Fatty Acid Synthase Inhibitors Retard Growth and Induce Caspase-dependent Apoptosis in Human Melanoma A-375 cells

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Abstract

Fatty acid synthase (FAS) is an enzyme crucial for endogeneous lipogenesis in mammals. It synthesizes fatty acids for body uses by catalyzing the formation of 16-carbon palmitate from condensation of malonyl-coenzyme A and acetyl-coenzyme A. FAS over-expression and hyperactivity have been characterized in various human malignancies but with a low expression level in normal tissues, suggesting that FAS is a novel target for cancer therapy. In this study, growth-inhibitory effects of the FAS pharmacological inhibitors cerulenin and C75 were first investigated on a number of human cancer cell lines of different histotypes, including liver HepG2, lung A-549, colon CaCo-2, Colo201, SW480 and SW620, cervix HeLa, prostate LNCaP and PC-3, breast MCF-7 and SK-BR-3, and skin A-375. Results from the MTT study revealed that the cancer cell proliferation and viability was reduced dose- and time-dependently by 20.8% to 89.4% of the control levels after 24 and 48 h of treatment with 10 to 160 μM of the inhibitor. Among the cancer cell lines tested, A-375 was found to be the most responsive one so that it was selected as the model to study the mechanism for growth inhibition.

At the doses which A-375 growth was prominently retarded, neither cerulenin nor C75 exerted cytotoxicity on the normal skin fibroblast HS68, suggesting that the inhibitors exerted preferential effect on the melanoma cells. DNA flow cytometric studies demonstrated that the FAS inhibitors accumulated G_2/M cells preceding the elevation of

sub G_1 or apoptotic cells with fragmented DNA. The proapoptotic effect of the FAS inhibitors was further confirmed using confocal microscopy with annexin-V FITC and propidium iodide staining. Results from the immunoblotting study further showed that poly(ADP-ribose) polymerase (PARP) was cleaved, which is a hallmark feature of apoptosis, and procaspase-3 was processed into the active and smaller 17 and 19 kDa subunits, with concomitant depletion of caspase-3 inhibitors XIAP and survivin. Besides caspase-3, executioner procaspase-6 and -7 were also found to be processed into their active subunits and cleavage of lamin A/C, a substrate for proteolysis by the active caspase, was observed. On the other hand, administration of the 100 μM pan-caspase inhibitor Z-VAD-FMK completely rescued the cells from PARP cleavage, indicating that caspase activations play significant functions in the cerulenin- and C75-induced apoptosis. The FAS inhibitors were found to trigger both the extrinsic death receptor and intrinsic mitochondrial apoptotic pathways. Death receptor DR5 was elevated, and procaspase-8 and 10 of the extrinsic pathway and procaspase-9 of the intrinsic pathway were processed into their active subunits. Flow cytometric TMRE studies revealed that both the inhibitors significantly altered the mitochondrial membrane potential together with the release of cytochrome *c* from mitochondria into the cytosol. Bid was depleted, possibly via proteolytic cleavage by caspase-8, indicating a possible linkage between both the pathways. To further support this hypothesis, administration of casapse-8 inhibitor Z-IETD-FMK

rescued nearly all the cells from apoptosis, while caspase-9 inhibitor Z-LEHD-FMK only rescued a small proportion of cells. This suggests that the death receptor pathway probably takes a leader role in the cerulenin- and C75-induced apoptosis. Besides the pharmacological inhibitors, blockade of FAS expression by its molecular inhibitor small interfering RNA also generated the similar effects, suggesting that it was the depletion of FAS activity that initiated the molecular changes in the apoptosis.

Results from this study suggest that FAS pharmacological and molecular inhibitors retard growth of melanoma A-375 cells, involving at least activation of caspase-dependent apoptosis. Further elucidations on the molecular mechanisms in apoptosis and cell cycle imposed by FAS inhibition would facilitate research on the synergism between FAS inhibition and some currently used drugs for more effective cancer treatments with less severe side effects.

中文摘要

在哺乳動物體內，脂肪酸合成酶對脂肪合成擔當很重要的角色。脂肪酸合成酶能催化輔酵素 A 與乙醯輔酵素 A 聚合成十六粒炭原單位的棕櫚酸酯，再製成脂肪酸供給體內不同細胞使用。一般而言，正常體細胞只需少量的脂肪酸合成酶合成體內所需的脂肪酸，而大部份的脂肪酸可從日常飲食中攝取到，最近有研究指出癌細胞內的脂肪酸合成酶有過量表現及失調的情況，讓癌細胞有足夠的脂肪酸快速生長，因此，脂肪酸合成酶近年成為癌症研究之重要對象。

本研究先找出脂肪酸合成酶抑制劑淺藍菌素、c75 對不同癌細胞的生長抑制效果，包括肝癌 HepG2、肺癌 A-549、結腸直腸癌 CaCo-2、Colo201、SW480 及 SW620、子宮頸癌 HeLa、前列腺癌 LNCaP 及 PC-3、乳癌 MCF-7 及 SK-BR-3 及黑色素腫瘤 A-375，發現抑制劑對黑色素腫瘤細胞 A-375 的抑制效果最強。在四甲基偶氮噬藍(MTT)法的細胞生長測試中，10 至 80 μM 脂肪酸合成酶抑制劑在 24 及 48 小時內有效抑制 A-375 共 20.8%至 89.4%之生長，其抑制效果更與時間及劑量相關。然而，20 至 80 μM 脂肪酸合成酶抑制劑不對纖維組織母細胞 HS68 構成毒性，証明抑制劑主力抑制癌細胞生長而已。

透過西方墨點法分析，淺藍菌素及 c75 在 24 小時內均能導致黑色素腫瘤細胞的多聚 ADP 核糖多聚胺 (PARP)分離；利用共軛焦顯微鏡技術

之磷脂結合蛋白 V 異硫氰酸熒光素(Annexin-V FITC)及碘化丙啶(PI)標記，顯示脂肪酸合成酶抑制劑能令細胞凋亡；流式細胞技術更証明脂肪酸合成酶抑制劑能誘導細胞發生 G_2/M 期阻滯和細胞凋亡；詳細的免疫轉漬分析指出脂肪酸合成酶抑制劑誘導硫胱氨酸蛋白酶-3 活化及分離成更細的 17 及 19kDa 的活化酶，從而減低凋亡蛋白 IAP 中的 XIAP 及 survivin 程度，抑制劑更誘導硫胱氨酸蛋白酶-6、7 及核纖層蛋白 A/C 的分離；然而，硫胱氨酸蛋白酶抑制劑 Z-VAD-FMK 能消除脂肪酸合成酶抑制劑所導致的 PARP 分離，証明脂肪酸合成酶抑制劑誘導黑色素腫瘤細胞進行硫胱氨酸蛋白酶活化性凋亡。

更多免疫轉漬分析指出脂肪酸合成酶抑制劑啟動外在的細胞膜死亡受體及內在的線粒體凋亡途徑。硫胱氨酸蛋白酶-8 及 10 (外在途徑) 及硫胱氨酸蛋白酶-9 (內在途徑) 均有活化及分離的現象；TMRE 標記指出脂肪酸合成酶抑制劑能誘導線粒體膜電位變化，及促進細胞色素 *c* 從線粒體釋放到胞質溶膠中，同時誘導凋亡蛋白 Bid 分離，証明外在的細胞膜死亡受體與內在的線粒體凋亡途徑相連；在硫胱氨酸蛋白酶抑制劑實驗中，發現硫胱氨酸蛋白酶-8 抑制劑 Z-IETD-FMK 較硫胱氨酸蛋白酶-9 抑制劑 Z-LEHD-FMK 更有效抑制劑消除脂肪酸合成酶抑制劑所導致的 PARP 分離，証明脂肪酸合成酶抑制劑先啟動外在的細胞膜死亡受體途徑才啟動內在的線粒體凋亡途徑。同樣地，如脂肪酸合成酶抑制劑，脂肪

酸合成酶小干擾性 RNA 也誘導黑色素腫瘤 A-375 細胞進行硫胱氨酸蛋白胨及 PARP 分離。

本研究先指出脂肪酸合成酶(FAS)抑制劑淺藍菌素及 c75 能有效誘導黑色素腫瘤 A-375 細胞進行硫胱氨酸蛋白胨活化性凋亡，這些研究結果對於研究脂肪酸合成酶抑制及現時西藥之間之協同作用去醫治癌症病人有很大的幫助。

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Table of Contents

List of Table

List of Figures

List of Abbreviations

ACC	acetyl coenzyme A carboxylase
Acetyl-CoA	acetyl coenzyme A
ACP	acyl carrier protein
AMC	7-Amino-4-methylcoumarin
Apaf-1	apoptosis protease activating factor
AT	acetyl transferase
ATP	adenosine triphosphate
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
C75	4-methylene-2-octyl-5-oxo-tetrahydro-furan-3-carboxylic acid
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
Cerulenin	(2R,3S), 2–3-epoxy-4-oxo-7,10-trans,transdodecadienamide
CPT-1	carnitine O-palmitoyltransferase-1
DAPK2	death-associated protein kinase 2
DE	dehydratase
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBV	epstein-barr virus
EDTA	ethylenediamine tetraacetic acid
EGCG	epigallocatechin-3-gallate
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay

ER	enoyl reductase
ERK	extraceullular signal-regulated kinase
$FADH_2$	reduced form of flavin adenine dinucleotide
FAS	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
Her-2/neu (HER2)	human epidermal growth factor receptor 2
HGF	hereditary gingival fibromatosis
IAPs	inhibitors of apoptosis protein
IC_{50}	50% inhibitory concentration
JNK	c-Jun N-terminal kinase
KR	β-ketoacyl reductase
KS	β-ketoacyl synthase
LDH	lactate dehydrogenase
Malonyl-CoA	malonyl coenzyme A
MAPK	mitogen-activated protein kinase
MMPs	matrix metalloproteinases
MT	malonyl transferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PDV	Paget's disease of the vulva
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase

pRb	retinoblastoma protein
PTEN	phosophatase and tensin homolog deleted on chromosome ten
RNAi (siRNA)	small interfering RNA
ROS	reactive oxygen species
SCID	severe combined immunodeficiency
SD	standard deviation
SREBP	sterol regulatory element-binding protein
TE	thioesterase
TMRE	tetramethylrhodamine ethyl ester
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TOFA	5-(tetradecyloxy)-2-furoic acid
TBS	Tris-buffered saline
XIAP	X-linked inhibitor of apoptosis

CHAPTER 1 General Introduction

1.1 Fatty Acid Synthase (FAS) – 7-domain multifunctional enzyme

Fatty Acid Synthase (FAS) is an enzyme crucial for endogenous lipogenesis in mammals. There are two types of FAS. Type I refers to a large, multifunctional enzyme with seven catalytic domains responsible for *de novo* lipogenesis in eukaryotic animal cells. On the other hand, Type II is a monofunctional enzyme which exists only in plants and prokaryotes.

1.1.1 Functions

FAS functions to synthesize fatty acids for body uses by catalyzing the synthesis of 16-carbon palmitate from the condensation of malonyl-CoA and acetyl-CoA. FAS plays a crucial role in energy homeostasis by converting excess dietary carbon intake into fatty acids for storage. When there is high metabolic need, these fatty acids provide energy for biochemical reactions via β-oxidation catalyzed by carnitine O-palmitoyltransferase-1 (CPT-1).

Since daily diet provides most fatty acids we need, endogenous synthesis is minimal. Therefore, FAS is expressed at low to even undetectable levels in most normal human tissues. Nevertheless, FAS is over-expressed in a large number of different human

cancer histotypes to support their rapid growth and proliferation, despite high levels of endogenous ambient fatty acids. (Baron *et al.*, 2004)

1.1.2 Structure

Each mammalian FAS monomer has 7 catalytic domains linked as an integral unit with eight catalytic sites. They include β-ketoacyl synthase (KS), malonyl (MT) / acetyl transferase (AT), dehydratase (DE), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE). In order to be activated, FAS situates two monomers head-to-tail to form a homodimer with the molecular size of around 250 kDa. (Menendez *et al.*, 2004b) (Fig.1.1)

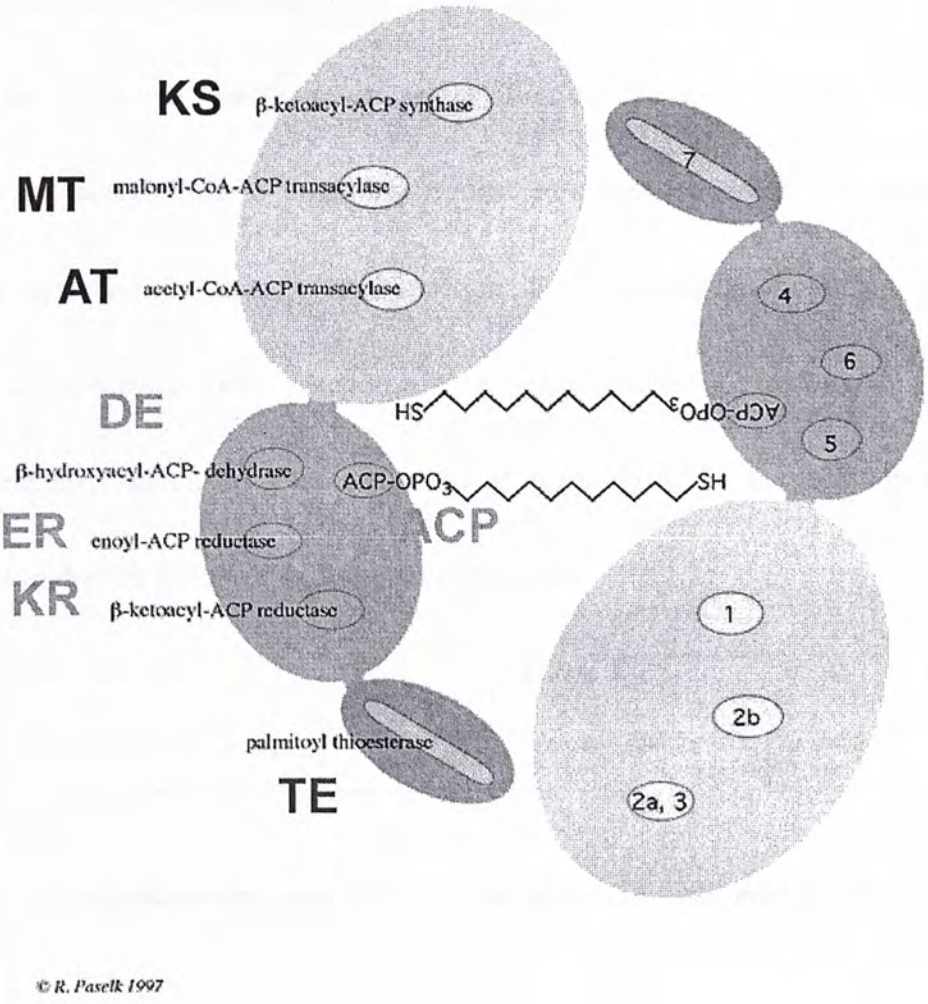


Fig. 1.1 Diagram showing a homodimer of animal FAS with seven catalytic domains

1.2 Fatty Acid biosynthesis reactions

De novo fatty acid biosynthesis involves a series of carboxylation, transacylation, elimination and reduction reactions, in which the condensation occurs between malonyl-CoA and acetyl-CoA to form end-product palmitate. In order to synthesize palmitate, the first and the most crucial reaction is to produce malonyl-CoA from endogenous acetyl-CoA. This reaction is a physiologically irreversible and rate-determining step which involves activation of acetyl-CoA, by the addition of a carbon dioxide under the action of Acetyl-CoA carboxylase (Fig 1.2)

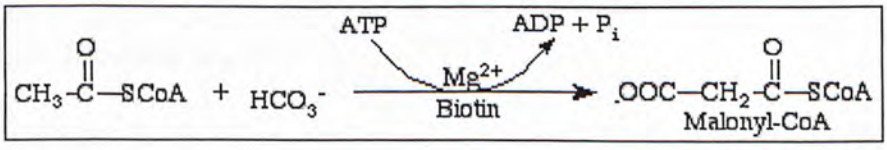


Fig. 1.2 Rate-determining step of *de novo* mammalian fatty acid synthesis

Then, Acetyl-CoA:ACP transacylase takes away an acetyl group from Acetyl-CoA in exchange for ACP to form Acetyl-ACP (STEP 1) (Fig. 1.3 and 1.4). Under action of β-ketoacyl-ACP synthase, Acetyl-ACP forms complex with enzyme to form acetyl-enzyme (STEP 2a). On the other hand, Malonyl-CoA:ACP transacylase enables the malonyl group to leave away from Malonyl-CoA and bond with SACP to form malonyl-SACP (STEP 2b) which incorporated into acetyl-enzyme to generate acetoacetyl-ACP (STEP 3). Now the carbon dioxide leaves the malonyl group, with the

electrons from its bond attacking the acyl group of Acetyl-ACP complex, generating a β-ketoacyl group ready to go through the reverse of the reactions of β-oxidation.

The keto-group of acetoacetyl-ACP is reduced to an alcohol using NADPH (b-ketoacyl-ACP reductase) (STEP 4), followed by the elimination of the alcohol (Enoyl-ACP hydrase) (STEP 5) to generate the *cis*-2,3-enoyl group. The enoyl is then reduced with NADPH substituting for $FADH_2$ (Enoyl-ACP reductase) to give the saturated 4-carbon butyryl-ACP (STEP 6). Then, the cycle repeats by shunting the reaction back to step 2a, although now the starting material has two additional carbon units than in the previous cycle. (Smith *et al.*, 2003)

After seven turns of the cycle, a 16-carbon palmitoyl-enzyme complex is formed, of which the ACP group is removed by palmityol thioesterase (STEP 7), forming palmitate as the predominant product of *de novo* FAS synthesis. Palmitate can then be utilized for lipogenesis in cells to make membrane-bounded structures, like plasma membrane, ER and Golgi apparatus, which has high turnover rate in lipogenic tissues or rapid-dividing tumor cells.

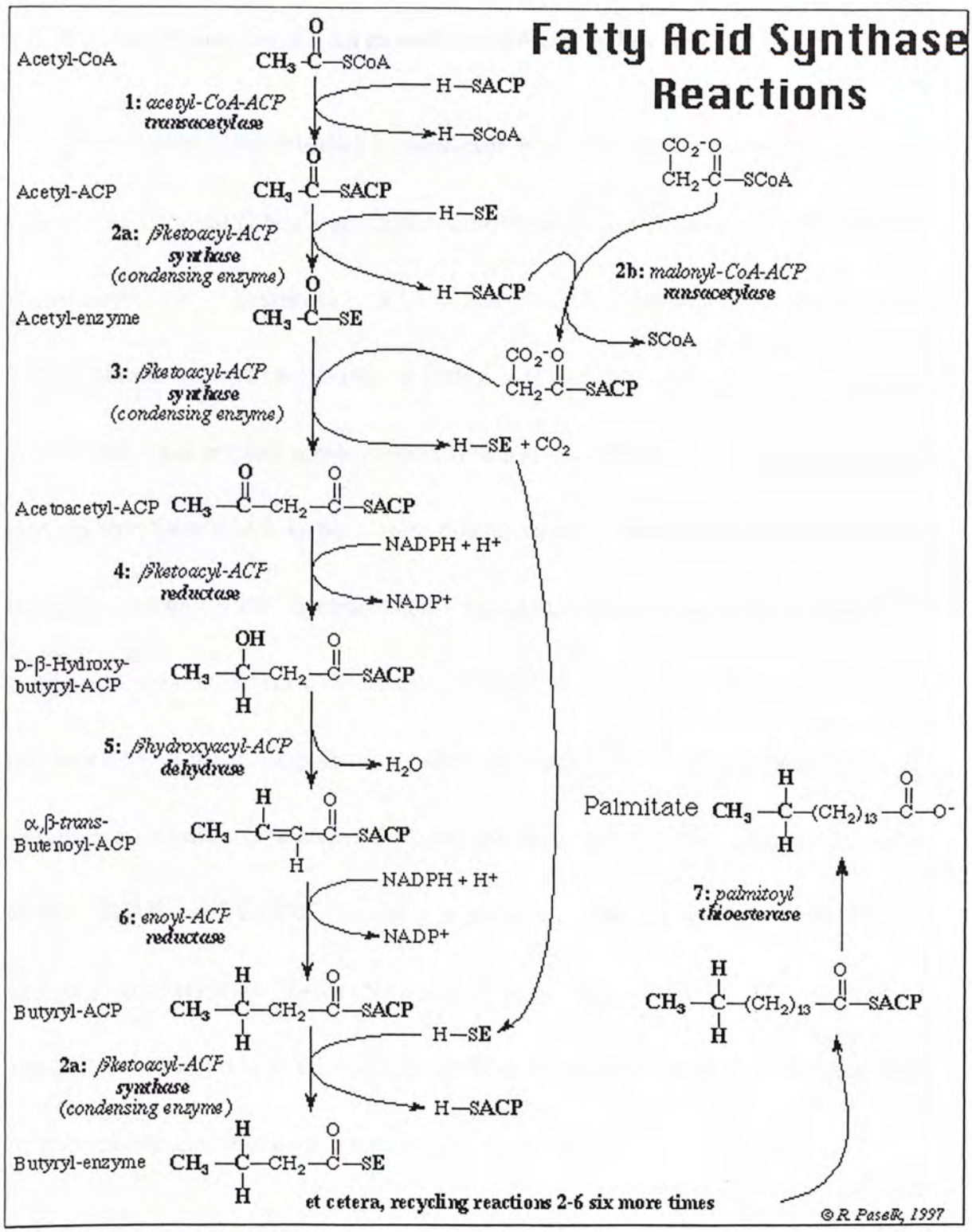


Fig. 1.3 Reaction sequence for biosynthesis of fatty acids *de novo* by the animal FAS

Acetyl-CoA + 7 malonyl-CoA +14 NADH
→ palmitate + 7 CO_2 + 14 NAD^+ + 8 CoA

Fig. 1.4 Overall equation for the *de novo* synthesis of palmitate from acetyl CoA

1.3 Malonyl Coenzyme A – An important mediator in lipogenesis

It was reported that substrate accumulation from FAS inhibition was responsible for cytotoxicity in breast (Zhou *et al.*, 2003) and ovarian cancer (Zhou *et al.*, 2007). Though supplement of exogenous FAS end-product palmitate could reverse FAS-inhibition-induced cytotoxicity in leukemia (Pizer *et al.*, 1996c), breast (Menendez *et al.*, 2004g) and prostate cancers (Brusselmans *et al.*, 2005a), more studies have shown that malonyl-CoA seems to be a more crucial target to mediate cytotoxicity by FAS inhibition in tumor cells. Pharmacological inhibition of FAS accumulated malonyl-CoA to trigger apoptosis in breast cancer cells (Thupari *et al.*, 2001 & 2002). In meanwhile, blockade of FAS mRNA expression by small interfering RNA in breast cancer cells also caused accumulation of malonyl-CoA and elevated levels of the proapoptotic genes BNIP3, TRAIL, and DAPK2, resulting in apoptosis (Bandyopadhyay *et al.*, 2006). In addition, administering 5-(tetradecyloxy)-2-furoic acid (TOFA), an inhibitor of acetyl-CoA carboxylase to relieve malonyl-CoA accumulation reduced the cytotoxicity by FAS inhibition in breast cancer cells (Pizer *et al.*, 2000).

