Baicalein Induces Caspase-dependent Apoptosis in Human Melanoma A375 Cells Associated with Elicitation of Intrinsic and Extrinsic Apoptotic Pathways

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Abstract of thesis entitled:

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Baicalein is a flavone isolated from the dried root of a traditional Chinese

medicinal herb *Scutelleria baicalensis* Gorgi, which is used in China for treating

cancers and inflammatory diseases. However, its mechanism of action has not been

fully elucidated. In this study, the growth inhibitory effect of baicalein was first

tested on a panel of cancer cell lines of different histotypes *in vitro*. Human

melanoma A375 was then found to be the most responsive cell line, substantiated by

the lowest IC_{50} of 37.5 μM.

Baicalein exhibited a dose-dependent growth inhibition on the melanoma cells

first arresting cell cycle at S phase. Apoptosis was then followed, indicated by of the

cleavage of PARP and DNA fragmentation. Caspase-8, the initiator caspase of the

extrinsic apoptotic pathway, takes the major role in eliciting the molecular cascade in

the apoptosis. This is evidenced by its activation at 24 h when PARP cleavage and

DNA fragmentation was first observed. On the other hand, caspase-9, the initiator

caspase of the intrinsic mitochondrial pathway, was only activated during the

exacerbation phase of apoptosis at 48 h (% of apoptotic cells> 20%). In addition,

sequential activation from caspase-8 to caspase-3 was illustrated by the inhibition of

caspase-3 activity after the co-administration of caspase-8 inhibitor,

Z-IE(OMe)TD(OMe)-FMK with baicalein. Elevation of the death receptors

TRAIL-R1 (DR4) and TRAIL-R2 (DR5), but not their ligand, TRAIL, was observed,

which was coherent with the caspase-8 activation. Furthermore, the sensitization of

the melanoma cells to sub-lethal dose of TRAIL (12.5 ng/ml) was observed after the

baicalein treatment, possibly due to the elevation of the death receptors.

Besides the extrinsic death receptor pathway, the intrinsic mitochondrial

pathway was also found to be activated during the exacerbation phase of apoptosis.

This is evidenced by mitochondrial membrane depolarization, release of cytochrome

c from mitochondria into the cytosol, and proteolytic activation of caspase-9.

Surprisingly, there was no observable change of Bcl-2 family proteins, including

antiapoptotic Bcl-2 and Bcl-X_L; proapoptotic Bax, Bad, Bim$_{EL}$ and Bid, which have

been widely recognized as the central mediators of the intrinsic apoptotic pathway.

On the other hand, reactive oxygen species (ROS), which has been reported

previously to cause mitochondrial dysfunction, was found to be elevated in the

baicalein-treated cells. Addition of antioxidant Trolox^{TM} was not only able to

scavenge the ROS produced, but also able to restore the depolarized mitochondrial

membrane and release of cytochrome *c*.

Findings from this study provide new knowledge about the mechanisms of baicalein in controlling cell growth, which is fundamental for the future development of this phytochemical as an anticancer regimen. Furthermore, the synergism between baicalein and TRAIL may open a new research direction to treat melanoma cells, which most of them are insensitive to TRAIL and result in poor prognosis.

黃芩素透過誘發外源性及內源性細胞凋亡途徑於人類惡性黑色素腫瘤細胞 A375 中引起由凋亡蛋白控制之凋亡

摘要

　　黃芩素乃其中一種由抗炎中藥黃芩所提取的黃酮類化合物。其抗炎及抗腫瘤的生物活性已被肯定。然而，其抑制癌細胞生長之機制仍未被清楚闡釋。為了分析黃芩素誘發細胞凋亡的機制，其抑制作用首先於不同種類之癌細胞上進行體外實驗測試。於六種不同的癌細胞當中，黃芩素對人類惡性黑色素腫瘤細胞的抑制作用最為明顯，其 IC_{50} 為 37.5 μM。故此，A375 黑色素腫瘤細胞便選定作進一步的分析。

　　黃芩素能中止 A375 腫瘤細胞的細胞週期於週期內之 S 期，並誘發細胞凋亡。黃芩素亦能活化細胞中之凋亡蛋白，其中凋亡蛋白-8 乃眾多凋亡蛋白中最先被活化。故此，我們推論出黃芩素可能透過誘發外源性細胞凋亡途徑而引起黑色素腫瘤細胞之凋亡。進一步的研究發現黃芩素能提升 A375 癌細胞之死亡受體 4 及死亡受體 5 之表達，並能增加黑色素腫瘤細胞對於腫瘤壞死因子相關凋亡誘導配體 (TRAIL) 所主導之凋亡作用之敏感性。

　　凋亡蛋白-9 之活化同時亦提示出黃芩素有可能從內源性細胞凋亡途徑引發起黑色素腫瘤細胞之凋亡。實驗結果顯示，黃芩素能引

起黑色素腫瘤細胞之線粒體膜之去極化，並引起細胞色素 c 從線粒體中釋放到至細胞質中。然而，從實驗結果中發現，黃芩素所造成粒線體外膜的通透性增加，並非由 Bcl-2 家族蛋白所調控，乃由黃芩素所引起之活性氧化物所做成。而抗氧化物 Trolox™ 之處理能逆轉黃芩素對黑色素腫瘤細胞之影響，反證活性氧化物對黃芩素引起之內源性細胞凋亡途徑的重要性。

綜合以上結果，黃芩素能於黑色素腫瘤細胞 A375 中同時誘發外源性及內源性細胞凋亡途徑，以達至抑制癌細胞生長之效果。我們寄望研究結果能進一步豐富對黃芩素引起的凋亡機制之知識，並希望將來進一步之研究能開啟對抗藥性癌細胞之嶄新之研究方向。

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List of Abbreviations

12-LOX	12-lipoxygenase
AMC	7-Amino-4-methylcoumarin
ANOVA	Analysis of variance
Ap	Apoptotic population
Apaf-1	Apoptotic protease activating factor-1
ATCC	American Type Culture Collection
Bai	Baicalein
BCA	Bicinchoninic acid
BH	Bcl-2 homology
BSA	Bovine serum albumin
COXs	Cyclooxygenases
CRD	Cysteine rich domain
Ctl	Control
Cu/ZnSOD	Copper/zinc superoxide dismutase
dATP	2'-deoxyATP
DCF	2',7'-dichlorofluorescein
DcR1	Decoy receptor 1
DcR2	Decoy receptor 2
DED	Death effector domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

DR3	Death receptor 3
DR4	Death receptor 4
DR5	Death receptor 5
DR6	Death receptor 6
EDTA	Ethylene diamine tetraacetic acid.
EMEM	Eagle's Minimum Essential Medium
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FDA	Food and Drug Administration
Fe^{2+} ions	Iron (II) ions
Fe-S proteins	Iron-sulfur proteins
H_2DCF	2',7'-dichlorodihydrofluorescein
H_2DCFDA	2',7'-dichlorofluorescin diacetate
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
JC-1	6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide
LOXs	lipoxygenases
MMP	Mitochondrial membrane permeabilization
MnSOD	Manganese superoxide dismutase
mtDNA	Mitochondrial DNA
MTT	3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
NADH	Reduced form of nicotinamide adenine dinucleotide
PAGE	Polyacrylamide gel electrophoresis

PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide
Rh123	Rhodamine123
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNase A	Ribonuclease A
ROS	Reactive oxygen species
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
SODD	Silencer of death domain
tBid	truncated Bid
TBS	Tris-buffered saline
TCM	Traditional Chinese Medicine
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TNFα	Tumor necrosis factor alpha
TRADD	TNFR-associated death domain
TRAF-2	TNFR-associated factor 2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R1	TRAIL -receptor 1
TRAIL-R2	TRAIL -receptor 2
TRAIL-R3	TRAIL -receptor 3
TRAIL-R4	TRAIL -receptor 4

TRAMP	TNF receptor-related apoptosis-mediating protein
VEGF	Vascular epidermal growth factor
WHO	World Health Organization
$\Delta\Psi_m$	Mitochondrial membrane potential

Chapter 1. General Introduction

1.1 Overview of Cancer

Every organism comprises of cells. Cell is the basic unit, for single-celled organisms like amoeba to complex organisms like human. In order to maintain the normal functions of an organism, myriad cellular activities such as proliferation, differentiation and cell death, have to be precisely coordinated and monitored (Sears and Nevins, 2002). Given the huge amount of events that need to be regulated within cells, abnormalities in these processes are not uncommon (Becker *et al.*, 2003).

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. The loss of balance of these two events will result in abnormal conditions (Thompson, 1995). The imbalance, if not corrected properly, eventually leads to the progressive increase in the number of dividing cells, forming tumors (neoplasm). Carcinogenesis, a term generally describes the formation and development of neoplasia, can be occurred spontaneously or induced by carcinogenic agents (Pitot and Dragan, 1991). Based on the differences in growth patterns, tumors are classified as either benign or malignant. Benign tumor belongs to the kind of neoplasm that eventually self-limited in its growth, and lacks of capabilities to invade other surrounding or distant tissues, i.e. metastasis (Cotran *et al.*, 1999). Contrarily, malignant tumor, also known as cancer, possesses the

metastatic ability that moves out form the origin, capable to settle at the endothelium

of secondary site, extravasating out of the vessels and forming autonomous lesion

(Egan *et al.*, 1991). Cancer, the outstanding example of pathological conditions of

uncontrolled cell growth, is therefore threatening the world population, which more

than 10 million cases were diagnosed per year globally (Surh, 2003).

Carcinogenesis is a multistep process that probably results from the action of

any one or a combination of chemical, physical, biologic, and genetic insults to cells.

The process of carcinogenesis may be divided into at least three stages: initiation,

promotion, and progression (Pitot, 1993). As carcinogenesis is a process that

characterized ultimately by aberrant, uncontrolled cell proliferation, non-lethal DNA

damage becomes a key requirement for the transformation of normal cells into

neoplastic cells (Malejka and Tretyakova, 2006). In addition to the presence of DNA

damage which is either spontaneous or caused by different types of carcinogens,

defects in DNA repair are also major contributors to malignant transformation. The

knowledge that differential DNA repair capacity exists is vital to our understanding

that defects in these repair processes play a vital role in carcinogenesis by increasing

the rate of mutation and thus the rate of neoplastic progression (Betram, 2000). In the

molecular aspect of carcinogenesis, cancer-causing genes may belong to either

oncogenes or tumor suppressor genes, and the initiation of carcinogenesis will be the

net effect after the complex interaction between activation of oncogenes and inactivation of tumor suppressor genes (Zhivotovsky and Orrenius, 2006). Oncogenes are usually derived from normal cellular counterparts, the proto-oncogenes, which function as essential components of intracellular signaling pathways, for instance, ligands, receptors, molecular switches and cytoplasmic signal transducers in a non-mutated state. Activation from proto-oncogenes to oncogenes mobilizes signaling cascade and leads to uncontrolled cell proliferation through the "change-of-function" mutations (Cline, 1996). Tumor suppressor genes, as the name suggested, protect cells from undergoing malignant transformation. Tumor suppressor genes function by one of the following mechanisms: protect the genome from mutagenic events, impede dysregulated progression through the cell cycle, induce apoptosis in cells that escape normal cell cycle controls, and inhibit cellular migration and metastasis. Historically, tumor suppressor genes have been described to acquire loss of function mutations or deletions leading to their inability to hinder malignant transformation (Hayslip and Montero, 2006). The carcinogenic mutations found in the two subsets of genes retain and perpetuate in neoplastic cells, most likely by the escape from regulatory mechanisms and the defects in DNA repair. This allows the accumulation of genetic alterations and thus more severe transformation to take place. This marks the state of progression in carcinogenesis. Although malignant

tumors are monoclonal in origin, by the time as they progress, metastasize and being detectable, the cell population has already become heterogeneous. The heterogeneity observed substantiates the significance of multiple alterations accumulated in different subclones of cancer cells with different characteristics (Yokota, 2000). Taken together, the genes involved in carcinogenesis comprise a specific subset of the genome whose products are involved in cellular activities that alter the normality of proliferation, such as the progression of a cell through the cell cycle, adhesion of a cell to its neighbors, apoptosis, and the repair of DNA damage (Karp, 2003). Regarding to the genome instability in carcinogenesis, restoration of genetic alternations back to their normal status represents important clues to re-transform the cancer cells back to normal (Aziz *et al.*,2003). Beside restoration of genomic normality, the elimination of the transformed cells also serve an attractive strategy in combating in cancer (Zönig et *al.*, 2001).

1.2   Apoptosis and cancer

Apoptosis is also known as programmed cell death. It represents a universal and exquisitely efficient cellular suicidal pathway. Apoptosis is a genetically-predeposited programme that is essential for embryonic development, immune-system function and the maintenance of tissue homeostasis in multicellular

organisms (Adams, 2003). Defective apoptotic pathways disrupt the balance between cell proliferation and cell death and thus lead to disease like cancer (Fesik, 2005). Apoptosis is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms (Elmore, 2007). Morphologically, apoptotic cells generally display characteristics such as cell shrinkage, cytoplasmic condensation, chromatin segregation and condensation, membrane blebbing, and the formation of membrane-bound apoptotic bodies (Loo and Rillema, 1998). The dissembled materials are packed inside the apoptotic bodies for subsequent engulfment by neighbouring cells and phagocytes, thus preventing unnecessary inflammatory response (Savill and Fadok, 2000). The morphological characteristics can be observed at different stage of apoptosis, and the detections of these changes have become solid evidence of apoptosis. The morphological changes are in fact resulted from the intrinsic biochemical changes that occur within apoptotic cells, such as DNA fragmentation and caspase activation. Caspases, or cysteine aspartyl-specific proteases, are the proteases that participate in the demolition process of apoptotic cells. The executioner caspases (caspase-3, -6 and -7) are ultimately responsible for the breaking down of cellular substrates, yet they needed to be first proteolytically activated by initiator caspases (caspase-8 and -9) (Thornberry, 1998; Adams, 2003). Apoptosis can be initiated by two major pathways:

involving either the release of cytochrome *c* in the mitochondria (the intrinsic mitochondrial pathway), or mediating by the activation of death receptors which sense the death signals by the binding with death-inducing ligands (the extrinsic death receptor pathway) (Bröker *et al.*, 2005). Both branches of apoptosis will be discussed in detail section 1.3a and 1.3b. Although the involvement of caspases has been regarded as an indispensable feature in apoptosis, more and more recent studies have demonstrated that caspase-independent apoptosis also played a role in eliminating unwanted cells in an organism. In the absence of caspase-activation, apoptosis can also be executed via the actions of other proteases that could also elicit apoptosis-like cell death (Stoka *et al.*, 2001). The discovery of various models of apoptosis opens an alternative research direction in the cell death pathways of cancer (Bröker *et al.*, 2005), which will not be discussed here.

Myriad studies have shown that many types of cancers are defective in eliciting the apoptotic pathway. The evasion of apoptosis has been regarded as one of the six hallmarks of cancer that endows the cancer cells with ability of uncontrolled growth and metastasis (Debatin and Krammer, 2004; Hanahan and Weinberg, 2000). For instance, the tumor suppressor protein p53 is often mutated in many types of cancer. Accumulation of the wild-type p53 protein in the cell results in upregulation of apoptosis or cell cycle arrest related proteins (Hailfinger *et al.*, 2007). It is therefore

not surprising that cancer cells are having mutated or even deleted expression of p53.

Another common apoptosis-related defects found in cancer cells would be the overexpression of a protein called Bcl-2. Bcl-2 is an antiapoptotic protein that facilitates survival of cells (details in section 1.3.2.i). Overexpression of Bcl-2 is commonly found in metastatic tumors, including breast (Lee *et al.*, 2007) and colon (Li *et al.*, 2007) carcinomas. Therefore, agents that are able to restore the normality of apoptotic pathway in cancer cells have the potential for effectively treating cancer, either use as a therapeutic drug or adjuvant therapy.

1.3.   Roles and regulations of caspase-dependent apoptosis

Caspases are a family of cysteine proteases that cleave their substrates specifically following an aspartate residue (Thornberry and Labzenik, 1998; Kumar, 2007). Fourteen caspases have been identified in mammalian cells, of which 11 enzymes are known in humans (Lamkanfi *et al.*, 2002). These enzymes are synthesized as precursor inactive zymogens that, upon receiving apoptotic signals, proteolytically process to generate subunits containing the active enzymes (Degterev *et al.*, 2003). Recent studies have revealed that the cleavage of the zymogen is not always a forcible requirement for caspase activation, but all activated caspases can be detected as cleaved fragments in apoptotic cells (Degterev *et al.*, 2003; Fuentes-Prior

and Salvesen, 2004). The caspase-dependent apoptotic cascade basically involves two subtypes of caspases: the initiator caspases (caspase-2, -8, -9 and -10) that activate the downstream effector caspases (caspase-3, -6 and -7) which are responsible for the direct destruction of cellular materials. From the two subsets, caspase-9 is regarded as the leading caspase in the intrinsic mitochondrial pathway; whereas caspase-8 is the key initiator of death-receptor-mediated apoptosis (Kumar, 2007). Other enzymes (caspase-1, -4, -5, -11, -12 and -14) are mainly involved in cytokine maturation and inflammation (Vermeulen *et al.*, 2005; Chang and Yang, 2000). By the action of caspases, two apoptotic pathways can be triggered: the extrinsic death-receptor pathway and the intrinsic mitochondrial pathway.

1.3.1 *Extrinsic death receptor pathway*

The extrinsic apoptotic pathway is activated by the engagement of death receptors on the cell surface with their death-inducing ligands. Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily. Twenty proteins are classified into this family which has broad range of biological functions including cell death and survival regulation, differentiation and immunoregulation (Walczak and Krammer, 2000). Besides sharing one to four cysteine rich domains (CRDs) extracellularly, members of this family also share an 80-amino-acid cytoplasmic domain called the 'death domain', which is of crucial importance in the transmission

of death signals to the intracellular signaling cascade (Bossen *et al.*, 2006). Six

members have been identified in this family including TRAMP (DR3/Apo-3), DR6,

and the best characterized members of the family CD95 (APO-1/Fas), TNF receptor

1 (TNFRI), TRAIL-R1 (DR4) and TRAIL-R2 (DR5). In order to elicit a cell death

signal from the cell surface to the cytoplasm, engagement of surface death receptors

to their corresponding death-inducing ligands is necessary. The TNF family members

CD95 ligand /FasL, TNFα and TNF-related apoptosis-inducing ligand (TRAIL) are

the corresponding ligands of the four best characterized receptors mentioned above.

With some less characterized exceptions, almost all death-inducing ligands are Type

II transmembrane proteins. The ligands can also exist in free, soluble form by the

proteolytic action of metalloproteases in the environment (Herr *et al.*, 2000). They

function in an autocrine and paracrine manner to mediate the ligation with the

surface death receptors (Sheikh, and Fornace Jr., 2000). Although ligation between

death receptor and corresponding ligand is believed to be the activation of extrinsic

pathway, some evidence has also suggested the possibility of ligand-independent

activation of death receptors (Sheikh *et al.*, 1998; Peter and Krammer, 2003)

Fig. 1.1 illustrates a simplified diagram of the extrinsic death receptor pathway.

Upon activation of death receptors by death-inducing ligands, the ligands first

trimerize to form a trimeric ligand cluster before ligation to corresponding death

receptors. The engagement of ligand cluster to death receptor further induces the trimerization of death receptors from the original monomeric conformation. Once the receptor is activated, the adaptor protein FAS-associated death domain (FADD) will then be recruited to the activated receptors via the interactions of there respective death domains (Sheikh and Huang, 2004). In case of the TNF-R1/TNFα death receptor-ligand pair, the recruitment of another adaptor protein to the death receptor takes place before the recruitment of FADD (Hsu et al., 1995), which will be discussed in detail in the next section. Once associated with the death receptor, the FADD molecule can further interact with procaspase-8 via the crosslinking between the death effector domain (DED) of FADD and procaspase-8 or -10, leading to activation of the initiator caspases by oligomerization-mediated activation (Askkenazi and Dixit, 1999). The association of the death-inducing ligand, death receptor, adaptor protein and initiator caspase forms an active, death inducing complex called death-inducing signaling complex (DISC). The DISC formation thus activates the downstream caspases and the subsequent apoptotic events by the active initiator caspases (Debatin and Krammer, 2004). It can either directly proteolytically activate the effector caspase-3 (type I cells) or cleave the carboxy-terminal part of a BH3 domain-only proapoptotic member of the Bcl-2 family namely Bid (type II cells) (Scaffidi et al., 1999). Consequently, translocation of the truncated Bid (tBid) to the

mitochondria activates, in combination with Bax, the intrinsic apoptosis pathway

described in section 1.3 2.i.
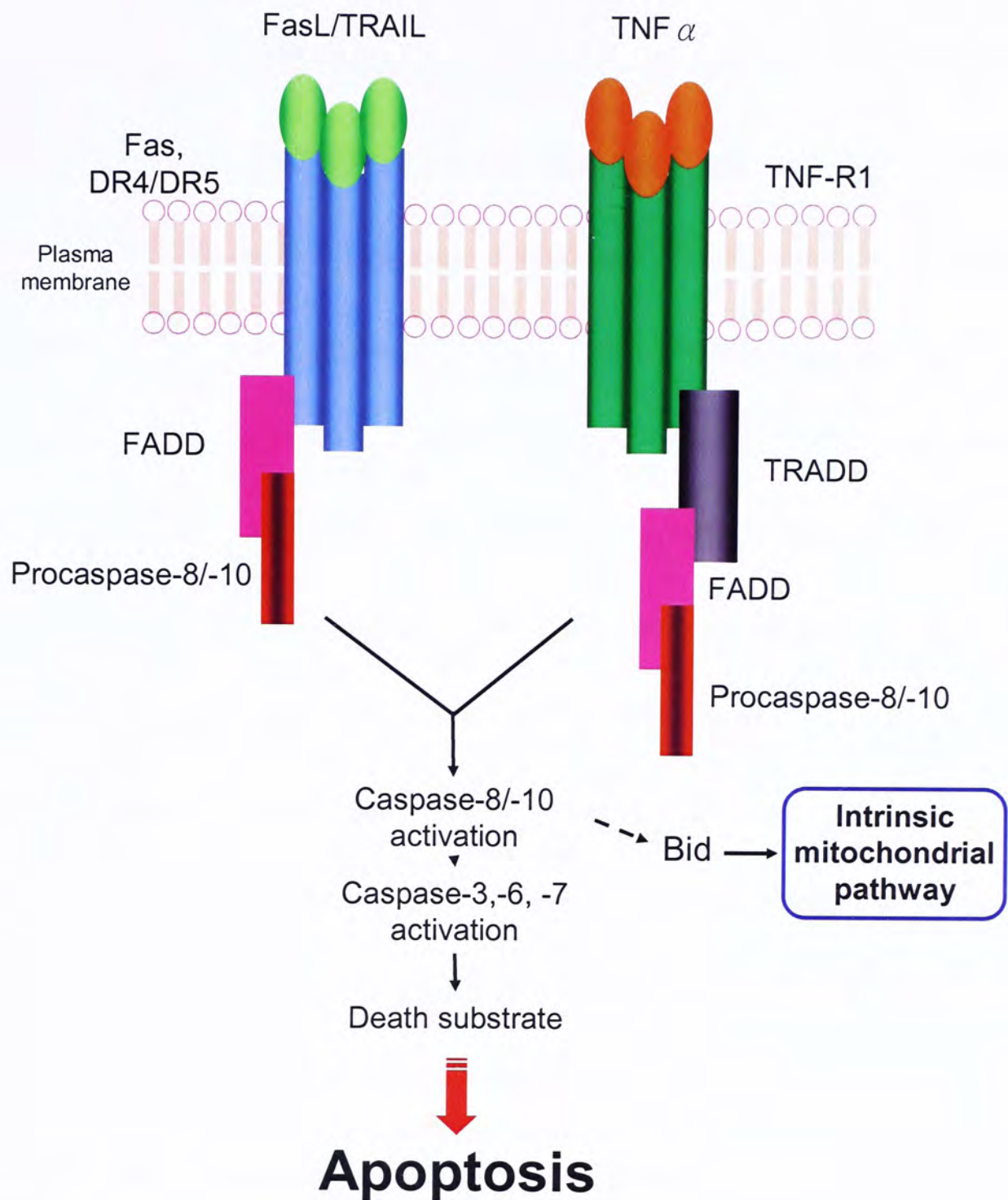
Fig. 1.1   Schematic diagram showing the components of the extrinsic death receptor

Three well-characterized death-inducing ligands, TNFα, FasL and TRAIL, induce death receptor-mediated apoptosis by the ligation to their corresponding receptors, recruitment of adaptor proteins and initiator caspase. Unlike FasL and TRAIL, TNFα recruits a specific adaptor protein, TRADD, prior to the DISC formation. (Modified from Sheikh and Fornace Jr., 2000; Sheikh and Huang, 2004)

i.   TNFR1 and TNFα

TNFα is protein which exists as a homotrimer in solution. Its bioactivity is

mainly regulated by soluble TNFα–binding receptors (Bemelmans *et al.*, 1996).

TNFα acts via two distinct receptors, TNFR1 and TNFR2. However, TNFR1

manipulates most of the biological activities of TNFα. Therefore, TNFR1 is in fact

expressed on all cell types, and is capable of inducing apoptotic cell death (van

Horssen *et al.*, 2006). Besides this apoptotic signaling, TNFR1 also possesses the

ability to transduce cell survival signals. The TNFR1-mediated apoptotic signaling is

elicited firstly by the death receptor-ligand ligation. Upon binding of the homotrimer

TNFα, the silencer of death domain (SODD) protein will be released and allows

TNFR1 to trimerize (Sheihk and Huang, 2004), TNFR-associated death domain

(TRADD) then binds to the death domain of TNFR-1 before recruiting the adaptor

proteins receptor interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and

eventually FADD (Rath and Aggarwal, 1999). FADD then binds to initiator caspases,

such as procaspase-8, and initiates a protease cascade leading to apoptosis.

Nonetheless, the involvement of FADD in TNFα-mediated signals transduction

becomes controversial, as suggested by Jin and El-Deiry (2006).

ii.   CD95/Fas and CD95 Ligand/FasL

Fas and FasL is another well-characterized death-receptor pair in the TNF receptor and TNF family. This pair of death receptor-ligand pair has been implicated in the maintenance of lymphocyte homeostatsis and immunoregulation. Fas is ubiquitously expressed yet FasL expression is only restricted to lymphoid organs and immune-privileged tissues (O'connell *et al.*, 2001). Both the cytokine and its receptor can exist in membrane and soluble forms, but only engagement of membrane-bound form leads to the activation of caspase-8 via DISC formation (Siegel *et al.*, 2000). The Fas/FasL death inducing signaling is very similar to that of TNFR1/TNFα, yet on the aspect of FADD recruitment, unlike TNFR1/TNFα, Fas/FasL ligation complex directly recruit FADD to form DISC, without recruitment of other adaptor proteins (Peter and Krammer, 2003; Sheikh and Huang, 2004). Beside this difference, the death signal induction pathway is similar to that of TNFR1/TNFα.

iii.   TRAIL-R1(DR4), TRAIL-R2 (DR5) and TRAIL

TRAIL and its corresponding receptor DR4 and DR5 is another pair of the death-inducing ligand receptor pair. Unlike TNFα and FasL, TRAIL appears to have selectivity in triggering apoptosis towards tumor cells, leaving normal tissue unaffected (Suliman *et al.*, 2001). The TRAIL resistance observed in normal tissues

is postulated to be the expression of TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which are the decoy receptors of TRAIL that lacks the cytoplasmic death domains and thus the ability to induce apoptosis (Zhang *et al.*, 2001). Some of the TRAIL-resistant cancer cells were also identified with overexpression of the decoy receptors, which compete with the death receptors for TRAIL engagement. The phenotypic composition of death and decoy receptors is therefore crucial in determining TRAIL sensitivity in cancer cells, which has been previously demonstrated in TRAIL-resistant prostate, breast and lung cancer cells (Sanlioglu *et al.*, 2007).

Having longer history than TRAIL, TNFα and Fas have been evidenced to have extensive antitumor activity *in vitro* and has been employed as a potential therapeutics *in vivo* (Ghobrial *et al.*, 2005). Unfortunately, both cytokines possess different degrees of toxicity that impair normal physiological functions. Recombinant TNFα has proven with extreme toxicity due to vascular inflammatory response (Wallach *et al.*, 1999; Zelster *et al.*, 2000). Meanwhile, tumor-derived FasL is found to induce severe toxicity in lymphoid organs of the host (Nagarkatti, 2000). The differential effects exhibited by the three members of TNF family have illustrated that TRAIL is an attractive potential therapeutics that worth-investigating.

The molecular pathway of activation of DR4 and DR5 is very similar to the Fas

counterpart, which also involves the homotrimer formation of TRAIL, trimerization

of death receptors, recruitment of FADD and the activation initiator caspases.

However, the actual activation of death receptor merely depends on the histotypes of

cancer cells and the type of death receptor inducers (Takeda *et al.*, 2007). For

instance, sulforaphane can sensitize osteosacroma cells to TRAIL through the

induction of DR5 expression (Matsui *et al.*, 2006); whereas in renal tumor cells, the

sensitization towards TRAIL by sub-lethal dose of doxorubicin was due to the

enhanced expression of DR4 (Jin *et al.*, 2007). It can be appreciated that although

TRAIL was found to be an efficacious therapeutic in treating cancers, the complexity

behind the apoptotic mechanisms induced in cancer cells is still awaiting for

explorations.

1.3.2   Intrinsic mitochondrial pathway

Unlike the extrinsic apoptotic pathway which responds primarily towards

extracellular death signals, the intrinsic apoptotic pathway is triggered by both

extracellular and intracellular stresses, such as growth-factor withdrawal, hypoxia,

DNA damage and oncogene induction (Crighton and Ryan, 2005). In response to

these stress-induced death signals, series of biochemical events are induced

ultimately in the mitochondria which include permeabilization of the outer

mitochondrial membrane, the release of cytochrome *c* and other apoptotic molecules,

the formation of apoptosome that comprised of cytochrome c, apoptotic protease activating factor 1 (Apaf-1) and caspase-9, as well as caspase activation (Budihardjo *et al.*, 1999). As mitochondrial dysfunction and the subsequent release of apoptogenic factors mark the "point of no return" in apoptosis (Hangartner, 2000). The normal functions and the integrity of the mitochondria should therefore be precisely regulated.

i.   Bcl-2 family of proteins

The Bcl-2 family of proteins has been regarded as the central regulator of mitochondrial pathway (Ghobrial et al., 2005). As an important apoptotic regulator, this family plays indispensable roles in caspase activation, and its opposing factions of anti- and pro-apoptotic members orchestrate the life-or-death decision (Skommer *et al.*, 2006). Being categorized in the same family, all proteins in the Bcl-2 family contain highly conserved domains, referred to as the Bcl-2 homology (BH) domain (BH1-4), which is essential for the homo- and hetero-complex formation among the family members that directly affecting the apoptosis-induction capacity. Structural and functional analyses revealed that the growing Bcl-2-homologous genes can be divided into at least three distinct subfamilies: the Bcl-2 and the Bax subfamily, and the BH3-only proteins (Adams and Cory, 1998).

The Bcl-2 subfamily, including Bcl-2 and its homolog Bcl-x_L, function as

potential oncogenes that promotes cell survival. The two antiapoptotic proteins modulate cell death primarily by affecting the mitochondrial sequestration of cytochrome c and several other proteins (Tudor *et al.*, 2000). By this, the formation of apoptosome and the subsequent activation of caspases are directly affected. Expression of Bcl-2 proteins also stabilizes the mitochondrial transmembrane potential by regulating the proton flux, inhibits generation of reactive oxygen species (ROS), and prevents cytoplasmic acidification (Schendel *et al.*, 1998).

