ANTI-CANCER EFFECTS OF ALOE-EMODIN: CELLULAR AND PROTEOMIC STUDIES

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SUMMARY

Aloe-emodin (AE) is a major bioactive hydroxyanthraquinone in Rhubarb (*Rheum palmatum*), a well known Chinese herbal medicine. This compound is known to exhibit multiple pharmacological and anti-cancer effects, although the precise molecular mechanisms were not well studied. AE was shown to have higher cytotoxicity in cancer cells, compared to its analogue emodin (EM). However, several studies suggested that AE, unlike EM, is a poor kinase inhibitor. In order to have a better understanding of the target molecules and relevant molecular pathways of AE, a systemic study integrating functional proteomics and conventional biochemical approaches was thus conducted on the anti-cancer effect of AE.

Our preliminary results showed that AE inhibited the growth of hepatoma cells *in vitro*. This action was cell line specific when compared to other non-tumorous cells. Furthermore, AE induced apoptosis and cell cycle arrest, which may account for its higher cytotoxicity compared to EM. Two-dimensional difference gel electrophoresis (2D-DIGE) proteomics analysis revealed that AE affected various proteins functionally associated with oxidative stress, cell cycle arrest, anti-metastasis and other anti-cancer activities. On the contrary, EM affected fewer proteins, consistent with its lower cytotoxicity. Further biochemical validation of the 2D-DIGE results revealed several novel anti-cancer functions of AE.

Firstly, antioxidant peroxiredoxins were found to be highly up-regulated while blocking peroxiredoxins expression by small interfering RNA (siRNA) sensitized AE-induced apoptosis, suggesting AE induced reactive oxygen species

(ROS)-dependent apoptosis. It was further found that AE induced excessive ROS generation and depleted the intracellular reduced glutathione. AE treatment also led to sustained activation of c-Jun N-terminal kinase (JNK), an important stress-responsive mitogen activated protein kinase (MAPK). Over-expression of antioxidant gene *sod1* significantly reduced AE-induced JNK activation and cell death, suggesting that oxidative stress-mediated JNK is one of the effector molecules in AE-induced apoptosis. More importantly, JNK deactivation by treatment of JNK inhibitor, JNK siRNA knockdown or over-expression of dominant negative JNK protected AE-induced apoptosis. In addition, the results demonstrated the critical role of apoptosis signal-regulating kinase1 (ASK1), a well established MAPK kinase kinase, in AE-induced JNK activation and apoptotic cell death. Finally, dissociation of inactive JNK-GST-pi complex was also involved in JNK activation through GST-pi oxidation. Taken together, these results clearly demonstrated that AE-induced apoptosis is mediated via oxidative stress and sustained JNK activation.

On the other hand, the 2D-DIGE result of the up-regulation of the tumor suppressor p16 was validated and confirmed to be responsible for AE-induced inhibition of DNA synthesis; while up-regulation of the metastasis suppressor, nucleoside diphosphate kinase A (NDKA), may account for AE-induced inhibition of cell migration/invasion.

In summary, the present study, which integrates the functional proteomics and biochemical approach, provides a comprehensive understanding of AE-induced anti-cancer effect.

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ABBREVIATIONS

2-DE	two dimensional electrophoresis
2D-DIGE	2D difference gel electrophoresis
7-AAD	7-amino-actinomycin D
ACN	acetonitrile
AE	aloe-emodin
ANT	adenine nucleotide translocase
AP1	activating protein 1
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAT	catalase
CDK	cyclin-dependent kinase
CK2	casein kinase II
CM-H_2DCFDA	chloromethyl-2 ,7 -dichlorofluorescein diacetate
Cu/Zn-SOD	copper/zinc superoxide dismutase
DAPI	4, 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DN	dominant negative
DNPH	2, 4-dinitrophenylhydrazine
ED_{50}	effective dose 50%
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
EM	emodin
ER	estrogen receptor
ERK	extracellular signal-regulated kinases
FBS	Fetal bovine serum
GPX	glutathione peroxidase
GR	glutathione reductase
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSH-MEE	glutathione monomethylester
GSSG	oxidized glutathione
GST-π	glutathione S-transferase π
IAPs	inhibitor of apoptosis proteins
IC_{50}	inhibitory dose 50%
IEF	isoelectric focusing
INF-γ	interferon-γ
I*κ*B	NF-*κ*B inhibitory protein
IKK	I*κ*B kinase
IL-1	Interleukin-1
iNOS	inducible nitric oxide synthase
IPG	immobilized pH gradient

JNK	c-Jun N-terminal kinases
LIMK	LIM motif-containing protein kinase
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MEK	MAPK/ERK kinase 1
MOMP	mitochondrial outer membrane permeabilization
MnSOD	manganese superoxide dismutase
MS	Mass spectrometry
NATs	*N*-acetyltransferases
NDKA	nucleoside diphosphate kinase A
NF-κB	nuclear factor kappaB
NO	Nitric oxide
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEITC	β-phenylethyl isothiocyanate
pI	isoelectric point
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PRDX	peroxiredoxin
P-SOH	protein sulfenic acid
P-SO_2H	protein sulfinic acid

P-SO_3H	protein sulfonic acid
Rb	retinoblastoma
PTP	protein tyrosine phosphatases
ROS	reactive oxygen species
SAPK	stress-activated protein kinases
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SOD	superoxide dismutase
TCM	traditional Chinese medicine
TFA	trifluoroacetic acid
TMRM	tetramethylrhodamine methyl ester
TOF	time of flight
TNF-α	tumor necrosis factor alpha
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Trx	thioredoxin
VDAC	voltage dependent ion channel

CHAPTER 1

INTRODUCTION

1.1 Aloe-emodin (AE)

1.1.1 Introduction: rhubarb and AE

Rhubarb root (also named as Da Huang in Chinese) is one of the earliest and best-known Chinese herbal medicines (Figure 1.1). The most commonly used species are *Rheum palmatum, Rheum palmatum var. tanguticum* and *Rheum officinale* of the Polygonaceae family. The first record of rhubarb can be traced back to Classic of the Materia Medica (Shen Nong Ben Cao Jing in Chinese) as early as 2500 years ago in China (Yang, 1997). In Traditional Chinese Medicine (TCM), rhubarb was ranked as a top medicinal herbal plant in Beng Cao Gang Mu (Li, 1982). Rhubarb was traditionally used for remedies of digestive system diseases, such as constipation (as a purgative agent), gastritis, enteritis, hepatitis, gastro-intestinal hemorrhage and ulcers. Currently, many of the Chinese herbal preparations also contain rhubarb.

The major bioactive constituents in rhubarb are hydroxyanthraquinone derivatives, including aloe-emodin (AE, ~1.5% of chemical content), emodin (EM, ~2.6%), rhein (~1.9%), chrysophanol (~1.9%), physcion (~0.8%), and danthron (<0.2%), along with di-O, C-glucosides of the monomeric reduced forms (rheinosides A–D), and dimeric reduced forms (sennosides A–F) (Cai *et al.*, 2004). Generally, rhubarb hydroxyanthraquinones are responsible for the main pharmacological effects of rhubarb. Chemical structures of the main rhuarb hydroxyanthraquinones are shown in Fig. 1.2. The presence and/or sites of the hydroxyl groups may be the main structural factors for the different biological activities of these hydroxyanthraquinone compounds.

AE and EM, two main rhubarb hydroxyanthraquinones, exhibit multiple biological functions *in vitro* and *in vivo*. Recently, both of them have aroused increasing research interests, due to their potent anti-cancer and pharmacological properties. It was found that both AE and EM can induce growth inhibition and apoptotic cell death either by itself or in combination with other cancer therapeutic agents in a variety of cancer cells (Huang *et al*., 2006a; Srinivas *et al*., 2006). Furthermore, a broader cancer-therapeutic value was credited to EM when EM was found to be effective in preventing cancer angiogenic and metastatic processes (Huang *et al.*, 2004; , 2005; Huang *et al.*, 2006b; Zhang *et al.*, 1998). Current molecular mechanistic studies suggested that the anti-cancer potency of EM is attributed to its inhibitory effect on protein tyrosine kinases, and a number of other kinases (e.g. CK2, PKC, and PI3K) (Huang *et al*., 2006a). Compared to EM, the molecular mechanism of AE has been less understood. It was believed that AE may share the same or similar anti-cancer properties with EM, due to the similarity in chemical structure of AE (1, 8-dihydroxy-3-hydroxylmethyl-anthraquinone) and EM (1, 3, 8-trihydroxy-6-methylanthraquinone, Fig. 1.2). Unexpectedly, some recent studies demonstrated that AE is a poorer kinase inhibitor against several pro-survival kinases than EM (Huang *et al*., 2006a; Sarno *et al*., 2002). These anti-cancer mechanisms of AE are still very much unknown.

In the following sections, the metabolism, pharmacological and anti-cancer properties of AE, including the recent reports on its mechanistic actions will be reviewed.

Fig. 1.1 Rhubarb plant

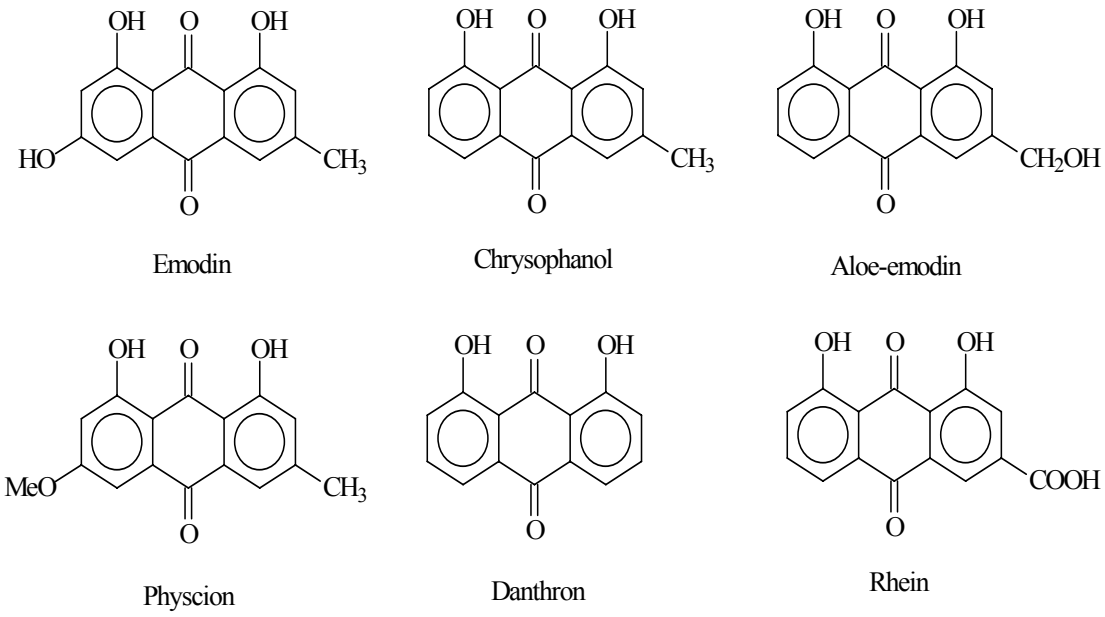


Fig. 1.2 Major hydroxyanthraquinones of rhubarb

1.1.2 Source and metabolism of AE

Aloe-emodin (AE) exists in many herbal plants besides rhubarb, e.g. Aloe Vera, Senna, etc (Thomson, 1971). Another important source for AE *in vivo* is the natural metabolic conversion from other anthraquinone derivatives (e.g. sennoside C, barbaloin, and chrysophanol). Sennoside C, the dimeric reduced forms of hydroxyanthraquinone, can be transformed by the action of intraluminal bacteria in mice into an active metabolite, aloe-emodin anthrone, which can subsequently be further auto-oxidized to AE (Yamauchi *et al*., 1992). Similarly, barbaloin, the glycosidic derivative precursor of AE, can be converted by human intestinal bacterial (*Eubacterium sp*. strain BAR) into purgative aloe-emodin anthrone (Akao et al., 1996). Besides, hydroxyanthraquinone chrysophanol can also be transformed to AE in a cytochrome P450-dependent oxidation manner (Mueller *et al*., 1998).

Absorption, excretion, tissue distribution and metabolism of AE have been studied after a single oral administration of [^{14}C] AE (4.5 mg/kg) to both male and female rats (Lang, 1993). The maximum plasma concentration of AE reached after 1.5-3 hr of treatment was 248 ng/ml (male rat) and 441 ng/ml (female rat) equivalents. The terminal half-life (for radioactivity) in blood was about 50 hr. Most of AE in rat plasma was found to be presented in a conjugated form, while 10% ^{14}C-activity in plasma was identified as free AE. Higher concentrations of AE were found to accumulate in liver, kidney and intestinal tract but its concentration were lower in ovaries and testes. 20-30% of the administrated dose was excreted in urine and the rest in feces. A similar study was carried in which a 25 mg single dose of AE was

administrated orally to albino rats of 120-150 g in weight (Maity *et al.*, 2001). About 15.4% of the administered AE was excreted while the rest was probably bound to serum proteins or metabolized in the rat.

1.1.3 Pharmacological properties of AE

Rhubarb has been used in TCM for thousands of years. Although accumulating data have shown that AE is potent in purgative, hepatoprotective and anti-microbial effects, it is still, however, far from being able to explain the multi-functional effects of AE.

1.1.3.1 Purgative activities

AE itself may not act directly as a purgative. It has been reported that intracaecal administration of AE (ED_{50} 246.3 μM/kg) and other anthraquinones (EM and chrysophanol) are less potent than rhein anthrone (ED_{50} 11.4 μM/kg) (Yagi and Yamauchi, 1999). Instead, the observed purgative effect of AE is probably executed through its precursor, aloe-emodin anthrone. Aloe-emodin anthrone, as an active metabolite transformed from sennoside C or barbaloin, can act independently or synergistically with rhein anthrone to exert purgative effects *in vivo* (Akao *et al.*, 1996; Yamauchi *et al.*, 1992).

1.1.3.2 Hepatoprotective effect

Studies have revealed that AE is capable of preventing induced hepatic damage and/or fibrosis. AE protected liver from carbon tetrachloride (CCl_4) induced hepatic damage. This protective action was reported to be associated with its antioxidant property by decreasing CCl_4-induced radical production and lipid peroxidation (Arosio

et al., 2000). AE may also inhibit hepatic fibrosis, as indicated by the finding that AE decreased PDGF-stimulated rat hepatic stellate cell activation and proliferation, a key process in the pathogenesis of hepatic fibrosis (Woo *et al*., 2002).

1.1.3.3 Anti-fungal, anti-protozoal and anti-bacterial effects

Similar to other hydroxyanthraquinones, AE has strong anti-fungal, and anti-bacterial properties. It has been reported that AE showed selective fungicidal activities against *B. cinerea and R. solani, C. albicans and T. mentagrophytes*, but did not inhibited the growth of *E. graminis, P. infestans, P. recondita, Py. Grisea, Cry. Neoformans*, and *S. schenckii* (Agarwal *et al*., 2000; Kim *et al*., 2004). Besides, AE treatment inhibit growth of protozean *T. b. brucei* (Camacho *et al*., 2000) and methicillin-resistant bacterial *Staphylococcus aureus* (Hatano *et al*., 1999).

On the other hand, the antibacterial effect of AE and its glycoside precursor barbaloin was compared and the result showed a stronger inhibitory effect of barbaloin against *E. coli.* It was suggested that the glycoside side chain may help barbaloin invade *E. coli* cells and enhance its cytotoxicity (Tian *et al*., 2003). Moreover, it has been recently reported that AE could inhibit 3C-like protease (3CLpro) of coronavirus in severe acute respiratory syndrome (SARS), which was found to be an important target for SARS therapy (Lin *et al*., 2005a). It may credit AE and other hydroxyanthraquinones as promising therapeutic agents in the treatment of SARS.

1.1.3.4 Other pharmacological effects

It was of great interest that AE may cause dose-dependent falls in mean arterial blood pressure in rats (Saleem *et al.*, 2001). This finding suggested that AE may work

as a potent hypotensive agent. On the other hand, another study (Yin and Xu, 1998) pointed out that AE may inhibit the proliferation of smooth muscle cells after arterial injury by decreasing protein and mRNA level of proliferating cell nuclear antigen. These two studies may indicate AE's potential protective role in the cardiovascular system, although more in-depth work is needed.

Similar to its analogue EM and the rhubarb extract, AE has been found to inhibit the formation of advanced glycation end products, that play a key etiologic role in the development of diabetic nephropathy (Nakagawa *et al.*, 2005). This finding may help to understand the clinical efficacy of rhubarb against renal failure.

1.1.4 Anti-cancer potential of AE

Besides its multiple pharmacological effects, AE has also attracted increasing research interests in its anti-cancer properties. These studies aimed to unravel both the potent activities of AE in cancer-prevention and cancer-therapy, and its mechanism actions. In the following sections, these findings will be summarized accordingly.

1.1.4.1 Anti-mutagenic and anti-carcinogenic effects

Among all hydroxyanthraquinones, only danthron was found to be capable of initiating cancer in animal models (Mori *et al.*, 1986). Whether AE initiates or prevents cancer is still debatable. Earlier studies had focused on the mutagenicity of AE. It had been reported that AE exhibited mutagenic activities in a battery of *in vitro* mutagenesis assays (e.g. bacterial cultures (*Salmonella typhimurium* assay) and mammalian cell cultures (chromosome aberration test in Chinese hamster ovary cells)). Contradictory non-mutagenic results, however, were found in other *in vitro* assays (mammalian cell

HGPRT test) and all *in vivo* assays (bone marrow assay (micronucleus test; chromosome aberration test), mouse spot test in melanoblast cells and unscheduled DNA synthesis test in male Wistar rats and NMRI mice) (Heidemann *et al.*, 1993; Heidemann *et al.*, 1996; Westendorf *et al.*, 1990). Therefore based on these findings, Heidemann *et al* concluded that it is unlikely for AE to be mutagenic and genotoxic *in vivo* (Heidemann *et al.*, 1996). Moreover, no rodent study has yet been reported to suggest that AE exhibited carcinogenic or tumor promoting property (Siegers *et al.*, 1993). It was believed that hydorxyanthraquinones including AE do not represent a genotoxic risk, if the estimated daily intake, concentration of hydroxyanthraquinones and the genotoxic potency, as well as protective effects of the food matrix in a balanced human diet were taken into consideration (Mueller *et al.*, 1999).

Besides the anti-mutagenesis effect, AE was found to be effective in inhibiting DNA adduct formation by decreasing *N*-acetyltransferases (NATs) expression level and activity in mice leukemia cells (Chung *et al.*, 2003) and human malignant melanoma cells (Lin *et al.*, 2005b). NATs are involved in the metabolic transformation of arylamine chemicals into carcinogenic intermediate metabolite and promoting the latter to induce DNA adducts formation and finally carcinogenesis. This action by AE was proposed to be cancer-preventive.

On the contrary, carcinogenic concern arose when Strickland and his colleagues reported that topical administration of AE in ethanol vehicle could switch ultraviolet-induced skin tumor to malignant melanoma in C3H/HeN mice, although in the absence of ultraviolet stimulation neither AE nor ethanol alone induced skin tumors (Strickland

et al., 2000). The same group (Badgwell *et al.*, 2004) later reported found that topical co-administration of AE and ethanol in the presence of ultraviolet exposure in mice may induce p53 mutation, which is in a similar spectrum to human p53 mutation. The precise molecular mechanism for that induced phototoxicity is still unknown. Besides, no epidemiological or case report could be found linking the usage of AE or rhubarb extract with skin cancer up to now. On the other hand, under some circumstances, the phototoxic effects of AE and other hydroxylanthraquinones can be beneficial. For example, some bis(ainoalkyl)-anthraquinones are photosensitizer agents and used in the medical treatment known as photodynamic therapy (Cardenas *et al.*, 2006).

Taken together, AE may not be carcinogenic and/or mutagenic by itself *in vivo*. Furthermore, AE may even exhibit cancer-preventive properties by inhibiting NATs activation. But topical administration of AE together with ethanol may pose a carcinogenic risk under stimulation of ultraviolet exposure, although no epidemiological report has ever been conducted on this important issue.

1.1.4.2 Inhibition of tumor cell growth and induction of cell cycle arrest

A number of *in vitro* studies have shown that AE exhibited high cytotoxicity against a variety of tumor cells, including human neuroectodermal tumor (Pecere *et al.*, 2000; Pecere *et al.*, 2003), lung carcinoma (Lee *et al.*, 2001), oral squamous cell carcinoma (Shi *et al.*, 2001), merkel carcinoma (Wasserman *et al.*, 2002), hepatoma (Shi *et al.*, 2001), leukemia (Chen *et al.*, 2004a), glioma (Mijatovic *et al.*, 2005a) and mouse L929 fibrosarcoma and rat astrocytoma cells (Mijatovic *et al.*, 2005a). More importantly, this cytotoxic effect of AE was cell line specific when compared with

non-carcinogenic normal human gingival fibroblasts (HGF) (Shi *et al.*, 2001), human lung fibroblast (MRC5) cells and hemopoietic progenitor cells (Pecere *et al.*, 2000), rat primary astrocytes and fibroblasts (Mijatovic *et al.*, 2004). It is also noteworthy to mention that this specific cytotoxicity against cancer cells was not found in barbaloin, a glycosidic derivative of AE (Fenig *et al.*, 2004; Pecere *et al.*, 2000; Wasserman *et al.*, 2002). The glycoside side chain in barbaloin may inhibit drug incorporation through cell membrane in mammalian cells, but promote the incorporation in *E. coli* (Tian *et al.*, 2003). Moreover, AE showed a high specificity for neuroectodermal tumor cells (Pecere *et al.*, 2000; Pecere *et al.*, 2003). According to Pecere and colleagues' report, specific energy-dependent drug incorporation of AE may account for the greater sensitivity of neuroectodermal tumor cells than normal human cells and other malignant cells (Pecere *et al.*, 2000). In the neuroectrodermal cells, after treatment with 5 μM AE for 45 min, the drug's intracellular concentration could reach to 2.5 mM (500 times).

Regulated cellular proliferation is essential for mammalian cell homeostasis. Instead, deregulated cell proliferation is a hallmark of cancer. Thus, one of the important approaches for cancer therapy is to re-regulate cell cycle progression. This regulation is mostly played via checkpoint proteins that control normal cell cycle progression. The effect of AE on G2/M cell cycle had been demonstrated in a number of cancer cells, including hepatoma (Kuo *et al.*, 2002; Shieh *et al.*, 2004), leukemia (Chen *et al.*, 2004a) and neuroectodermal cells(Pecere *et al.*, 2003). Similarly, G1/S cell cycle arrest was also found in human colon carcinoma (Schorkhuber *et al.*, 1998), hepatoma (Kuo *et al.*, 2004; Kuo *et al.*, 2002), lung carcinoma (Yeh *et al.*, 2003), glioma

(Acevedo-Duncan *et al.*, 2004; Mijatovic *et al.*, 2005a) and leukemia cells (Chen *et al.*, 2004a). However, lower dose (less than 10 μM) of AE may increase DNA synthesis, which was suggested as a cellular response for AE's cytotoxicity (Wolfle *et al.*, 1990).

Some studies suggested that p53 and p21 pathways may be involved in the cell cycle arrest induced by AE (Kuo *et al.*, 2002; Pecere *et al.*, 2003). These studies, however, mainly focused on the apoptotic role of p53 and p21 (discussed below). The exact molecular mechanism for AE-induced cell cycle arrest is still unclear.

1.1.4.3 Induction of cell death in cancer cells

The process of apoptosis (also named as programmed cell death) is fundamental in the developmental and homeostatic maintenance of complex biological systems. Deregulation of normal apoptotic mechanisms may contribute to cell malignant transformation and provide a growth advantage to cancer cells against surrounding normal cells (Evan and Vousden, 2001). Apoptosis is morphologically characterized by cell shrinkage, chromatin condensation, DNA fragmentation and enzymatic activation of specific cysteine proteases known as caspases. Two pathways converge on the key apoptosis executor caspase-3: one involving extrinsic cell death receptor activation and caspase-8 activation, and the other involving intrinsic mitochondrial disruption and activation of caspase-9 (Fischer and Schulze-Osthoff, 2005).

Pecere and colleagues firstly reported that AE induced DNA fragmentation (one of the biomarkers of apoptosis) at 48 hours after G2/M arrest in neuroectodermal tumor cells (Pecere *et al.*, 2000). It was also found that AE could induce apoptosis in many other cancer cells derived from human lung carcinoma (Lee, 2001; Lee *et al.*, 2001),

hepatoma (Kuo *et al.*, 2002), leukemia (Chen *et al.*, 2004a) and bladder carcinoma (Lin *et al.*, 2006). Moreover, the induction of apoptosis by AE was found to be irreversible, as evidenced by the finding that replacement of fresh drug-free medium after 4 hr treatment of AE cannot decrease the induced apoptosis level (Lee *et al.*, 2001).

Furthermore, several groups have investigated whether AE initiates apoptosis from mitochondria-involved intrinsic or death receptor-involved extrinsic apoptotic pathways or both. In neuroblastoma cells, the intrinsic mitochondrial-mediated but not extrinsic death receptor-mediated apoptotic pathway activation was found to be involved in AE-induced apoptosis (Pecere *et al.*, 2003). Similarly in transformed rat hepatic stellate cells, AE treatment also failed to activate caspase-8, the key caspase in extrinsic apoptotic pathway (Lian *et al.*, 2005). Although in lung carcinoma CH27 cells the activation of caspase-3, -8, and -9 were all found in AE-induced apoptosis, caspase-8 activation occurred later than that of caspase-3 and -9 (Lee *et al.*, 2001). Therefore, all the above results indicate that mitochondria-involved intrinsic apoptotic pathway played a more important role in apoptosis induced by AE.

To further explore the intrinsic mitochondria–mediated apoptotic pathway induced by AE, several groups examined the role of Bcl-2 family members. The Bcl-2 family proteins consist of both antiapoptotic (Bcl-2, Bcl-XL) and proapoptotic (Bax, Bak) proteins and they are well-characterized regulators of apoptosis, especially the intrinsic apoptotic pathway (Cory and Adams, 2002). The antiapoptotic Bcl-2 members (Bcl-2, Bcl-XL) was found to be down-regulated upon AE treatment on CH27 (Lee *et al.*, 2001) and H460 cells (Yeh *et al.*, 2003). In contrast, proapoptotic members (Bax and Bak)

were found to be up-regulated in hepatoma cells (Kuo *et al.*, 2002). Moreover, translocation of Bax and Bak from cytosol to mitochondria is an important event to initiate mitochondria-mediated apoptosis. CH27 cells exhibited such translocation of Bax and Bak upon treatment of AE (Lee *et al.*, 2001).

In addition to the caspase-dependent apoptosis, AE was once reported to induce autophagy (caspase-independent type II programmed cell death) in glioma cells (Mijatovic *et al.*, 2005a). But this finding was made based on two indirect evidence. One was the formation of acidic vesicle (possible indication for autophagic vacuole). And the other was that pan-caspase inhibitor z-VAD-FMK could not protect AE-induced growth inhibition as detected by the MTT assay. However, the second observation could not exclude possible involvement of cell cycle arrest and/or senescence because both of them were AE-inducible and capable of decreasing cell proliferation. Moreover, whether the formation of acidic vesicle played a role in survival or cell death was still unknown. Thus, more direct experiments are needed to establish the role of autophagy (e.g. knocking-down the key executor atg-5 in autophagy (Kroemer and Jaattela, 2005)) induced by AE treatment.

1.1.4.4 Sensitization effect

Acquired chemoresistance (or relapse from cancer therapy) is a significant impediment for effective chemotherapy for various tumors. Different combinations of chemotherapeutic drugs may offer great potential for improving anti-cancer responses and decreasing off-target side effects in various carcinomas (Nakanishi and Toi, 2005).

AE has been reported to potentiate the cytotoxic effect of some common

chemotherapeutic agents (cisplatin, doxorubicin, and 5-fluoroucil) in Merkel carcinoma cells (Fenig *et al.*, 2004). However, the exact mechanism for the synergistic effect is less studied. Furthermore, AE can sensitize apoptosis in this cell line when co-treated with a tyrosine kinase inhibitor ST1 571 (Fenig *et al.*, 2004). As mentioned above, AE coexists with EM in rhubarb and EM is commonly used as a tyrosine kinase inhibitor (Huang *et al.*, 2006a). One interesting question to be raised is whether these two compounds have some synergistic effects on cell death, although no relevant studies had been conducted. In addition, AE as well as EM and rhubarb extract, have been reported to sensitize tumor cells to arsenic trioxide-induced apoptosis (Yang *et al.*, 2004) and this action was attributed to their potential in inducing oxidative stress.

On the contrary, controversies arose as there were two reports indicated that AE could protect some tumor cells from cell growth inhibition induced by other chemotherapeutic agents. Mijatovic *et al* reported that AE decreased the cytotoxicity induced by interferon-γ (INF-γ) and interleukin-1 (IL-1) in mouse fibrosarcoma cells (L929), although AE itself can inhibit L929 cell growth (Mijatovic *et al.*, 2004). This protective action was linked to the antioxidant property of AE in decreasing transcription of inducible nitric oxide synthase (iNOS) and inhibiting production of nitric oxide (NO). But AE, being not a direct NO-scavenger, failed to protect SIN-1 (nitric oxygen donor) induced cytotoxicity. Paradoxically this combined effect was only found in L929 cells but not rat C6 astrocytoma cells (Mijatovic *et al.*, 2004). Whether this effect would occur *in vivo* is yet to be confirmed. On the other hand, as the authors mentioned in their report, this anti-NO effects by AE may be beneficial

under certain circumstances (e.g. treatment of NO-resistant tumors or protection of deleterious NO release). Another study done by the same group (Mijatovic *et al.*, 2005b) showed that AE can reduce slightly the cytotoxicity of cisplatin in L929 and C6 cells. The deactivation of ERK kinase by AE was proposed to play a protective role in that reaction. Paradoxically the same authors reported that ERK inhibition was a key process leading to apoptosis in AE-treated C6 cells (Mijatovic *et al.*, 2005a). Since ERK convey both cell death and cell survival signals, AE's role in tumor cell may depend on cell type and treatment.