1.4 FAS expression in different histotypes

1.4.1 FAS in normal cells

FAS is expressed at low to even undetectable levels in most normal human tissues, yet FAS-dependent fatty acid synthase still functions crucially in highly lipopgenic tissues, for instance: liver, lactating breast and adipose tissues. FAS is responsible for (1) storage of excess energy intake, e.g. fat, (2) synthesis of fat from other carbohydrate or protein sources once the diet is low in fat, (3) synthesis of fat for lactation of the pregnant. In well-nourish and healthy adults, FAS works primarily for energy storage by converting excess carbohydrate to fatty acids which are then stored as triacylglycerols. Specialized physiological functions include milk lipid production in lactating breast tissues as well as surfactant in infant lungs (Menendez *et al.*, 2004b).

1.4.2 FAS in pathological cells

Hereditary gingival fibromatosis (HGF) is a genetic disease of a progressive enlargement of the gingival. Recent researches showed that FAS was expressed by gingival fibroblasts and highly expressed in proliferative HGF cells. Moreover, FAS inhibition significantly reduced both normal gingiva and HGF fibroblast growth, indicating that androgen-driven fatty acid biosynthesis takes part in their proliferation. (Almeida *et al.*, 2005)

Paget's disease is a malfunction of bone remodeling, in which bone breaks down more quickly, and when it grows again, softer than normal bone. Paget's disease diversely affects many bone structures, like the skull, the hip and pelvis and bones in the legs and back. Clinical studies showed that increased FAS expression was observed in 87% of patients with invasive Paget's disease of the vulva (PDV) and 75% with microinvasive PDV. Statistical analysis revealed that FAS over-expression was highly associated with invasive PDV (Alo *et al.*, 2005).

1.4.3 Tumor-associated FAS (Oncogenic antigen-519) in cancer cells

Over-expression and hyperactivity of FAS have been characterized in various human malignancies, including carcinoma of breast (Alo *et al.*, 1996), ovary (Gansler *et al.*, 1997), oral tongue (Krontiras *et al.*, 1999), colorectum (Visca *et al.*, 1999), lung (Piyathilake *et al.*, 2000), prostate (Bull *et al.*, 2001), oesophagus (nemoto *et al.*, 2001), stomach (Kusakabe *et al.*, 2002), skin (Innocenzi *et al.*, 2003) and bladder (Visca *et al.*, 2003), with low expression levels in normal tissues.

Increased FAS expression closely correlates malignancy of cancer. In the animal model, high FAS expression and activity in transgenic adenocarcinoma of mouse prostate (TRAMP) increased with age, tumor progression, and in metastatic lesions. (Pflug *et al.*, 2003). Immunohistochemical studies illustrated that expression of fatty acid synthase was

stronger in malignant melanomas in comparison to conventional nevi and Spitz nevi, and was the highest for metastatic melanoma (Kapur *et al.*, 2005). Yang *et al.* (2002) also claimed of the need of FAS activation during neoplasmic lipogenesis and transformation from epithelial cells into metastatic cancer cells. Epstein-Barr virus (EBV) is a human gamma-herpes virus found associated with a variety of cancers, including Burkitt's lymphoma, nasopharyngeal carcinoma, gastric carcinoma. It was also found that FAS expression was significantly induced by the Epstein-Barr virus immediate-early protein BRLF1 and was required for lytic viral gene expression (Li *et al.*, 2004). FAS works closely with EBV to trigger malignancy of several cancer types.

Increased FAS expression also correlates with poor prognosis and survival. Statistical analysis revealed that FAS-positive lung cancer patients had an overall lower prognostic value while FAS-negative expression in stage I patients had better survival. FAS seemed to be a marker of increased risk of recurrence in lung carcinoma (Visca *et al.*, 2004).

Availability of fatty acids may modulate proliferation of FAS-overexpressing cancer cells either by (1) supplying a larger pool of available substrates for membrane synthesis in rapid cell division or (2) by regulating membranous second messengers required for cell replication. (Baron *et al.*, 2004) Essential fatty acids are not only energy-rich molecules, but also an important component of the membrane bilayer. A

report showed that arachidonic acid (an omega-6 fatty acid) played an important role in stimulation of growth-related genes and proliferation, via PI3K signaling and NF-kappaB translocation to the nucleus in PC-3 human prostate cancer cells. (Hughes-Fulford *et al.*, 2006). This may indicate the importance of fatty acids in cancer development.

1.5 FAS signaling models in breast and prostate cancers

1.5.1 Association between FAS and PI3K/Akt pathway

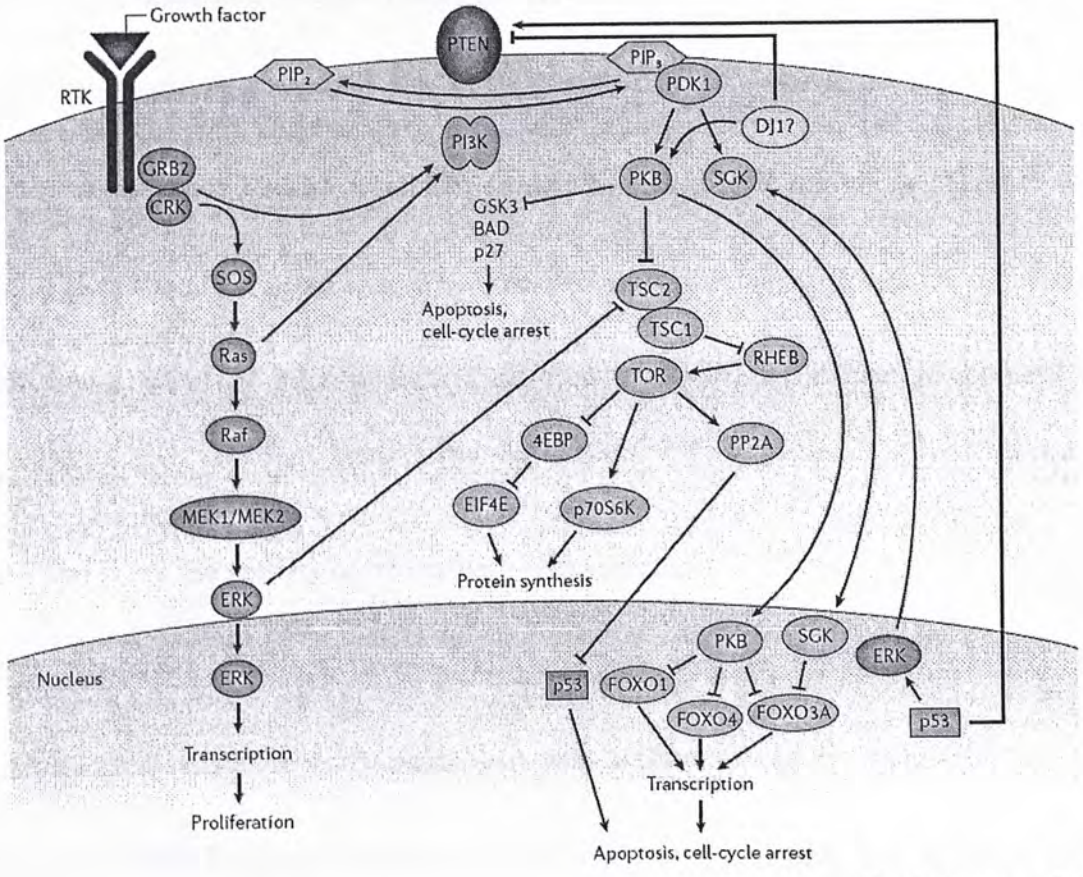
FAS over-expression and hyperactivity were highly associated with PI3K/Akt pathway (Fig 1.5). In LNCaP prostate carcinoma, administering LY294002, an inhibitor of the PI3K pathway, caused a dramatic decrease in FAS protein expression. Reintroduction of PTEN, a negative PI3K regulator, resulted in decreased levels of FAS expression which was dependent on its lipid phosphatase activity (Van de Sande *et al.*, 2002). Likewise, inhibition of PI3K pathway in prostate cancer worked synergistically with FAS siRNA to enhance cell death (Bandyopadhyay *et al.*, 2005). Recent studies revealed that high-level expression of fatty acid synthase was linked to activation and nuclear localization of Akt/PKB by envision detection technique on well-preserved frozen prostate needle biopsies (Van de Sande *et al.*, 2005). In addition, positive feedback regulation between AKT activation and fatty acid synthase expression was found in ovarian carcinoma cells (Wang *et al.*, 2005).

Although novel studies have indicated the possible role of MAPK/Erk pathway in fatty acid biosynthesis, the conclusion is inconsistent (Yang *et al.*, 2002) (Menendez *et al.*, 2004d) and it probably needs further elucidation of the mechanism behind.

1.5.2 Hypothetical model of FAS hyperactivity in breast and prostate cancer cells

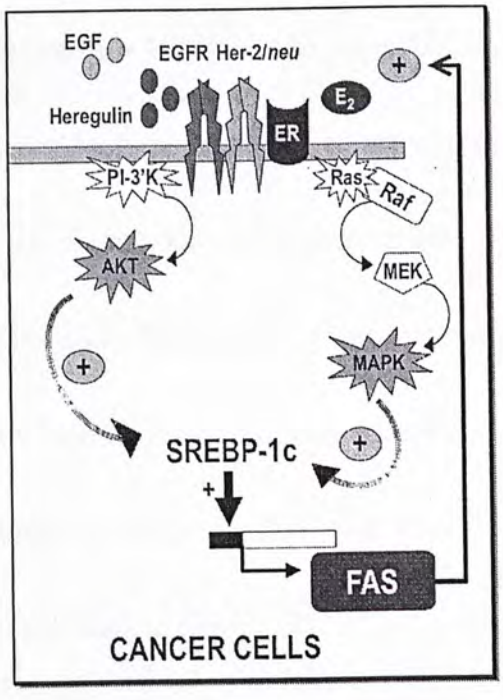
Different hypothetical models of FAS regulation has been proposed for normal and cancer cells. In normal lipogenic cells like hepatocytes and adipocytes, FAS-catalyzed *de novo* fatty acid biosynthesis is nutrient-dependent. FAS activity can be stimulated by a high carbohydrate diet, and inhibited by dietary fatty acid or during fasting. Hormones, like insulin, leptin, also take part in regulation of FAS expression via modulation of the transcriptional factor sterol regulatory element-binding protein (SREBP)-1c and MAPK ERK1/2 and PI-3'K/AKT signaling cascades. (Menendez *et al.*, 2004d)

Nevertheless, in breast and prostate cancer cells, SREBP-1c expression is driven by pathological conditions, like overexpression of growth factors (e.g. EGF, heregulin), and/or overexpression of growth factor receptors (e.g. Her-2/neu, EGFR), leading to hyperactivation of PI3k/Akt pathway, rather than responding normally to physiological regulators (e.g. insulin, leptin) as in normal lipogenic cells. The constitutive activation of oncogenic cascades would then hinder FAS gene to respond to a normal fatty acid's down-regulatory actions, and with a positive feedback loop, thus resulting in the observed gigantic levels of FAS in cancer cells (Menendez *et al.*, 2004b) (Fig. 1.6).



(Van de Sande *et al.*, 2002)

Fig. 1.5 Phosphatidylinositol 3-kinase (PI3K) signaling pathway



(Menendez *et al.*, 2004b)

Fig. 1.6 Proposed FAS regulatory positive-feedback loop in breast and prostate cancer cells

1.6 FAS inhibition to tackle cancer cell growth

1.6.1 FAS inhibitors

Apart from obesity treatment studies by using FAS inhibitors (Thupari *et al.*, 2002) (Leonhardt & Langhans, 2004), researches in the recent few years focus on the anti-cancer effect of FAS inhibition on growth of different cancer histotypes. With head-to-tail dimer conformation, targeted inhibition of one of the enzymatic domains of FAS can block the activity of one or both FAS subunits.

Specific inhibitors investigated nowadays include cerulenin and C75, with triclosan and orlistat recently discovered with anti-tumor activity (Kridel *et al.*, 2004) (Russell, 2004). Triclosan inhibits enoyl-reductase of type I fatty acid synthase *in vitro* and exert cytotoxic effect to MCF-7 and SKBr-3 breast cancer cells. (Liu *et al.*, 2002) Orlistat (Xenicaltrade mark), a US Food and Drug Administration (FDA)-approved drug for bodyweight loss, has been demonstrated to act against breast cancer by blockade of cell cycle progression, promotion of apoptosis and PEA3-mediated transcriptional repression of Her2/*neu* (erbB-2) oncogene. (Menendez *et al.*, 2005 e & f) Nevertheless, researches on these two inhibitors are just preliminary and the underlying mechanisms of action have been merely on start. On the other hand, cerulenin and C75 are the two inhibitors recognized and studied for decade.

1.6.1.1 Cerulenin

Cerulenin [(2R,3S), 2–3-epoxy-4-oxo-7,10-trans,transdodecadienamide] is an mycotoxin discovered by Hata *et al.* in 1960 from fungus *Cephalosporium caerulens* (Omura, 1976). Cerulenin is a natural specific noncompetitive inhibitor of the β-ketoacyl synthase activity of FAS, functions by covalently modifying the active site cysteine, resulting in dead-end FAS inhibition (Menendez *et al.*, 2004b) (Fig. 1.5).

It was found that cerulenin induced apoptosis in different wild-type p53 and mutant p53 tumor cell lines *in vitro*, through accumulation of tumor suppressor p53, up-regulation of apoptotic Bax protein, activation of caspases 3 and 9, as well as accompanied by release of cytochrome *c* from mitochondria. In contrast, normal human keratinocytes and fibroblasts were resistant to the apoptotic effect by cerulenin (Heiligtag *et al.*, 2002). In addition, *in vivo* studies showed that fatty acid synthesis inhibition delayed tumor progression in a xenograft model of ovarian cancer. Cerulenin significantly hindered tumor cell fatty acid biosynthesis *in vivo*, ablated established ascites tumor, attenuated ascites incidence, delayed onset of ascites and markedly increased survival (Pizer *et al.*, 1996b).

1.6.1.2 C75

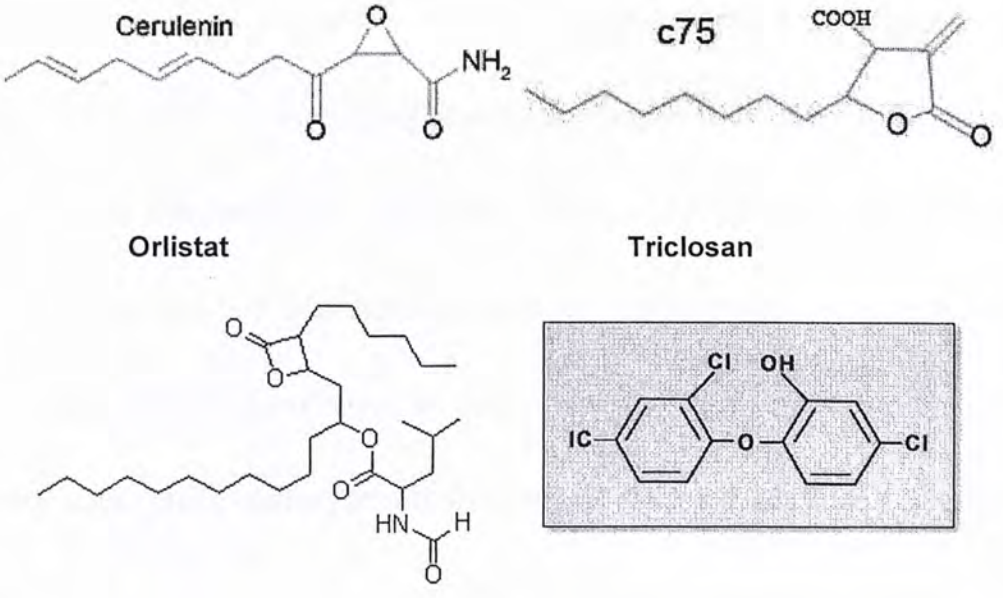
C75 [4-methylene-2-octyl-5-oxo-tetrahydro-furan-3-carboxylic acid], a synthetic analog of cerulenin, is a α-methylene-butyrolactone inhibitor of FAS (Fig. 1.7).

Treated with C75, subcutaneous xenografts of MCF-7 breast carcinoma in nude mice showed significant fatty acid synthesis inhibition, apoptosis, and tumor growth inhibition to less than 1/8 of control volumes, without comparable toxicity in normal tissues (Pizer *et al.*, 2000). It was also found that C75 caused marked delay in tumor progression in *neu*-N transgenic mice of mammary cancer (Alli *et al.*, 2005).

1.6.2 Small interfering RNA

Apart from post-translational modification of FAS, FAS hyperactivity and over-expression can also be down-regulated by small interference RNA technique in which translation of cytosolic FAS mRNA is blocked by enzymatic degradation. It was reported that RNA interference-mediated silencing of the FAS gene reduced growth and induced morphological changes and apoptosis of LNCaP prostate cancer cells (De Schrijver *et al.*, 2003). On the other hand, silencing of the ACC-alpha gene, the enzyme which provides the malonyl-CoA substrate, also resulted in cell growth inhibition and induction of caspase-mediated apoptosis in highly lipogenic LNCaP prostate cancer cells as similarly as when treated with FAS RNAi (Brusselmans *et al.*, 2005a).

(a)



(b)

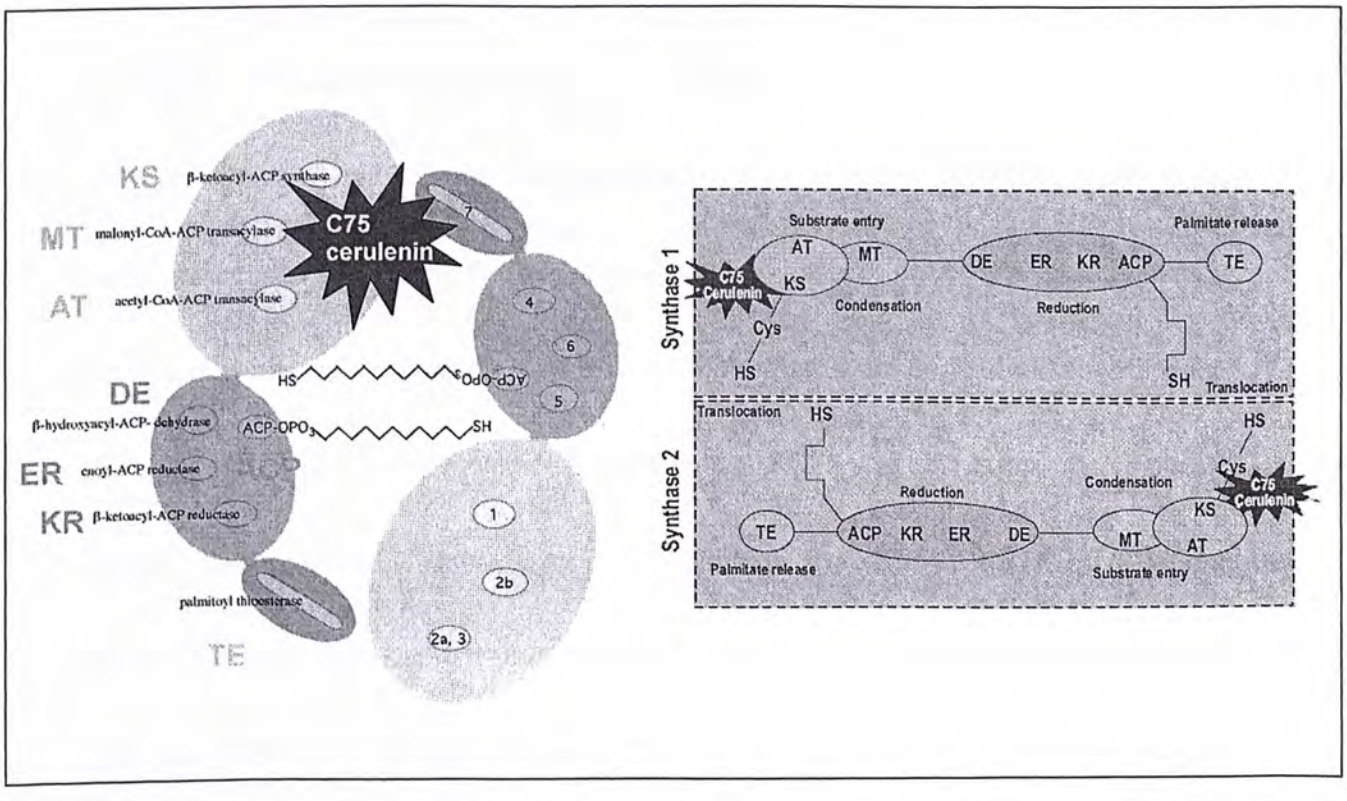


Fig. 1.7 Pharmacological FAS inhibitors used in biological research nowadays.

(a) Molecular structures of cerulenin, C75, triclosan and orlistat, (b) FAS inhibitors cerulenin and C75 act on different catalytic domains of FAS homodimer

1.7 FAS inhibition to enhance chemoresistant cancer cells sensitivity to drugs

Chemotherapeutic anti-cancer drugs always poses harmful side effects on normal cells. Toxicity of taxol always bring bone marrow suppression (principally neutropenia), alopecia, and hypersensitivity reactions to cancer patients (Markman, 2002), while vinorelbine triggers febrile neutropenia including, neurotoxicity and thrombocytopenia (Crown *et al.*, 2002). Monotherapy of recently-developed drug trastuzumab on Her2/neu overexpressing breast cancer patients also induced cardiac dysfunction (Campone *et al.*, 2004).

Apart from killing cancer cells directly, blockage of FAS expression potentiates sensitivity of chemoresistant cancer cells towards these anti-cancer therapeutic drugs. Pharmacological and small interference RNA-mediated inhibition of breast cancer-associated fatty acid synthase synergistically enhances Taxol (Paclitaxel)-induced cytotoxicity, alleviating possible side effects when drugs were applied alone (Menendez *et al.*, 2005d). FAS activity blockade by cerulenin and C75 was also found synergistically enhance apoptosis-inducing activity of vinorelbine in MCF-7, MDA-MB-231 and SK-BR-3 breast cancer cells (Menendez *et al.*, 2004a). Likewise, the dual targeting of FAS and HER2 by specific chemical FAS inhibitors and the humanized antibody trastuzumab (Herceptin) which directed against HER2, were synergistically cytotoxic towards HER2-overexpressing breast carcinoma (Menendez *et al.*, 2004h).