The Bax subfamily includes the proapoptotic proteins Bax and Bak which act as accelerators of apoptosis. In cells, Bax and Bak change conformation near the time of commitment to cell death and synchronize with the release of cytochrome c. This conformational change of Bax/Bak plays an essential role in the perforation of mitochondrial outer membrane (Antignani and Youle, 2006). Consequently, ion fluxes and consecutive loss of the mitochondrial membrane potential occur.

As the name suggested, the BH3-only proteins carry only one BH3 domain. The well-studied members of this subfamily include Bad and Bid. Proapoptotic Bad protein interacts via its BH3 domain with Bcl-2 and Bcl-x_L to facilitate the actions of apoptotic stimuli (Tudor *et al.*, 2000). Another BH-3 only protein Bid plays a role in linking the death-receptor signaling to the mitochondrial pathway. During the death-receptor mediated apoptosis, Bid is proteolytically cleaved by caspase-8 to a

pro-apoptotic form, the truncated Bid (tBid), from the inactive, intact Bid. tBid appears to interact physically with Bax to mediate a conformational change in the Bax N-terminus (Danial and Korsmeyer, 2004). This event triggers insertion of cytosolic Bax into the outer mitochondrial membrane and thus disrupts the mitochondrial integrity (Eskes *et al.*, 2000).

ii.   Reactive oxygen species (ROS)

ROS has been recently identified to play a regulatory role in apoptosis (Ott *et al.*, 2007). ROS in fact refers to small and highly reactive molecules with unpaired valence shell electrons. ROS includes oxygen ions, free radicals and peroxides (Sen, 2003).It is formed as a natural by-product of the normal metabolism of oxygen, mitochondria, the power house of the cell, would therefore be the primary site of production of ROS by oxidative phosphorylation (Storz, 2007).

The overall system of oxidative phosphorylation includes five large multienzyme complexes, designated as complexes I, II, III, IV and ATP synthase .There are two main sites of superoxide generation in this system: NADH dehydrogenase at complex I, and the interface between CoQ and complex III at the inner membrane of mitochondria (Nishikawa *et al.*, 2007). Once the superoxide is formed, it will rapidly discharge through the mitochondrial matrix to the cytosol where it changes to hydrogen peroxide (H_2O_2). Under normal circumstances, the

cellular antioxidant defence system functions to eradicate the excess ROS produced.

Manganese superoxide dismutase (MnSOD) detoxifies cells from superoxide released into the mitochondrial matrix, and the copper/zinc superoxide dismutase (Cu/ZnSOD) from cytosolic superoxide (Crompton, 1999). Moreover, water- and lipid- soluble vitamins are also powerful antioxidant to serve the purpose. Mitochondria itself can also contribute to the antioxidant defence by the oxidation of succinate (Orrenius et al., 2007).

Physiological problems arise only when the damaging ROS cannot be eradicated by the innate antioxidative mechanisms, which leads to oxidative stress to the cells (Liu et al., 2002). Being such a reactive molecule present in the cells, the accumulation of ROS exerts devastating effects on the cells. Besides oxidizing the polyunsaturated fatty acids in phospholipids on membranous structure (Hernández-Hernández, 2005), damaging genomic DNA (Tsuzuki et al., 2007) and altering protein conformation (Sohal, 2002), ROS also disturbs the normality of mitochondria, leading to the onset of mitochondrial mediated apoptotic pathway.

The first mitochondrial target of ROS is mitochondrial DNA (mtDNA). mtDNA is essential in encoding proteins and RNAs which are indispensable in regulating electron transfer and oxidative phosphorylation (Fleury et al., 2002). Due to the close proximity between the site of ROS production and mtDNA, the histone-free mtDNA

is more susceptible to ROS damage, which directly leads to mitochondrial

dysfunction (Orrenius *et al.*, 2007)

The second target is the oxidation of mitochondrial proteins that are essential in

the functions of electron transport chain. Superoxides are found to impose toxicity by

direct oxidation and inactivation on iron-sulfur (Fe-S) proteins, such as aconitases,

and therefore leading to the release of iron (Fridovich, 1997). The simultaneous

release of Fe^{2+} ions after oxidation of aconitases is accompanied by the release of

H_2O_2, which pose further oxidative burden to the cells (Liochev and Fridovich, 1999).

Besides, oxidation of the enzyme also generates the potent hydroxyl radical, which

can oxidize mitochondrial proteins, DNA, and lipids, thereby exacerbating the

overall oxidative damage.

The mitochondrial lipid peroxidation is the third target of ROS. Lipid peroxides

alter vital mitochondrial functions, such as respiration and oxidative phosphorylation,

inner membrane barrier properties, maintenance of mitochondrial membrane

potential ($\Delta \Psi_m$), and mitochondrial Ca^{2+} buffering capacity (Cejas *et al.*, 2004;

Venditti *et al.*, 2004), which all leads to the impairment of mitochondrial functions.

In the mediation of mitochondrial apoptotic pathway, the release of cytochrome

c is undoubtedly crucial (Slee *et al.*, 1999). This event is mediated by ROS by the

peroxidation of cardiolipin (Ott *et al.*, 2007). Cardiolipin is one of the major

components of phospholipids which, in the mitochondrial membranes, functions to stabilized cytochrome c and other electron transport chain-related proteins present in the intermembrane space (Tuominen *et al.*, 2002) and confers fluidity and mobility of the mitochondrial membrane (Ott *et al.*, 2007). It is found that cardiolipin oxidation decreases its binding affinity for cytochrome c and facilitates cytochrome c mobilization from the intermembrane space (Ott *et al.*, 2002), which is mediated by intracellular ROS accumulations.

The role of ROS in the induction of apoptosis, particularly the mitochondrial mediated pathway, has been postulated and supported by numerous scientific evidences. Being highly toxic molecules, ROS mediates cell death pathway via a variety of cell death paradigms that wait to be fully elucidated. Mitochondrion, as a primary source of cellular ROS and a site of damages, is therefore tightly regulated by ROS on apoptosis induction.

1.4 Phytochemicals from Traditional Chinese Medicine (TCM) as a source of new therapeutics

Conventional therapeutic and surgical approaches have not been able to control the incidence of most of the cancer types. Despite the continuous efforts made on new anticancer drug discoveries, some of the cancers remain poorly treated. The

obstacles faced by the treating of cancers attributes to the occurrence of multidurg

resistance (Gottesman *et al.,*2002), the metastatic properties of cancer cells (Aziz *et*

al., 2003; Eccles and Welch, 2007) and the cytotoxicity of anticancer drug (de Bono

et al. 2003; Crighton and Ryan, 2004) which limits the drug dosage to a tolerable yet

effective level. Moreover, the heterogeneity of cancer cells and the complex signal

transduction pathways behind nullify the efficacy of single, conventional

chemotherapy. Thus, there is an urgent need to develop novel agents that provide

promising therapies for the management of cancer.

Natural organisms have evolved complex chemical defence and signaling

systems that are designed for protection purpose and provide other biological

benefits. These organisms thus produce substances containing novel chemotypes that

may have beneficial effects for humans. In fact, over half of the current anticancer

agents in clinical use are natural products or are derived from natural products

(Cragg *et al.*, 2005). Among the anticancer drugs approved by the United States Food

and Drug Administration since 1960, over 50% of them were originated from the

natural resources, especially from terrestrial plants (Kim and Park, 2002). Important

anticancer agents such as paclitaxel and camptothecin are the renowned examples of

plant-derived chemotherapeutic drugs, which are both discovered from terrestrial

plants *Taxus brevifolia* and *Camptotheca acuminata* respectively (Braña and

Sánchez-Migallón, 2006). Besides the discovery of therapeutics, plant-derived

chemicals also serve as chemopreventive agents in cancer prevention. An increasing

number of evidence has suggested that many dietary phytochemicals are efficacious

in halting or reversing the perpetuation of carcinogenesis at different stages (Surh,

2004). In this context, it can be appreciated that chemicals extracted from plants are

particularly important, both as a potential therapeutics and as a chemopreventive

agent (Liu *et al.*, 2004).

In the search of novels phytochemicals with potential anti-neoplastic effects,

Traditional Chinese Medicine (TCM) would represent a rich and attractive pool of

bioactive chemicals that deserves further investigation (Chen *et al.*, 2006). Because

of the growing interest in TCM-based therapeutic agents, increasing effort has been

directed towards scientific proof, clinical evaluation and molecular analysis of

components in the herbal medicine (Chen *et al.*, 2003; Cohen *et al.*, 2002). One of

the scientifically well-established TCM candidates would be the medicinal fungus

'Ling Zhi', *Ganoderma Lucidum*. It has long been used as a folk remedy for

promotion of health and longevity (Lin and Zhang, 2004), and the dried powder of

Ganoderma lucidum was popular as a cancer chemotherapy agent in ancient China

(Sliva, 2003). The immunomodulatory and anti-tumor activities of the fungus have

been extensively studied, revealing the possible mechanisms by modulating

functions of immune cells. The polysaccharide and triterpene fractions of *G. Lucidum* exhibited significant anti-tumor effect in several tumor-bearing animals mainly through its immunoenhancing activity (Lin *et al.*, 2003; Gao *et al.*, 2005). Supporting by large amount of scientific evidence, the use of Ganoderma has been enforced in preclinical studies, which the combinatory use of *G. Lucidum* with hypercholesterolemia drug lovastatin have demonstrated promising effect on tumor regression (Shiao, 2003). The successful scientific profiling of this medicinal fungus and the identification of the bioactive components within have illustrated the potential of TCM in the development of novel anticancer regimens that deserves attention.

1.5   Biological effects of baicalein

Baicalein (5,6,7-trihydroxyl-2-phenyl-chromen-4-one) is a flavone extracted from the dried root of a Chinese medicinal plant called *Scutellaria baicaleinsis* (Huang Qin). Huang Qin is one of the important medicinal herbs widely used for the treatment of various diseases. Based on the theory of TCM, this drug is considered to be a detoxification and damp-removing agent and unblocks the lung and stomach meridians (Ye *et al.*, 2002). In scientific language, this herbal medicine was traditionally used as an anti-inflammatory agent, which is effective in treating

inflammation-related illnesses such as respiratory tract infection, diarrhoea, jaundice and hepatitis (Zhang *et al.*, 2003). Besides, Huang Qin has also been used as a traditional antitumor herb. The anticancer activity possessed by the herbal medicine has been investigated, either alone or in combination with other herbs. Its antitumor activities has been verified in different types of cancers, including breast, hepatocellular, pancreatic, prostate, and urothelial carcinoma cell lines (Yano et al., 1994; So *et al.*,1997). Being an effective anti-tumor herb, Huang Qin is therefore one of the ingredients of the herbal formulation PC-SPES, which is a mixture of several Chinese herbs and one American herb *Serenoa repens*. PC-SPES is in fact a popular herbal supplement consumed by patients diagnosed with prostate cancer in the US (Hsieh *et al.*, 2002)

From the medicinal plant *Scutellaria baicaleinsis*, three major bioactive phenolic compounds have been identified, namely wogonin, baicalein and baicalin (Chen *et al.*, 2001). Among the three bioactive flavanoids, baicalein has shown the strongest antiproliferative effects in several types of cancer *in vitro* (Chang et al., 2002; Ma *et al.*, 2005).
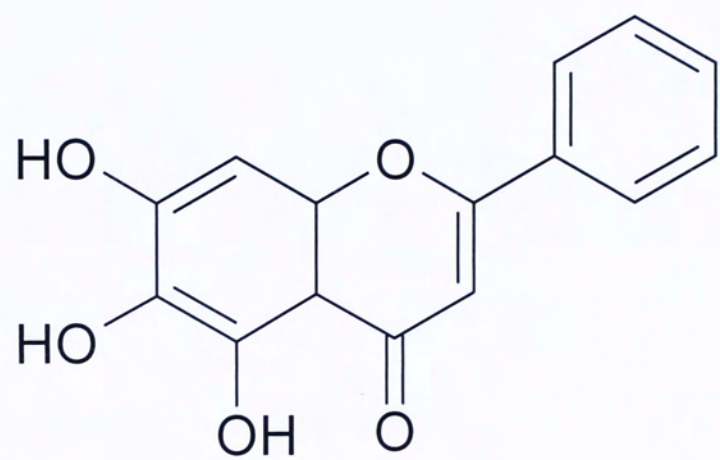
Fig. 1.2   Chemical structure of baicalein

1.5.1. Role of baicalein as a lipoxygenase inhibitor

One of the possible mechanisms behind the antiproliferative effects of baicalein would probably be the inhibitory effect on lipoxygenases (LOXs). The function of LOXs is similar to the functions of cyclooxygenases (COXs) in the metabolism of polyunsaturated fatty acids, which both classes of enzymes oxidize the essential fatty acids such as arachidonic and linoleic acids to form a wide array of bioactive chemicals like prostaglandins and leukotrienes that are important in carcinogenesis, metastasis and angiogenesis (Kashfi and Ragas, 2005). Therefore, the COX and LOX pathways are often upregulated in cancer cells, and the use of COX and LOX inhibitors has been regarded as a possible direction in suppressing carcinogenesis and tumor progression (Ding et al., 2003). Baicalein is widely recognized as a potent inhibitor of the pro-carcinogenic 12-LOX which frequently employs in the study of lipoxygenase inhibition. The 12-LOX inhibition by baicalein has shown to attenuate the growth of prostate cancers (Ding et al., 1999) and gastric cancer (Wong et al., 2001) in vitro and in vivo.

1.5.2 Dual roles of baicalein as an antioxidant and prooxidant

Similar to other bioactive flavonoids, baicalein possesses antioxidant and free-redical scavenging activities that are able to repress stress-induced cell death (Lin et al., 2007). Baicalein is able to induce the expression of stressed-induced

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heme oxygenase 1 and the modulation of ERK signal transduction pathway, which is essential in inhibiting H_2O_2-induced apoptosis. The H_2O_2-induced apoptosis mimics the oxidative-stress-induced apoptosis that are commonly found after treatment of chemotoxic agent (Chen *et al.*, 2006). As an antioxidant that can inhibit stress-induced cell death, baicalein also served as a potent free-radical scavenger by the suppression of xanthine oxidase activity (Shieh *et al.*, 2000).

Interestingly, besides acting as an antioxidant, baicalein is also capable in apoptosis induction via the upregulation of intracellular ROS production. Baicalein has been reported to induce ROS-mediated mitochondrial dysfunction in human promyeolytic leukemia by two different groups (Wang *et al.*, 2004; Makino *et al.*, 2006). The criteria behind the switch of the dual roles played by baicalein are not yet elucidated.

1.5.3 Roles of baicalein as an anti-carcinogenic, anti-proliferative and anti-metastatic agent

Baicalein is able to exert its anticancer effect on the initiation phase of cancerous lesions. This is achieved by the modulation of enzyme activities resulting in the decreased carcinogenicity of xenobiotics. Baicalein is capable in activating the phase II detoxifying enzymes, which are essential in the detoxification of carcinogen. Being a bioactive flavone, baicalein also regulates the activity of aromatase (CYP19),

thus decreasing estrogen biosynthesis and producing antiestrogenic effects, which are important in the tumorigenesis and progression of breast and prostate cancers (Moon et al., 2006). Moreover, the administration of baicalein is capable in inhibiting cell shedding from primary tumor as well as the subsequent invasion at secondary site, supporting by the study using confrontation culture model consisting of multicellular tumor spheroids and embryoid bodies (Günther et al., 2007). In the context of vascular network development in cancer cells, baicalein is able to inhibit angiogenesis in prostate cancer cells, which is probably due to its inhibitory effect of 12-LOX that in turn reduces the expression of vascular epidermal growth factor (VEGF) in cancer cells (Nie et al., 2006)

1.6 Aims of current study

The demands for the discovery of new anticancer drugs have never been slowed down, despite the continuous efforts made on new drug development. The complexity of cancers and the practical difficulties in anticancer strategies have left many of the cancers poorly treated. Over 50% of the currently used anticancer drugs are originated from natural sources, particularly the terrestrial plants (Kim and Park, 2002). This suggests the great potentials of further discovery of new therapeutics from the plant kingdom. In this context, TCM would represent a rich and attractive

pool of bioactive chemicals that deserved further investigation (Chen *et al.*, 2006). Many studies have identified the bioactive compounds in some renowned TCM and have been enacted as complementary therapy. In this study, the antiproliferative effects of the TCM-derived flavone, baicalein, is investigated. Despite the previous researches on the antiproliferative mechanisms of baicalein in other types of cancer cells, the antiproliferative effects of baicalein on human melanoma A375 cells have never been reported by other researchers. Moreover, the various modes of actions demonstrated by baicalein on cancer cell growth inhibition previously have indicated the potentials of the discovery of new antiproliferative mechanisms.

The application of analytical techniques, such as flow cytometry and immunoblotting, in the study of cell proliferation, apoptosis, and their regulatory gene expression are the mode of choice to provide new and important information on how baicalein exerts its *in vitro* anticancer activities on the melanoma cells. Findings from the experimental results are of crucial importance on the deduction of novel signal transduction pathways. We anticipate that the results from this study would allow more understandings on the anticancer mechanisms of baicalein on the melanoma cell line, the results of which may open a new direction in the research in melanoma treatment. As baicalein is derived from an inexpensive herb Huang Qin which is easily available, it merits in further research and development as a potent

cancer adjuvant therapeutic agent.

Chapter 2. Effect of Baicalein on Growth and Survival of Human Cancer Cells

2.1 Introduction

In the evaluation on the cytotoxic effects of a potential compound, *in vitro* antiproliferative study has provided us with fast and inexpensive cytotoxic testings. Different assays for quantifying cell population have been established to assess the cell viability and proliferation of the drugs on cancer cells, based on different features of viable and non-viable cells. Among these parameters, the most prominent parameter associated with cell viability and proliferation would be the change in metabolic activity. Cellular damages will inevitably result in a loss of ability of the cell to maintain and provide energy for metabolic functions and growth, therefore based on this premise, metabolic activity can be assayed as a reflection of cell viability.

In 1983, Mosmann described a rapid colorimetric assay for the measurements of cell growth and metabolic activity, which was called MTT assay (Mosmann, 1983). This assay, which is usually applied for adhesive cell line, is based on the reduction of the yellow water-soluble substrate 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a water insoluble dark blue formazan product by active mitochondrial dehydrogenases in living cells. As yellow water soluble MTT is

only reduced by active dehydrogenases in the functional mitochondria, the amount of formazan produced is therefore directly proportional to the viable cell number, and the result is found to be reliable over a wide range of drug concentrations (Pagé, 2004).

The degree to which anticancer agents selectively target cancer cells is a key determinant in successful therapeutic outcomes (Durvvri, 2006). A potential efficacious compound should also possess specificity in actions that preferentially targeted to cancer cells. Having indefinite proliferative and metastatic ability, cancerous cells present very different genotypic and phenotypic characteristics when compared with the controlled, normal counterparts. It is the differences that keep the two types of cells apart and provide therapeutic targets in combating cancer. Therefore as an attractive potential antitumor compound, it should display differential activities on cancer cells and normal cells, which only the cancer cells are being suppressed, leaving the normal, health cells slightly or even not affected.

In the present study, MTT assay was applied for the preliminary screening of the growth inhibitory effect of baicalein on different cancer cells and a normal skin fibroblast cell line. The concentration of baicalein with 50 % inhibition on cell proliferation was also estimated for the comparison of growth inhibitory effects as well as for further investigation of the mechanistic actions accounting for its growth

inhibitory effects on the cancer cells.

2.2 Materials and Methods

2.2.1 Cell culture

Six human cancer cell lines including malignant melanoma A375, colorectal carcinoma Caco-2, hepatocellular carcinoma HepG2, lung carcinoma A549, prostate carcinoma PC-3 and breast carcinoma MCF7 cells; and one human skin fibroblast cell line Hs68 were used to study the cytotoxic and cytostatic effects of baicalein in this study. All cell lines were provided by American Type Culture Collection (ATCC; Rockville, MD). All media used, unless otherwise stated, were purchased from Gibco (Rockville, MD). A375, Caco-2 and Hs68 cells were grown in Dulbecco's Modified Eagle Medium (DMEM). A549 and PC-3 cells were grown in F12K medium (Sigma, St. Louis, MO) while Hep-G2 and MCF7 cells were grown RPMI-1640 medium and Eagle's Minimum Essential Medium (EMEM), respectively. All media were supplemented with 0.2 % sodium bicarbonate (Sigma), 10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml of streptomycin (all from Gibco). In addition to the above supplements, the growth medium for MCF7 cells was further supplemented by 1 mM sodium pyruvate and 0.01 mg/ml bovine insulin (both from Sigma). The cells were maintained under a fully humidified atmosphere with 5 % CO_2 at 37 °C, and passaged three times a week. .

2.2.2 Measurement of growth and survival of various cell lines

MTT assay was applied to examine the effects of baicalein on cell growth control. Different cancer cells and the normal skin fibroblast cell line were seeded in 96-well flat-bottomed culture plates at an initial number of 5×10^3 cells and 1×10^4 cells per 100 µl respectively. The cells were acclimatized for 24 h before incubation with 0 - 100 µM of baicalein (Sigma) for 48 h. After incubation, 20 µl of the MTT solution was added to each well and further incubated for 5 h. The MTT solution was freshly prepared by dissolving MTT (Sigma) in filtered phosphate-buffered saline (PBS) at 5 mg/ml. After 5 h of incubation, the wells were carefully aspirated and the blue formazan formed was dissolved by adding 150 µl dimethyl sulphoxide (DMSO). All the wells were then mixed by pipetting until all crystals were dissolved completely. Absorbance of the blue formazan solution generated was measured at 570 nm by the microplate reader. The inhibitory effects of baicalein on the tested cell lines were expressed as percentage inhibition, which was calculated by the following equation:

$$\left(1 - \frac{\text{Average absorbance of treatment group}}{\text{Average absorbance of control group}} \right) \times 100\%$$

The percentage inhibition calculated was plotted against the corresponding

concentration of baicalein tested. The concentration of baicalein with 50 % inhibition on the growth and survival (i.e. IC_{50}) on each cell line was then estimated from the growth-inhibitory plot.

2.2.3 Statistical analysis

Results were expressed as mean ± standard deviation (S.D.) and analyzed by Student's *t*-test. The level of significance was taken at p=0.05, p=0.01 and p=0.001.

2.3 Results

2.3.1 Baicalein retards the growth and survival of human melanoma A375 and colorectal carcinoma Caco-2

Effects of baicalein on the growth and survival of a panel of human cancer cells of different histotypes were studied with MTT assay. Among the six cancer cell lines tested, baicalein only reduced the growth and survival of human melanoma A375 and colorectal carcinoma Caco-2 in a dose-dependent manner, while no significant growth inhibition was observed in the other four cell lines, namely hepatocellular carcinoma Hep-G2, lung carcinoma A549, prostate carcinoma PC-3 and breast carcinoma MCF7 cells. At the highest concentration of 100 µM after 48 h of incubation, baicalein reduced the growth and survival of A375 and Caco-2 cells by

76.0 % and 67.3 % respectively, when compared to their control levels (Fig. 2.1).

The values of IC_{50} of baicalein on A375 and Caco-2 were then estimated as 37.5 and

84.0 μM, respectively. Since melanoma A375 was found to be the most responsive

cell line to the inhibitory effect of baicalein, further studies were performed on this

cancer cell line.
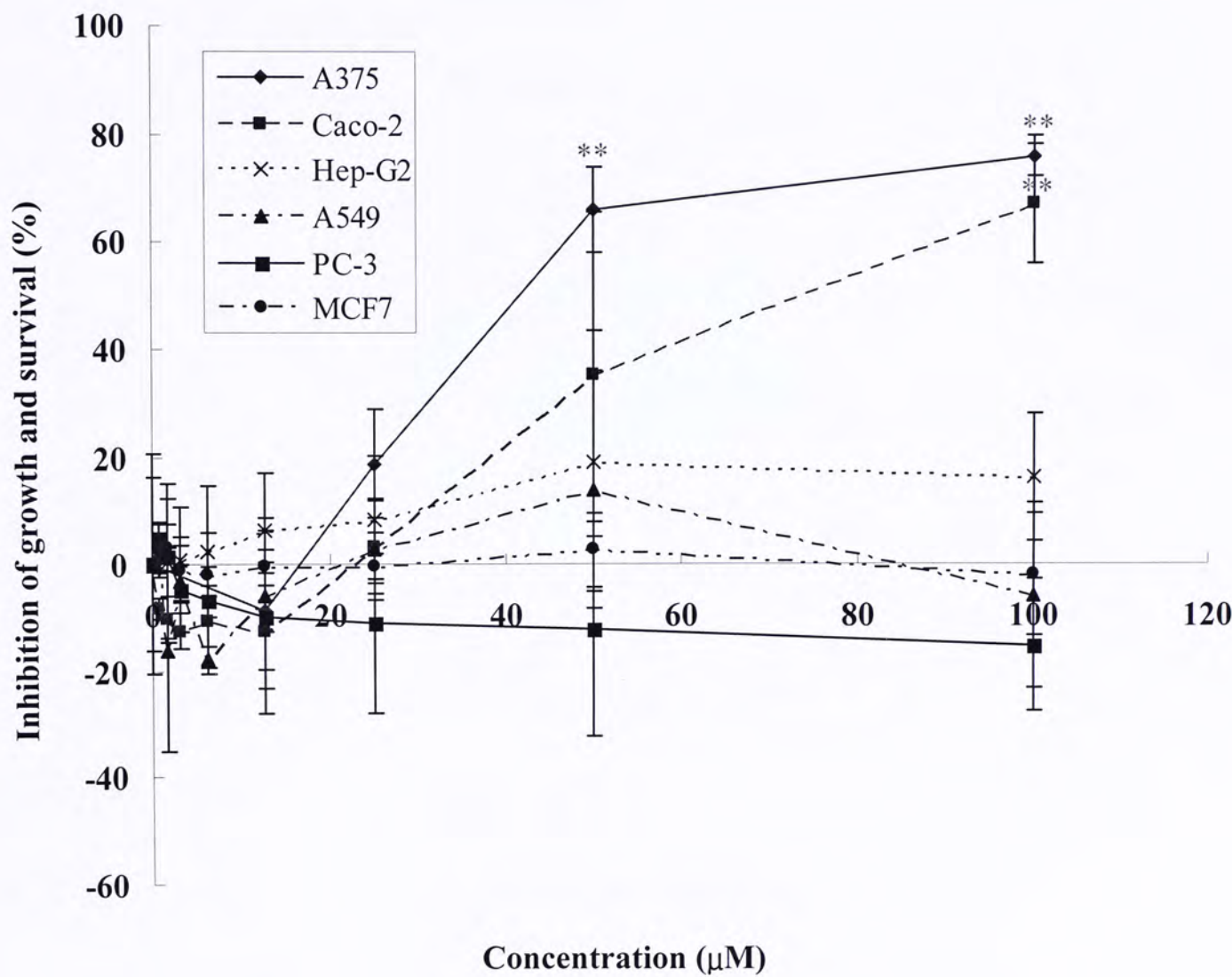
Fig. 2. 1   **Effects of baicalein on growth and survival of various human cancer cell lines**

The growth and metabolic activities of A375, Caco-2, Hep-G2, A549, PC-3 and MCF7 were measured by MTT assay after incubation with 0-100 μM of baicalein for 48 h. Baicalein only reduced the growth and survival of A375 and Caco-2 cells but not the other cancer cell lines. Results are expressed as mean ± S.D. (n=4). ** $p<0.01$, compared to the control level by Student's t-test.

2.3.2 Baicalein reduces the growth and survival of melanoma A375 but not in normal skin fibroblast Hs68 cells

As mentioned in section 2.3.1, baicalein significantly reduced the growth and survival of melanoma A375 cells. In this study, the inhibitory effect of baicalein on the normal skin fibroblast Hs68 cells was investigated. Interestingly, as illustrated by MTT assay, lower dose of baicalein, i.e. 6.25-50 μM, were stimulatory instead of inhibitory on the growth and survival of the skin fibroblast after 48h of incubation (Fig. 2.2). Although 100μM baicalein seemed to reduce the growth and survival of the normal cells by 11.8% of the control level, the inhibition was not statistically significant. At the same concentration, baicalein was able to reduce the growth and survival of the melanoma cells by 76%, indicating that the inhibitory effect of baicalein might be specific.
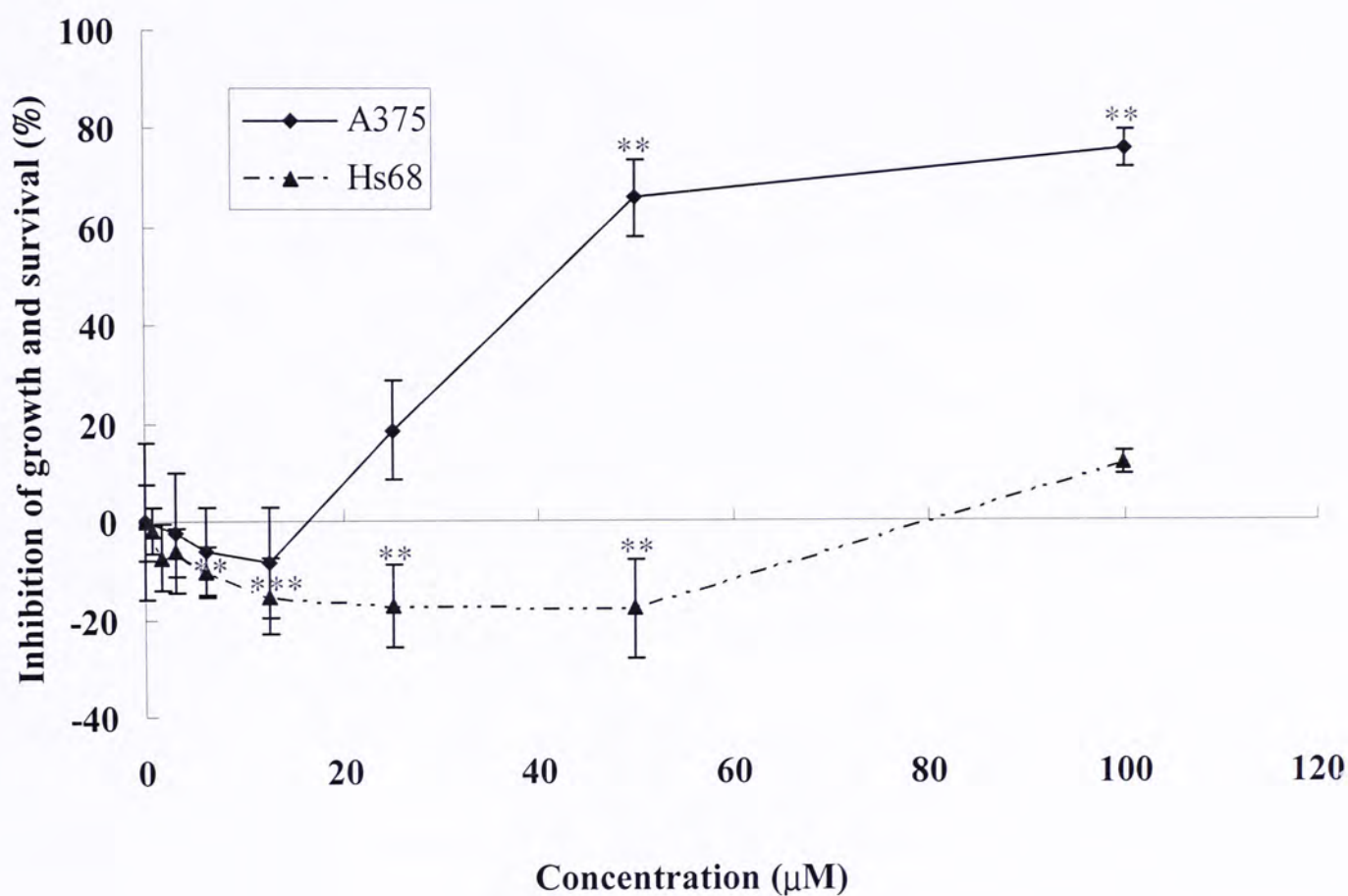
Fig. 2.2   **Effects of baicalein on growth and survival of human melanoma A375 and human skin fibroblast Hs68 cells**

The growth and metabolic activities of the melanoma and the skin fibroblast were measured by MTT assay after incubation with 0-100 μM of baicalein for 48 h. Results are expressed as mean $\pm$ S.D. (n=4) *, ** and *** represent statistical significance levels at $p<0.05$, $p<0.01$ and $p<0.001$ respectively, when compared to the control level by Student's t-test.