1.1.4.5 *in vivo* anti-cancer effect

Only a few studies on the *in vivo* anti-neoplastic effect of AE have been reported. Kupchan and his co-workers found that AE exhibited tumor-inhibitory activity in mice against P-388 lymphocytic leukemia (Kupchan, 1976; Kupchan and Karim, 1976). However, the authors stated that this anti-leukemic activity was observed only when AE was administered as a suspension in acetone-Tween 80. In addition, Pecere *et al.* reported that treatment of 50 mg/kg/day AE significantly reduced xenograft neuroectodermal tumor (IMR5) growth without any acute or chronic toxic effects (Pecere *et al.*, 2000). Under the same condition, AE failed to inhibit the growth of human colon carcinoma cells, LoVo109, thus suggesting that the anti-neoplastic effect of AE was highly specific to neuroectodermal tumors cells.

1.1.5 Molecular mechanism of anti-cancer action of AE

There are only a few studies examining the molecular mechanism of anti-cancer action of AE, as compared to its analogue, EM. It is believed that AE might share the

same or similar molecular pathways with EM. This conclusion might be right if we considered only their shared pathway in the induction of p53 and oxidative stress. However, the anti-cancer potency of EM was at least partially attributed to its inhibition on prosurvival protein tyrosine kinases, and a number of other kinases (e.g. CK2, PKC, and PI3K) (Huang *et al.*, 2006a). In fact, AE, by comparison, was found to be a poor kinase inhibitor in many studies. Thus it might be of great interest to examine how AE, a poorer kinase inhibitor, is able to exhibit similar or higher cytotoxicity as EM (Fenig *et al.*, 2004; Lee, 2001; Shi *et al.*, 2001). In the next three subsections, these three aspects: p53 pathway, oxidative stress, and kinase inhibition, will be discussed in detail. In addition, some of the AE-affected molecular mechanisms will be discussed in comparison with EM.

1.1.5.1 Tumor suppressor gene p53

Tumor suppressor gene p53 is an important "gatekeeper" molecule in the process of cancer development (Sherr, 2004). It regulates cancer cell death and cell cycle arrest pathways. Many tumor cells evade apoptosis and cell cycle arrest via mutation of p53. In response to stress stimuli (such as DNA damage), p53 is stabilized and translocated to the nucleus and then transactivates many target genes (e.g. p21, Bax, CD95). In certain cells, activation of p53 leads to apoptosis and activation of another tumor suppressor gene p21, which contributes to cell cycle arrest in G1 phase by inhibiting cyclin-CDK complex. One of the important chemotherapeutic strategies is thus to restore the p53 expression and function.

Cells with wild-type and deficient/mutant p53 have been used to study the function

of p53 in AE-induced anti-cancer effects. Kuo and his colleagues reported that AE can induce apoptosis in both HepG2 (wild-type p53) and Hep3B cells (deficient p53) (Kuo *et al.*, 2002). In HepG2 cells (cytotoxic IC_{50}: 41 μM) induction of apoptosis and G1/S arrest was accompanied with up-regulation of p53 and p21. Although p53-deficient Hep3B cells were more resistant to AE (cytotoxic IC_{50}: 56 μM), the p53-independent transcription of p21 and up-regulation of Bax were suggested to be responsible for AE-induced apoptosis (Kuo *et al.*, 2002). Another report by Pecere *et al*. convincingly revealed that p53 conveyed the sensitivity of neuroblastoma cells to AE (Pecere *et al.*, 2003). In their study, neuroblastoma SJ-N-KP cells (wild-type p53, IC_{50}: 4.2 μM) were seven-fold more sensitive than SK-N-BE(2c) cells (deficient in p53 nuclear transcriptional activity, IC_{50}: 29.1 μM) to AE treatment, although similar amount of drug was uptaken by these two different types of neuroblastoma cells. p53 wild type SJ-N-KP cell underwent p53 transcriptional-dependent apoptosis, while p53 mutant SK-N-BE(2c) cells succumbed to p53 transcription-independent apoptosis via translocation of p53 to mitochondria (Pecere *et al.*, 2003). Taken together, these two studies suggested that p53 is a key protein governing the cell sensitivity to AE. In cells with deficient or mutated p53, other pathways (e.g. p21 and mitochondria-mediated cell death) may be responsible for AE-induced apoptosis, although the precise mechanisms are yet to be determined.

Similarly, EM has been shown to induce the accumulation of p53 in HepG2 cells with the resultant increase in p21 expression and subsequent cell cycle arrest (Shieh *et al.*, 2004). The underlying mechanism on how EM increases p53 level is still under

investigation. One hypothesis suggested this action was attributed to the inhibitory effect of EM on kinase CK2 (Uhle *et al.*, 2003). Deactivation of CK2 by EM may block CK2-directed phosphorylation and subsequent ubiquitin-mediated degradation of p53, and thus lead to p53 accumulation. Unfortunately, AE may not follow the same pathway because AE was found to be a poor inhibitor of kinase CK2 (discussed below).

1.1.5.2 Oxidative stress

Sustained oxidative stresses are maintained in cancer cells (Toyokuni *et al.*, 1995). This high but tolerable production of reactive oxygen species (ROS) may help cancer cells survive and proliferate through activating redox-sensitive transcription factors and responsive genes (e.g. NF-*k*B and AP1). However, when intolerable level of ROS production (e.g. induced by therapeutic agents) reaches a certain threshold, such as irreversible DNA damage, cell may switch to senescence or apoptotic cell death (Buttke and Sandstrom, 1995). Through manipulation of the redox balance, some phytochemicals, such as hydroxyanthraquinones from rhubarb (Huang *et al.*, 2006a), and polyphenols from grapes (Delmas *et al.*, 2003) seem to be good candidates for a direct or combined application in cancer chemotherapeutics and/or chemoprevention.

AE was found to be effective in preventing induced oxidative stress *in vitro*, such as peroxidation of linoleic acid catalyzed by soybean 15-lipoxygenase (Malterud *et al.*, 1993), and oxidative modification of low-density lipoprotein formed with 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (Iizuka *et al.*, 2004). Moreover, it could also inhibit IFN-γ- and IL-1-stimulated NO production and apoptosis (Mijatovic *et al.*, 2004). This inhibitory action on NO production was found to be a consequence of

a suppressed expression of iNOS gene, but not the direct interference of NO or iNOS enzyme activity (Mijatovic *et al.*, 2004). In addition, the *in vivo* hepatoprotective effect of AE against carbon tetrachloride (CCl_4) was also proposed to be correlated with its antioxidant properties, because AE decreased CCl_4-induced lipid peroxidation and liver damage (Arosio *et al.*, 2000).

However, similar to other so-called "antioxidant" hydroxyanthraquinones, AE may also induce oxidative stress in cells. In human lung carcinoma H460 cells, AE induced DNA single strand damage through generation of reactive oxygen species (Lee *et al.*, 2006a). Similarly, its analogue, EM has been reported to induce excessive ROS generation and ROS-dependent cell death (Jing *et al.*, 2002; Su *et al.*, 2005; Yi *et al.*, 2004), although one report suggested that in leukemia HL60 cells, the antioxidant NAC, catalase, and SOD failed to protect EM-induced apoptosis (Chen *et al.*, 2002). Furthermore, it has been found that AE also induced phototoxicity via oxidative stress. Thus AE could efficiently generate single oxygen when irradiated with ultraviolet light and lead to decreased cell survival (Vath *et al.*, 2002). In addition, Vargas *et al*. also showed that AE and other two anthraquinones (EM and rhein) were photolabile when stimulated with visible light under aerobic conditions. Indeed, many other phytochemicals, such as curcumin (Atsumi *et al.*, 2007), were found to exhibit similar phototoxic actions. This photoexcitabe property of AE was suggested to be beneficial as a potential candidate drug in photodynamic therapy (Cardenas *et al.*, 2006), although more research work needs to be conducted.

Collectively, AE may exhibit a dual-role in radical species production and

subsequent oxidative stress. On the one hand, its phenolic structure enables AE to counteract the harmful oxidative injury induced by other strong oxidants; whilst on the other; therapeutic dose of AE can selectively induce oxidative stress in cancer cells. The option to be an antioxidant or oxidant for AE thus depends on the basal cellular oxidant/antioxidant level, the dose of AE, and other crucial factors.

1.1.5.3 Modulation of kinase activity

Compared to EM, AE has been less reported as a kinase inhibitor. In this section, some protein tyrosine kinases and other Ser/Thr kinase pathways (e.g. PKC, MAPK) reported to be affected by AE will be discussed in detail in parallel with EM.

1.1.5.3.1 Direct inhibition of kinase activity

AE is a poor kinase inhibitor when compared with its analogue EM. The inhibitory effect of EM against several prosurvival protein kinases (e.g. protein tyrosine kinase, HER2/neu, CK2, PI3K) was well-established (Battistutta *et al.*, 2000; Frew *et al.*, 1994; Jayasuriya *et al.*, 1992; Zhang *et al.*, 1995; Zhou *et al.*, 2006). On the contrary, AE is less studied with respect to the direct inhibition of kinase activity. The inhibitory IC_{50} of AE against many kinases (e.g. IC_{50} of 28 μM for CK2) was much higher than that of EM (IC_{50} of 2.0 μM for CK2) (Sarno *et al.*, 2002), suggesting that the inhibitory effect of AE on cell proliferation is unlikely to act through the inhibition of certain kinases. Instead, short-term treatment of AE (20 μM) has been reported to increase protein tyrosine phosphorylation in SW480 colon carcinoma cells and VACO235 adenoma cells (Schorkhuber *et al.*, 1998). Moreover, a recent paper revealed that unlike inactive EM, aloe-emodin-8-*O*-β–D-glucopyranoside (IC_{50}: 26.6 μM), together with rhein-8-*O*-β-D-

glucopyranoside and chrysophanol were mild inhibitors against protein tyrosine phosphatase 1B (hPTP1B) *in vitro* (Li *et al.*, 2006), although the precise mechanisms have not been well elucidated. Therefore, AE may be less potent than EM in terms of kinase inhibition.

1.1.5.3.2 Protein kinase C (PKC)

Protein kinase C (PKC) is a family of serine/threonine kinases that have important roles in cell-cycle regulation, apoptosis and malignant transformation (Griner and Kazanietz, 2007). There are almost 11 PKC genes. Among them, PKC α, δ and ε are widely expressed in mammalian cells but the other PKC members are largely cell-type specific. Of particular interest, PKC ε has been shown to be up-regulated, while PKC α and δ are down-regulated in various types of cancers (Griner and Kazanietz, 2007). The effects of distinct PKCs are still unclear and some of them even have opposing effects. For example, active cleavage of PKC δ by caspase-3 has been reported to be important for caspase-3 activation (Emoto *et al.*, 1995) whereas PKC ε is mainly involved in cell survival, chemotherapeutic resistance and invasive metastasis (Cacace *et al.*, 1996; Pan *et al.*, 2006; Tachado *et al.*, 2002).

Similar to EM, AE decreased protein expression of PKC δ and ε in lung carcinoma CH27 (Lee, 2001) and H460 cells (Yeh *et al.*, 2003). Surprisingly, unlike EM, AE treatment increased the total PKC kinase activity instead of decreasing it (Lee, 2001) and PKC inhibitor (forskolin) failed to protect AE-induced apoptosis (Yeh *et al.*, 2003). Moreover, caspase-3 inhibitor could prevent caspase-3-dependent cleavage of PKC δ in EM-treated cells, but not in AE-treated cells (Lee, 2001), suggesting that other

caspase-3-independent mechanisms may be responsible for the decreased expression of PKC δ by AE treatment. The involvement of PKC after AE treatment was determined in glioma cells by another lab (Acevedo-Duncan *et al.*, 2004). Down-regulation of most PKC isozymes (except anti-apoptotic PKC ι), together with inhibition of PKC activity were executed by AE. Therefore, AE may promote apoptosis through modulation of PKC activities, most probably via the down-regulation of PKC δ and ε.

1.1.5.3.3 Mitogen-activated protein kinases (MAPK)

Another Ser/Thr kinases mitogen-activated protein kinases (MAPK) play a central role in regulating cell proliferation, apoptosis and migration (Chen *et al.*, 2001; Reddy *et al.*, 2003). The MAPK members consist of three major classes: the c-jun N-terminal kinases (JNKs), the extracellular signal regulated proteins kinase (ERKs) and p38. They are well-established redox-sensitive pathways (discussed below in Section 2.6.2).

Both AE and EM could disrupt ERK activation/phosphorylation. AE treatment inhibited ERK activation and subsequently induced cell differentiation and cell death in rat C6 glioma cells (Mijatovic *et al*., 2005a). This ERK deactivation by AE was then found to be associated with cell differentiation but not cell death, because treatment of PD98059 (ERK upstream kinase MEK inhibitor) could mimic AE-induced cytotoxicity and differentiation effects but not apoptosis. Moreover, inhibition of ERK by AE was further found to decrease cisplatin-induced cytotoxicity in C6 cells (Mijatovic *et al*., 2005b). Thus, the involvement of ERK pathway on AE's cytotoxicity may depend on cell type and treatment dose, due to the complicated ERK involvement in both apoptotic and survival pathway. On the other hand, several studies revealed that EM may not be a

direct kinase inhibitor of ERK and its inhibition is stimulator-dependent. EM could inhibit EGF-, TNF-α- IGF- or TPA-stimulated ERK activation/phosphorylation (Huang *et al*., 2004; Kwak *et al*., 2006; Lee *et al*., 2006b; Zhou *et al*., 2006), but not PDGF-stimulated ERK activation (Zhou *et al*., 2006).

Compared to ERK, proapoptotic p38 and JNK pathways are less studied in AE-treated cells. It has been reported that exposure of lung carcinoma H460 cells to 40 μM AE resulted in the degradation of p38 (Yeh *et al.*, 2003). Unexpectedly, pretreatment of p38 inhibitor (SB202190) prevented AE-induced p38 degradation and apoptosis, suggesting a proapoptotic role of p38 in AE-induced apoptosis (Yeh *et al.*, 2003). And in this study JNK pathway was unaffected by AE. However, the action by EM on p38 pathway is somewhat dissimilar to AE. EM alone has been found to be incapable of affecting p38 activation (Kaneshiro *et al.*, 2006; Su *et al.*, 2005). But EM may inhibit EGF- or streptozotocin-stimulated p38 activation (Kwak *et al.*, 2006; Wang *et al.*, 2006a). Similarly, EM can inhibit TPA- or TNF-α-stimulated JNK activation (Huang *et al.*, 2004; Lee *et al.*, 2006b), although JNK activation was found to be induced in EM and rhein-treated cells (Lin *et al.*, 2003; Olsen *et al.*, 2007).

1.1.5.4 Inhibition of other non-kinase biomolecules

Biomolecules other than kinases may also be targeted by AE. AE and other anthraquinones may interact with DNA (Pecere *et al.*, 2003). This property prompted researchers to investigate whether AE, like anthracycline drugs, inhibit topoisomerase II activity. It was because these widely-used anti-cancer anthracycline drugs are derived from the anthraquinone structure and capable of inhibiting topoisomerase II catalytic

functions by intercalating themselves into the DNA structure and interacting with topoisomerase II (Froelich-Ammon and Osheroff, 1995). Topo II inhibition may in turn promote DNA cleavage by inhibiting the DNA resealing reaction after double nucleic acid cleavage. However, AE, unlike EM, showed poor inhibition on topoisomerase II activity (IC_{50} of 741 μM for AE vs.7 μM for EM respectively) in a cell-free decatenation assay (Mueller and Stopper, 1999). Furthermore, AE was found to inhibit but not stimulate double-stranded DNA breaks formation (Pecere *et al.*, 2003).

Nuclear factor kappaB (NF-*κ*B) is a ubiquitous transcription factor that conveys inflammatory, anti-apoptotic and prosurvival signals (Hoffmann *et al.*, 2006; Karin, 2006). This transcription factor is constitutively activated in tumor cells and is subject to be further activated upon stress. There are less studies on AE's effect on activation of NF-κB. Mijatovic and colleagues reported that AE could not affect INF-γ and IL-1-induced I*κ*Bα phosphorylation (key factor for NF-κB activation) at any treatment time point in mouse fibrosarcoma L929 and rat astrocytoma C6 cells (Mijatovic *et al*., 2004), although the possibilities that AE might interfere downstream of NF-κB activation can not be excluded in that paper. On the contrary, EM has been shown to inhibit TNF-α- and TPA-stimulated NF-*κ*B activation (Huang *et al.*, 2004; Kumar *et al.*, 1998). The stabilization of inhibitory binding subunit I*κ*Bα by EM may account for this deactivation of NF-*κ*B (Huang *et al.*, 2004; Kumar *et al.*, 1998). Furthermore, EM at high concentration (50-500 μM) could directly inhibit the binding efficiency of NF-*κ*B to DNA through oxidation of NF-*κ*B p50 (Jing *et al*., 2006). It is noteworthy that this inhibitory action by EM depends highly on the stimulator used, since Bhat-Nakshatri *et*

al reported that activation of NF-κB by heregulin through EGFR and Erb3 phosphorylation was unaffected by EM (Bhat-Nakshatri *et al.*, 2002).

Monoamine oxidase is an important metabolic enzyme for a wide range of monoamine neurotransmitters, such as dopamine (Youdim *et al.*, 2006). Screening of plant-derived anthraquinones had been conducted for identification of potential inhibitors for monoamine oxidase in treatment of Parkinson's disease (Kong *et al.*, 2004). Only EM was found to be active as an inhibitor against monoamine oxidase B (IC_{50}: 35.4 μM), while AE, rhein, chrysophanol, physcion were inactive.

Phytoestrogens may counteract or replace estrogen for the binding of estrogen receptor (ER). Synthetic estrogen-replacement therapy has been reported to be effective for the treatment of osteoporosis, hypercholesteremia and symptoms of menopause (Messina *et al.*, 2006). In a search of phytoestrogens from herb root of *Polygonum cuspidatum*, EM was found to exhibit strong affinity to human estrogen receptors (IC_{50} for ER-α: 2.7 μM and IC_{50} for ER-β: 5.2 μM), while AE failed to bind to either ER-α or ER-β even at a higher concentration of 40 μM (Matsuda *et al.*, 2001).

Taken together, previous reports suggested that AE executed its anti-cancer activity through induction of p53 and oxidative stress. However, it is still unclear why AE, as a poor kinase inhibitor, exhibit similar or higher cytotoxicity compared to its analogue EM. In the next Sections, some relevant molecular pathways to be covered in the present study, in terms of oxidative stress and redox-sensitive signaling in apoptosis and cancer, will be discussed.

1.2 Reactive oxygen species (ROS) and protein oxidative modifications

1.2.1 ROS generation and antioxidant defense systems

The major reactive oxygen species (ROS) include superoxide anion (O^{2-}), hydroxyl radical (·OH) and the nonradical hydrogen peroxide (H_2O_2). These redox radicals can react with and oxidatively modify key cellular macromolecules: DNA, proteins and lipids. In terms of immunology, ROS are regarded as host defending radicals generated by neutrophil to attack exogenous pathogens (Pryor, 1982). Recent studies increasingly indicate that ROS are important signaling transduction molecules.

The redox imbalance between the elevated generation of ROS and impaired ability of biological system to neutralize them in favor of the former causes oxidative stress (Benhar *et al*., 2002; Poli *et al*., 2004). The aberrant ROS homeostasis is thus generally thought to be associated with a variety of physiological and pathological processes, including ischemia/reperfusion, cardiovascular diseases, neurodegenerative diseases, ageing and cancer (Boonstra and Post, 2004; Otani, 2004; Poli *et al*., 2004; Stadtman and Berlett, 1998; Toyokuni *et al*., 1995; Ueda *et al*., 2002; Wu, 2006).

In mammalian cells, several enzyme systems are capable of producing intracellular ROS, including the mitochondrial electron transport chain, cytochrome P450 in endoplasmic reticulum, NADPH oxidase complex, xanthine oxidase, cyclooxygenase, lipoxygenase, and peroxisomes (Inoue *et al*., 2003). Among them, mitochondrial oxygen metabolism via electron transport chain is the major source of cellular ROS. Any disruption of the mitochondrial electron transport system can drastically increase superoxide (O^{2-}) production (Fig. 1.3, reaction ①).

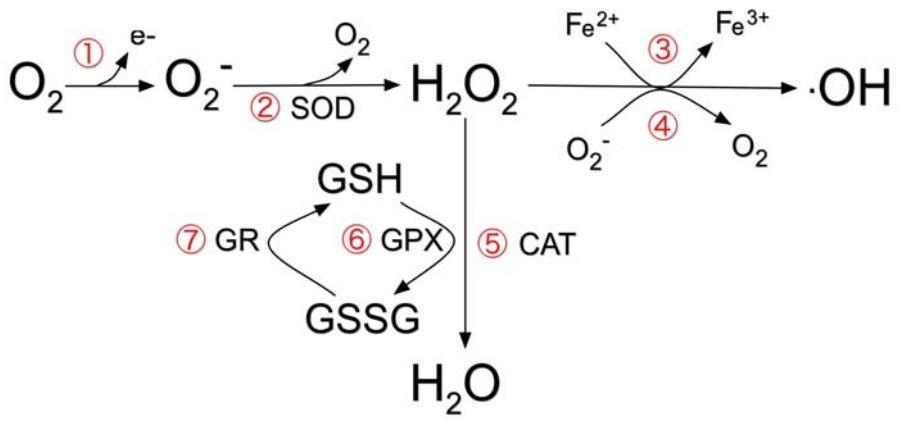


Fig. 1.3 Main cellular pathways of ROS formation from superoxide anion. SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione. (Adapted from Halliwell and Cross. Environ Helath Perspect. 1994; 102 suppl 10: 5-12)

Normally, O^{2-} is rapidly converted to nonradical H_2O_2 and O_2 (Fig. 1.3, reaction ②) by manganese superoxide dismutase (MnSOD) present in mitochondria and copper/zinc superoxide dismutase (Cu/Zn-SOD) in cytosol. Nonradical H_2O_2 can be further detoxified to water by the enzymes catalase (Fig. 1.3, reaction ⑤) and glutathione peroxidase (Fig. 1.3, reaction ⑥). Alternatively, H_2O_2 reacts with reduced transition metal Fe^{2+} or Cu^{+} via Fenton reaction (Fig. 1.3, reaction ③) to form the highly reactive hydroxyl radicals (·OH). In addition, ·OH can also be generated by the metal catalyzed Haber-Weiss reaction (Fig. 1.3, reaction ④). Unlike O^{2-} and H_2O_2, reactive ·OH is unable to be eliminated by an enzymatic reaction. It is probably because of its short half-life, which is too fast to diffuse to the enzyme's active site that ·OH reacts with any oxidizable compound in its close proximity and thus causes oxidative damage to all types of macromolecules.

Under normal conditions, the adverse effects caused by excessive ROS can be minimized by both enzymatic and non-enzymatic antioxidant defenses (Kern and Kehrer, 2005; Loo, 2003; Sun and Oberley, 1996). The enzymes SOD (MnSOD and Cu/Zn-SOD) and catalase belong to the primary defense system used to destroy the radical superoxide and nonradical H_2O_2, respectively. Another major enzymatic antioxidant defense system against H_2O_2 is the glutathione redox cycle. This defense system consists of glutathione (GSH), glutathione peroxidase (GPX) and glutathione reductase (GR), and it resides in both the cytosol and mitochondria. This is different from catalase, which is present only in the peroxisome. Glutathione peroxidase (Fig. 1.3, reaction ⑥), for example, catalyses the reduction of H_2O_2 and other peroxidases by oxidizing cofactor GSH to its oxidized disulfide form GSSG. In contrast, glutathione reductase, with its cofactor NADPH, reverses this reaction by reducing GSSG back to GSH (Fig. 1.3, reaction ⑦).

In addition, cells are also equipped with other non-enzymatic antioxidative proteins (e.g. thioredoxin, glutathione-S-transferase π, peroxiredoxins, etc) and some small-molecule antioxidants (e.g. Vitamin E). These antioxidants reduce excessive ROS mainly by oxidizing themselves. In general, all the redox-based antioxidants are interconnected with each other to form an "antioxidant network" that comprised of both enzymatic and non-enzymatic reactions. In this way, antioxidants can be regenerated and/or recycled by cellular biological reductants. In addition, in response to oxidant insults, cells tend to up-regulate antioxidative enzymes or stress-responsive proteins to counteract the harmful effects caused by excessive ROS production.

1.2.2 Protein oxidative modifications

Oxidative stress often results in reversible or irreversible oxidative modifications of cellular macromolecules, including proteins (as carbonyl derivatives), lipids (as isoprostanes, age pigment) and DNA (as 8-hydroxy-2-deoxyguanosine). Proteins are the major targets for reactive oxidants and other radicals *in vivo* (Davies *et al.*, 1999). It has been estimated that proteins can scavenge up to 50-75% of reactive radicals (Davies *et al.*, 1999). Protein oxidation, as well as lipid peroxidation, is generally more stable than oxidant radicals themselves, since the latter has short half-lives and are highly reactive (Dalle-Donne *et al.*, 2005). Protein oxidation retains the feature of oxidative damage, and is generally regarded as a sensitive and stable biomarker for oxidative stress *in vivo* (Dalle-Donne *et al.*, 2005). More importantly, protein oxidation is frequently found to be associated with induction of redox-sensitive signaling pathways and a variety of pathophysiological processes and diseases, such as cardiovascular diseases, ageing and cancer (Davis and Dean, 1997).

Oxidative stress can reversibly or irreversibly modify sensitive proteins (Fig.1.4) and in turn modulate cellular signaling pathways. This process is termed as redox regulation (Stadtman and Berlett, 1998). Reversible modifications usually occur at cysteinyl residues. These residues may not only protect key cellular macromolecules from intolerable irreversible oxidative damage, but also modulate a number of protein functions. On the contrary, irreversible oxidative modifications, such as protein-protein cross-linking and protein carbonylation, may lead to protein loss-of-function, degradation or accumulation into cytoplasmic inclusions (Baty *et al.*,

2005; Butterfield and Lauderback, 2002; Giasson *et al.*, 2000; Grune *et al.*, 2003; Picklo *et al.*, 2002). In general, protein oxidative modifications can modulate a number of biochemical properties of proteins, such as enzymatic activity (Oliver, 1987; Starke *et al.*, 1987), DNA binding activity of transcription factors (Clive and Greene, 1996; Hainaut and Milner, 1993) and proteolytic degradation rates (Banan *et al.*, 2000; Zaffagnini *et al.*, 2007). Although the association between protein oxidation and ageing has been suggested (Stadtman, 1992), little is known about how oxidative modification of individual protein affects the pathophysiologic processes.

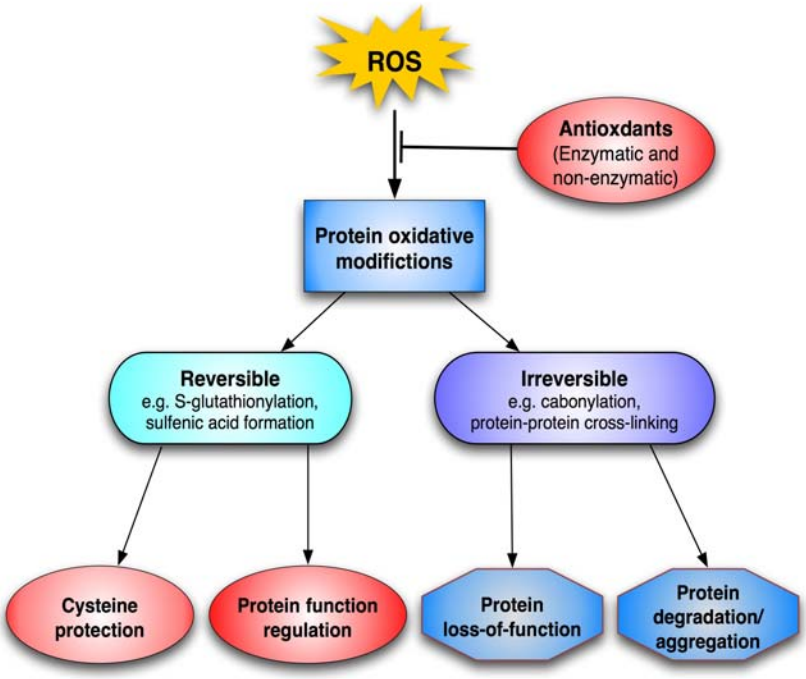


Fig. 1.4 Interplay of ROS and antioxidants results in protein redox regulation. Redox imbalance in favor of excessive ROS generation may cause reversible or irreversible chemical modifications of sensitive proteins. This may in turn lead to protein functional regulation, loss-of-function or degradation. (Adapted from Ghezzi and Bonetto. Proteomics 2004: 3, 1145-1153.)

1.2.2.1 Oxidative modification of protein thiols

Thiols of cysteinyl (Cys) residues in proteins can be oxidized into reversible S-glutathionylation, sulfenic acid (P-SOH), and intra- or inter-molecular disulfides. These chemical modifications may have different pathophysiological properties, depending on the oxidation level, the nature of affected proteins and oxidation sites.

S-glutathionylation refers to the reaction of protein thiols conjugated with low-molecular-weight compounds under mild oxidative stress. In this reaction, GSH is a predominant ligand to be conjugated because of its high concentration (0.5-10 mM) in mammalian cells (Halliwell and Gutteridge, 1999). Generally, S-glutathionylation is regarded as a protective mechanism against further irreversible protein thiol oxidation (Klatt and Lamas, 2000; Schafer and Buettner, 2001). However, if the Cys residue in a protein is functionally critical, S-glutathionylation will render this protein inactive (Eaton *et al.*, 2002). Various systems can reverse this reaction, such as thioredoxin (Trx) and protein disulfide isomerase, using GSH or NADPH as a reducing donor.

Another thiol oxidation product, sulfenic acid, is extremely unstable. This oxidative modification could be easily reduced by intracellular reductants. Alternatively, strong oxidative insults are ready to further oxidize sulfenic acid to sulfinic ($P\text{-}SO_2H$) and sulfonic acid ($P\text{-}SO_3H$). For example, 2-Cys peroxiredoxins could be oxidized further into the form of $P\text{-}SO_2H$ and/or $P\text{-}SO_3H$. This reaction was found to be a molecular switch controlling the transcriptional response to increasing level of hydrogen peroxide (Bozonet *et al.*, 2005; Wood *et al.*, 2003). Unlike P-SOH, formation of $P\text{-}SO_2H$ and $P\text{-}SO_3H$ cause protein loss-of-function and this modification

generally cannot be reversed by metabolic processes. However, an exception has recently been observed that the sulfinic inactive form of peroxiredoxin-1 could be reduced through an unknown mechanism (Woo *et al.*, 2003).

