cells after treatment with 100 μM 18β-glycyrrhetinic acid was shown in Figure 5.11. The results showed that the expressions of PKC α, δ, ε, ζ and λ isoforms remained fairly constant throughout the time period examined.

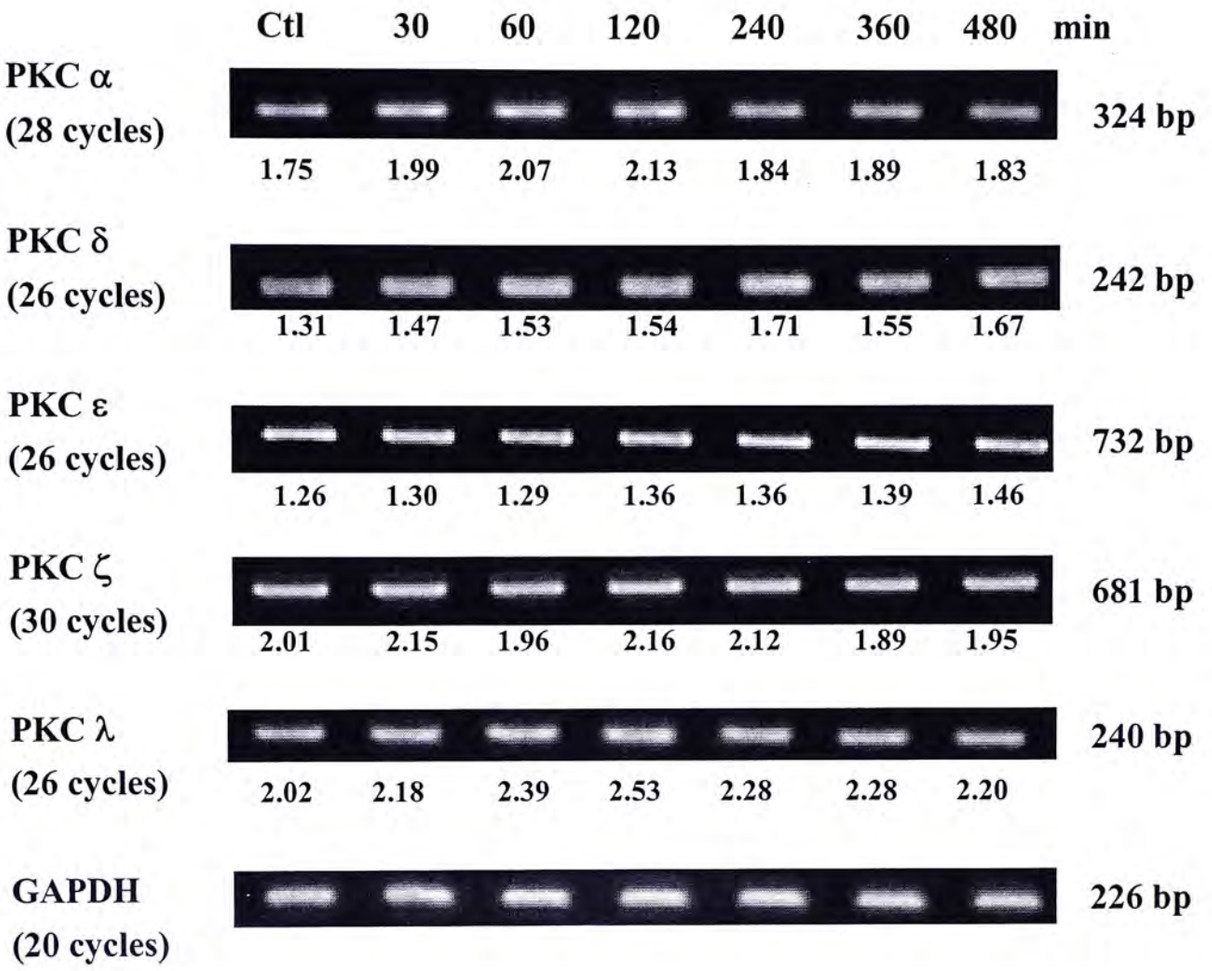


Figure 5.11: Effect of 18β-glycyrrhetinic acid on the mRNA expression of PKC isoforms in BU-1 cells. BU-1 cells (10^6) were cultured overnight and treated with either 0.17% DMSO (Ctl) or 100 μM 18β-glycyrrhetinic acid for various time periods (30 minutes to 8 hours). Total RNA was isolated and subjected to RT-PCR as described under Materials and Methods. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide. The amounts of the PCR cDNA products were semi-quantified by the ImageQuant software (Molecular Dynamics). The value below each band represents the relative intensity after normalization with respect to GAPDH. The number of PCR cycles completed for each set of primers is indicated in the parentheses and the number on the right indicates the size of the band in base pairs (bp).

5.2.6 The Possible Involvement of Protein Kinase C in the Anti-proliferative and Differentiation-Inducing Effects of 18β-Glycyrrhetinic Acid on the Neuroblastoma BU-1 Cells

Modulation of PKC activity was shown to be correlated with differentiation of neuroblastoma cells (Minana *et al.*, 1990; Fagerstrom *et al.*, 1996; Mak *et al.*, 2001). To gain better insights into the underlying signal transduction processes, the effects of modulators of protein kinase activity on the proliferation and differentiation of BU-1 cells cultured with 18β-glycyrrhetinic acid were investigated. In the present study, the PKC activators [PMA, phorbol 12,13-diacetate (PDA)] and PKC inhibitors (staurosporine, calphostin C, Gö 6976) were employed in combination with 18β-glycyrrhetinic acid in the proliferation assay (^{3}H-TdR incorporation assay) and morphological study to determine whether the anti-proliferative and differentiation-inducing effects of 18β-glycyrrhetinic acid were PKC-dependent or not.

Combined treatment of BU-1 cells with PMA, PDA, staurosporine, and 18β-glycyrrhetinic acid did not result in any significant change in the anti-proliferative effect of 18β-glycyrrhetinic acid when compared with the growth inhibition of cells treated with 80 μM 18β-glycyrrhetinic acid alone (data not shown). However, combined treatment of BU-1 cells with calphostin C or Gö 6976 with 18β-glycyrrhetinic acid slightly enhanced the anti-proliferative effect of 18β-glycyrrhetinic acid (Table 5.1 & 5.2).

In addition, combined treatment of BU-1 cells with PMA, PDA and

18β-glycyrrhetinic acid did not modulate the differentiation-inducing effect of 18β-glycyrrhetinic acid compared with those treated with 80 μM 18β-glycyrrhetinic acid alone (data not shown). In contrast, combined treatment of BU-1 cells with 1 nM staurosporine, a broad spectrum PKC inhibitor and 80 μM 18β-glycyrrhetinic acid significantly reduced the number of differentiated cells compared with BU-1 cells treated with 18β-glycyrrhetinic acid alone (Figure 5.12). Moreover, Figure 5.13 shows that calphostin C used at 150 nM clearly prevented the neurite outgrowth and cell size enlargement induced by 80 μM 18β-glycyrrhetinic acid. Furthermore, treatment of BU-1 cell with 80 μM 18β-glycyrrhetinic acid in the presence of 4 nM Gö 6976, a PKC inhibitor that inhibits Ca^{2+}-dependent isozymes α and βI at nanomolar concentrations without affecting the kinase activity of Ca^{2+}-independent PKC δ, ε and ζ isozymes even at micromolar level (Martiny-Baron *et al.*, 1993), effectively blocked the differentiation-inducing effect of 18β-glycyrrhetinic acid with a marked decrease in the number of differentiated cells (Figure 5.14).

Table 5.1: Combined effect of 18β-glycyrrhetinic acid and calphostin C on the proliferation of BU-1 cells.