2.4 Discussion

Being a rapid, economical and simple assay (Pagé, 2004), MTT assay was employed to study the inhibitory effects of baicalein on a panel of cancer cell lines of different histotypes *in vitro*. Human melanoma A375, colorectal carcinoma Caco-2, hepatocellular carcinoma HepG2, lung carcinoma A549, prostate carcinoma PC-3 and breast carcinoma MCF7 cells were included in the testing panel. The results from MTT assay were illustrated in a growth-inhibitory plot, where the IC_{50}, i.e., the concentration of compound that causes 50% of cell growth reduction compared to the control level, of baicalein was estimated. The lower the value of IC_{50}, the more efficacious the bioactive compound would be against the cell growth reduction (Budman *et al.*, 2003). Baicalein exhibited differential effects on different cell lines. Only human melanoma A375 and colorectal carcinoma Caco-2 showed significant growth inhibition after baicalein treatment, with the value of IC_{50} of 37.5 and 84.0 μM, respectively (Fig 2.1). Meanwhile, hepatocellular carcinoma HepG2, lung carcinoma A549, prostate carcinoma PC-3 and breast carcinoma MCF7 did not respond prominently to baicalein treatment even at maximum dose of 100 μM. Consequently, human melanoma A375, being the most responsive cancer cell line, was chosen for further studies.

Before moving on to investigate the inhibitory mechanism of baicalein for A375

cells, the specificity of baicalein on cancer cells was investigated by comparing its effect on the melanoma cell line with that on a normal human skin fibroblast cell line Hs68 (Fig. 2.2). This normal cell line had also been previously employed to evidence the specific antiproliferative effect of bioactive chemicals on hepatoma cells (Chan *et al.*, 2004). Interestingly, instead of inhibiting cell growth and survival, baicalein stimulated the growth of the fibroblast cell line. All these results substantiate that baicalein is generally non-toxic and possesses specific growth-inhibitory effect on the melanoma cells.

Chapter 3. Effects of Baicalein on Cell Cycle and the Apoptosis in Human Melanoma A375 Cells

3.1 Introduction

Ultimately, cancer is the consequence of the loss of balance between cell proliferation and cell death (Thompson, 1995). Baicalein has been shown to retard both cell proliferation and viability of the human malignant melanoma A375 cells (Chapter 2.1); however, the underlying mechanisms remain unclear, thus different aspects of its cell growth inhibitory activity were further explored in this study.

The proliferation of eukaryotic cells is governed by series of events comprised the cell cycle. Any factors that affect the genes and/or proteins regulating the cell-cycle progression will cause a delay or an arrest of the cell-cycle phase, leading to delayed proliferation. Cell cycle analysis therefore provides a useful tool to evaluate the effects of exogenous compounds on cell proliferation. Thus, how different lengths of baicalein treatment affect the cell cycle profile of the melanoma cells, in terms of cell cycle distribution, was evaluated by the application of flow cytometry. Furthermore, as baicalein inhibited the growth of the melanoma, the inhibitory effect might probably due to the perturbing of cell cycle progression, and the subsequent induction of cell death. The induction of cell death by baicalein could also be visualized in the DNA flow cytometric analysis concerning the characteristics

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of apoptosis, DNA fragmentation (Bedner, 1999). By the appearance of the apoptotic

peak on the DNA histogram, the modes of cell death triggered by baicalein could be

distinguished. During apoptosis, chromosomal DNA will be fragmented into

specifically 50- to 200-kb fragments, then later into nucleosomal units of DNA

fragments (Nagata, 2000). Therefore, cells undergoing apoptosis will appear as

discrete peak (sub-G_1 peak) on the left of G_1 peak (Compton, 1992; McConkey et al.,

1996). Unlike apoptosis, necrosis does not trigger extensive DNA degradation

comparable to that of apoptosis; also the products of degradation are heterogenous in

size therefore fail to form discrete peaks on the DNA histogram (Darzynkiewicz et

al., 1997). As a result, the observation of discrete sub-G_1 peaks may serve as an

evidence of the occurrence of apoptosis.

DNA Flow Cytometric Analysis

One of the important applications of DNA flow cytometry is to study the hypo-

and hyperdiploidy of cancer cells (Vermes et al., 2000), which is based on the

measurement of fluorescent signals emitted by DNA-binding dye, such as propidium

iodide (PI). PI is a fluorogenic DNA dye that intercalates within double-stranded

DNA in a specific stoichiometric manner (i.e. the extent of staining is directly

proportional to the amount of DNA within the cells) (Darzynkiewicz et al., 1992).

After staining the fixed cells with PI, the red fluorescence from the dye can be

measured at 620 to 740 nm after excitation by laser beam at 488 nm by flow cytometer. A DNA histogram is thus generated to illustrate the DNA content of the entire cell population.

DNA histogram is the frequency distribution generated from the relative PI fluorescence of thousands of individual cells detected by the photomultiplier tubes of flow cytometer. The x-axis represents the channel number. Fluorescence of the light pulse from each signal is digitalized and converted into a channel number. Therefore, the channel number thus reflects the relative amount of DNA in each nuclear unit. The y-axis refers to the number of counts per channel, which corresponds to the relative number of cells. The DNA histogram is constructed by plotting the relative number of cells against the increasing channel numbers, therefore representing the DNA content distribution of cells from the cell population (Rabinovitch, 1994).

A typical DNA histogram is shown in Fig. 3.1 with the shape of two peaks separated by a trough. The first peak represents the G_0/G_1 phase, in this period; the DNA content is the least in the cell cycle which represents the DNA content of non-dividing cells. The second peak represents the G_2/M phase in which the DNA content of the cells is duplicated and is ready to divide into respective daughter cells. In between these two peaks is the S phase in which DNA is actively synthesized, where the DNA content is increasing. In the case of apoptotic cells, a peak with

lower DNA content on the left of G_0/G_1 peak will be detected, which is termed as apoptotic peak (Ap). During apoptosis, endonuclease(s) may be activated and results in the DNA fragmentation at internucleosomal regions generating nucleosomal and oligonucleosomal DNA (Nagata, 2000). These DNA fragments are extracted and the remaining DNA within the apoptotic cells becomes lower in content than those of G_1. The red fluorescence intensity from the apoptotic cells is thus lower, and exists as the apoptotic peak on the left of G_1 peak in DNA histogram.

DNA histogram analysis allows an objective approximation for the population of cells in the G_0/G_1, S an G_2/M phases of cell cycle which acts as a crude index of the proliferative activity of the tumor. In addition, the incidence of apoptosis can also be detected.
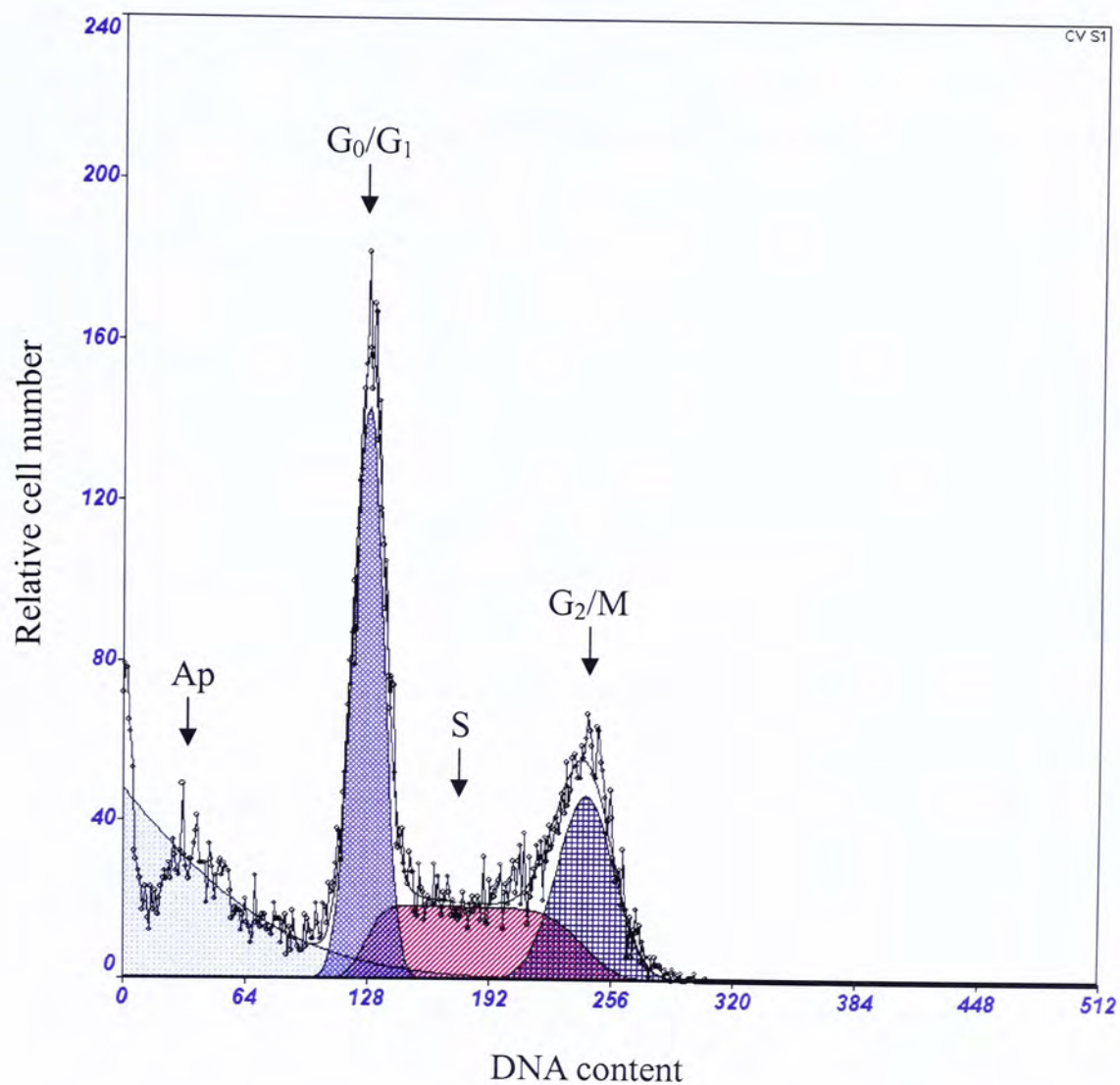
Fig. 3.1   **A DNA histogram of flow cytometry**

A eukaryotic cell cycle is divided into G_0/G_1, S and G_2/M phases. An apoptotic peak (Ap) is also found on the left of the G_0/G_1 peak.

Besides the physical characteristics, biochemical characteristics of apoptosis are also prominent indicators readily available for the detection of apoptosis. Caspase activation is one of the important biochemical features commonly found in apoptotic cells. Being able to self-activated or activated by other caspases, caspases are responsible for the initiation, execution and amplification of the apoptotic cascade (Kumar, 2007). Therefore the detection of activated caspase is a convincing piece of evidence supporting the occurrence of apoptosis. One of the duties of active caspase is the demolition of unwanted cellular materials, which include the proteolytic inactivation of poly(ADP-ribose) polymerase (PARP). PARP is an enzyme that governs the process of poly(ADP-ribosylation), which is a post-translational modification of proteins that, in eukaryotic cells, plays a crucial role in DNA repair and replication, transcription and cell death, and represents a cellular emergency reaction (Scovassi and Poirier, 1999). In the late phase of apoptosis, PARP is inactivated by caspases in order to halt the DNA repair machinery and to signal the cells to massive apoptosis. To verify and confirm if the baicalein-induced apoptosis is a caspase-dependent event, protein expression of various caspases and PARP was investigated by immunoblotting and the application of specific caspase inhibitor in this study prior to the detail investigation of the possible mechanisms behind.

Immunoblotting

Immunoblotting, also known as Western blotting, is an analytical method commonly employed in the analysis of protein expression of cells. It comprises basically two steps: sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of the fractionated proteins from the gel to nitrocellulose membrane.

Electrophoresis is a widely used and powerful technique to fractionate proteins, which depends on the ability of charged molecules to migrate when placed in an electric field. The separation of proteins is usually accomplished using polyacrylamide gel electrophoresis (PAGE) in which the electrophoretic move of proteins is carried out through a gelated matrix, polyacrylamde, which is the polymer of acrylamide that are cross-linked to form a molecular sieve. The larger the protein, the more it becomes entangled, and the slower it migrates. PAGE is usually carried out in the presence of the negatively charged detergent sodium dodecyl sulfate (SDS). The electrostatic repulsion between the bound SDS molecules causes the proteins to unfold into similar rodlike shape, thus eliminating differences in shape as a factor in separation. Furthermore, the negatively changed SDS molecules bind to protein molecules stochiometrically which mask the intrinsic charges of proteins. Consequently, each protein species, regardless of its size, has an equivalent charge

density and is driven through the gel with the same force. As a result, proteins become separated by SDS-PAGE on the basis of a single property, which is the molecular mass (Lodish *et al.*, 2000).

3.2   Materials and Methods

3.2.1   Determination of cell cycle changes and quantification of apoptosis

To determine the cell cycle changes after baicalein treatment, the melanoma cells were seeded at an initial density so that at the end of treatment 1×10^6 cells were harvested. After 24 h acclimatization, the cells were treated with or without 37.5 μM baicalein for 24, 36 48, 60 and 72 h. After treatment, the cells were harvested and washed with PBS and fixed in 70% ethanol at -20 °C for 24 h. The cells were then incubated in dark for 30 min with 1 mg/mL ribonuclease A (RNase A; Sigma) and 10 μg/mL propidium iodide (PI; Sigma) in PBS. The DNA content in the stained cells was thus analyzed by measuring PI fluorescence using the Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL) at excitation wavelength > 625 nm. Different populations of cells exhibiting different DNA content were interpreted as cells in different phases of cell cycle. Simultaneously, cells with hypodiploid DNA content were quantified and regarded as the apoptotic population.

3.2.2 Immunoblotting

a. Protein extraction

Preparing whole cell lysates, cells were harvested and homogenized for 30 minutes on ice in a protein lysis 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). Organelles and cell debris in the cell lysates were precipitated and removed by centrifugation at 4 °C for 15 minutes at 15 000g. The resulting supernatants were frozen at -20 °C or used immediately. Aliquots of the supernatants were used for protein concentration determination by the standard bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL).

b. Determination of protein concentration

After cell lysates preparation, the protein concentration of the lysate was determined by BCA assay kit (Pierce, Rockford, IL). Briefly, standard curve was first prepared with 0, 25, 50, 100, 200 and 400 µg/ml of bovine serum albumin (BSA) per 50 µl in microcentrifuge tubes for calibration. Meanwhile, 1 µl of total cell lysates was diluted with 49 µl ultrapure water for protein content measurement. 1 ml of freshly-prepared BCA assay solution was added to each microcentrifuge tube before incubation at 37 °C for 30 min. After the incubation, the tubes were cooled to room temperature and the absorbance was measured at 562 nm by spectrophotometer

(Eppendorf, Hamburg, Germany). A calibration curve was constructed with the absorbance of the standards and the protein concentration of the sample cell lysate was therefore estimated.

c.   *Sodium Dodecyl Sulphate-Polyarcylamide Gel Electrophoresis (SDS-PAGE)*

Samples (30 – 80 µg protein) were denatured in Laemmli loading dye at 95°C before resolving on 8 or 13% polyacrylamide gel. Resolved proteins on the gels were then transferred onto nitrocellulose membranes (Hybond ECL; Amersham, Buckinghamshire, UK) under an electric field of 105 V for 1 h.

d.   *Immunodetection*

The membranes adhered with proteins were then blocked for 1 h in Tris-buffered saline (TBS) with 0.1% Tween-20 containing 5% non-fat dry milk. Immunodetection of was performed by overnight incubation at 4 °C with primary antibodies diluted in the same blocking buffer. In this experiment, primary antibodies against PARP, caspase-8, caspase-3, caspase-9, and Bid (Cell Signalling Technology, Beverly, MA) were used under dilution factor of 1:1000 and antibody against β-actin (Sigma) were used under dilution factor of 1:5000. The blots were then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signalling Technology) for 1 hour at room temperature at a dilution of 1:2000, and proteins were visualized by enhanced chemiluminescence (Cell Signalling

Technology) and captured by X-ray films.

3.2.3 Inhibition of caspase-8 by caspase-8 inhibitor

The consequences after inhibition of caspase-8 in A375 cells was studied by the administration of caspase-8 inhibitor (Z-IE(OMe)TD(OMe)-FMK) (Calbiochem). Firstly, 1×10^6 cells were acclimatized for 24 h before baicalein treatment. The cells were then pre-treated with 40 μM caspase-8 inhibitor for 1 h. After pre-incubation, baicalein at IC_{50} was added to further incubate for 48 h. The cells were then harvested for immunoblotting (section 3.2.2) and for caspase-3 activity assay (section 3.2.4)

3.2.4 Fluorometric measurement of caspase-3 activity

Caspase-3 activity was determined using Caspase-3 activity Kit (BD Biosciences). Briefly, cell lysates were prepared from 1×10^6 cells after incubation with baicalein at IC_{50} for 48 h. After thorough washing with PBS, cell pellet was resuspended in cold cell lysis buffer (provided) and incubated for 30 min on ice. Subsequently, 25 μl cell lysate and 5 μl fluorogenic caspase-3 substrates (Ac-DEVD-AMC) were mixed in a HEPES reaction buffer (provided), 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. Fluorescence values generated in each treatment was converted into fold changes of the control.

3.2.5   Statistical analysis

Results were expressed as mean ± S.D. and the statistical significance of the difference between control and the treatment groups was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The level of significance was taken at $p=0.05$.

3.3   Results

3.3.1.   Baicalein induces S-phase arrest in cell cycle and triggers apoptosis

As mentioned in section 2.3.1, baicalein reduced the growth survival of A375 by 50 % at 37.5 μM after 48 h of incubation. In this study, by using DNA flow cytometry, cells in different cell-cycle phases were measured in the melanoma cells and thus the regulation of baicalein on cell cycle progression was elucidated. The cancer cells were treated without, i.e., control, or with 37.5 μM of baicalein, i.e., IC_{50}, and incubated for 24 to 72 h. Baicalein induced time-dependent accumulation of S-phase cells and depletion of $G_{0/1}$- phase cells, so that both the accumulation and the

depletion peaked at 60 h (Fig. 3.2). At 60 h of incubation with baicalein, the proportion of cells at S phase increased from the control 30.36 % to 50.93 %; the cells in $G_{0/1}$ phase, in meanwhile, reduced from 53.57% to 33.07% (Fig. 3.3).

Besides regulating the cell cycle, baicalein also induced apoptosis, in A375 cells. This was evidenced by the appearance of hypodiploid cells in the DNA histogram, indicating that cells were undergone DNA fragmentation, and lost their DNA content (Fig. 3.2). This population of cells, named as sub-G_1 population, is a possible indicator of apoptosis. After the baicalein treatment, there was a time-dependent increase of sub-G_1 population, i.e. the apoptotic population (Ap), concomitant with the time-dependent increase of S-phase and decrease of $G_{0/1}$-phase cell population. The apoptotic population increased especially prominent from 36 h onwards, so that 51.33 % of the cancer cells had undergone apoptosis at 72 h of incubation (Fig. 3.3)

Fluorescence level from PI (DNA content)
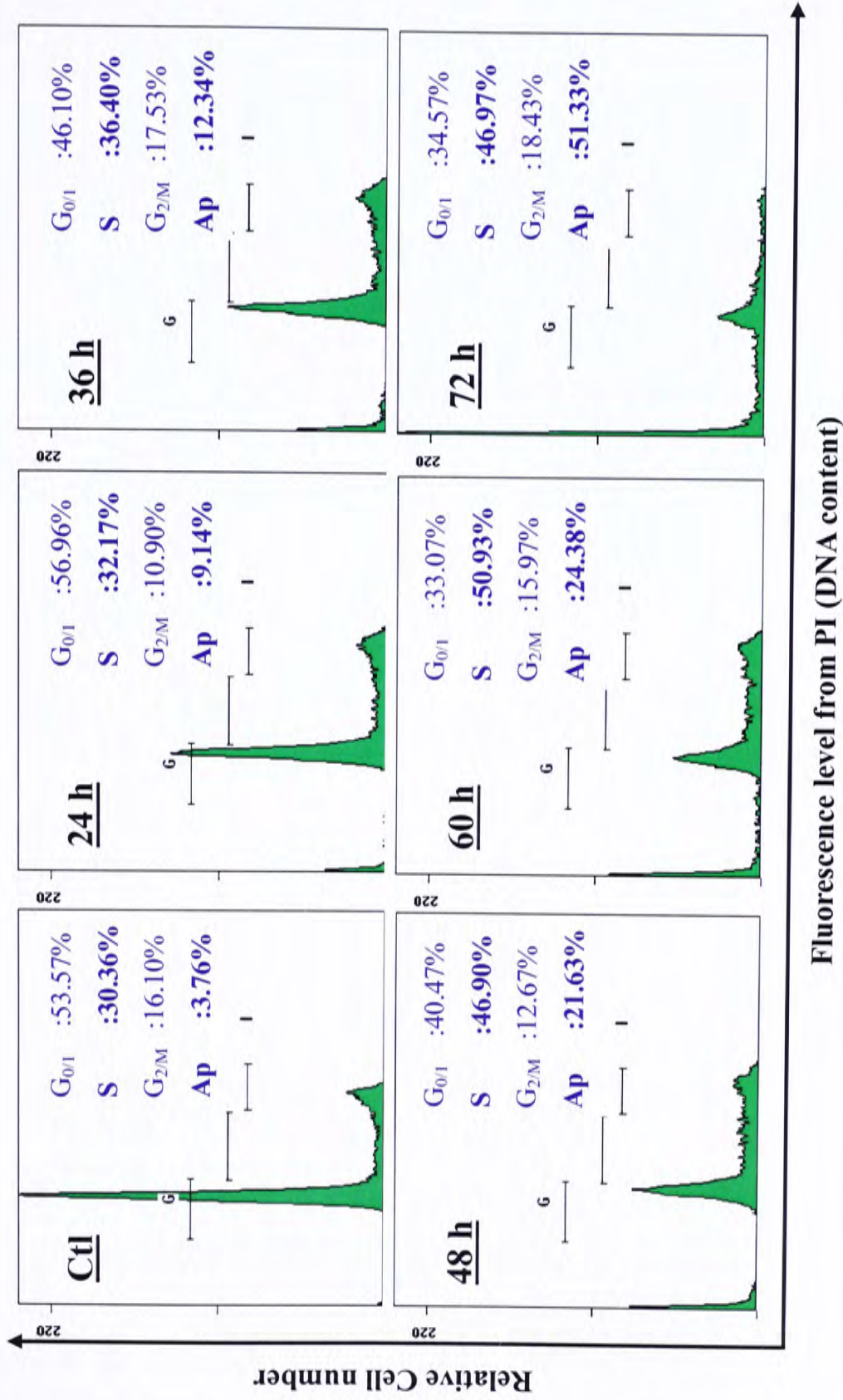
Fig. 3.2   **Representative DNA histogram showing the effects of baicalein on cell cycle and apoptosis in A375 cells**

The melanoma cells were incubated without (Ctl) and with IC_{50} (37.5 μM) of baicalein for 24, 36, 48, 60 and 72 h. Cells in different phases of cell cycle, i.e., $G_{0/1}$, S and $G_{2/M}$, and in apoptosis, i.e., Ap, were then measured by DNA flow cytometry. Baicalein induced time-dependent accumulation of S-phase cells, depletion of $G_{0/1}$ cells and elevation of apoptotic cells in the melanoma. Proportion of cells in different cell cycle phases and apoptosis were also shown.
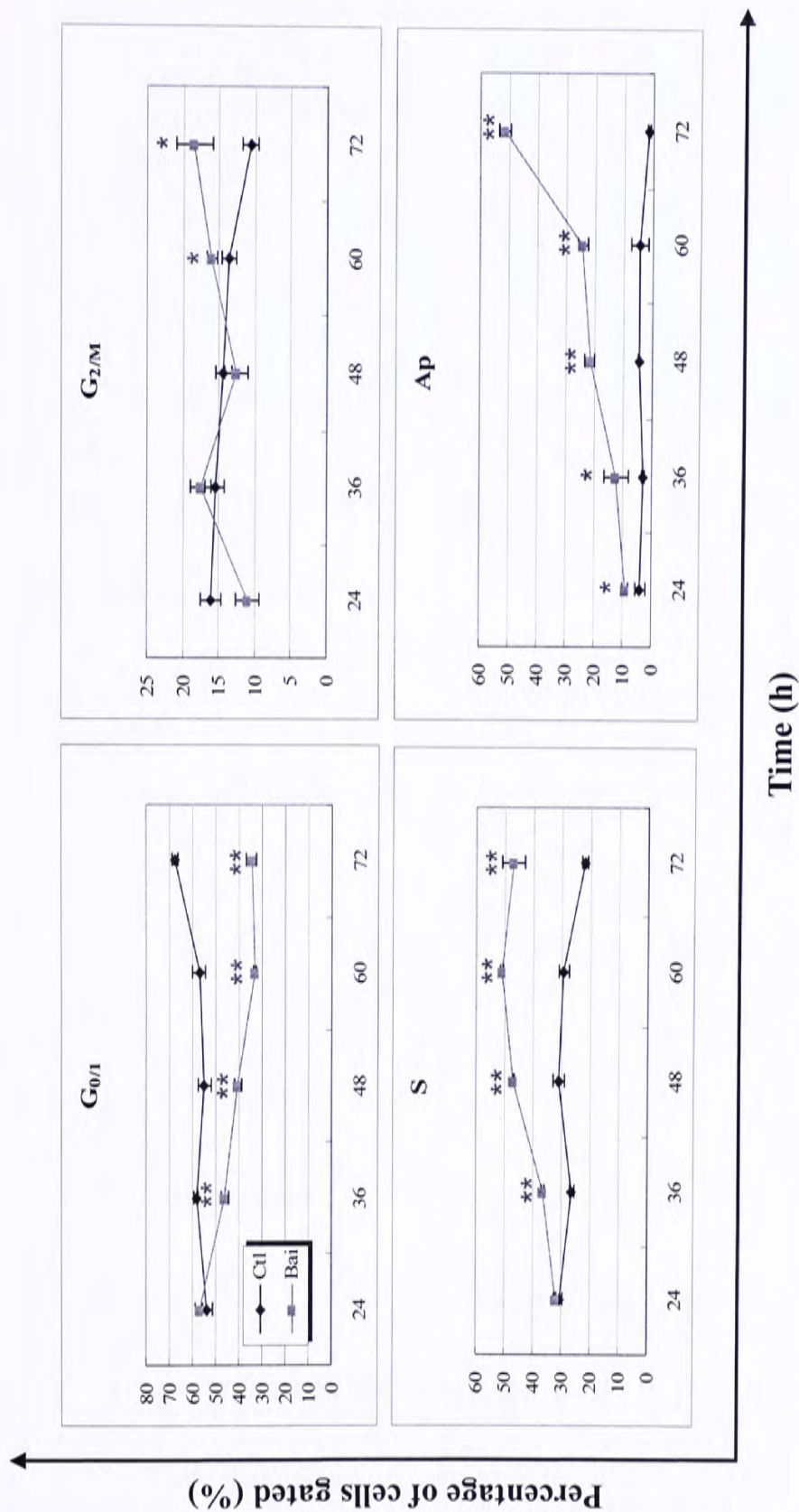
Fig. 3.3   **Effects of baicalein on cell cycle distribution and apoptosis**

Numeric data from DNA-PI flow cytometry showing the proportion of $G_{0/1}$, S, $G_{2/M}$, and apoptotic (Ap) cells in the melanoma cells treated without (Ctl) or with baicalein (Bai) at IC_{50} (37.5μM) for 24 to 72 h. Baicalein induced time-dependent accumulation of S-phase cells, depletion of $G_{0/1}$ cells and elevation of apoptotic cells. Results were expressed as mean ± SD of three independent experiments. $*p<0.05$ and $**p<0.01$, compared to the control levels by Student's t-test.

3.3.2. Baicalein induces proteolytic inactivation of PARP and activation of caspases

As hypodiploid cells with fragmented DNA were observed in the DNA histogram after flow cytometric analysis, effect of baicalein on the proteolytic inactivation of PARP was therefore investigated by immunoblotting to substantiate the occurrence of apoptosis. At 36 h incubation with baicalein, proteolytic inactivation of PARP was initiated in the melanoma cells, as the p89 cleaved PARP fragment was detected by immunoblotting. This indicated the incidence of apoptosis. From 36 h onwards, the cleavage was intensifying time-dependently so that at 60 h and 70 h, intact PARP was absent in the immunoblotting (Fig 3.4, *the most upper panel*). Therefore, both DNA flow cytometry and PARP immunoblotting showed that 37.5 µM baicalein induced time-dependent apoptosis in A375 cells starting from 36 h of incubation.

Activation of caspases is another hallmark of apoptotic cell death. To investigate whether the baicalein-induced apoptosis is a caspase-dependent event, cell lysate was immunoblotted against specific antibodies for caspase-8, caspase-3 and caspase-9. After the baicalein treatment, the three studied caspases were activated sequentially. Caspase-8 possessed the proteolytic activation as early as 24 h of baicalein treatment as early as 24 h of baicalein treatment, which was evidenced by the presence of the active p18 fragment. Thereafter, caspase-3 and caspase-9 were both activated at 48 h. This was indicated by the presence of the activated p17 caspase-3 fragment, and the

activated p37 and p35 caspase-9 fragments from 48 h onwards (Fig 3.4, *lower panels*).