Labeled thiol reactive reagents were applied in the identification of protein thiol modifications. Proteomic approaches utilizing radioactively [35S]-labeled GSH (Fratelli *et al.*, 2002) and non-radioactive biotinylated GSH ester (Eaton *et al.*, 2003) have been developed for the detection of S-glutathionylated proteins. Furthermore, the instability of disulfide formation in these methods using labeled GSH was overcome by a more sophisticated strategy (Lind *et al.*, 2002). In this method, selective reduction and then stable alkylation of glutathionylated proteins with biotin-labeled *N*-ethylmaleimide (NEM) were conducted instead, which could be finally enriched by avidin-based affinity chromatography.

1.2.2.2 Oxidative formation of protein carbonylation

Protein carbonylation is an irreversible oxidative modification. It occurs at many sites of a protein (especially side chains of Pro, Arg, Lys and Thr) and by different mechanisms upon oxidative stress. Introduction of carbonyl groups into protein side-chains generally leads to loss of catalytic or structural functions in the oxidized proteins, which is associated with ageing and neurodegenerative diseases (Levine, 2002). Normal cellular processes may also utilize this modification on specific proteins as a mechanism for protein degradation (Levine, 2002; Nystrom, 2005).

Because the increase in the number of protein carbonyls correlates well with the increased protein damage (Shacter *et al.*, 1994), assessment of protein carbonyl has

been used widely as a marker for oxidative stress (Dalle-Donne *et al.*, 2003; Levine *et al.*, 2000). The pioneering analysis of 2, 4-dinitrophenylhydrazine (DNPH) derivatization developed by Levine and coworkers is the most widely utilized methodology for detection of protein carbonyls (Levine *et al.*, 1990; Levine *et al.*, 1994). Biochemically, protein carbonyls could be derivatized with DNPH, which thus enables them to be recognizable and quantifiable by an immunoassay using commercially-available anti-DNP IgG. Since Reinheckel and coworkers (Reinheckel *et al.*, 2000) introduced post-IEF DNP derivatization in 2DE into the measurement of protein carbonyls, this relative comprehensive and reproducible method greatly facilitated the discovery of new carbonylated proteins in many oxidative stressed (Mostertz and Hecker, 2003; Reinheckel *et al.*, 2000) or diseased samples (Castegna *et al.*, 2002; Choi *et al.*, 2002; Rabek *et al.*, 2003). Furthermore, double staining of 2DE immunoblots (e.g. anti-DNP immunoblot in combination with SYPRO Ruby fluorescent total protein staining) enabled the determination of the oxidation ratio of susceptible proteins (Korolainen *et al.*, 2002).

1.2.2.3 Other oxidative modifications

Similar to thiol groups, methioninyl residues in a protein are highly susceptible to oxidation by redox radicals. Since this modification is enzymatically reversible, methionine oxidation has been proposed to serve as an endogenous antioxidant defensing mechanism (Levine *et al.*, 2000). However, recent studies reveled that methionine oxidation may also be associated with protein loss-of-function, such as DJ-1 and actin (Choi *et al.*, 2006; Dalle-Donne *et al.*, 2002).

1.3 Oxidative stress in apoptosis

1.3.1 Apoptosis

Apoptosis, or apoptotic cell death, is well characterized as a physiologically 'suicidal' programmed cell death. Dys-regulation of apoptosis has been shown in various pathological processes, including neurodegenerative diseases, autoimmunity and cancer. It has also been well-recognized that defects in apoptosis account for not only carcinogenesis, but also resistance to cancer therapeutics (Jacobson *et al.*, 1997; Kerr *et al.*, 1972; Okada and Mak, 2004).

Apoptosis was initially found as a series of distinct morphological changes, including plasma membrane blebbing, nuclear fragmentation and chromatin condensation, degradation of apoptotic cells and formation of membrane-enclosed apoptotic bodies (Jacobson *et al.*, 1997). With the discovery of the key molecules (e.g. caspase, bcl-2, etc.) which govern the apoptotic processes, apoptosis is proven to be well-regulated by a chain of biochemical reactions. Among these changes, cleavage/activation of caspases (a family of cysteine proteases) is central for apoptosis (Alnemri *et al.*, 1996; Riedl and Shi, 2004; Thornberry and Lazebnik, 1998). Caspases are normally kept inactive as precursor procaspases. Upon apoptotic stimulation, the initiator caspases (caspase-8 and -9) are activated by oligomerization to form the active caspases, which subsequently cleave/activate the precursor form of executor caspases (caspase-3 and -7). Activated executor caspases in turn cleave a specific set of key cellular substrates, resulting in the well-defined biological and morphological changes (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998).

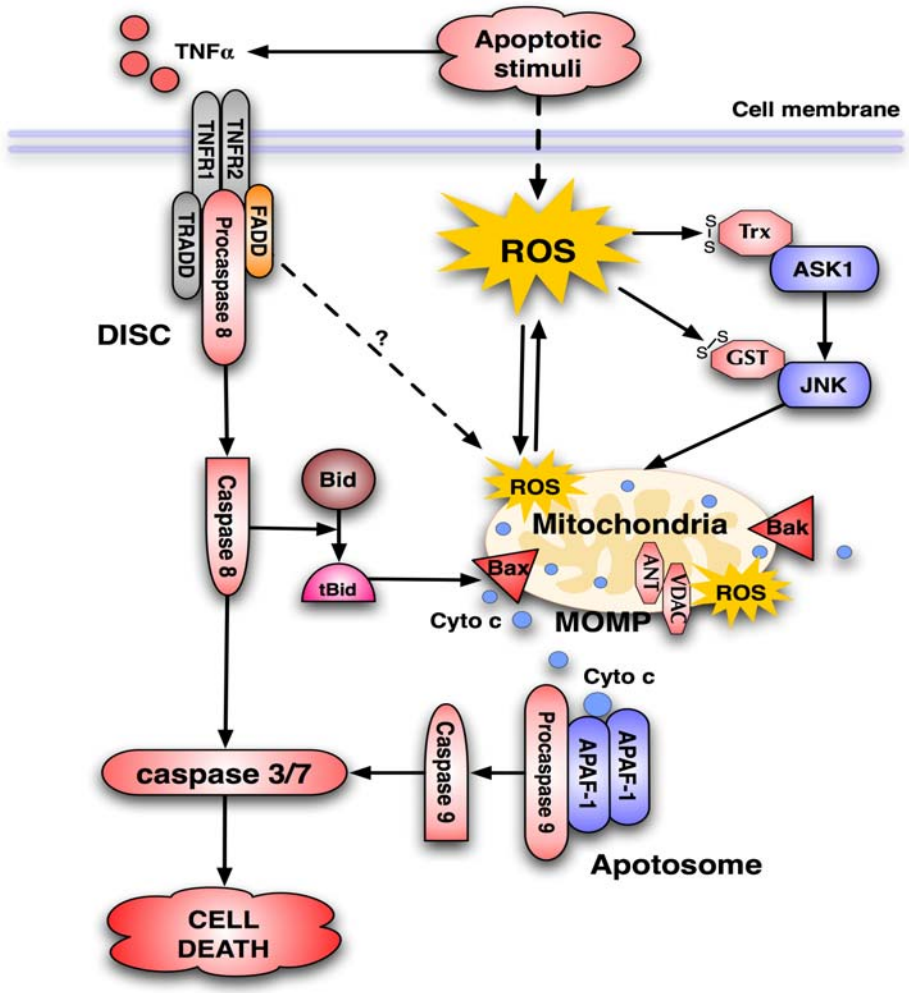


Fig. 1.5 ROS involvement in extrinsic and intrinsic apoptotic pathways. Upon apoptotic stimuli, extrinsic death receptor (TNFR1/2)- and intrinsic mitochondria-mediated apoptosis pathways trigger caspase activation cascade from formation of DISC and apoptosome complex, activation of initiator caspases (caspase 8 and 9) to activation of executor caspase (caspase 3/7). Excessive ROS generation from mitochondria and other sources promoted MOMP and release of sequestered cyt c to form apoptosome. ASK1 and JNK are redox-sensitive molecules to transduce ROS to induce mitochondria-mediated apoptosis. (Adapted from Shen and Liu. Free Radical Bioloby & Medicine. 2006; 40; 928-939.)

There are two apoptotic pathways (extrinsic and intrinsic, see Fig. 1.5) by which caspase activation is triggered (Boatright and Salvesen, 2003). The extrinsic pathway is mediated by signaling cascade of cell surface death receptors (e.g. Fas and TNF receptor) upon stimulatory binding of ligands such as FasL and TNF-α, respectively. The activation of death receptor in turn induces the formation of the death-induced signaling complex (DISC), further recruiting FADD, TRADD and procaspase-8. The recruitment of procaspase-8 on the DISC promotes caspase-8 oligomerization/ activation and apoptotic cascade that follows. On the other hand, the intrinsic pathway is mitochondria-mediated (Garrido *et al.*, 2006; Kroemer and Reed, 2000; Spierings *et al.*, 2005; Youle and Karbowski, 2005). It could be activated by various extracellular and intracellular stresses including oxidative stress, nutrient deprivation, and cytoskeletal disruption. Upon apoptosis stimulation, a series of biochemical changes lead to mitochondrial outer membrane permeabilization (MOMP), a pivotal event in apoptosis. Proteins sequestered in the mitochondrial intermembrane space (between the inner and outer mitochondrial membrane) thus are released and accessible to other proteins in the cytosol. This action is essential for the intrinsic apoptotic pathway, as demonstrated in cell-free system (Liu *et al.*, 1996; Newmeyer *et al.*, 1994).

Proteins that are released to the cytosol in the process of MOMP, comprise cytochrome *c*, Smac/DIABLO, Omi, and other proapoptotic molecules (Kroemer and Reed, 2000; Spierings *et al.*, 2005; Youle and Karbowski, 2005). Among them, cytochrome *c* binds to cytosolic, monomeric apoptotic protease activating factor-1 (APAF-1). This action promotes APAF-1 oligomerization to form a protein complex

called “apoptosome” (Zou *et al.*, 1999). The latter further recruits initiator procaspase-9 and then activates it by dimerization. The active caspase-9 is capable of cleavage/activation of the executor caspase-3 and -7. Similarly, Smac/DIABLO and Omi can bind to cytosolic IAPs (inhibitor of apoptosis proteins). IAPs repressively bind to caspases in normal conditions while released Smac/DIABLO and Omi could sequester IAPs from the inhibitory binding to caspase (Du *et al.*, 2000; Hegde *et al.*, 2002; Martins *et al.*, 2002; Suzuki *et al.*, 2001; Verhagen *et al.*, 2000).

1.3.2 ROS involvement in apoptosis

ROS are involved in both intrinsic and extrinsic apoptosis. On the one hand, ROS may be essential for the extrinsic apoptotic pathway (Shen and Pervaiz, 2006). Ligation of TNF-α or other death signals to the death receptor is capable of inducing ROS production through the mitochondria, while scavenging ROS (by addition of pharmacological antioxidants or genetic over-expression of SOD and Trx) is able to abolish caspase activation and apoptosis that follows (Delhalle *et al.*, 2002; Devadas *et al.*, 2003; Goossens *et al.*, 1999; Kasahara *et al.*, 1997; Lee *et al.*, 2002; Schulze-Osthoff *et al.*, 1993; Wong and Goeddel, 1988). The molecular mechanism linking death receptors to ROS production from the mitochondrial source is yet unclear. A recent study comparing JNK knockout cells with wild-type cells suggested that TNF-α-induced JNK activation may account for the mitochondrial-derived ROS production (Ventura *et al.*, 2004). Furthermore, the generated ROS may promote death receptor-mediated apoptosis through increased expression of death receptors on cell surface (Jung *et al.*, 2005; Kim *et al.*, 2006; Minana *et al.*, 2002).

On the other hand, ROS may directly activate the mitochondria-mediated intrinsic apoptotic pathway (Fruehauf and Meyskens, 2007; Kroemer and Reed, 2000; Youle and Karbowski, 2005). This effect is primarily mediated through opening of the mitochondrial permeability transition pore complex in the process of MOMP. The pore complex consists of an inner membrane segment, adenine nucleotide translocase (ANT), and an outer membrane segment, voltage dependent ion channel (VDAC), as well as cyclophilin D and intermembrane creatine kinase. These two mitochondrial membrane proteins VDAC and ANT were proposed to account for the induction of MOMP and apoptosis that follows. VDAC functions in regulating superoxide flux from the mitochondria to the cytosol, making itself vulnerable to superoxide-mediated MOMP (Han *et al.*, 2003; Shoshan-Barmatz *et al.*, 2006). It has been well recognized that upon oxidative stress, pore-opening proteins (such as Bax and Bak, together with chaperone tBid, and Bim) are up-regulated and target VDAC to destabilize it (Scorrano and Korsmeyer, 2003). In contrast, Bcl-2 and Bcl-X_L exert antiapoptotic effects by stabilizing VDAC configuration (Scorrano and Korsmeyer, 2003). VDAC destabilization in turn promotes ion influx, and ultimately induction of MOMP and the release of proteins normally sequestered in the mitochondrial inter-membrane space. While in terms of ANT, its three cysteinyl residues (Cys 57, 160, and 257) are redox-sensitive and are ready to be oxidized to form intramolecular Cys cross-links upon oxidative stress (Bauer *et al.*, 1999; Costantini *et al.*, 2000; Faustin *et al.*, 2004). ANT with such oxidative modifications alters its structural conformation and becomes incapable of binding nucleotide and allowing calcium entry, which finally induces

MOMP and pore opening. This biochemical chain of MOMP, especially ANT oxidation, could be blocked by mitochondrial antioxidant, such as GSH and Trx (Armstrong and Jones, 2002; Wudarczyk *et al.*, 1996).

It is, however, noteworthy that direct treatment with redox radicals, such as hydrogen peroxide and redox-active quinones, can initiate either apoptosis when given in lower doses or necrosis when given in higher doses (Hampton and Orrenius, 1997). The switch from apoptotic to necrotic cell death in increasing level of oxidative stress has been proposed to be a result of the oxidative deactivation of caspases (such as thiol oxidation (Nobel *et al.*, 1997; Samali *et al.*, 1999) and S-nitrosylation (Melino *et al.*, 1997)) and/or dys-regulated ATP depletion (Leist *et al.*, 1999).

1.4 Oxidative stress in cancer: double-edged sword

Oxidative stress have long been believed to be associated with cancer (Jacobson *et al.*, 1997; Kerr *et al.*, 1972; Okada and Mak, 2004). Current understanding also favors the idea that lowering oxidative stress may have a health benefit from cancer. However, it is still controversial for the clinical usage of antioxidant supplements to prevent and/or treat cancer. Two large, blind, clinical trials of supplementation with beta-carotene (a moderate antioxidant) yielded the same result that beta-carotene supplementation even increased the risk of lung cancer in current and former smokers (Group, 1994; Omenn *et al.*, 1996). Although other tenable explanations may account for that risk, the role of ROS in cancer initiation and/or progression seems more complex than previously expected. Actually a dual role (so called “doubled-edged sword” effect) of oxidative stress in cancer is suggested, according to current findings.

1.4.1 Constitutive oxidative stress in cancer cells

ROS has long been associated with carcinogenesis and malignant transformation. Toyuki and coworkers proposed the concept of "persistent oxidative stress in cancer" over 10 years ago (Toyokuni *et al.*, 1995). This concept was based on the observations of increased generation of ROS in human tumor cells *in vitro* (Szatrowski and Nathan, 1991), formation of DNA oxidative adduct *in vivo* (8-hydroxy-2'- deoxyguanosine (Jaruga *et al.*, 1994; Malins and Haimanot, 1991; Olinski *et al.*, 1992)) but decreased antioxidants expression in tumor tissues (e.g. down-regulation of catalase in hepatoma (Sato *et al.*, 1992)). Considering the growth-stimulatory effect of ROS in growth factor signaling pathways, these observations suggest that the increase in basal level of redox signaling may confer tumor cells higher proliferative potentials than their parental normal cells (Klaunig and Kamendulis, 2004; Loo, 2003). It is, however, difficult to validate the results obtained from these types of comparisons *in vitro* or *in vivo*, because of the lack of genetically comparable normal control cells.

Wallace hypothesized that dysfunctional mutations in either nuclear or mitochondrial genes encoding components of mitochondrial electron transport chain may account for excessive ROS generation, genetic instability and carcinogenesis that follows (Wallace, 2005). Recently, increasing generation of ROS was found to occur in genetically transformed cells (Trachootham *et al.*, 2006). The authors introduced either oncogenic *H-RAS* or *Bcr-Abl* to form tumorigenic cells from an immortalized but tumor-incapable epithelial cell line (T72 cells). Their results indicated a significant elevation of ROS generation in the transformed cells compared to T72 control cells.

These results supported the hypothesis that oxidative stress occur in neoplastic transformation. However, it is still difficult to answer the question whether this high ROS generation is only a response to high-rate metabolism in fast proliferating cells or a causative factor for carcinogenesis. The natural role of increased ROS generation in cells during carcinogenesis is yet to be determined.

1.4.2 Therapeutic or suicidal level of ROS: beyond the breaking point

Contrary to the above model favoring the carcinogenesis-stimulatory effect of ROS, some other models had suggested a therapeutic or suicidal effect could result from increased ROS generation. It is common to observe an exaggerated oxidative stress in cell death triggered by a variety of cancer therapeutic drugs, such as redox cycling agents (e.g. anthracycline (Muller *et al.*, 1998) and arsenic (Kang *et al.*, 2004)), histone deacetylase inhibitors (Rosato *et al.*, 2003), proteosome inhibitors (Perez-Galan *et al.*, 2006). These findings all suggest that cancer cells are susceptible to oxidative stress. Thus it has been proposed that the already elevated level of ROS in cancer cells may make these cells more vulnerable than normal cells, when the induced oxidative stress by therapeutic agents reached an intolerable level (Benhar *et al.*, 2002; Trachootham *et al.*, 2006). Cell death and/or senescent pathways as responses are sensitized in such circumstances. This notion is consistent with the dual effects of H_2O_2 treatment. H_2O_2 may either promote or inhibit growth proliferation in cancer cells, depending on the intracellular concentration of H_2O_2 (Rhee *et al.*, 2003).

According to the above model, modulation of the intracellular ROS level in cancer cells may be effective in controlling cancer proliferation. For example, in the

transformed tumorigenic T72 cells with increased ROS level (as mentioned above), treatment with β-phenylethyl isothiocyanate (PEITC) further increased ROS generation and induced cell death (Trachootham *et al.*, 2006). It was demonstrated that the augmented oxidative stress is not only from the increased ROS generation but also from the impaired detoxifying antioxidant system (depletion of intracellular reduced GSH and inhibition of glutathione peroxidase). By contrast, the normal T72 cells only exhibited a smaller increase of ROS generation and cell death upon PEITC treatment. Therefore, this difference in cell death is possibly a result of the lower baseline ROS level in normal cells, compared to the tumorigenic cells. The depletion of GSH by PEITC presumably had no observable effect for the cellular redox environment in normal cells but potentiated cell death in tumorigenic cells with a high level of ROS.

The cell may itself also be equipped with a suicidal ROS-mediated anti-cancer mechanism even without exogenous treatment. Two recent papers disclosed the enforcing effect of ROS on senescence and cell death. The study by Takahasi *et al* showed that higher level of ROS was maintained in senescent cells and treatment of ROS scavenger could block this hard-to-reverse process (Takahashi *et al.*, 2006). They proposed that in normal cells E2F-dependent transcription switched off senescence and detoxified excessive ROS. But under oxidative stress, this action was inactivated. Excessive ROS thus potentiated PKC-mediated inhibition of cytokinesis, which may account for the induction of senescence. In another paper, Dolado and coworkers found that excessive ROS could inhibit carcinogenesis via a selective sensor p38 MAPK (Dolado *et al.*, 2007). p38 deficient cells formed subcutaneous tumors much

faster than wild–type cells. Importantly these actions could be rescued by reintroducing ectopic expression of p38. In their model, p38 appears to sense oncogene-stimulated oxidative stress and p38 activation protect cells from ROS-induced oncogenic transformation by induction of apoptosis and growth arrest.

Taken together, constitutive oxidative stress is maintained in cancer cells and functions for oncogenic transformation and higher proliferative potentials. But this elevation of ROS may make cancer cells vulnerable to higher level of ROS by inducing cell death and cell growth arrest. Accordingly, addition of any agent that either increase ROS production or impair the intracellular detoxifying antioxidant system may push tumor cells move beyond the breaking point for a suicidal or therapeutic purpose. Due to the complexity of cancer etiology, it is however still unknown whether this "double-edged sword" effect of ROS works in all types of cancers (e.g. chemical or virus-induced carcinoma).

1.5 Cellular signaling by ROS

A wide range of intracellular molecules involved in gene transcription, cell growth arrest, senescence and apoptosis (or necrosis) are susceptible to redox radicals. But unlike other second messengers or small-molecular kinase inhibitors, redox radicals are unlikely to bind specifically to certain proteins, because they are too simple in chemical structure to be recognized. It has been suggested that proteins with low-p*Ka* cysteinyl residues are highly vulnerable to oxidative stress (Rhee *et al.*, 2003). In addition, the extent and duration of oxidative stress, in combination with the level of cellular detoxifying antioxidants influence the cell fate. Thus the effect of

redox radicals on proteins and relevant molecular pathways is highly dependent on the susceptibility of the specific protein (Rhee *et al.*, 2003). In the following sections, a few redox-sensitive pathways (e.g. NF-*κ*B and MAPK) will be discussed.

1.5.1 Nuclear factor-κB (NF-κB)

It has been well-established that nuclear factor-*κ*B (NF-*κ*B) and AP-1 are the most important transcription factors affected by redox radicals. Activation of these two transcription factors can induce genes involved in either cell proliferation or cell death (Bubici *et al.*, 2006; Clive and Greene, 1996; Gloire *et al.*, 2006; Loo, 2003).

As an example, NF-*κ*B is a ubiquitously expressed transcription factor for a variety of genes involved in cell proliferation, differentiation and inflammation (Gloire *et al.*, 2006; Hoffmann *et al.*, 2006; Karin, 2006). An increasing number of evidence indicates the association of NF-*κ*B activation with cancer development and progression (Karin, 2006). A recent mice study revealed that activation of NF-*κ*B was essential for inflammation-initiated cancer development in later stages (Lavon *et al.*, 2003; Pikarsky *et al.*, 2004). It was further proposed that NF-*κ*B executed this action through its negative-feedback regulation of ROS-initiated apoptosis (Bubici *et al*., 2006; Karin, 2006). In addition, NF-*κ*B could reduce ROS through transcriptional regulation of antioxidant proteins (e.g. sod1) (Bubici *et al*., 2006; Gloire *et al*., 2006).

Normally NF-*κ*B is kept in an inactive state in the cytosol by binding to the inhibitor of NF-*κ*B (I*κ*B). In response to various extracellular stimuli, NF-*κ*B gets activated and translocated to the nucleus by IKK (I*κ*B kinase)-dependent phosphorylation and degradation of I*κ*B. Early models for NF-*κ*B activation by ROS

suggested that most stimuli (e.g. TNFα, IL-1, TPA, lipopolysaccharide and H_2O_2) activate NF-κB through generation of ROS and more importantly, pretreatment of ROS scavengers can significantly prevent the induced activation of NF-κB (Baeuerle and Henkel, 1994; Bowie and O'Neill, 2000; Michiels *et al.*, 2002; Schoonbroodt and Piette, 2000). However, these models were challenged by some recent findings that activation of NF-κB is independent of ROS in some cell lines and under some stimulations (Brennan and O'Neill, 1995; Gloire *et al.*, 2006; Li and Karin, 1999).

On the contrary, oxidative stress, above a certain threshold, may negatively affect NF-κB activation through oxidation of NF-κB (Cys62) and interruption of its DNA binding activity (Matthews *et al.*, 1992; Nishi *et al.*, 2002; Pineda-Molina *et al.*, 2001). In addition, inactivation of NF-κB can also be obtained by H_2O_2 treatment through negative oxidative modification of its upstream kinase IKK (Cys179) and subsequent stabilization of IκB (Korn *et al.*, 2001). Therefore, ROS here exhibits a dual action on NF-κB: low level of ROS stimulate NF-κB activation while excessive ROS may directly oxidize NF-κB and interrupt its DNA-binding property.

1.5.2 Mitogen-activated protein kinase (MAPK)

The mitogen-activated protein kinase (MAPK) pathway is one of the most well-established redox-sensitive cell signaling pathways (Benhar *et al.*, 2002). Three MAPKs subgroups have been found: ERK (extracellular signal-regulated kinases), JNKs (c-Jun N-terminal kinases) and p38. Among them, JNK and p38 mainly play a pro-apoptotic function in response to various cellular stress including redox radicals, while ERK subgroup is primarily involved in mitogen-activated proliferative

responses (Kyriakis and Avruch, 2001; Lewis *et al.*, 1998; Shen and Liu, 2006). In the classical model, activation of these three MAPK members is mainly through a series of phosphorylation/activation of the MAPK modules including MAP kinase kinase kinase (MPAKKK), MAP kinase kinase (MAPKK) and MAPK (see Fig. 1.6).

1.5.2.1 c-Jun N-terminal kinases (JNK)

It has been proposed that the reciprocal, negative cross-talk between pro-survival NF-κB and pro-apoptotic JNK is critical for cell decision in entering either survival or apoptosis (Bubici *et al.*, 2006). In this model, ROS is regarded as a central player in determining the activity of JNK and NF-κB. Also named as stress-activated protein kinases (SAPK), JNK are activated by various environmental stresses including oxidative stress, chemotherapeutic agents and cytokines. JNK are primarily activated through phosphorylation at sites Thr 183 and 185 by two upstream MAPKK (JNNK1/MKK4/SEK1 and JNKK2/MKK7). Activated JNK regulate various cellular processes comprising cell proliferation, apoptosis, and differentiation.

Two different modes of JNK activation (transient pro-survival and sustained pro-apoptotic) have now been well recognized (Shen and Liu, 2006; Shen and Pervaiz, 2006). Signaling cascades of pro-inflammatory cytokines (such as TNF-α and IL-1) are responsible for the earlier and transient mode of JNK activation. Under non-stressed circumstances, transient JNK activation mediates pro-survival responses. This effect was clearly demonstrated in some transgenic and biochemical studies (Behrens *et al.*, 2000; Chen *et al.*, 2001; Lamb *et al.*, 2003). In contrast, excessive ROS may account for the delayed and sustained mode of JNK activation. Sustained

JNK activation leads to stress-induced apoptotic cell death (Tournier *et al.*, 2000). Thus the pro-apoptotic effect of JNK depends on the extent and duration of its activation by exogenous stress.

Oxidative stress is of particular importance in modulation of sustained JNK activation and JNK-mediated apoptosis (Bubici *et al.*, 2006; Shen and Liu, 2006; Torres, 2003). The main activator of JNK pathway has been proposed to be apoptosis signal-regulating kinase-1 (ASK1), another redox-sensitive pro-apoptotic kinase (Saitoh *et al.*, 1998; Shen and Liu, 2006; Tobiume *et al.*, 2002). ASK1 is a ubiquitously expressed MAPKKK that activate both JNK and p38 by phosphorylating respective MAPKKs. In the presence of ROS, ASK1 was activated by oligomerization after dissociation from its inhibitory binding subunit thioredoxin (Trx) (Liu *et al.*, 2000; Saitoh *et al.*, 1998; Tobiume *et al.*, 2002). Trx functions as an antioxidant protein, but when it is exposed to excessive redox radicals, it can be oxidized and deactivated on residues Cys32 and Cys35 and form a intramolecular disulfide bond (Liu and Min, 2002; Saitoh *et al.*, 1998). This oxidation may release ASK1 and then activate it. It is noteworthy that several other redox-sensitive molecules (GST-μ, glutaredoxin and heat shock protein 72) could activate ASK1 in a similar mode (Cho *et al.*, 2001; Park *et al.*, 2002; Song *et al.*, 2002).

Furthermore, JNK itself was also regulated by inhibitory binding with another redox-sensitive protein glutathione S-transferase π (GST-π) (Adler *et al.*, 1999). Upon oxidative stress, GST-π was oxidized and underwent oligomerization, thus losing its inhibitory binding with JNK. Importantly, this module of GST-π oxidation-involved

modulation of JNK is independent of the ASK1/JNK module mentioned above. Thus, ROS are capable of activating JNK through multiple redox-sensitive pathways.

The precise mechanism for JNK-mediated apoptosis has yet to be determined, although current understanding favors the role of mitochondria as its main target. Upon apoptotic stress, activated cytosol JNK may directly translocate to mitochondria and cause the release of sequestered apoptogenic factors such as cytochrome *c* from mitochondrial intermembrane space (Aoki *et al.*, 2002; Kharbanda *et al.*, 2000). JNK also executes its pro-apoptotic role through its modulation of the Bcl-2 family members. This action may be a result of JNK-dependent negative phosphorylation of anti-apoptotic Bcl-2 (Yamamoto *et al.*, 1999) and myeloid cell leukemia 1 (Inoshita *et al.*, 2002). JNK may also activate pro-apoptotic Bim by phosphorylation (Putcha *et al.*, 2003) and promote Bax mitochondrial translocation (Tsuruta *et al.*, 2004).