Treatment of BU-1 cells with	% Inhibition of ^{3}H-TdR Incorporation
50 nM calphostin C	4.19 ± 1.10
150 nM calphostin C	7.59 ± 0.42
80 μM 18β-glycyrrhetinic acid	48.13 ± 0.42
50 nM calphostin C + 80 μM 18β-glycyrrhetinic acid	54.61 ± 1.08 *
150 nM calphostin C + 80 μM 18β-glycyrrhetinic acid	59.06 ± 0.42 **

BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated alone with 80 μM 18β-glycyrrhetinic acid, calphostin C (50 and 150 nM), or with combinations of 18β-glycyrrhetinic acid and calphostin C at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity in counts per minutes (cpm) was measured by liquid scintillation counting. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using untreated BU-1 cells as a control. The data shown are the mean ± standard error of quadruplicate wells. *$p < 0.01$, **$p < 0.001$ vs. 18β-glycyrrhetinic acid alone.

Table 5.2: Combined effect of 18β-glycyrrhetinic acid and Gö 6976 on the proliferation of BU-1 cells.

Treatment of BU-1 cells with	% Inhibition of ^{3}H-TdR Incorporation
3 nM Gö 6976	18.20 ± 2.49
4 nM Gö 6976	52.83 ± 3.41
80 μM 18β-glycyrrhetinic acid	48.72 ± 0.55
3 nM Gö 6976 + 80 μM 18β-glycyrrhetinic acid	53.70 ± 0.44 *
4 nM Gö 6976+ 80 μM 18β-glycyrrhetinic acid	61.26 ± 1.56 *

BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated alone with 80 μM 18β-glycyrrhetinic acid, Gö 6976 (3 and 4 nM), or with combinations of 18β-glycyrrhetinic acid and Gö 6976 at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity in counts per minutes (cpm) was measured by liquid scintillation counting. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using untreated BU-1 cells as a control. The data shown are the mean ± standard error of quadruplicate wells. *$p < 0.01$ vs. 18β-glycyrrhetinic acid alone.

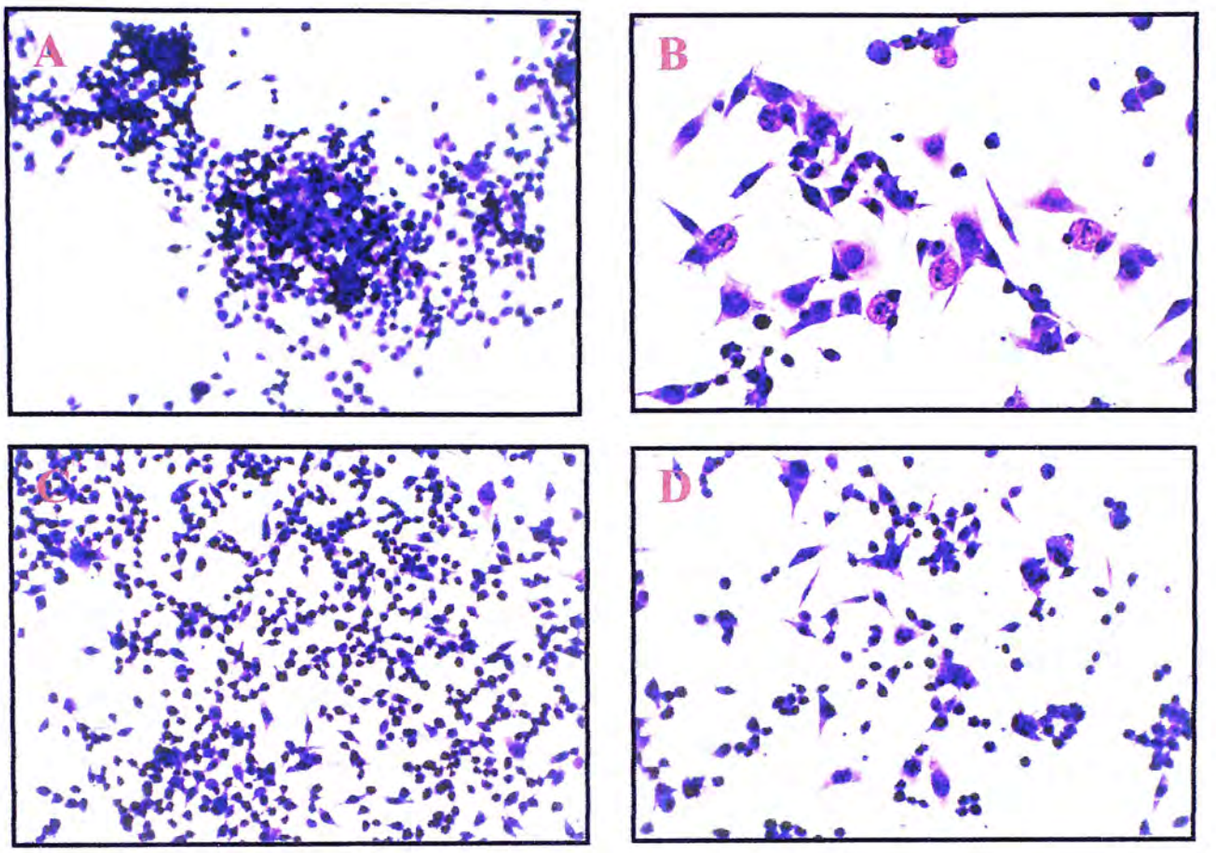


Figure 5.12: Combined effect of 18β-glycyrrhetinic acid and staurosporine on the differentiation of BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.13% DMSO), (B) 80 μM 18β-glycyrrhetinic acid, (C) 1 nM staurosporine, or (D) the combination of 80 μM 18β-glycyrrhetinic acid and 1 nM staurosporine at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