Encapsulating the above findings, it was found that baicalein could induce caspase-dependent apoptosis, revealed by the inactivation of the genome guarding enzyme PARP and the activation of caspase-8, -3 and -9 at different times of treatment.
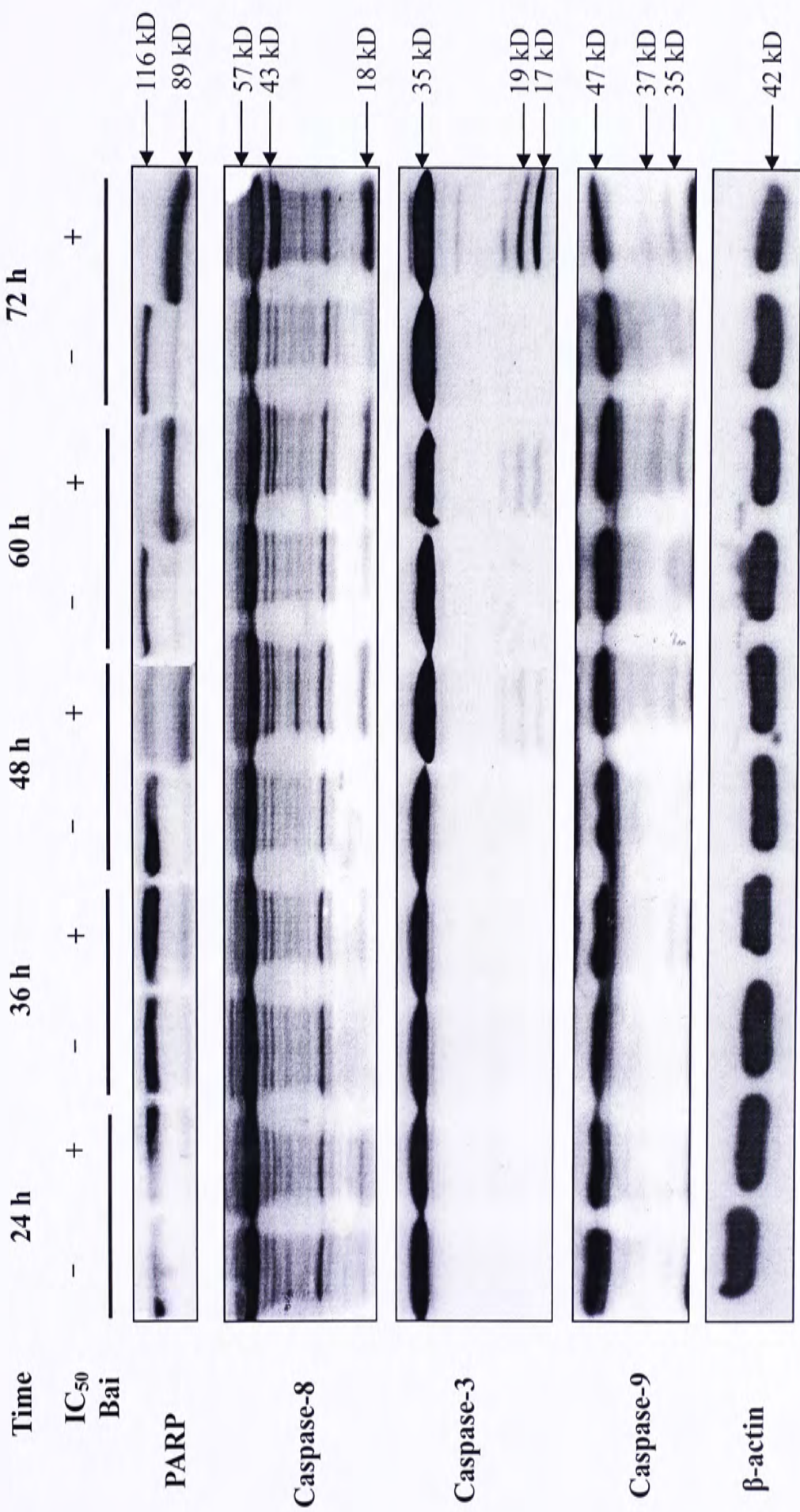
Fig. 3.4 **Representative immunoblots showing the effects of baicalein on proteolytic inactivation of PARP and activation of caspases**

The melanoma cells were incubated in the absence, i.e., control (–), or in the presence of baicalein at IC_{50} (+) for 24 to 72 h. Baicalein (Bai) induced time-dependent PARP cleavage and inactivation starting from 36 h of incubation. Baicalein also induced time-dependent cleavage and activation of caspase-8 from 24 h, caspase-3 and caspase-9 from 48h onwards. β–actin was also detected as internal control to normalize expressions of other proteins.

3.3.3. Caspase-8 is the major initiator caspase eliciting the baicalein-induced apoptosis

In the previous section (section 3.3.2), it was found that baicalein could induce apoptotic cell death in A375 cells by sequential activation of caspases, in which caspase-8 was activated as early as 24 h of treatment. Since prominent apoptosis was only found at 36 h of baicalein incubation, it was hypothesized that caspase-8 would be the major initiator caspase responsible for the onset of baicalein-induced apoptotic cascade. To verify the hypothesis, 40 µM caspase-8 inhibitor (Z-IE(OMe)TD(OMe)-FMK) was pre-treated with A375 cells 1 h prior to baicalein treatment. After further incubation with baicalein at IC_{50} for 48 h, expressions of PARP, and the two down-stream substrates of caspase-8, i.e. caspase-3 and Bid, were studied by immunoblotting. It was revealed that after caspase-8 inhibition, the level of PARP inactivation, indicated by the amount of p89 inactive fragment, was reduced and became comparable to the control level. (Fig 3.5). Besides, caspase 3 was also de-activated after co-incubation of caspase-8 inhibitor with baicalein, indicating a correlation between caspase-8 activity and caspase-3 activation. Bid, another substrate of caspase 8, did not show any significant change either after baicalein treatment or after capsase-8 inhibitor co-administration with baicalein. Since truncated Bid (tBid), produced via cleavage of Bid by caspase 8, had been shown previously in activating the intrinsic apoptotic pathway, this implied that caspase-8 activity did not take a major role in the proteolytic activation of caspase-9. The

ways how baicalein induces the intrinsic apoptotic pathway in A375 cells will be

further discussed in Chapter 5.

63
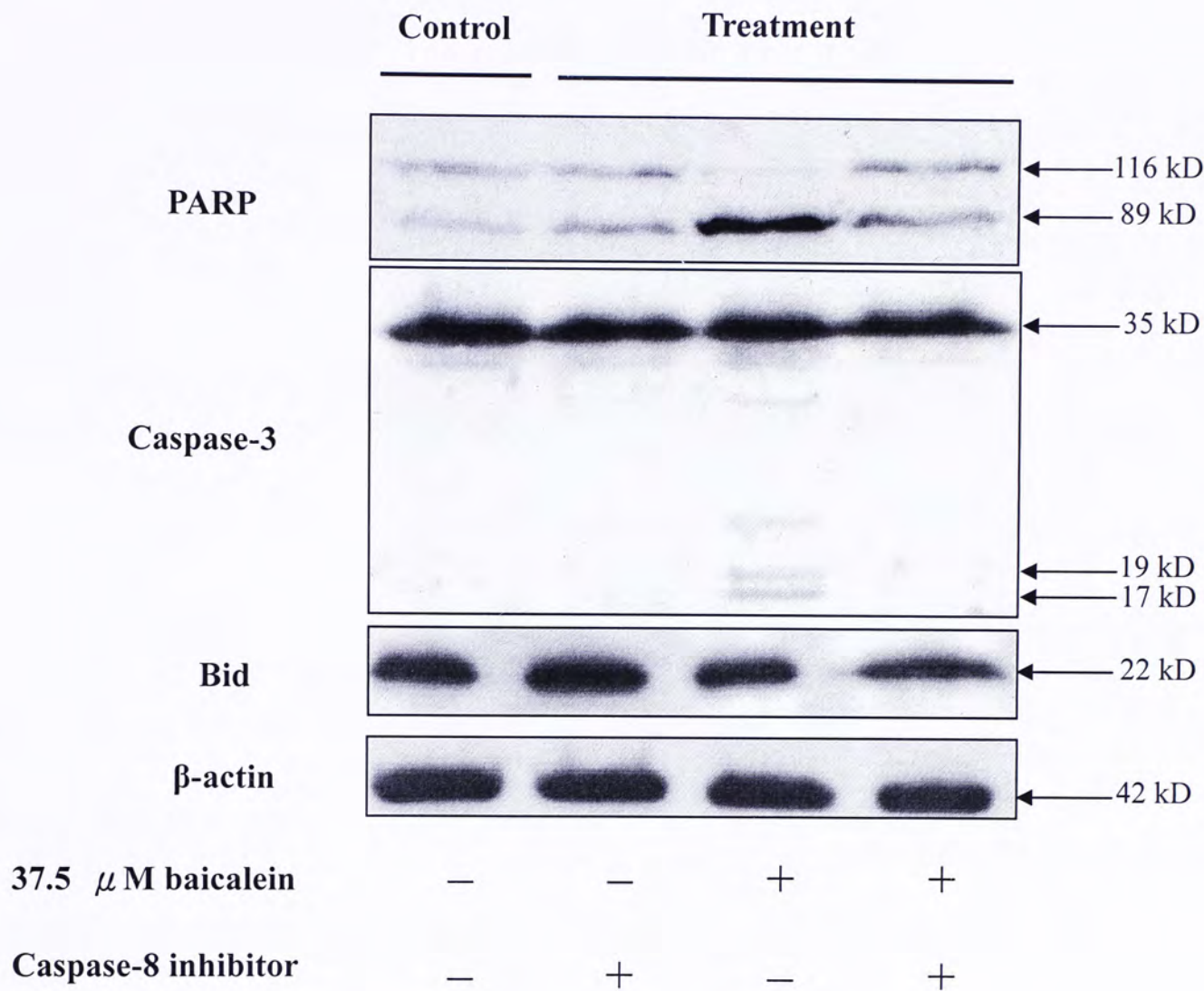
Fig. 3.5   **Representative immunoblots showing the effect of casapse-8 inhibitor on baicalein-induced apoptosis**
The melanoma cells were incubated in the absence, i.e., control, or in different combinations of baicalein at IC_{50} and 40μM caspase-8 inhibitor (Z-IE(OMe)TD(OMe)-FMK) for 48 h. The caspase-8 inhibitor retarded the baicalein-induced PARP and caspase-3 cleavage so that they became comparable to the control level. Neither baicalein treatment alone nor co-administration with caspase-8 inhibitor affected Bid expression.

β–actin was also detected as internal control to normalize the expressions of other proteins.

To consolidate the findings that caspase-8 involves in the activation of caspase-3 and subsequently the induction of apoptosis, caspase-3 activity was measured after different combinations of baicalein and caspase-8 inhibitor treatment. Specific caspase-3 fluorogenic substrate, Ac-DEVD-AMC, was then added to the cells, and the activity of caspase-3 was measured according to the intensity of fluorescence of free AMC released by active caspase-3. After baicalein treatment alone, caspase-3 activity was significantly elevated to 3.6 fold of the control level, which was agreed with the immunoblotting result mentioned previously. After 1 h prior treatment with the caspase-8 inhibitor, the baicalein-induced caspase-3 activity diminished to only 2 fold of the control level. (Fig. 3.6)

To sum up, caspase-8, the earliest caspase being activated by baicalein, plays an initiating role in the baicalein-induced, caspase-dependent apoptosis. This was evidenced by the reversal of PARP inactivation and de-activation of caspase-3 after the pre-treatment of caspase-8 inhibitor with baicalein.
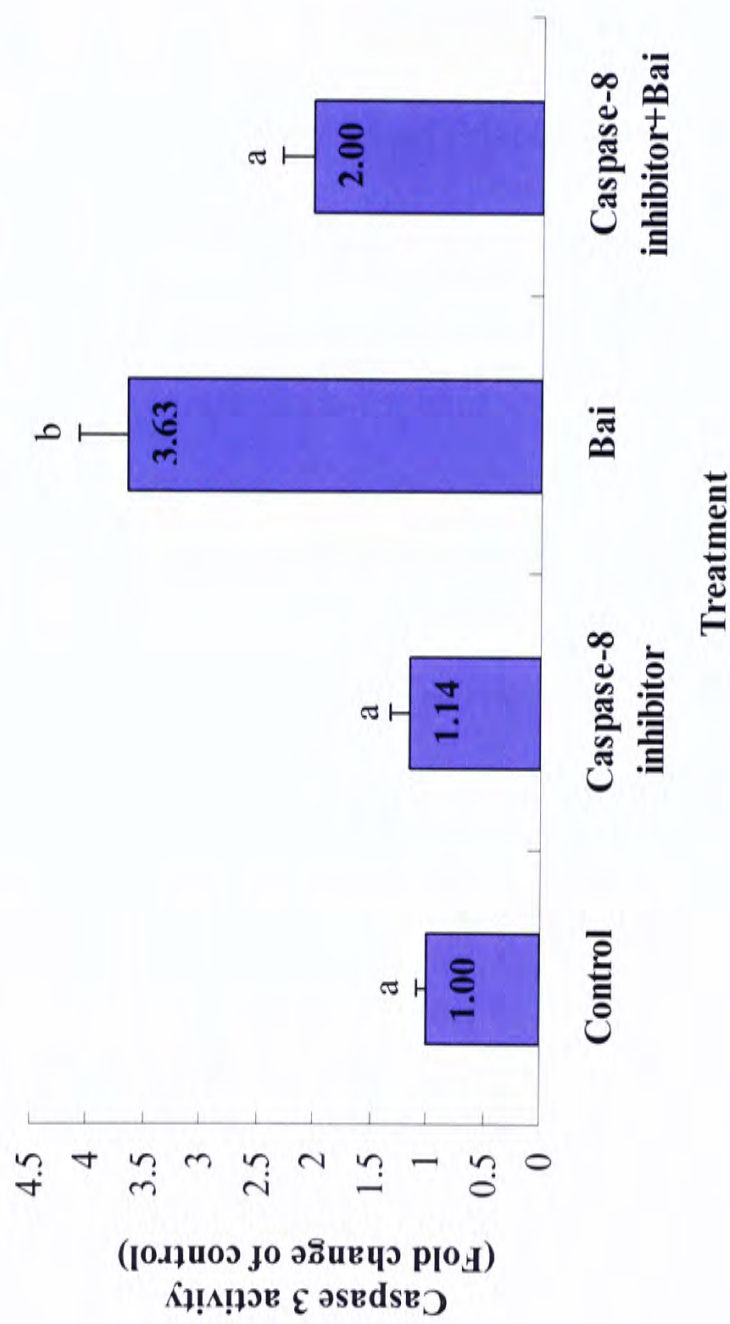
Fig. 3.6

Effects of baicalein and caspase-8 inhibitor on caspase-3 activity

Different combinations of baicalein (Bai) and caspase-8 inhibitor (Z-IE(OMe)TD(OMe)-FMK) were incubated with the melanoma cells and caspase-3 activity was measured by the fluorescence of AMC cleaved from the caspase-3 substrate Ac-DEVD-AMC. The caspase-8 inhibitor retarded the baicalein-induced activation of caspase-3.

Data are shown as fold change of control level. Results are expressed as mean ± S.D of three independent experiments. a and b represent the statistical significance ($p=0.05$) of the difference between control and the treatment groups, which was determined by one-way ANOVA followed by Tukey's test for multiple comparisons.

3.4 Discussion

As mentioned in chapter 1, cellular homeostasis is maintained by the balance between cell proliferation and cell death. A network of overlapping molecular mechanisms is involved in the exquisite control of cell proliferation and programmed cell death, i.e. apoptosis. Any aberrant changes in either branches of the cellular homeostasis may result in increased cellular multiplicity, resulting in the formation of neoplasia that may become cancerous (Bertram, 2001).

Uncontrolled cell proliferation is one of the profound characteristics that can be observed in cancer cells. Proto-cancer cells often acquired mutations that drive uncontrolled cell-cycle progression so that the mutated cells can escape from the homeostatic restraints and growth autonomously (Lowe *et al.*, 2004). As a result, many anticancer drugs are developed aiming at the deregulated cell cycle in cancer cells (Donovan *et al.*, 2005) In order to further assess the growth-inhibitory effect of baicalein on A375 cells, cell cycle after baicalein treatment was investigated, aiming at revealing capability of the phytochemical to halt the uncontrolled cell cycle progression and thus hinder cell growth. There are four serial biologically defined phases in cell cycle: G_1 (first gap), S (DNA synthesis), G_2 (second gap), and M (mitosis). Among and within different phases of the cell cycle, checkpoints are present to ensure proper completion of the steps in the previous phase prior proceeding to the next phase (Eastman, 2004). The change of cell cycle was studied

by DNA flow cytometry, and the cells at different phases of cell cycle were determined by their difference in DNA content. As shown in the DNA histograms, baicalein initiated a prominent S-phase arrest in the melanoma cells after 36 h of incubation. The S-phase arrest persisted and the cells were accumulating continuously up to 72 h of incubation. The baicalein-induced S-phase arrest was accompanied by the progressive decreases of $G_{0/1}$ cells. This further illustrated that the treated melanoma cells were able to pass through the G_1 checkpoint prior to the DNA synthesis, but could not pass through the G_2 checkpoint after doubling the DNA content and undergo mitotic division, i.e. entering the M phase of cell cycle. Similar S-phase arrest in A375 cells was also been reported after treatment with a catechin derivative (Lozano *et al.*, 2005).

Prolonged cell cycle arrest will initiate cells to undergo apoptosis, so that the hypodiploid cells may appear in DNA histogram because of DNA fragmentation, which is a characteristic feature of apoptosis (Fischer and Schulze-Osthoff, 2005). At the early stage of apoptosis, chromosomal DNA will be fragmented into 50- to 200-kb fragments, then later into nucleosomal units of DNA fragments (Nagata, 2000). Therefore, cells undergoing apoptosis will appear as discrete peak (sub-G_1 peak) on the left of G_1 peak, indicating the presence of hypodiploid nucleosomes in the DNA histogram (Compton, 1992; McConkey *et al.*, 1996; Tanuma and Shiokawa, 1996). The sub-G_1 population apparently appeared after 36 h of baicalein incubation.

This apoptotic population was then increasing synchronously with the S-phase-arrested cells, so that more than 50% of the cells had undergone apoptosis at 60 h of treatment.

To further manifest the occurrence of apoptosis in baicalein-treated cells, proteolytic inactivation of PARP was studied by immunoblotting. PARP is an enzyme that governs the post-translational modification of proteins involved in DNA repair, cell death and cellular emergency reactions (D'Amours *et al.*, 1999; Pieper *et al.*, 1999). This reaction, named as poly(ADP-ribosyl)lation, is important in maintaining genome integrity, particularly at times when DNA nicks were detected (Scovassi and Poirier, 1999). At the early stage of apoptosis, when DNA fragmentation begins, PARP activity is first stimulated for DNA repair; however, when a massive apoptotic DNA fragmentation occurred which signals a "no-return" cell death, PARP will be cleaved and become inactivated (Scovassi *et al.*, 1998). Consequently, immunodetection of the cleaved products of PARP has been regarded as a prominent evidence of apoptotic cell death (Soldani *et al.*, 2001). From the immunoblotting result, occurrence of apoptosis is evidenced by the presence of the cleaved p89 fragment of PARP. The cleaved fragment started to appear after 36 h of treatment. The proteolytic inactivation was then intensified with prolonged baicalein incubation, as shown by the increasing intensity of the p89 fragment. Starting from 60 h and afterwards, massive apoptosis took place which signalled the subsequent degradation

of intact PARP so that no intact 116-kD PARP, but only the p89 proteolytic product

was detected.

After confirming the induction of apoptosis by baicalein, we were interested in

the upstream signalling events that lead to it. Caspases have been regarded as the

enzymes of apoptosis (Zönig *et al.*, 2001; Crighton and Ryan, 2004). This family of

intracellular proteases can either be self-activated or activated by other caspases

before participating in the proteolytic cascade in apoptosis (Cryns and Yuan, 1998;

Thornberry and Lazebnik, 1998). According to their functions in the apoptotic

cascade, caspases can be divided into two subclasses: the initiator caspases, including

caspase-8 and -9; and executioner caspases, including caspase-3, -6 and -7. Nearly all

caspases have been reported previously to be able to proteolytically inactivate PARP

in vitro (Soldani *et al.*, 2001). Therefore, the activation of various caspases was

studied by immunoblotting. Interestingly, we found that caspase-8, -3 and -9 were

activated at different times of baicalein treatment. Caspase-8 was found to be the

earliest caspase activated after 24 h of treatment, whereas both caspase-3 and -9 were

activated after 48 h. This sequential activation suggested that the extrinsic apoptotic

pathway, in which active caspase-8 is an initiator caspase of the pathway (Ghobrial *et*

al., 2005), was elicited after baicalein treatment. Involvement of the extrinsic

pathway in the baicalein-induced apoptosis was further evidenced by the

pharmacological inhibition of caspase-8 so that the activation of its downstream

substrate caspase-3 was terminated (Budihardjo *et al.*, 1999). Cleavage of PARP was also rescued consequently.

From these results, it is revealed that baicalein induces apoptosis in A375 cells first through S-phase cell cycle arrest prior to apoptosis. The apoptosis is caspase-dependent and the death receptor signalling pathway is elicited.

Chapter 4. Effects of Baicalein on the Extrinsic Apoptotic Pathways in Human Melanoma A375 Cells

4.1 Introduction

Besides acting as an evidence to support the elicitation of apoptosis, activation of different caspases also serves as an indicator which the apoptotic pathways involved can be identified. In the previous chapter, we have demonstrated that caspase-dependent apoptosis was elicited by baicalein in A375 cells. Among the caspases activated, caspase-8 was activated at the earliest time of treatment.

Caspase-dependent apoptosis can be triggered via two pathways: the extrinsic death-receptor pathway and the intrinsic mitochondrial pathway. Caspases involved in apoptosis can basically divide into two subtypes: the initiator caspases (caspase-2, -8, -9 and -10), which responsible to the detection of death signals prior to self-activation, and the effector caspases (caspase-3, -6 and -7) which are responsible for cell demise after activated by the initiator caspases. From the two subsets, caspase-9 is regarded as the leading caspase in the intrinsic mitochondrial pathway; whereas caspase-8 is the key initiator of death-receptor mediated apoptosis (Kumar, 2007). The early activation of caspase-8 in baicalein-induced apoptosis has indicated the possible activation of the extrinsic, death-receptor-mediated pathway.

The extrinsic apoptotic pathway is elicited by ligation of the death-inducing ligands to the death receptors which are present on the cell surface. Death receptors

are members of the TNF receptor superfamily which have been identified to have broad range of biological functions including cell death and survival regulation (Walczak and Krammer, 2000). Members of this family are entrusted with the ability to transmit death signals by the possession of an 80-amino-acid cytoplasmic domain called the 'death domain' (Bossen *et al.*, 2006). Six members have been identified in this family, in which four of them have been well characterized in the induction of apoptosis: CD95 (APO-1/Fas), TNF receptor 1 (TNFRI), TRAIL-R1 (DR4) and TRAIL-R2 (DR5). By the engagement of death receptors to their corresponding ligands, which are CD95 ligand /FasL, TNFα and TRAIL, cell death signals from the cell surface to the cytoplasm can thus be elicited (Herr *et al.*, 2000).

As potential therapeutic targets, the three death-receptor/ligand pairs have been extensively studied. Having longer history than TRAIL, TNFα and Fas were found to possess antitumor activity *in vitro* and has been employed as a potential therapeutics *in vivo* (Ghobrial *et al.*, 2005). Unfortunately, both cytokines possess different degrees of toxicity that impairs normal physiological functions, as discussed previously in Chapter 1. As a result, the tumor-specific TRAIL-mediated pathway becomes an attractive potential therapeutics, and has been receiving enormous attention.

TRAIL appears to have specificity in triggering apoptosis towards tumor cells but not in normal cells (Suliman *et al.*, 2001; Boehrer *et al.*, 2006). The TRAIL

resistance observed in normal tissues is postulated to be the expression of the decoy

receptors of TRAIL, DcR1 andDcR2, which lack the ability to induce apoptosis

(Zhang *et al.*, 2001). Some of the TRAIL-resistant cancer cells were also identified

with overexpression of the decoy receptors. On the other hand, it was found that the

functional receptors, DR4 and DR5, were widely expression in different tissues, yet

the expression was highly elevated when compared to that of normal cells (Kendrick

et al., 2007). As a result, one of the determinants of TRAIL sensitivity of the cell is

the net effect of death and decoy receptors that express and function on the cell

surface.

Owing to its minimal toxicity, TRAIL has been therefore targeting for

therapeutic development. One of the therapeutic routes would be the administration

of recombinant TRAIL. Several previous studies have suggested that recombinant

TRAIL exhibited poor specificity for tumor cells and significant hepatotoxicity,

which was related to the preparation method of the recombinant TRAIL. One

construct of recombinant TRAIL, Apo2L/TRAIL (PRO1762), is currently being

evaluated in phase I clinical trials. (Genentech, South San Francisco, CA; Amgen,

Thousand Oaks, CA)   (Almasan and Ashkenazi, 2003).

Being a promising therapeutic target, the synergism between TRAIL-mediated

apoptosis and conventional chemotherapy has been extensively investigated.

Accumulating evidence suggests that several cytotoxic agents, such as cisplatin,

paclitaxel, and adriamycin, administered in combination with TRAIL *in vitro* and *in vivo* enhance apoptosis (Komdeur *et al.*, 2004; Lane *et al.*, 2004). Furthermore, TRAIL-mediated apoptosis has also been shown to enhance the effects of ionizing radiation (Chinnaiyan *et al.*, 2000; Buchsbaum *et al.*, 2003). Observed from these findings, TRAIL would be a promising candidate for the development of combined anticancer regimen which may give rise to novel and effective treatments.

As caspase-8 was found to be elicited in the baicalein induced apoptosis, the components of the extrinsic death receptor pathway were thus studied by immunoblotting. Moreover, exogenous TRAIL was added to the baicalein-treated cells to see if the phytochemical treatment could sensitize the cells to TRAIL-mediated apoptosis, which is evidenced to be a safe and effective mode of apoptosis.

4.2 Materials and Methods

4.2.1 Immunoblotting

Detailed procedures of immunoblotting were described in section 3.2.2 with some modifications. In this section, primary antibodies against DR4 and TRAIL (both from BD Biosciences) were used under the dilution of 1:500 and 1:300 respectively; primary antibodies against DR5 and β-actin (both from Sigma) were used under dilution factor of 1:500 and 1:5000 respectively.

4.2.2 Determination of sub-lethal dose of exogenous TRAIL

The sub-lethal dose of exogenous TRAIL on A375 cells was determined by MTT assay (details in section 2.2.2). Briefly, 1×10^4 cells per 100 µl were seeded in each well of a flat-bottomed 96-well plate. After 24 h acclimatization, the cells were challenged with 0- 400 ng/ml TRAIL (Calbiochem, La Jolla, CA) for 12 h. MTT assay was then performed and the sub-lethal dose of TRAIL was estimated from the growth-inhibitory plot.

4.2.3 Determination of the combinatory effect of exogenous TRAIL and baicalein

The combinatory effect of exogenous TRAIL and baicalein on the growth and survival of A375 cells was also determined by MTT assay (details in section 2.2.2). Briefly, 5×10^3 cells per 100 µl were seeded in each well of a flat-bottomed 96-well plate. After acclimatization, the cells were then treated with 0- 100 µM of baicalein for 48 h. Twelve hours before the cells were harvested (i.e. after 36 h of incubation), the sub-lethal dose of TRAIL was added in each well of the plate and incubated for further 12 h. MTT assay was then performed and the combinatory effect of exogenous TRAIL and baicalein was compared using the growth-inhibitory plots.

4.2.4 Statistical analysis

Results were expressed as mean $\pm$ S.D. and analyzed by Student's t-test. The level of significance was taken at $p=0.05$, $p=0.01$ and $p=0.001$.

4.3 Results

4.3.1 Baicalein upregulates the expressions of death receptor 4 (DR4) and death receptor 5 (DR5)

As mentioned in section 3.3.3, caspase-8 was found to be the major initiator of the apoptotic cascade induced by baicalein. As caspase-8 is the initiator caspase in the death receptor signaling branch of apoptosis, we therefore postulated that other components of the death receptor signaling pathway would also be modulated after the baicalein treatment. In this study, immunoblotting was used to determine the effects of baicalein on the expressions of two death receptors, DR4 and DR5, as well as their corresponding death signal ligand, TRAIL. The melanoma cells were incubated with baicalein at IC_{50} for 24 to 72 h. As shown in Fig. 4.1, both DR4 and DR5 levels elevated at 24 h of baicalein treatment, and the elevation persisted over the next 48 h when compared to their corresponding time controls. On the other hand, the TRAIL level was not significantly affected even after 72h of baicalein treatment. These findings demonstrated the selective effect of baicalein on the different

components in the death receptor apoptotic pathway. Also, it seemed that the

phytochemical affected the receptors rather than the ligand of the TRAIL pathway.
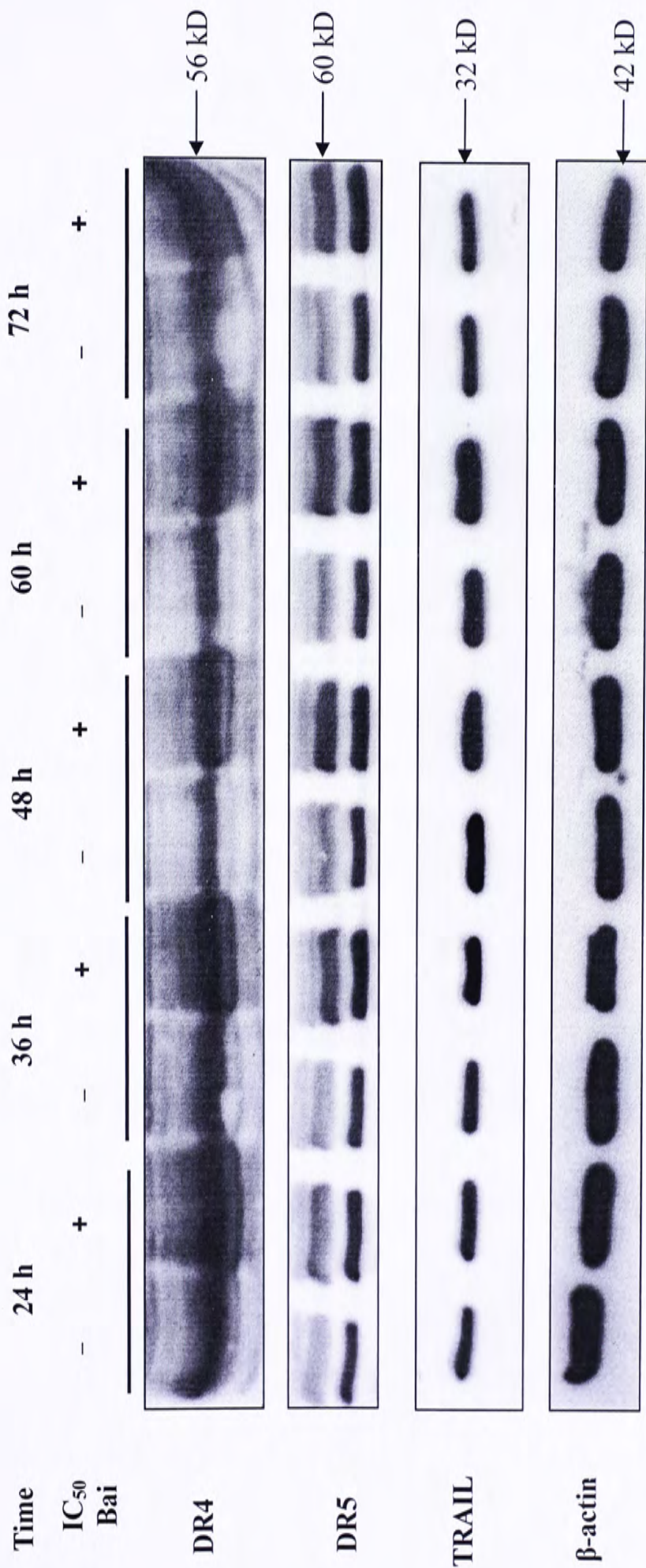
Fig. 4.1 **Representative immunoblots showing the effects of baicalein on the expressions of DR4, DR5 and TRAIL**
The melanoma cells were incubated in the absence, i.e., control (−), or in the presence of baicalein (Bai) at IC_{50} (+) for 24 to 72 h. Baicalein induced elevation of DR4 and DR5 levels and did not affect TRAIL level persistently over time. β-actin was also detected as internal control to normalize the expressions of other proteins.