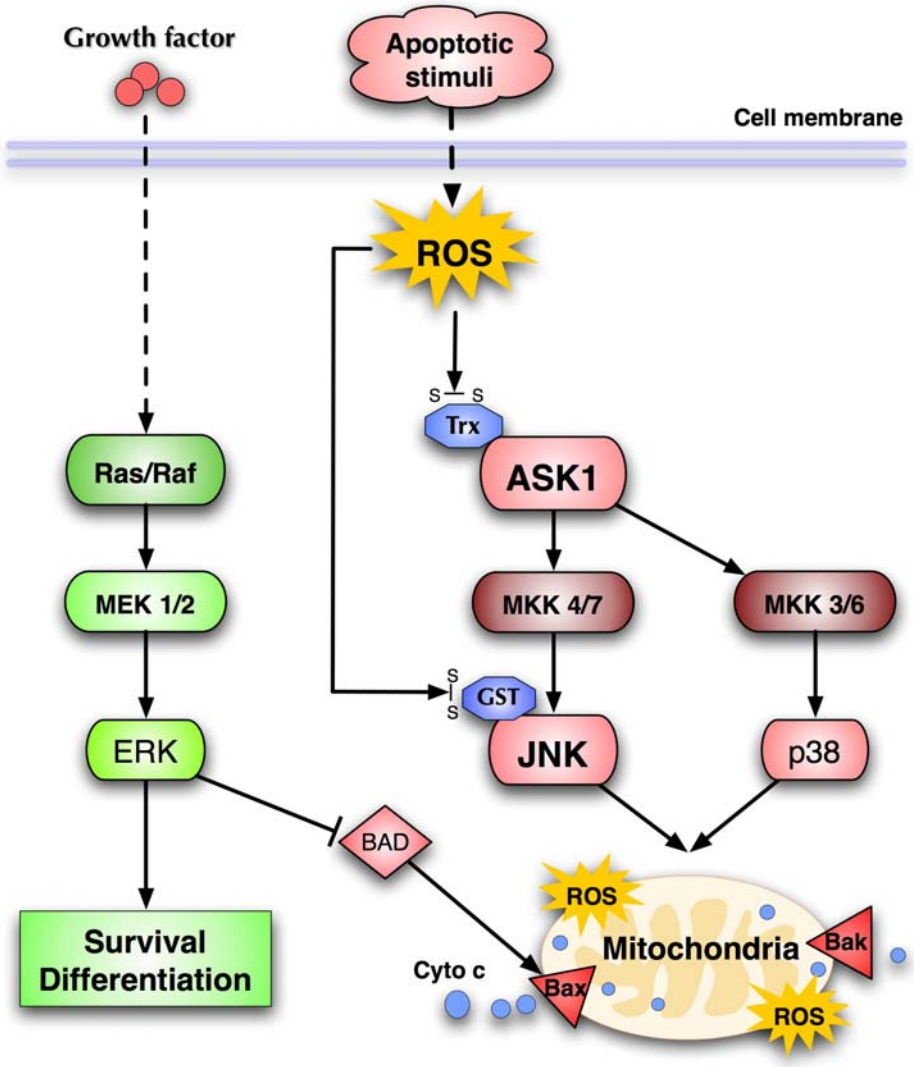
1.5.2.2 p38

Similar to JNK, p38 (also known as SAPK2) is another redox-sensitive MAPK member (Otani, 2004; Torres, 2003; Ueda *et al.*, 2002). Although p38 has different upstream MAPKKs (MKK3 and MKK6) from JNK, it shares some similar upstream and downstream pathways in terms of ROS-mediated apoptosis. For example, the signaling pathway of ROS-mediated JNK activation through ASK1 also applies to p38 (Ichijo *et al.*, 1997). Beside its primary function in inflammatory diseases, p38 also works as a tumor suppressor. For example, Dolado and coworkers had reported that p38 MAPK was a selective sensor for oncogene-stimulated ROS and it could protect cells from oncogenic transformation by induction of apoptosis and growth arrest

(Dolado *et al.*, 2007). Under oxidative stress, p38 and JNK may exhibit redundant functions in apoptosis. However their cross-talk *in vivo* is yet to be determined.

1.5.2.3 Extracellular signal-regulated kinases (ERK)

Different from JNK and p38, extracellular signal-regulated kinases (ERK) is a pro-survival MAPK member (Reddy *et al.*, 2003; Torres, 2003). Activation of ERK is generally associated with the regulation of meiosis, mitosis in differentiated cells, in response to a variety of stimuli, including growth factors, cytokines, virus infection and carcinogens. Furthermore, oncogenic Ras activate ERK pathways, which accounts for the increased proliferative potential of tumor cells. Activation of ERK by oxidant radicals has been reported in a number of studies (Dong *et al.*, 2004; Gupta *et al.*, 1999; Guyton *et al.*, 1996; Tournier *et al.*, 1997). However, the mechanism for ERK activation by ROS is still unclear. It was proposed that stimulation of growth factor receptor through oxidative deactivation of phosphatase may account for ERK activation (Knebel *et al.*, 1996). Pharmacological deactivation of ERK usually sensitizes cells to ROS-induced cell death, suggesting the anti-apoptotic role of ERK (Ikeyama *et al.*, 2002; Mijatovic *et al.*, 2005a; Torres, 2003). This action may be due to the fact that activation of ERK contributes to phosphorylation and inactivation of pro-apoptotic BAD (Hayakawa *et al.*, 2000; Scheid *et al.*, 1999). However, contradictory results have been reported that ERK could also promote apoptosis depending on the cell type, the stimulus, and the duration of ERK activation (Ramachandiran *et al.*, 2002; Tikoo *et al.*, 2001; Zhuang and Schnellmann, 2006).



1.5.3 Fig. 1.6 ROS involvement in MAPK pathways. Upon apoptotic stress, increased generation of ROS potentiated cells for apoptotic cell death through signaling cascade of ASK1-JNK and ASK1-p38 activation. Oxidation and dea**ctivation of thioredoxin (Trx) and GST-π (GST) release ASK1 and JNK from their inhibitory binding, respectively. Sustained activation of JNK and p38 may in turn result in mitochondria-involved apoptosis. In contrast, ERK generally accounts for cell survival and differentiation.** (Adapted from Shen and Liu. Free Radical Biology & Medicine. 2006; 40; 928-939.)

Other redox-sensitive signaling pathways

Besides the above redox-sensitive pathways, redox radicals could increase protein tyrosine phosphorylation by negatively oxidizing protein phosphatase. Active Cys215 of protein tyrosine phosphatases (PTP) has been found to be reversibly oxidized to Cys-SO_2H after stimulation of epidermal growth factor (EGF) (Lee *et al.*, 1998). The requirement of PTP oxidation in EGF-, PDGF- or insulin-induced protein tyrosine phosphorylation indicated the important role of oxidative modification and inactivation of PTP (Lee *et al.*, 1998; Mahadev *et al.*, 2001; Meng *et al.*, 2002).

In conclusion, ROS are important signal transduction molecules involved in cell proliferation, senescence and apoptosis (necrosis). ROS mediate these actions through reversible or irreversible oxidative modification of key cellular macromolecules. Proteins, as the major targets of radical oxidants, are sensitive markers for oxidative stress and signaling mediators for the redox-sensitive pathways. Upon oxidative stress, low ROS generation may potentiate cell proliferation and malignant transformation. However, when ROS reaches intolerable level, pro-apoptotic pathways, such as the well-established redox-sensitive ASK1-JNK pathways, are ready to be activated. Thus, ROS appear to be a "double-edged sword" on cancer cells, although the precise mechanisms are yet to be determined. If this hypothesis works under *in vivo* conditions in human, therapies could be developed accordingly through delicate redox modulation: either to keep a relative reducing environment or to strengthen ROS generation beyond the tolerable limit to induce apoptotic cell death in vulnerable cancer cells.

1.6 Objectives of the study

Presently, the exact anti-cancer molecular mechanism for AE is still unknown. Although previous investigations focusing on a single pathway disclosed some of the anti-cancer molecular mechanisms of AE, no comprehensive and large-scale studies have ever been carried out before. Therefore, we proposed to perform a system-wide investigation using a high-throughput proteomics approach followed by biochemical validation to identify the functional targets of AE, which are responsible for its anti-cancer properties.

Thus the main objectives of this study are

1. to comprehensively identify the anti-cancer protein targets of AE by proteomic approaches *in vitro*;
2. to characterize and validate the affected targets and involved molecular pathways by conventional biochemical and cellular approaches;

The integration of the proteomic and biochemical approaches may help to elucidate the relevant molecular pathways affected by AE treatment in hepatoma cells. These results will thus lead to a better understanding of the multi-functional effects of AE, such as apoptosis, cell cycle arrest, hepatoprotection, anti-metastatic effect, etc. In addition, comparison of the affected pathways between AE and EM may help to reveal why these two chemically similar hydroxyanthraquinones behave so differently biochemically. This comparison might also be useful to understand the combined effect of these two hydroxyanthraquinones in the usage of Rhuarb in TCM.

CHAPTER 2

ALOE-EMODIN INHIBITED TUMOR CELL GROWTH BY AFFECTING MULTIPLE PROTEINS

2.1 Introduction

Aloe-emodin (AE) and its analogue emodin (EM) are two main hydroxyanthraquinones in Rhubarb root (Huang *et al.*, 2006a). Several earlier studies suggested that both AE and EM could inhibit cancer cell growth directly or synergistically with other anti-cancer agents (Kuo *et al.*, 2002; Pecere *et al.*, 2000; Shieh *et al.*, 2004; Yi *et al.*, 2004; Zhang *et al.*, 1995). The anti-cancer potency of EM was at least partially attributed to its inhibition on protein tyrosine kinases, and a number of other kinases (e.g. CK2, PKC, and PI3K) (see Chapter 1). *In vitro* evidence also revealed that AE could suppress cell proliferation through induction of cell cycle arrest and apoptosis in several cancer cell lines, including human hepatoma (Kuo *et al.*, 2002), glioma(Mijatovic *et al.*, 2005a), leukemia (Chen *et al.*, 2004a) and lung carcinoma cells (Lee *et al.*, 2005), although the mechanisms are yet unclear. In general, the anti-cancer effect of AE, as compared to EM, is less well studied.

Recently, both AE and EM have received much attention because of their potent hepatoprotective (Arosio *et al.*, 2000; Lin *et al.*, 1996) and anti-hepatoma effects (Kuo *et al.*, 2002; Shieh *et al.*, 2004). In Traditional Chinese Medicine, rhubarb extract had been used to treat hepatitis, one of the main causes of hepatoma (Feng *et al.*, 1994). Both AE and EM were also capable of protecting liver from injury induced by carbon tetrachloride or D-galactosamine (Arosio *et al.*, 2000; Lin *et al.*, 1996). Furthermore, AE had been reported to inhibit stellate cell transformation, which was known to play a key role in the pathogenesis of hepatic fibrosis (Woo *et al.*, 2002). Taken together, these findings revealed the hepatoprotective capability of AE.

To uncover the anti-cancer mechanism of AE, a proteomic approach (2D-difference gel electrophoresis (2D-DIGE) coupled with MALDI TOF/TOF MS/MS) was adopted to obtain a global view of the differences in protein expression levels and/or post-translational modifications in the human hepatoma celll lines, HepG2, after AE treatment. This strategy, together with cellular and biomolecular studies may reveal the main protein targets of AE.

2.2 Material and methods

2.2.1 Chemicals and reagents

AE, EM and other chemicals were purchased from Sigma-Aldrich (St Louis, MO). Cy3, Cy5 and Cy2 N-hydroxysuccinamide ester DIGE dyes, 4-7 linear IPG strips and buffer were from GE Healthcare (Bucks, UK). z-VAD-FMK and Ac-DEVD-AFC were obtained from Calbiochem (San Diego, CA).

2.2.2 Cell lines and cell culture

The human hepatoma cell lines, HepG2 and HCC-M cells were cultured as described previously (Liang *et al.*, 2002; Neo *et al.*, 2005). Briefly, cells were grown in the DMEM medium (with 100 U/ml penicillin, 100 μg/ml streptomycin, 3.7 g/L sodium bicarbonate, pH 7.4) plus 10% FBS in standard incubator conditions (37 $^{\circ}$C, 5% CO_2). Similarly, three immortal non-tumorous normal cell lines, including the opossum kidney proximal tubule OK cell line, human keratinocyte HACAT cell line and human airway epithelial BEAS-2B cell line were cultured under the same condition.

2.2.3 Cell viability determination by trypan blue exclusion

The number of total cells and number of dead cells (with damaged cell membrane) were counted by the trypan blue exclusion assay. In brief, after the indicated treatment, cells were collected by trypsinization, re-suspended in PBS and then incubated with 0.2 % (w/v) trypan blue (in Hank's balanced salt solution) for 5 min. The number of total cells and number of stained cells were counted using a hemacytometer under an inverted light microscope.

The number of cells and percentage of stained cells were calculated as follows:

Number of cells per ml = cell number per quadrant * Dilution factor * 10000.

Percent dead cells = (Number of stained cells / Total number cells) *100.

2.2.4 Cell viability determination by MTT assay

Cell proliferation was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). In brief, after the indicated treatment, cells were re-incubated in fresh medium plus 25 μl MTT solution (5 mg/ml in PBS) for 1.5 hr in 37 $^{\circ}$C, and lysed in 100 μl lysis buffer (50% N,N-dimethyl formamide, 10% (w/v) SDS, 1% acetic acid, pH 4.6) for another 0.5 hr. The MTT OD was measured at 590 nm using a TECAN SpectraFluor Plus® fluorescence and absorbance microplate reader (Tecan Austria GmbH, Austria).

2.2.5 DNA content determination by flow cytometry

The DNA content was determined by flow cytometry (Yang *et al.*, 2000). Briefly, after the indicated treatment, trypsinized cells were re-suspended, fixed in pre-chilled 70% ethanol for at least 2 hr, and incubated with 20 μg/ml propidium iodide (with 0.2

mg/ml RNAse A and 0.1% triton X-100 in PBS) for 15 min. Ten thousand cells per sample were counted with a flow cytometer (BD Pharmingen, San Diego, CA) and the DNA content was analyzed by WinMDI 2.8 software.

2.2.6 Apoptotic cell death determination by DAPI staining

Nuclear condensation/fragmentation as a morphological marker for apoptosis was determined by DAPI (4, 6-diamidino-2-phenylindole) staining (Shi *et al.*, 2005). In brief, after the indicated treatment, cells were fixed with ice-chilled 70% ethanol for 30 min before staining with DAPI (300 nM in PBS) and visualized under an inverted fluorescence microscope. Cells with clear condensed nuclei were considered as apoptotic cells. Ten randomly selected fields with more than 200 cells were examined. The percentage of apoptotic cells to total cells was presented as means ± SD.

2.2.7 Caspase 3/7 activity assay

Caspase 3/7 activity was measured as described previously (Wenzel *et al.*, 2004). Briefly, after the indicated treatment, the cytosolic supernatants were extracted in 0.5% NP-40, 20 mM HEPES pH 7.4, 84 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM PMSF and 1X protease inhibitor cocktail (Roche Applied Science**,** Indianapolis, IN). 20 µl of cell lysate was then incubated with fluorogenic caspase 3/7 tetrapeptide-substrate Ac-DEVD-AFC (50 µM) in the activity buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 mM DTT). Cleavage of the caspase 3/7 substrate was determined at an emission wavelength of 510 nm after excitation at 410 nm using a TECAN SpectraFluor Plus® fluorescence and absorbance microplate reader (Tecan Austria GmbH, Austria).

2.2.8 Protein preparation for 2D-DIGE

After the designated treatment, both adherent and floating HepG2 cells were collected and lysed in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 25 mM NaF, 1 mM Na_3VO_4, 1X HALT protease inhibitor (Pierce, Rockford, IL, USA), 50 μg/ml DNAse I and 50 μg/ml RNAse A). The protein concentration was estimated using the Coomassie® Plus Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

2.2.9 2D-DIGE

To ensure reproducibility, three different batches of control, AE- and EM-treated cells were used (Table 2.1). Duplicate experiments were also performed for each sample. Protein labeling for 2D-DIGE was conducted according to the Ettan DIGE user manual (GE Healthcare, Bucks, UK). The control, treated and pooled standard (which contained all control and treated samples in equal portions) samples were labeled with 200 *p*mol Cy3, Cy5 and Cy2 respectively. In each 2D-DIGE gel, 75 μg of protein sample mixtures, including the Cy3-labeled control, Cy5-labeled treated and Cy2-labeled pooled standard samples (each in 25 μg), were prepared for loading.

The first dimensional electrophoresis step, isoelectric focusing (IEF), was conducted as described previously (Neo *et al*., 2005) with minor modifications, on precast 18 cm pH 4-7 linear Immobilized pH gradient (IPG) strips using the IPGphor IEF unit (GE Healthcare, Bucks, UK),. Briefly, the pre-labeled samples were loaded using the cup-loading method near the anodic end of the strips in the universal strip holders. IEF was then carried out as follows: (i) 200 V, 200 Vhr; (ii) 500 V, 250 Vhr; (iii) 1000 V, 500 Vhr; (iv) 1000 V – 8000 V, 2250 Vhr and (v) 8000 V, 36000 Vhr.

Table 2.1 Experimental plan for 2D-DIGE. Altogether, three different batches of samples (each in duplicate) were analyzed by 2D-DIGE. C1, C2, and C3 were control samples treated with DMSO for 24 hr; AE1, AE2 and AE3 were samples treated with 40 μM AE 24 hr; EM1, EM2 and EM3 were samples treated with 40 μM EM for 24 hr. Pooled standards were combinations of all samples and then labeled with Cy2.

Batch No.	Gel No.	Cy2-standard	Cy3-Control	Cy5-Treated
1	1, 2	pooled standards	C1	AE1
1	3, 4	pooled standards	C1	EM1
2	5, 6	pooled standards	C2	AE2
2	7, 8	pooled standards	C2	EM2
3	9, 10	pooled standards	C3	AE3
3	11, 12	pooled standards	C3	EM3

After reduction with 1% DTT and alkylation with 2.5% iodoacetamide of the IEF strips, the second dimensional separation (SDS-PAGE) was conducted using 1 mm thick, 12.5%T polyacrylamide gel in a Protean II XL electrophoresis cell (Bio-Rad, Hercules, CA). Twelve gels were run together in dark at a setting of 30 mA per gel.

2.2.10 Silver staining

Vorum Silver Staining was conducted as described previously (Liang *et al.*, 2002; Neo *et al.*, 2005). In brief, after fixation in 120 ml of fixing buffer (50% methanol, 12 % acetic acid, 0.05 % formalin) overnight, the SDS-PAGE gels were subsequently washed in 120 ml of 35% ethanol for 20 min thrice, sensitized in sensitization buffer (0.2% silver nitrate 0.076% formalin) for 20 min, developed in developing buffer (6%

sodium carbonate, 0.004% sodium thiosulphate, 0.05% formalin) till the silver spots appear. And finally the reaction was stopped in 1.46% sodium EDTA for 20 min.

2.2.11 Protein visualization

Gel Images of the separated proteins were obtained using the Molecular Dynamics Typhoon 9400 variable mode imager (GE Healthcare, Bucks, UK) with optimal excitation/emission wavelength for each DIGE fluor (Cy2 488/520 nm; Cy3 532/580 nm; Cy5 633/670 nm, respectively) and viewed with the ImageQuant software (GE Healthcare, Bucks, UK). After image acquisition, gels were re-stained by silver staining and scanned on an UMAX ImageScanner (Fremont, CA, USA).

2.2.12 Image analyses and quantitation

The DIGE images were analyzed using the DeCyder V 5.02 software (GE Healthcare, Bucks, UK) according to the Ettan DIGE User Manual. In brief, the DIGE images underwent spot detection and quantification in the differential in-gel analyses module, with an average of 1600 spots per gel being detected. Next, the matched images were analyzed in the biological variation analyses module, which provided statistical data of the differentially-expressed spots. Statistically significance ($p < 0.01$, Student's t-test) and consistent presence in at least 5 out of 6 gels were two criteria for the acceptance of the differentially-expressed protein spots. The selected spots were filtered based on an average expression level change of at least two-fold.

2.2.13 In-gel tryptic digestion

After protein spots were excised manually from silver stained gels, in-gel tryptic digestion procedure was conducted (Neo *et al.*, 2005). In brief, each gel plug was

washed with H_2O, incubated with 150 µl washing buffer [2.5 mM ammonium bicarbonate, 50% v/v acetonitrile (ACN)] at 4 °C overnight, reduced with 10 mM DTT at 37 °C for 1 hr, alkylated with 55 mM iodoacetamide in the dark at room temperature for 45 min, and finally digested in 10 µl trypsin solution (20 µg/ml trypsin in 20 mM ammonium bicarbonate) at 37 °C for at least 16 hr. Peptide mixture were then extracted with 10 µl of extraction buffer [50% v/v ACN, 0.1% trifluoroacetic acid (TFA)] and finally concentrated by Speedvac to about 1-2 µl.

2.2.14 Mass spectrometry and database searching

Peptide mixtures were analyzed by MALDI-TOF/TOF in an Applied Biosystems 4700 Proteomics Analyzer (Foster City, CA), after the trypsin digest were crystallized with alpha-cyano-4-hydroxy cinnamic acid matrix solution (5 mg/ml, with 50% ACN, 0.1% TFA) and spotted onto a MALDI target (192-well) plate. The MS results were automatically acquired with a trypsin autodigest exclusion list and the five most intense ions selected for MS/MS analysis. The collision gas was atmospheric air and the energy was 1 kV. Interpretation was carried out using the GPS Explorer software (Applied Biosystems, Foster City, CA) and database searching using the in-house MASCOT program (Matrix Science, London, UK). Both combined MS/MS/MS and MS/MS searches were conducted with the following settings: MSDB database (http://csc-fserve.hh.med.ic.ac.uk/msdb.htm), all entries, peptide tolerance at 200 ppm, MS/MS tolerance at 0.3 Da, carbamidomethylation of cysteine (fixed modification), phosphorylation at serine, threonine, and tyrosine (variable modifications), methionine oxidation (variable modifications) and N-terminus acetylation (variable modification).

2.3 Results

2.3.1 AE, but not EM induced specific cytotoxicity in hepatoma cells

As shown in Fig. 2.1A and 2.1C by the MTT assay, AE treatment dose- and time-dependently inhibited the cell proliferation in HepG2 cells. Similar results were obtained in another hepatoma HCC-M cells (Fig. 2.1B and 2.1D); suggesting that such an effect was not cell-type specific. These results were consistent with the other reported findings that AE was cytotoxic to hepatoma cells (Kuo *et al.*, 2002; Shieh *et al.*, 2004). In contrast, its analogue EM was less cytotoxic. The IC_{50} of EM treatment for both HepG2 and HCC-M cells was more than 40 μM (Fig. 2.1A and 2.1B), which was twice that for AE (20 μM). This higher cytotoxicity of AE versus EM was found to be similar in other cancer cells, e.g. lung carcinoma cells (Lee, 2001) and Merkel carcinoma cells (Fenig *et al.*, 2004).

To evaluate whether AE could specifically inhibit hepatoma cells against other non-tumorous normal cells, trypan blue exclusion assay was carried out after treatment of AE in several immortal normal cells, including HepG2 cells. As shown in Fig. 2.2A, AE dose-dependently decreased the total HepG2 cell number but increased the percentage of dead cells (these cells failed to exclude trypan blue due to cell membrane damage). On the contrary, higher concentration (up to 100 μM) of AE treatment did not induce evident cytotoxicity in BEAS-2B (human airway epithelial cell line), OK (opossum kidney proximal tubule cell line), and HACAT (human keratinocyte cell line) cells. These findings suggested that AE treatment inhibited hepatoma cell growth specifically.

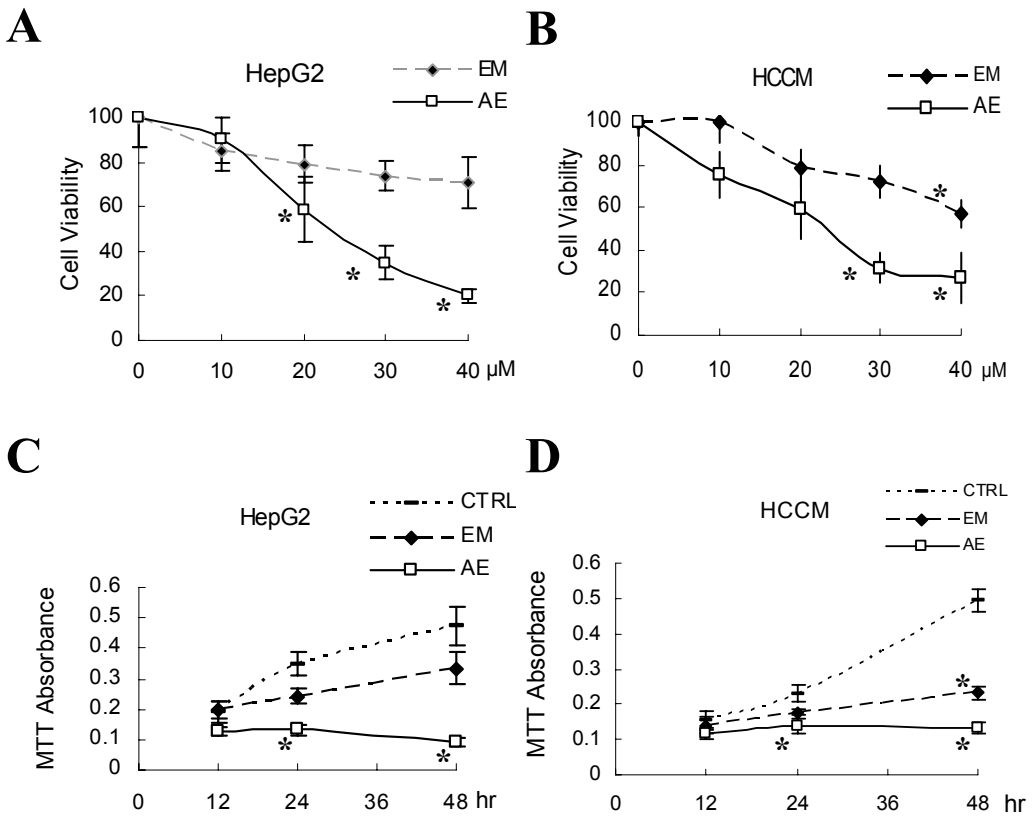


Fig. 2.1 AE induced higher cytotoxicity than EM. The HepG2 and Hcc-M cell viability was measured by MTT assay in the absence (control) or presence of AE or EM as indicated. The control cells were treated with same volume of DMSO vector (<0.2%). (A, B). HepG2 and HCC-M cells were treated with increasing doses of AE or EM for 48 hr. The percentage of viable cell compared with the control cells was presented as means ± SD. (C, D). HepG2 and HCC-M cells were treated with 40 μM AE or EM for the indicated times from 12 to 48 hr. The MTT absorbance from each group was presented as means ± SD.

* $p<0.01$ when compared to control group.

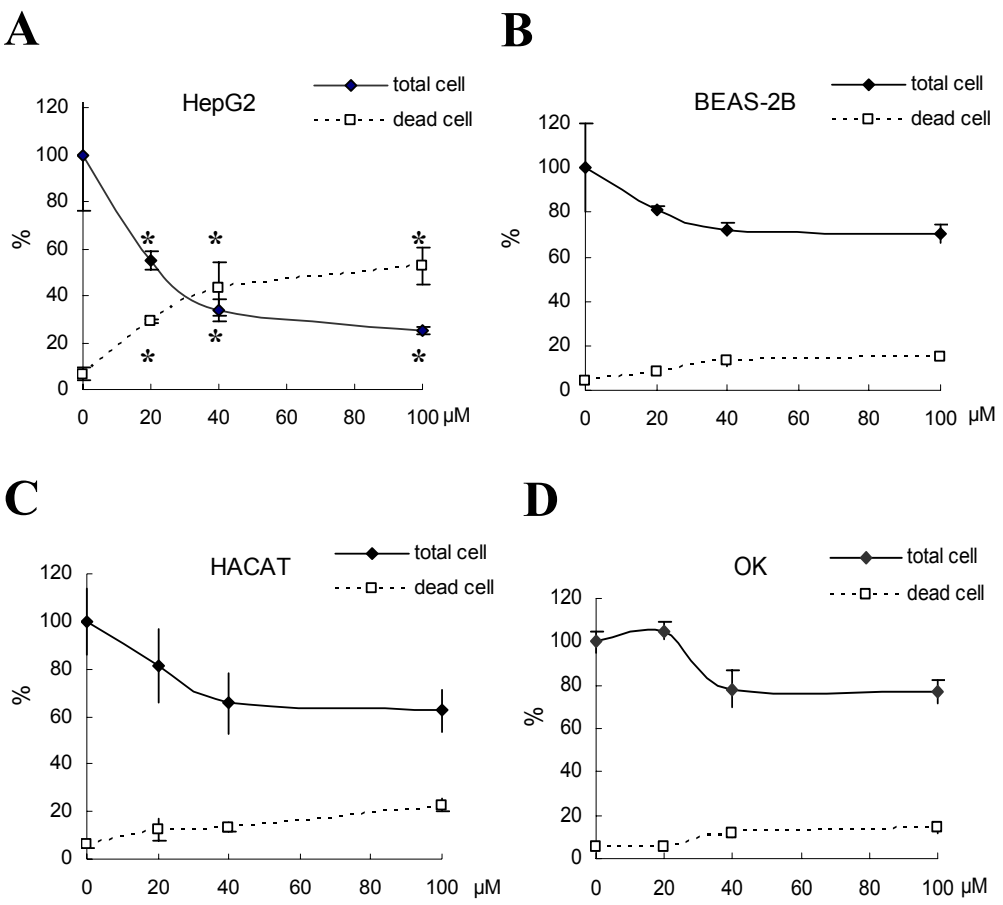


Fig. 2.2 Specific cytotoxic effects of AE on HepG2 cells as compared to other normal immortal cells. AE treatment (20, 40 and 100 μM for 48 hr) was conducted in human hepatoma HepG2 cells (A), human airway epithelial BEAS-2B cells (B), human keratinocyte HACAT cells (C), and opossum kidney proximal tubule OK cells (D). Cell viability (total cells) and the percentage of dead cells with damaged cell membrane to exclude tryphan blue were determined by trypan blue exclusion assay. At least three batches of samples were tested. The percentage of viable cell and damaged cells was compared with the control cells, and presented as means ± SD.

2.3.2 AE induced apoptotic cell death and G2/M arrest

The preliminary morphological observation revealed that AE treatment induced an increasing number of shrunk cells and floating cells, which was an indication of cell death. As shown in Fig. 2.3A and 2.3B by DAPI nuclear staining, AE treatment induced typical apoptotic nuclear fragmentation and chromatin condensation in HepG2 cells time-dependently. This induced apoptosis was further found to be caspase-dependent, because pretreatment with the pan-caspase inhibitor z-VAD could substantially protect AE-induced apoptosis (Figs. 2.3A, B). Consistently, AE treatment time-dependently increased the caspase-3/7 activity from 12 hr till 48 hr time-dependently (Fig. 2.3C). The result of DNA content analysis by flow cytometry provided further evidence that AE induced nuclear fragmentation, as shown by the increased sub-G1 proportions in AE-treated cells (Fig. 2.4). In addition, AE also increased the G2/M proportion, indicating its role in G2/M cell cycle arrest. All these effects by AE treatment were consistent with earlier reports reported for lung carcinoma (Lee *et al.*, 2005), glioma (Mijatovic *et al.*, 2005a) and leukemia cells (Chen *et al.*, 2004a).