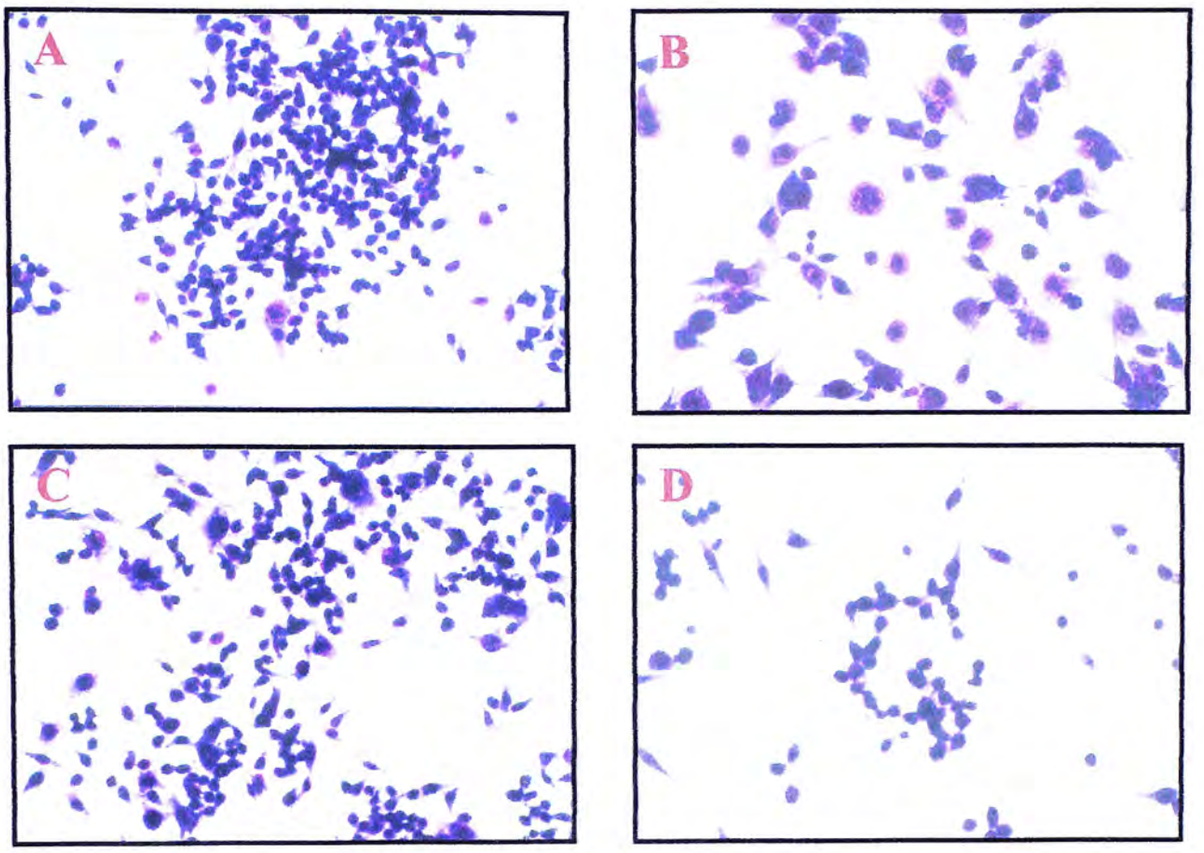


Figure 5.13: Combined effect of 18β-glycyrrhetinic acid and calphostin C on the differentiation of BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.13% DMSO), (B) 80 μM 18β-glycyrrhetinic acid, (C) 150 nM calphostin C, or (D) the combination of 80 μM 18β-glycyrrhetinic acid and 150 nM calphostin C at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

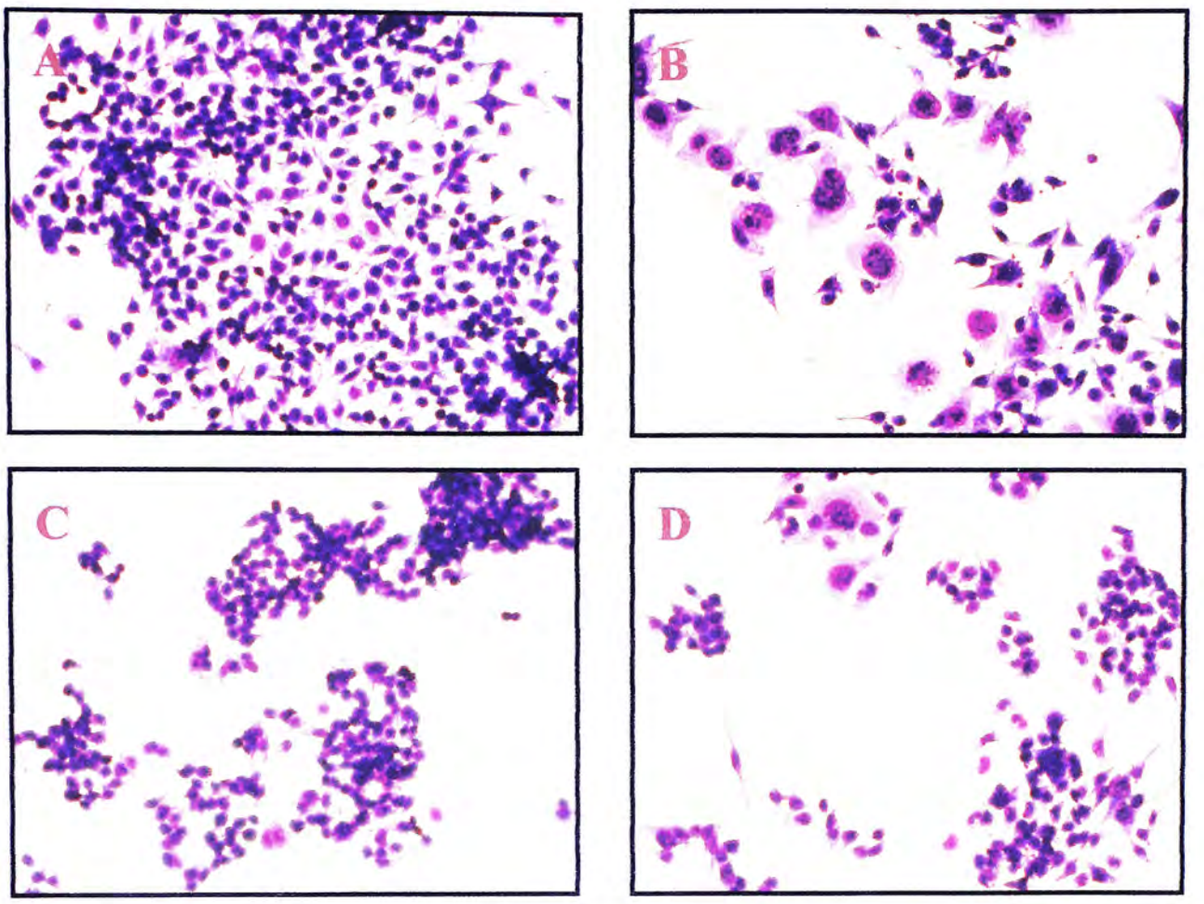


Figure 5.14: Combined effect of 18β-glycyrrhetinic acid and Gö 6976 on the differentiation of BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.13% DMSO), (B) 80 μM 18β-glycyrrhetinic acid, (C) 4 nM Gö 6976, or (D) the combination of 80 μM 18β-glycyrrhetinic acid and 4 nM Gö 6976 at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

5.2.7 The Possible Involvement of Protein Kinase A in the Anti-proliferative and Differentiation-Inducing Effects of 18β-Glycyrrhetinic Acid on the Neuroblastoma BU-1 Cells

To investigate whether PKA signaling pathway is involved in the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation of BU-1 cells, several PKA activators (Sp-isomer, db-cAMP and forskolin) or inhibitors (Rp-isomer, H-89, KT5720 and 14-22 amide) were used in combination with 18β-glycyrrhetinic acid to see whether modulation of PKA activity would affect the effects of 18β-glycyrrhetinic acid. The results showed that treatment of BU-1 cells with Rp-isomer, H-89, KT5720 and 14-22 amide at pharmacological concentrations that are known to inhibit the PKA activity did not alter the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation of BU-1 cells (data not shown). In contrast, the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation of BU-1 cells were markedly augmented by PKA activators. As shown in Table 5.3 - 5.5, incubation of BU-1 cells with PKA activators alone slightly inhibited the growth of BU-1 cells while combined treatment of PKA activators and 18β-glycyrrhetinic acid significantly enhanced the anti-proliferative effect of 18β-glycyrrhetinic acid in a dose-dependent manner. The potentiation effect of PKA activators could also be applied to the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells. In general, for the dose of PKA activators employed in this study, treatment of cells with PKA activators alone did not result in any morphological changes of BU-1 cells (Figure 5.15D, 5.16D & 5.17D). Combined treatment of BU-1 cells with 70 μM 18β-glycyrrhetinic acid and PKA activator significantly induced extensive differentiation of BU-1 cells while treatment of cells with either 70 μM

18β-glycyrrhetinic acid or PKA activator alone did not induce significant differentiation (Figure 5.15 – 5.17). Combined treatment of cells with PKA activators and 80 μM 18β-glycyrrhetinic acid also significantly enhanced the extent of differentiation compared with cells that were treated with 80 μM 18β-glycyrrhetinic acid alone (Figure 5.15 - 5.17).

Table 5.3: Combined effect of 18β-glycyrrhetinic acid and Sp-isomer on the proliferation of BU-1 cells.

Treatment of BU-1 cells with	% Inhibition of ^{3}H-TdR Incorporation
60 μM Sp-isomer	4.01 ± 0.78
80 μM Sp-isomer	5.16 ± 0.97
80 μM 18β-glycyrrhetinic acid	62.81 ± 0.75
60 μM Sp-isomer + 80 μM 18β-glycyrrhetinic acid	80.88 ± 0.06 **
80 μM Sp-isomer + 80 μM 18β-glycyrrhetinic acid	85.43 ± 0.49 **

BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated alone with 80 μM 18β-glycyrrhetinic acid, Sp-isomer (60 and 80 μM), or with combinations of 18β-glycyrrhetinic acid and Sp-isomer at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity in counts per minutes (cpm) was measured by liquid scintillation counting. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using untreated BU-1 cells as a control. The data shown are the mean ± standard error of quadruplicate wells. **$p < 0.001$ vs. 18β-glycyrrhetinic acid alone.