4.3.2. **Baicalein sensitizes the melanoma cells to sub-lethal dose of exogenous**

TRAIL

As elevation of the two death receptors, DR4 and DR5, were observed as early as

24 h of baicalein treatment, which was concomitant with the activation of caspase-8,

we hypothesized that the elevation might be able to sensitize the melanoma cells to

the death signal. To testify the hypothesis, we first determined the sub-lethal dose of

exogenous TRAIL by challenging the melanoma cells with various doses of TRAIL,

from 0 to 400 ng/ml for 12 h. Incubation with exogenous TRAIL caused a

dose-dependent inhibition of growth and survival in A375 cells, yet the inhibition

became plateau after 25 ng/ml so that 44% inhibition of growth and survival was

attained at the maximum dose of 400 ng/ml (Fig. 4.2). From the result, a sub-lethal

dose of 12.5 ng/ml TRAIL was chosen to further investigate the sensitization effect of

baicalein on the melanoma cells. At 12.5 ng/ml TRAIL, approximately 5% inhibition

of cell growth and survival was detected, although the inhibition was not statistically

significant.

The melanoma cells were then challenged with 12.5 ng/ml TRAIL and various

concentrations of baicalein to study the sensitization effect of the phytochemical on

TRAIL treatment. Although the sub-lethal dose of TRAIL did not induce significant

decrease in cell growth and survival in A375 cells, baicalein prominently sensitized

the melanoma cells to the TRAIL treatment. The sensitization effect of baicalein on

TRAIL is depicted in Fig 4.3. The combinatory effect was the most prominent with 12.5-50 μM baicalein, at which the growth inhibition was at most doubled after exogenous TRAIL addition, when compared to the baicalein treatment alone.

Taken together, baicalein can induce cell death in A375 cells via modulation of the extrinsic apoptotic pathway, in which the levels of death receptors DR4 and DR5 are elevated. This elevation has functional significance since it can sensitize the cells to the TRAIL death signal. Therefore, combinatory effect does exist between baicalein and TRAIL on reducing growth and survival of the melanoma cells.
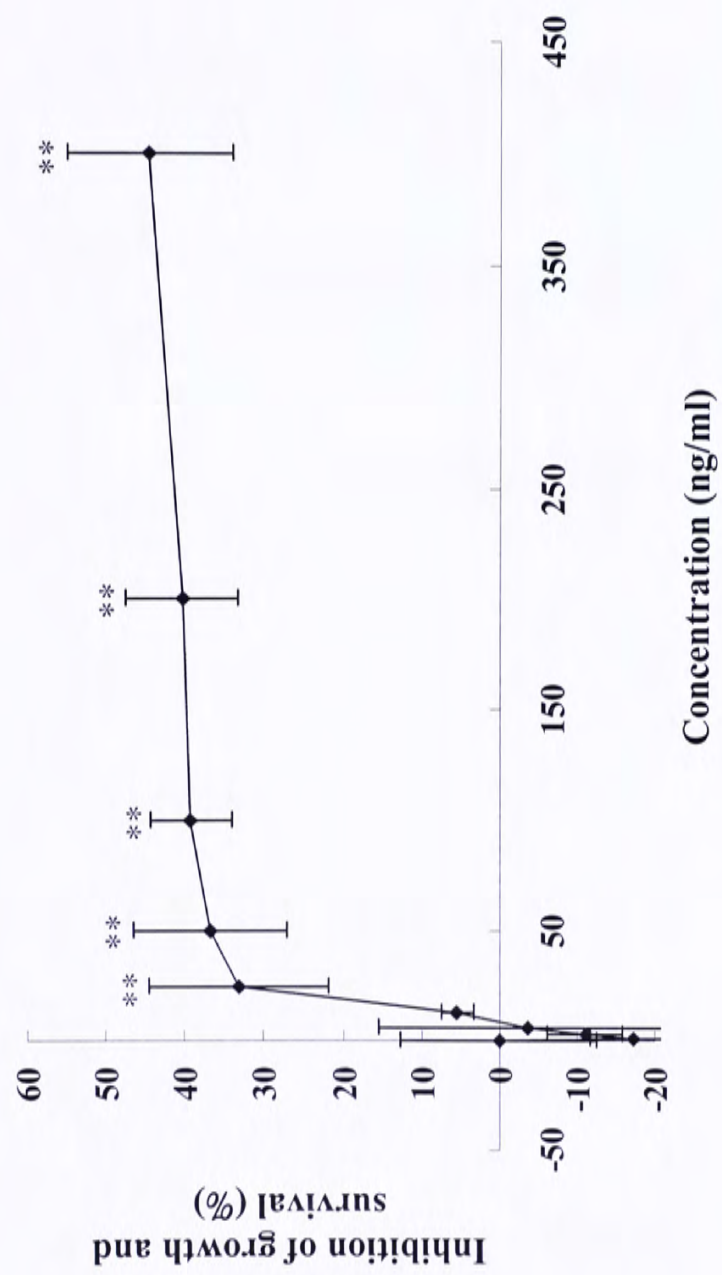
Fig. 4.2

Effect of TRAIL on growth and survival of A375 cells

Growth and survival of the melanoma cells was measured by MTT assay after incubation with 0-400 ng/ml of TRAIL for 12 h. The inhibition became plateau at 25 ng/ml TRAIL. Since 12.5 ng/ml TRAIL did not induce significant inhibition on growth and survival of the melanoma cells, it was chosen for further study. Results are expressed as mean ± S.D. (n=3)

** $p < 0.01$, when compared to the control level by Student's t-test.

Concentration of baicalein (μM)
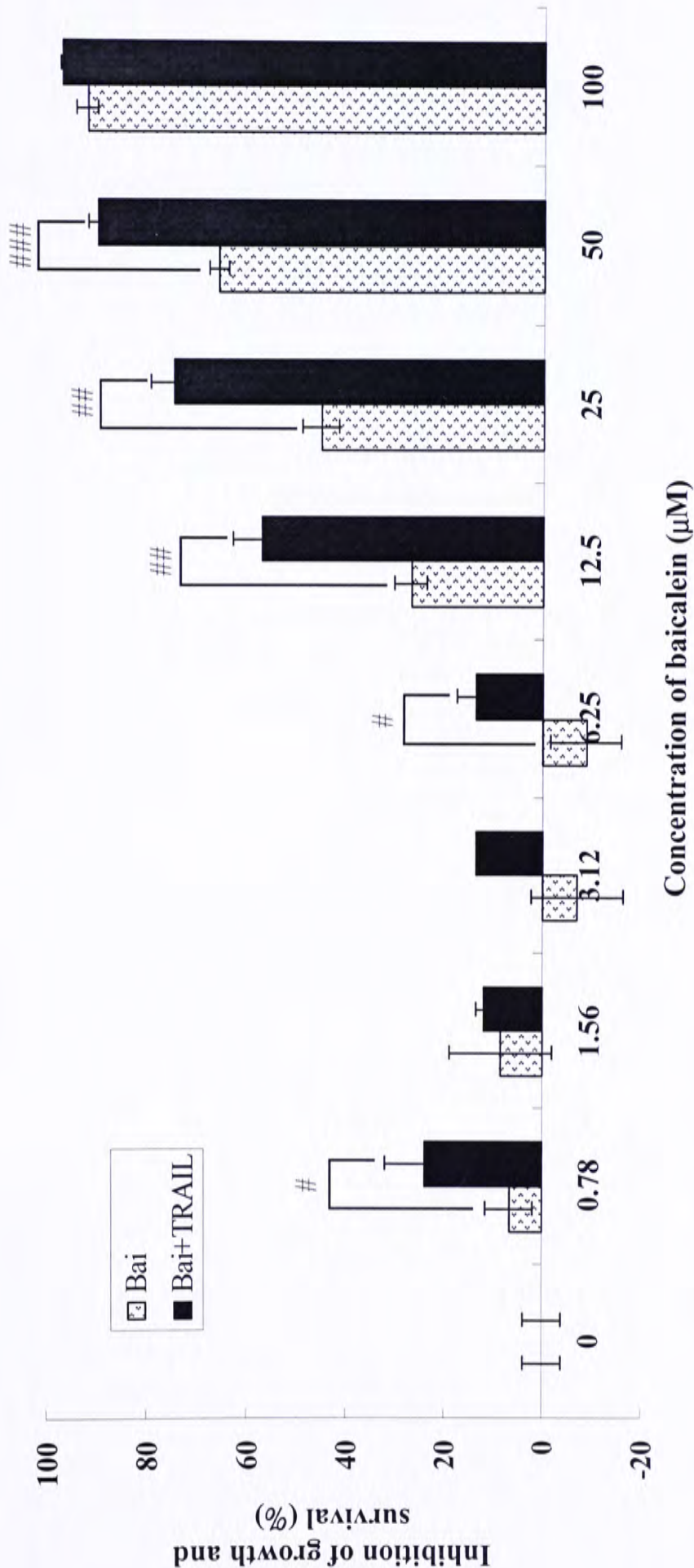
Fig. 4.3

Sensitization effect of baicalein on TRAIL treatment

The growth and survival of A375 cells was measured by MTT assay after incubation with 0-100 μM of baicalein for 48 h in the absence (Bai) or in the presence of 12.5 ng/ml TRAIL (Bai+TRAIL). The sensitization was most prominent with of 12.5 to 50 μM baicalein. Results are expressed as mean ± S.D. (n=3) # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ represent statistical significance, when compared to the control levels (i.e. treatment under the same concentration of baicalein) by Student's t-test.

4.4   Discussion

In chapter 3, we demonstrated baicalein induced caspase-dependent apoptosis after cell cycle arrest in A375 cells. As caspase-8 was the earliest caspase activated in the signaling cascade, which suggested the involvement of death receptor-mediated signal transduction, other components of the extrinsic pathway were therefore studied. Being an initiator caspase, caspase-8 transduces various death signals through the formation of death-inducing signaling complex (DISC). After binding of the death ligand to its receptor, the complex is formed by the interaction between the death effector domain (DED) of procaspase-8 and the adaptor protein Fas-associated death domain (FADD) so that the inactive zymogen of caspase-8 is activated (Debatin and Krammer, 2004; Nuñez et al., 1998). As the first step to evaluate the effect of baicalein on the receptor-mediated pathway, the expression of two death receptors, DR4 and DR5, as well as their corresponding death-inducing ligand, TRAIL were studied. Unlike other death-inducing ligands such as FasL and TNFα, which are generally toxic, TRAIL shows selective cytotoxic effect on cancer cells but not on normal cells (LeBlanc and Ashkenazi, 2003; Koschny et al.,2007). This differential response can be explained by the expression of death receptors of TRAIL only on cancer cells (Hersey and Zhang, 2001) and thus makes TRAIL mediated-apoptosis an attractive target to investigate and develop. Nonetheless, TRAIL has not yet been a commonly used anticancer agent due to the innate or

acquired resistance against TRAIL observed in many cancers (Ivanov and Hei, 2006), such as melanoma (Griffith *et al.*, 1998), primary lymphoma and glioblastoma (Koschny *et al.*, 2007).

As shown in the immunoblotting results, baicalein elevated both DR4 and DR5 in the melanoma cells as early as 24 h of treatment. This elevation was compatible to the activation of caspase-8 in a temporal manner which indicated a causal link might exist between death receptor elevation and caspase-8 activation in the baicalein-induced apoptosis. Ivanov and Hei (2006) proposed that an effective TRAIL-mediated signaling in cancer cells should have the following requirements: 1) The death-inducing ligand, i.e. TRAIL, either exogenous or induced surface expression; 2) sufficient surface expression of DR4/5 to accomplish effective apoptosis induction by receptor oligomerization or DISC formation and 3) effective repression of anti-apoptotic proteins. With reference to these three major criteria of TRAIL-induced apoptosis, baicalein seems to enhance initiation of the extrinsic apoptotic pathway through upregulation of death receptors. As more DR4 and DR5 were expressed in the baicalein-treated cells, trimerization of the receptors and thus the subsequent DISC formation were facilitated. This resulted in the activation of caspase-8 and onset of the apoptotic cascade.

To testify the functional significance of the baicalein-induced expression of DR4 and DR5, the baicalein-treated melanoma cells were challenged with a

sub-lethal dose of exogenous TRAIL to see if baicalein could sensitize the melanoma towards a non-cytotoxic dose of TRAIL. Since the cellular response towards TRAIL depends on the histotype of cells and the preparation method of the death ligand (LeBlanc and Aschkenazi, 2003), the sub-lethal dose of TRAIL specific for A375 cells in this study was first determined. Revealing from the dose-response curve of A375 to TRAIL, the growth-inhibition occurred dose-dependently, which become plateau with 25 ng/ml or higher doses. A similar pattern of dose-response of A375 cells to TRAIL was also reported (Kurnabov et $al.$, 2007). From our results, the sub-lethal dose of 12.5 ng/ml TRAIL was selected, which was the maximum concentration of TRAIL that did not cause a statistically significant inhibition of the growth and viability of A375 cells (>95%).

Human malignant melanoma A375 has been previously studied of having a consistent expression of both DR4 and DR5, and was classified to be a TRAIL-sensitive cell line (Kurbanov et $al.$, 2005). This is coherent with our findings that both DR4 and DR5 were constitutively expressed in untreated A375, as well as its responsiveness to TRAIL inhibition. As predicted from our hypothesis, baicalein sensitizes the melanoma cells and exhibits a combinatory effect with TRAIL. For instance, after the cells have been treated with 12.5 µM baicalein, even the sub-lethal dose of TRAIL could further reduced the growth and survival of the cells by 2-fold when compared to the baicalein-treated cells.

The combinatory effect between baicalein and TRAIL may be explained by the elevated DR4 and DR5 expression induced by baicalein. Several phytochemicals have also been reported to enhance DR4 and DR5 expression in cancer cells, such as sulforaphane which is abundant in cruciferous vegetables (Matsui *et al.*, 2006); and quercetin that can be widely found in vegetables and fruits (Chen *et al.*, 2007). However, according to our knowledge, we are the first group to show that baicalein can sensitize melanoma cell and exhibit combinatory effect with TRAIL to inhibit its growth.

Taken together, baicalein is able to augment the apoptotic effect of TRAIL in A375 cells by elevating DR4 and DR5 levels. This suggests an attractive anticancer mechanism that, being a neoplasm-specific cytotoxic agent (section 2.3.2), baicalein is able to upregulate expression of DR4 and DR5 in the melanoma cells, and thus sensitizes the cancer cells to a non-cytotoxic level of TRAIL. Combinatory effect of this phytochemical with the novel therapeutic agent TRAIL is therefore worth investigating for development of new anticancer regimen.

Chapter 5. Effects of Baicalein on the Intrinsic Apoptotic Pathways in Human Melanoma A375 Cells

5.1 Introduction

The apoptotic route that undergone by dying cells could be delineated by analyzing the activation of various caspases, as previously mentioned in Chapter 3. This led our investigation in the extrinsic apoptotic pathway in the baicalein-treated melanoma cells, owing to the early activation of caspase-8. To this end, the baicalein-induced apoptosis was found to be mediated via the extrinsic death-receptor pathway, primarily through the elevation of death receptors.

In addition to the activation of caspase-8 in baicalein-treated cells, caspase-9 was also found to be activated (Chapter 3). Besides the initiation role played by caspase-8 in the extrinsic apoptotic pathway, caspase-9 is also an initiator caspase in the intrinsic mitochondrial pathway (Kumar, 2007). Similar to the activation of caspase-8, the activation of caspase-9 also suggested the possibility of the elicitation of the intrinsic mitochondrial pathway.

The intrinsic apoptotic pathway is triggered by different kinds of stresses, compared with that of the extrinsic pathway which is ligand-receptor mediated (Crighton and Ryan, 2005). Upon the reception of the death-inducing stress signals, series of biochemical events occur and ultimately converge in the mitochondria. There are several morphological and biochemical features characterized the

mitochondrion-mediated apoptosis such as the occurrence of mitochondrial membrane permeabilization (MMP) and the release of cytochrome c (Budihardjo *et al.*, 1999). As mitochondrial dysfunction and the subsequent release of apoptogenic factors mark the "point of no return" in apoptosis (Hangartner, 2000). Therefore, the loss of mitochondrial membrane integrity is an indication of the onset of the mitochondrion-mediated apoptosis.

There are several analytical methods available for the detection of MMP, yet the most commonly employed method is the detection of mitochondrial membrane depolarization. Under normal physiological condition, the inner mitochondrial membrane is nearly impermeable to all ions, which allows the building up of proton gradient necessary for oxidative phosphorylation (Kroemer *et al.*, 1998). Therefore, the maintenance of the proton gradient within the mitochondria is an indicator of normal mitochondrial functions. Once the intrinsic apoptotic pathway is initiated, the mitochondrial membrane is perforated and therefore the mitochondrial membrane potential can no longer be maintained. Therefore the dissipation of mitochondrial membrane potential is a good indicator of MMP, which is a hallmark of intrinsic mitochondrion-mediated cell death (Marchetti *et al.*, 1996).

JC-1 flow cytometric analysis

The detection of mitochondrial membrane depolarization by flow cytometry with cationic lipophilic dyes has been a convenient analytical tool to assess the

occurrence of MMP. There are several dyes available for the purpose, such as

Rhodamine123 (Rh123), $DiOC_6$ and JC-1. However, drawbacks of some dyes have

been identified. For instance, apoptotic cells tend to exhibit an increase in

fluorescence obtained with Rh123 caused by mitochondrial swelling (Zuliani *et al.*,

2003). As a result, the choice of membrane integrity fluorochromes would be critical.

In this context, the use of fluorochrome JC-1 (6'-tetrachloro-1, 1', 3,

3'-tetraethylbenzimidazolylcarbocyanine iodide) would be an appropriate choice for

the detection of mitochondrial membrane dissipation. Under normal circumstances,

JC-1 exists as J-aggregates in the inner, polarized mitochondrial membrane and emits

red fluorescence of 590 nm. As the mitochondrial membrane potential drops under

apoptotic conditions, J-aggregates disintegrate and JC-1 exists as its

green-fluorescent monomeric form, emitting fluorescent signal at 527 nm. Therefore

the dissipation process can be visualized on the bivariate DNA histogram by

observing the shift of red color to green color under the same excitation wavelength

of 488nm (Salvioli *et al.*, 1997; Zuliani *et al.*, 2003). The ratio of green to red

fluorescence emitted by JC-1 is therefore an indicator of extend of mitochondrial

membrane depolarization (Bedner *et al.*, 1999).

To investigate the mitochondrial membrane depolarization induced by baicalein

in A375 cells, JC-1 was applied to examine the mitochondrial membrane potential. It

is well known that several stimuli induce a decrease in the mitochondrial

transmembrane potential, and this indicates the mitochondrial membrane is permeabilized and thus allows the release of apoptogenic factors, such as cytochrome c (Crompton, 1999). Therefore in order to substantiate the occurrence of MMP, detection of cytosolic cytochrome c was also performed by immunoblotting.

The Bcl-2 family of proteins has been regarded as the central regulator of mitochondrial pathway (Ghobrial et al., 2005). As a result, the expression of several well-characterized Bcl-2 family proteins in baicalein-treated cells was also investigated in the present study by immunoblotting. The Bcl-2 family can be sub-divided into anti-apoptotic (e.g. Bcl-2, Bcl-xL etc.) and pro-apoptotic (e.g. Bax, Bad etc.). The activated members from the two sub-group of the Bcl-2 family exert their functions at mitochondria by interacting with one another and induce permeabilization of the outer mitochondrial membrane. The extrinsic pathway can be connected the intrinsic pathway via the Bcl-2 family, BH-3 only member Bid. Activated caspase-8 of the extrinsic pathway, Bid can be cleaved and truncated (i.e. tBid). The tBid formed then translocates to mitochondria and enhances the oligomerization of the pro-apoptotic Bcl-2 family members, such as Bax and Bak, leading to outer mitochondrial membrane permeabilization (Kandasamy *et al.*, 2003; Schwarz *et al.*, 2007). The induction of apoptosis by baicalein via the modulation of the Bcl-2 family proteins has been previously report in other types of cancer. For instance, the apoptosis induced by baicalein in breast cancer cells was elicited via the

reduction of expression of the antiapoptotic Bcl-2 and Mcl-1 (Tong *et al.*, 2002). Nonetheless, the regulation of Bcl-2 family proteins might not be obligatory in the baicalein-induced mitochondrial pathway, especially the induction of MMP, as previously reported by Wang *et al.* (2004).

Though Bcl-2 family proteins are the central regulator of mitochondrial dysfunction, other mechanisms that leads to MMP may also elicited by baicalein in the melanoma cells. In this context, the role of ROS in the mitochondrion-mediated apoptosis should therefore be investigated. Reactive oxygen species (ROS) has been recently identified to play a regulatory role in apoptosis (Ott *et al.*, 2007). It is formed as a natural byproduct of the normal metabolism of oxygen. Mitochondria, the power house of the cell, would therefore be the primary site of production of ROS by oxidative phosphorylation (Storz, 2007). Despite the available antioxidative systems that can eradicate the excess ROS from the cells, the imbalance of the production and removal of ROS will lead to devastating damages. Ironically, the major producer of cellular ROS, the mitochondria would be the primary sites damaged by plethora of ROS.

ROS exerts its devastating effects by targeting different machineries in the mitochondria. The mitochondrial DNA (mtDNA), which is free of histone and in close proximity with the ROS produced, is more vulnerable to oxidative damage than other components in the same organelle(Orrenius *et al.*, 2007). ROS also perturbs the

normal electron transport chain by oxidixing the ion-carrying proteins and related enzymes (Fridovich, 1997). As a potent oxidizing agent, ROS is capable in oxidizing the phospholipids on the mitochondrial membrane, which directly affects the membrane integrity. Consequently, the mitochondrial membrane potential can no longer be maintained, and leads to the impairment of mitochondrial functions (Venditti *et al.*, 2004).

To this end, the role of ROS in the mitochondrion-mediated pathway in baicalein-treated cells was also elucidated, aiming at revealing any possible mechanisms involved. To begin with, the intracellular ROS level was first detected by the application of a flurogenic probe, 2',7'-dichlorofluorescin diacetate (H_2DCFDA) and the addition of antioxidant Trolox^{TM} to delineate the possibility of baicalein-induced ROS on mitochondrial membrane depolarization and cytochrome *c* release.

ROS production assay by H_2DCFDA

The use of H_2DCFDA is a popular method for measuring the production of ROS by a variety of cell types, in both flow-cytometric and spectrofluorometric systems. This method is frequently employed due to its simplicity and high sensitivity. Also, it is relatively inexpensive when compare to the traditional approaches of ROS measurement, such as assaying for the reduction of cytochrome c by superoxide (Krejsa and Schieven, 2000). H_2DCFDA is able to cross the plasma

membrane and enter the cell, in which the probe will be hydrolyzed by cellular esterases to produce 2',7'-dichlorodihydrofluorescein (H_2DCF). The de-acetylated form of the probe is then susceptible to oxidation, generating a fluorescent product, 2',7'-dichlorofluorescein (DCF). Accumulation of DCF therefore indicates the production of redox-active substances (Brubacher and Bols, 2001). The resulting output of ROS is measured as the increase in fluorescence associated with oxidation of the probe, where this relationship between DCF and fluorescence is linear over a broad concentration range, allowing for expression of the relative level of ROS production in arbitrary units.

5.2 Materials and methods

5.2.1 Analysis of mitochondrial membrane potential

The change of mitochondrial membrane potential after baicalein treatment was detected by using the fluorescent potential sensor probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcanbocyanine iodide (JC-1; Molecular Probes, Eugene, OR). After harvest and thorough washing, 1×10^6 treated or untreated cells were incubated with 10µg/ml JC-1 for 15 minutes at 37 °C prior to analysis on a flow cytometer under an excitation wavelength of 488 nm. Data were expressed as the change of red/green fluorescence intensity ratio.

5.2.2 Fractionation of cell lysates into cytosolic and mitochondrial fractions for

immunoblotting

To obtain mitochondrial and cytosolic fractions, the melanoma cells were

homogenized and fractionated by the Cytosol-Mitochondria Fractionation kit

(Calbiochem). Briefly, 1×10^7 cells were harvested and resuspended in cytosol

extraction buffer (provided) supplemented with protease inhibitors for 10 minutes on

ice, and disrupted using a dounce tissue grinder. Homogenates were then clarified at

700g for 10 minutes at 4 °C. The supernatants were further centrifuged at 10 000g for

30 minutes at 4 °C. The resulting supernatants were recovered as cytosolic fraction

and the pellets were resuspended in mitochondrial extraction buffer (provided) to

yield the mitochondrial fraction. The protein content in the fractions were quantified

by BCA assay (details in section 3.2.2b) and were stored under -20°C prior use.

5.2.3 Immunoblotting

Detailed procedures of immunoblotting were described in section 3.2.2 with

some modifications. In this section, primary antibodies against Bcl-xL, Bax, Bad,

Bim, Bid and cytochrome c (all from Cell Signaling) were used under the dilution of

1:1000; whereas primary antibodies against Bcl-2 (BD Bioscience) and β-actin

(Sigma) were used under dilution factor of 1:250 and 1:5000 respectively.

5.2.4   Determination of cellular reactive oxygen species (ROS) production

Total cellular ROS production was measured using 2',7'-dichlorofluorescin diacetate (H_2DCFDA; Molecular Probes). Briefly, 5×10^5 cells were harvested and washed before staining with 10 μM H_2DCFDA in dark at 37 °C for 1 h. Cells were pre-treated with 500 mM hydrogen peroxide (H_2O_2; Sigma) 5 h prior collection of cells, which acts as a positive control. After incubation, the cells were washed and the fluorescence generated was measured by a fluorescence plate reader (Tecan) with an excitation wavelength of 460 nm and an emission wavelength of 530 nm. Fluorescence values generated in each treatment was converted into fold changes of the control.

5.2.5   Verification of ROS generation via the addition of Trolox[TM]

Trolox[TM] (Sigma), a water soluble vitamin E analogue, was co-administrated with baicalein to verify the effect of cellular ROS. Before baicalein treatment, cells were pre-treated with 100 μM Trolox[TM] for 1 h. After pre-incubation, baicalein at IC_{50} was added to further incubate for 48 h. The cells were then harvested for cytosolic/ mitochondrial cell fractionation (section 5.2.2) followed by immunoblotting (section 3.2.2) as well as for measurement of ROS (section 5.2.3).

5.2.6   Statistical analysis

Results were expressed as mean ± S.D. and the statistical significance of the difference between control and the treatment groups was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The level of significance was taken at p=0.05.

5.3   Results

5.3.1.   Baicalein induces mitochondrial membrane depolarization

As mentioned in section 3.3.2, baicalein first induced activation of caspase-8, which is the initiator caspase of the extrinsic or death receptor apoptotic pathway; followed by caspase-9, which is the initiator caspase of the intrinsic or mitochondrial pathway. To further substantiate the ability of baicalein in activating the mitochondrial apoptotic pathway, mitochondrial membrane depolarization, an early hallmark event of mitochondrial-mediated apoptosis, was investigated by JC-1 flow cytometry. Effect of baicalein on the mitochondrial membrane potential was determined after 24, 48 and 72 h of incubation. Under normal circumstances, JC-1 exists as J-aggregates in the inner, polarized mitochondrial membrane and emits red fluorescence. As the mitochondrial membrane potential drops under apoptotic conditions, J-aggregates disintegrate and JC-1 exists as its green-fluorescent

monomeric form. The ratio of green to red fluorescence emitted by JC-1 is therefore

an indicator of extend of mitochondrial membrane depolarization (Bedner *et al.*,

1999).

As shown in Fig. 5.1, control cells showed a low mean ratio of green to red

fluorescence after staining with JC-1, At 48 h of treatment, baicalein induced a shift

of cell population to the right, indicating that more green fluorescence was emitted

and the mitochondrial membrane was depolarized [Fig. 5.1 a ii.) vs b ii.)], For a

longer period of baicalein treatment, i.e., 72 h, there was even a drop of mean red

fluorescence level, suggesting that the mitochondria were not able to function

properly so that they were no longer negatively charged to accumulate the

J-aggregates of JC-1 in the mitochondria. This showed that late phase apoptosis or

necrosis had occurred and massive cell death was eluted.
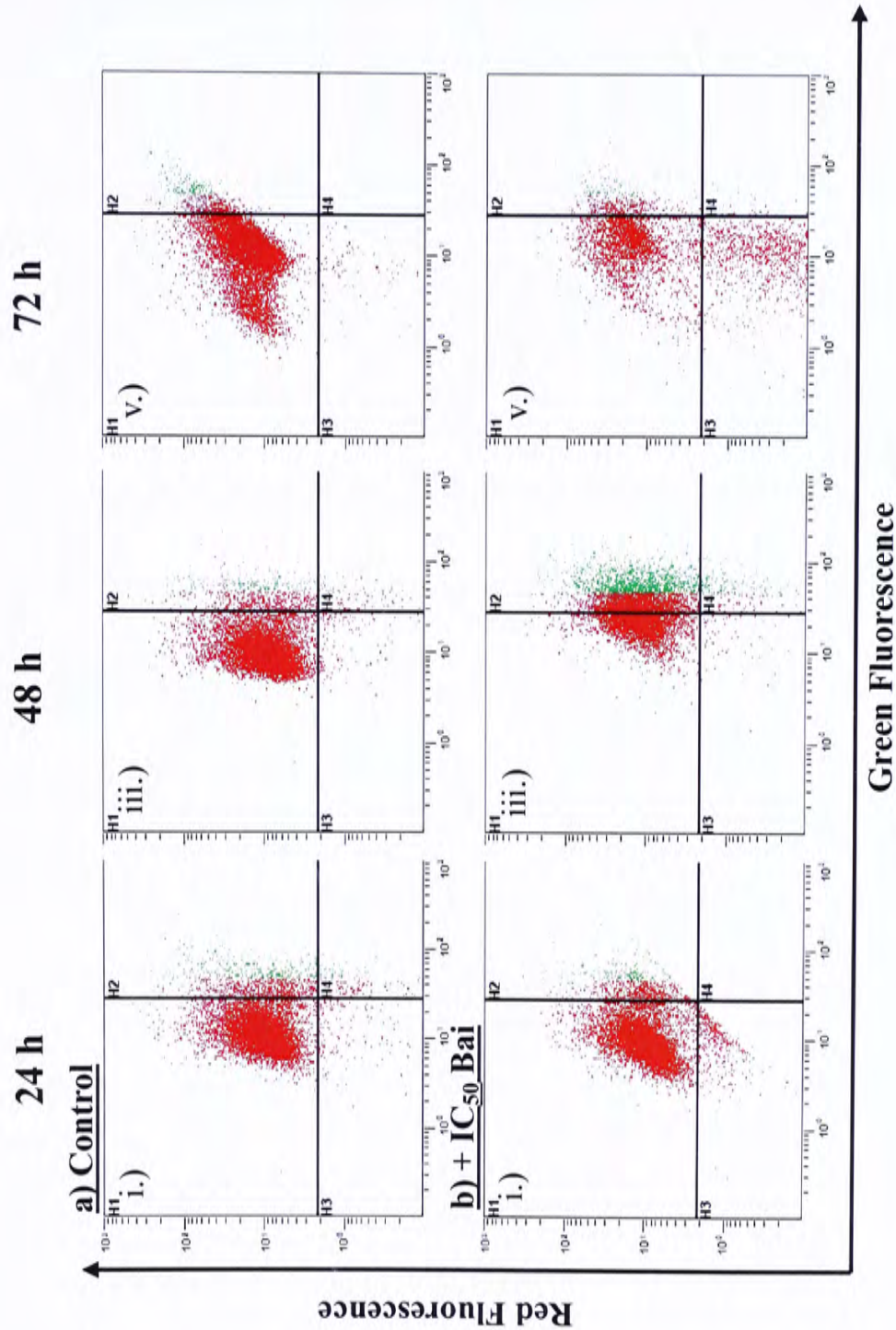
Fig. 5.1   **Representative bivariate dot plots showing the effects of baicalein on mitochondrial membrane potential in A375 cells**
The melanoma cells were incubated a) in the absence (control) or b) in the presence of baicalein (Bai) at IC_{50} for 24, 48 and 72 h, and the mitochondrial membrane potential was measured by JC-1 flow cytometry. At 48 h, baicalein induced mitochondrial membrane depolarization so that more green fluorescence was emitted from the cell.