On the contrary, although EM also increased the caspase 3/7-like activity (Fig. 2.3C), it induced a lower level of apoptotic nuclear fragmentation (Fig. 2.3 B) and minimal G2/M cell cycle arrest (Fig. 2.4). The lower percentage of apoptotic cells induced by EM was apparently in line with EM's lower cytotoxicity as determined by the MTT assay (Fig. 2.1).

Taken together, the above results suggested that AE had a higher potential in inducing marked apoptotic cell death and cell cycle arrest than EM.

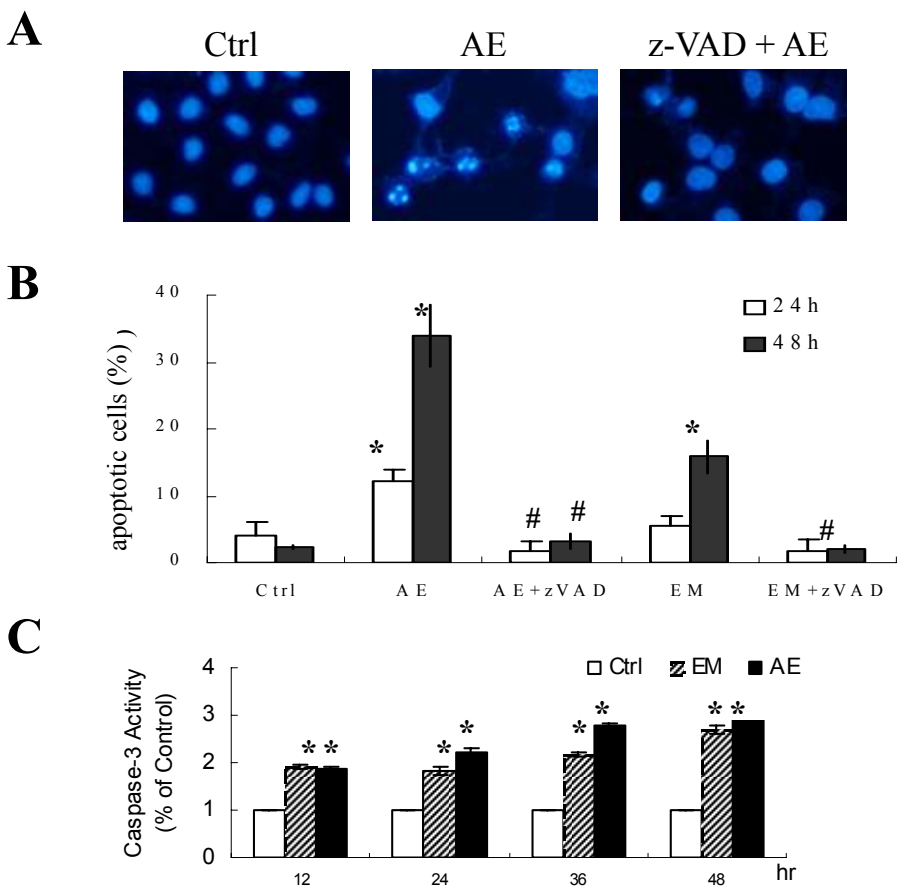


Fig. 2.3 AE induced apoptotic cell death. HepG2 cells were pretreated with/without 25 μM pan-caspase inhibitor z-VAD-FMK (z-VAD, 30mins) and then treated with 40 μM AE or EM as indicated. The nuclear morphology was checked by DAPI staining under a fluorescent microscope. (A), Representative DAPI-stained nuclei after the indicated treatment were shown. Cells with clear condensed nuclei were considered as apoptotic cells. (B), Ratio of the number of apoptotic to total cells were presented (n=3). Ten randomly selected fields for more than 200 cells were examined. (C), After the indicated treatment with 40 μM AE or EM, caspase-3/7 activity was determined by measurement of the cleavage of Ac-DEVD-AFC.

# $p<0.01$ when compared to treatment only group.

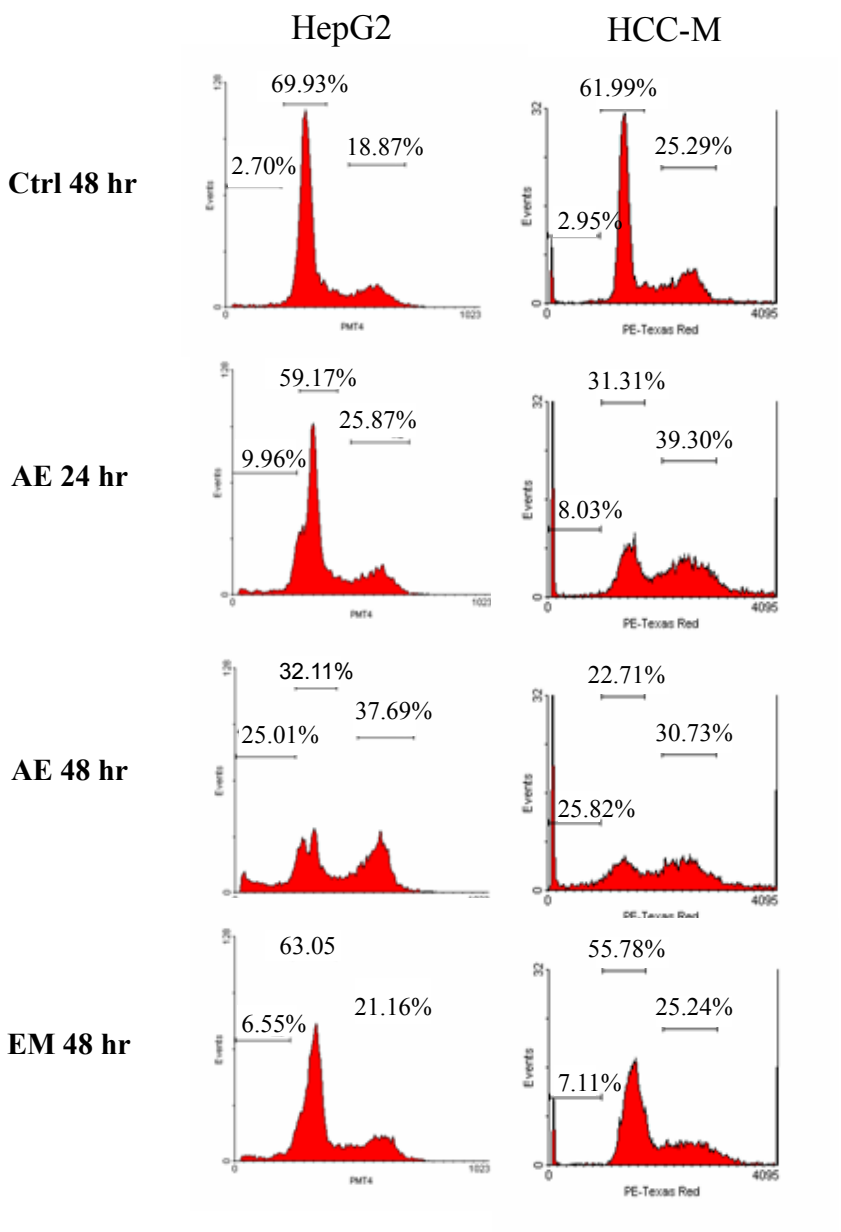


Fig. 2.4 AE induced DNA fragmentation and G2/M cell cycle arrest. HepG2 and HCC-M cells were treated with 40 μM AE (or EM) for 24 hr or 48 hr as indicated. After trypsinization and PI staining, the cell DNA content (subG1, G1/S and G2/M) was analyzed by flow cytometry. The percentage of each DNA phase was indicated (n=4).

2.3.3 AE affected the expression of multiple proteins.

To obtain a comprehensive understanding of the anti-cancer effects of AE, the HepG2 cell proteome after treatment with/without 40 μM AE for 24 hr were analyzed by 2D-DIGE. As reported earlier, under this treatment condition, only a small proportion of cells (10%) succumbed to cell death (Fig. 2.1B), thus ensuring that the changes of protein expression were a direct result of AE treatment but not from the induced apoptosis. In parallel, the cell proteome after EM treatment was also analyzed and compared with that of AE.

Since the preliminary 2-DE experiment using pH 3-10 non-linear IPG strips showed that most of the differentially-expressed proteins were located in the pH 4-7 region (data not shown), 18 cm pH 4-7 linear IPG zoom strips were subsequently chosen for 2D-DIGE experiments. In total, over 1600 proteins were separated by 2D-DIGE. As shown in Fig 2.5A and Table 2.2, AE treatment affected the expression level of multiple proteins. In these cells, 40 protein spots with an expression ratio higher than 2.0 were found to be differentially expressed. 22 proteins (from 27 spots) were successfully identified by MALDI TOF/TOF MS/MS (Table 2.2). A representative MS/MS analysis and database search using the MASCOT program for the matched peptides of PRDX6 was shown in Fig. 2.6. On the contrary, EM affected the expression level of much fewer protein spots than AE (Fig. 2.5B), which was in line with EM's lower cytotoxicity (Fig. 2.1). The differentially-expressed proteins affected by AE or EM treatment were annotated on the representative 2D-DIGE gels, respectively (Fig. 2.5).

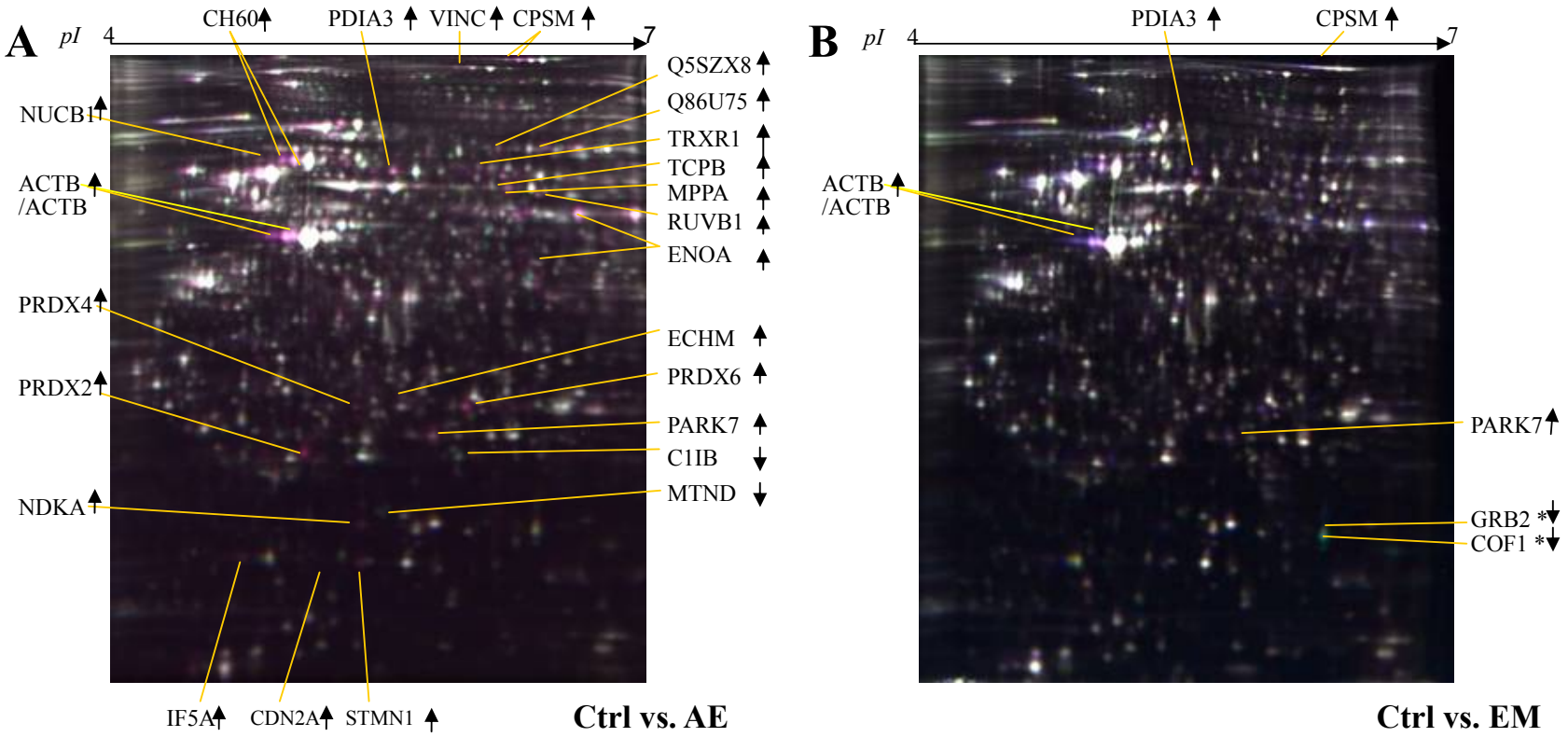


Fig. 2.5 AE and EM treatment affected multiple proteins. The proteome of HepG2 cells treated with/without AE (A) or EM (B) were analyzed by 2D-DIGE. Significantly differentially-expressed proteins were labeled as Swiss-Prot entry number (e.g. ACTB stand for ACTB_HUMAN), with ↑ to indicate up-regulation and ↓ to indicate down-regulation.

Table 2.2 List of differentially expressed proteins in AE- or EM-treated cells identified by MALDI TOF/TOF MS/MS. Average volume ratio (Av. Vol. ratio) were obtained from the DeCyder software. Apparent M_r and pI was estimated using in-house program. M_r in kDa.

Accession No. [a]	Protein name	Theoretical	Apparent	Av.Vol. Ratio [b]		Score	Matched Peptides Sequences[c]	Sequence Coverage (%)
		M_r/pI	M_r/pI	AE	EM			
PRDX2_HUMAN	Peroxiredoxin 2	22/5.7	28/5.4	5.86	1.46	359	92-109; 120-127; 140-150	18
PRDX6_HUMAN	Peroxiredoxin 6	25/6.0	29/6.0	3.44	1.45	300	84-96; 97-105; 132-141; 144-154; 155-161; 182-198	71
ACTG_HUMAN or ACTB_HUMAN	Actin, cytoplasmic 1 or 2	42/5.3	50/5.1	2.60	1.21	99	85-95; 239-254;	38
			50/5.15	3.67	1.50	192	85-95; 239-254	41
			50/5.2	2.99	1.50	246	19-28; 85-95; 96-113; 197-206; 239-254; 292-312	54
			50/5.25	2.80	1.48	164	29-39; 85-95; 96-113; 239-254; 292-312	55
NUCB1_HUMAN	Nucleobindin 1	54/5.1	76/5.1	2.98	1.04	308	54-69; 88-97; 153-163; 190-199; 311-323; 350-357	14
CH60_HUMAN	60 kDa heat shock protein, mitochondrial	61/5.7	66/5.2	2.98	1.27	507	61-72; 253-270; 271-292; 373-389; 432-448	14
			66/5.3	2.84	1.49	114	97-121; 251-268; 269-290; 302-309	38

Accession No. [a)]	Protein name	Theoretical	Apparent	Av.Vol. Ratio [b)]		Score	Matched Peptides Sequences[(c)]	Sequence Coverage (%)
		M_r/pI	M_r/pI	AE	EM			
STMN1_HUMAN	Stathmin	17/5.8	20/5.4	2.90	1.38	63	14-26	33
NDKA_HUMAN	Nucleoside diphosphate kinase A (p16)	20/5.4	24/5.5	2.85	1.49	153	7-18; 57-66; 106-114	50
CPSM_HUMAN	Carbamoyl-phosphate synthase [ammonia], mitochondrial	165/6.3	160/5.3	2.65	1.36	89	588-603; 815-826; 1445-1453	21
			160/5.35	2.65	1.50	337	254-279; 588-603; 815-826; 1248-1259; 1310-1317; 1361-1373; 1445-1453	28
PARK7_HUMAN	protein DJ-1	20/6.3	28/5.9	2.47	1.56	127	13-27; 33-48;	68
TCPB_HUMAN	T-complex protein 1, beta subunit	57/6.0	60/6.1	2.43	1.26	65	120-130; 181-188; 358-375; 501-515	41
MPPA_HUMAN	Mitochondrial processing peptidase alpha subunit	58/6.4	60/6.15	2.43	1.26	176	84-99; 159-177; 251-268; 270-288	37
VINC_HUMAN	Vinculin	124/5.5	120/6.0	2.38	1.24	114	114-132; 548-561; 608-629; 833-853;	40
PDIA3_HUMAN	Protein disulfide-isomerase A3	57/6.0	72/5.7	2.37	1.60	155	336-344; 352-363; 472-282	35
PRDX4_HUMAN	Peroxiredoxin 4	30/5.9	29/5.6	2.33	1.32	100	187-200; 213-223	9
RUVB1_HUMAN	RuvB-like 1	50/6.0	54/6.3	2.33	1.36	187	318-333; 340-357	7
IF5A1_HUMAN	Eukaryotic translation initiation factor 5A-1	17/5.1	17/5.0	2.29	1.27	111	117-139	46
ECHM_HUMAN	Enoyl-CoA hydratase, mitochondrial	31/8.3	31/5.8	2.19	1.45	81	119-127; 158-178	39

Accession No. [a)]	Protein name	Theoretical	Apparent	Av.Vol. Ratio [b)]		Score	Matched Peptides Sequences[(c)]	Sequence Coverage (%)
		M_r/pI	M_r/pI	AE	EM			
Q5SZX8_HUMAN	Chaperonin Containing TCP1, subunit 3 isoform b	61/6.1	72/6.1	2.04	1.26	206	203-215; 427-437; 438-448	6
Q86U75_HUMAN	Dihydropyrimidinase-like 2	68/5.6	72/6.2	2.03	1.08	64	174-189; 190-211	6
CD2A1_HUMAN	Cyclin-dependent kinase inhibitor 2A	16/5.2	17/5.4	2.01	1.04	193	30-46; 88-99; 113-124	50
MTND_HUMAN	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	22/5.6	24/5.6	-2.20	-1.41	100	2-15; 83-96; 97-108; 109-116;	68
ABHEB_HUMAN	Abhydrolase domain-containing protein 14B	22/5.9	26/6.0	-2.40	-1.06	168	9-22; 33-42; 43-56	32
COF1_HUMAN [d)]	Cofilin-1	19/8.2	22/6.4	-1.12	-4.33	249	35-45; 54-73; 74-81; 82-92; 96-112; 133-144	72
GRB2_HUMAN [d)]	Splice isoform GRB3-3 of growth factor receptor-bound protein 2	20/5.5	23/6.4	1.09	-2.78	110	84-95; 102-108	71

a). Represents entry numbers from Swiss-Prot database.

b). Positive value signifies up-regulation against control samples and negative value signifies down-regulation in terms of fold-differences. All ratio is statistically significant with $t<0.01$ (Student's t test).

c). The matched peptides are shown in relation to the amino acid sequences of individual protein.

d). Represent differentially-expressed proteins only found in EM-treated cells.

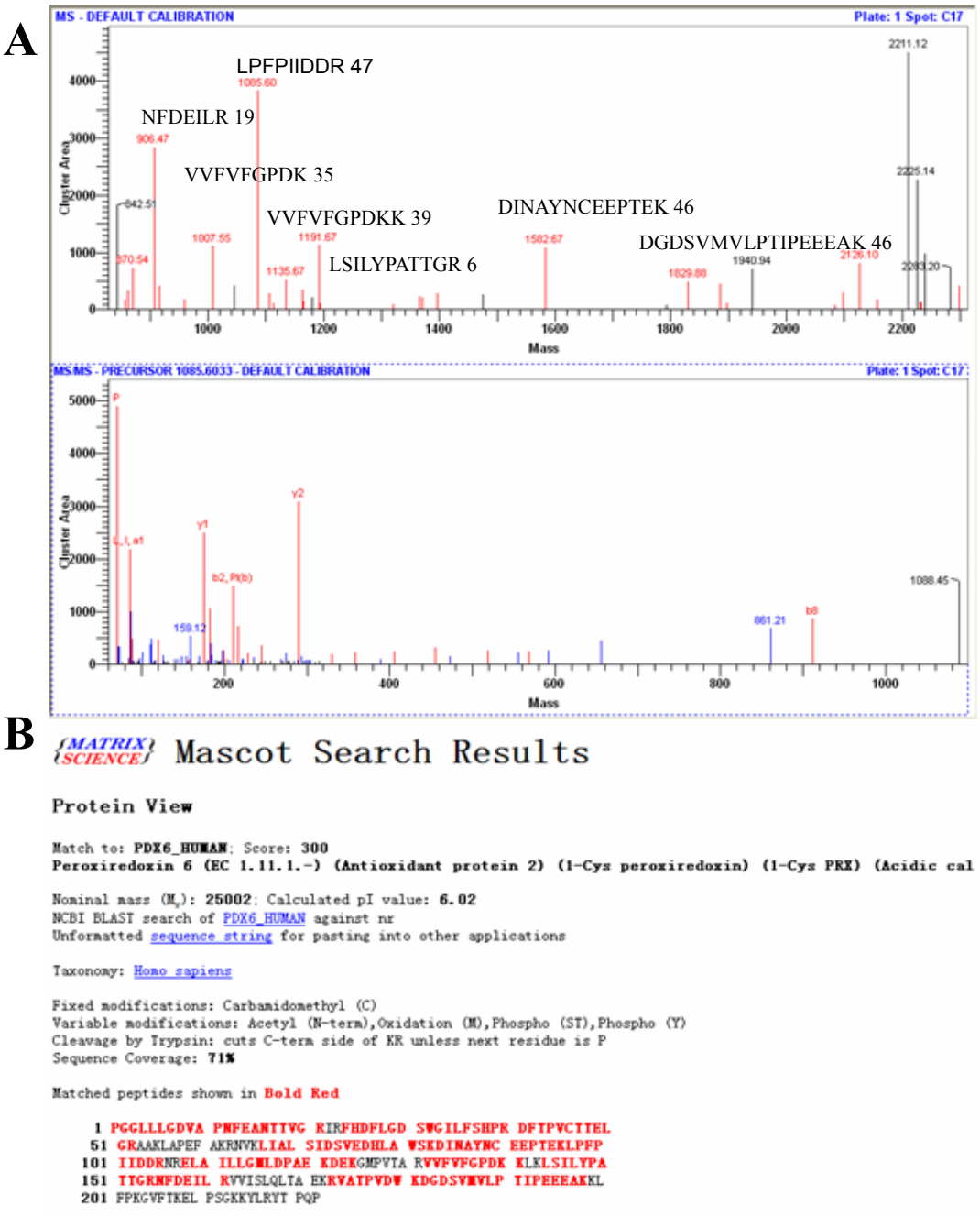


Fig. 2.6 A representative TOF-TOF MS/MS analysis of PRDX6. After trypsin digestion, protein spots were analyzed by MS/MS and database search using the MASCOT program. The matched peptides were annotated with amino acid sequence and score. The bottom panel of (A) showed the MS/MS results of one selected peptide.

2.3.4 General functional classification of AE-affected proteins

In HepG2 cells, AE treatment altered the expression level of multiple proteins. In terms of subcellular location, most differentially-expressed proteins resided in the cytoplasm. Others may predominantly resided in the mitochondrion (CH60, CPSM, MPPA, ECHM), nucleus (NDKA, PARK7, IF5A1, ABHEB), endoplasmic reticulum (PDIA3) or Golgi apparatus (NUCB1).

On the other hand, in terms of known protein function, these affected proteins were found to be involved in different aspects of cellular functions, including redox regulation, protein folding, cell cytoskeleton and adhesion, metabolism, and transcription/translation. It is noteworthy that in AE-treated cells, many differentially expressed proteins were found to be functionally related to oxidative stress and cancer initiation/progression (Fig. 2.7).

On the contrary, EM treatment only affected a few proteins (Fig. 2.5 and Table 2.2), which was in line with its lower cytotoxicity (Fig. 2.1). Among the affected proteins, redox-sensitive proteins PARK7, PDIA3, ACTG were up-regulated by EM, which was similar to AE treatment. However the up-regulation levels for these three proteins by EM were much lower than that for AE (Table 2.2). Whether up-regulation of these proteins was a direct result of the treatment by rhubarb hydroxyanthraquinone (EM and AE) or indirectly as a consequence of the induced oxidative stress was yet to be determined. It is noteworthy that cofilin was significantly down-regulated by EM, but not AE. Although this protein has been found to be associated with both apoptosis

and metastasis (Chua *et al.*, 2003; Wang *et al.*, 2006b), the significance of the down-regulation of cofilin after EM treatment had to be determined.

Taken together, AE differentially affected the expression level of multiple proteins with known anti-cancer properties, thus indicating the possible functional effects of AE. In contrast, EM affected fewer proteins and also at a lower expression levels, probably in consonant with its lower cytotoxicity.

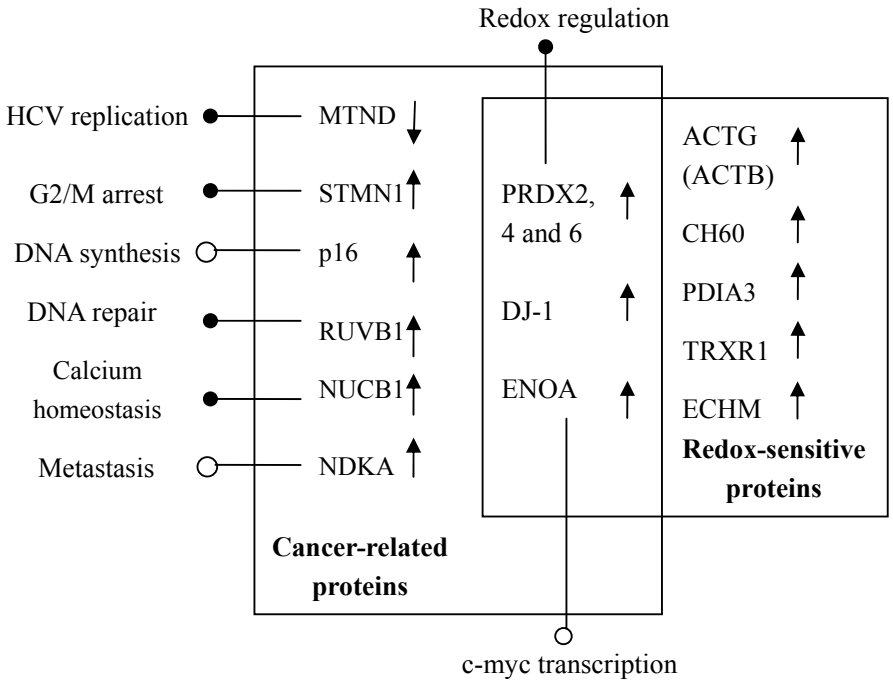


Fig. 2.7 Schematic representation of the affected proteins in AE-treated cells. AE affected multiple proteins which have been reported to be involved in oxidative stress and/or cancer. Some cancer–related proteins were labeled with their known functions.

↑ up-regulation ↓ down-regulation ○— inhibition ●— induction

2.4 Discussion

The present study demonstrated that AE treatment affected multiple proteins that are functionally associated with oxidative stress and cancer.

AE exhibited a higher cytotoxicity in hepatoma cells than in several immortal normal cells, including human airway epithelial BEAS-2B cells, opossum kidney proximal tubule OK cells and human keratinocyte HACAT cells (Fig. 2.2). This specific anti-cancer effect of AE has been consistently demonstrated in several earlier *in vitro* cell studies, including human oral squamous cell carcinoma (HSC-2) and salivary gland tumor (HSG) cell lines *vs.* human gingival fibroblasts (HGF) cells (Shi *et al.*, 2001), human neuroectodermal tumor cells *vs.* normal human lung fibroblast MRC5 cells and hemopoietic progenitor cells (Pecere *et al.*, 2000), mouse L929 fibrosarcoma and rat C6 astrocytoma cells *vs.* primary astrocytes and fibroblasts (Mijatovic *et al.*, 2004). Taken together, this anti-cancer specificity may potentiate AE as a specific chemotherapeutics drug. It is noteworthy that endothelial cells (bovine aortic endothelial BAEC cells and human umbilical vein endothelial HUVEC cells) were recently reported to be sensitive to AE (Cardenas *et al.*, 2006). This property was suggested to be useful for modulation of angiogenesis and anti-cancer effects (Cardenas *et al.*, 2006).

Furthermore, AE exhibited a higher cytotoxicity than its analogue EM in hepatoma cells (Fig. 2.1). This result of AE's higher cytotoxicity is consistent with previous studies conducted in Merkel cell carcinoma (Fenig *et al.*, 2004), lung carcinoma CH27 (Lee, 2001), human oral squamous cell carcinoma (HSC-2) and

salivary gland tumor (HSG) cell lines (Shi *et al.*, 2001). It would be immensely informative to understand the molecular mechanism responsible for this higher cytotoxicity of AE over EM, since AE had been reported to be less competent in inhibiting most kinases than EM (reviewed in section 1.1.5).

AE was shown to induce apoptosis and G2/M cell cycle arrest (Fig. 2.3 and 2.4), but the underlying mechanism for this effect is yet to be determined. In order to elucidate this phenomenon, a proteomics investigation was conducted to obtain a global view of the affected proteins by AE treatment. More importantly, proteomic approaches can also monitor post-translational modifications of proteins (e.g. protein phosphorylation and ubiquitination, key regulations for protein activity and turnover), considering that AE and EM may affect protein phosphorylation by inhibiting different kinases activities. In this study, 2D-DIGE, a quantitative 2-DE method was used. In 2D-DIGE, the same amount of control samples and treated samples were labeled with fluorescent dyes Cy3 or Cy5 respectively and loaded together in the same gel to minimize the gel-to-gel variance. Moreover, the introduction of a pooled internal standard (which consisted of equal amount of all control and treated samples and labeled with Cy2 dye) enabled accurate quantification and matching between different gels (Alban *et al.*, 2003). The fluorescence intensities (as indicators for the protein expression levels) were then determined by the DeCyder V 5.02 software. This method had greatly increased data quality and confidence.