Table 5.4: Combined effect of 18β-glycyrrhetinic acid and db-cAMP on the proliferation of BU-1 cells.

Treatment of BU-1 cells with	% Inhibition of ^{3}H-TdR Incorporation
10 μM db-cAMP	6.81 ± 1.37
100 μM db-cAMP	15.63 ± 1.27
80 μM 18β-glycyrrhetinic acid	70.72 ± 0.26
10 μM db-cAMP + 80 μM 18β-glycyrrhetinic acid	82.71 ± 0.38 **
100 μM db-cAMP + 80 μM 18β-glycyrrhetinic acid	94.83 ± 0.04 **

BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated alone with 80 μM 18β-glycyrrhetinic acid, db-cAMP (10 and 100 μM), or with combinations of 18β-glycyrrhetinic acid and db-cAMP at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity in counts per minutes (cpm) was measured by liquid scintillation counting. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using untreated BU-1 cells as a control. The data shown are the mean ± standard error of quadruplicate wells. **$p < 0.001$ vs. 18β-glycyrrhetinic acid alone.

Table 5.5: Combined effect of 18β-glycyrrhetinic acid and forskolin on the proliferation of BU-1 cells.

Treatment of BU-1 cells with	% Inhibition of ^{3}H-TdR Incorporation
1 μM forskolin	4.05 ± 1.40
5 μM forskolin	9.45 ± 0.78
80 μM 18β-glycyrrhetinic acid	67.29 ± 0.72
1 μM forskolin + 80 μM 18β-glycyrrhetinic acid	84.64 ± 0.28 **
5 μM forskolin + 80 μM 18β-glycyrrhetinic acid	90.22 ± 0.28 **

BU-1 cells (7.5 x 10^4 cells/ml) were cultured overnight and incubated alone with 80 μM 18β-glycyrrhetinic acid, forskolin (1 and 5 μM), or with combinations of 18β-glycyrrhetinic acid and forskolin at 37°C for 48 hours. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 6 hours before harvest. Radioactivity in counts per minutes (cpm) was measured by liquid scintillation counting. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using untreated BU-1 cells as a control. The data shown are the mean ± standard error of quadruplicate wells. **p < 0.001 vs. 18β-glycyrrhetinic acid alone.

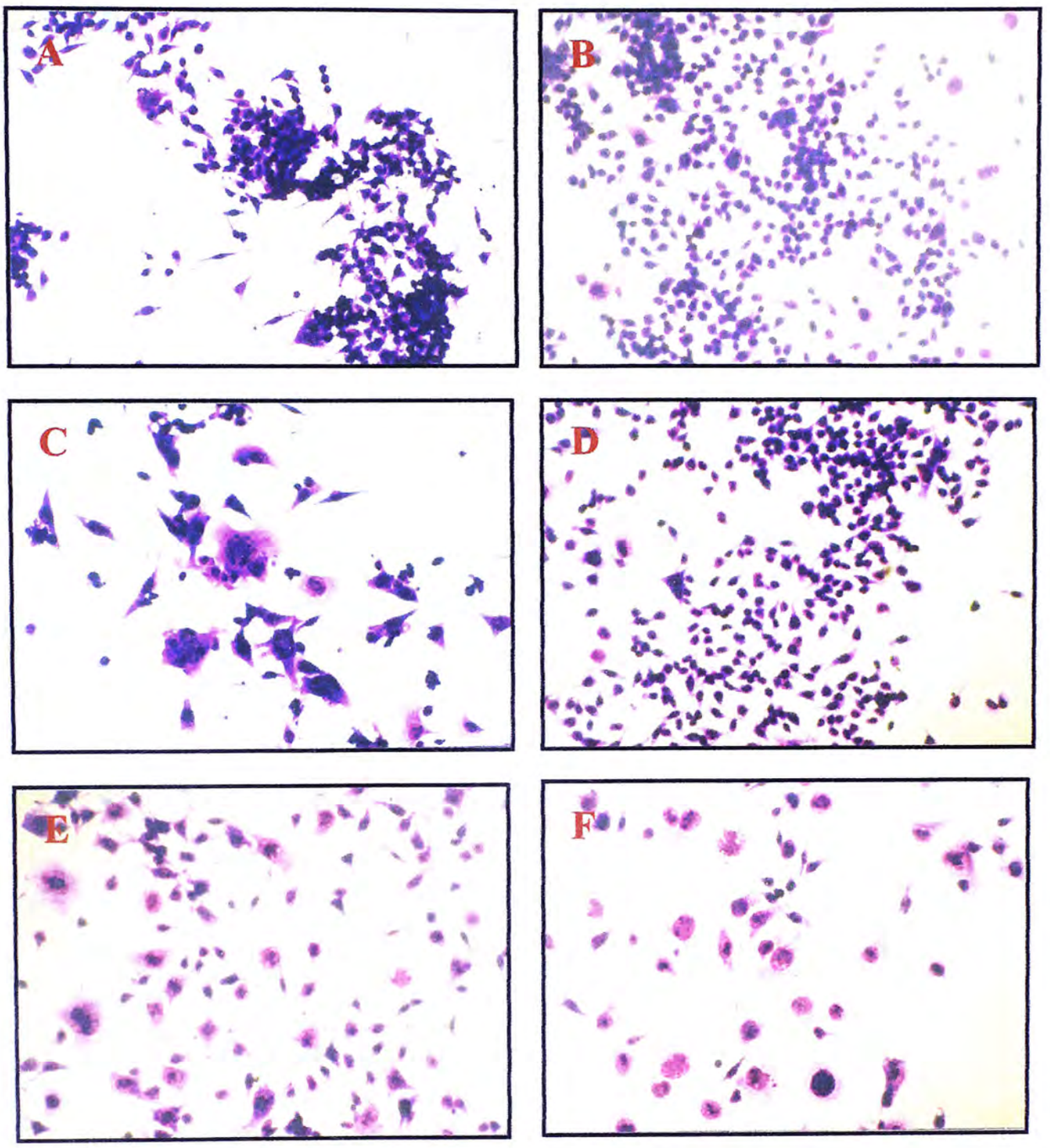


Figure 5.15: Combined effect of 18β-glycyrrhetinic acid and Sp-isomer on the differentiation of BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.13% DMSO), (B) 70 μM 18β-glycyrrhetinic acid, (C) 80 μM 18β-glycyrrhetinic acid, (D) 80 μM Sp-isomer, (E) the combination of 70 μM 18β-glycyrrhetinic acid and 80 μM Sp-isomer or (F) the combination of 80 μM 18β-glycyrrhetinic acid and 80 μM Sp-isomer at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