1.8 Hypothesis

FAS has been the molecular target for anticancer therapy, but still, most of the studies of FAS inhibition mainly focused on FAS-overexpressing breast and prostate cancer. Though various cancer histotypes highly expressed FAS, and they were reported to induce programmed cell death by pharmacological FAS inhibitors or small interference RNA, the detailed molecular pathway of such suicidal cell death was not fully elucidated. We aim to screen through a panel of different cancer cell lines to choose the most sensitive one as the cell line model, then investigating exactly how FAS inhibition modulates signaling cascades and finally causing programmed cell death in cancer cells. Identification of such signaling cascades provides more information on targeting FAS, and probably develops synergy with other chemotherapeutic agents, to cure cancer patients in the near future.

CHAPTER 2 Materials and Methods

2.1 Chemicals and antibodies

Cerulenin, C75 and all other chemicals used in this study were obtained from Sigma Chemcial (St. Louis, MO), unless otherwise stated. Mouse anti-human β-actin and DR4, DR5, TRAIL, Bax, fatty acid synthase antibodies were obtained from BD PharMingen. Other antibodies were obtained from Cell Signaling Technology (Danvers, MA).

2.2 Cell cultures

Hepatocellular carcinoma HepG2, melanoma A-375, lung carcinoma A-549, colorectal adenocarcinoma CaCo-2, Colo201, SW480 and SW620, cervix adenocarcinoma HeLa, prostate carcinoma LNCaP and PC-3, breast adenocarcinoma MCF-7 and SK-BR-3, and normal fibroblast HS68 were all provided by American Type Culture Collection (Rockville, MD). MCF-7, HepG2 and HS68 were cultured in RPMI 1640 medium, PC-3 and A-549 in F12K medium, A-375 and CaCo-2 in DMEM medium, SW480 and SW620 in L15 medium, HeLa in DMEM, SK-BR-3 in McCoy's 5a medium, LNCaP in the RPMI medium with 4g/L glucose. All the cultures were supplemented with 0.25% sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) and 2% penicillin-streptomycin (Gibco, Rockville, MD); and were incubated at 95% relative humidity, 5% CO_2 and 37°C. The cultures were passaged at least three times a week.

2.3 MTT assay

Cancer cells were seeded in 96-well flat-bottom plates at 2.5×10^3 per well for 24 h. The cells were then incubated with cerulenin or C75 at 20, 40 and 80 μM for 24 and 48 h. After incubation, cell proliferation and viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based assay (Roche, Mannheim, Germany) (Loo and Rillema, 1998). Twenty microliters of MTT solution (5 mg/ml in PBS) was added to the cells for 4 h at 37°C in CO_2 incubator. The formazan salt formed was then dissolved by DMSO. Color intensity of the formazan solution, which reflects the cell growth condition, was determined by reading optical density at 570 nm by a microplate reader. Reductions of cell growth were plotted against cerulenin or C75 concentrations, and the value of IC_{50}, i.e. the concentration at which the cell growth was inhibited by 50% of the control level, was estimated from the plot.

2.4 5-Bromo-2'-deoxyuridine (BrdU)-labeling cell proliferation assay

Cancer cells were seeded in 96-well flat-bottom plates at 2.5×10^3 per well for 24 h. The cells were then incubated with cerulenin or C75 at 20, 40 and 80 μM for 24 and 48 h. All the procedures, including BrdU incorporation, fixation, washing and measurement were followed exactly as those described in the user's guide, distributed with the cell proliferation ELISA, BrdU (Chemiluminescence) kit from Roche, Mannheim, Germany.

2.5 Cytotoxicity detection assay of LDH release

Human melanoma A-375 cells, in parallel with human normal fibroblast HS68 cells were seeded in the 96-well flat-bottom plates at 2.5×10^3 and 5×10^3 respectively per well overnight. The cells were then incubated with cerulenin or C75 at 20, 40 and 80 μM for 24 h. After incubation, cell cytotoxicity was measured by the LDH detection assay (Roche, Mannheim, Germany). 2% Trizol Reagent (Invitrogen) was used to permeabilize all the cell membrane and acted as the high control. Reagent mixtures (according to user's guide) were added to the cells for 1 h at 37°C in CO_2 incubator. Active LDH released from dead and plasma membrane-damaged cells, which was indicated by conversion of a yellow tetrazolium salt into a red, formazan-class dye, was then measured at 490 nm by a microplate reader. LDH release was expressed as fold increase with the control.

2.6 DNA flow cytometry

Human melanoma A-375 cells, at number 5×10^5, were seeded in T25 culture flask for 24 h. The cells were then treated with 20, 40 and 80 μM of cerulenin or C75 for 24 h. After incubation, the cells were trypsinized, washed with PBS and fixed overnight in ice-cold 70% ethanol. After fixation, the cells were washed twice with 1% BSA in PBS, resuspended in 1 ml DNA-binding propidium iodide (PI) solution (10 mg/ml in PBS, containing 0.05 mg/ml RNase A) and analyzed with EPICS XL flow cytometer (Beckman

Coulter, Miami, FL). Cell cycle was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). $SubG_1$ or apoptotic cells and cells with different phases in cell cycle were measured using the control software of flow cytometer.

2.7 Confocal micocropy

Human melanoma A-375 cells, at number 1×10^4 per 200 μl of medium, were seeded in each chamber of the 8-chamber polystyrene vessel tissue culture treated glass slide (Becton Dickinson, NJ, USA) and incubated for 24 h at 37°C in CO_2 incubator. The cells were then incubated with cerulenin or C75 at 20, 40 and 80 μM for 24 h. The vessel and the silicon borders were then removed, and the slide was rinsed with PBS and stained with 100 μl of Annexin-V-FLUOS Labeling Solution (Roche, Mannheim, Germany) for 20 minutes at 37°C in CO_2 incubator. The slide was then analyzed by the Bio-Rad Radiance 2100 MP Scanning System equipped with 488nm argon laser (Hertfordshire, England). Green fluorescence from fluorescein (FITC) on apoptotic cells and red fluorescence from propidium iodide (PI) in necrotic cells were measured at 518 nm and 617 nm, respectively.

2.8 Immunoblot analysis

2.8.1 Preparation of protein lysates

For preparation of total protein lysates, cells with different treatment, were harvested

and suspended for 2 h on ice in an extraction buffer (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.2% BSA, 1% Triton X-100) supplemented with protease inhibitors (BD Biosciences, San Jose, CA). Lysates were obtained by centrifugation at 4 °C for 10 minutes at 13 000 *g*. The resulting supernatants were stored at -20 °C or used immediately. Aliquots of the supernatants were used for protein concentration determination by the standard bicinchoninic acid assay kit (Pierce, Rockford, IL).

To obtain mitochondrial and cytosolic fractions, homogenates from melanoma cells were fractionated by the Cytosol-Mitochondria Fractionation kit (Calbiochem). Briefly, 1 × 10^7 cells were harvested and resuspended in cytosol extraction buffer supplemented with protease inhibitors for 15 minutes on ice, and disrupted using a Dounce homogenizer. Homogenates were then centrifuged at 700*g* at 4 °C for 10 minutes. The supernatants were further centrifuged at 10 000*g* for 30 minutes at 4 °C. The resulting supernatants (cytosolic fraction) and pellets (mitochondrial fraction) were stored at -20 °C or used immediately.

2.8.2 Immunoblotting

50 μg protein content of the cell lysate from different treatment was resolved by 10% SDS-polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). The membrane was firstly blocked with 5% non-fat milk in TBS and 0.1% Tween 20 (Bio-Rad, Hercules, CA), and

then incubated with antibodies with agitation at 4°C overnight. β-Actin was also measured as loading control. After incubation, the membrane was washed by TBS and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG polyclonal antibodies (Cell Signaling Technology) for 1 h with agitation at room temperature. Finally, the membrane was washed by TBS, incubated with 10 ml LumiGLO Substrate (Cell Signaling Technology), and then exposed to X-ray film. Antibodies were stripped from the membrane by 5 minute-wash of distilled water, followed by 5-minute-wash of 0.2 M sodium hydroxide solution and 10 minutes of distilled water wash, before blocked with 5% non-fat milk in TBS and 0.1% Tween 20 for re-probing of another antibody.

2.9 Caspase inhibitor studies

The melanoma cells were incubated with or without 100 μM of pan-caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) (BD PharMingen, Franklin Lakes, NJ) for 2 h. The cells were then treated with or without 40 μM cerulenin or C75 for 24 h. After treatment, protein lysates were obtained and subjected to the PARP immunoblot analysis as described above.

2.10 Analysis of mitochondrial membrane potential

Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was detected by using the

potential-sensitive fluorescent probe, tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR). Treated or untreated cells were washed with PBS, then incubated with 25 nM TMRE for 15 minutes at 37 °C prior to analysis on a flow cytometer. As a positive control, cells were incubated with 100 μM potential-disrupting agent, carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma) for 24 hours. Data are displayed as the percentage of cells with reduction in mitochondrial membrane potential.

2.11 Determination of caspase activities

Caspase-3 activities were determined by using the fluorogenic peptide substrates, DEVD-AMC (50 μM) (Calbiochem). Cell lysates from 1×10^6 cells and substrates were mixed in a standard reaction buffer (20 mM HEPES, 10% glycerol, 2 mM dithiothreitol (DTT), pH 7.5), and incubated for 1 hour at 37 °C. The amount of enzyme-catalyzed AMC release was measured by a fluorescence plate reader (Tecan, Grödig, Austria) with an excitation wavelength of 380 nm and an emission wavelength of 440 nm, and expressed as the fold increase to the control.

2.12 siRNA transfection

Individual siRNAs against the *FAS* gene were designed (sense sequences: CCCUGAGAUCCCAGCGCUGdTdT, antisense sequence: CAGCGCUCCCAUCUCAGGGdTdT) and custom synthesized by Dharmacon, Inc. (Lafayette, CO). SMARTpool siRNAs

synthesized by Dharmacon, Inc. were used for negative control. The siRNA was transfected into the melanoma cells using DharmaFECT 1 Transfection Reagent (Dharmacon, Lafayette, CO) according to the manufacturer's protocol. Protein lysates were obtained and subjected to immunoblot analysis as described above.

2.13 Statistical analysis

Results are expressed as mean ± S.D. Means were compared by Student's *t*-test. *n* is the number of replicates for the experiment. *p* is the probability of random error of the treatment group. * is denoted for $p<0.05$, meaning that the difference of the treatment group with the control is 95% due to the effect of the treatment while 5% due to random error, and is the confidence level generally accepted to claim as significantly different. ** is denoted for $p<0.005$ for very significantly different treatment group.

Chapter 3 Results

3.1 Cytostatic & cytotoxic studies of FAS inhibitors on human cancer cells

3.1.1 Cerulenin and C75 suppress cell growth of different cancer histotypes

Over-expression and hyperactivity of FAS have been characterized in various human malignancies. In order to test for the inhibitory effect of FAS inhibitors on growth of different cancer histotypes, a panel of cancer cell lines, including hepatocellular carcinoma HepG2, melanoma A-375, lung carcinoma A-549, colorectal adenocarcinoma CaCo-2, Colo201, SW480 and SW620, cervix adenocarcinoma HeLa, prostate carcinoma LNCaP and PC-3, and breast adenocarcinoma MCF-7 and SK-BR-3 were subjected to 10 to 160 μM cerulenin and C75 treatment for 48 h. The cell proliferation and viability was then measured by MTT assay.

3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT)-based assay is a colorimetric assay measuring the reduction of a tetrazolium component MTT into a dark blue formazan by mitochondrial succinate dehydrogenase of viable cells. The number of viable cells is correlated to the amount of formazan formed. The assay revealed that both cerulenin and C75 inhibited proliferation and viability of all the tested cancer cell lines, with IC_{50} values ranging from 21.86 to 240.66 μM at 48 h of treatment (Table 3.1). Apart from colorectal Colo201, cerulenin was more potent than C75 in

suppressing growth of cancer cells. Amongst the 13 tested cell lines, lung carcinoma A-549 cells were the least susceptible to the growth inhibition by the two FAS inhibitors (cerulenin IC_{50} = 143.26 μM; C75 IC_{50} = 240.66 μM). On the other hand, melanoma A-375 was found to be the most sensitive cancer cell line to the FAS inhibitors (cerulenin IC_{50} = 21.86 μM; C75 IC_{50} = 32.43 μM). Further studies on the mechanisms of how the FAS inhibitors retarded cell growth were performed on A-375 cells.

Cancer cell lines	Histotypes	Values of IC_{50} (μM) cerulenin	C75
HepG2	Hepatocellular carcinoma	75.24	97.55
A-375	Malignant melanoma	21.86	32.43
A-549	Lung carcinoma	143.26	240.66
CaCo-2	Colorectal adenocarcinoma	59.77	186.93
Colo201	Colorectal adenocarcinoma*	52.57	38.33
SW480	Colorectal adenocarcinoma	37.33	52.14
SW620	Colorectal adenocarcinoma	27.75	45.91
HeLa	Cervix adenocarcinoma	85.74	79.11
LNCaP	Prostate carcinoma*	33.36	73.41
PC-3	Prostate carcinoma*	30.18	67.86
MCF-7	Breast adenocarcinoma*	47.61	84.05
SK-BR-3	Breast adenocarcinoma*	42.43	78.78

** Metatstatic histotypes*

Table 3.1 **IC_{50} values of cerulenin and C75 on growth of cancer cell lines from different tissues at 48 h of treatment measured by the MTT-based assay.**

The cells were incubated with 10 - 160 μM cerulenin or C75 for 48 h. Malignant melanoma A-375 had the lowest IC_{50} values among the cancer cell lines tested in this study.

3.1.2 Cerulenin and C75 suppress cell growth of A-375 dose- and time-dependently

Human melanoma cell line A-375 was chosen for further mechanistic studies. To study the time effect of FAS inhibitors on cell growth and viability, A-375 was incubated with 20, 40 and 80 μM cerulenin or C75 for 24 and 48 h, by MTT-assay and BrdU-labeling assay.

MTT-assay revealed that cerulenin have already retarded the cell proliferation and viability dose-dependently at 24 h (Fig. 3.1). The proliferation and viability was reduced by 20.78% to 61.71% of the control level by 20 to 80 μM cerulenin treatment. At 48 h, the growth inhibition was even more prominent so that the cell proliferation and viability was reduced by 54.09% to 83.43%. Similar time effect was also observed on C75 treatment, although 20 μM of the inhibitor did not reduce the cell growth significantly. The cell proliferation and viability was reduced by 16.15% and 46.70% by 40 to 80 μM C75 respectively at 24 h. At 48 h, the growth was reduced by 44.28% and 75.58%. Overall speaking, C75 was less effective than cerulenin in suppressing the cell growth at both 24 and 48 h of treatment.

To further evidence the inhibitory effect of the FAS inhibitors on melanoma A-375, 5-Bromo-2'-deoxyuridine(BrdU)-labeling cell proliferation assay was employed to study specifically the effect of the FAS inhibitors on cell proliferation. BrdU-labeling assay measures the uptake of synthetic nucleoside thymidine analogue BrdU during DNA

synthesis, so that the more BrdU is found in the more actively-proliferating cells. The results showed that both cerulenin and C75 inhibited A-375 cell proliferation dose- and time-dependently (Fig. 3.2). At 24 h, the proliferation was reduced by 29.48% to 86.71% of the control level by cerulenin and by 19.62% to 47.03% by C75. At 48 h, the proliferation was more prominently reduced by 62.54% to 90.30% by cerulenin and by 30.20% to 90.30% by C75.

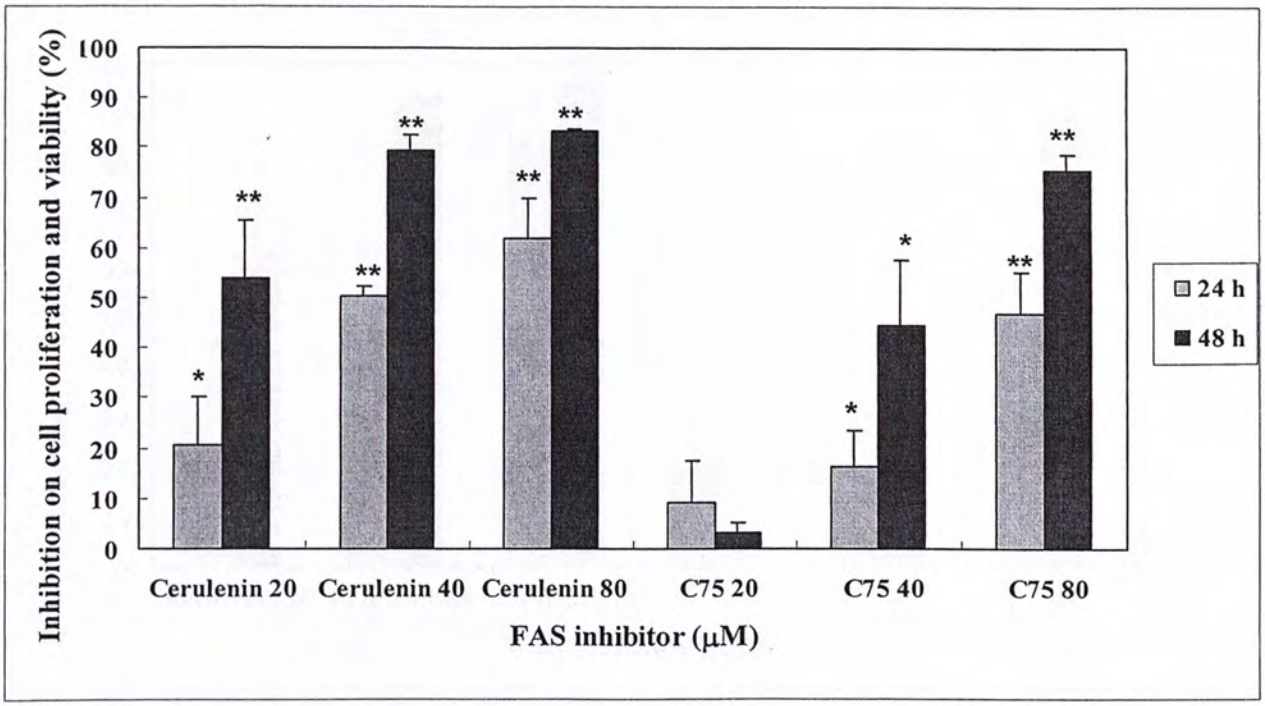


Fig. 3.1 Effect of FAS inhibitors on proliferation and viability of melanoma A-375 cells measured by the MTT-based assay.

The cells were incubated with 20 - 80 μM cerulenin or C75 for 24 h and 48 h. Both cerulenin and C75 reduced cell proliferation and viability, with the more prominent effect at 48 h of treatment. Results are expressed as mean ± SD (n=4). *p<0.05; **p<0.005, Student's t-test.

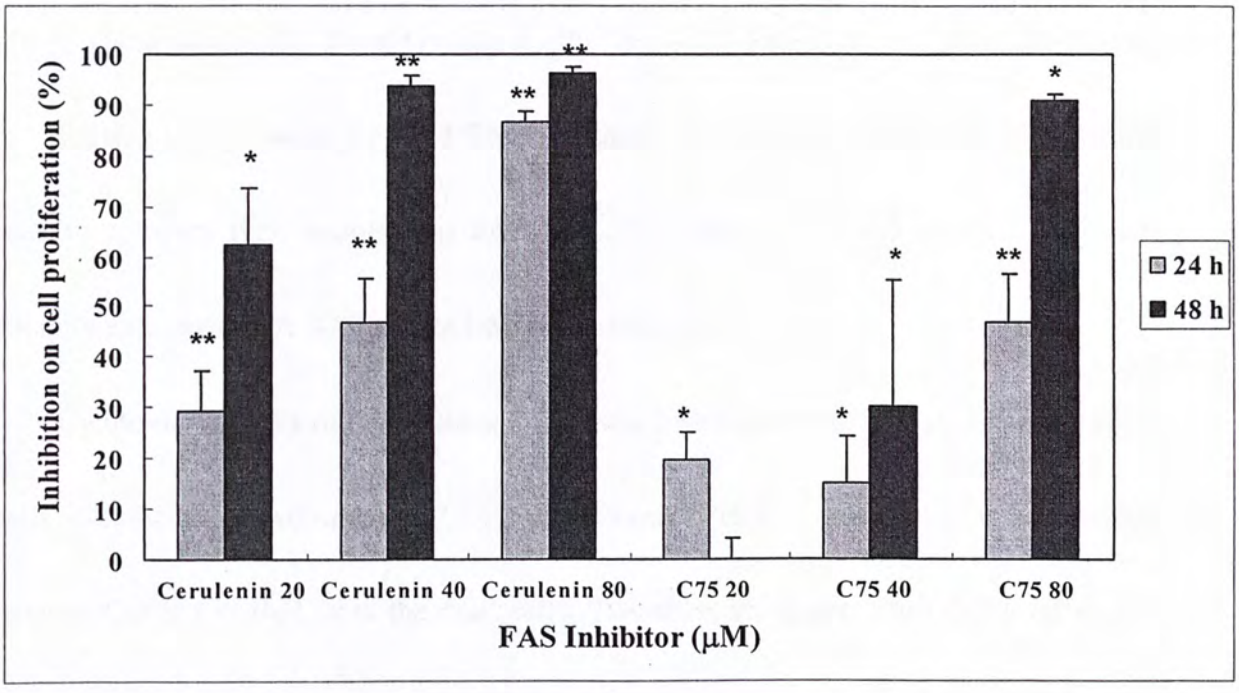


Fig. 3.2 Effect of FAS inhibitors on proliferation of melanoma A-375 cells measured by the BrdU-labeling assay.

The cells were incubated with 20 - 80 μM cerulenin and C75 for 24 h and 48 h. Both cerulenin and C75 hindered proliferation of A-375 cells dose- and time-dependently. Results are expressed as mean ± SD (n=4). $*p<0.05$; $**p<0.005$, Student's *t*-test.

3.1.3 Cerulenin and C75 exert cytotoxic effect on A-375 but not normal skin HS68 cells

Protein lysates were prepared from melanoma A-375 and normal fibroblast HS68 cells to compare their endogenous FAS level. The immunoblot showed that FAS was strongly expressed in A-375 but not HS68 cells (Fig. 3.3).

Cytotoxicity effect of cerulenin and C75 was investigated on both A-375 and HS68 cells with lactate dehydrogenase (LDH) cytotoxicity detection assay. LDH is a cytosolic enzyme that is released from the dead cells. Therefore, the higher the LDH level in the culture medium, the lower the cell viability and the higher the cytotoxicity of the tested sample. When the melanoma cells were treated with 20, 40 and 80 μM cerulenin or C75 for 24 h, a dose-dependent increase of LDH release from the dead cells was found (Fig. 3.4). On the contrary, the FAS inhibitors, especially C75, imposed much lesser toxicity on the normal fibroblast cells. It was noteworthy that although cerulenin was more effective than C75 in retarding growth of the melanoma cells, 80 μM of this inhibitor was also toxic to the normal skin cells after 24 h of treatment.