The 2D-DIGE result (Fig. 2.5) showed that AE treatment affected several proteins, which are functionally associated with oxidative stress and cancer

initiation/progression (Fig. 2.7). Redox-sensitive proteins, including peroxiredoxin-2, -4 and -6, protein disulfide-isomerase A3 (PDIA3), actin (ACTG or ACTB), 60 KD heat shock protein (CH60), protein DJ-1 (PARK7) and enoyl-CoA hydratase (ECHM) were among those markedly up-regulated in AE-treated cells (Table 2.2). The up-regulation of these redox-sensitive proteins (Fig. 2.7, Box: redox-sensitive proteins) has been frequently reported in several earlier studies in which oxidative stress was involved (Cesaratto *et al.*, 2005; Fratelli *et al.*, 2002; Fratelli *et al.*, 2003). These up-regulation of these proteins are common cellular responses and function to reduce the induced oxidative stress, thus suggesting the involvement of oxidative stress in AE treatment in the present study. Besides, two other redox-sensitive proteins with volume ratio slightly less than 2 that were up-regulated in AE-treated cells were alpha enolase (ENOA, up-regulated in two spots, 1.94 and 1.89 fold) and thioredoxin reductase 1 (TRXR1, up-regulated 1.86 fold) (Fig. 2.5). These two redox-sensitive proteins have also been reported to be up-regulated and oxidized in many *in vitro* and *in vivo* systems under oxidative stress (Cesaratto *et al.*, 2005; Fratelli *et al.*, 2002). Besides, alpha enolase, as a tumor suppressor, is an inhibitory binding protein of c-myc promoter and in turn suppress transcription and cell growth (Feo *et al.*, 2000). Furthermore, the MALDI TOF/TOF MS/MS results suggested that methionine oxidation was found in the peptide fragments of some proteins (e.g. protein DJ-1, ENOA, TRXR1, and GARS protein) in the AE-treated but not in the untreated cells (data not shown), further supporting the involvement of oxidative stress.

Oxidative stress may cause reversible or irreversible damage to certain redox-sensitive proteins and in turn affect the cell fate by inducing cell death or cell senescence (see section 1.3.2). For example, oxidation of 2-Cys peroxiredoxin was recently found to be a molecular switch controlling the transcriptional response to the increasing level of hydrogen peroxide (Bozonet *et al.*, 2005; Wood *et al.*, 2003). Of the differentially-expressed proteins affected by AE treatment, peroxiredoxins (e.g. PRDX2, PRDX4 and PRDX6) are among the list of most highly up-regulated proteins. It is worth noting that PRDX2, PRDX4 and PRDX6 are also named as thioredoxin peroxidase 1, thioredoxin peroxidase AO372 and non-selenium glutathione peroxidase, respectively. Peroxiredoxins have also been reported to be increased in cells stressed with oxidants such as H_2O_2 (Cesaratto *et al.*, 2005; Fratelli *et al.*, 2002) or ionizing radiation (Wang *et al.*, 2005). These proteins share a conserved sequence region that contained a reactive cysteinyl residue near the N-terminus of the proteins, which may probably act as a reaction intermediate to reduce the peroxide. PRDX-2 and -4 belonged to the 2-Cys subgroup of peroxiredoxins while PRDX 6 belonged to the 1-Cys subgroup, based on their cysteinyl residue site. Under oxidative stress, the reactive Cys residues are ready to be oxidized to form cysteine sulfenic acid (Cys-SOH), and further oxidized to cysteine sulfinic acid (Cys-SO_2H) or cysteine sulfonic acid (Cys-SO_3H) (Kwak *et al.*, 2006). Recently, it was reported that oxidation of peroxiredoxins in the form of Cys-SO_2H and/or Cys-SO_3H is a sensitive marker of oxidative stress in HepG2 cells *in vitro* and transplanted livers during ischemia/reperfusion *in vivo* (Cesaratto *et al.*, 2005). Taken

together, the up-regulation and oxidation of these redox-sensitive proteins found in the present study suggested that oxidative stress might be involved in the cytotoxicity of AE. But whether AE directly induce oxidative stress and the role of oxidative stress in AE-induced apoptotic cell death are yet to be determined. This part will be discussed in the next Chapter.

Besides these redox-sensitive proteins, the other proteins affected by AE are related to cancer initiation and/or progression (Fig. 2.7, Box: cancer-related proteins). For example, up-regulation of p16 and stathmin are associated with DNA synthesis inhibition (Ivanchuk *et al.*, 2001; Serrano, 2000) and G2/M cell cycle arrest (Kong *et al.*, 1992) respectively. Tumor suppressor protein p16 functions in inhibiting CDK4/6 activity and DNA replication (Sherr, 2004). On the other hand, stathmin is known to play a critical role in the regulation of the microtubule filament system. In its active hypophosphorylated form, it can promote depolymerization of microtubules and/or preventing polymerization of tubulin heterodimers, which plays a key role in cell mitosis (see review (Kong *et al.*, 1992)). Disruption of stathmin function either by forced or inhibited expression both results in reduced cellular proliferation and G2/M cycle arrest (Luo *et al.*, 1998; Marklund *et al.*, 1994). It is of interest to note that the up-regulation of stathmin has also been reported to be associated with G2/M arrest in pancreatic ductal carcinoma cell lines treated with trichostatin-A, a potent inhibitor of histone deacetylases (Cecconi *et al.*, 2003). Thus, up-regulation of these two proteins may be responsible for the anti-proliferative effect of AE.

In the study here, 1, 2-dihydroxy-3-keto-5-methylthiopentene dioxygenase

(MTND) was found to be down-regulated by AE. Considering that this protein had been reported to be essential for hepatitis C virus replication (Yeh *et al.*, 2001), the findings in this study suggested that AE could have an anti-hepatitis potential. This effect may account for the anti-hepatitis effect of Rhubarb application in TCM.

Interestingly, AE treatment up-regulated the expression level of nucleoside diphosphate kinase A (NDKA, or nm23 as gene name), a metastasis suppressor (Fig. 2.5 and Table 2.2). Low expression of NDKA had been reported to be correlated with poor patient prognosis and survival in several epidemiological cohort studies, including hepatocellular carcinoma (Hartsough and Steeg, 2000). The present finding of up-regulation of NDKA by AE thus suggested that AE may also have an anti-metastatic capability, although its analogue EM had been shown to inhibit invasion (Huang *et al.*, 2004), migration (Huang *et al.*, 2005) and adhesion (Huang *et al.*, 2006b) in human carcinoma cells. Thus, AE treatment altered the expression level of multiple proteins, which may contribute to its anti-cancer potentials, including inhibition of DNA synthesis and HCV replication as well as cancer metastasis. Some of these representative proteins have been selected for further functional studies and these findings are reported in Chapter 4.

On the contrary, EM, although structurally similar to AE, only caused mild up-regulation of the redox-sensitive proteins DJ-1, actin and protein disulfide isomerase (Fig. 2.5 and Table 2.2). This probably corresponding to its lower cytotoxicity in HepG2 cells (Fig. 2.1). Besides, cofilin was significantly down-regulated in EM-treated cells but not in AE-treated cells (Fig. 2.5 and Table 2.2).

It is worth noting that in the 2D-DIGE map, this protein was located at around pI 6.4. However, its theoretical pI is 8.2 (which is beyond the pI range of 4-7 IPG strip). It indicated that this acidic form of cofilin (pI 6.4) may be a phosphorylated form of cofilin. Cofilin has been reported to be a member of the cofilin/actin depolymerization factor family. It regulates actin dynamics by promoting the rate of actin depolymerization and facilitating actin filament turnover (Chen et al., 2000). Cofilin has also been recently reported to be directly associated with invasion, intravasation, and metastasis of mammary tumors (Wang et al., 2006b). On the other hand, cofilin can also function as a pro-apoptotic protein. Mitochondrial translocation of dephosphorylated cofilin was found to be an essential step in apoptosis induction before cytochrome c release (Chua et al., 2003). Transfection of mutated cofilin (at its phosphorylation site) can suppress its mitochondrial translocation and subsequent apoptosis-stimulating action (Chua et al., 2003). However in the present study, the decrease of acidic form of cofilin in EM-treated cells but not in AE-treated cells may not be crucial for apoptosis, because EM can only induce mild apoptotic cells death at a latter time point when compared with AE. This down-regulation of (phosphorylated) cofilin may just be derived from the inhibition of its upstream kinase (e.g. Lim kinase), since EM is capable of inhibiting many kinases. The role of this protein in EM-induced cell death will be further discussed in Chapter 4.

In the present study, the pH 4-7 IPG strips were chosen for 2D-DIGE investigation, because most affected proteins were found in this region in a preliminary study using pH 3-10 none-linear IPG strips. The usage of 18cm pH 4-7

zoom strips double the gel resolution in this region compared with that of pH 3-10 strips and is helpful to separate proteins with similar pI, Mr in this range. Nevertheless, other proteins that possibly account for AE-induced anti-proliferative effects may reside outside of this pI range. Those proteins will not be present in the gel of 2D-DIGE using pH 4-7 zoom strips. On the other hand, considering the wide dynamic range of protein concentration (10 to 12 order of magnitude) in cells, 2D-DIGE may not be sensitive enough to detect proteins with low abundance. Thus these proteins would not be studied here.

In summary, AE induced higher cytotoxicity in hepatoma cells than EM through induction of apoptosis and cell cycle arrest. Using 2D-DIGE, we revealed that AE treatment affected several proteins that are functionally associated with oxidative stress, cell cycle arrest, anti-metastatic, and other anti-cancer activities. Some of these proteins were then selected for further functional studies and will be reported in the subsequent chapters.

CHAPTER 3

ALOE-EMODIN INDUCED APOPTOSIS THROUGH OXIDATIVE STRESS AND SUSTAINED JNK ACTIVATION

3.1 Introduction

In the previous Chapter of proteomic investigation, AE was found to alter the expression of a number of proteins involved in oxidative stress, cell cycle arrest, anti-metastasis and apoptosis. Among the affected proteins, redox-sensitive proteins (e.g. PRDX and DJ-1) were found to be highly up-regulated, suggesting that oxidative stress may be involved in AE-induced cytotoxicity. This should not be surprising, as the quinone structure of AE is highly redox active. It could, for example, form a redox cycle with its semiquinone radicals, leading to the formation of ROS (Pryor, 1982; Watanabe and Forman, 2003). Furthermore, AE was recently reported to induce DNA damage via excessive ROS production in human lung cancer cells (Lee *et al.*, 2006a).

To minimize oxidative damage resulting from excessive production of ROS, cells are equipped with both enzymatic and non-enzymatic antioxidant defenses, including superoxide dismutase (SOD), catalase, and a variety of low molecular weight antioxidants such as glutathione and vitamins. Particularly, the GSH / GSSG couple provides an efficient protective system against the redox imbalance (Chandra *et al.*, 2000). Many chemotherapeutic drugs may induce apoptotic cell death by exhausting the intracellular thiol buffer system through depletion of GSH (Ghibelli *et al.*, 1995) or redistribution of GSH (Voehringer *et al.*, 1998). The unbalanced redox states may further trigger downstream cellular signaling events, such as mitochondrial dysfunction and other signaling pathways which lead to apoptotic cell death (Chandra *et al.*, 2000).

Among the various redox-activated cell signaling pathways are the

well-established mitogen-activated protein kinases (MAPKs) (Benhar *et al.*, 2002). There are three subgroups of MAPKs: ERK (extracellular signal-regulated kinases), JNKs (c-Jun N-terminal kinases) and p38. It has been well-established that JNK and p38 mainly play a pro-apoptotic function in response to various cellular stress, while the ERK subgroup is primarily involved in proliferative responses (Kyriakis and Avruch, 2001; Lewis *et al.*, 1998; Shen and Liu, 2006). AE has been reported as an inhibitor of the ERK pathway (Mijatovic *et al.*, 2005a) and an inducer of p38 pathway (Yeh *et al.*, 2003) in human carcinoma cells. However, the involvement of JNK pathway in AE-induced oxidative stress and apoptosis has not been reported yet.

The present study aims to investigate the role of oxidative stress and MAPK pathway in AE-induced apoptotic cell death. The results obtained demonstrated that JNK are the key effector molecules in AE-induced apoptosis in response to AE-mediated oxidative stress and protein oxidation.

3.2 Materials and methods

3.2.1 Reagents, antibodies and plasmid

AE, EM, hydrogen peroxide, GSH-monomethylester, buthionine-L-sulfoximine, mercaptosuccinic acid and other chemicals were purchased from Sigma-Aldrich (St Louis, MO). Cell permeable JNK inhibitor III was from Merck (San Diego, CA).

Antibodies against cytochrome *c* and GST-π were purchased from BD Pharmingen (San Diego, CA). The following antibodies were bought from Cell Signaling (Beverly, MA): caspase-3, caspase-8, caspase-9, PARP, phospho-cJun (ser63), phospho-cJun (ser73), Cu/Zn-SOD, ASK1, phospho-ERK (Thr202/Tyr204)

and COX IV. Antibodies against DNP, peroxiredoxins, phospho-JNK (Thr183/Tyr185), JNK1, JNK2, Trx and tubulin, together with small interfering RNA (siRNA) specific against human peroxiredoxin 2 and total peroxiredoxin, JNK1, JNK2 and control (non-silencing) siRNA were from Santa Cruz (Santa Cruz, CA). Anti-peroxiredoxin-SO_3 IgG was from Labfrontier (Seoul, Korea). Rainbow-colored protein molecular weight marker was obtained from GE Healthcare (Amersham, Bucks, UK). The SuperSignal Dura ECL kit was from Pierce (Rockford, IL).

The pEGFP-N1 expression vector was from Clontech (Palo Alto, CA). The pEGFP-C3/sod1 vector was a kind gift from Dr. J. J. Bower at West Virginia University. The dominant-negative JNK1 and JNK2 expression vectors (JNK1-DN and JNK2-DN) and constitutive active JNNK2-JNK1 vector were generous gifts from Dr. A.G. Porter (Li *et al.*, 2004). The wild-type HA-ASK1, C-terminal coiled-coil domain deletion mutant of ASK1 expression vector and the anti-phospho-ASK1 (thr845) antibody were graciously provided by Dr. H. Ichijo (Tobiume *et al.*, 2002).

3.2.2 Cell culture and treatments

The human hepatocellular carcinoma cell lines, HepG2, HCC-M and Hep3B cells were cultured as described previously (Liang *et al.*, 2002; Neo *et al.*, 2005). Briefly, the hepatoma cells were grown in the DMEM or MEM medium (with 100 U/ml penicillin, 100 μg/ml streptomycin, 3.7 g/L sodium bicarbonate, pH 7.4) plus 10% FBS in standard incubator conditions (37℃, 5% CO_2). AE was dissolved in DMSO as a stock solution of 100 mM. In all treatments, the control group was treated with the same amount of DMSO.

3.2.3 Determination of apoptosis

Nuclear fragmentation and chromatin condensation as a marker for apoptotic cell death was determined in section 2.2.6.

3.2.4 Analysis of intracellular glutathione (GSH/GSSG)

Intracellular glutathione were analyzed as described previously (Zhang *et al.*, 2004a). Briefly, after the indicated treatment, the same number of cells were collected and lysed in lysis buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 8.0). After cell homogenization using an ultrasonic probe, proteins were precipitated by mixing with 25% metaphosphoric acid. The supernatant was then collected and the GSH contents were measured by adding o-phthaladehyde (50 μg/ml) for 15 min before fluorescence excitation at 350 nm and detection at 420 nm. For the detection of GSSG, the same amount of supernatant was reacted with N-ethylmaleimide (0.04M) for 30 min to eliminate free GSH. Then the solution was diluted 10 times with 0.1 M NaOH before the *o*-phthaladehyde (50 μg/ml) reaction and fluorescence measurement.

3.2.5 Measurement of ROS production in cells

The intracellular production of ROS was estimated using chloromethyl-2,7-dichlorofluorescein diacetate (CM-H_2DCFDA, Molecular Probe, Eugene, OR) as described previously with minor modifications (Zhang *et al.*, 2004a). Briefly, after designated treatments, cells were trypsinized and incubated with CM-H_2DCFDA (5 μM) at 37 $^{\circ}$C for 30 min. Afterwards, cells were collected and washed three times with PBS. 10000 cells were immediately measured by flow cytometry (BD Pharmingen, San Diego, CA) with excitation at 488 nm and emission at 525 nm.

3.2.6 Measurement of mitochondrial outer membrane potential

The mitochondrial outer membrane potential (MOMP) was estimated using tetramethylrhodamine methyl ester (TMRM, Molecular Probe, Eugene, OR) (Zhang *et al.*, 2004a). Briefly, at the end of each designated treatments, trypsinized cells were incubated with 200 nm TMRM at 37 $^{\circ}$C for 15 min. Afterwards, cells were washed three times with PBS before detection with excitation/emission at 488/535 nm.

3.2.7 Cell subfractionation and detection of the release of mitochondrial proteins

Cell subfractionation were conducted as described previously (Zhang *et al.*, 2004b). In brief, after the indicated treatment, cells were re-suspended in ice-chilled isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 10 mM Tris, pH 7.4) for 10 min. After passing through gauge #27 needles for 20 times to break the cell membrane, the cell homogenates were subjected to a series of centrifugation steps (50 × *g* for 10 min, 500 × *g* for 20 min and finally 15000 × *g* for 20 min) to fractionate unbroken cells, heavy nuclear fraction and mitochondria fraction, respectively. The mitochondria rich fractions were lysed with Western blotting lysis buffer (see below). After a final centrifugation step at 60,000*g* at 4°C for 20 min, an equal amount of mitochondrial and cytosolic proteins were subjected to Western blotting for detection of cytochrome *c*.

3.2.8 Co-immunoprecipitation

The co-immunoprecipitation was conducted as described previously (Huang *et al.*, 2005). In brief, after the designated treatment, cells were collected and treated with the co-immunoprecipitation lysis buffer (50mM HEPES, pH 7.6, 250 mM NaCl, 0.1%

NP-40, 5mM EDTA, 0.5mM PMSF and 1X Complete® proteinase inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)) for 30 min on ice. Equal amount of protein samples (500 μg) were incubated with 2 μg IgG antibodies against JNK1/2 or Trx respectively and rotated overnight at 4°C. The protein-antibody complexes were then incubated with protein A/G agarose beads (Roche Molecular Biochemicals, Indianapolis, IN) for 1 hr. The beads bound with protein-antibody complexes were washed with iced-cold lysis buffer five times and then eluted with SDS sample loading buffer before SDS-PAGE.

3.2.9 Western blotting

Western blotting were conducted as described previously (Huang *et al.*, 2005). After the indicated treatment and cells collection, protein samples were treated with lysis buffer (20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 0.5 mM sodium vanadate and 1X Complete® protease inhibitor). For analysis of MAPK molecules, cells were lysed in SDS lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 1X Complete® protease inhibitor).

Protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were then fractionated on 11% SDS-PAGE in the Mini-PROTEAN II system (BioRad, Hercules, CA) and blotted onto PVDF membranes (Millipore, Bedford, MA). It is worth noting that oligomerization of GST-π was analyzed in non-reducing conditions (i.e. no DTT in lysis buffer and no β-mercaptoethanol in SDS sample loading buffer) (Adler *et al.*, 1999).

After blocking with 5% nonfat milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 hr at room temperature, the transblotted membrane was probed with various first antibodies and then second antibodies individually. The signals were then visualized with SuperSignal® West Dura Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Densitometric measurements were performed using the Kodark 1D 3.5 Image Station (Kodark, Rochester, NY).

3.2.10 Derivatization of protein carbonyls for 1-DE and 2-DE Western blotting

Protein samples were derivatized with 2, 4-dinitrophenylhydrazine (DNPH) by two methods for either 1-DE or 2-DE Western blotting. For 1-DE Western blotting, cells were collected as mentioned above and lysed in 6% SDS lysis buffer (62.5 mM Tris-HCl, pH 6.8, 6% SDS, 10% glycerol and 1X Complete® protease inhibitor). Equal amount of protein extract was incubated with 10 mM DNPH (in 5% trifluoroacetic acid) and vortexed for 20 min at room temperature. The reaction was then terminated by adding 1.5 volumes of neutralizing solution (2 M Tris, 30% glycerol).

On the other hand, post-isoelectric derivatization of protein carbonyls was conducted for 2-DE samples as described previously (Conrad *et al.*, 2001). In brief, the IPG strips were incubated with/without 10 mM DNPH for 20 min. The reaction was then stopped by incubation with the neutralizing solution (2 M Tris, 30% glycerol) for 15 min. Following this, the second dimensional separation was conducted as described in section 2.2.9.

3.2.11 Colloidal silver staining

The colloidal silver stain was used to visualize the protein profiles after the Western blotting step (Liang *et al.*, 2002; Neo *et al.*, 2005). After washing the membranes with fresh water thrice (5 min each), they were incubated with 25 ml of staining solution (2 mg/ml silver nitrate, 2 mg/ml sodium citrate, 8 mg/ml iron sulfate) for 15-30 min until a clear image appeared.

3.2.12 Gene transient transfection for over-expression or knocking-down

Gene transient over-expression and knocking-down experiment were conducted by electroporation using the Nucleofector® purchased from Amaxa (Gaithersburg, MD) according to the manufacturer's user manual. In brief, 1-5 X 10^6 cells were collected and re-suspended in 100 μl of Nucleofector® solution. The cell suspension was then mixed with 1-5 μg DNA (gene construct or empty vectors) or 0.5-3 μg siRNA as designated and immediately transferred into an Amaxa cuvette. The electroporation was performed using the Nucleofector® program T-28. Afterwards, cells were re-suspended in fresh DMEM medium. Designated treatment was conducted for 36 hr after transfection in the gene over-expression assay or 48 hr in the knockdown study.

3.3 Results

3.3.1 AE induced mitochondrial-mediated apoptosis

The results in Chapter 2 revealed that AE was capable of inducing caspase-dependent apoptosis in human hepatoma cells. Here the cell death signaling pathway was further investigated. As shown in Fig. 3.1A, AE induced time- and

dose-dependent cleavage/activation of caspase-9 and caspase-3. Consistently, AE induced increasing cleavage of poly (ADP-ribose) polymerase (PARP), one of the caspase-3 substrates. However, there was no evidence of cleavage/activation of caspase-8 (Fig. 3.1A), the initiator caspase for the death receptor-mediated extrinsic apoptotic pathway. Next, there was drastic translocation of cytochrome *c* from mitochondria to cytosol, preceding activation of caspase 9 and 3 (Fig. 3.1B). In addition, AE treatment decreased mitochondrial outer membrane potential (MOMP) in the TMRM assay, indicating that mitochondrial membrane integrity/function was impaired (Fig. 3.2C). These results thus suggested that AE-induced apoptosis was mainly executed via the mitochondria-mediated intrinsic cell death pathway. And this conclusion was comparable to the results conducted in neuroblastoma cells, for which mitochondrial-dependent intrinsic but not extrinsic apoptotic pathway activation was involved in AE-induced apoptosis (Pecere *et al.*, 2003).

Another two hepatoma cell lines (HCC-M and Hep3B cells) were studied to determine whether mitochondrial-mediated apoptosis by AE was HepG2 cell specific. It is noteworthy that both p53 wide-type cells (HepG2 and HCC-M) and p53 deficient cells (Hep3B) succumbed to AE-induced apoptosis, as evidenced by the time-dependent increase of nuclear condensation (Fig. 3.1C) and the cleavage of caspase-9, caspase-3 and PARP, but not caspase-8 (Fig. 3.8A). Therefore these findings suggested that AE-induced apoptosis is not cell line specific and it is believed that the apoptosis process involved the intrinsic pathway in human hepatoma cells.

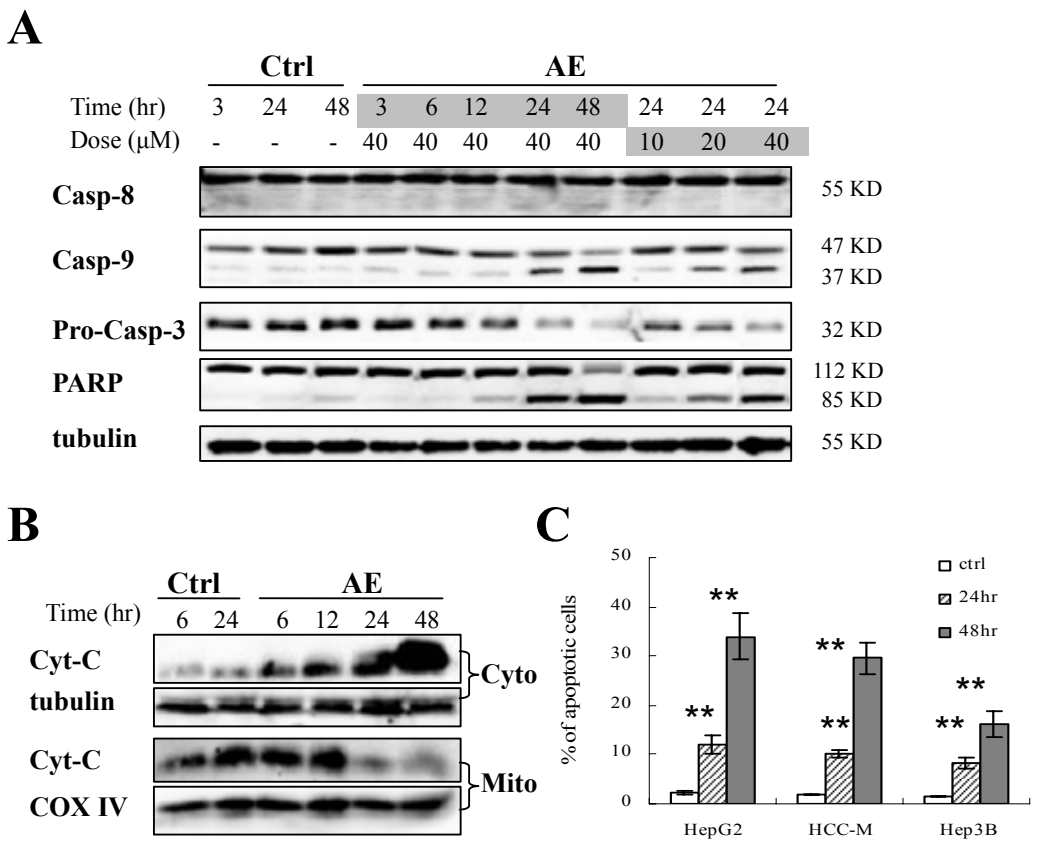


Fig. 3.1 AE induced mitochondrial-mediated apoptosis. (A). After the indicated treatment, HepG2 cells were collected and subjected to Western blotting for detection of cleavage of procaspase-3, -8, -9 and PARP. (B). Mitochondrial and cytosolic fractions from cells treated with/without AE were fractionated as described in Materials and Methods. Cytochrome c was then probed in each fraction, with COX IV as mitochondrial loading control and tubulin as the cytosolic loading control. (C). Three different hepatoma cells (HepG2, HCC-M and Hep3B) were incubated with 40 μM AE for 24 and 48 hrs. Cell death was determined by DAPI staining as above.

** $p<0.05$ when compared with control cells.

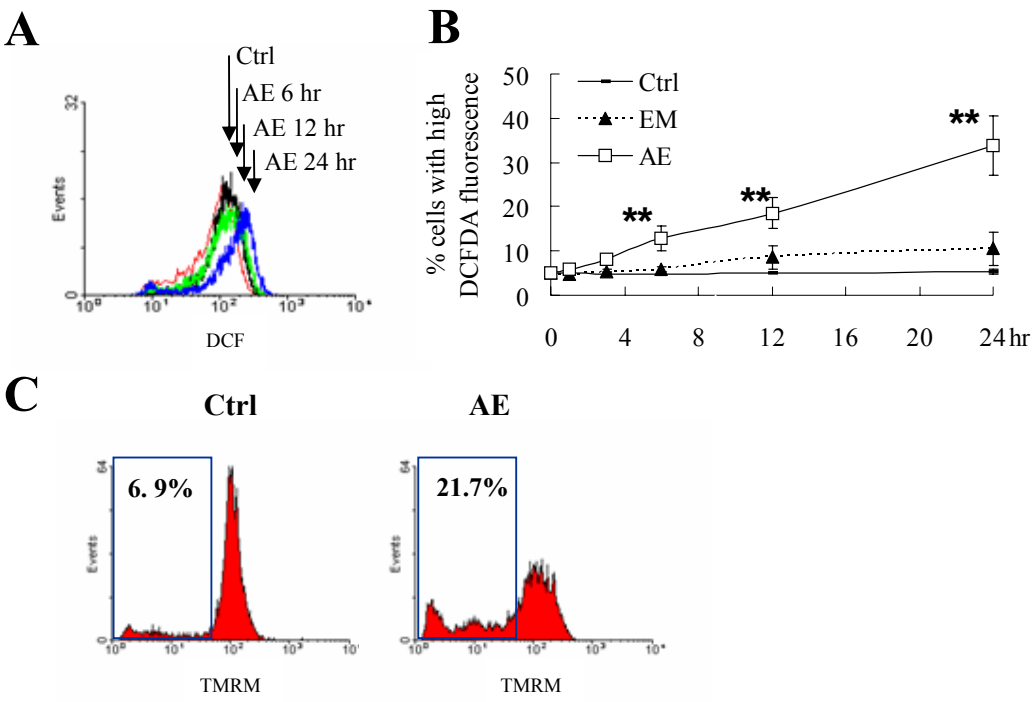


Fig. 3.2 AE increased ROS generation but decreased MOMP. (A) Kinetics of AE-induced ROS generation in HepG2 cells was measured using CM-H_2DCFDA (5μM). Cells were treated with 40 μM AE as indicated. The cells were stained with CM-H_2DCFDA (5 μM, 30mins) and then washed with PBS before measurement of the DCFDA intensity by flow cytometry. (B). DCFDA assay were carried out as (A). The percentage of cells with high DCFDA fluorescence in cells treated with 40 μM AE, EM or DMSO (for control cell) for the indicated time is shown as means ± SD (n=5). ** $p<0.01$ when compared to control group. (C). AE decreased mitochondrial outer membrane potential (MOMP). HepG2 cells were treated with/without 40 μM AE for 24 hr. Cells were then collected and stained with 200 nM TMRM for the determination of MOMP.