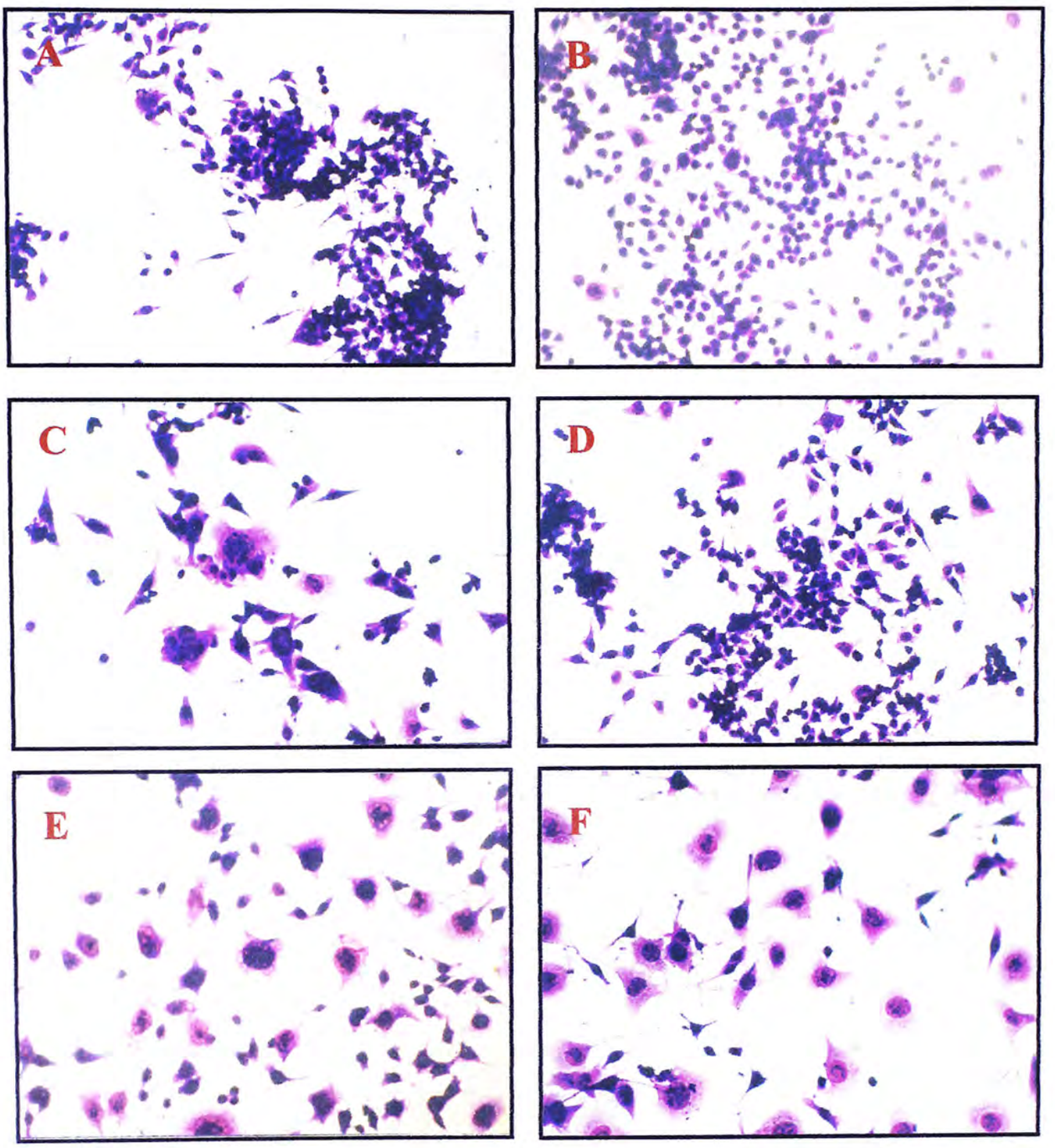


Figure 5.16: Combined effect of 18β-glycyrrhetinic acid and db-cAMP on the differentiation of BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.13% DMSO), (B) 70 μM 18β-glycyrrhetinic acid, (C) 80 μM 18β-glycyrrhetinic acid, (D) 100 μM db-cAMP, (E) the combination of 70 μM 18β-glycyrrhetinic acid and 100 μM db-cAMP or (F) the combination of 80 μM 18β-glycyrrhetinic acid and 100 μM db-cAMP at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

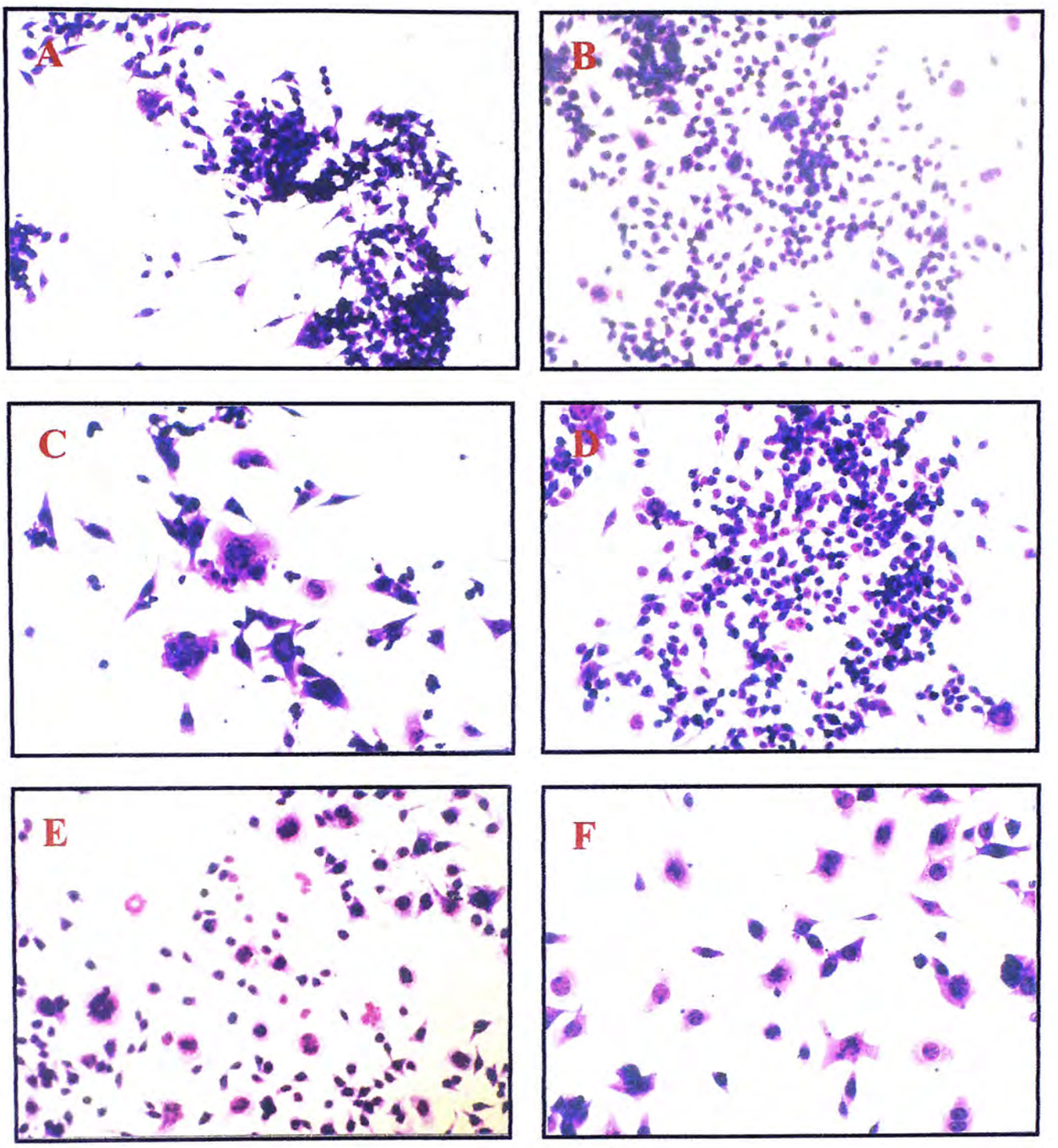


Figure 5.17: Combined effect of 18β-glycyrrhetinic acid and forskolin on the differentiation of BU-1 cells. The BU-1 cells (5,000 cells/well) grown overnight on coverslips in the wells of a 24-well plate were treated with (A) control solvent (0.13% DMSO), (B) 70 μM 18β-glycyrrhetinic acid, (C) 80 μM 18β-glycyrrhetinic acid, (D) 5 μM forskolin, (E) the combination of 70 μM 18β-glycyrrhetinic acid and 5 μM forskolin or (F) the combination of 80 μM 18β-glycyrrhetinic acid and 5 μM forskolin at 37°C for 4 days. The treated cells were then stained with Hemacolor solutions (Magnification x 200).