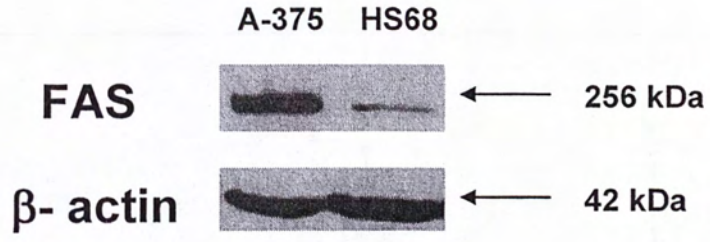


Fig. 3.3 Immunoblots of FAS expression in human melanoma A-375 and normal skin fibroblast HS68 cells.

A-375 cells had a very much higher FAS expression level than HS68 cells.

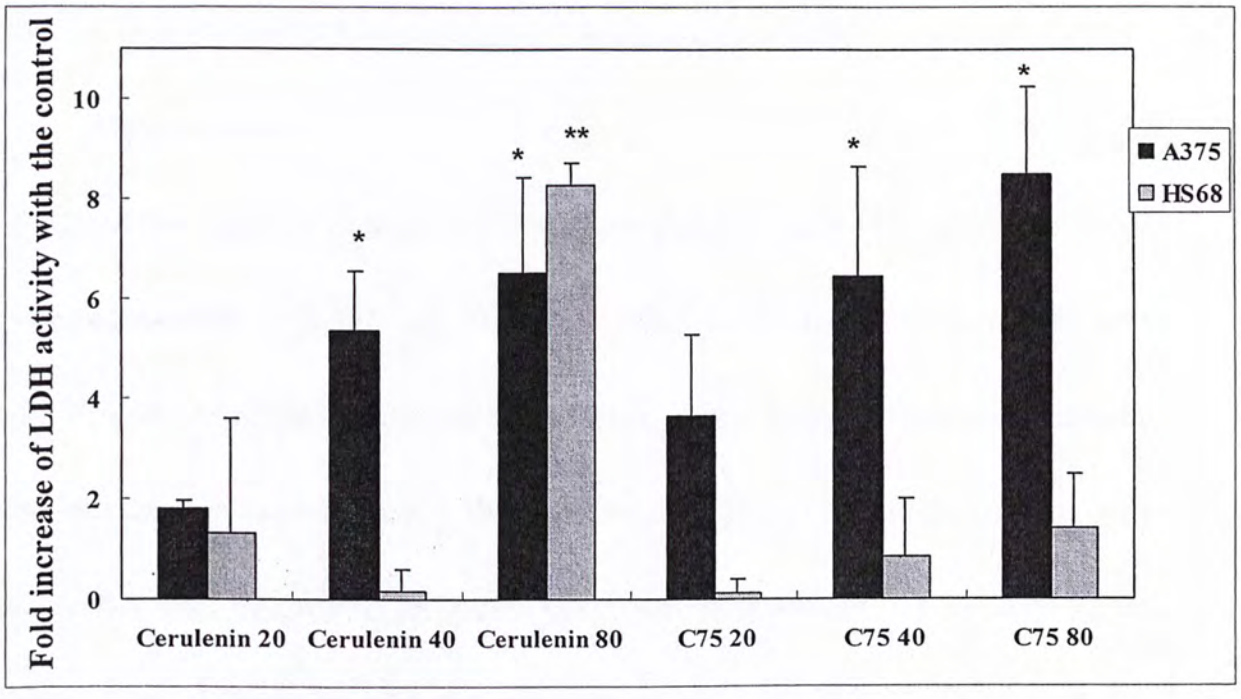


Fig. 3.4 Differential cytotoxic effects of the FAS inhibitors on the viability of melanoma A-375 cells and normal skin fibroblast HS68 cells measured by the LDH release assay.

The cells were treated with 20, 40 and 80 μM cerulenin or C75 for 24 h, and cytotoxicity of the FAS inhibitors was measured by LDH release assay. Although 80 μM cerulenin was toxic to both the melanoma and normal skin fibroblast, the FAS inhibitors exhibited very much lesser toxicity on the normal skin cells than the melanoma cells. Numeric data indicate fold of increase of LDH released from dead cells. Results are expressed as mean ± SD (n=3). *p<0.05; **p<0.005, Student's t-test.

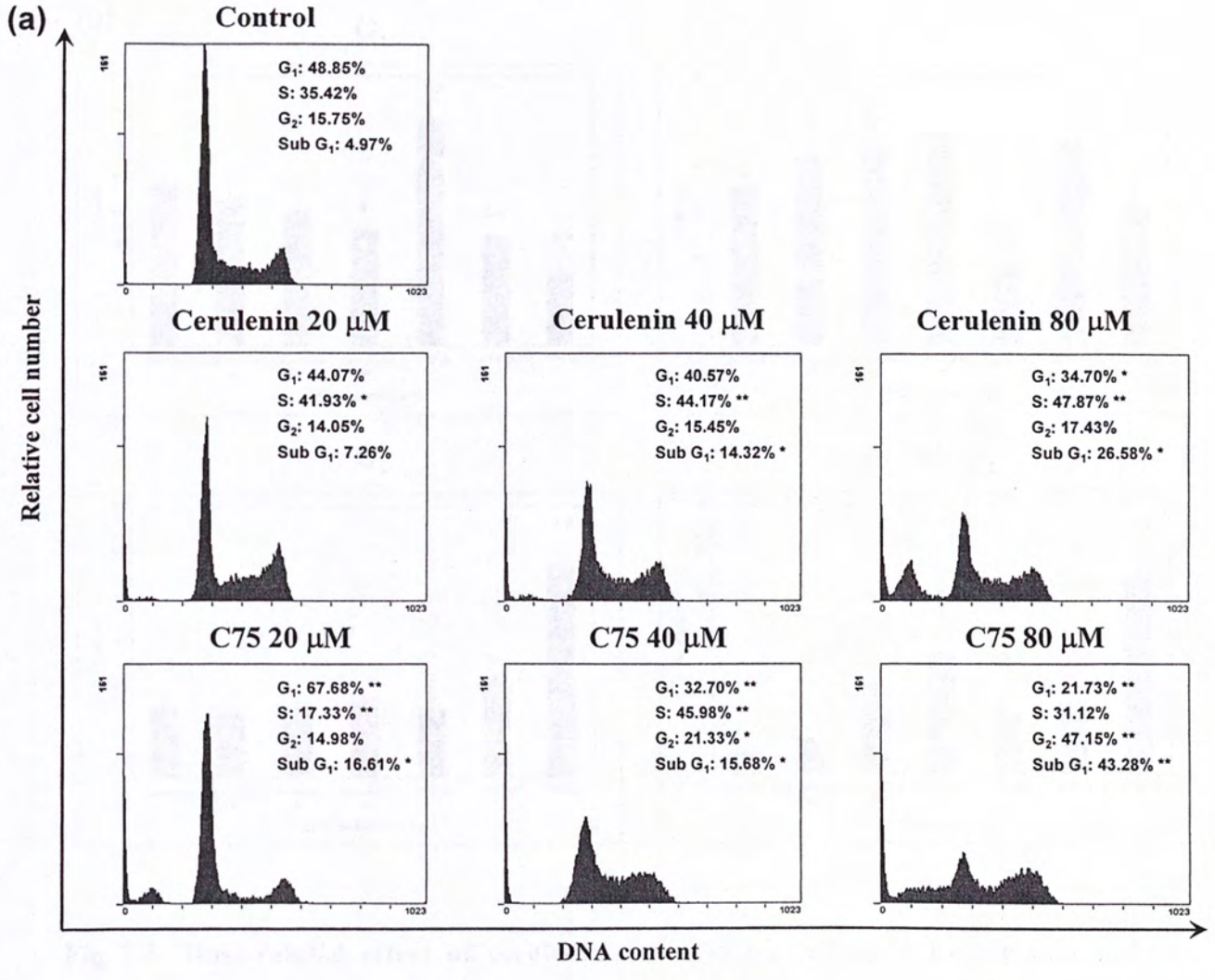
3.1.4 Cerulenin and C75 arrest cell cycle progression and induce apoptosis with DNA fragmentation

DNA flow cytometry was used to investigate the effects of the FAS inhibitors on cell cycle and apoptosis in A-375 cells. Dose effect was first investigated by treating the cells with 20 – 80 μM of the inhibitor for 24 h. Representative DNA histograms and numeric data are shown in Figs. 3.5a and 3.5b respectively. At 24 h, cerulenin arrested cell cycle progression dose-dependently so that S phase cells increased by 118 to 135% of the control level at 20 to 80 μM of the inhibitor (Fig. 3.5b). This S phase arrest was accompanied by depletion of G_1 cells. Although C75 is a synthetic analog of cerulenin, it affected the cell cycle differently and the effect seemed to be dependent on the dose applied. At 20 μM, C75 elevated G_1 cells by 38.55% of the control level, with depletion of S cells; at 40 μM, the inhibitor increased S and G_2/M cells, with decrease of G_1 cells; and at 80 μM, it elevated G_2 cells prominently by 199.37%, with depletion of G_1 cells (Fig. 3.5b). Besides arresting cell cycle progression, both cerulenin and C75 significantly induced DNA fragmentation, a hallmark of apoptosis, in the melanoma cells (Fig. 3.5a). At 80 μM, cerulenin and C75 elevated subG_1 or apoptotic cells by 5.35-fold and 8.71-fold of the control level, respectively (Fig. 3.5b).

Time effect of the FAS inhibitor on inducing apoptosis was further investigated by treating the cells with 40 μM of the inhibitor for 24 and 48 h. DNA fragmentation was

more prominent at 48 h drug treatment (Fig. 3.6a). At 40 μM cerulenin, proportion of $subG_1$ or apoptotic cells increased by 5.50-fold at 24 h and by 19.13-fold of the control level at 48 h. Likewise, 40 μM C75 elevated the apoptotic cells by 4.49- and 15.58-fold at 24 and 48 h respectively (Fig. 3.6b).

Besides DNA flow cytometry, annexin V-FITC/PI staining and confocal microscopy were used to confirm the apoptotic effect of the FAS inhibitors. Apoptosis was triggered by cerulenin so that annexin V-FITC bound onto the melanoma cells and emitted green fluorescence (Fig. 3.7a). Although to lesser extents, cerulenin also induced necrosis so that PI penetrated into the cells and emitted red fluorescence (Fig. 3.7a). Similar observations were also found with the C75-treated cells (Fig 3.7b). Therefore, confocal microscopy studies illustrated that cerulenin and C75 induced apoptosis in A-375 cells.


(a)
Control
G1: 48.85%
S: 35.42%
G2: 15.75%
Sub G1: 4.97%
Cerulenin 20 μM
G1: 44.07%
S: 41.93% *
G2: 14.05%
Sub G1: 7.26%
Cerulenin 40 μM
G1: 40.57%
S: 44.17% **
G2: 15.45%
Sub G1: 14.32% *
Cerulenin 80 μM
G1: 34.70% *
S: 47.87% **
G2: 17.43%
Sub G1: 26.58% *
C75 20 μM
G1: 67.68% **
S: 17.33% **
G2: 14.98%
Sub G1: 16.61% *
C75 40 μM
G1: 32.70% **
S: 45.98% **
G2: 21.33% *
Sub G1: 15.68% *
C75 80 μM
G1: 21.73% **
S: 31.12%
G2: 47.15% **
Sub G1: 43.28% **
Relative cell number
DNA content

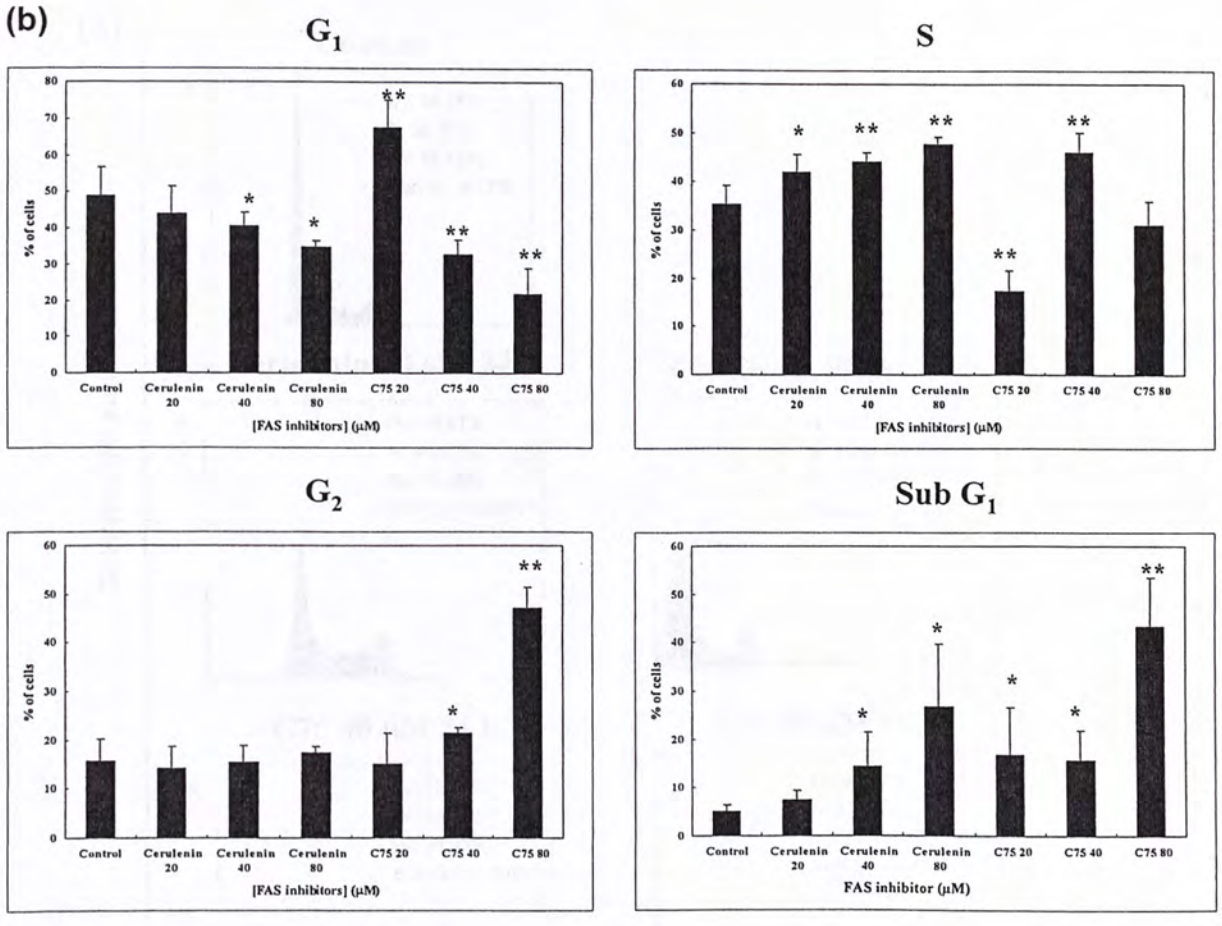
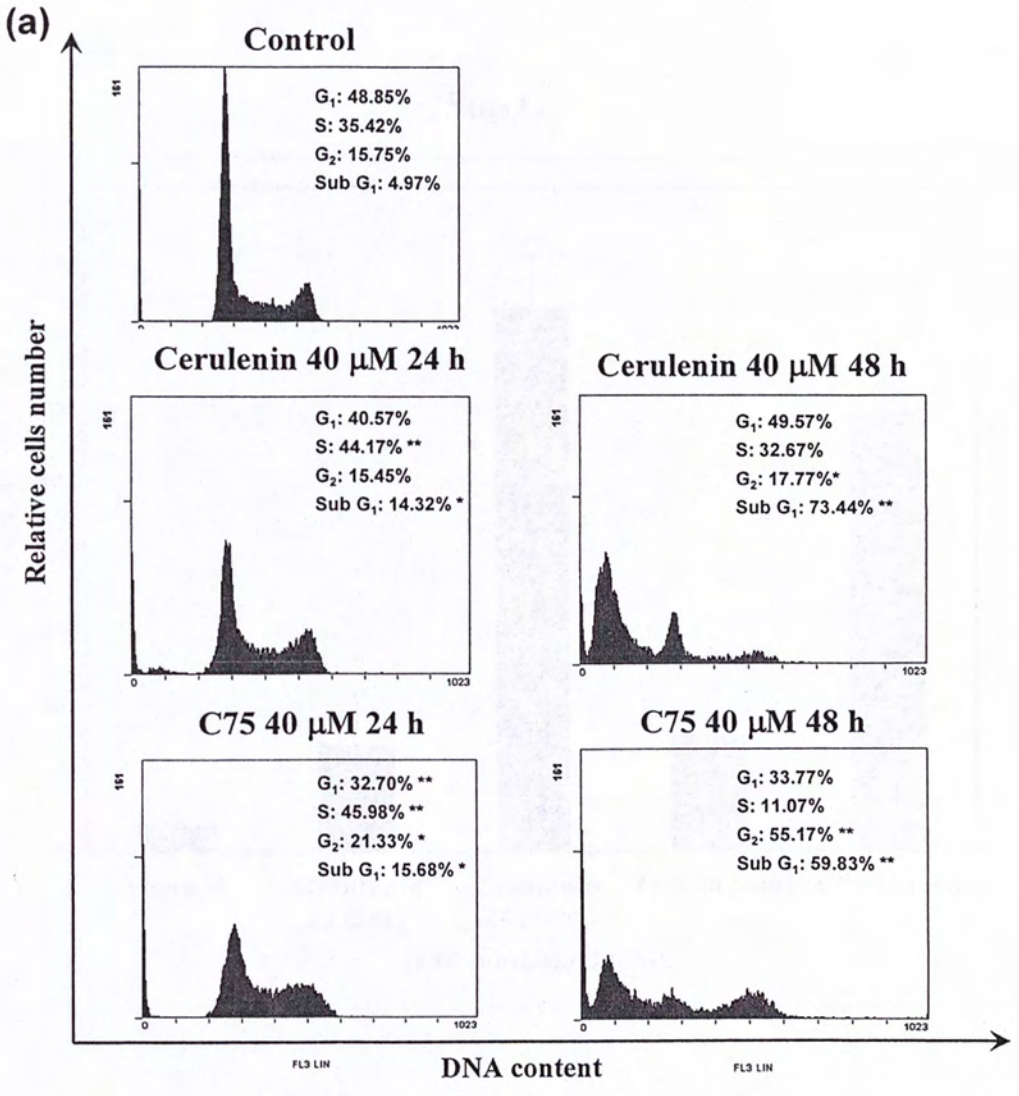


Fig. 3.5 Dose-related effect of cerulenin and C75 on cell cycle progression and apoptosis in A-375 cells.

The cells were treated with 20, 40 and 80 μM cerulenin or C75 for 24 h, and were then subjected to DNA flow cytometry. (a) Representative DNA histograms showing that cerulenin and C75 accumulated cells at S and G_2/M phases, respectively. Both cerulenin and C75 elevated subG_1 cells with fragmented DNA or apoptotic cells. (b) Numeric data showing proportion of cells in different cell cycle phases and apoptosis. Results are expressed as mean ± SD (n=3). *p<0.05; **p<0.005, Student's t-test.


(a)
Control
G1: 48.85%
S: 35.42%
G2: 15.75%
Sub G1: 4.97%
Cerulenin 40 μM 24 h
G1: 40.57%
S: 44.17% **
G2: 15.45%
Sub G1: 14.32% *
Cerulenin 40 μM 48 h
G1: 49.57%
S: 32.67%
G2: 17.77%*
Sub G1: 73.44% **
C75 40 μM 24 h
G1: 32.70% **
S: 45.98% **
G2: 21.33% *
Sub G1: 15.68% *
C75 40 μM 48 h
G1: 33.77%
S: 11.07%
G2: 55.17% **
Sub G1: 59.83% **
Relative cells number
DNA content
FL3 LIN
FL3 LIN

(b)

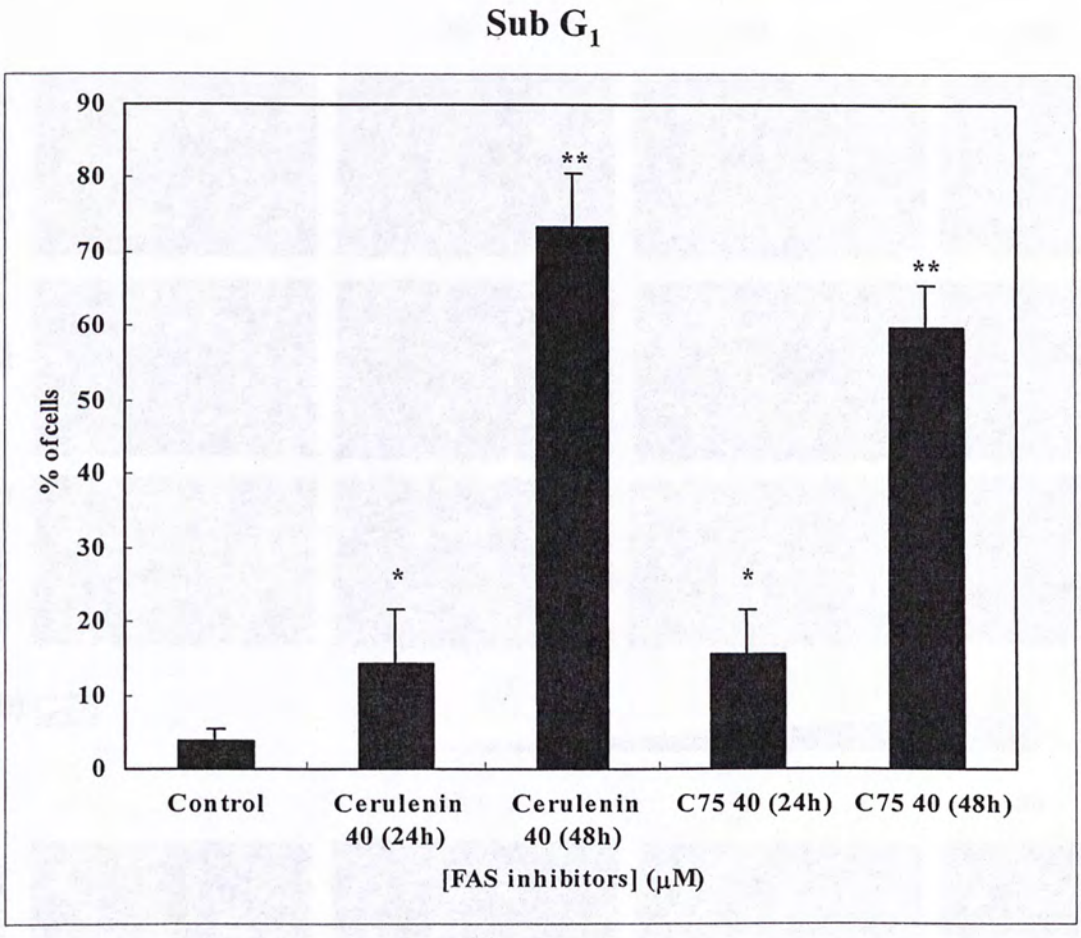


Fig. 3.6 Time-dependent induction of apoptosis induced by cerulenin and C75 on apoptosis in A-375 cells.