5.3.2. Cytochrome *c* is released in the baicalein-induced mitochondrial membrane depolarization

As mitochondrial membrane depolarization was observed at 48 h after baicalein treatment in A375 cells (section 5.3.1), release of apoptogenic factors from the mitochondrial intermembrane space to the cytosol was therefore investigated to consolidate the occurrence of mitochondrial membrane depolarization. Cytochrome *c* is an electron carrier found normally in mitochondria and is released into the cytosol as an apoptogenic factor when mitochondria-mediated apoptosis occurred (Yang *et al.*, 1997) Release of cytochrome *c* into the cytoplasm was therefore studied by immunoblotting in this study. This was achieved by fractionating the cell lysate into cytosolic and mitochondrial fractions through specific lysis, mechanical disruption of cells and differential centrifugation. Specific antibody against cytochrome *c* was used to detect the relative amount of cytochrome *c* in both fractions. At 48 h of treatment, more cytosolic cytochrome *c* was found in the baicalein-treated cells than in the control cells, together with a decrease of mitochondrial cytochrome *c* in baicalein-treated cells when compared to the control cells. (Fig. 5.2)

Taken together, it was found that baicalein had induced the intrinsic apoptotic pathway in A375 cells, evidenced by occurrence of mitochondrial membrane depolarization and the release of cytochrome *c* into the cytoplasm.
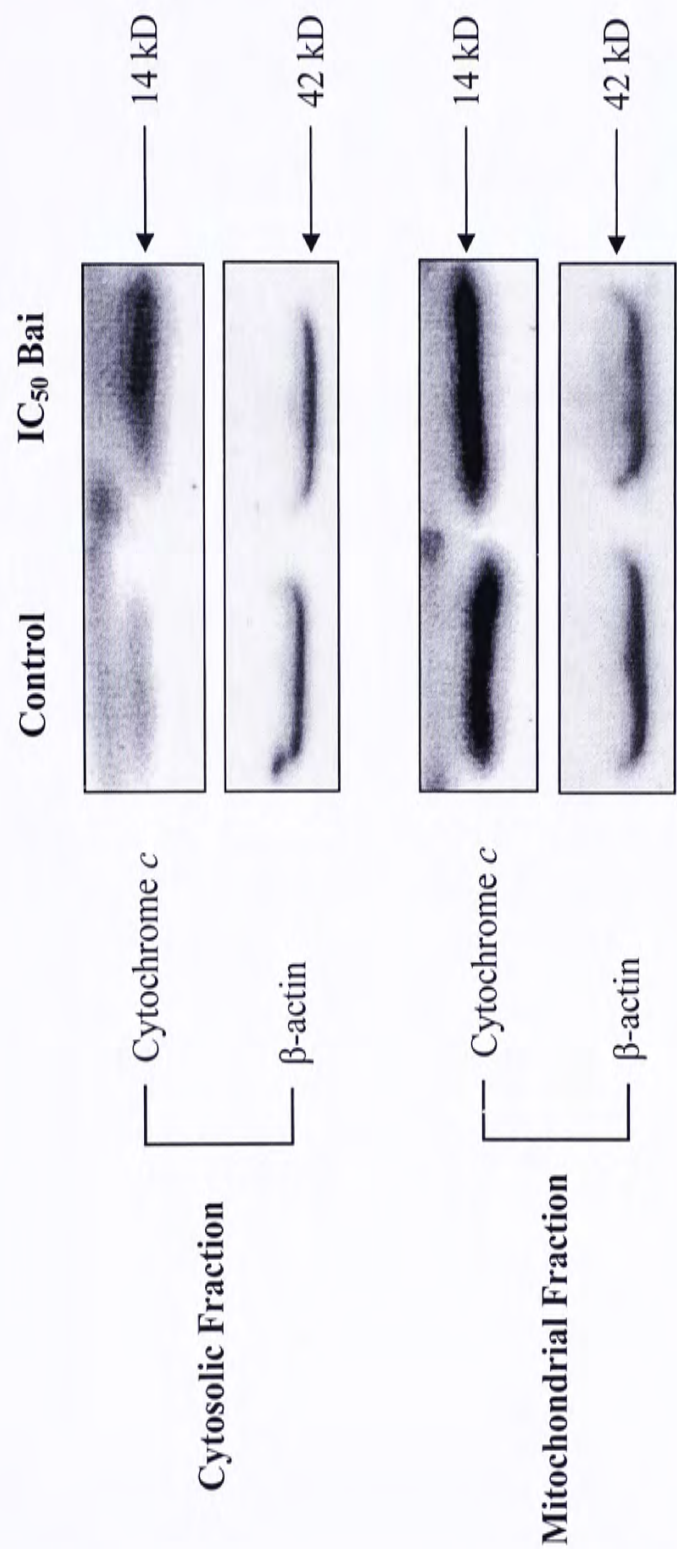
Fig. 5.2 **Representative immunoblots showing the effects of baicalein on cytochrome *c* level in the cytosolic and mitochondrial fractions of A375 cells**

The melanoma cells were incubated in the absence, i.e., control, or in the presence of baicalein (Bai) at IC_{50} for 48 h. The cells were then harvested and disrupted mechanically to yield the cytosolic and mitochondrial fractions. More cytosolic cytochrome *c* was found in the baicalein-treated cells than in the control cells. β–actin was also detected as internal control to normalize the expressions of other proteins.

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5.3.3. Baicalein does not elicit the intrinsic apoptotic pathway via modulation of some better-characterized Bcl-2 family proteins in A375 cells

As mentioned in section 3.3.2 and 5.3.1, baicalein may found to elicit the mitochondrial apoptotic pathway and thus activation of caspase-9. Since the mitochondrial apoptotic pathway is controlled by Bcl-2 family proteins (Tudor et al., 2000), six better-characterized proteins were measured in the baicalein-treated cells. The melanoma cells were treated with baicalein at IC_{50} for 24 to 72 h, and expression of anti-apoptotic members Bcl-2 and Bcl-x_L; and the pro-apoptotic members Bax, Bad, Bim$_{EL}$, and Bid was revealed by immunoblotting. However, baicalein neither significantly depleted level of the anti-apoptotic proteins (Fig. 5.3) nor prominently elevated level of the pro-apoptotic proteins (Fig. 5.4).

Time	24 h	36 h	48 h	60 h	72 h
IC$_{50}$ Bai	− +	− +	− +	− +	− +

Bcl-2 ← 28 kD

Bcl-x$_L$ ← 30 kD

β-actin ← 42 kD
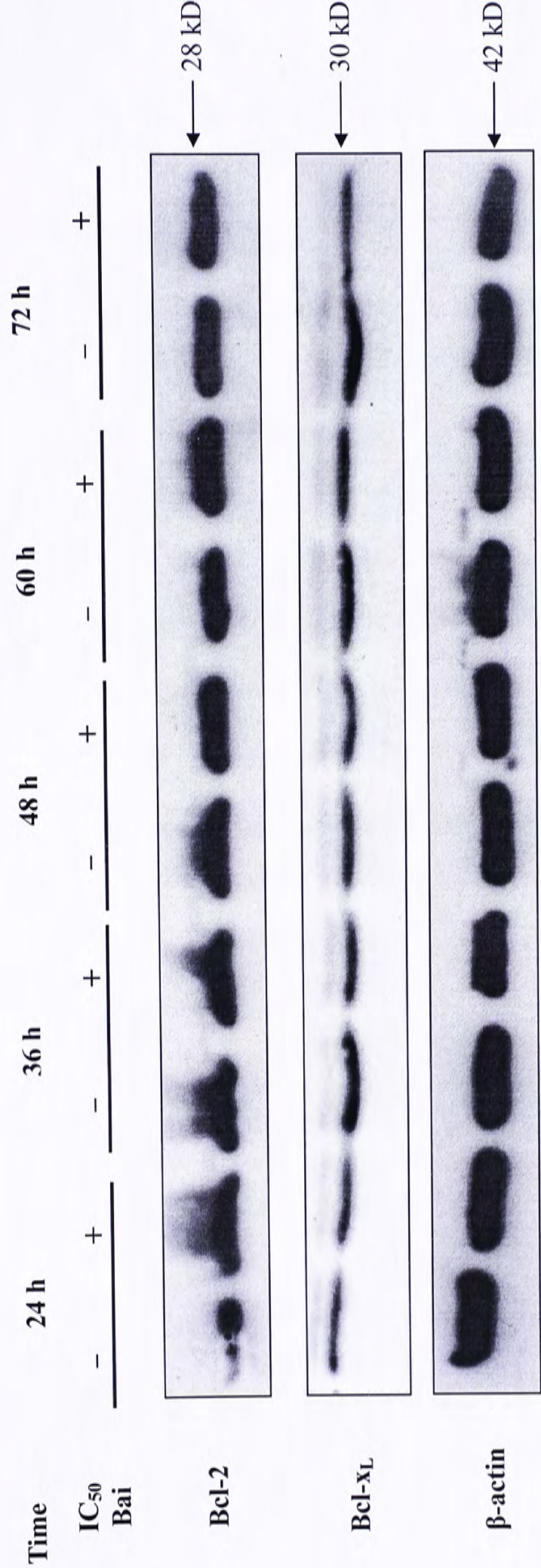
Fig. 5.3 **Representative immunoblots showing the effect of baicalein on expression of some anti -apoptotic members of Bcl-2 family proteins**

The melanoma cells were incubated in the absence, i.e., control (−), or in the presence of baicalein at IC$_{50}$ (+) for 24 to 72 h. Baicalein (Bai) did not significantly decrease expression of Bcl-2 and Bcl-x$_L$ even after 72 h of incubation, when compared to their corresponding controls.

β-actin was also detected as internal control to normalize the expressions of other proteins.

Time	24 h	36 h	48 h	60 h	72 h
IC$_{50}$ Bai	− +	− +	− +	− +	− +

Bax — 21 kD

Bad — 23 kD

Bim$_{EL}$ — 23 kD

Bid — 22 kD

β-actin — 42 kD

Fig. 5.4   **Representative immunoblots showing the effect of baicalein on expression of some pro-apoptotic members of Bcl-2 family proteins**

The melanoma cells were incubated in the absence, i.e., control (−), or in the presence of baicalein at IC$_{50}$ (+) for 24 to 72 h. Baicalein (Bai) did not significantly increase expression of Bax, Bad, Bim$_{EL}$ and Bid even after 72 h of incubation, when compared to their corresponding controls.

β-actin was also detected as internal control to normalize the expressions of other proteins.

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5.3.4. Baicalein induces ROS production

As mentioned in section 5.3.3, baicalein did not modulate at least some better-characterized Bcl-2 proteins to release cytochrome c from mitochondria. In addition to the well-established role of the Bcl-2 family proteins that regulate the mitochondrial release of apoptogenic factors, ROS-triggered mitochondrial permeability transition also provides an alternative for the release of apoptogenic factors from mitochondria (Rosse *et al.*, 199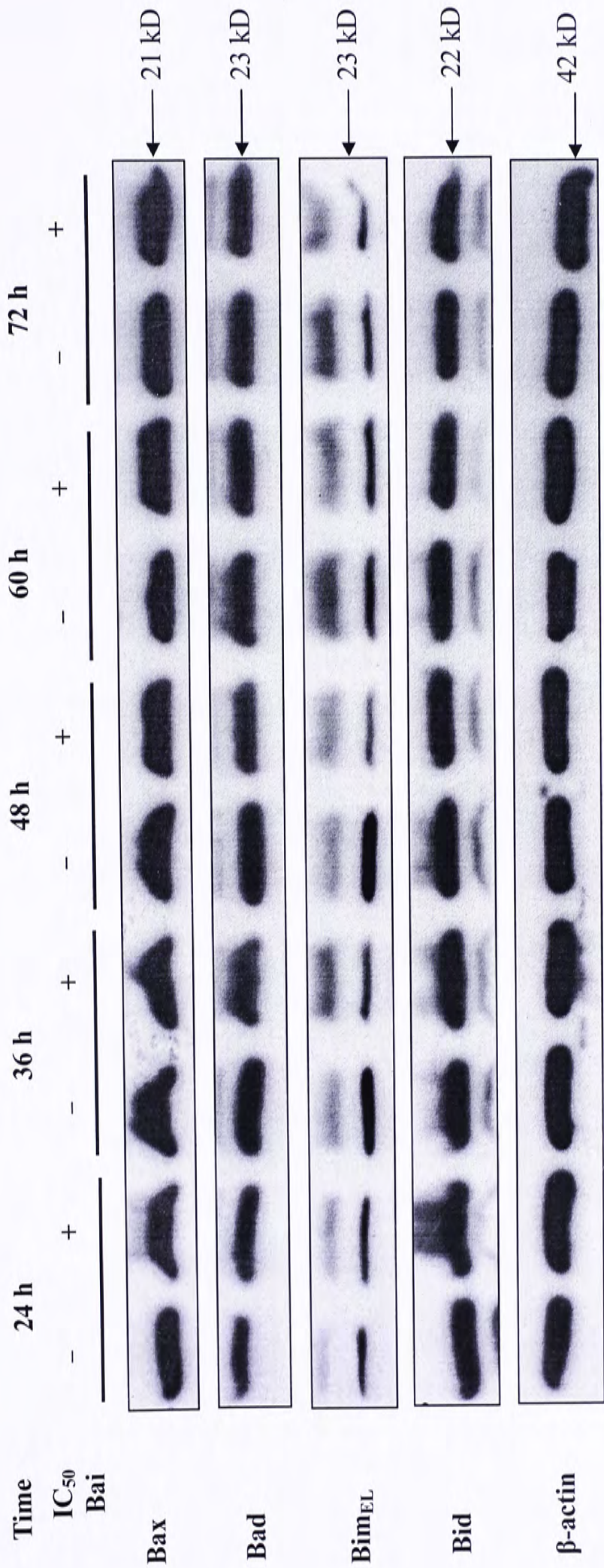8). Owing to the lack of evidence in the modulation of Bcl-2 family proteins by baicalein, we therefore hypothesized that the mitochondrial membrane perforation and the subsequent cytochrome c release would be a ROS-mediated event. To test this hypothesis, ROS level in the baicalein-treated cells was measured by DCF spectrofluorometry. Furthermore, Trolox^{TM}, a water soluble vitamin E analogue that can penetrate and protect cells from ROS, was also given to the baicalein-treated cells to retard generation of and any response caused by ROS. The amount of ROS produced in A375 cells after incubation with baicalein and Trolox^{TM} was directly measured by H$_2$DCFDA. H$_2$DCFDA is a cell penetrating dye that will be oxidized by ROS to highly fluorescent DCF, so that the intensity reflects the level of cellular ROS production. As illustrated in Fig 5.5, the addition of baicalein prominently elevated the total cellular ROS level by 1.7-fold of the control level, suggesting that the phytochemical did induce ROS production in the cells. Addition of Trolox^{TM}, on the other hand, suppressed the baicalein-induced ROS

production by more than 50%. Similar suppression by Trolox^{TM} was also observed in H$_2$O$_2$-treated cells, which was included in the experimental setup as a positive control.
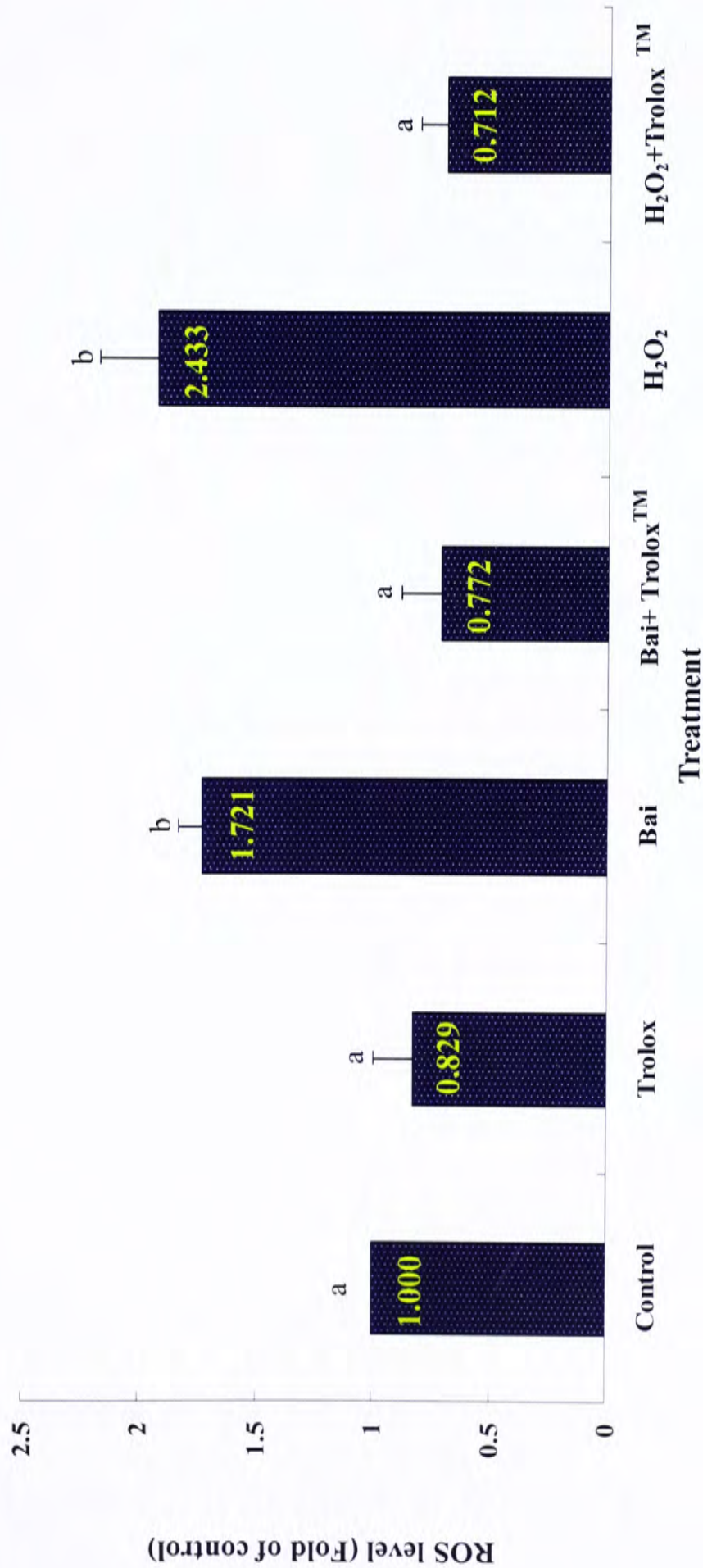
Fig. 5.5

Effects of baicalein and Trolox[TM] on cellular ROS level

The melanoma cells were treated with different combinations of baicalein (Bai) at IC_{50} and 100 μM Trolox[TM] for 48 h. Some melanoma cells were also treated with 500 mM H_2O_2 and Trolox[TM] as positive controls. Baicalein significantly elevated the ROS level in the melanoma cells that could be restored to the control level of Trolox[TM]. Similar findings were also observed with H_2O_2 and trolox treatment.

Data are shown as fold change of control level. Results are expressed as mean ± S.D of three independent experiments. a and b represent the statistical significance (p=0.05) of the difference between control and the treatment groups, which was determined by one-way ANOVA followed by Tukey's test for multiple comparisons.

5.3.5. Baicalein induces mitochondrial membrane permeabilization via ROS-mediated mechanisms

As found in section 5.3.4, baicalein induced ROS production in A375 cells that could be inhibited by Trolox^{TM}. To study the role of ROS in the baicalein-induced mitochondrial apoptosis, the melanoma cells were incubated with different combinations of baicalein and Trolox^{TM}, and the mitochondrial membrane depolarization, a key step in the mitochondrial apoptotic pathway was measured. Result from JC-1 analysis revealed that addition of Trolox^{TM} could inhibit the baicalein-induced mitochondrial membrane depolarization. After 48 h baicalein incubation, about 89% of the melanoma cells had depolarized mitochondrial membrane potential (Fig. 5.6). Co-incubation of 100 μM Trolox^{TM} with baicalein at IC$_{50}$ drastically reduced depolarized cells to about 27% only.

Compatible result was also obtained in the mitochondrial cytochrome *c* release after baicalein and Trolox^{TM} treatments. As demonstrated in Fig 5.7, the baicalein -induced release of cytochrome *c* after mitochondrial membrane depolarization was restored back to the control level by Trolox^{TM} co-treatment.

In summary, baicalein induced apoptosis in A375 cells, mediated by the intrinsic apoptotic pathway with mitochondrial membrane depolarization and release of cytochrome *c* into the cytoplasm. However, modulation of proteins from the Bcl-2 family, a common initiator of intrinsic apoptotic pathway, was not observed. Instead,

cellular ROS level was elevated and behaved as an initiator of the mitochondrial

apoptotic pathway that could be inhibited by anti-oxidant.

	Control		Treatment	
Baicalein	−	−	+	+
Trolox[TM]	−	+	−	+
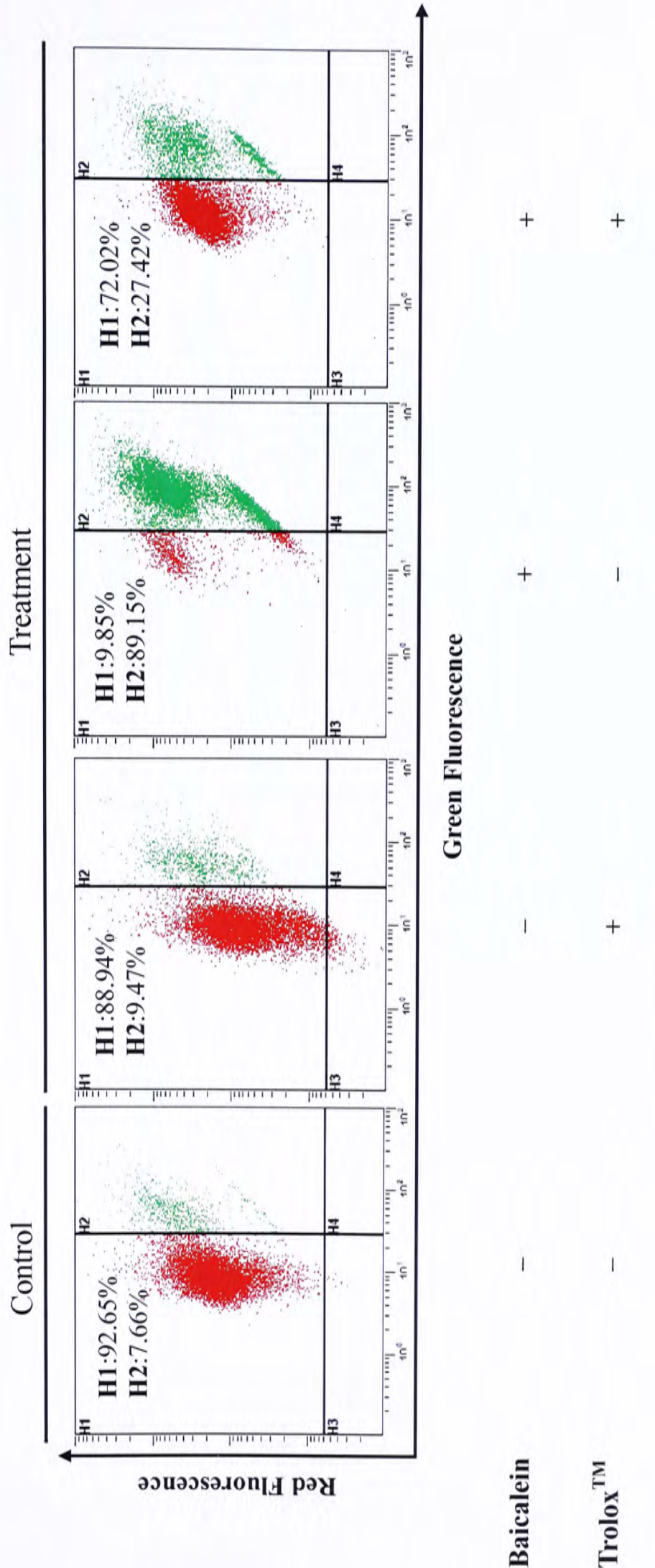
Fig. 5.6   **Effects of baicalein and Trolox[TM] on mitochondrial membrane potential in A375 cells**

Representative bivariate JC-1 analysis demonstrates the mitochondrial membrane potential in A375 cells after incubation with different combination of baicalein at IC_{50} (i.e. 37.5 μM) and 100μM Trolox[TM] for 48 h by flow cytometry. Diagrams of one representative out of three experiments were shown.
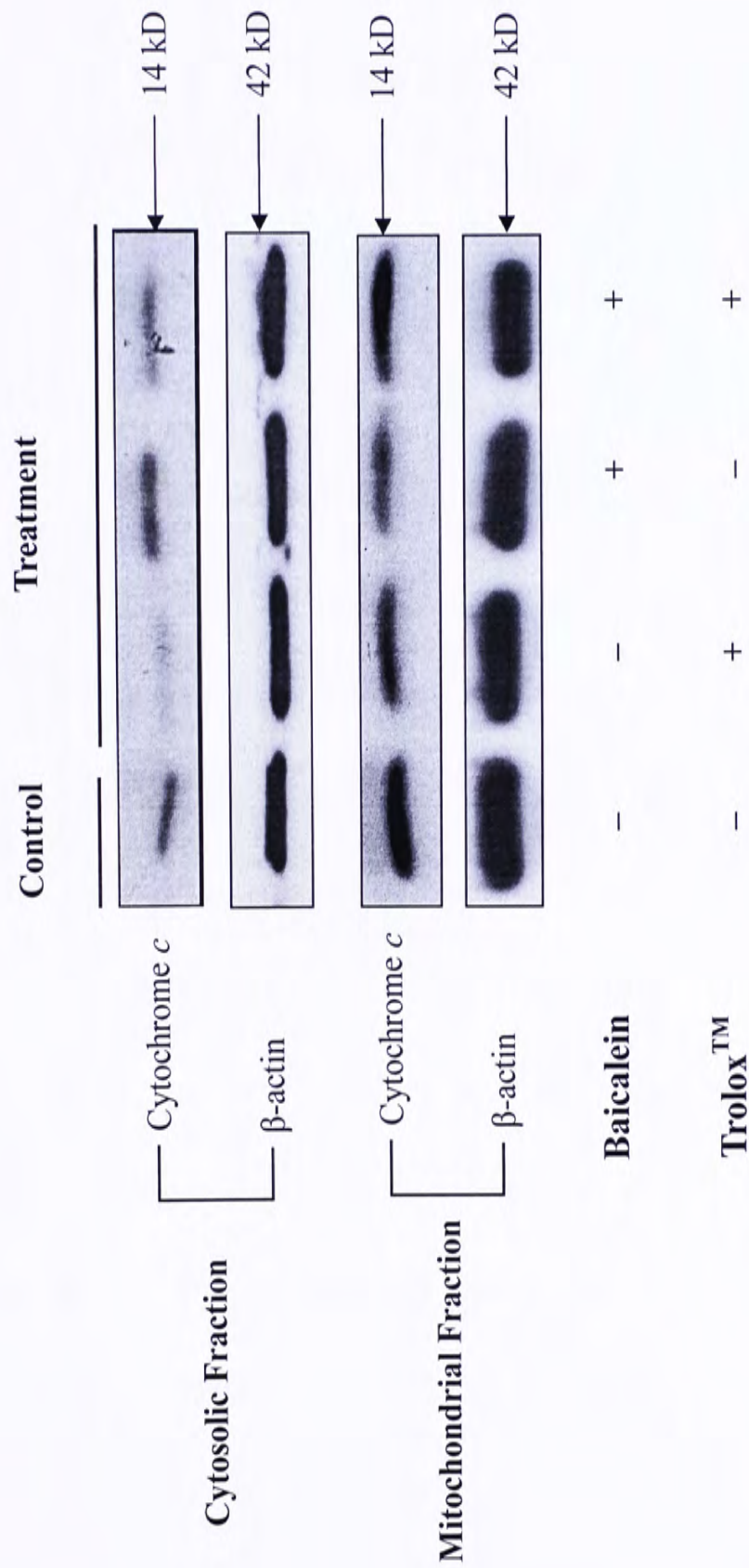
Fig. 5.7 **Representative immunoblots showing the effects of baicalein and Trolox™ on cytochrome *c* level in the cytosolic and mitochondrial fractions of A375 cells**

The melanoma cells were incubated different combinations of 100 μM Trolox™ and baicalein at IC$_{50}$ for 48 h. The cells were then harvested and disrupted mechanically to yield the cytosolic and mitochondrial fractions. The baicalein-induced mitochondrial release of cytochrome *c* was restored to the control level by Trolox™. β–actin was also detected as internal control to normalize the expressions of other proteins.

5.4 Discussion

In chapter 4 we demonstrated that the early phase of the baicalein-induced apoptosis was executed via the death receptor pathway, with the elevation of death receptors DR4 and DR5, and activation of caspase-8. Furthermore, it seemed that during the exacerbation phase of the cell death, the mitochondrial pathway was involved.

The activation of caspase-9 is the first proteolytic signal in the intrinsic apoptotic pathway (Riedl and Salvesen, 2007). Unlike the initiator caspase-8 in the extrinsic apoptotic pathway that is activated by transmembrane receptor, caspase-9 is activated by the cytosolic complex called apoptosome. Apoptosome composes of several molecules, including Apaf-1, cytochrome c, ATP or 2'-deoxyATP (dATP) (Shi, 2006). The apoptosome formed will then recruit and activate caspase-9, which performs the subsequent cleavage of apoptotic substrates. In this circumstance, the release of cytochrome c from mitochondria into the cytosol becomes critical, not only because cytochrome c is one of the components of apoptosome, but also it triggers the self-inhibited and monomeric Apaf -1 molecules to oligomerize and initiate the apoptosome formation (Riedl et al., 2005). Hence, the release of mitochondrial cytochrome c to the cytosol is a key event of execution of the intrinsic apoptotic pathway.

Cytochrome c is an electron carrier that participates in the oxidative

phosphorylation under normal physiological conditions (Slee *et al.*, 1999). As a mobile electron carrier, cytochrome *c* binds electrostatically to negatively charged surfaces of respiratory complexes at the outer face of the inner membrane (Kannt *et al.*, 1998). Since electrons flow rapidly down the respiratory chain, cytochrome *c* can freely shuttle among each complex and is not tightly bound. Therefore, it is normally restricted to the intermembrane space by the integrity of the mitochondrial membrane (Crompton, 1999). During the execution of apoptosis, the mitochondrial membrane will lose its integrity and therefore apoptogenic factors, such as cytochrome *c* will be released to the cytosol. There are several proposed mechanisms for this mitochondrial membrane permeabilization (MMP), and the actual mechanisms underlying the release of apoptogenic factors seem complex and controversial. MMP can be further sub-divided into permeabilization of inner and outer membranes, which have distinct characteristics and significance in the mitochondrion-mediated apoptosis (Schwarz *et al.*, 2007). Nonetheless, the ultimate consequences would be the loss of mitochondrial membrane integrity and release of apoptogenic factors (Kroemer *et al.*, 2007). In this aspect, we detected the change of mitochondrial potential with flow cytometry as an MMP indicator.