5.3 Discussion

In the previous two chapters, glycyrrhizin and 18β-glycyrrhetinic acid had been shown to exert anti-proliferative and differentiation-inducing effects on the murine neuroblastoma BU-1 cells *in vitro*. However, the mechanisms by which they exerted their effects on the BU-1 cells had not been fully elucidated. In the present study, the effects of glycyrrhizin and 18β-glycyrrhetinic acid on the cell cycle progression of BU-1 cells were studied by flow cytometry. The results showed that both glycyrrhizin and 18β-glycyrrhetinic acid could induce cell cycle arrest of BU-1 cells at the G_2/M phase accomplished with a decrease of cells in the G_0/G_1 phase and S phase although the cell cycle arrest of glycyrrhizin-treated cells occurred in a delayed fashion when compared with the 18β-glycyrrhetinic acid-treated cells. Therefore, it can be speculated that the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid on BU-1 cells might be mediated through the blockade of the cell cycle progression. Moreover, the absence of sub-G1 peak in the DNA histogram is consistent with the observation in chapter three that glycyrrhizin and 18β-glycyrrhetinic acid are incapable of triggering apoptosis in the neuroblastoma BU-1 cells.

The mammalian cell cycle consists of two major phases known as S and M phases, during which DNA synthesis and mitosis occurs respectively, and two intervening phases called G_1 and G_2 in which the cell is preparing to initiate DNA synthesis and cell division respectively. Complexes of cyclins with cyclin dependent kinases (CDK) play a central role in the control of the cell cycle progression by phosphorylating specific substrates that is thought to be critical in the regulation of an

ordered sequence of events leading to DNA replication and chromosomal segregation. Progression through the cell cycle depends on the expression of certain proteins at appropriate times. Cyclins are activators of CDK and their expressions are tightly regulated. Cyclin A is implicated in the control of the S phase as well as mitosis. Cdc2 forms complexes with cyclin A, allowing the progression through S into M phase. The cyclin B/cdc2 complexes which are also known as the maturation-promoting factor (MPF), accumulate in the G_2 phase and induce mitosis as a consequence of phosphorylation of target proteins such as the nuclear membrane lamins and various cytoskeletal proteins (Puri *et al.*, 1999; Denhardt, 1999). Since glycyrrhizin and 18β-glycyrrhetinic acid were shown to arrest the cell cycle at the G_2/M phase and 18β-glycyrrhetinic acid was more potent than glycyrrhizin, therefore, the effect of 18β-glycyrrhetinic acid on the expression of G_2 cyclins and cyclin dependent kinase was further investigated by RT-PCR and Western blot. The results showed that 18β-glycyrrhetinic acid down-regulated the expression of cyclin A, cyclin B and cdc2 dose- and time-dependently at both transcriptional and translational levels, suggesting that one of the mechanisms by which 18β-glycyrrhetinic acid might arrest the growth of BU-1 cells was via the down-regulation of these cell cycle regulatory proteins.

Results in the previous two chapters showed that glycyrrhizin and 18β-glycyrrhetinic acid were able to inhibit the growth and induce differentiation of BU-1 cells. Therefore, we aimed to determine whether combined treatment of glycyrrhizin with 18β-glycyrrhetinic acid would result in synergistic or additive effect on growth inhibition and differentiation of neuroblastoma cells. The results in this chapter showed that combined treatment of glycyrrhizin and 18β-glycyrrhetinic acid

greatly enhanced the growth inhibition of BU-1 cells when compared with treatment with either compound alone. Synergistic effect of glycyrrhizin and 18β-glycyrrhetinic acid could also be found on the differentiation of BU-1 cells. Combined treatment of glycyrrhizin and 18β-glycyrrhetinic acid at the dose that did not induce differentiation alone significantly induced differentiation of BU-1 cells with an increase in cell size and outgrowth of neurites. This finding provides a rational basis for the potential use of glycyrrhizin for the differentiation therapy of neuroblastoma since 18β-glycyrrhetinic acid is the major metabolite of glycyrrhizin and both glycyrrhizin and 18β-glycyrrhetinic acid could be detected in the plasma after intravenous administration of glycyrrhizin and the presence of glycyrrhizin and 18β-glycyrrhetinic acid would thus greatly enhance the growth inhibition and differentiation of the neuroblastoma cells. However, the mechanism by which glycyrrhizin and 18β-glycyrrhetinic acid exert synergistic effect is still unclear and will require further investigations. Besides the synergistic effect between glycyrrhizin and 18β-glycyrrhetinic acid, 18β-glycyrrhetinic acid was also found to act in synergy with ATRA in both growth inhibition and differentiation of BU-1 cells, implying that combination of 18β-glycyrrhetinic acid and ATRA might have potential therapeutic value in the treatment of some forms of neuroblastoma. The actions of retinoic acid are thought to be mediated through two types of nuclear retinoid receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). ATRA binds with high affinity to RARs and these receptors function as transcription factors in a ligand-dependent manner as heterodimers with ligand-independent RXRs (Allegretto *et al.*, 1993). Moreover, recent study showed that ATRA treatment of SH-SY5Y neuroblastoma cells rapidly activated the PI3K/Akt signaling pathway and caused a decrease in the expression of differentiation-inhibiting genes and an increase

in the expression of differentiation-promoting genes (Lopez-Carballo *et al.*, 2002). Furthermore, the PI3K/Akt signaling pathway is involved in the ATRA-induced differentiation of SH-SY5Y cells and in the transcriptional down-regulation of differentiation-inhibiting genes. Lopez-Carballo *et al.* (2002) hypothesized that ATRA may activate PI3K through an atypical extragenomic action of its receptor. Since the action mechanism of ATRA is quite complicated by itself, further investigation is required to elucidate the exact mechanism of the synergistic action of 18β-glycyrrhetinic acid and ATRA on the proliferation and differentiation of the neuroblastoma BU-1 cells.

PKC is a family of serine-threonine kinases that have been shown to play an important role in the growth, survival and differentiation of cells (Goekjian & Jirousek, 2001). The conventional PKC isoforms were shown to be involved in growth and survival of neuroblastoma cells while the novel isoforms seemed to play a role in neurite outgrowth (Fagerstrom *et al.*, 1996; Zeidman *et al.*, 1999a; Zeidman *et al.*, 1999b; Svensson *et al.*, 2000). Recent studies have indicated that changes in the expression of PKC isoforms and/or their activities are associated with differentiation of neuroblastoma cells (Minana *et al.*, 1990; Zeidman *et al.*, 1999b; Mak *et al.*, 2001). Our results showed that the mRNA expression of certain PKC isoforms remained fairly constant throughout the time period examined. However, due to the failure of proper amplification of PKC β (formation of primer dimers) by RT-PCR although various annealing temperature had been tried, the mRNA expression level of PKC β was not shown. Since PKC β may play a role in mediating the 18β-glycyrrhetinic acid induced effect, a new primer set for PKC β should be redesigned in order to test whether PKC β expression could be modulated by 18β-glycyrrhetinic acid.