The cells were treated with 40 μM cerulenin or C75 for 24 h and 48 h, which were then subjected to DNA flow cytometry. (a) Representative DNA histograms showing that both the FAS inhibitors elevated subG$_1$ cells with fragmented DNA or apoptotic cells time-dependently. (b) Numeric data showing proportion of cells in apoptosis. Results are expressed as mean ± SD (n=3). *p<0.05; **p<0.005, Student's t-test.

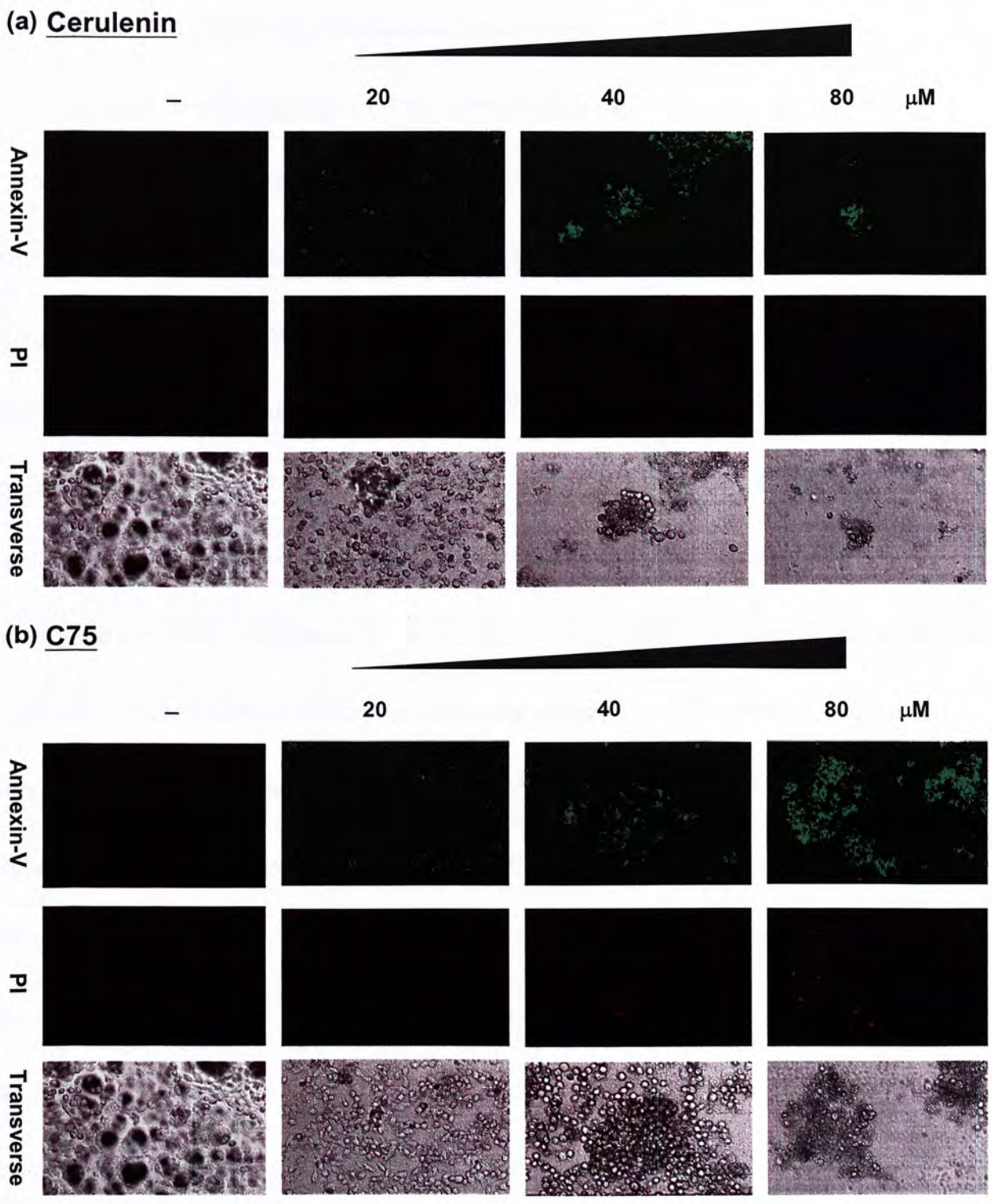


Fig. 3.7 Effects of cerulenin and C75 on apoptosis and necrosis in A-375 cells.
The cells were treated with 20, 40 and 80 μM (a) cerulenin or (b) C75 for 24 h, followed by annexin V-FITC/PI staining and confocal microscopy. Green fluorescence was emitted from both necrotic and apoptotic cells while red fluorescence was emitted from necrotic cells. Both the FAS inhibitors induced apoptosis in the melanoma cells.

3.2 Mechanistic studies of FAS inhibitors in A-375 cells

3.2.1 Cerulenin and C75 induce caspase-dependent apoptosis

Caspase-3 is one of the key executioners of apoptotic cell death. Activation of caspase-3 requires cleavage of 35 kDa inactive zymogen procaspase-3 into small activated units and is the hallmark for caspase-dependent apoptosis. When HS68 cells were treated with 20, 40 and 80 μM cerulenin or C75 for 24 h, no significant cleavage of procaspase-3 could be detected by immunoblot studies (Fig. 3.4). Cerulenin and C75 at least did not trigger caspase-dependent apoptosis on normal fibroblast HS68 cells.

After treating with or without 20, 40 and 80 μM of cerulenin or C75 for 24 h, FAS, PARP and caspase-3 were determined in the melanoma cells with immunoblot analysis. Although both cerulenin and C75 seemed to deplete FAS expression, the magnitude of depletion was not very prominent (Fig. 3.8a). PARP involves in DNA repair to maintain cell viability in response to environmental stress, and cleavage of which causes cellular disassembly (Oliver *et al.*, 1998). Caspase-3 may be activated during apoptosis and cleaves PARP (Fernandes-Alnemri *et al.*, 1994). Both cerulenin and C75 induced cleavage of procaspase-3 (35 kDa) into its smaller active subunits (17 and 19 KDa) dose-dependently in A-375 cells (Fig. 3.8a). The caspase-3 activation was associated with cleavage of full length 116kDa PARP into its smaller inactive fragment (89 KDa). This PARP cleavage and inactivation also occurred dose-dependently (Fig. 3.8a). On the

contrary, both the FAS inhibitors did not induce caspase-3 activation in normal skin fibroblast HS68 (Fig. 3.8b), suggesting that the caspase activation seemed to be specific in the melanoma cells.

Caspase-3 activity assay was used to confirm the caspase activation by the FAS inhibitors. Compatible to the PARP study, the assay showed that caspase-3 activity was induced by cerulenin and C75 dose-dependently. Cerulenin significantly activated caspase-3 by 2.11-fold of the control level at 40 μM and 5.70-fold at 80 μM, while C75 elevated the activity by 1.08-, 2.90- and 24.63-fold at 20, 40 and 80 μM respectively (Fig. 3.8c). Therefore, both the caspase-3 immunoblotting and activity assay showed that cerulenin- and C75-induced apoptosis involved caspase-3 activation.

In addition to caspase-3, other downstream executioner caspases, including caspase-2, caspase-6 and caspase-7, might also play crucial roles in executing the FAS inhibitor-induced apoptosis. Immunoblots showed that both cerulenin and C75 induced proteolytic activation of all these executioner caspases (Fig. 3.9). Lamins, a substrate for activated caspase-6, was also cleaved into small fragment of 28 kDa, showing the caspase activation (Fig. 3.9). Cerulenin and C75 probably triggered caspase-dependent apoptosis, through activation of many downstream executioner caspase-2, 3, 6, 7, to induce PARP and lamin a/c cleavage to cause DNA repair defect and nuclear membrane deformation, finally bringing the cells to the demise. To further confirm involvement of other caspases

in the induced apoptosis, the melanoma cells were pre-treated with 100 μM pan-caspase inhibitor Z-VAD-FMK for 2 h, followed by the cerulenin or C75 treatment at 40 μM for 24 h. The caspase inhibitor completely rescued the cells from the PARP cleavage induced by cerulenin or C75 in immunoblot studies (Fig. 3.10a), and the FAS inhibitor-induced growth inhibition in MTT assay (Fig. 3.10b), suggesting that the apoptosis triggered by the FAS inhibitors was mediated by caspase activation.

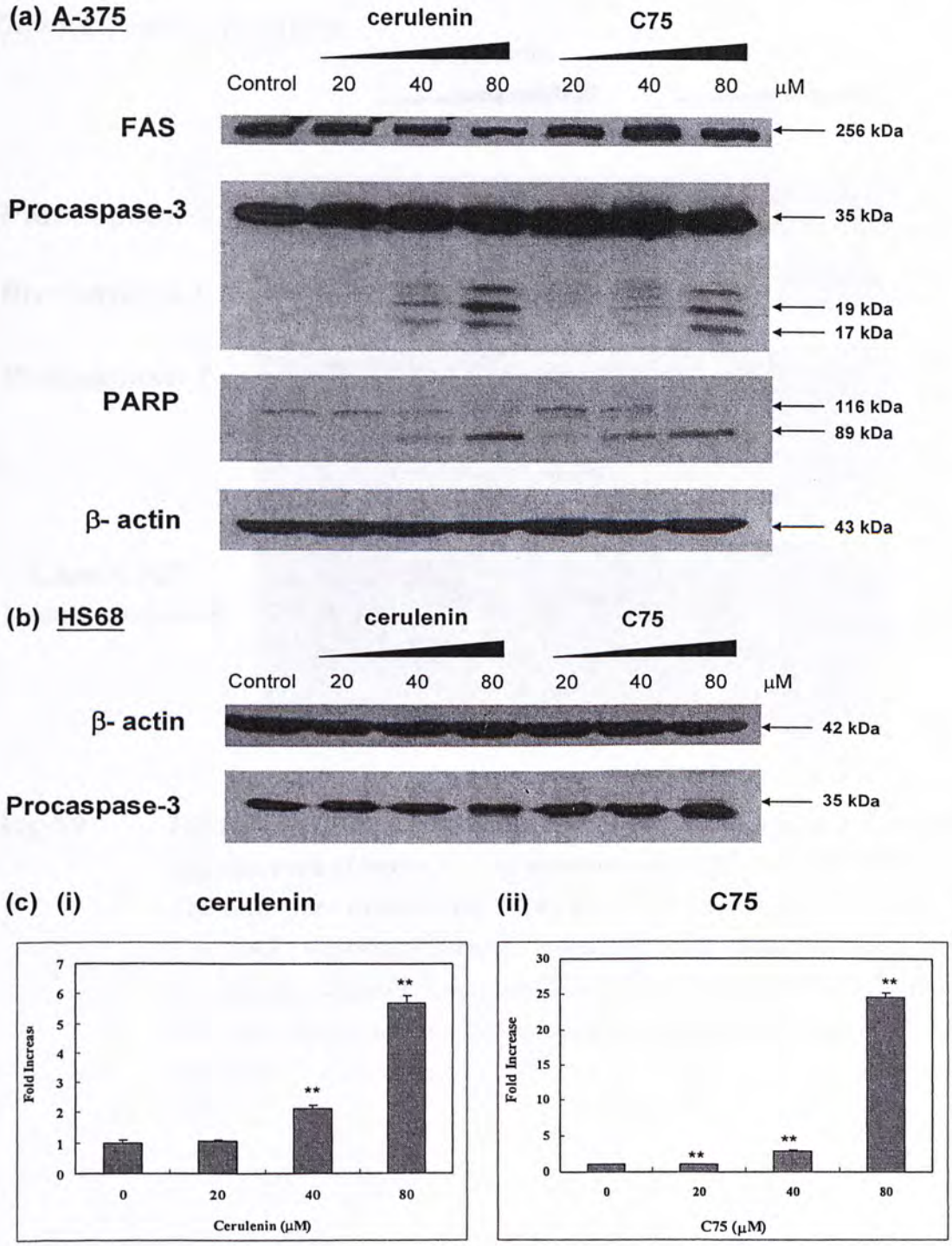


Fig. 3.8 Induction of caspase-3 activation by cerulenin and C75 in A-375 cells.
The cells were treated with 20, 40 and 80 μM cerulenin or C75 for 24 h. (a) The melanoma A-375 cells did not show prominent change of FAS level after the treatment. However, both caspase-3 and PARP showed dose-dependent cleavage, suggesting that the FAS inhibitors might activate caspase-3 to cleave PARP. (b) The normal skin fibroblast HS68 did not show caspase-3 proteolytic cleavage after the treatment. (c) Both cerulenin and C75 induced dose-dependent elevation of caspase-3 activity.

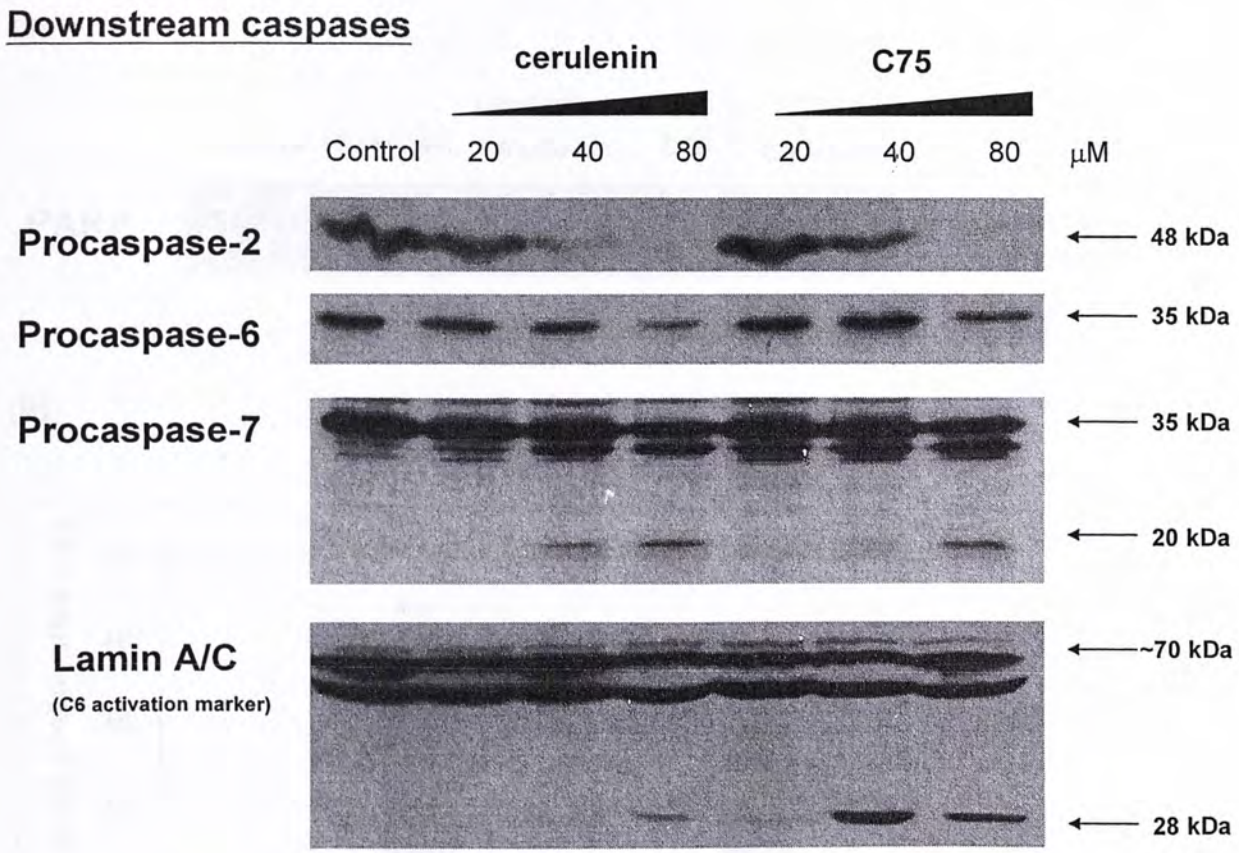
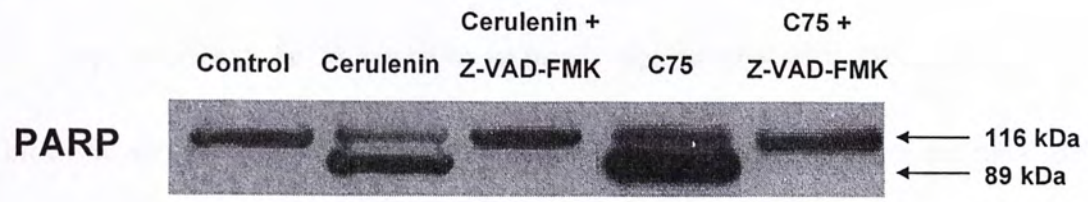


Fig. 3.9 **Induction of proteolytic activation of caspase-2, caspase-6, caspase-7 and cleavage of lamin A/C by cerulenin and C75 in A-375 cells.**
The cells were treated with 20, 40 and 80 μM cerulenin or C75 for 24 h. The FAS inhibitors induced proteolysis and thus activation of the executioner caspases. Lamin A/C, a substrate of the activated caspase-6 was also found to be cleared, suggesting that the caspase had been activated.

(a)



(b)

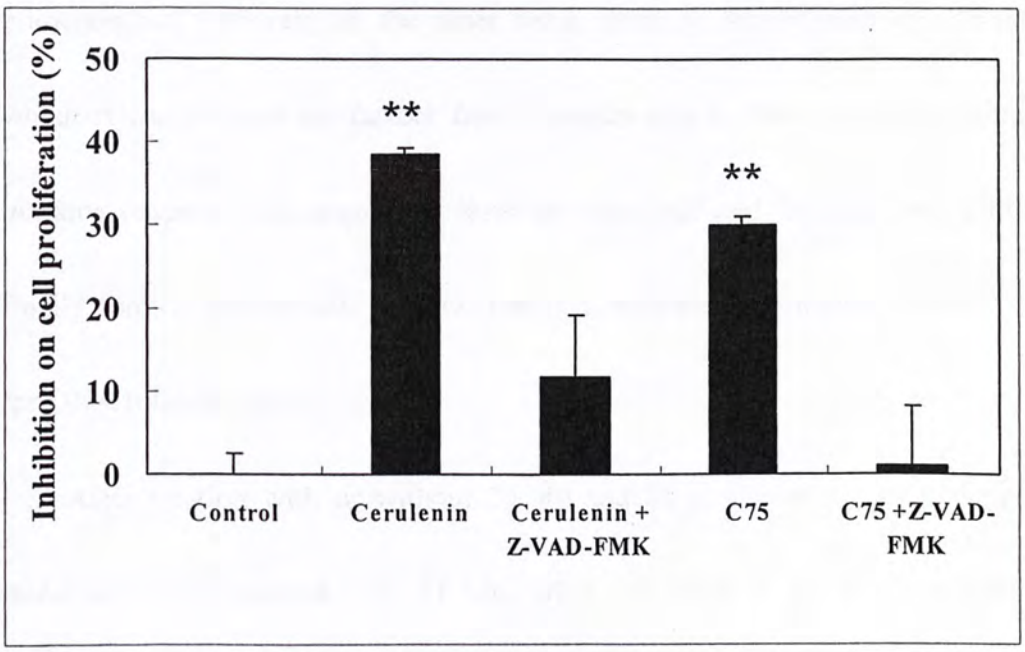


Fig. 3.10 **Effect of pan-caspase inhibitor on cerulenin- and C75-induced PARP cleavage or growth inhibition in A-375 cells.**

The cells were pretreated with 100 μM pan-caspase inhibitor Z-VAD-FMK for 2 h before the cerulenin or C75 treatment. (a) The inhibitor completely rescued the cells from the FAS inhibitor-induced PARP cleavage in immunoblot studies. (b) the inhibitor rescued the cells from the FAS inhibitor-induced growth inhibition in MTT assay, suggesting that the apoptosis was solely caspase-dependent.

3.2.2 Cerulenin- and C75-induced apoptosis involve extrinsic death receptor pathway

Apoptosis can be triggered by extrinsic signals (the death receptor pathway) and intrinsic signals (the mitochondrial pathway). The extrinsic death receptor pathway involves some external death ligands which bind to receptor domains on the membrane surface to activate the initiator caspases (e.g. caspase-8 and -10). The intrinsic mitochondrial pathway, on the other hand, involves the induced mitochondrial stress which releases apoptotic factors from mitochondria to the cytosol to activate another initiator caspase, i.e. caspase-9. Both the extrinsic and intrinsic apoptotic pathways finally convert their signals to activation of downstream executioner caspases responsible for DNA fragmentation.

After treating with or without 20, 40 and 80 μM cerulenin or C75 for 24 h, the molecules (Procaspase-8, -10, TRAIL, DR4 and DR5) in the death receptor apoptotic pathway were determined in the melanoma cells. Immunoblots showed that either cerulenin or C75 induced proteolytic processing of procaspase-8 dose-dependently (Fig. 3.11). In comparison, procaspase-10 was only depleted at 80 μM cerulenin. Furthermore, the FAS inhibitors elevated death receptor DR5 level dose-dependently, but did not affect levels of death receptor DR4 and death ligand TRAIL. Using specific caspase-8 inhibitor, the crucial role of caspase-8 in the induced apoptosis was further confirmed (will be discussed later in section 3.2.3).

To test the relation of elevated DR5 by the FAS inhibitors to activation of extrinsic apoptotic pathway, A-375 cells were at first treated with 10, 20, 40 and 80 μM of cerulenin or C75 for 24 h, followed by administration of 12.5 ng/ml TRAIL molecules 12 h prior to cell harvest. 12.5 ng/ml TRAIL molecules enhanced sensitivity of A-375 cells to apoptosis induced by 10 and 20 μM of cerulenin, and 10, 20 and 40 μM of C75 (Fig. 3.12). We therefore suggested that cerulenin and C75 triggered extrinsic apoptotic pathway by upregulating DR5 expression, which in turn activated proteolytic processing of procaspase-8 into active subunit to elicit the downstream signaling pathway.