3.3.2 AE induced oxidative stress

The up-regulation of several redox-sensitive proteins found by 2D-DIGE suggested that oxidative stress might be involved in the cytotoxicity of AE. To confirm this, CM-H_2DCFDA, a ROS indicator was utilized to study the production of ROS. As shown in Fig. 3.2 A and B, HepG2 cells exhibited a time-dependent increase of ROS production. Similar results were also obtained in the HCC-M and Hep3B cells (Fig. 3.8B). This result was comparable to a report for human lung carcinoma cells (Lee *et al.*, 2006a), thus suggesting AE was capable of inducing oxidative stress in cancer cells. It is interesting to note that although EM is similar in structure to AE, it only caused minimal ROS production in HepG2 cells, corresponding to its ability of inducing a less extensive and lower level of redox-sensitive proteins, as shown by the 2D-DIGE result (Fig. 2.5 and Table 2.2).

3.3.3 AE induced protein carbonyl formation

Oxidative stress may cause reversible or irreversible damage to DNA, lipid and/or certain proteins and in turn affectes the cell fate by inducing cell death or cell senescence. It has been reported that AE induced DNA damage through ROS generation (Lee *et al.*, 2006a), but the authors did not show whether DNA damage accounted for AE-induced cytotoxicity or not. On the other hand, oxidatively modified proteins had been shown to play an important role in the modulation of protein function, cellular signaling and a number of human diseases (Nystrom, 2005). Assessment of the irreversibly protein-bound carbonyls, a widely used biomarker for protein oxidative modification (Levine, 2002), was thus conducted in this study.

As shown in Fig. 3.3A, in HepG2 cells AE treatment time-dependently increased the content of protein carbonyls, suggesting that AE treatment caused irreversible protein oxidation. Unfortunately, in 1D Western blotting, the proteins with carbonyl modifications were hard to be identified. However, as shown in Fig. 3.3B, the proteins with protein carbonyl were well separated and detected in 2D Western blotting. The positions of these proteins in the 2D map of HepG2 cells indicated that beta-tubulin (TBB1), 60 KD heat shock protein (CH60) and protein disulfide-isomerase A3 (PDIA3) were oxidatively modified via protein carbonyl formation (Fig. 3.3B). Protein oxidation by carbonyl formation in HSP60 and beta-tubulin under oxidative stress had been reported before (Aksenov *et al.*, 2001; Levine, 2002). Protein carbonyls may escape proteolysis and form cytotoxic high molecular weight aggregates (Nystrom, 2005), but the exact functional implications of protein carbonyl formation in AE-induced cytotoxicity has yet to be determined.

3.3.4 AE induced peroxiredoxin oxidation

The oxidative property of AE caused protein oxidation in cells, but how this affected AE-induced cytotoxicity is still unknown. The 2D-DIGE revealed that the most highly up-regulated proteins induced by AE (Table 2.2) were peroxiredoxins, which have recently been reported to confer resistance to cell death caused by oxidative stress (Chang *et al.*, 2004; Kim *et al.*, 2000; Veal *et al.*, 2004). On the other hand, oxidation of these proteins was regarded as a sensitive marker of oxidative stress in HepG2 cells *in vitro* and liver tissue *in vivo* (Cesaratto *et al.*, 2005). The role of these proteins and their effects upon oxidation were determined in this section.

As shown in Fig. 3.4A, AE treatment caused time-dependent oxidation in peroxiredoxins when probed by a specific monoclonal antibody which recognized the sulfinic and sulfonic forms of the 2-Cys peroxiredoxin. Moreover, 2D Western studies confirmed the oxidation of PRDX-2 and -4 in the 2D map (Fig. 3.4B). It is noteworthy that the total peroxiredoxin level remained unchanged (Fig. 3.4A), suggesting that the up-regulated peroxiredoxins were predominantly in the oxidized form. In contrast, EM and DMSO vector as negative control had no effect on the oxidation of peroxiredoxins (Fig. 3.4A), which was consistent with their low cytotoxicity (Fig.3.1). Oxidation of PRDX-6 was not detected in this experiment, because PRDX-6 belonged to the 1-Cys subgroup which could not be detected by this antibody.

It is interesting to note that protein DJ-1 could also be detected by this monoclonal antibody against oxidized peroxiredoxins (Fig. 3.4B). Protein DJ-1 has a similar homology to the carboxyl-terminal domain of catalase in *Escherichia coli*. In human umbilical vein endothelial cells, DJ-1 was also found to be sensitive to H_2O_2-mediated oxidation (Kinumi *et al.*, 2004). Recently, it has benn reported that protein DJ-1 can scavenge H_2O_2 by oxidizing itself and in turn cause cell death (Kinumi *et al.*, 2004). This non-specific binding in the present study may be due to the fact that all three cysteines (Cys-46, -53 and -106) in DJ-1 could be oxidized to cysteine sulphonic acid (Kinumi *et al.*, 2004). Collectively, these findings provided convincing evidence that AE treatment could lead to oxidative stress and oxidation of 2-Cys peroxiredoxins.

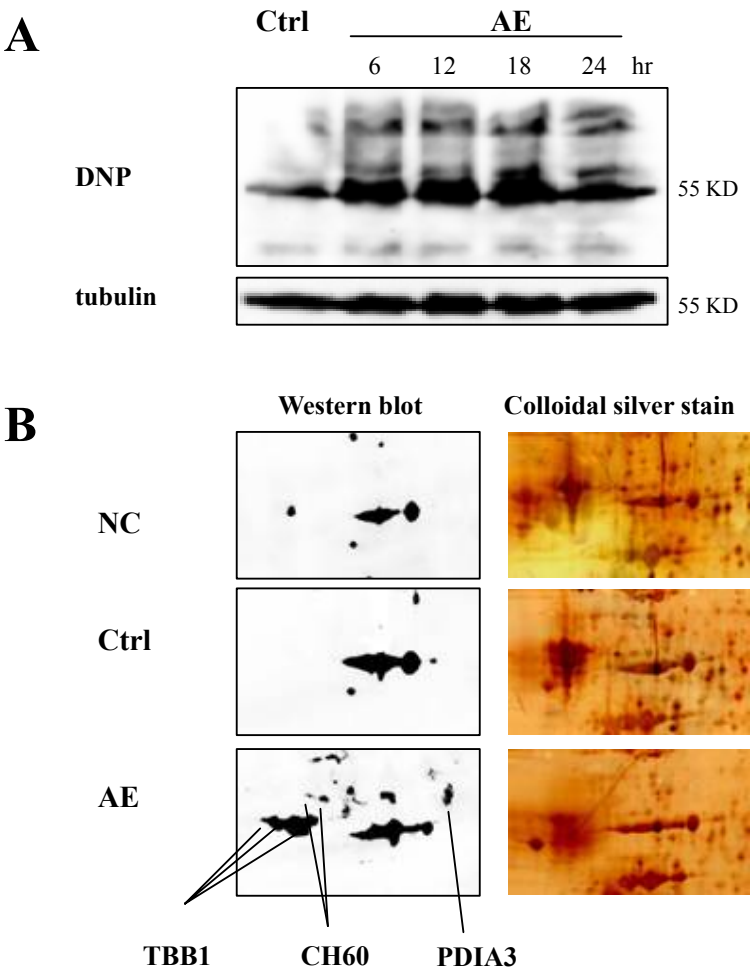


Fig. 3.3 AE induced protein carbonyl formation. (A). Protein carbonyl formation was measured by 1D Western blotting as indicated. (B). 200 μg proteins from control and AE-treated cells were loaded in 18 cm pH 4-7 IPG strips for IEF. Then the strips were incubated with (for Ctrl and AE) or without (for NC) DNPH solution before SDS-PAGE and transbloting. NC which was incubated without DNPH was used as negative control. The left panel is the Western-blot image of proteins with carbonyl modification. The right panel is the colloidal silver stained images after Western blotting of the same PVDF membrane, which represented the overall protein profile.

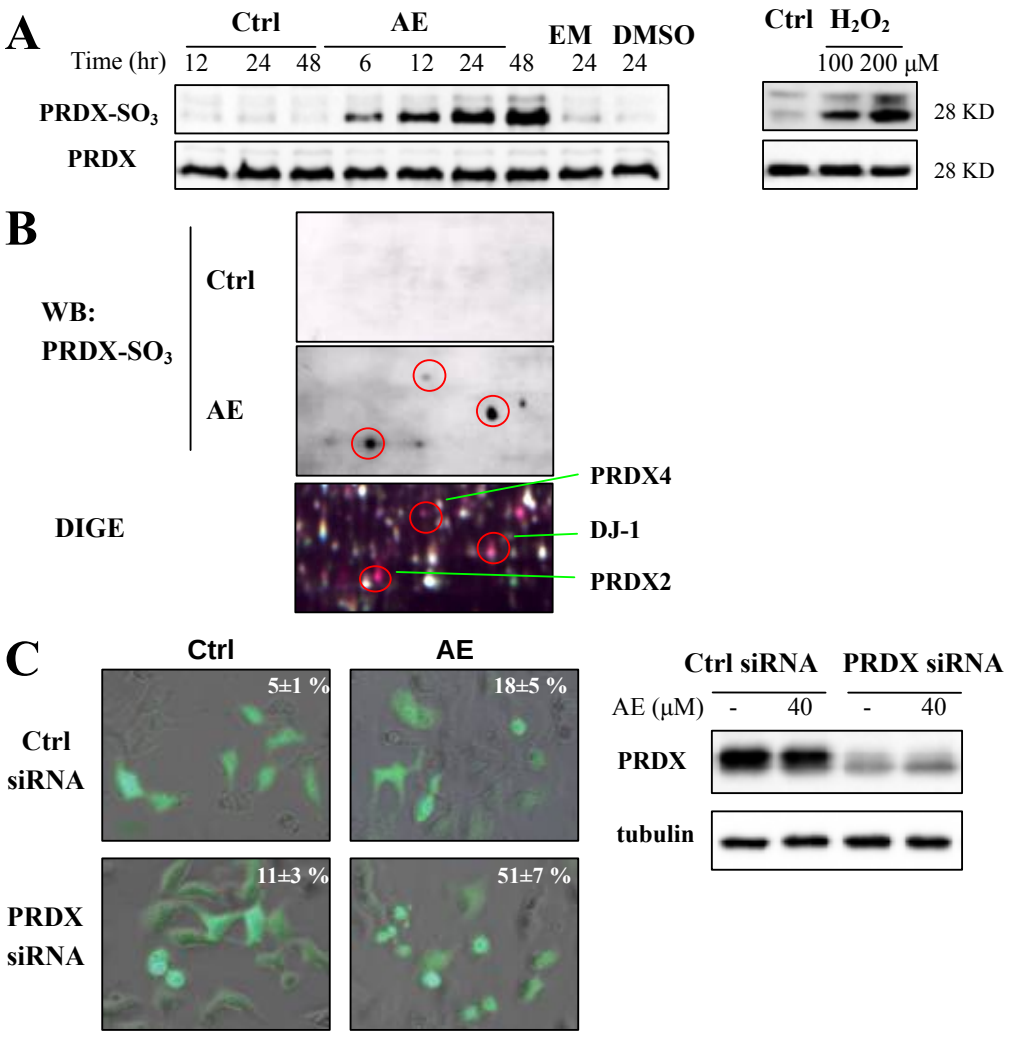


Fig. 3.4 AE induced peroxiredoxin oxidation. (A). After the indicated treatment (with H_2O_2 as a positive control), peroxiredoxin oxidation was detected by 1D Western blotting. (B). 2D-Western of peroxiredoxin oxidation. Peroxiredoxin oxidation in cells treated with AE (40 μM, 24 hr) was analyzed by 2D-Western blot. The bottom panel is a representative 2D-DIGE image labeled with identified proteins as indicated. (C). HepG2 cells were transfected with PRDX siRNA or control siRNA, together with EGFP as a transfection marker. The cells after treatment were visualized under fluorescent microscopy and harvested for Western blotting.

3.3.5 AE induced cell death by exhausting intracellular GSH

Peroxiredoxin functions as a protein antioxidant by oxidizing itself. As expected, transient knocking-down of antioxidant peroxiredoxins by siRNA sensitized HepG2 cells to cell death induced by AE (Fig. 3.5C). For example, in cells with a lower expression of peroxiredoxins, a drastic increase of apoptotic cell death was induced by AE treatment, when compared with cells with normal level of peroxiredoxins (Fig. 3.5C). This finding indicated that AE treatment induced ROS-dependent apoptotic cell death. Since the cysteinyl residues of peroxiredoxins were being oxidized, it might be important to determine whether AE also affected the intracellular GSH, the predominant small-molecule antioxidant.

As shown in Fig. 3.5A, AE decreased the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) as early as after 2hr. The involvement of the GSH buffer system in AE-induced apoptosis was then examined. Pretreatment with buthionine-L-sulfoximine (BSO, 500 μM for 24hrs), a specific GSH synthesis inhibitor (Shen *et al.*, 2000), significantly decreased the intracellular GSH level (Fig. 3.5B) and augmented apoptosis by AE (Fig. 3.5C). In contrast, a GSH donor glutathione monomethyl ester (GSH-MEE, 5 mM for 2hrs) (Kannan *et al.*, 2004) significantly enhanced the intracellular GSH level (Fig. 3.5B) and subsequently blocked AE-induced apoptosis (Fig. 3.5C).

Collectively, the above results suggested that AE treatment may induce apoptotic cell death by inducing oxidative stress and exhausting the intracellular GSH buffer system.

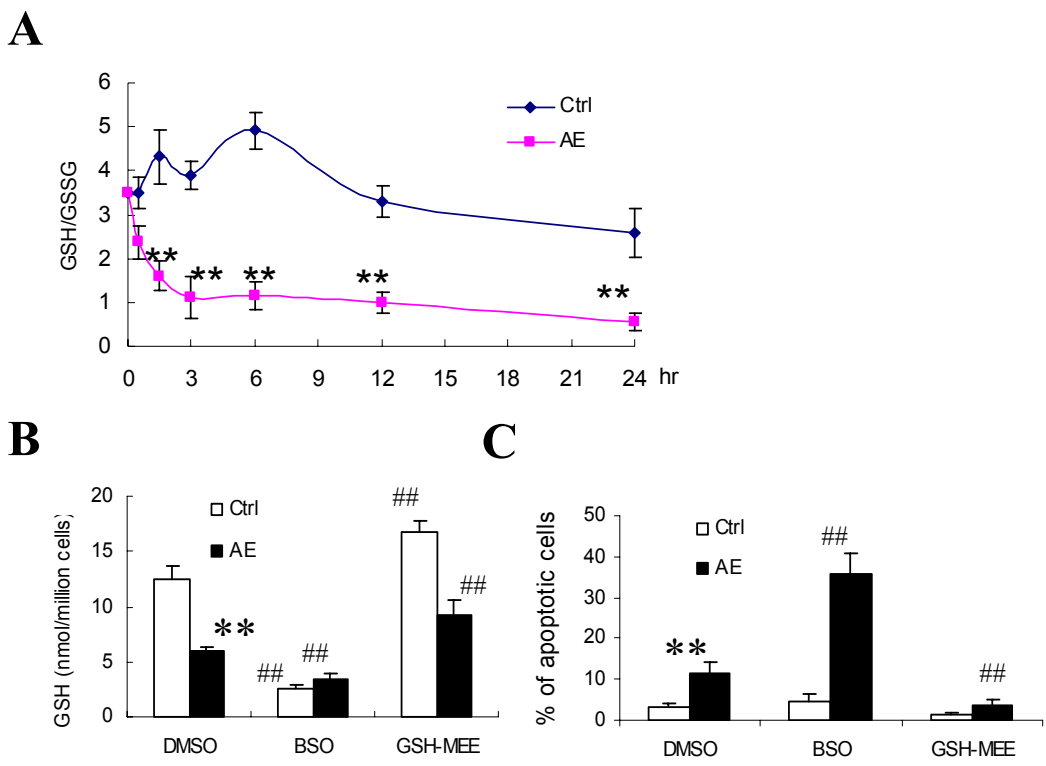


Fig. 3.5 AE induced cell death by exhausting intracellular GSH. (A). At the end of the indicated treatments, the ratio of intracellular GSH and GSSG level was analyzed, as described in Material and Methods. (B, C). After pretreatment with GSH synthesis inhibitor, buthionine-L-sulfoximine (BSO, 500 μM for 24 hr) or GSH donor, glutathione monomethylester (GSH-MEE, 5 mM for 2 hr), cells were additionally treated with AE for 6 hr to determine the intracellular GSH level (B) or 24 hr to determine apoptotic cell death (C). The GSH and GSSG content were expressed in nanomoles per million cells. The percentage of apoptotic cell death were determined and counted by DAPI staining as mentioned above.

** indicates $p<0.05$ when compared with control cells.

indicates $p<0.05$ when compared with cells treated with AE only.

3.3.6 AE induced sustained activation of JNK

It has been well established that MAPKs such as JNK are redox sensitive and are involved in apoptosis (Kyriakis and Avruch, 2001; Lewis *et al.*, 1998; Shen and Liu, 2006). Thus it might be important to examine whether the redox-sensitive MAPK pathways (JNK, p38 and ERK) were involved in AE-induced hepatoma cell apoptosis. As shown in Fig. 3.6, AE treatment induced sustained phosphorylation/activation of JNK from 3 hr up to 48 hr, which is also evidenced by the phosphorylation of its downstream substrate c-Jun. On the contrary, no evident JNK and c-Jun phosphorylation could be found within the first 1 hr (Fig. 3.7). Thus AE-induced JNK activation was a delayed and sustained action, but not an early and transient process. On the other hand, phosphorylation/activation of p38 remained unchanged by AE (Fig. 3.6), although AE had been reported to induce apoptosis in human lung non small cell carcinoma cell line H460 via the p38 pathway (Yeh *et al.*, 2003). The pro-survival ERK pathway, however, was found to be inhibited by AE, as evidenced by the decreased phosphorylation/activation of ERK. This inhibitory effect was similar to the finding carried out using glioma cells (Kannan *et al.*, 2004). It is noteworthy that peroxiredoxin oxidation occurred coincidently with JNK activation and ERK inhibition, implying an inherent association of oxidative stress and JNK activation and/or ERK deactivation (Fig. 3.6). The JNK activation in HCC-M and Hep3B cells were also investigated. Similar results of sustained JNK activation, together with caspase cleavage/activation and peroxiredoxin oxidation were observed (Fig. 3.8A); suggesting that sustained JNK activation may contribute to AE-induced cell death.

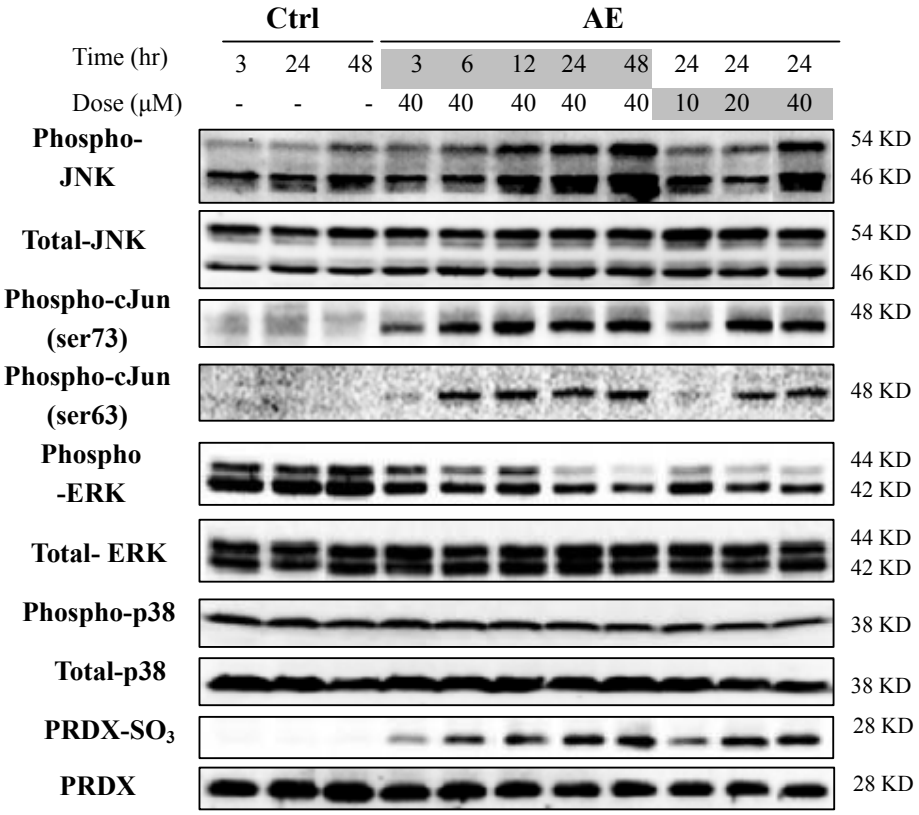


Fig. 3.6 AE induced sustained JNK activation and ERK inhibition**.** HepG2 cells were treated with the indicated dose of AE for a series of times as indicated. The phosphorylation/activation of JNK, ERK, p38 and peroxiredoxin oxidation was detected by Western blotting.

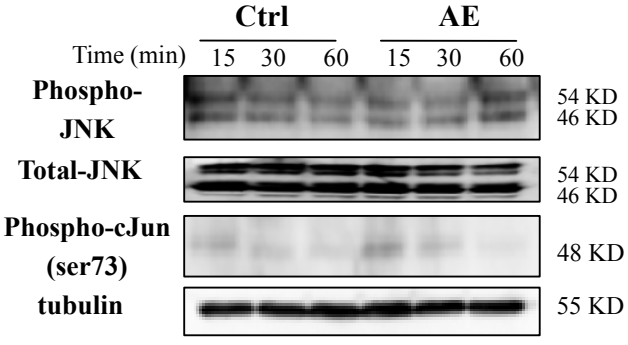


Fig. 3.7 AE was incapable of activating JNK within 1 hr**.** HepG2 cells were treated with 40 μM AE for the indicated time. The phosphorylation/activation of JNK and c-Jun was detected by Western blotting.

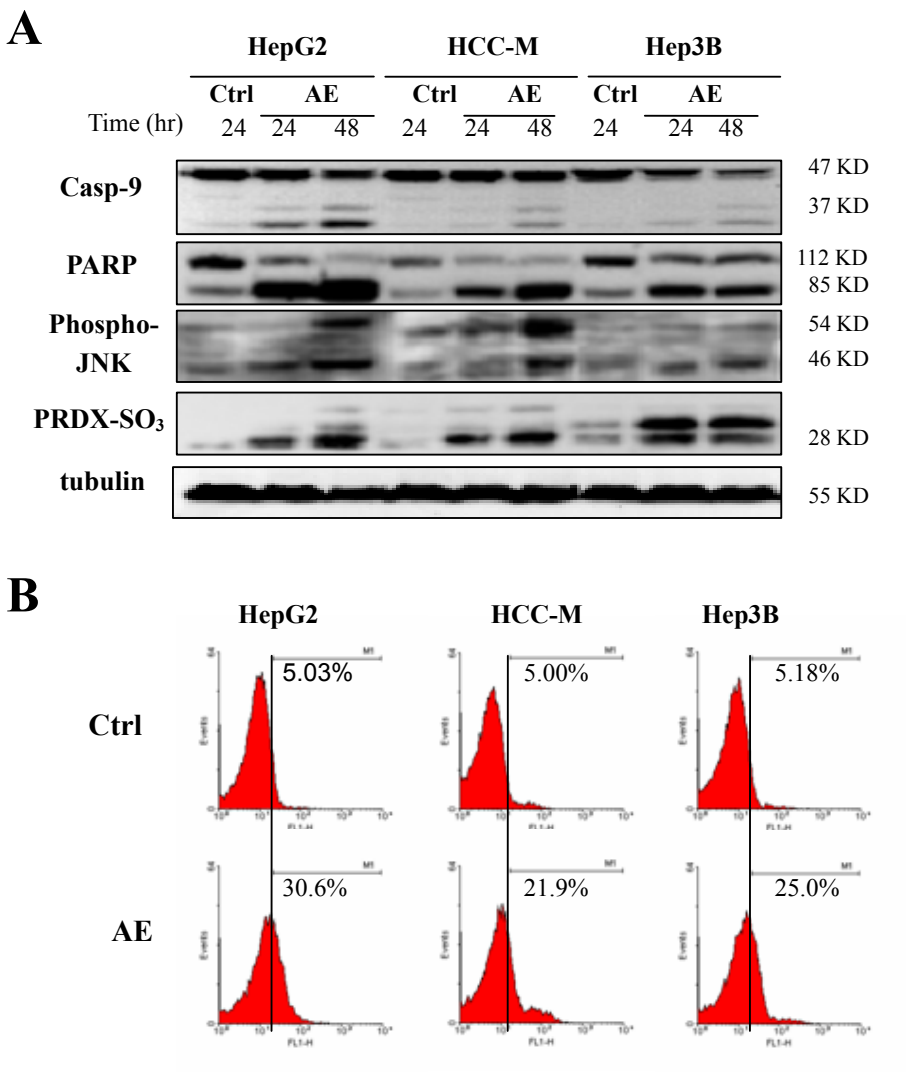


Fig. 3.8 AE induced oxidative stress, JNK activation and apoptosis in hepatoma cells**.** (A) HepG2, HCC-M and Hep3B cells were treated with 40 μM AE for the indicated times. At the end of treatment, the cells were collected and lysed. The caspase cleavage/activation, JNK phosphorylation/activation, and peroxiredoxin oxidation were detected by Western blotting. (B) HepG2, HCC-M and Hep3B cells were treated with 40 μM AE for 24 hr. The ROS generation was detected by DCF assay as indicated in the Material and Methods.

3.3.7 AE-induced apoptosis and JNK activation was ROS-dependent

To further investigate the association of oxidative stress with MAPK activation and apoptosis, antioxidant sod1 (pEGFP-C3/sod1) was over-expressed in HepG2 cells. Over 90% cells were transfected with EGFP-sod1 and the expression level sustained as long as 72 hr (data not shown). As shown in Fig. 3.9A, over-expression of antioxidant sod1 efficiently prevented AE-induced oxidative stress, as indicated by the significant decrease of peroxiredoxin oxidation (Fig. 3.9A). Coincidently, over-expression of sod1 decreased AE-induced JNK phosphorylation/activation (Fig. 3.9B). As a result of the decreased oxidative stress and JNK activation, sod1 over-expression substantially protected HepG2 cells against AE-induced apoptosis, as shown by the decrease of caspase-9 activation and PARP cleavage (Fig. 3.9A) and the percentage of cells with nuclear condensation (Fig. 3.9B). Taken together, the above results suggested that oxidative stress-mediated JNK may be the effector molecule in AE-induced apoptosis.

On the contrary, AE-induced ERK deactivation was unaffected by sod1 over-expression (Fig. 3.9A), although the level of AE-induced apoptosis was comparatively lower in sod1 over-expressed cells. It may suggest that ERK inhibition by AE was ROS-independent. It may also indicate that ERK inhibition was dispensable for AE-induced apoptosis. Whether AE could directly inhibit ERK or its upstream kinase has yet to be established, but its analogue EM had been shown to be a direct kinase inhibitor of ERK (Zhou *et al.*, 2006).

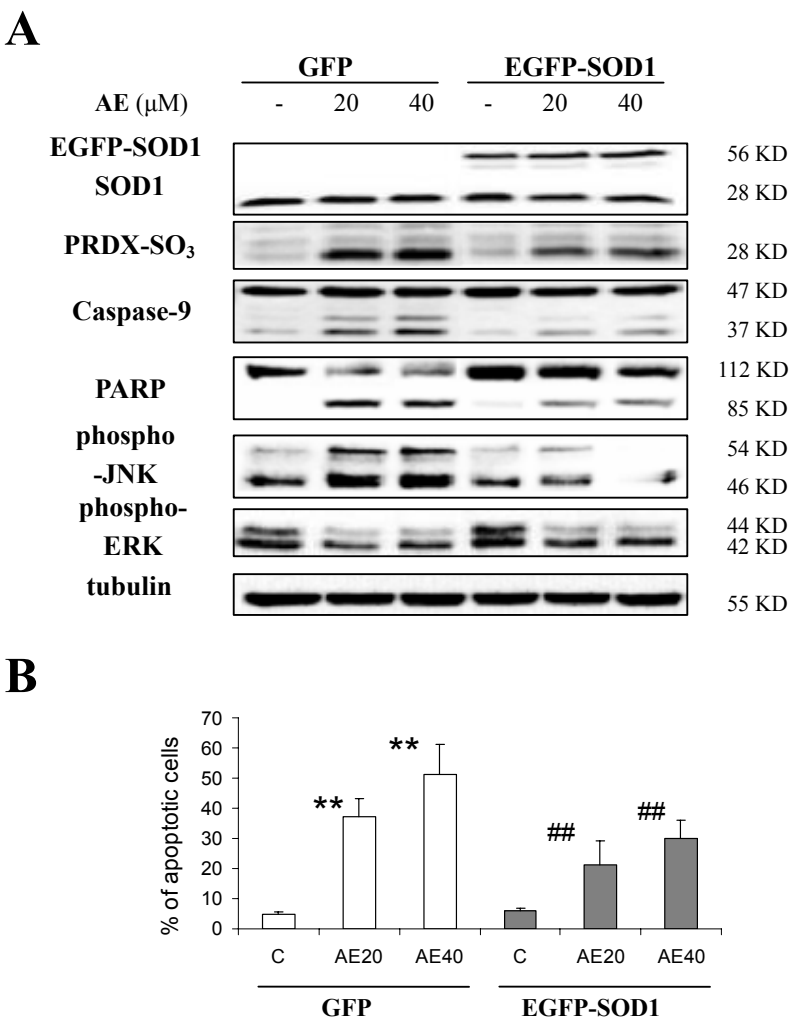


Fig. 3.9 Over-expression of antioxidant sod1 reduced AE-induced JNK activation and apoptosis by AE. HepG2 Cells were electro-transfected with sod1-GFP. After 36 hr, cells were treated with AE or DMSO for 24 hr. (A). Cleavage of caspase-9 and PARP, oxidation of peroxiredoxin and phosphorylation/activation of JNK were investigated by Western blotting. (B) The percentage of apoptotic cells (with shrunk cell morphology) among the transfected cells was determined from a total of 200 randomly selected cells.