Although, the protein levels and the enzymatic activities of the PKC isoforms had not been investigated, the possible involvement of PKC in the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation of BU-1 cells was examined by incubation of BU-1 cells with 18β-glycyrrhetinic acid (80 μM) in the presence or absence of PKC activators or inhibitors. Our results showed that for all PKC activators and inhibitors examined, no antagonistic effect could be found on the anti-proliferative effect of 18β-glycyrrhetinic acid. However, some inhibitors such as calphostin C and Gö 6976 even slightly potentiated the growth inhibitory effect of 18β-glycyrrhetinic acid. This potentiation effect may reflect that PKC activity is important for the growth and/or survival of neuroblastoma cells and inhibition of PKC would inhibit the growth of neuroblastoma cells (Zeidman *et al.*, 1999b). Moreover, treatment of cells with PKC inhibitors alone inhibited the growth of BU-1 cells dose-dependently (data not shown), supporting the hypothesis that PKC activity is required for the growth and/or survival of BU-1 cells. Since no antagonistic effect was observed on the proliferation of BU-1 cells when PKC activator or inhibitor was combined with 18β-glycyrrhetinic acid compared with treatment of cells with 18β-glycyrrhetinic acid alone, we cannot conclude whether the anti-proliferative effect of 18β-glycyrrhetinic acid is PKC dependent or not.

On the other hand, staurosporine, a broad spectrum protein kinase inhibitor, was found to be capable of suppressing neurite outgrowth and decreasing the number of differentiated BU-1 cells, suggesting that protein kinase(s) might be involved in the differentiation-inducing effect of 18β-glycyrrhetinic acid. Staurosporine inhibits PKC with an IC_{50} value of approximately 0.7 nM and inhibits myosin light chain kinase, protein kinase A, protein kinase G and calmodulin kinase at higher

concentrations. Therefore, in order to confirm whether PKC was involved in the differentiation-inducing effect of 18β-glycyrrhetinic acid, more specific protein kinase C inhibitors including calphostin C and Gö 6976 were used. Calphostin C is a highly specific inhibitor of protein kinase C that interacts with the protein's regulatory domain by competing at the binding site of diacylglycerol and phorbol esters with an IC_{50} value of 50 nM and inhibits myosin light chain kinase, protein kinase A and protein kinase G at much higher concentrations. The results showed that calphostin C almost completely blocked the differentiation-inducing effect of 18β-glycyrrhetinic acid. On the other hand, Gö 6976 is a PKC inhibitor that selectively inhibits Ca^{2+}-dependent PKC isoforms (PKC α and βI) at nanomolar concentrations. Our results showed that Gö 6976 markedly inhibited the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells, suggesting that the conventional PKC isoforms (PKC α and βI) might be involved in the differentiation induced by 18β-glycyrrhetinic acid. This finding was in agreement with a recent report showing that conventional PKC isoforms play a role in euxanthone-induced neuritogenesis in the BU-1 cells (Mak *et al.*, 2001).

Recently, the role of PKC novel isoforms especially PKC ε in neurite outgrowth has been suggested (Fagerstrom *et al.*, 1996; Zeidman *et al.*, 1999b; Zeidman *et al.*, 2002). Overexpression of the regulatory domain of PKC ε has resulted in the induction of neurite-like processes (Zeidman *et al.*, 1999a). Moreover, PKC ε has been shown to play a role in the regulation of phosphorylation of microtubule-associated protein tau (Ekinci & Shea, 1997). Our present results indicated that calphostin C, which inhibits both the conventional and novel PKC

isoforms, exerted a much more pronounced effect in suppressing the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells. This observation suggests that novel PKC isoforms may also be involved in the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells. The finding that inhibition of PKC reduced the 18β-glycyrrhetinic acid-induced differentiation implies that the differentiation-inducing effect of 18β-glycyrrhetinic acid on the BU-1 cells may be PKC-dependent. However, the major problem in determining which PKC isoform mediates the differentiation-inducing effect is the lack of highly-specific PKC isoform inhibitors. Perhaps the use of antisense oligonucleotides can provide better insights for the involvement of certain PKC isoforms in neuronal cell differentiation.

O'Brian *et al.* (1990) reported that concentrations of 18β-glycyrrhetinic acid below 200 μM slightly enhanced the PKC activity in the presence of Ca^{2+} and phosphatidylserine. However, it had no effect on the PKC activity in the absence of these co-factors, suggesting that 18β-glycyrrhetinic acid may activate the conventional and novel PKC isoforms. In the present study, the combined treatment of BU-1 cells with PKC activators and 18β-glycyrrhetinic acid failed to potentiate the differentiation-inducing effect of 18β-glycyrrhetinic acid. In addition, incubation of BU-1 cells with PKC activators alone could not induce differentiation in BU-1 cells (data not shown), suggesting that PKC may be necessary for the differentiation induced by 18β-glycyrrhetinic acid but activation of PKC alone was insufficient to induce differentiation in the BU-1 cells. Therefore, factors other than PKC would be involved in the differentiation-inducing effect of 18β-glycyrrhetinic acid and this awaits further investigations. Although BU-1 is a subclone of the murine neuroblastoma Neuro-2a, the role of PKC in the differentiation of BU-1 and Neuro-2a

cells seems to be different. An earlier report showed that inhibition of protein kinase C by a PKC inhibitor H7 induced neuritogenesis in Neuro-2a and the neuritogenesis was prevented and reversed by PMA, an activator of PKC (Minana *et al.*, 1990). Moreover, a recent report demonstrated that treatment of Neuro-2a cells with staurosporine (20 nM) resulted in marked differentiation towards the neuronal phenotype (Mohan *et al.*, 1999). On the contrary, another study showed that calphostin C, a more specific inhibitor of PKC, inhibited the proliferation of Neuro-2a cells but did not induce morphological differentiation (Minana *et al.*, 1992). The discrepancy of these findings remains unclear; perhaps, it may be due to the intrinsic differences in responses among the clonal variants or due to the differences in the specificity of the inhibitors employed in these studies. Since H7 and staurosporine are broad spectrum PKC inhibitors, perhaps the induction of differentiation promoted by H7 and staurosporine in Neuro-2a cells may not be directly linked to the inhibition of PKC and the non-specific inhibition of protein kinases other than PKC may cause the differentiation.

The involvement of cyclic AMP-dependent protein kinase (PKA) on growth inhibition and/or neuronal differentiation has been shown on several neuronal cell lines (Kim *et al.*, 2000; Kim *et al.*, 2002). To determine whether the PKA signaling pathway is involved in the 18β-glycyrrhetinic acid-induced growth inhibition and differentiation of BU-1 cells, PKA activators or PKA inhibitors were used in combination with 18β-glycyrrhetinic acid in the ^{3}H-TdR incorporation assay and morphological study. Interestingly, all PKA inhibitors tested (Rp-isomer, H-89, KT5720 and 14-22 amide) failed to modulate the growth-inhibitory and differentiation-inducing effects of 18β-glycyrrhetinic acid (data not shown). On the

other hand, when combined with 18β-glycyrrhetinic acid, the cAMP-elevating agent such as forskolin, and the cAMP analogs, Sp-isomer and dibutyryl cAMP (db-cAMP), markedly augmented the growth inhibition and differentiation of BU-1 cells induced by 18β-glycyrrhetinic acid. These results suggest that the growth-inhibitory and differentiation-inducing effects of 18β-glycyrrhetinic acid on BU-1 cells were not mediated directly by an increase in PKA activity but the 18β-glycyrrhetinic acid signaling pathway might be cAMP-dependent. Similarly, it has been reported that induction of neurite outgrowth in PC12 cells by the bacterial nucleoside N^6-methyldeoxyadenosine is mediated through a cAMP-dependent/PKA-independent signaling pathway (Charles *et al.*, 2003). It is possible that the promotion of neurite outgrowth by 18β-glycyrrhetinic acid could be mediated by a signal transduction pathway which involves the generation of endogenous cAMP but independent of PKA. However, whether 18β-glycyrrhetinic acid could directly stimulate the adenylyl cyclase and increase the endogenous cAMP level in BU-1 cells awaits further investigations.