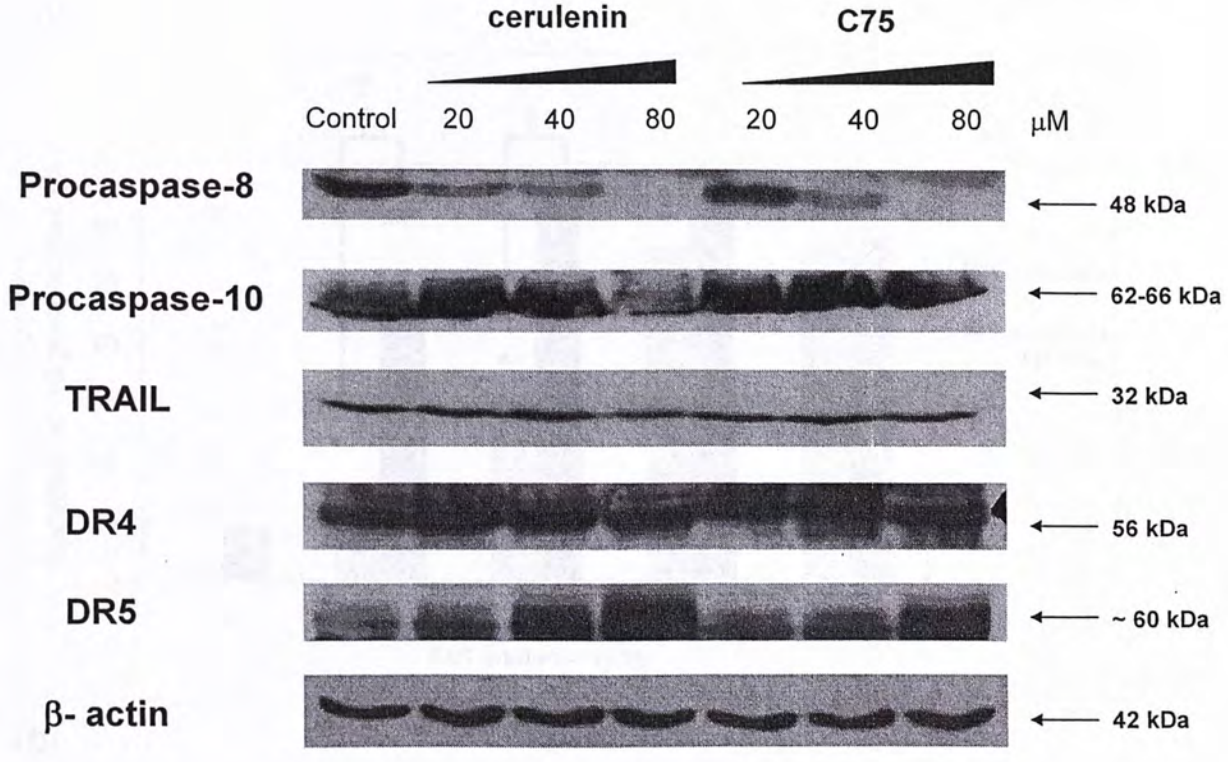


Fig. 3.11 **Immunoblots showing the effects of cerulenin and C75 on the molecules in the death receptor apoptotic signaling pathway in A-375 cells.**

A-375 cells were treated with 20, 40 and 80 μM of cerulenin or C75 for 24 h. Molecules in the death receptor pathway were then determined by immunoblot analysis. Either cerulenin or C75 induced proteolytic processing of procaspase-8 and elevation of DR5.

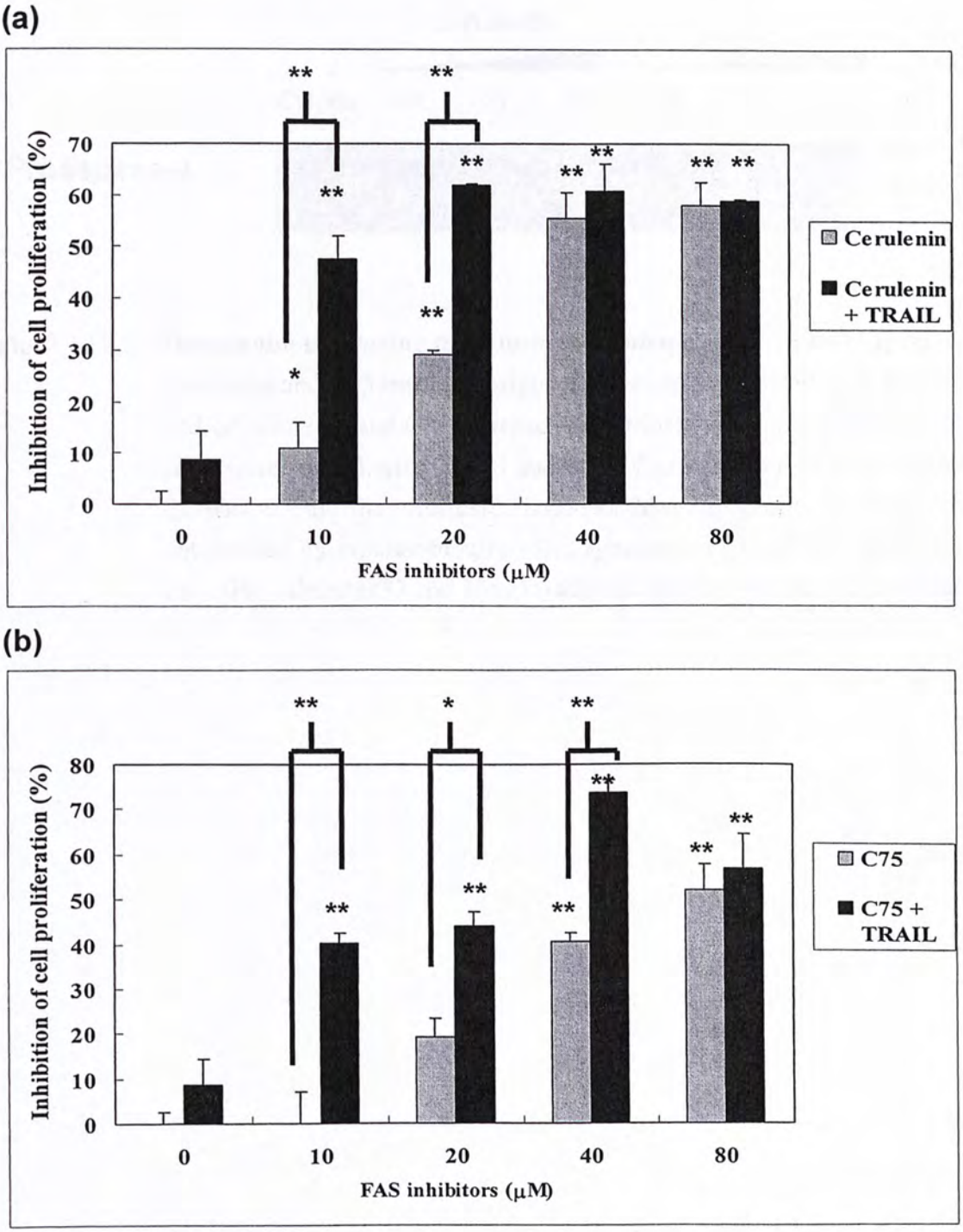


Fig. 3.12 **Effect of TRAIL molecules on cerulenin- and C75-induced apoptosis on TRAIL-resistant A-375 cells.**

A-375 cells were treated with 10, 20, 40 and 80 μM of (a) cerulenin or (b) C75 for 24 h, with administration of 12.5 ng/ml TRAIL molecules 12 h prior to cell harvest. TRAIL molecules enhanced sensitivity of TRAIL-resistant A-375 cells to cerulenin- and C75-induced apoptosis. Results are expressed as mean ± SD (n=4). *p<0.05; **p<0.005, Student's t-test.

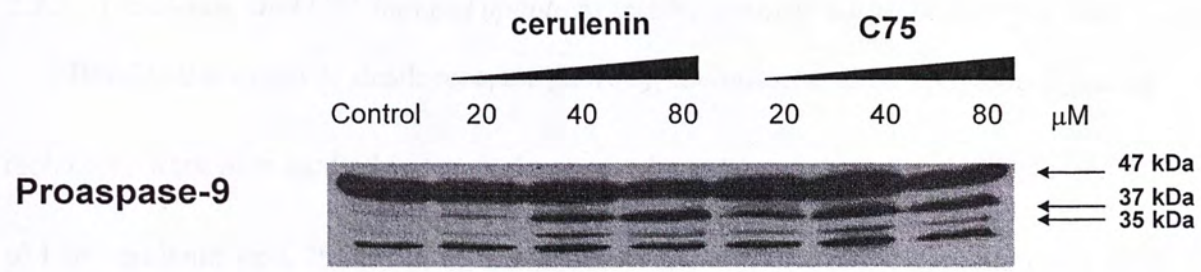
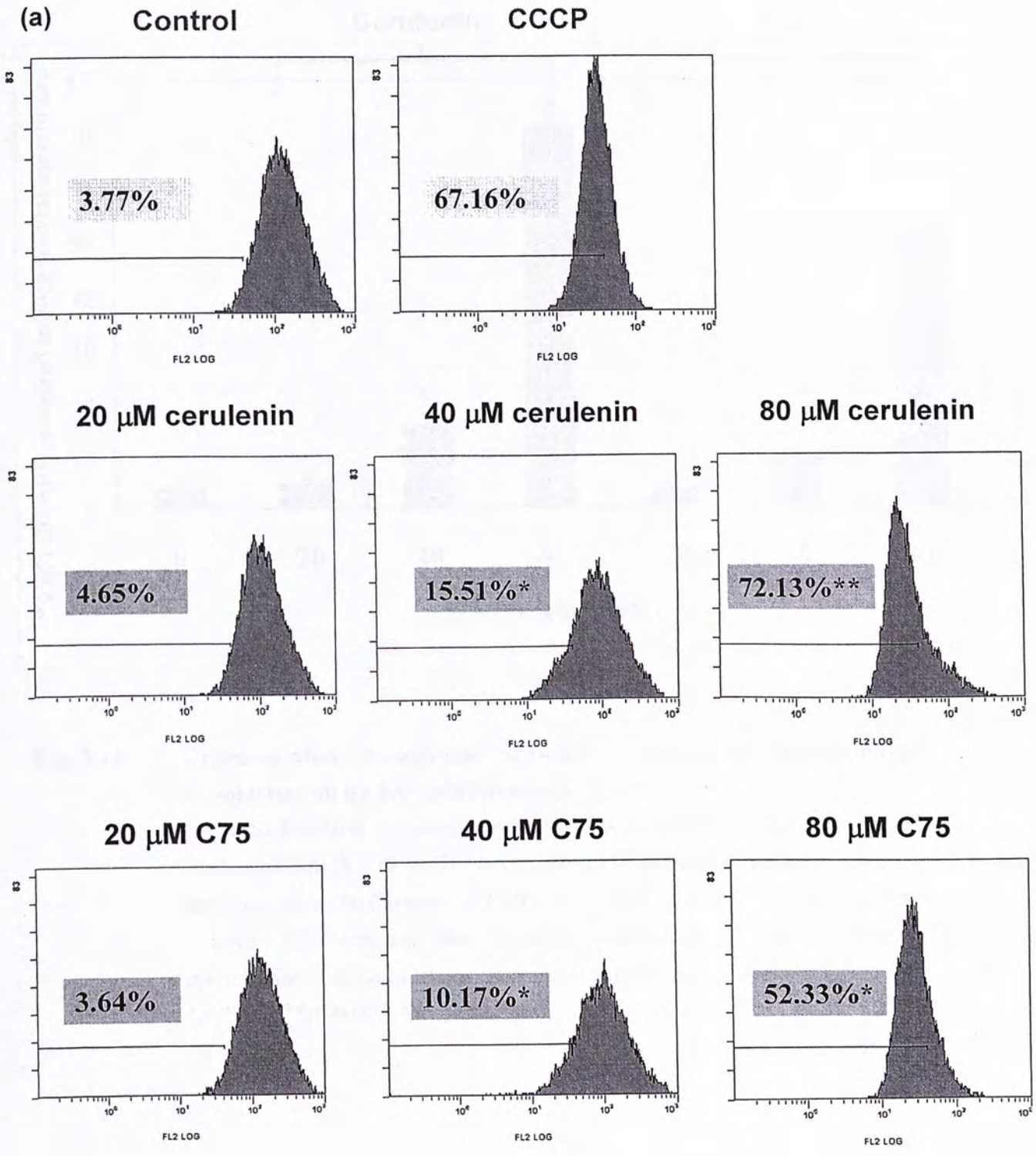


Fig. 3.13 **Immunoblots showing the effects of cerulenin and C75 on caspase-9.** Cerulenin and C75 treatment triggered procaspase-9 cleavage at 40 and 80 μM of cerulenin and C75 to switch on intrinsic apoptotic pathway. A-375 cells were treated with 20, 40 and 80 μM of cerulenin or C75 for 24 h. Caspase-9 in the intrinsic mitochondrial apoptotic pathway was determined by immunoblotting. The zymogen (47 kDa) was cleaved into its active subunits (37 and 35 kDa) after treatment with the FAS inhibitors.

3.2.3 Cerulenin- and C75-induced apoptosis involve intrinsic mitochondrial pathway

Besides the extrinsic death receptor pathway, the mitochondrial apoptotic signaling molecules were also studied in the melanoma cells. After treatment with 20, 40 and 80 μM of cerulenin or C75 for 24 h, immunoblots showed that either cerulenin and C75 triggered proteolytic processing of procaspase-9 so that it was cleaved into the active subunits (37 and 35 kDa) (Fig. 3.13).

According to the current concept, upon firing of mitochondrial apoptotic signaling pathway, the mitochondrial membrane is first depolarized by the Bcl-2 family proteins so that apoptotic factor cytochrome *c* is released out of mitochondria. Cytochrome c then binds to procaspase-9/Apaf 1 complex in the cytosol for caspase-9 activation (Rodriguez and Lazebnik, 1999). To verify whether the caspase-9 activation was induced by mitochondrial depolarization, tetramethylrhodamine ethyl ester (TMRE) was employed as the probe for semi-quantitative measurement of mitochondrial membrane potential. When mitochondrial membrane depolarized, the cells are not able to uptake the probe so that the fluorescence peak in membrane potential histogram shifts to the left. Fig. 3.14 shows a positive control with the emission peak shifted to the left by mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Upon FAS inhibitor treatment, mitochondrial membrane depolarization was observed. Cerulenin at 40 and 80 μM caused 15.51 % and 72.13 % of the cells to exhibit mitochondrial membrane depolarization (Fig. 3.14 a & b). Likewise, 10.17 % and 52.33 % of the cells were depolarized by 40 and 80 μM C75, respectively.


(a)
Control
CCCP
3.77%
67.16%
FL2 LOG
20 μM cerulenin
40 μM cerulenin
80 μM cerulenin
4.65%
15.51%*
72.13%**
20 μM C75
40 μM C75
80 μM C75
3.64%
10.17%*
52.33%*

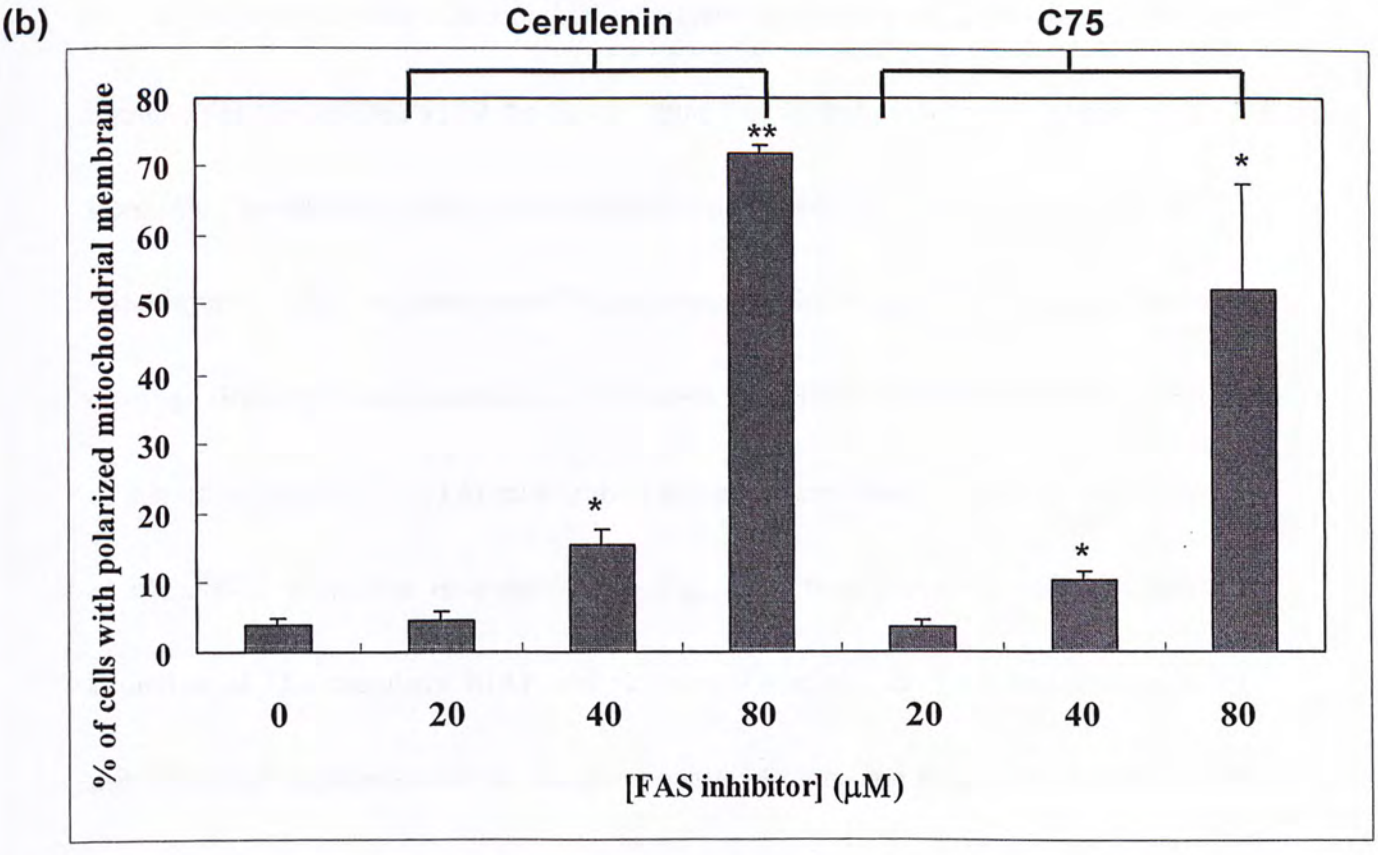


Fig. 3.14 **Representative histograms showing induction of mitochondrial depolarization by FAS inhibitors and CCCP.**

(a) mitochondrial depolarization was observed after cerulenin and C75 treatment on A-375 cells. Mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) was also included as the positive control. (b) Numeric data showing percentage of cells exhibiting mitochondrial depolarization. Results are expressed as mean ± SD (n=3). *p<0.05; **p<0.005, Student's t-test.

In conjunction with mitochondrial membrane depolarization, it was found from the immunoblot studies that apoptotic factors smac/Diablo and cytochrome *c* were released from mitochondria into the cytosol dose-dependently by the FAS inhibitors (Fig. 3.15). Cytochrome *c* binds to procaspase-9/Apaf 1 complex in cytosol to activate procaspase-9 cleavage (Rodriguez and Lazebnik, 1999), while smac/Diablo interacts with the inhibitors of apoptosis protein (IAPs) to relieve their the inhibitory effect on caspases (Srinivasula *et al.*, 2001). From the immunoblots in Fig. 3.16, both cerulenin and C75 induced depletion of IAP members XIAP and survivin. Therefore, the FAS inhibitors induced mitochondrial depolarization to release apoptotic factors and at the same time caused depletion of IAPs so that the caspases might be activated by them altogether.

Mitochondrial membrane depolarization is regulated by the Bcl-2 protein family, in which pro-survival members (e.g. Bcl-2, Bcl-xL, Mcl-1) antagonize pro-apoptotic proteins (e.g. Bax, Bim) and BH3-only proteins (e.g. Bad, Bmf) to produce a "net" death-promoting or death-suppressing cell fate. In this study, effects of the FAS inhibitors on some better-characterized Bcl-2 proteins were investigated. Fig. 3.17 showed that cerulenin and C75 caused depletion of anti-apoptotic members Bcl-xL at higher doses (40 and 80 μM) and Mcl-1 at lower doses (20 and 40 μM). However, no significant changes of the pro-apoptotic Bax and BH3 only proteins were observed. Our studies suggested that the FAS inhibitors might trigger the intrinsic mitochondrial apoptotic pathway in

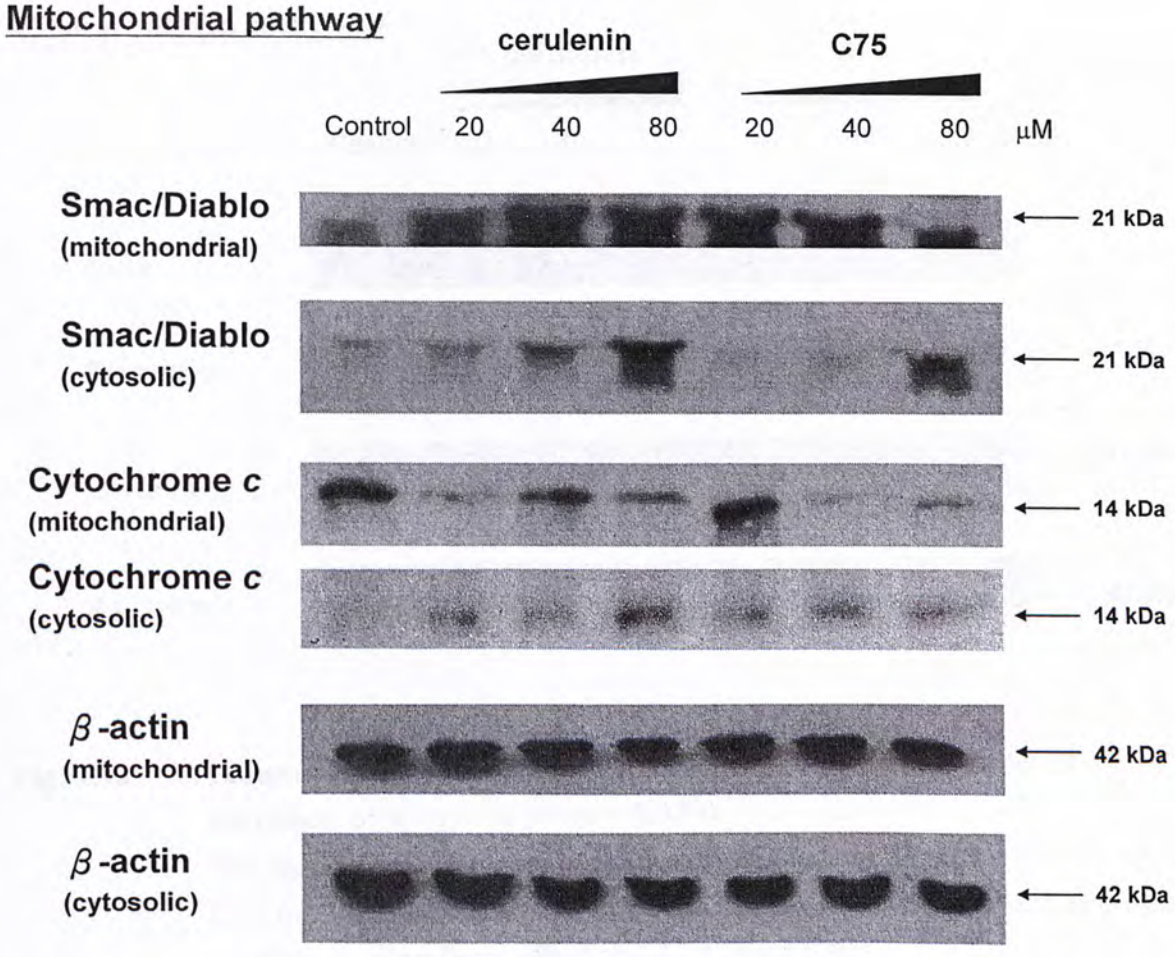


Fig. 3.15 **Immunoblots showing the effects of cerulenin and C75 on the mitochondrial and cytosolic levels of apoptotic factors Smac/Diablo and cytochrome *c*.**

The melanoma cells were treated with 20, 40 and 80 μM cerulenin and C75 for 24 h. The cells were then lysed and subjected to mitochondrial/cytosolic fractionation. The fractions were then immunoblotted for the above apoptotic factors. Cerulenin and C75 caused release of Smac/Diablo and cytochrome *c* from mitochondria to the cytosol.