Under normal physiological condition, the inner mitochondrial membrane is nearly impermeable to all ions, including protons. This allows the building up of proton gradient necessary for oxidative phosphorylation which drives ATP synthesis.

The inner mitochondrial transmembrane potential ($\Delta\Psi_m$) is a result of the charge imbalance across the inner membrane. Therefore, the maintenance of the proton gradient within the mitochondria is of vital importance for cellular survival (Kroemer *et al.*, 1998). Although a temporary loss of the $\Delta\Psi_m$ may occasionally occur in normal conditions (Zoratti and Szabo, 1995), a long-lasting or permanent $\Delta\Psi_m$ dissipation is often associated with cell death apoptosis (Marchetti *et al.*, 1996). As a result, MMP is also a good indicator of apoptosis.

During the exacerbation phase of the baicalein-induced apoptosis, involvement of caspase-9 was further substantiated by cytochrome *c* release and loss of mitochondrial membrane potential. It was found that after 48 h of baicalein treatment, there was a prominent loss of mitochondrial membrane potential. The loss of potential was accompanied by the release of cytochrome *c* into the cytosol, indicating the possible formation of apoptosome and thus activation of caspase-9. Supporting by these findings, we believed that baicalein induced not only the extrinsic death receptor pathway (Chapter 4), but also the intrinsic mitochondrial apoptotic pathway, especially during the exacerbation phase of the induced apoptosis, in A375 cells.

After validating the elicitation of the intrinsic mitochondrial pathway in the baicalein-induced apoptosis, the next question await to be answered would be the regulators that initiate this signaling cascade. Bcl-2 family proteins are well-studied for their central regulatory roles in the mitochondrial-mediated apoptosis. Bcl-2

family proteins share conserved domains, namely the Bcl-2 homology (BH) domains. This family can be sub-divided into anti-apoptotic (e.g. Bcl-2, Bcl-xL etc.) and pro-apoptotic (e.g. Bax, Bad etc.) proteins so that they interact with one another to regulate apoptosis. The activated pro-apoptotic members of the Bcl-2 family exert their function at mitochondria by inducing permeabilization of the outer mitochondrial membrane. This permeabilization results in the release of proapoptotic factors from the intermembrane space, such as cytochrome c, to activate effector caspases (Wang, 2001). The extrinsic pathway can be connected the intrinsic pathway via the Bcl-2 family, BH-3 only member Bid. Under the action of activated caspase-8 in the extrinsic pathway, Bid can be cleaved and truncated (i.e. tBid). The tBid formed then translocates to mitochondria and enhances the oligomerization of the pro-apoptotic Bcl-2 family members, such as Bax and Bak, leading to outer mitochondrial membrane permeabilization (Kandasamy *et al.*, 2003; Schwarz *et al.*, 2007). In the extrinsic pathway, this permeabilization serves as an amplification loop for the activation of effector caspases (Gross *et al.*, 1999).

In this study, several proteins of the Bcl-2 family, which have been commonly reported to be the central regulators of mitochondrial apoptosis, were studied by immunoblotting. These include anti-apoptotic Bcl-2 and Bcl-x$_L$; pro-apoptotic Bax, Bad and Bim$_{EL}$; as well as the pathway-linker Bid. Out of our expectation, none of the Bcl-2 family members studied had significant change after baicalein treatment,

even after prolonged treatment of 72 h when extensive apoptosis occurred (chapter 3). The immunoblotting results seemed not compatible to the previous findings and traditional beliefs about the loss of mitochondrial integrity and release of cytochrome *c*, by the Bcl-2 family members.

In fact, besides Bcl-2 family proteins, intracellular ROS has been recently identified to play a regulatory role in apoptosis (Ott *et al.*, 2007). The undesirable condition brought by ROS is the pose of oxidative stress on the cells. The generation of ROS is unavoidable, and the major source of ROS is the oxidative phosphorylation in mitochondria. With damaging effects on cellular macromolecules, ROS is constantly eradicated by antioxidative systems. Problems arise at times when imbalance occurred between generation of ROS and antioxidative systems, resulting in the generation of oxidative stress (Liu *et al.*, 2002). The ability of ROS to initiate apoptosis is its devastating effect on cellular components, particularly those inside the mitochondria (Orrenius *et al.*, 2007). The destructive effects include lipid oxidation that mostly affects membrane structures; damage of both nuclear and mitochondrial DNA and protein molecules (Fleury *et al.*, 2002). In addition, ROS also perturbs the mitochondrial ionic balance and causes dissipation of the membrane potential, resulting in the release of apoptogenic factors from within (Kowaltowski, 1996).

The possibility that baicalein induces mitochondrial apoptosis via ROS

generation further is substantiated by previous findings. Baicalein has been reported

to induce ROS-mediated mitochondrial dysfunction in human promyeolytic leukemia

(Wang *et al.*, 2004; Makino *et al.*, 2006). We therefore hypothesized that ROS might

involve in the initiation of the mitochondrial pathway in the baicalein-induced

apoptosis. It was found that ROS was prominently elevated in the baicalein-treated

cells. To confirm the involvement of ROS in the mitochondrial apoptosis, Trolox^{TM}

was used in the study. Trolox^{TM} is the water soluble analogue of α- tocopherol, and is

frequently used as a standard antioxidant in biochemical studies to compare the

antioxidative capacity of compounds of interest (Poljšak and Raspor, 2007).

Trolox^{TM} was co-administered with baicalein to the melanoma cells to see if the

antioxidant was able to reverse the mitochondrial apoptotic features brought by

baicalein. Firstly, we found that Trolox^{TM} was able to eradicate the elevation of ROS

in baicalein-treated cells. For comparison, we also showed that Trolox^{TM} could lower

hydrogen peroxide (H_2O_2), which we had added to the cells to act as a positive

control of ROS production. The antioxidant then rescued the mitochondrial

membrane depolarization and release of cytochrome *c* caused by baicalein in A375

cells. The restoration of normal mitochondrial potential gradient also ceased the

cytochrome *c* release.

Recapitulate the above findings, baicalein is able to induce apoptosis, firstly via

the extrinsic death receptor pathway as previously discussed during the initiation

phase, which is then followed by the intrinsic mitochondrial pathway through the

production of ROS during the exacerbation phase.

Chapter 6 General Discussion

Cancer has become one of the most devastating diseases worldwide. According to the World Health Organization (WHO), malignant tumors were responsible for 12 per cent of the nearly 56 million deaths worldwide from all causes in the year 2000 (Mathers *et al.*, 2001). Despite the continuous efforts made on new anticancer drug discoveries, some of the cancers remain poorly treated. The obstacles faced by cancer treatment attribute to the occurrence of multidurg resistance (Gottesman and Paston, 1993), the metastatic properties of cancer cells (Aziz *et al.*, 2003; Eccles and Welch, 2007) and the lack of selective cytotoxicity of anticancer drug (de Bono *et al.* 2003; Crighton and Ryan, 2004) which limit the drug dosage to a tolerable yet effective level. As a result, the urge of discovery of new antiproliferative therapeutics and cancer regimen has never been slowing down.

Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Over 50% of anticancer drugs approved by the United States Food and Drug Administration (FDA) since 1960 were originated from the natural resources, especially from terrestrial plants (Kim and Park, 2002). Chemicals derived from plants have been used as major chemotherapeutics, such as taxol; or as adjuvants facilitating the treatments by conventional anticancer drugs. As a preventive measure, the importance of phytochemicals has been increasingly

recognized (Aziz *et al.*, 2003; Surh, 2003).

Regarding to the importance of new anticancer drug discovery from the natural source, Traditional Chinese Medicine (TCM) would represent a rich and attractive pool of bioactive chemicals that worth investigating (Chen *et al.*, 2006). Because of the growing interest in therapeutic agents based on TCM, increasing effort has been directed towards scientific proof, clinical evaluation and molecular analysis of TCM (Chen *et al.*, 2003). In fact, many phytochemicals have been isolated from TCM and have gained much attention in the medical community. Many of the selected phytochemicals have been extensively studied for the anticancer properties and health beneficial effects. The medicinal fungus 'Ling Zhi', *Ganoderma Lucidum*, has long been used as a folk remedy for promotion of health and longevity (Lin and Zhang, 2004). Extensive studies have unraveled the possible antitumor mechanisms of the fungus. Different bioactive constituents, such as the polysaccharide and triterpene fractions exert antiproliferative effects by immunomodulation (Lin *et al.*, 2003). In this study, the antiproliferative properties of another bioactive phytochemical, baicalein, was investigated. Baicalein is a flavone isolated from an anti-inflammatory TCM called Huang Qin, which is actually the dried root of medicinal herb *Scutelleria baicalensis* Georgi (Wang *et al.*, 2004). In mainland China, *S. baicalensis* is used for treating upper respiratory infections, diphtheria,

nephritis, dysentery, scarlet fever and hepatitis. Being one of the bioactive components of the herb, baicalein has been reported to possess anti-viral, antioxidant and anti-inflammatory activities (Li *et al.*, 2004). Recent studies have also shown that baicalein retarded cancer cell growth *in vitro* and *in vivo*. Baicalein was able to induced apoptosis *in vitro* in bladder cancer cell lines (Ikemoto *et al.*, 2000) and breast cancer cell line (Po *et al.*, 2002); in animal models, tumor regression was also observed for prostate carcinoma (Guo *et al.*, 2004) and breast cancer (So *et al.*, 1996). Despite of the extensive studies of the antitumor effects of baicalein, comprehensive investigation on its mechanisms are very limited. Being an efficacious phytochemical with potent anticancer and other beneficial effects, a detailed study on its mechanism should be elucidated to enrich our knowledge in drug discovery from the natural source.

Baicalein was shown firstly in the present investigation to inhibit the growth of different human cancer cell lines, including malignant melanoma A375 and colorectal carcinoma Caco-2. However, baicalein did not induce significant growth inhibition in hepatocellular carcinoma HepG2, lung carcinoma A549, prostate carcinoma PC-3 and breast carcinoma MCF7. This differential activity of baicalein demonstrates that baicalein is not a broad-spectral cytotoxic compound, and its actions may depend on the histotype or molecular constituents of the cells. The

differential activity of baicalein was further validated with the normal skin fibroblast cell line Hs68. At the doses which baicalein prominently inhibited the melanoma cell growth, the normal skin cells were not significantly affected. This further substantiates the specificity on cell growth inhibition by baicalein. Consequently, human melanoma A375, being the most responsive cell line, was chosen for further studies.

Responses of A375 cells to the antiproliferative and cytotoxic actions of baicalein seem to occur in a dose-dependent manner. To further analyze, we used DNA flow cytometry to examine the time effects of baicalein on cell cycle and apoptosis, the two main processes that could modulate tumor development. The results illustrated that the antiproliferative action of baicalein was associated with the primitive accumulation of cells at S phase of cell cycle shortly after 36 h of incubation. The S-phase arrest was accompanied by depletion of G_1 cells, suggesting that some cells were delayed or forced to complete the cell cycle without re-entering G_1 phase. This aberrant termination of cell cycle designated some of the arrested cells to undergo apoptosis. In parallel with the S-phase arrest, the apoptotic population accumulated exponentially under prolonged baicalein treatment. The appearance of apoptotic cells shortly after S-phase arrest suggested the importance of apoptosis induction in the cell growth control induced by baicalein. As a result, the

apoptotic mechanisms of baicalein-induced cell death were further investigated.

The incidence of apoptosis was revealed in the flow cytometric analysis by the presence of hypodiploid or apoptotic population in the DNA histogram. The apoptotic population was identified by flow cytometry according to the occurrence of DNA fragmentation, which is one of the morphological hallmarks of apoptosis. Apoptosis was characterized by several physical and biochemical features. Apoptotic cells would have a characteristic morphology with chromatin condensation, DNA fragmentation, and cell shrinkage (Ziegler and Groscurth, 2004). Biochemically, caspases will be activated to execute the subsequent proteolysis and breaking down of the cells (Gilmore *et al.*, 2000). The baicalein-induced apoptosis was confirmed biochemically by the study of caspase activation via immunoblotting. It was found that baicalein induced caspase-dependent apoptosis of the melanoma cells, with activations of caspase-8, caspase-9 and caspase-3. Among the three activated caspase, caspase-8 was the earliest caspase to be activated, i.e. after 24 h treatment, whereas caspase-9 and caspase-3 were both activated after 48 h of treatment. This sequential activation has delineated the possible apoptotic cascade involved. In this case, as caspase-8 was the earliest activated initiator caspase, the extrinsic apoptotic pathway was then investigated.

Caspase-dependent apoptosis is executed by the initiation of two pathways: the

death-receptor-mediated extrinsic pathway and the mitochondrion-mediated intrinsic pathway. Caspase-8 is the initiator caspase of the extrinsic pathway, and it is activated by the DISC formation with the death-inducing ligand, death receptor, death-domain containing molecule and procaspase-8 (Kurbanov *et al.*, 2005). As caspase-8 was found to be activated, we hypothesized that the death receptor/ligand interaction might be enhanced. To testify this hypothesis, expression of the components of the death receptor pathway was investigated by immunoblotting. In this study, TRAIL-mediated pathway was chosen for further studies. TRAIL is a member of the TNF family of cytokines that can initiate cell death. Unlike other family members such as TNFα and FasL which possess general cytotoxicity, TRAIL seems to exert its death-inducing effect specifically in neoplastic cells, rather than in normal cells (MacFarlane, 2003). This specificity in action has imposed an attractive character for TRAIL to be developed into new therapeutic regimen. Results from the immunoblotting illustrated that baicalein elevated the two TRAIL receptors, DR4 and DR5, but not their ligand TRAIL. This finding supports our postulation that baicalein may upregulate DR4 and DR5 to activate caspase-8 and the extrinsic apoptotic pathway.

In order to evaluate the functional significance of the elevated receptors, sub-lethal dose of TRAIL, i.e.12.5 ng/ml, was co-administered to see if baicalein

could sensitize A375 cells to the growth-inhibitory effect of TRAIL. According to our knowledge, we are the pioneer group to show that synergism exists between baicalein and TRAIL so that the growth inhibition on the melanoma cells could be enhanced by almost 2-fold if they are administered together. This sensitization may probably due to the enhanced expression of DR4 and DR5 after baicalein treatment, yet further investigation is needed to prove the hypothesis.

The activation of caspase-8 has led us to the investigation of the extrinsic pathway and so do the activation of caspase-9. The activation of caspase-9 is a strong indicator of the elicitation of the mitochondrion-mediated apoptosis. Self-activation of caspase-9 relies on the close proximity between procaspase-9 molecules that brought by apoptosome (Hajra and Liu, 2004). Apoptosome composes of several molecules of Apaf-1, cytochrome c and ATP molecules (Shi, 2006). For apoptosome formation, presence of cytosolic cytochrome c is critical. Release of cytochrome c from mitochondria indicates mitochondrial dysfunction, and molecules for apoptosome formation. Hence, the release of mitochondrial cytochrome c to the cytosol is a key event of execution of the intrinsic apoptotic pathway. To detect the execution of the mitochondrial pathway in baicalein-induced apoptosis, mitochondrial membrane potential and cytosolic cytochrome c were measured by flow cytometry and immunoblotting, respectively. We further demonstrated in this

study that there was mitochondrial membrane depolarization as early as 48 h of treatment, with concomitant release of cytochrome c. This implies that besides the death receptor pathway, the mitochondrial pathway is also executed in the baicalein-induced apoptosis in A375 cells.

The next question awaits to be revealed would be what the regulators of mitochondrial dysfunction are in the baicalein-induced apoptosis. The Bcl-2 family is the central regulator of apoptosis because its members integrate diverse survival and death signals into the mitochondrial membranes. In this family of proteins, members are sub-divided into anti-apoptotic members (such as Bcl-2, Bcl-x_L, etc.) and pro-apoptotic members (such as Bax, Bad, etc.). Members from the two branches interact with each other and regulate different aspects of apoptosis. The well-characterized candidates such as Bcl-2 and Bax have been reported to be involved in mitochondrial membrane depolarization and thus facilitate the release of cytochrome c (Rosse $et\ al.$, 1998). Having such an important role in the mitochondrion-mediated apoptosis, some better characterized members of the Bcl-2 family were studied. They are the anti-apoptotic Bcl-2 and Bcl-x_L, proapoptotic Bax, Bad and Bim$_{EL}$, as well as the linker of the two apoptotic pathways Bid. Out of our expectation, none of the Bcl-2 family proteins had significant change after the baicalein treatment, even after the prolonged 72 h incubation. This indicates that the

above Bcl-2 family proteins do not participate in the elicitation of the mitochondrial pathway, although we cannot exclude the possibility that other Bcl-2 proteins, which have been less-studied, may participate in the pathway.

ROS has been recently identified to play a regulatory role in apoptosis (Ott *et al.*, 2007). Generation of intracellular ROS is unavoidable, yet problems arise only when the damaging ROS cannot be eradicated by the innate antioxidative systems, resulting in oxidative stress to the cells (Liu *et al.*, 2002). Besides oxidizing the lipids in membranous structures and damaging DNA (Orrenius *et al.*, 2007), ROS also disturbs the normal mitochondrial proton gradient, causing loss of membrane potential and release of apoptogenic factors (Kowaltowski, 1996). Since the Bcl-2 family proteins did not change after the baicalein treatment, we hypothesized that the mitochondrial dysfunction might be caused by other factors, including ROS. It was found that ROS was elevated in the baicalein-treated cells, and the antioxidant Trolox^{TM} was able to eradicate the ROS produced inside the cells. Furthermore, the mitochondrial membrane depolarization and release of cytochrome *c* were also rescued by the antioxidant. The results suggested that ROS is al least one of the major regulators of the mitochondrial dysfunction in the baicalein-induced apoptosis.

In conclusion, results from this study have proposed a comprehensive mechanism for the growth-inhibitory effect of baicalein on A375 cells. Baicalein

retards the cancer growth by first disturbing cell cycle progression by arresting the cells at S-phase. Prolonged cell cycle arrestment leads to apoptosis. Both the extrinsic and intrinsic apoptotic pathways are activated. In mediating the extrinsic pathway, baicalein elevates the death receptors DR4 and DR5 that enhanced activation of the initiator caspase-8. Later on, the initiator caspase-9 of the intrinsic pathway is also activated and ROS seems to be a regulator, that results in the mitochondrial membrane depolarization and cytochrome c release. The two pathways finally converge to into the activation of the effector caspase-3, PARP cleavage and DNA fragmentation (Fig. 5.1). Interestingly, the synergism between baicalein and TRAIL may allow the future development of this phytochemical as an adjuvant agent that can restore or enhance the TRAIL sensitivity, especially in those resistant cancer cells. Being an abundant and inexpensive, yet efficacious phytochemical, the development of baicalein as a potent anticancer agent is valuable and deserved.
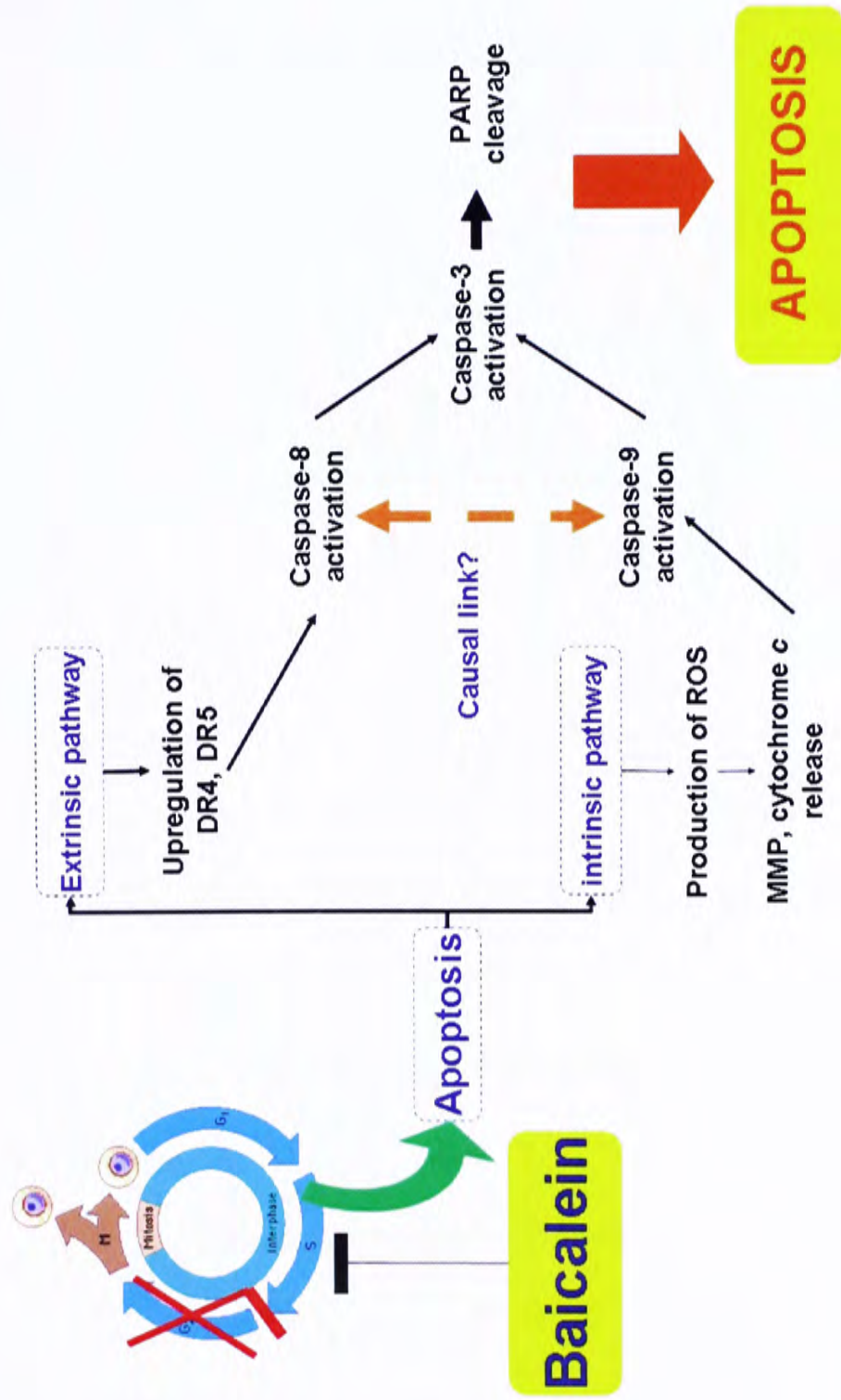
Fig. 6.1

Overview of proposed mechanism in baicalein-induced apoptosis in A375 cells

Baicalein induced S-phase arrest and subsequent apoptosis in A375 cells via both the extrinsic and intrinsic pathway. Baicalein elevated the expression of DR4 and DR5 that enhanced the activation of caspase-8. On the other hand, baicalein induced the production of ROS which act as the major regulator controlling the intrinsic apoptotic pathway. Both the death-receptor and mitochondrial pathway, though the presence of a direct linkage remains unclear, initiated activation of effector caspases and the progression of apoptosis.

References

Adams, J. M. and Cory, S. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science* **281**:1322–1326.

Adams, J.M. 2003. Ways of dying: multiple pathways to apoptosis. *Genes & Development* **17**: 2481-2495.

Almasan, A. and Ashkenazi, A. 2003. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine and Growth Factor Reviews* **14**: 337–348.

Antignani, A. and Youle, R.J. 2006. How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? *Current Opinions in Cell Biology* **18**: 685-689.

Askkenazi, A. and Dixit, V.M. 1999. Apoptosis controlled by death and decoy receptors. *Current Opinions in Cell Biology* **11**: 255-260.

Aziz, M. H., Kumar, R. and Ahmad, N. 2003. Cancer chemopreventive by resveratrol: *In vitro* and *in vivo* studies and the underlying mechanisms (Review). *International Journal of Oncology* **23**: 17-28.

Becker, W. M., Kleinsmith, L. J. and Hardin, J. 2003. The cell cycle: DNA replication, mitosis, and cancer. *The World of the Cell*, 5th Ed. Benjamin Cummings, San Francisco. pp. 523-572.

Bedner, E., Li, X., Gorczyca, W., Melamed, M.R. and Darzynkiewicz, Z. 1999. Analysis of apoptosis by laser scanning cytometry. *Cytometry* **35**: 181-195.

Bemelmans, M.H., van Tits, L.J. and Buurman, W.A. 1996. Tumor necrosis factor: function, release and clearance. *Critical Review in Immunology* **16**:1–11.

Bertram, J.S. 2000. The molecular biology of cancer. *Molecular Aspects of Medicine* **21**: 167-223.

Blagosklonny, M.V. 2007. Mitotic arrest and cell fate: why and how mitotic inhibition of transcription drives mutually exclusive events. *Cell Cycle* **6**: 70-74.

Boehrer, S., Nowak, D., Hoelzer, D., Mitrou, P.S. and Chow, K.U. 2006. The molecular biology of TRAIL-mediated signaling and its potential therapeutic exploitation in hematopoietic malignancies. *Current Medicinal Chemistry* **13**: 2091-2100.

Bossen, C., Ingold, K., Tardivel, A., Bodmer, J.L., Gaide, O., Hertig, S., Ambrose, C., Tschopp, J. and Schneider, P. 2006. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *Journal of Biological Chemistry* **281**: 13964-13971.

Braña, M.F. and Sánchez-Migallón, A. 2006. Anticancer drug discovery and pharmaceutical chemistry: a history. *Clinical and Translational Oncology* **8**: 717-728.

Bröker, L.E., Kruyt, F.A.E. and Giaccone, G. 2005. Cell death independent of caspases: a review. *Clinical Cancer Research* **11**: 3155-3161.

Brubacher, J.L. and Bols, N.C. 2001. Chemically de-acetylated 2',7' -dichlorodihydrofluorescein diacetate as a probe of respiratory burst activity in mononuclear phagocytes. *Journal of Immunological Methods* **251**: 81-91.

Buchsbaum, D.J., Zhou, T., Grizzle, W.E., Oliver, P.G., Hammond, C.J., Zhang, S., Carpenter, M. and LoBuglio, A.F. 2004. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy

and/or radiation therapy in a human breast cancer model. *Clinical Cancer Research* **9**:3731–3741.

Budman, D.R., Calvert, A.H. and Rowinsky, E.K. 2003. The role of in vitro cell line, human xenograft and mouse allograft models in anticancer drug development. In: *Handbook of Anticancer Drug development* (Pine, J. Ed.). Lippincott Williams and Wilkins, Baltimore. pp 131-135.

Budihardjo, I.I., Poirier, G.G. and Kaufmann, S.H. 1998. Apparent cleavage of poly(ADP-ribose) polymerase in non-apoptotic mouse LTA cells: An artifact of cross-reactive secondary antibody. *Molecular and Cellular Biochemistry* **178**: 245–249.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. 1999. Biochemical pathways of caspase activation of apoptosis. *Annual Review of Cell and Developmental Biology* **15**: 269-290.

Cejas, P., Casado, E., Belda-Iniesta, C., De Castro, J., Espinosa, E., Redondo, A., Sereno, M., García-Cabezas, M.A., Vara, J.A., Domínguez-Cáceres, A., Perona, R. and González-Barón, M. 2004. Implications of oxidative stress and cell membrane lipid peroxidation in human cancer (Spain). *Cancer Causes & Control* **15**: 707-719.

Chan, J.Y.W., Cheung, J.Y.N., Luk, S.C.W., Wu, Y.J., Fung, S.F. and Fung, K.P. 2004. Anti-cancer and pro-apoptotic effects of an herbal medicine and Saccharomyces cerevisiae producy (CKMB) on human hepatocellular Carcinoma HepG2 in vitro and in vivo. *Immunopharmacology and Immunotoxicology* **26**:597-609.

Chang, H.Y. and Yang, X. 2000. Proteases for Cell Suicide: Functions and

Regulation of Caspases. *Microbiology and Molecular Biology Reviews* **64**: 821-846.

Chang, W.H., Chen, C.H. and Lu, F.J. 2002. Different effects of baicalein, baicalin and wogonin on mitochondrial function, glutathione content and cell cycle progression in human hepatoma cell lines. *Planta Medica* **68**: 128-132.

Chen, C.Y., Shen, S.C., Chen, L.G., Lee, T.J.F. and Yang, L.L. 2001. Wogonin, baicalein and baicalein inhibition of inducible nitric oxide synthase and cyclooxgenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. *Biochemical Pharmacology* **61**: 1417-1427.

Chen, W., Wang, X., Zhuang, J., Zhang, L. and Lin, Y. 2007. Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. *Carcinogenesis* [In press].

Chen, X., Ung, C.Y. and Chen, Y. 2003. Can an in silico drug-target search method be used to probe potential mechanisms of medicinal plant ingredients? *Natural Product Report* **20**: 432–444.

Chen, X., Zhou, H., Liu, Y.B., Wang, J.F., Li, H., Ung, C.Y., Han, L.Y., Cao, Z.W. and Chen, Y.Z. 2006. Database of traditional Chinese medicine and its application to studies of mechanism and to prescription validation. *British Journal of Pharmacology* **149**: 1092-1103.

Chinnaiyan, A.M., Prasad, U., Shankar, S., Hamstra, D.A., Shanaiah, M., Chenevert, T.L., Ross. B.D. and Rehemtulla, A. 2000. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proceedings of the National Academy of Science of the United*

Sates of America **97**:1754–1759.

Cline, M.J. 1996. Overview: the nature of malignancy. 1996. In: *Apoptosis and Cell Cycle Control in Cancer* (Thomas N.S.B. Ed.). BIOS Scientific Publishers Limited. Oxford. pp. 131-135.

Cohen, I., Tagliaferri, M. and Tripathy, D. 2002. Traditional Chinese medicine in the treatment of breast cancer. *Seminars in Oncology* **29**: 563-574.

Cragg, G.M., Kingston, D.G. I. and Newman, D.J. 2005. *Anticancer Agent from Natural Products*. CRC press, Boca Raton. pp. 1-4.

Crompton, M.M. 1992. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Reviews* **11**:105-119.

Crompton, M. 1999. The mitochondrial permeability transition pore and its role in cell death. *The Biochemical Journal* **341**: 233-249.

Cotran, R., Kumar, V. and Collins, T. 1999. *Robbins Pathologic Basis of Disease,* 6th Ed. W.B. Saunders, Philadelphia. pp. 260-265.

Crighton, D. and Ryan, K.M. 2004. Splicing DNA-damage responses to tumor cell death. *Biochimica et Biophysica Acta* **1705**: 3-15.

Cryns, V. and Yuan, J. 1998. Protease to die for. *Genes & Development* **12**:1151-1170.

D'Amours, D., Desnoyers, S., D'Silva, I. and Poirier, G.G.. 1999. Poly (ADP-ribosyl) ation reaction in the regulation of nuclear functions. *The Biochemical Journal* **342**: 249–268.

Danial, N.N. and Korsmeyer, S.J. 2004. Cell death: critical control points. *Cell* **116**: 205-219.

Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczycam W., Hotz, M.A., Lassota, P. and Traganos, F. 1992. Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**: 795-808.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T. and Traganos, F. 1997. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* **27**: 1-20.

Debatin, K. and Krammer, P.H. 2004. Death receptor in chemotherapy and cancer. *Oncogene* **23**: 2950-2966.

de Bono, J.S., Tolcher, A.W. and Rowinsky, E.K.2003. The future of cytotoxic therapy: selective cytotoxicity based on biology is the key. *Breast Cancer Research* **5**:154-9.