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

Licorice (*Radix Glycyrrhizae*) is generally referred to as Gancao (甘草) by the Chinese and is one of the most widely used medicinal herbs since ancient time. It is also utilized extensively in foods and tobacco products as a sweetener and as a flavoring agent, respectively. Glycyrrhizin, a water soluble triterpenoid saponin, is the major active component of licorice root, and accounts for 6 - 14% by weight of the licorice extract. 18β-glycyrrhetinic acid, the aglycone of glycyrrhizin, is also present in the root of licorice (0.5 - 0.9%). After oral administration in humans, glycyrrhizin is primarily metabolized into 18β-glycyrrhetinic acid by the β-glucuronidase of intestinal bacteria. Research in the past two decades showed that licorice exhibits diverse biological and pharmacological activities, including anti-inflammatory, hepatoprotective, anti-carcinogenic, anti-tumor, anti-viral, immunomodulatory, and mineralocorticoid effects. Many of these activities have been attributed mainly, if not all, to glycyrrhizin and its aglycone, 18β-glycyrrhetinic acid. Although the anti-tumor effects of glycyrrhizin and 18β-glycyrrhetinic acid on various cancer cells have been demonstrated by *in vivo* and *in vitro* studies, yet the direct anti-tumor action mechanisms of these two natural products have not been fully elucidated.

In the present study, glycyrrhizin and 18β-glycyrrhetinic acid were found to exhibit differential anti-proliferative effect on one murine (Neuro-2a BU-1) and two human (SK-N-DZ and SH-SY5Y) neuroblastoma cell lines in a dose-dependent manner. 18β-glycyrrhetinic acid was found to be more potent than glycyrrhizin as it exerted its anti-proliferative activity at micromolar concentrations. The murine neuroblastoma BU-1 cells also responded time-dependently to the growth-inhibitory effect of glycyrrhizin and 18β-glycyrrhetinic acid. However, the anti-proliferative

effect of glycyrrhizin and 18β-glycyrrhetinic acid depended both on the duration of drug treatment and the drug concentration used. Exposure of the BU-1 cells to 18β-glycyrrhetinic acid for 24 hours had resulted in a significant decrease in the proliferating potential of drug-treated cells despite the drug was removed. However, the anti-proliferative effect of glycyrrhizin and 18β-glycyrrhetinic acid cannot be attributed to the direct cytotoxicity of the drugs on the neuroblastoma cells as they were not cytotoxic in nature and both of them also failed to induce apoptosis in the BU-1 cells.

Besides the anti-proliferative effect, glycyrrhizin and 18β-glycyrrhetinic acid were found to be capable of inducing differentiation in the neuroblastoma cells. Both glycyrrhizin and 18β-glycyrrhetinic acid could induce morphological as well as biochemical changes in the BU-1 cells as judged by the neurite outgrowth and increased expression of neuronal differentiation markers MAP2 and neurofilament-200 in drug-treated cells. Moreover, the differentiation induced by 18β-glycyrrhetinic acid in BU-1 cells was accompanied with a down-regulation of N-myc gene expression and an up-regulation of the expression of the Bcl-2, NF-κB/p50 and c-fos genes. It is possible that the drug-induced changes in these gene expressions are responsible for the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells. Nevertheless, the mechanisms by which 18β-glycyrrhetinic acid could alter the expression of these genes have not been elucidated and await further investigations. The anti-proliferative and differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid suggest that these two drugs have potential use for the differentiation therapy of neuroblastoma, however, whether the pharmacologically effective concentrations of

glycyrrhizin and 18β-glycyrrhetinic acid could be achieved *in vivo* need to be addressed.

In this study, attempts had also been made to elucidate the underlying mechanisms for the anti-proliferative and differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid on BU-1 cells and strategies that could lower the effective concentrations of these two drugs were sought. Both glycyrrhizin and 18β-glycyrrhetinic acid were found to be able to induce cell cycle arrest of BU-1 cells at the G_2/M phase accompanied with a significant decrease of cells in the G_0/G_1 and S phases. Therefore, one of the underlying mechanisms by which glycyrrhizin and 18β-glycyrrhetinic acid exert their anti-proliferative effect would be due to the arrest of the cell cycle progression at the G_2/M phase. Further studies with the 18β-glycyrrhetinic acid had revealed that the cell cycle arrest of BU-1 cells may be mediated via the down-regulation of several cell cycle regulatory proteins since treatment of BU-1 cells with 18β-glycyrrhetinic acid caused a down-regulation of cyclin A, cyclin B and cdc2 both transcriptionally and translationally. On the other hand, combined treatment of the BU-1 cells with glycyrrhizin and 18β-glycyrrhetinic acid or 18β-glycyrrhetinic acid and all-*trans* retinoic acid (ATRA) had resulted in synergistic action on the proliferation and differentiation of the BU-1 cells. Therefore, it is conceivable that the combined used of glycyrrhizin, 18β-glycyrrhetinic acid and ATRA would be one of the effective ways to reduce the effective therapeutic concentrations of glycyrrhizin and 18β-glycyrrhetinic acid, and thus minimizing their side effects on the cancer patients.

Since protein kinases play important roles in many cellular events such as cell

growth and differentiation, the involvements of protein kinase C (PKC) and protein kinase A (PKA) in the anti-proliferative and differentiation-inducing effects of glycyrrhizin and 18β-glycyrrhetinic acid on the neuroblastoma BU-1 cells were also studied. Our data showed that the mRNA expression of various PKC isoforms was not significantly altered by 18β-glycyrrhetinic acid throughout the time periods examined. Interestingly, treatment of BU-1 cells with PKC inhibitors (staurosporine, calphostin C and Gö 6976) clearly antagonized the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells while treatment of BU-1 cells with PKC activators (PMA and PDA) failed to potentiate the neuronal cell differentiation induced by 18β-glycyrrhetinic acid. These results suggest that the 18β-glycyrrhetinic acid-induced differentiation may be dependent on the conventional and novel PKC isoforms but the activation of PKC alone was insufficient to induce differentiation in the BU-1 cells. However, the protein levels and enzyme activities of the various PKC isoforms in drug-treated BU-1 cells had not been examined in this study, and further experiments should be performed to elucidate whether 18β-glycyrrhetinic acid could enhance the activity of certain PKC isoforms in BU-1 cells. In addition, whether other factors are also involved in the 18β-glycyrrhetinic acid-induced differentiation of BU-1 cells is an intriguing aspect that is worthy of future investigations.

Surprisingly, the growth-inhibitory and differentiation-inducing effects of 18β-glycyrrhetinic acid on BU-1 cells were enhanced by treatment of BU-1 cells with PKA activators (Sp-isomer, db-cAMP and forskolin) but treatment of BU-1 cells with PKA inhibitors (Rp-isomer, H-89, KT5720 and 14-22 amide) was incapable of reducing the 18β-glycyrrhetinic acid-induced growth inhibition and neuronal cell

differentiation. One hypothesis is that the anti-proliferative and differentiation-inducing effects of 18β-glycyrrhetinic acid may be mediated by the cAMP signaling pathway but is independent of PKA since cAMP elevating agent (forskolin) or cAMP analogs (Sp-isomer and db-cAMP) clearly potentiated the growth-inhibitory and differentiation-inducing effects of 18β-glycyrrhetinic acid. However, whether 18β-glycyrrhetinic acid could stimulate the adenylyl cyclase and increase the endogenous cAMP level remains unclear and the downstream signaling pathway(s) is an intriguing area that requires further in-depth investigations.

In conclusion, since licorice and its active constituent, glycyrrhizin, are still widely used not only for medicinal purposes throughout the world but also as important ingredients in food industry, therefore, more researches should be conducted in the future to gain more insights on their action mechanisms, efficacy and therapeutic potentials. By unraveling the cellular and molecular mechanisms by which glycyrrhizin and 18β-glycyrrhetinic acid might modulate the proliferation and differentiation of neuroblastoma cells, it is hoped that novel treatment for some forms of neuroblastoma, based on the inhibition of neuronal cell proliferation and induction of cell differentiation, can be developed with higher efficacy and minimal toxicity in the treatment of neuroblastoma.

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