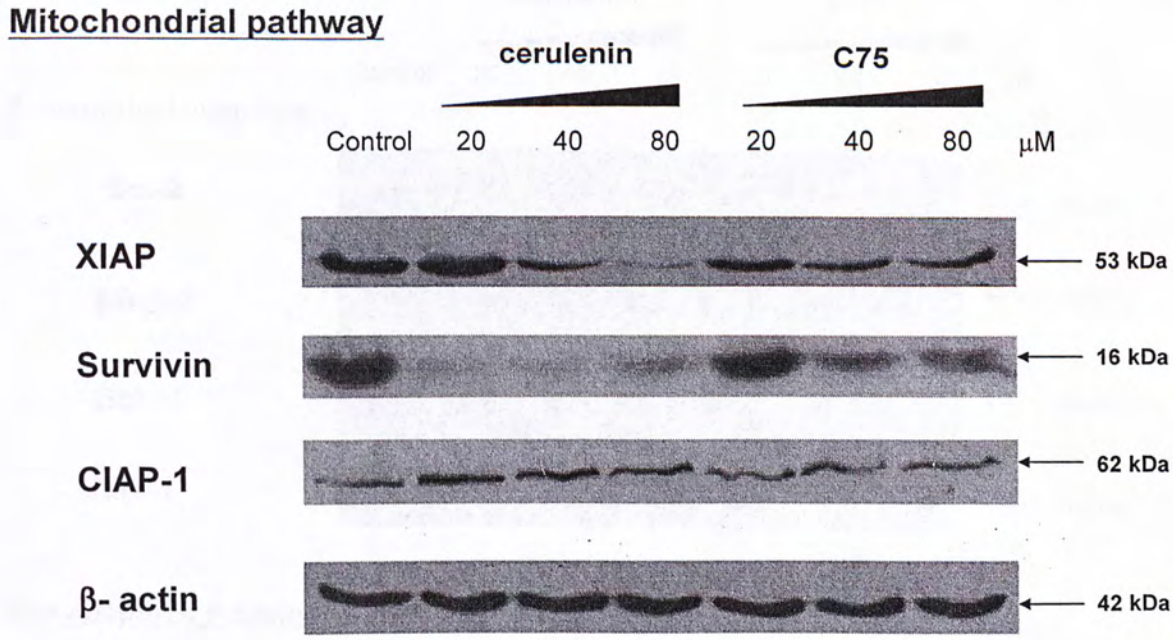


Fig. 3.16 **Immunoblots showing the effects of cerulenin and C75 on levels of inhibitors of apoptosis protein (IAPs).**

The melanoma cells were treated with 20, 40 and 80 μM cerulenin and C75 for 24 h. Cerulenin and C75 induced depletion of XIAP and survivin without any significant effect on CIAP-1 expression.

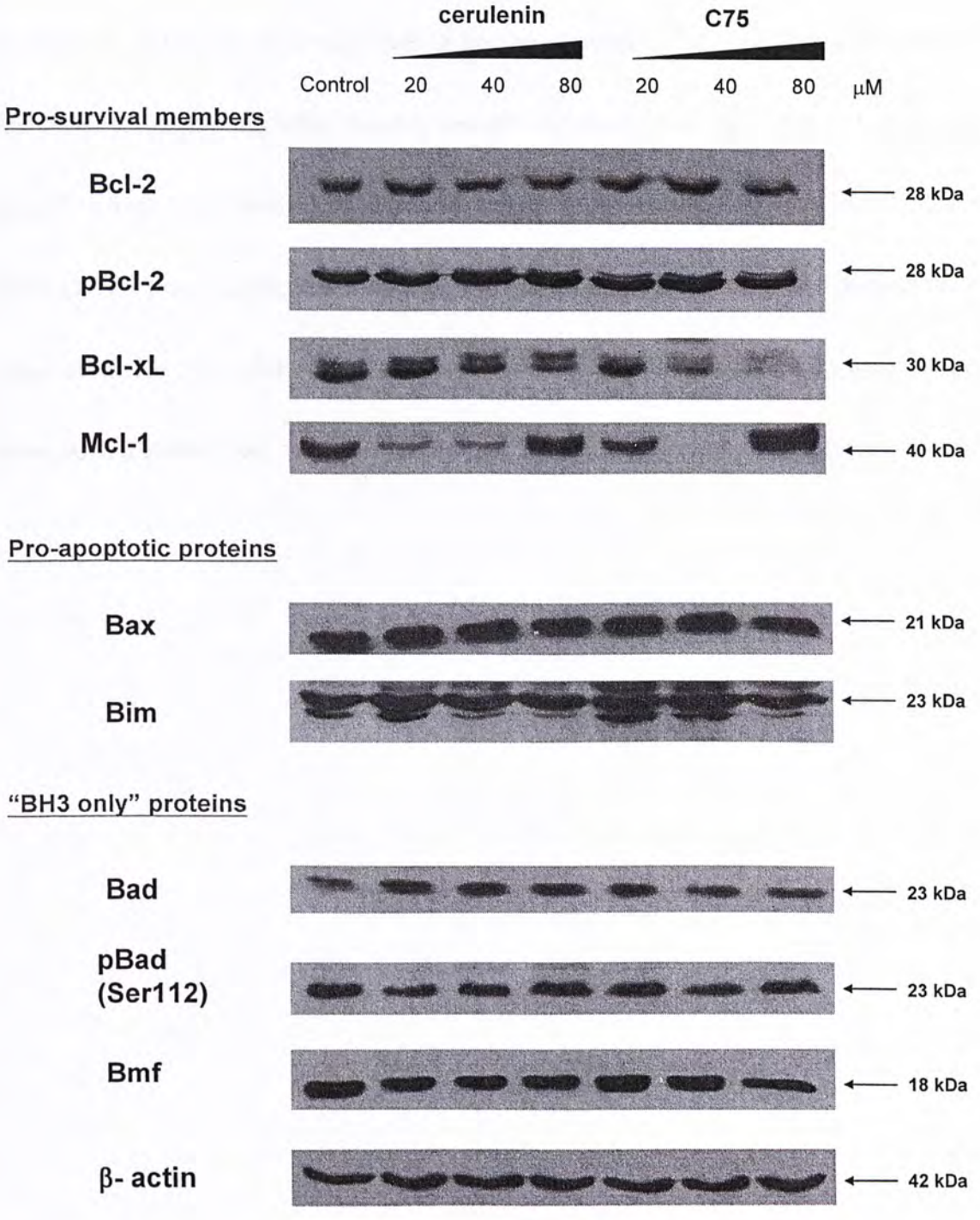


Fig. 3.17 Immunoblot showing the effects of cerulenin and C75 on the levels of the Bcl-2 family proteins in A-375 cells.

The melanoma cells were treated with 20, 40 and 80 μM cerulenin and C75 for 24 h, and the levels of the Bcl-2 family proteins were measured by immunoblot analysis. The FAS inhibitors only depleted the level of anti-apoptotic Bcl-xL and Mcl-1 and did not affect the level of other pro-apoptotic members.

A-375 cells at least by depleting level of the pro-survival Bcl-xL and Mcl-1, resulting in the net death-promoting effect to alter the mitochondrial membrane potential. Then, the mitochondrial membrane depolarization triggered the release of cytochrome *c* and smac/Diablo from mitochondria into the cytosol responsible for caspase-9 activation. In meanwhile, the FAS inhibitors also caused depletion of IAPs that favors the activation of downstream executioner caspases, resulting in DNA fragmentation of apoptosis.

3.2.4 Extrinsic death receptor pathway serves as a pioneer and links with intrinsic mitochondrial pathway in cerulenin- and C75-induced apoptosis

Bid, the BH3 domain-only protein, is proteolytically cleaved by caspase-8 to truncated Bid which translocates to mitochondria to sensitize mitochondrial damage and cytochrome *c* release. Bid, therefore, relays extrinsic apoptotic signal from the cell surface to intrinsic apoptotic signal in mitochondria. From immunoblots in Fig. 3.18, it was found that cerulenin and C75 caused depletion of full size Bid (22kDa), possibly via cleavage by caspase-8, suggesting that a crosstalk might exist between the death receptor and mitochondrial apoptotic pathways in the induced apoptosis.

Caspase inhibitor studies were then performed to find out which apoptotic pathway took the initiative in the cerulenin- and C75-induced apoptosis. Pre-treating the cells with pan-caspase inhibitor (Z-VAD-FMK) completely rescued them from having the PARP cleavage induced by the FAS inhibitor, indicating the induced apoptosis was solely caspase-dependent (Fig. 3.19). Furthermore, in the cerulenin-treated cells, caspase-8 inhibitor (Z-IETD-FMK) completely rescued the cells from having PARP cleavage and caspase-9 inhibitor only partially reduced the cleavage level. In the C75-trteated cells, on the other hand, the caspase-8 inhibitor could not rescue the cells completely but was still more effective than the caspase-9 inhibitor. Therefore, both the Bid immunoblotting and caspase inhibitor studies supported that caspase-8 was the initiative caspase that might activate caspase-9, possibly through the Bid cleavage, and thus depleted its level.

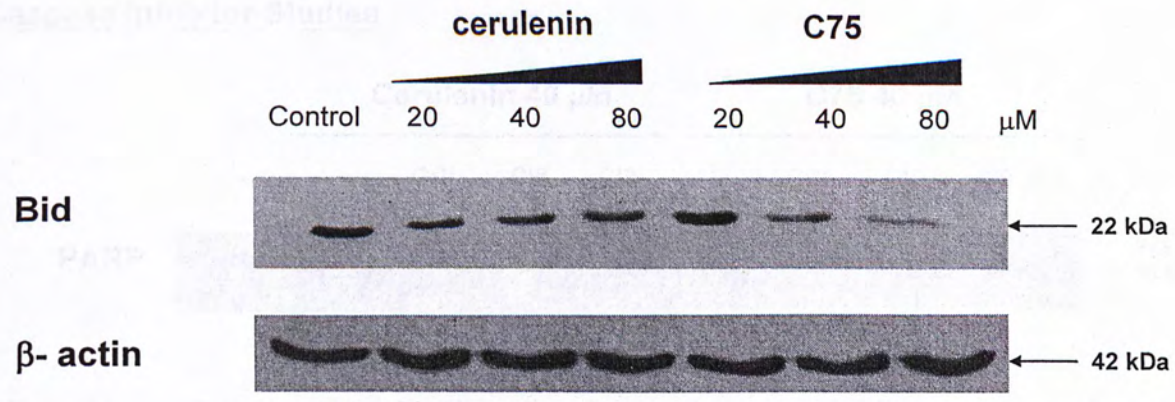


Fig. 3.18 **Immunoblots showing the effects of cerulenin and C75 on Bid.** The melanoma cells were treated with 20, 40 and 80 μM cerulenin and C75 for 24 h. Both the FAS inhibitors caused depletion of Bid protein and the depletion was more prominent and dose-dependent in the C75-treated cells.

Caspase Inhibitor Studies

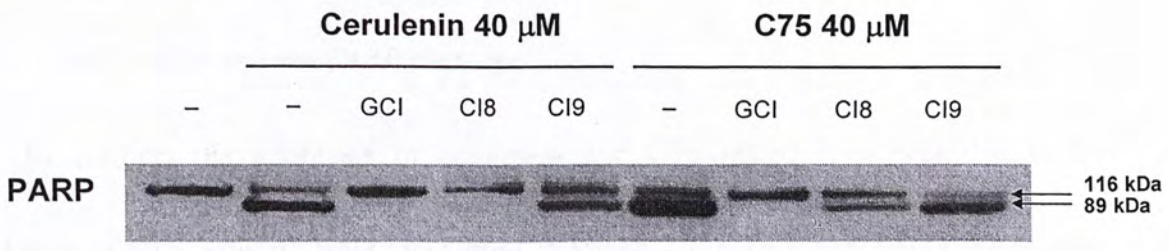


Fig. 3.19 **Immunoblot showing the effects of caspase inhibitors on cerulenin and C75-induced PARP cleavage.**

The cells were first treated with 100 μM pan-caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-FMK) or caspase-9 inhibitor (Z-LEHD-FMK) for 1 h, followed by incubation with 40 μM cerulenin or C75 for 24 h. Pan-caspase inhibitor completely rescued the cells from having the FAS inhibitor-induced PARP cleavage. In the cerulenin-treated cells, caspase-8 inhibitor completely rescued the cells from having PARP cleavage as the pan-caspase inhibitor did. Caspase-9 inhibitor could only partially rescue the cells. In the C75-treated cells, both caspase-8 and caspase-9 inhibitors could only partially rescue the cells and caspase-8 inhibitor was more effective. Therefore, caspase-8 seemed to be the initiative caspase in the induced apoptosis and might activate caspase-9.

3.3 Small interfering RNA on Fatty Acid Synthase (FAS siRNA)

3.3.1 FAS siRNA induces PARP cleavage

To confirm the apoptosis in cerulenin- and C75-treated cells was due to the depletion of FAS activity, RNAi was used to knock down FAS expression temporarily inside the cells. To knock down FAS expression, A-375 cells were transfected with 100 or 200 nM FAS siRNA for 72 h or 96 h. Immunoblots showed that the siRNA was effective to downregulate FAS expression at 72 h and there was a retrieval of the expression at 96 h (Fig. 3.20). In meanwhile, the transfected cells also had PARP cleavage at 100 nM and 200 nM siRNA after 72 h and 96 h of treatment (Fig. 3.20). Therefore, transfection with 100 nM siRNA for 72 h was chosen for further studies. FAS siRNA probably induced apoptosis by reducing PARP level to hinder its role in DNA repair and so DNA fragmented for the cell to demise.

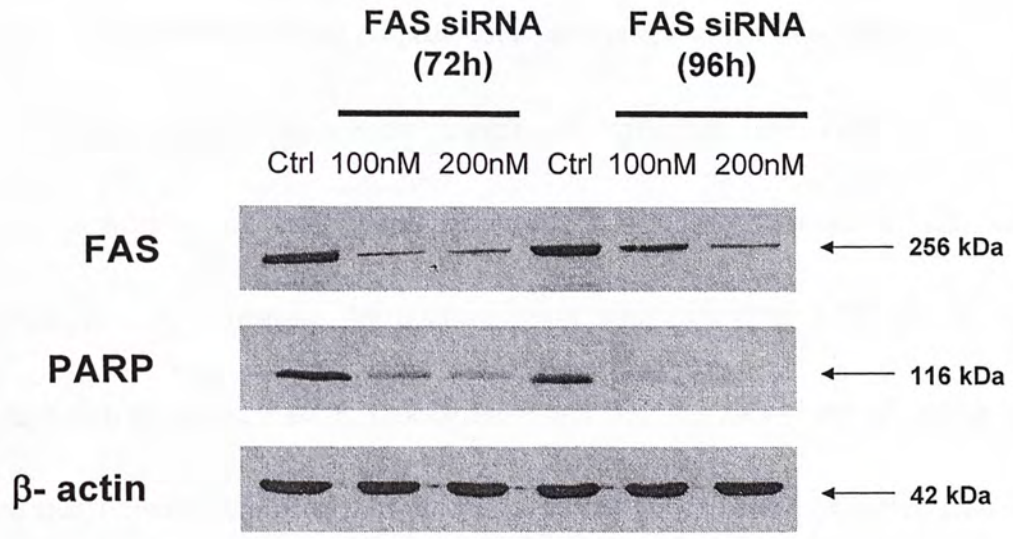


Fig. 3.20 **Immunoblots showing the effects of FAS siRNA on FAS and PARP expression.**

The cells were treated with or without 100 or 200 nM FAS siRNA for 72 or 96 h. The FAS expression was downregulated at 72 h but retrieved at 95 h of treatment. PARP depletion was observed with FAS depletion.

3.3.2 FAS siRNA triggers caspase-dependent apoptosis as FAS inhibitors

When A-375 cells were transfected with 100 nM FAS siRNA for 72 h, downregulation of PARP was observed, which was associated with cleavage of procaspase-3, indicating the occurrence of apoptosis (Fig. 3.21). In the meanwhile, depletion of pro-caspase-8, procaspase-9 and Bid was also observed, which was similar to that found in the cerulenin- and C75-treated cells. Therefore, either pharmacological inhibition or molecular depletion of FAS resulted in apoptosis with similar molecular mechanism and activation of caspases in A-375 cells.

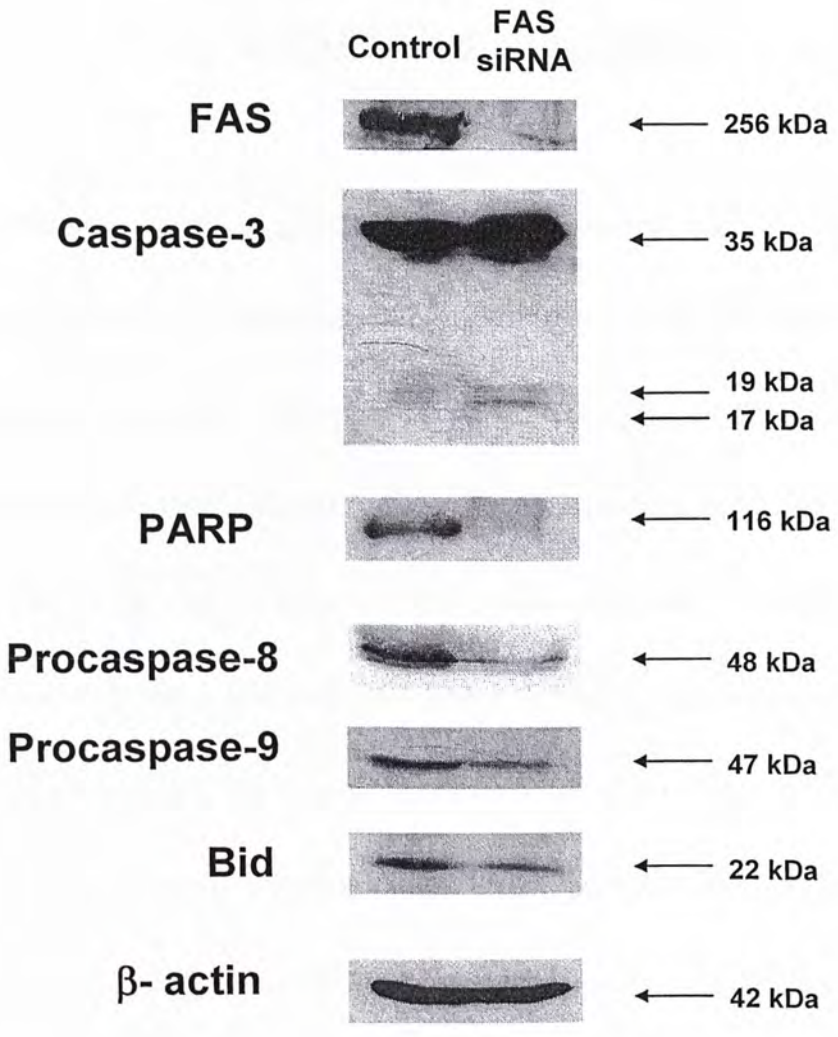


Fig. 3.21 **Immunoblots showing the effects of FAS siRNA on several key molecules in apoptosis found in the FAS inhibitor studies.**

The cells were treated with or without 100 nM FAS siRNA for 72 h. FAS and several key molecules in the apoptotic pathway induced by cerulenin and C75 were then immunoblotted. The siRNA downregulated FAS expression and induced cleavage of procaspase-3 into its active fragments and of PARP, indicating the incidence of apoptosis. Similar to the apoptosis caused by the FAS inhibitors, depletion of procaspase-8, procaspase-9 and Bid was also found, suggesting that either pharmacological or molecular inhibition of FAS activity would lead to the apoptosis with similar molecular cascade.

CHAPTER 4 Discussion

FAS inhibitors inhibited growth of various cancer cell lines *in vitro*. Huang *et al.* (2000b) demonstrated that cerulenin selectively exerted growth inhibitory effect on colonic cancer, but not on normal fibroblast. Likewise, Chen *et al.* (2000) showed that FAS inhibitors selectively inhibited proliferation of K562 leukemia cells dose-dependently. We found in this study that, besides colonic cancer and leukemia cells, FAS inhibitors cerulenin and C75 also inhibited the growth of cells from other cancers, including hepatocellular carcinoma, melanoma, lung carcinoma, colorectal adenocarcinoma, cervix adenocarcinoma, prostate carcinoma and breast adenocarcinoma (Table 3.1). Since melanoma A-375 was found to be the most sensitive cell line to the growth inhibition, it was used as the model to investigate the mechanism in the growth inhibition.

Since FAS strongly expresses in melanoma A-375 but weakly expresses in normal skin fibroblast HS68 cells (Fig. 3.3), we speculated that the FAS inhibitors should have higher toxicity on the cancer than on the normal cells. We demonstrated that the FAS inhibitors, especially C75, was selectively cytotoxic to the melanoma cells (Fig 3.4). However, cerulenin was found in this study to be toxic to both A375 and HS68 cells at 80 μM (~18 μg/ml). Previous studies also showed that cerulenin was harmless only at concentrations lower than 5 μg/ml in gingival fibroblast (Zhang *et al.*, 2005) and cervical

fibroblast (Pizer *et al.*, 1996b).

Several reports have shown that cerulenin has lower specificity than C75 as a fatty acid inhibitor, which may explain why cerulenin exerts toxicity on normal cells at high concentration. Cerulenin contains a very reactive epoxide group which interacts with proteins other than FAS. Cerulenin also inhibits protein palmitoylation, a process of posttranslational modification for key signaling proteins to attach to the plasma membrane (Jochen *et al.*, 1995; Lawrence *et al.*, 1999), which is more related to cancer cell growth inhibition than inhibition of FAS activity (Lawrence *et al.*, 1999). Moreover, cerulenin hinders cholesterol synthesis (Goldfine *et al.*, 1978; Malvoisin *et al.*, 1990) and inhibits proteolysis (Ikuta *et al.*, 1986; Moelling *et al.*, 1990). In addition, the use of cerulenin as a FAS inhibitor is limited due to its chemical instability and more stable C75 has recently been developed (Kuhajda *et al.*, 2000).