Degterev, A., Boyce, M. and Yuan, J. 2003. A decade of caspases. *Oncogene* **22**: 8543–8567.

Ding XZ, Iversen P, Cluck MW, Knezetic JA, Adrian TE. 1999. Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. *Biochemical and Biophysical Research Communications* **261**: 218-223.

Ding, X.Z., Hennig, R. and Adrian, T.E. 2003. Lipoxygenase and cyclooxygenase metabolism: new insights in treatment and chemoprevention of pancreatic cancer. *Molecular Cancer* **2**: 1-12.

Donovan, J.C.H., Slingerland, J. and Tannock, I.F. 2005. Cell Proliferation and Tumor Growth. In: *The Basic Science of Oncology* (Tannock, I.F., Hill, R.P., Bristow, R.G. and Harrington, L. Eds.) 4th Ed. McGraw-Hill, New York. pp. 167-193.

Duvvuri, M., Konkar, S., Hong, K.H., Blagg, B.S. and Krise, J.P. 2006. A new

approach for enhancing differential selectivity of drugs to cancer cells. *ACS Chemical Biology* **1**: 309-315.

Eastman, A. 2004. Cell cycle checkpoints and their impact on anticancer therapeutic strategies. *Journal of Cellular Biochemistry* **91**: 223-231.

Eccles, S.A. and Welch, D.R. 2007. Metastasis: recent discoveries and novel treatment strategies. *Lancet* **369**: 1742-1757.

Egan, S.E., Wright, J.A. and Greenberg, A.H. 1991. Molecular determinants of metastatic transformation. *Environmental Health Perspectives* **93**: 91-95

Elmore, S. 2007. Apoptosis: A Review of Programmed Cell Death. *Toxicology Pathology* **35**: 495-576.

Eskes, R., Desagher, S., Antonsson, B. and Martinou, J. C. 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Molecular Cell Biology* **20**: 929-935.

Fesik, S. W. 2005. Promoting apoptosis as a strategy for cancer drug discovery. *Nature Review Cancer* **5**: 876-885.

Fischer, U. and Schulze-Osthoff, K. 2005. New approaches and therapeutics targeting apoptosis in disease. *Pharmacological Reviews* **57**: 187-215.

Fleury, C., Mignotte, B. and Vayssière, J. 2002. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* **84**: 131-141.

Fridovich, I. 1997. Superoxide anion radical (O_2^-.), superoxide dismutases, and related matters. *Journal of Biological Chemistry* **272**:18515-18517.

Fuentes-Prior, P., Salvesen, G.S. 2004. The protein structures that shape caspase activity, specificity, activation and inhibition. *The Biochemical Journal* **384**:

201-232.

Gao, Y., Tang, W., Dai, X., Gao, H., Chen, G., Ye, J., Chan, E., Koh, H.L., Li, X. and Zhou, S. 2005. Effects of water-soluble Ganoderma lucidum polysaccharides on the immune functions of patients with advanced lung cancer. *Journal of Medicinal Food* **8**: 159-168.

Ghobrial, I.M., Witzig, T.E. and Adjei, A.A. 2005. Targeting apoptosis pathways in cancer therapy. *CA: A Cancer Journal for Clinicians* **55**: 178-194.

Gilmore, A. P., Metcalfe, A. D., Romer, L. H. and Streuli, C. H. 2000. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformational and subcellular localization. *Journal of Cell Biology* **149**: 431-446.

Gottesman, M.M. and Pastan, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry* **62**: 385-427.

Griffith, T.S., Chin, W.A., Jackson, G.C., Lynch, D.H. and Kubin, M.Z. 1998. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *Journal of Immunology* **161**:2833-2840.

Gross, A., Yin, X.M., Wang, K. Wei, M.C., Jockel, J., Milliman C., Erdjument-Bromage, H., Tempst, P., Korsmeyer, S.J. 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome *c* release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *The Journal of Biological Chemistry* **274**:1156–1163.

Günther S, Ruhe C, Derikito MG, Böse G, Sauer H, Wartenberg M. 2007. Polyphenols prevent cell shedding from mouse mammary cancer spheroids

and inhibit cancer cell invasion in confrontation cultures derived from embryonic stem cells. *Cancer Letters* **250**: 25-35.

Guo, Q.L., Ding, Q.L. and Wu, Z.Q. 2004. Effect of baicalein on experimental prostatic hyperplasia in rats and mice. *Biological & Pharmaceutical Bulletin* **27**: 333-337.

Hailfinger, S., Jaworski, M., Marx-Stoelting, P., Wanke, I. and Schwarz, M. 2007. Regulation of P53 stability in p53 mutated human and mouse hepatoma cells. *International Journal of Cancer* **120**: 1459-1464.

Hajra, K.M. and Liu, J.R. 2004. TRAIL-induced signalling and apoptosis. *Apoptosis* **9**:691-704.

Hanhana, D. and Weinberg, R.A. 2000. The hallmarks of cancer. *Cell* **100**: 57-70.

Hangartner, M.O. 2000. The biochemistry of apoptosis. *Nature* **407**: 770-776.

Hayslip, J. and Montero, A. 2006. Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review. *Molecular Cancer* **5**:44.

Hernández-Hernández, A., Garabatos, M.N., Rodríguez, M.C., Vidal, M.L., López-Revuelta, A., Sánchez-Gallego, J.I., Llanillo, M. and Sánchez-Yagüe, J. 2005. Structural characteristics of a lipid peroxidation product, trans-2-nonenal, that favour inhibition of membrane-associated phosphotyrosine phosphatase activity. *Biochemica et Biophysica Acta* **1726**: 317-325.

Herr, I., Posovszky, C., Di Marzio, L.D., Cifone, M.G., Boehler, T., Debatin, K.M. 2000. Autoamplification of apoptosis following ligation of CD95-L, TRAIL and TNF-alpha. *Oncogene* **19**: 4255-4262.

Hersey, P. and Xhang, X.D. 2001. How melanoma cells evade TRAIL-induced

apoptosis. *Nature Review Cancer* **1**: 142-150.

Hsieh, T., Lu, X., Chea, J. and Wu, J.M. 2002. Prevention and Management of Prostate Cancer Using PC-SPES: A Scientific Perspective. *Journal of Nutrition* **132**: 3513S.

Hsu, H., Xiong, J. and Goeddel, D.V. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* **81**: 495-504.

Ikemoto, S., Sugimura, K., Yoshida, N., Yasumoto, R., Wada, S., Yamamoto, K. and Kishimoto, T. 2000. Antitumor effects of Scutellariae radix and its components baicalein, baicalin, and wogonin on bladder cancer cell lines. *Urology* **55**: 951-955.

Ivanov, V.N. and Hei, T.K. 2006. Sodium arsenite accerlerates TRAIL-mediated apoptosis in melanoma cells through upregulation of TRAIL-R1/R2 surface levels and downregulation of cFLIP expression. *Experimental Cell Research* **312**: 4120-4138.

Jin X, Wu XX, Abdel-Muneem Nouh MA, Kakehi Y. 2007. Enhancement of death receptor 4 mediated apoptosis and cytotoxicity in renal cell carcinoma cells by subtoxic concentrations of doxorubicin. *The Journal of Urology* **177**: 1606.

Jin, Z. and El-Deiry, W.S. 2006. Distinct signaling pathways in TRAIL- versus tumor necrosis factor-induced apoptosis. *Molecular and Cellular Biology* **26**: 8136-8148.

Jones, R.A., Johnson, V.L., Hinton, R.H., Poirier, G.G., Chow, S.C. and Kass, G.E.N. 1999. Liver poly(ADP-ribose)polymerase is resistant to cleavage by caspases. *Biochemical and Biophysical Research Communications* **256**: 436–441.

Kandasamy, K., Srinivasula, S.M., Alnemri, E.S., Thompson, C.B., Korsmeyer, S.J., Bryant, J.L. and Srivastava, R.K. 2003. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. *Cancer Research* **63**: 1712-1721.

Kannt, A., Lancaster, C.R. and Michel, H. 1998. The role of electrostatic interactions for cytochrome *c* oxidase function. *Journal of Bioenergetics and Biomembranes* **30**: 1-6.

Karp, G. 2003. Cellular reproduction. *Cell and molecular biology: concepts and experiments*, 3rd Ed. John Wiley & Sons, Inc., New York. pp. 580-627.

Kashifi, K. and Rigas, B. 2005. Non-COX-2 targets and cancer: expanding the molecular target repertoire of chemoprevention. *Biochemical Pharmacology* **70**: 969-986.

Kendrick, J.E., Estes, J.M., Straughn Jr., J.M., Alvarez, R.D. and Buchsbaum, D.J. 2007. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its therapeutic potential in breast and gynecologic cancers. *Gynecologic Oncology* [In press].

Kim, J. and Park, E.J. 2002. Cytotoxic anticancer candidates from natural resources. *Current Medicinal Chemistry. Anticancer Agents* **2**: 485-537.

Komdeur, R., Meijer, C., Van Zweeden, M., De Jong, S., Wesseling, J., Hoekstra, H.J. and van der Graaf, W.T., 2004. Doxorubicin potentiates TRAIL cytotoxicity and apoptosis and can overcome TRAIL-resistance in rhabdomyosarcoma cells. *International Journal of Oncology* **25**: 677–684.

Koschny, R., Walczak, H. and Ganten, T.M. 2007. The promise of TRAIL — potential and risks of a novel cancer therapy. *Journal of Molecular Medicine* [In press].

Kowaltowski, A.J., Castilho, R.F.and Vercesi, A.E. 1996. Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca^{2+} is dependent on mitochondrial-generated reactive oxygen species. *FEBS Letters* **378**:150-152.

Kroemer, G., Dallaporta, B., Resche-Rigon, M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis. *Annual Review of Physiology* **60**: 619–642.

Kroemer, G., Galluzzi, L. and Brenner, C. 2007. Mitochondrial membrane permeabilization in cell death. *Physiological Reviews* **87**: 99-163.

Kubanov, B.M. Geilen, C.C., Fecker, L.F., Orfanos, C.E. and Ebrele, J. 2005. Efficient TRAIL-R1/DR4 mediated apoptosis in melanoma cells by tumor necrosis factor-related apoptosis inducing ligand (TRAIL). *The Journal of Investigative Dermatology* **125**: 1010-1019.

Kufe, D., Pollock, R., Weichselbaum, R., Bast, R., Gansler, T., Holland, J. and Frei, E. 2003. Oncogenes in the initiation and progression of neoplasia. *Holland-Frei Cancer Medicine 6.* 6th Eds. BC Decker Inc, Hamilton. pp. 617-632.

Kumar, S. 2007. Caspase function in programmed cell death. *Cell Death and Differentiation* **14**: 32-43.

Kurbanov, B.M., Fecker, L.F., Geilen, C.C., Sterry, W. and Eberie, J. 2007. Resistance of melanoma cells to TRAIL dose not result from up-regulation of

antiapoptotic proteins by NFκB but is related to downregulation of initiator caspases and DR4. *Oncogene* **26**: 3364-3377.

Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X and Vandenlebeele, P. 2002. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death and Differentiation* **9**: 358-361.

Lane, D., Cartier, A., L'Espérance, S., Côté, M., Rancourt, C. and Piché, A. 2004. Differential induction of apoptosis by tumor necrosis factor-related apoptosis-inducing ligand in human ovarian carcinoma cells. *Gynecologic Oncology* **93**: 594-604.

LeBlanc, H.N. and Ashkenazi, A. 2003. Apo2L/TRAIL and its death and decoy receptors. *Cell Death and Differentiation* **10**: 66-75.

Lee, K.H., Im, S.A., Oh, D.Y., Lee, S.H., Chie, E.K., Han, W., Kim, D.W., Kim, T.Y., Park, I.A., Noh, D.Y., Heo, D.S., Ha, S.W. and Bang, Y.J. 2007. Prognostic significance of bcl-2 expression in stage III breast cancer patients who had received doxorubicin and cyclophosphamide followed by paclitaxel as adjuvant chemotherapy. *BMC Cancers* **7**: 63-65.

Li, Y.C., Tyan, Y.S., Kuo, H.M., Chang, W.C., Hsia, T.C. and Chung, J.G. 2004. Baicalein induced in vitro apoptosis undergo caspases activity in human promyelocytic leukemia HL-60 cells. *Food and Chemical Toxicology* **42**: 37-43.

Li, Q., Dashwood, W. M., Zhong, X., Nakagama, H. and Dashwood, R.H. 2007. Bcl-2 overexpression in PhIP-induced colon tumors: cloning of the rat Bcl-2 promoter and characterization of a pathway involving beta-catenin, c-Myc and E2F1. *Oncogene* [In press].

Lin HY, Shen SC, Lin CW, Yang LY, Chen YC. 2007. Baicalein inhibition of hydrogen peroxide-induced apoptosis via ROS-dependent heme oxygenase 1 gene expression. *Biochimica et Biophysica Acta* **1773**: 1073-1086.

Lin, S.B., Li, C.H., Lee, S.S. and Kan, L.S. 2003. Triterpene-enriched extracts from Ganoderma lucidum inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sciences* **72**: 2381-2390.

Lin, Z.B. and Zhang, H.N. 2004. Anti-tumor and immunoregulatory activities of Ganoderma lucidum and its possible mechanisms. *Acta Pharmacological Sinica* **25**: 1387-1395.

Liochev, S.I. and Fridovich, I. 1999. The relative importance of HO* and ONOO− in mediating the toxicity of O*. *Free Radical Biology and Medicine* **26**: 777-778.

Liu, R.H. 2004. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. *The Journal of Nutrition* **134**: 3479S.

Liu, Y., Fiskum, G. and Schubert, D. 2002. Generation of reactive oxygen species by the mitochondrial electron transport chain. *Journal of Neurochemistry* **80**: 780-787.

Lodish, H. Berk, A., Zipurskey, L.S., Matsudaira, P., Baltimore, D. and Darnell, J. 2000. *Molecular Cell Biology*. 4th Ed. W.H. Freeman, New York. pp. 362-365

Loo, D.T. and Rillema, J.R. 1998. Measurement of cell death. *Methods in Cell Biology* **57**: 251-264.

Lowe, S.W., Cepero, E. and Evan, G. 2004. Intrinsic Tumor Supperssion. *Nature* **432**: 307-315.

Lozano, C., Torres, J.L., Juliá, L., Jimenez, A, Centelle, J.J. and Cascante, M. 2005. Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Letters* **579**: 4219-4225.

Ma, Z., Otsuyama, K., Liu, S., Abroun, S., Ishikawa, H., Tsuyama, N., Obata, M., Li, F.J., Zheng, X., Maki, Y., Miyamoto, K. and Kawano, M.M. 2005. Baicalein, a component of Scutellaria radix from Huang-Lian-Jie-Du-Tang (HLJDT), leads to suppression of proliferation and induction of apoptosis in human myeloma cells. *Blood* **105**: 3312-3318.

MacFarlane, M. 2003. TRAIL-induced signalling and apoptosis. *Toxicology Letters* **139**: 89-97.

Makino, T., Tsubouchi, R., Murakami, K., Haneda, M. and Yoshino, M. 2006. Generation of reactive oxygen species and induction of apoptosis of HL60 cells by ingredients of traditional herbal medicine, Sho-saiko-to. *Basic & Clinical Pharmacology & Toxicology* **98**: 401-405.

Malejka-Giganti, D. and Tretyakova, N. 2006. Molecular mechanisms of Carcinogenesis. In: *Carcinogenic and Anticarcinogenic Food Components* (Baer-Dubowska, W., Bartoszek, A. and Malejka-Gianti, D. Eds.). Taylor&Francis. Boca Raton. pp. 13-36.

Marchetti, P., Castedo, M., Susin, S.A., Zamzami, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, F., Geuskens, M. and Kroemer, G. 1996. Mitochondrial permeability transition is a central coordinating event of apoptosis. *Journal of Experimental Medicine* **184**: 1155–1160.

Mathers, C.D., Boschi-Pinto, C., Lopez, A.D. and Murray, C.J.L. 2001. Cancer incidence, mortality and survival by site for 14 regions of the world. Global

Programme on Evidence for Health Policy Discussion Paper No. 13. Geneva, WHO.

Matsui, T., Sowa, Y., Yoshida, T., Murata, H., Horinaka, M., Wakada, M., Nakanishi, R., Sakabe, T., Kubo, T. and Sakai, T. 2006. Sulforanphane enhances TRAIL-induced apoptosis through the induction of DR5 expression in osteosarcroma cells. *Carcinogenesis* **27**: 1768-777.

McConkey, D. J., Chandra, J., Wright, S., Plunkett, W., McDonnell, T. J., Reed, J. C. and Keating, M. 1996. Apoptosis sensitivity in chronic lymphocytic leukemia is determined by endogenous endonuclease content and relative expression of bcl-2 and bax. *The Journal of Immunology* **156**: 2624-2630.

Moon YJ, Wang X, Morris ME. 2006. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicology In Vitro* **20**: 187-210.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *Journal of immunological Methods* **65**: 55-63.

Nagarkatti, N. 2000. Tumor-derived Fas ligand induces toxicity in lymphoid organs and plays an important role in successful chemotherapy. *Cancer Immunology, Immunotherapy* **49**: 46-55.

Nagata, S. 2000. Apoptotic DNA fragmentation. *Experimental Cell Research* **256**: 12-18.

Nie, D., Krishnamoorthy, S., Jin, R., Tang, K., Chen, Y., Qiao, Y., Zacharek, A., Guo, Y., Milanini, J., Pages, G. and Honn, K.V. 2006. Mechanisms regulating tumor angiogenesis by 12-lipoxygenase in prostate cancer cells. *Journal of Biological Chemistry* **281**: 18601-18609

Nishikawa T, Kukidome D, Sonoda K, Fujisawa K, Matsuhisa T, Motoshima H, Matsumura T, Araki E. 2007. Impact of mitochondrial ROS production on diabetic vascular complications. *Diabetes Research and Clinical Practice* [In press].

Nuñez, G., Benedict, M.A., Hu, Y. and Inohara, N. 1998. Caspases: the protease of the apoptotic pathway. *Oncogene* **17**: 3237-3245.

O'connell, J., Houston, A., Bennett, M.W., O' Sullivan, G.C. and Shanahan, F. 2001. Immune privilege or inflammation? Insights into the Fas enigma. *Nature Medicine* **7**: 271-274.

Orrenius, S., Gogvadze, V. and Zhivotovsky, B. 2007. Mitochondrial oxidative stress: Implications for cell death. *The Annual Review of Pharmacology and Toxicology* **47**: 143-183.

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. 2002. Cytochrome *c* release from mitochondria proceeds by a two-step process. *Proceedings of the National Academy of Science USA* **99**: 1259-1263.

Ott, M., Gogvadze, V. and Orrenius, S. 2007. Mitochondria, oxidative stress and cell death. *Apoptosis* **12**: 913-922.

Pagé, M. 2003. High-volume Screening. In: *Cancer Drug discovery and Development Anticancer Drug Development Guide: Preclinical Screenig, Clinical Trail and Approval.* (Teicher, B.A. and Andrews, P.A. Eds.). 2nd Ed. Human Press Inc., New Jersey. pp 3-21.

Peter, M.E. and Krammer, P.H. 2003. The CD95 (APO-1/Fas) DISC and beyond. *Cell Death and Differentiation* **10**: 26-35.

Pieper, A.A., Verma, A., Zhang, J. and Snyder, S.H. 1999. Poly(ADP-ribose)

polymerase, nitric oxide and cell death. *Trends In Phamacological Science* **20**: 171–181.

Pitot, H.C. and Dragan, Y.P. 1991. Facts and Theories Concerning the Mechanisms of Carcinogenesis. *The FASEB Journal* **5**: 2280-2286.

Pitot, H.C. 1993. The molecular biology of carcinogenesis. *Cancer* **72**: 962-970.

Po, L.S., Chen, Z.Y., Tsang, D.S and Leung, L.K. 2002. Baicalein and genistein display differential actions on estrogen receptor (ER) transactivation and apoptosis in MCF-7 cells. *Cancer Letters* **187**: 33-40.

Poljšak, B. and Raspor, P. 2007. The antioxidant and pro-oxidant activity of vitamin C and trolox in vitro: a comparative study. *Journal of Applied Toxicology* [In press].

Rabinovitch PS. 1994. DNA content histogram and cell-cycle analysis. *Methods in Cell Biology* **41**: 263-96.

Rath, C. and Aggarwal, B.B.1999. TNF-induced signaling in apoptosis. *Journal of Clinical Immunology* **19**:350-364.

Riedl, S. J., Li, W., Chao, Y., Schwarzenbacher, R. and Shi, Y. 2005. Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* **434**: 926-933.

Riedl, S.J. and Salvesen, G.S. 2007. Apoptosome: the signaling platform for cell death. *Nature Review Molecular Cell Biology* **8**: 405-413.

Rosse, T. Oliver, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B. and Borner, C. 1998. Bcl-2 prolongs cell survival after bax-induced release of cytochrome c. *Nature* **391**: 496-499.

Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. 1997. JC-1, but not DiOC6(3)

or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Letters* **411**: 77-82.

Sanlioglu, A.D., Koksal, I.T., Ciftcioglu, A., Baykara, M., Luleci, G. and Sanlioglu, S. 2007. Differential expression of TRAIL and its receptors in benign and malignant prostate tissues. *The Journal of Urology* **177**: 359-364.

Savill, J. and Fadok, V. 2000. Corpse clearance defines the meaning of cell death. *Nature* **407**: 784-788.

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S.J., Krammer, P.H. and Peter, M.E. 1999. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *Journal of Biological Chemistry* **274**: 22532-22538.

Schendel, S. L., Montal, M. and Reed, J. C. 1998. Bcl-2 family proteins as ion-channels. *Cell Death and Differentiation* **5**: 372-380.

Schwarz, M., Andrade-Narvarro, D.A. and Gross, A. 2007. Mitochondrial carriers and pores: Key regulators of the mitochondrial apoptotic program? *Apoptosis* **12**: 869-876.

Scovassi, A.I. and Poirier, G.G. 1999. Poly(ADP-ribosylation) and apoptosis. *Molecular and Cellular Biochemistry* **199**: 125-137.

Scovassi, A.I., Denegri, M., Donzelli, M., Rossi, L., Bernardi, R., Mandarino,A., Frouin, I. and Negri, C. 1998. Poly(ADP-ribose) synthesis in cells undergoing apoptosis: An attempt to face death before PARP degradation. *European Journal of Histochemistry* **4**: 251–258.

Sears, R.C. and Nevins, J.R. 2002. Signaling Network that Link Cell Proliferation and Cell Fate. *Journal of Biological Chemistry* **277**: 11617-11620.

Sen, C.K. 2003. The general case for redox control of wound repair. *Wound Repair and Regeneration* **11**: 431-438.

Sheikh, M.S., Antinore, M.J., Huang, Y. and Fornace, A.J. Jr. 1998. Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis receptor 1. *Oncogene* **17**: 2555-2563.

Sheikh, M.S. and Fornance Jr., A.J. 2000. Death and decoy receptors in p53-mediated apoptosis. *Leukemia* **14**: 1509-1514.

Sheikh, M.S. and Huang, Y. 2004. Death receptors as target of cancer therapeutics. *Current Cancer Drug Targets* **4**: 97-104.

Shi, Y. 2006. Mechanical aspects of apoptosome assembly. *Current Opinion in Cell Biology* **18**: 677-684.

Shiao, M.S. 2003. Natural products of the medicinal fungus Ganoderma lucidum: occurrence, biological activities, and pharmacological functions. *Chemical Record* **3**: 172-180.

Shieh, D.E., Liu, L.T. and Lin, C.C. 2000. Antioxidant and free radical scavenging effects of baicalein, baicalin and wogonin, *Anticancer Research.* **20**: 861-2865.

Siegel, R.M., Chan, F.K., Chun, H.J. and Lenardo, M.J. 2000. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nature Immunology* **1**:469-74.

Skommer, J., Wlodkovic, D. and Deptala, A. 2006. Larger than life: Mitochondria and the Bcl-2 family. *Leukemia Research* **31**: 277-286.

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S., Green, D.R. and

Martin, S.J. 1999. Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2,-3, -6, -7, -8, and-10 in a caspase-9 -dependent manner. *The Journal of Cell Biology* **144**: 281-292.

Sliva, D. 2003. Ganoderma lucidum (Reishi) in cancer treatment. *Integrative Cancer Therapies* **2**: 358-364.

So, F.V., Guthrie, N., Chambers, A.F., Moussa, M. and Carroll, K.K. 1996. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutrition & Cancer* **26**: 167-181.

So, F., Guthrie, N., Chambers, A., and Carroll, K. 1997. Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. *Cancer Letters* **112**: 127–133.

Sohal, R.S. 2002. Role of oxidative stress and protein oxidation in the aging process. *Free Radical biology & Medicine* **33**: 37-44.

Soldani, C., Lazzè, M.C., Bottone, M.G., Tognon, G., Biggiogera, M., Pellicciari, C.E. and Scovassi, A.I. 2001. Poly(ADP-ribose)polymerase cleavage during apoptosis: When and where? *Experimental Cell Research* **269**: 193–201.

Stoka, V., Turk, B. Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., Bromme, D., Krajewski, S., Reed ,J.C., Yin, X.M/, Turk, V. and Salvesen, G.S. 2001. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *The Journal of Biological Chemistry* **276**: 3149-3157.

Storz, P. 2007. Mitochondrial ROS--radical detoxification, mediated by protein kinase D. *Trends in Cell Biology* **17**: 13-18.

Suliman, A., Lam, A., Datta, R. and Srivastava, K. 2001. Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and –independent pathway. *Oncogene* **20**: 2122-2133.

Surh, Y.J. 2003. Cancer Chemoprevention with Dietary Phytochemicals. *Nature Reviews Cancer* **3**:768-780.

Takeda, K., Stagg, J., Yagita, H., Okumura, K. and Smyth, M.J. 2007. Targeting death-inducing receptors in cancer therapy. *Oncogene* **26**: 3745-3757.

Tanuma, S. and Shiokawa, D. 1996. An endonuclease responsible for apoptosis. *Apoptosis* (Kuchino, Y. and Muller, W. E. G., Eds.). Springer-Verlag, New York. pp. 1-12.

Thompson, C.B. 1995. Apoptosis in the Pathogensis and Treatment of Disease. *Science* **267**: 1456-1462.

Thornberry, N.A. 1998. Caspase: key mediators of apoptosis. *Chemistry & Biology* **5**: R97-R103.

Thornberry, N.A. and Lazebnik, Y. 1998. Caspase: enemies within. *Science* **281**: 1312-1316.

Tsuzuki, T., Nakatsu, Y. and Nakabeppu, Y. 2007. Significance of error-avoiding mechanisms for oxidative DNA damage in carcinogenesis. *Cancer Science* **98**: 465-470.

Tudor, G., Aguilera, A., Halverson, D.O., Laing, N.D. and Sausville, E.A. 2000. Susceptibility to drug-induced apoptosis correlates with differential modulation of Bad, Bcl-2 and Bcl-xL protein levels. *Cell Death and Differentiation* **7**: 574-586.

Tuominen, E.K., Wallace C.J. and Kinnunen, P.K. 2002. Phospholipid cytochrome *c*

interaction: evidence for the extended lipid anchorage. *Journal of Biological Chemistry* **277**: 8822–8826.

van Horssen, R., Ten Hagen, T.L. and Eggermont, A.M. 2006. TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. *Oncologist* **11**: 397-408.

Venditti, P., De Rosa, R.and Di Meo, S. 2004. Effect of cold-induced hyperthyroidism on H_2O_2 production and susceptibility to stress conditions of rat liver mitochondria. *Free Radical Biology and Medicine* **36**: 348-358.

Vermes I, Haanen C, Reutelingsperger C. 2000. Flow cytometry of apoptotic cell death. *Journal of Immunological Methods* **243**: 167-190.

Vermeulen, K., Van Bockstaele, D.R. and Berneman, Z.N. 2005. Apoptosis: mechanisms and relevance in cancer. *Annals of Hematology* **84**: 627-639.

Walczak, H. and Krammer, P.H. 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) Apoptosis Systems. *Experimental Cell Research* **256**: 58-66.

Wallach, D., Varfolomeev, E.E., Malinin, N.L., Golstsev, Y.V., Kovalenko, A.V. and Boldin, M.P. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annual Review of Immunology* **17**: 331-367.

Wang, J., Yu, Y., Hashimoto, F., Sakata, Y., Fuji, M. and Hou, D. 2004. Baicalein induces apoptosis through ROS-mediated mitochondrial dysfunction pathway in HL-60 cells. *International Journal of Molecular Medicine* **14**: 627-632.

Wang, X. 2001. The expanding role of mitochondria in apoptosis. *Genes & Development* **15**:2922–2933.

Wong, B.C., Wang, W.P., Cho, C.H., Fan, X.M., Lin, M.C., Kung, H.F. and Lam, S.K. 2001. 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer

cells. *Carcinogenesis* **22**: 1349-1354.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T., Jones, D.P. and Wang, X. 1997. Prevention of apoptosis of Bcl-2: Release of cytchrome *c* blocked. *Science* **279**: 1129-1132.

Yano, H., Mizoguchi, A., Fukuda, K., Haramaki, M., Ogasawara, S., Momosaki, S.,and Kojiro, M. 1994. The herbal medicine Sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0-G1 phase. *Cancer Research* **54**: 448–454.

Ye, F., Xui, L., Yi, J., Zhang, W. and Zhang, D.Y. 2002. Anticancer activity of Scutellaria baicalensis and its potential mechanism. *Journal of Alternative and Complementary Medicine* **8**: 567-572.

Yokota, J. 2000. Tumor progression and metastasis. *Carcinogenesis* **21**: 479.

Zelster, R., Leticia, V. Tanck, C., Holyst, M.M., Ritchlin, C. and Gaspari, A.A. 2001. Clinical, histological and immunophenotypic characteristics of injection site reactions associated with etanercept: a tumor necrosis factor alpha receptor:Fc fusion protein. *Archives of Dermatology* **137**: 893-899.

Ziegler, U. and Groscurth, P. 2004. Morphological features of cell death. *News in Physiological Sciences* **19**: 124-128.

Zhang DY, Wu J, Ye F, Xue L, Jiang S, Yi J, Zhang W, Wei H, Sung M, Wang W, Li X. 2003. Inhibition of cancer cell proliferation and prostaglandin E2 synthesis by Scutellaria baicalensis. *Cancer Research* **63**: 4037-4043.

Zhang, X.D., Zhang, X.Y., Gray, C.P. Nguyen, T. and Hersey, P. 2001. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of human melanoma is regulated by Smac/DIABLO release from mitochondria. *Cancer*

Research **61**: 7339-7348.

Zhivotovsky, B. and Orrenius, S. 2006. Carcinogenesis and apoptosis: paradigms and paradoxes. *Carcinogenesis* **27**: 1939-1945.

Zoratti, M and Szabo, I.1995. The mitochondrial permeability transition. *Biochimica et Biophysica Acta* **1241**: 139–176.

Zuliani, T., Duval, R,, Jayat, C., Schnébert, S., André, P., Dumas, M. and Ratinaud, M.H. 2003. Sensitive and reliable JC-1 and TOTO-3 double staining to assess mitochondrial transmembrane potential and plasma membrane integrity: interest for cell death investigations. *Cytometry. Part A* **54**: 100-108.

Zönig, M., Hueber, A., Baum, W. and Evan, G. 2001. Apoptosis regulator and their role in tumorigenesis. *Biochemica et Biophysica Acta* **1551**: F1-F37.