Transformation of melanocytes to melanoma stems from abnormal changes in cell cycle regulatory mechanisms, including pRb and cyclin D_1, which assist cell cycle phase transition, and negative regulators, like p16, p27, p53 and p21, which cause cell cycle arrest (Lee and Yang, 2001; Li *et al.*, 2006). Drugs that target these proteins and induce cell cycle arrest would be effective tools against melanoma development. Several reports have shown that FAS inhibitors retard cell growth by inducing cell cycle arrest. Orlistat blocked breast cancer cells at G_1/S checkpoint by downregulating E3 ligase Skp2 (Knowles *et al.*,

2004). When treated with cerulenin and C75, RKO colon carcinoma cells exhibited a biphasic stress response with S phase and G_2 arrest, resulting in delay of cell cycle progression (Li *et al.*, 2001). Cerulenin elevated expression of cyclin-dependent kinase inhibitors p21 and p27, downregulated cyclin D_1 and arrested prostate cancer cells at G_2/M phase (Furuya *et al.*, 1997). C75 inhibited FAS activity and induced G_2 phase arrest in $HepG_2$ and SMMC7721 cells and triggered G_1 phase arrest in Hep3B cells (Gao *et al.*, 2006). Moreover, time- and dose-dependent S phase arrest was found in glioma cells after cerulenin treatment (Zhao *et al.*, 2006a). Other reports demonstrated that FAS inhibitors arrested cells at S phases in myeloma (Wang *et al.*, 2006), leukemia and beast carcinoma (Pizer *et al.*, 1998a). Likewise, our flow cytometric studies also showed that cerulenin and C75 redistributed the melanoma A375 cells into S and G_2 phases, with significant depletion of G_1 cells (Fig. 3.5), in parallel with dose-dependent reduction of cell growth.

Pizer *et al.* (1998b) claimed a direct linkage between fatty acid synthesis and DNA synthesis in proliferating tumor cells. FAS inhibition actually deregulates adenine nucleotide biosynthesis, causing defect in DNA replication and nuclear division (Chung *et al.*, 2006). Apart from DNA synthesis, Swinnen *et al.* (2003) also suggested the importance of FAS overexpression to dysregulation of membrane composition in tumor cells. It was found that fatty acid synthase inhibition completely prevented lipid accumulation and hindered preadipocyte differentiation (Schmid *et al.*, 2005). Cerulenin and C75 probably

exert their growth-inhibitory effect via cell cycle arrest by interfering with the DNA replication machinery and deregulating different cell cycle phases. Cessation of rapid growth, lipogensis of tumor-associated cell membranes and proliferation that melanoma cells originally possessed is thus resulted.

Apoptosis is a programmed cell death characterized by chromatin condensation, nucleosome-sized DNA fragments, membrane blebbing, cell shrinkage, and compartmentalization of the dead cells into membraneous apoptotic bodies (Darzynkiewicz *et al.*, 1997). Apoptosis brings homeostasis by maintaining optimum cell number in our body. Cells that are diseased, malfunctioning or under stress may undergo apoptosis and be replaced by healthy ones. Over the past few decades, apoptosis has been regarded as a novel target for cancer chemotherapy, and a variety of apoptosis pathway-targeted drugs are in clinical trials (Cummings *et al.*, 2004).

Cancer cell proliferation requires FAS overexpression since FAS synthesizes fatty acids for membraneous structures in rapidly-dividing cancer cells (Swinnen *et al.* 2003). FAS blockade by pharmacological inhibitors or small interfering RNA induce apoptosis in different cancer cells, including myeloma (Wang *et al.*, 2006), glioma (Zhao *et al.*, 2006a), oral squamous cell carcinoma (Zhang *et al.*, 2005), pediatric medulloblastoma (Slade *et al.*, 2003), colonic carcinoma (Huang *et al.*, 2000 a,c), leukemia (Chen *et al.*, 2000) prostate (Furuya *et al.*, 1997), breast (Pizer *et al.*, 1996a) and endometrical cancers (Lupu *et al.*,

2006), with PARP cleavage and DNA fragmentation. We are the pioneer group to show that cerulenin and C75 also triggered apoptosis in melanoma A375 cells, indicated by strong green florescent annexin V-FITC signal in confocal microscopy (Fig. 3.7), dose-dependent cleavage of downstream caspase-3 and PARP in immunoblotting (Fig. 3.8a), and dose-dependent and time-dependent elevation of subG$_1$ cell proportion in DNA flow cytometry (Fig 3.5 & 3.6). Heiligtag *et al.* (2002) demonstrated that cerulenin triggered mitochondrial apoptotic pathway with caspase-3 activation in a panel of wild-type p53 and mutant p53 tumor cell lines. We also showed in this study that administering pan-caspase inhibitor Z-VAD-FMK completely rescued cells from cerulenin- and C75-induced apoptosis, indicating such apoptosis is solely caspase-dependent (Fig. 3.10). Overall speaking, present and previous studies support that FAS pharmacological inhibitors retard cancer cell growth effectively and caspase-dependent apoptosis plays an important role to bring cells to demise.

Apoptosis can be triggered by extrinsic signals (death receptor pathway) and intrinsic signals (mitochondrial pathway). Mitochondrial apoptotic pathway involves complex signaling cascades and is tightly regulated by the Bcl-2 family proteins in which different members promote or block cell death at different stages to control cell fate (Reed *et al.*, 1997). In mammalian cells, the Bcl-2 family could be divided into three groups upon their functions and sequence homology: the pro-survival Bcl-2 subfamily includes Bcl-2, Bcl-xL,

Mcl-1, A1 and Bcl-w; the pro-apoptotic Bax subfamily includes Bax, Bak, and Bok; and the death-promoting BH3-only subfamily includes Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, and Puma (Zamzami and Kroemer, 2001). These molecules either homodimerize or heterodimerize with molecules of opposing functions, and the ratio of pro-apoptotic and anti-apoptotic proteins decides between cell survival and apoptosis. Mitochondria take a crucial role in apoptosis, and in case apoptosis occurs, the mitochondrial membrane will be depolarized and permeabilized to release apoptotic factors into cytosol to transducer the apoptotic cascades downstream (Kromer and Reed, 2000). Our TMRE studies indicated that both cerulenin and C75 induced the mitochondrial membrane depolarization in A375 cells (Fig. 3.14). In meanwhile, apoptotic factors Smac/Diablo and cytochrome *c* were also found in the cytosol (Fig. 3.15). All these findings evidenced the initiation of the mitochondrial pathway by the FAS inhibitors.

Previous reports have also shown that FAS inhibitors trigger the mitochondrial-mediated apoptosis in a variety of cancer cell lines, though with different Bcl-2 family proteins to trigger the depolarization. It was found that cerulenin caused apoptotic cell death and PARP cleavage in U251 and SNB-19 glioma cells independent of the death-receptor pathway, and overexpressing Bcl-2 inhibited the cell death (Zhao *et al.*, 2006a). *In vitro* studies on a panel of wild-type and mutant p53 cell lines have shown that the cerulenin-induced apoptosis associated with release of cytochrome *c* and cleavage of

Bax (Heiligtag *et al.*, 2002). Likewise, nude mice transplanted with colonic carcinoma LoVo cells showed elevation of Bax and reduction of Bcl-2 proteins after cerulenin administration (Huang *et al.*, 2000a).

With the immunoblots on the Bcl-2 family proteins in this study, we are the pioneer group to illustrate that FAS inhibitor significantly depletes Mcl-1 and Bcl-xL proteins in the melanoma cells (Fig. 3.17). Mcl-1 is anti-apoptotic, interacting with and antagonizing pro-apoptotic Bcl-2 members to maintain mitochondrial integrity and cell viability in cancer cells (Mandelin and Pope, 2007). Mcl-1 is highly expressed to resist apoptosis in melanoma cells and has been regarded as a therapeutic target for melanoma since enhanced killing on melanoma existed by simultaneously targeting Mcl-1 and Noxa (Qin *et al.*, 2006). Mcl-1 antisense therapy also sensitized human melanoma in a SCID mouse xenotransplantation model (Thallinger *et al.*, 2003). Anti-apoptotic Bcl-xL is to maintain metabolite exchange across the outer mitochondrial membrane under stress conditions, and malfunction of which causes membrane depolarization and apoptosis (Vander Heiden *et al.*, 2001). It has been recently shown that Bcl-xL is associated with cisplatin chemoresistance in cancer cells (Villedieu *et al.*, 2007), and drug sensitization occurred with introducing Bcl-xL small interfering RNA (Lei *et al.*, 2007). Interestingly, our data suggested possible sensitization on melanoma towards apoptosis by simultaneously targeting anti-apoptotic Mcl-1 and Bcl-xL with FAS inhibitor.

Cytochrome *c*, the respiratory component in mitochondria, is one of the apoptotic factors discharged into the cytosol, recruiting apoptosis protease activating factor (Apaf-1) and procaspase-9 to form apoptosome. This activates caspase-9, downstream caspase-3 and other effector molecules to bring about cell death (Rodriguez and Lazebnik, 1999). We found in this study that after the FAS inhibitor treatment, cytochrome *c* was released (Fig. 3.15) and caspase-9 was activated (Fig. 3.13), indicating the execution of the mitochondrial apoptotic pathway. Apart from cytochrome *c*, the FAS inhibitor also induced Smac/Diablo release (Fig. 3.15).

Smac/Diablo is a 21kDa mammalian mitochondrial protein which is released into the cytosol upon mitochondrial stress (Du *et al.*, 2000; Verhagen *et al.*, 2000) and competes with caspases for binding of IAPs (inhibitor of apoptosis proteins) to relieve the inhibitory effect of the IAPs on caspases (Srinivasula *et al.*, 2001). In mammalian cells, the IAP family members include C-IAP1, C-IAP2, XIAP, survivin, livin and NAIP. Recent reports have shown that IAP proteins are over-expressed in cancer cell lines and primary tumors and play a very crucial role in cancer progression (Altieri *et al.*, 1999; Tamm *et al.*, 2000). Our data showed that cerulenin and C75 significantly depleted survivin and XIAP proteins (Fig. 3.16). Survivin, the smallest mammalian member of IAP with molecular mass of 16.5 kDa, is highly expressed in fetal development and malignant cancer cells (Reed & Reed, 1999). Through caspase-3 inhibition, survivin interacts with microtubules in mitosis,

overcomed G_2/M-phase checkpoint to favour aberrant progression of transformed cells (Li *et al.*, 1998). *In vivo* studies also showed that apoptosis was inhibited with transgenic expression of survivin in melanoma cells (Grossman *et al.*, 2001). Blockade of survivin expression by introducing small interfering RNA induced caspase-dependent apoptosis in colorectal cancer (Williams *et al.*, 2003). Besides survivin, as mentioned before, XIAP was also found to be depleted after the inhibitor treatment.

XIAP (X-chromosome-linked IAP) is a 57kDa protein that suppresses cell death by inhibiting certain downstream caspases, targeting pro-apoptotic proteins for degradation and activating prosurvival signaling cascade via TAK1 (MAPKKK) (Liston *et al.*, 2003). Treatment on cancer malignancy by antisense oligonucleotdies against XIAP has been shown effective *in vitro* and is currently under evaluation in clinical trials (Schimmer *et al.*, 2006). Specific downregulation of XIAP and Bcl-2 by RNAi sensitized multi-drug resistant MCF-7 human breast cancer cells to chemotherapeutic agents (Lima *et al.*, 2004). Molecular analysis has revealed that the amino-terminal residues of Smac/Diablo interact with the BIR3 region of XIAP to antagonize it from inhibiting caspase activation (Liu *et al.*, 2000). As found in this study, the FAS inhibitor caused mitochondrial release of Smac/Diablo, so that it might compete with IAPs survivin and XIAP from binding to caspase substrates and so rendered the caspases active. The caspase activations might also be enhanced due to simultaneous depletion of IAPs.

Previous studies on cerulenin- and C75-induced apoptosis mostly focused on the mitochondria-mediated apoptotic signaling cascade. Recently, Bandyopadhyay *et al.* (2006) investigated the apoptotic pathway by using FAS siRNA and microarray analysis, and colleagues showed recently that the death receptor pathway was also important in the induced apoptosis in the breast cancer cells, with elevation of mRNA levels of the proapoptotic genes BNIP3, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and death-associated protein kinase 2 (DAPK2). Our data also showed that both cerulenin and C75 elicited the death-receptor apoptotic pathway in A-375 cells, with elevated tumor necrosis factor receptor DR5, rather than TRAIL, and activation of caspase-8 (Fig 3.11).

A-375 and other human melanoma cell lines are TRAIL-resistant. Exogenous administering TRAIL/Apo2 did not induce PARP cleavage and DNA fragmentation (Chawla-sarkar *et al.*, 2002). The mechanisms of how malignant cells and normal cells become resistant to TRAIL-induced apoptosis have not yet been completely elucidated and several factors are known to alter their responses to TRAIL, including hypoxia, matrix metalloproteinases (MMPs) and cytokines present in the tumor microenvironment (Mace *et al.*, 2006). DR5 is one of the receptors for TNF-related TRAIL. DR5 mediates death receptor apoptotic pathway and has been targeted for cancer therapy in variety of cell types (Thorburn, 2004). It was reported that deletion of DR5 developed resistance to

TRAIL-mediated apoptosis and response to DNA-damaging stimuli (Wu *et al.*, 2000). Nevertheless, our studies showed that cerulenin and C75 elevated DR5 level (Fig. 3.11), and administering TRAIL molecules enhanced sensitivity to apoptosis induced by 10 and 20 μM of FAS inhibitors in TRAIL-resistant A-375 cells (Fig. 3.12). At least, such findings can prove the important role of DR5 in FAS inhibitor-induced apoptotic signaling cascade and its role to abolish TRAIL resistance in A-375 cells.

Bid is the BH3 domain-only protein that acts as a death agonist of the Bcl-2 family (Wang *et al.*, 1996). Bid is localized in the cytosol as an inactive precursor (Luo *et al.*, 1998) and is cleaved from its full length 22 kDa to its active form 15 kDa by caspase-8 in the death-receptor-mediated signaling pathway. Truncated Bid is then translocated to mitochondria to sensitize mitochondrial permeabilization and cytochrome *c* release (Gross *et al.*, 1999), relaying an apoptotic signal from the cell surface to mitochondria. Although we could not detect the truncated Bid in the immunoblot, we found in this study depletion of full length Bid in the cerulenin- and C75-treated melanoma cells (Fig 3.18). On the other hand, our caspase inhibitor studies showed that caspase-8 played an initiative role in the caspase-dependent apoptosis induced by the FAS inhibitor (Fig. 3.19). Taken together, we hypothesized that a crosstalk may exist between the extrinsic death-receptor pathway and the intrinsic mitochondrial apoptotic pathway possibly with Bid as the linker. However, the precise molecular mechanism for truncated Bid translocation and the subsequent release of

cytochrome *c* remains to be elucidated.

Finally, we also showed that the apoptotic cascade initiated by the FAS inhibitors was at least caused by their inhibition on FAS activity since molecular depletion of FAS with siRNA also elicited a similar cascade.

In conclusion, the inhibitor retarded FAS activity that resulted in elevation of DR5 and sensitized the melanoma cells to apoptosis. Caspase-8 activation and Bid truncation then relayed the signals from the death receptors to mitochondria. Depletion of Bcl-2 and Mcl-1 was resulted which triggered the mitochondrial membrane depolarization and release of cytochrome *c* to activate caspase-9. In meanwhile, the release of Smac/Diablo and depletion of IAPs relieved the inhibitory effect on executioner caspase-3. Caspase-3, together with other executioner caspases, induced PARP cleavage and caused DNA fragmentation to bring the cells to demise (Fig 4.1).

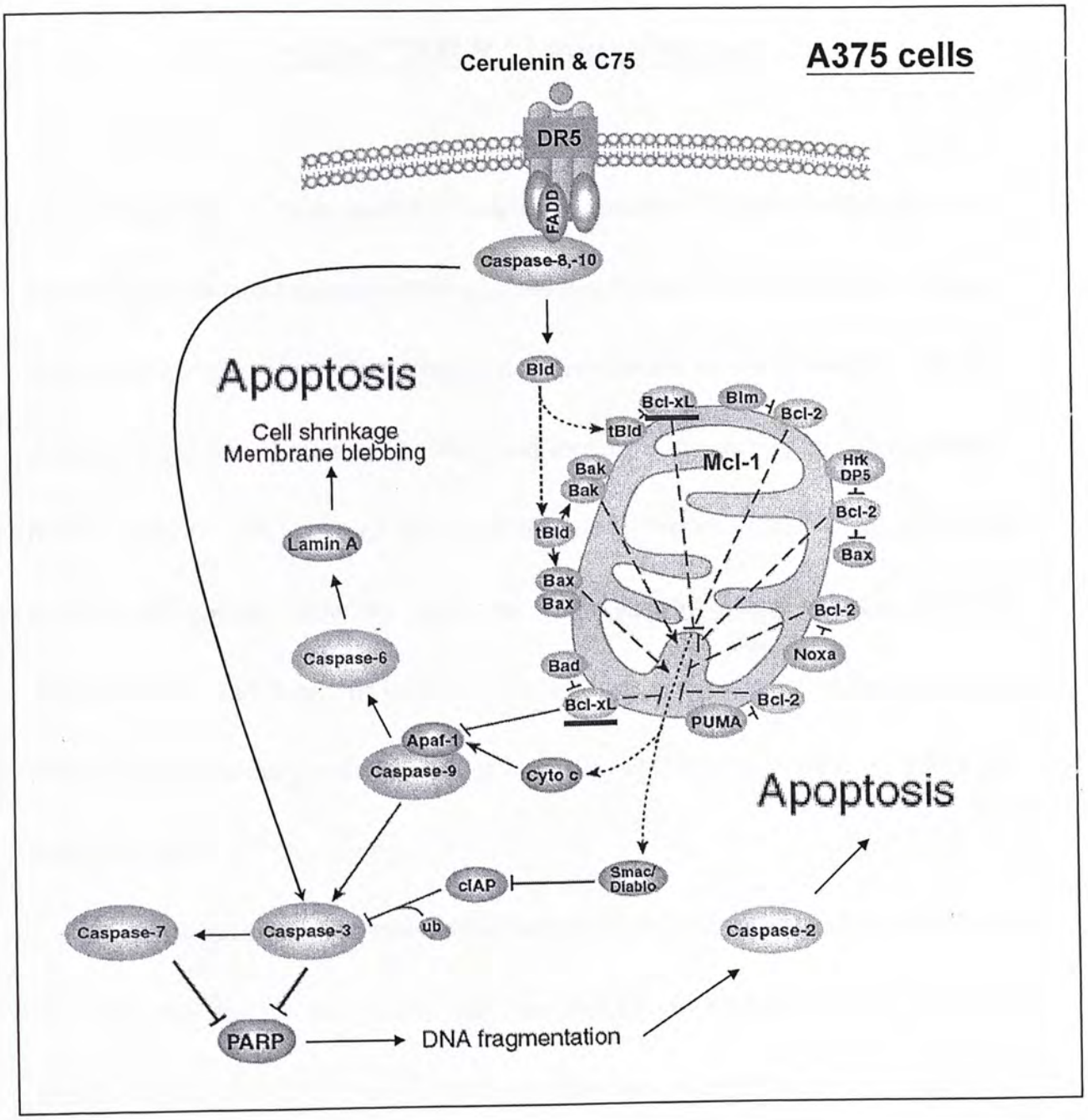


Fig. 4.1 The proposed molecular mechanisms of apoptosis induced by FAS inhibition on melanoma A-375 cells.

CHAPTER 5 Future Prospect

Amongst the currently-used FAS inhibitors cerulenin, C75, triclosan and orlistat, cerulenin was chemical unnstable (Kuhajda, 2000) and exerted effects other than fatty acid inhibition (Lawrence *et al.*, 1999). Despite a synthetic mammalian FAS inhibitor, C75 also induced weight loss (Loftus *et al.*, 2000) and stimulated carnitine palmitoyltransferase-1 (CPT-1) activity with increased fatty acid oxidation (Thupari *et al.*, 2004). Effects of triclosan and orlistat were not promising. Even though 5 alpha-reductase inhibitor dutasteride and COX-2-specific inhibitor celecoxib were found to suppress lipogenesis, the studies were preliminary and not tested on normal counterparts (Schmidt *et al.*, 2007) (Lu & Archer, 2007).

Scientists have paid concerted efforts on developing and discovering more specific novel FAS inhibitors to treat cancer with promising effects. McFadden *et al.* (2005) had recently synthesized C93, a novel small-molecule FAS inhibitor candidate that did not induce weight loss or stimulate fatty acid oxidation. In addition, C93 also exhibited significant antitumor activity and apoptosis against SKOV3 xenografts in athymic mice without significant cytotoxicity to proliferating cellular compartments such as bone marrow, gastrointestinal tract, or skin (Zhou *et al.*, 2007). Detailed molecular pathway of C93-induced apoptosis needed further investigation.

Anticancer research nowadays focused more on chemoprevention with dietary phytochemicals (Surh, 2003) than using pharmacological therapeutic agents. Epigallocatechin-3-gallate (EGCG), an antioxidant and chemopreventive polyphenol highly abundant in green tea, was identified to be a potent natural FAS inhibitor by selectively suppressing lipogenesis and inducing apoptosis in prostate cancer cells (Brusselmans *et al.*, 2003). Further signaling studies revealed that EGCG-induced apoptosis involved JNK activation, with elevated Bax expression, mitochondrial membrane potential changes, and activation of caspase-9 and caspase-3, in breast cancer cells (Zhao *et al.*, 2006b) (Vergote *et al.*, 2002). EGCG also took part in ROS generation and suppressed intracellular ATP levels in MCF-7 cells, leading to either apoptosis or necrosis (Hsuuw and Chan, 2007). In addition to epigallocatechin-3-gallate, five other flavonoids, namely luteolin, quercetin, kaempferol, apigenin, and taxifolin, also markedly suppressed cancer cell lipogenesis and induced PARP cleavage in both breast and prostate cancers (Brusselmans *et al.*, 2005b). Supplement of exogenous FAS end-product palmitate, nevertheless, rescued the cells from flavonoid-induced apoptosis. This provided insights that more specific and less harmful FAS inhibitors might be identified in natural food products in the near future.

Recent studies have focused on the synergistic effects between molecular targets in carcinogenesis and some currently used anticancer drugs, so as to enhance efficacies of the

drugs on cancer patients with lesser side effects. Some cancer cells have got defects in apoptotic signaling, rendering the cells resistant to drug-induced cell death. Several reports have shown that blockage of FAS expression sensitized these chemoresistant cancer cells towards some anticancer drugs, and new pathways of FAS inhibition relating p53 and estrogen receptor have been proposed (Menendez *et al.*, 2004 a-h) (Menendez *et al.*, 2005 a-f). However, researches targeting FAS for cancer therapy have mainly focused on breast and prostate cancers, and there is no such similar report on melanoma cells, though with moderately high FAS level. We are the first group to identify the molecular mechanisms of how FAS inhibition induces apoptosis using A-375 as a model melanoma cell line. Further elucidations on the molecular mechanisms of action in apoptosis and cell cycle imposed by FAS inhibition can facilitate further research on the synergism between the currently used anticancer drug and potential FAS inhibitors from natural products for effective treatment of melanoma patients in the near future.

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