3.3.8 JNK activation played a crucial role in AE-induced apoptosis

The role of the JNK pathway in apoptosis is both cell type- and stimulus-dependent. The proapoptotic effect of JNK also depended on the extent and duration of its activation (Shen and Liu, 2006). Thus it is important to evaluate the role of JNK activation in AE-induced apoptosis through both pharmacological and genetic approaches. On the one hand, it was found that AE–induced apoptosis could be partially prevented by the cell permeable JNK inhibitor III (Fig. 3.10A).

On the other hand, AE-induced apoptosis could be blocked by either knocking-down JNK or exogenously expressing dominant negative JNK. Knocking-down of both JNK-1 and -2 genes by specific siRNA significantly decreased JNK expression level as well as its phosphorylation/activation status, when compared with cells transfected with control siRNA (Fig. 3.10B). As expected, AE-induced apoptotic cell death was also inhibited, as evidenced by the decline in the cleavage of caspase-9, PARP (Fig. 3.10B) and the percentage of cell death (Fig. 3.10C). Taken together, JNK activation was involved in AE-induced apoptosis. This hypothesis was further supported by the protective effect of over-expressing dominant negative JNK1 and JNK2 (DN-JNK1/2). Similar to the result of knocking-down assay, inhibition of JNK activity by over-expression of DN-JNK1/2 protected cells from AE-induced apoptosis (Figs. 3.10D and E). On the contrary, over-expression of constitutively active JNKK2-JNK1, which maintained sustained JNK1 activation, consistently augmented AE-induced apoptosis (Fig. 3.12A). In summary, the above findings indicated that sustained JNK activation was crucial for AE-induced apoptosis.

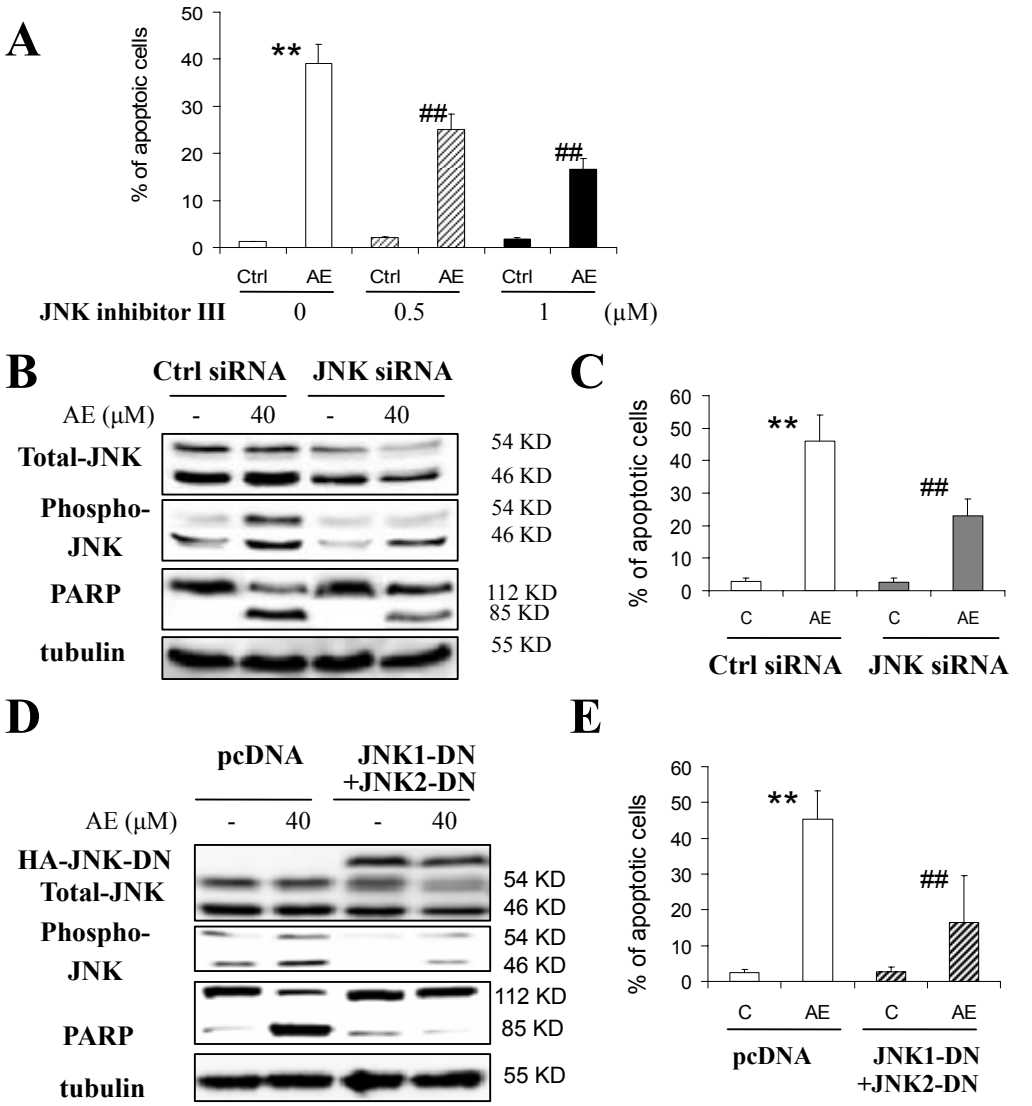


Fig. 3.10. JNK activation by AE was crucial for AE-induced apoptosis. (A). Cell death was determined after pretreatment with cell permeable JNK inhibitor III for 1 hr and then 40 μM AE for 48 hr. (B, C). JNK1 and JNK2 (JNK1/2) were transiently knocked-down in HepG2 cells by specific siRNA. After 48 hr, transfected cells were treated with AE for 24 hr. (D, E) HepG2 cells were transiently transfected with dominant negative JNK1 and JNK2 protein vector (HA-JNK-DN) and then treated with AE for 24 hr. Cleavage of caspase-9 and PARP, oxidation of peroxiredoxin and phosphorylation/activation of JNK were investigated by Western blotting.

3.3.9 ASK1 enhanced JNK activation and AE-induced apoptosis

It is well-established that oxidative stress can induce JNK activation by activating its upstream apoptosis signal-regulating kinase1 (ASK1) (Shen and Liu, 2006). The activation of redox-sensitive ASK1 has been reported to be initiated by ASK1 oligomerization after dissociation from its inhibitory binding partner Trx in the presence of oxidative stress (Saitoh *et al.*, 1998). Here the role of redox-sensitive ASK1 in AE-induced apoptosis was examined. As shown in Fig. 3.11A, AE induced sustained ASK1 activation from 6 hr to 24 hr by phosphorylation at Thr845, a conserved site for ASK1 activation by ROS (Tobiume *et al.*, 2002), while the total ASK1 expression level remained unchanged. Coincidently, ASK1 that co-precipitated with Trx was also decreased (Fig. 3.11B); suggesting that ASK1 activation is initiated by its dissociation from Trx.

To further assess the role of ASK1 activation in AE-induced sustained JNK activation and apoptosis, transient over-expression of mutant ASK1 with the C-terminal coiled-coil domain deletion (a dominant negative mutant for ASK1 oligomerization (Tobiume *et al.*, 2002)) was carried out. As expected, over-expression of the redox-insensitive mutant ASK1 failed to phosphorylate JNK (Fig. 3.11C) and in turn prevented AE-induced apoptosis (Fig. 3.11D). On the contrary, over-expression of the wild-type ASK1 augmented JNK phosphorylation/activation and sensitized cells to AE-induced apoptosis (Fig. 3.12B), similar to the action of over-expression of constitutively active JNKK2-JNK1. Collectively, the above data suggested that ASK1 was crucial for AE-induced sustained JNK activation and apoptosis.

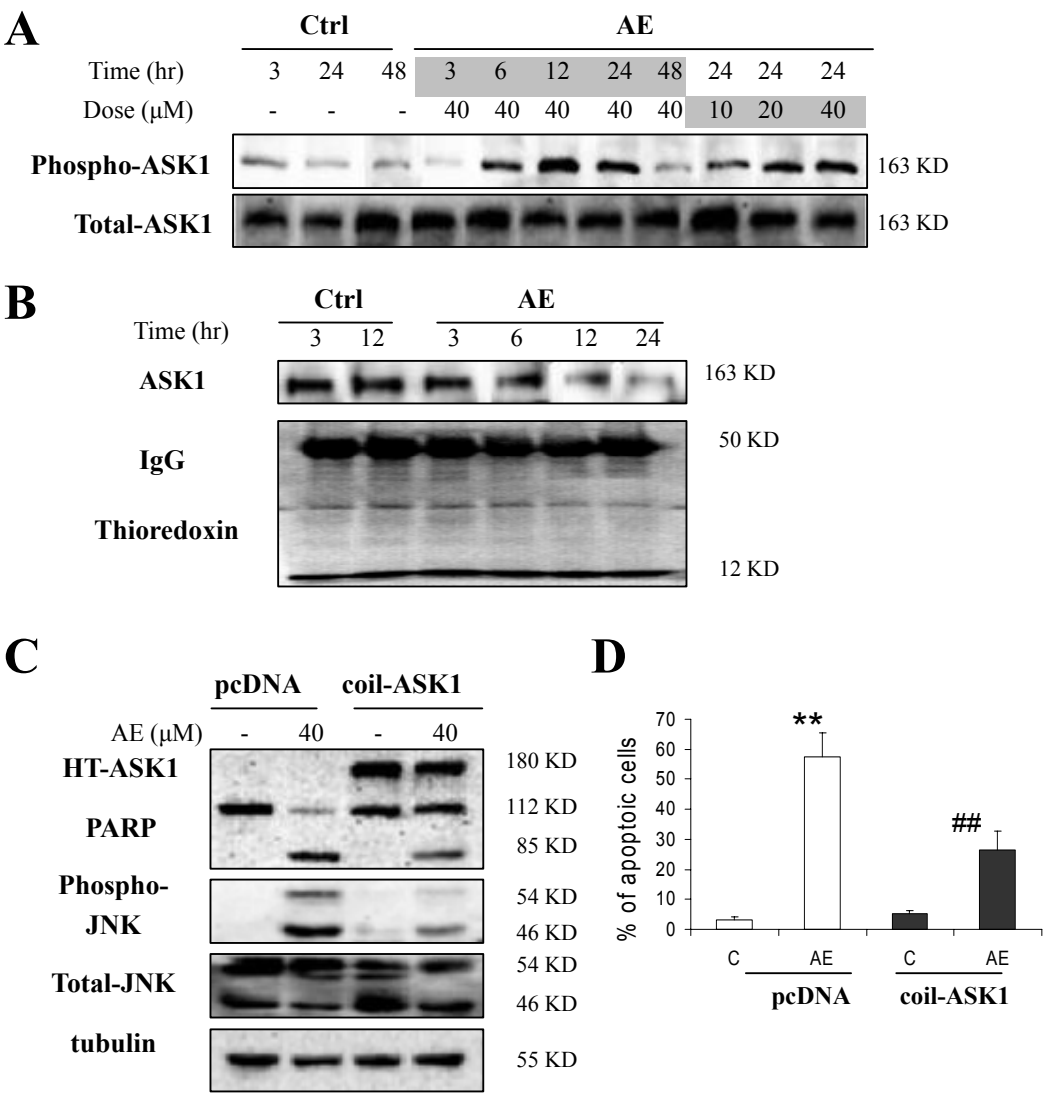


Fig. 3.11 ASK1 contributed to AE-induced JNK activation and apoptosis. (A). Active phosphorylation/activation of ASK1 (thr845) in AE-treated cells was analyzed by Western blotting. (B). ASK1 co-precipitated with Trx was decreased by AE treatment as determined by co-immunoprecipitation assay. (C, D). HepG2 cells were transiently electro-transfected with empty vector or dominant negative coiled-coil deletion mutant of ASK1 (coil-ASK1). After 36 hr, transfected cells were treated with AE for 24 hr. Western blotting showed activation of ASK1 and JNK, and cleavage of PARP, caspase-9 by AE.

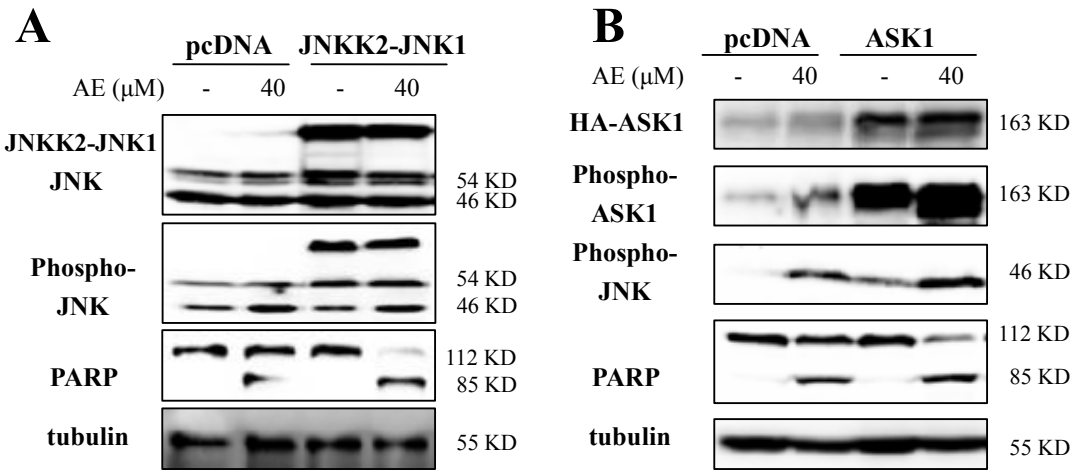


Fig. 3.12 Over-expression of JNKK2-JNK1 and ASK1 sensitized cells to AE-induced apoptosis. Cells were over-expressed with/without JNKK2-JNK1 (A) or wild type ASK1 (B) vectors before treatment with AE for 12 hr.

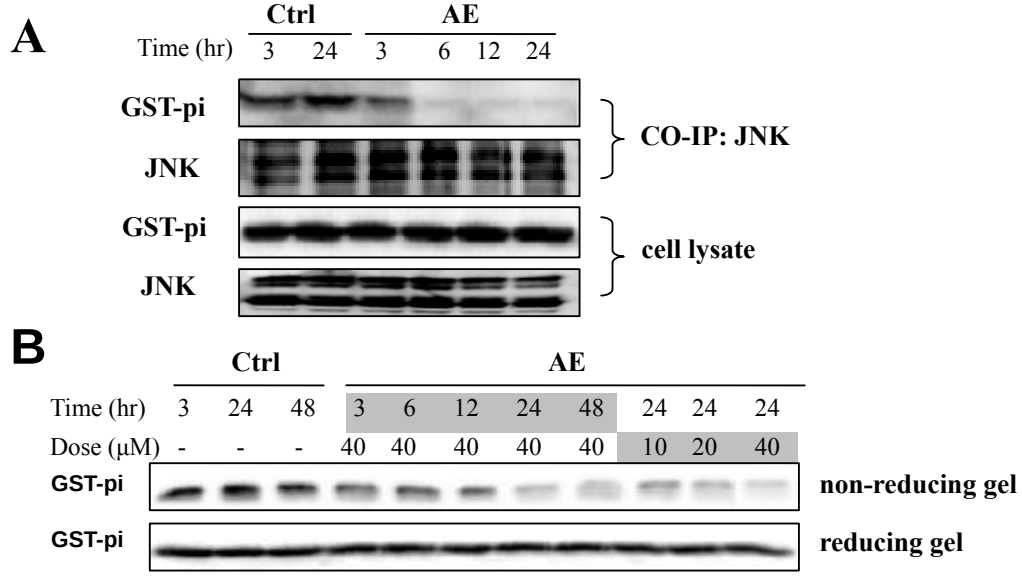


Fig. 3.13 GST-π oxidation by AE contributed to JNK activation. (A) After the indicated treatment, GST-π co-precipitated with JNK was probed as indicated. (B). After the indicated treatment, cells were lysed and loaded onto non-reducing or reducing gels as described in Material and Methods. The reduced form of GST-π in non-reducing gel and total GST-π in reducing gel was determined by Western blotting.

3.3.10 Dissociation of GST-π from JNK was involved in JNK activation

Similar to ASK1, JNK could be activated by another redox-sensitive pathway. Resembling ASK1-Trx, JNK can form an inactive complex with GST-π (Adler *et al.*, 1999). Upon oxidative stress, protein oxidation of the cystinyl residues of GST-π promoted GST-π oligomerization and released it from the inactive complex with JNK. Importantly, this module is independent of the ASK1/JNK pathway. Thus, it might be important to assess whether AE treatment could induce JNK activation via dissociation of its inhibitory binding subunit GST-π from the inactive JNK-GST-π complex, another redox-sensitive mechanism for JNK activation (Adler *et al.*, 1999).

As shown in Fig. 3.13A, the inhibitory binding of GST-π with JNK decreased from as early as 3 hr, which was similar to the time of JNK activation (Figure 3.6). This dissociation had been reported to be initiated from the oxidative cross-linking of GST-π (Adler *et al.*, 1999). Next, as shown in Fig. 3.13B, it was found that the reduced monomeric form of GST-π in the non-reducing gel but not the total GST-π in reducing gel was decreased upon treatment of AE from 3 hrs, suggesting that GST-π aggregation mediated by cysteinyl oxidation occurred upon AE treatment. This was consistent with the dissociation of inhibitory Trx from inactive ASK1-Trx complex (Fig. 3.11B). Therefore, AE may also initiate JNK activation by dissociation of the inactive JNK-GST-π complex via oxidative stress.

3.4 Discussion

In this study, it was found that AE induced apoptosis via oxidative stress and sustained JNK activation in hepatoma cells.

Sustained oxidative stresses are maintained in cancer cells (Toyokuni *et al.*, 1995). This high but tolerable production of reactive oxygen species (ROS) may help cancer cells survive and proliferate through activating redox-sensitive transcription factors and responsive genes (e.g. NF-*k*B and AP1). However, when intolerably high levels of ROS production (e.g. induced by therapeutic agents) reached certain threshold, such as irreversible DNA damage, cells may switch to senescence or apoptotic cell death (Buttke and Sandstrom, 1995). Through manipulation of the redox balance, some phytochemicals, such as anthraquinones from rhubarb (Huang *et al.*, 2006a), and polyphenols from grapes (Delmas *et al.*, 2003) seemed to be good candidates for a direct or combined application in cancer chemotherapeutics and/or chemopreventics. But on the one hand, AE and other anthraquinones can counteract the harmful oxidative injury caused by other strong oxidants (Arosio *et al.*, 2000; Lin *et al.*, 1996; Malterud *et al.*, 1993). On the other hand, therapeutic dose of AE and other anthraquinones can selectively induce apoptosis via sustained and site-directed oxidative stress (Lee *et al.*, 2006a).

Owing to its quinone structure, AE may generate ROS in cells. This effect had been confirmed recently (Lee *et al.*, 2006a) and by the present study. To exclude the possibility that the cytotoxicity induced by AE was derived from an artificial production of H_2O_2 in the culture medium, the amount of H_2O_2 in the culture medium (without cell) was determined by ferrous oxidation-xylenol orange FOX assay (Nourooz-Zadeh *et al.*, 1994). Less than 10 μM H_2O_2 could be detected (data not shown) when AE was incubated alone in DMEM medium for as long as 24 hr. Thus,

AE was unlikely to induce its cytotoxicity via artificial production of H_2O_2 in the culture medium.

The results in the present study showed that exposure of AE interrupted the redox balance and decreased the intracellular GSH/GSSG ratio and in turn induced the intrinsic apoptotic pathway. Pretreatment of the cell-permeable GSH synthesis inhibitor BSO sensitized AE-induced cell death, while the glutathione-donor GSH-MEE completely protected it (Fig. 3.5). Consistently, over-expression of antioxidant enzyme sod1 effectively protected cells from AE-induced apoptosis (Fig. 3.9). These results clearly suggested the crucial proapoptotic role of oxidative stress in AE-induced apoptosis.

Protein oxidation, especially on the cysteinyl residues, may be crucial for AE-induced apoptosis. In most eukaryotes, peroxiredoxins are highly conserved and are highly abundant antioxidant enzymes (Wood *et al.*, 2003). Oxidation of peroxiredoxins, as a sensitive marker for protein oxidation in HepG2 cells *in vitro* and liver *in vi*vo (Cesaratto *et al.*, 2005), was found to increase dose- and time-dependently after AE treatment in the present study (Fig. 3.4A). It has been reported that oxidation of the unique peroxiredoxin homolog Tpx1 in Schizosaccharomytes *pombe* was essential for peroxide-induced activation of the JNK/p38 homologue Sty1 (Veal *et al.*, 2004). Wood and his colleagues (Wood *et al.*, 2003) suggested that eukaryotic peroxiredoxins evolved to act as a “floodgate”: peroxiredoxins are sensitive to H_2O_2 but can be rapidly inactivated to allow increasing H_2O_2 levels to stimulate signal transduction. In the present study, knocking-down of

peroxiredoxins sensitized the cells to AE-induced apoptosis (Fig. 3.4B); suggesting that peroxiredoxin oxidation may play an anti-apoptotic role. Although peroxiredoxins normally function to protect cells from oxidative stress-induced apoptotic cell death (Chang *et al.*, 2004; Kim *et al.*, 2000), the physiological association of peroxiredoxins and JNK in mammalian cells and the proposed "floodgate" effect of endogenous peroxiredoxins (Wood *et al.*, 2003) remained to be further investigated.

In addition, protein oxidation in terms of extra-molecular cross-linking occurred in GST-π and decreased its reduced monomeric form (Fig. 3.13B), which had been reported as an essential redox-sensitive pathway for JNK activation (Adler *et al.*, 1999). The coincidence in the decrease of GST-π co-precipitation with JNK and JNK activation suggested that JNK activation was initiated by AE-induced GST-π oxidation (Fig. 3.13A). In addition, ASK1 (the JNK upstream kinase) could be activated in a similar mode by dissociation from its inhibitory binding subunit Trx in the presence of ROS (Saitoh *et al.*, 1998). Consistently, Trx that co-precipitated with ASK1 was decreased from 6 hrs onwards (Fig. 3.11B), which may in turn activate ASK1. Taken together, AE-induced oxidative stress may activate JNK and ASK1 by oxidizing their respective redox-sensitive inhibitory binding subunit GST-π and Trx.

It has now been well recognized that activation of JNK consisted of two different modes. The earlier and transient activation was through the signaling cascade of proinflammatory cytokines (tumor necrosis factor-α and interleukin-1), while the delayed and sustained activation was mediated by ROS (Shen and Liu, 2006; Shen and Pervaiz, 2006). On the other hand, the proapoptotic effect of JNK depended on the

extent and duration of its activation: sustained JNK activation may lead to apoptotic cell death, whereas transient activation was not capable of inducing apoptosis and even have an anti-apoptotic effect. The sustained JNK activation was suggested to be mainly due to its upstream kinase ASK1, another redox-sensitive proapoptotic kinase (Shen and Liu, 2006). ASK1 is a ubiquitously expressed MAPKKK that activate both JNK and p38 by phosphorylating the respective MAPKKs. In the presence of ROS, ASK1 itself was activated by oligomerization after dissociation from its inhibitory binding subunit Trx (Tobiume *et al.*, 2002).

Induction of differentiation but not apoptosis had been observed to be accompanied with ERK inhibition (within 4 hr) in rat C6 glioma cells by AE treatment, although JNK and p38 pathways were not involved (Mijatovic *et al.*, 2005a). However, in that study the delayed and sustained mode of JNK activation was not investigated. In the present study, AE treatment resulted in sustained activation of JNK which was responsible, at least partially, for apoptosis induction in the hepatoma cells. It is clearly demonstrated by the findings that AE-induced apoptosis could be reversed by both pretreatment of pharmacological JNK inhibitor and genetic manipulation of JNK expression. This action by AE could also be found in some other dietary phytochemicals. For example, diallyl disulfide (an oil-soluble compound in garlic extracts), curcumin,(a polyphenolic pigment in *Curcuma longa*), phenethyl isothiocyanate (PEITC, from watercress) and epigallocatechin-3-gallate (EGCG, from green tea extract) were found to be capable of inducing apoptosis via ROS-dependent JNK signaling cascade in neuroblastoma (Filomeni *et al.*, 2003), colon cancer (Collett

and Campbell, 2004; Hu *et al.*, 2003) and pancreatic cancer cells (Qanungo *et al.*, 2005), respectively. Results from these studies and the present finding therefore suggested that certain phytochemicals may activate the therapeutic redox-sensitive pathways (e.g. JNK, p38) and apoptosis in cancer cells, via increasing ROS production and/or decreasing ROS scavenging capacity.

The ERK deactivation by AE was found to be ROS-independent and not involved in AE-induced apoptosis because over-expression of antioxidant sod1 could not reverse AE-induced ERK deactivation (Fig. 3.9). But the exact mechanism of ERK inhibition by AE is yet to be determined. One possibility is that AE might directly inhibit ERK and/or its upstream kinase, and this action is ROS-independent, because its analogue EM had been found to moderately inhibit the ERK kinase assay (Sarno *et al.*, 2003).

In conclusion, the present study elucidated a novel anticancer function of AE in hepatoma cells. These findings suggested that AE may induce apoptosis via marked oxidative stress and sustained JNK activation.

CHAPTER 4

BIOCHEMICAL CHARACTERIZATION OF OTHER ANTI-CANCER PROTEINS AFFECTED BY ALOE-EMODIN

4.1 Introduction

Accumulating data had shown that AE was involved in a myriad of functions, including anti-cancer, hepatoprotective and other effects, although the precise molecular mechanisms are not fully understood. One effective strategy is to find the key molecular pathways that are involved in these molecular functions via proteomics. This approach had been successfully applied in the study of lung cancer cells (Lee *et al.*, 2005). However the author focused on only one affected protein, nucleophosmin. It was suggested that the release and degradation of nucleophosmin from the nucleus to the cytoplasm was associated with AE-induced apoptosis. Unfortunately, the other differentially- expressed proteins in that study were not characterized.

As described in Chapter 2, a more reliable and quantitative 2D method, 2D-DIGE, was used to obtain a global view of the differentially- expressed proteins in hepatoma cells affected by AE here. Our results revealed that several proteins were associated with oxidative stress and/or anti-cancer effects (Fig. 2.7). On the one hand, the oxidative stress and protein oxidation induced by AE treatment was found to account for the sustained activation of the proapoptotic ASK1/JNK pathway and subsequent apoptotic cell death.

On the other hand, the other differentially expressed proteins may also be important for the AE-induced anti-cancer actions. For example, p16 is a well-established CDK4 inhibitor and capable of inhibiting DNA synthesis (Kim and Sharpless, 2006; Sherr, 2004), while nucleoside diphosphate kinase A (NDKA) is a potent metastasis inhibitor (Hartsough and Steeg, 2000). These affected proteins

indicated that the anti-cancer effects of AE might be conducted through multiple signaling pathways. It is noteworthy that these affected pathways may work synergistically, but not exclusively. However, how AE execute anti-cancer effect via these proteins is still unknown. In this Chapter, some representative proteins affected by AE will be further validated and characterized.

In Chapter 2, EM was also subjected to 2D-DIGE analysis. It was observed that EM affected fewer proteins with lower expression level changes, which is in line with its lower cytotoxicity in HepG2 cells. Among those affected proteins, phosphorylated (or acidic) form of cofilin is the most down-regulated protein (Fig. 2.5 and Table 2.2). It has been reported that active dephosphorylation of cofilin is associated with cancer metastasis (Wang *et al.*, 2006b). In addition, mitochondrial translocation of dephosphorylated cofilin was an early step in apoptosis (Chua *et al.*, 2003). Thus, in the present Chapter, the role of cofilin in EM-treated cells will be further investigated.

4.2 Materials and methods

4.2.1 Chemical and reagents

AE, EM, crystal violet and other chemicals were purchased from Sigma-Aldrich (St Louis, MO). Recombinant human epithelial growth factor (EGF) was purchased from Calbiochem (San Diego, CA). The matrigel BD Falcon® 8.0 μm Cell Culture Inserts were bought from BD (Becton Dickinson, Franklin Lakes, N.J.). Antibodies against p16, cofilin and phospho-cofilin (ser 3) were obtained from Cell Signaling Technology (Beverly, Mass). Antibodies against Rb, phospho-Rb (pRb, at site ser795), NDKA and tubulin were from Santa Cruz Technology (Santa Cruz, Calif).

4.2.2 Cell culture

The human hepatoma cell lines HepG2 and HCC-M and human breast cancer cell line MD-MBA-231 cells were cultured in DMEM medium (with 100 U/ml penicillin, 100 μg/ml streptomycin, 3.7 g/L sodium bicarbonate, pH 7.4) plus 10% FBS in standard incubator conditions (37 °C, 5% CO_2).

4.2.3 BrdU incorporation assay

Bromodeoxyuridine (BrdU) incorporation assay was carried out according to the BrdU Flow Kits instruction manual (BD Pharmingen, San Diego, CA). In brief, at the end of the designated treatment period, HepG2 cells were incubated and labeled with 10 μM BrdU for 30 min at 37 °C. The newly synthesized DNA was thus incorporated with BrdU within the above incubation step. Labeled cells were then collected and fixed/permeabilized in 100 μl BD Cytofix/Cytoperm buffer for 15 min at room temperature twice. After washing with BD Perm/Wash buffer, cells were resuspended in 100 μl DNase (300 μg/ml) for 1 hr at 37 °C to expose the incorporated BrdU. Afterwards, cells were incubated with FITC-coupled anti-BrdU IgG for 20 min and later counter-stained with 7-AAD (7-amino-actinomycin D) to show the BrdU incorporation rate and total DNA content, respectively. Finally, cells were added with 1 ml staining buffer and kept in the dark at 4 °C before measurement of the FITC intensity and total DNA content by flow cytometry. The excitation/emission wavelength for detection of FITC-coupled BrdU was 488/525 nm, while for detection of 7-AAD is at 488/660 nm. The result was analyzed by WinMDI 2.8 software (Scripps Institute, La Jolla, CA).

4.2.4 Wound healing assay

Wound healing assay was conducted to study the migration potential of cancer cells upon AE or EM treatment as described previously (Huang *et al.*, 2004; , 2005). In brief, HepG2 and MD-MBA-231 cells were grown on glass coverslips in a cell culture incubator in standard condition until formation of a confluent monolayer. The cells were then starved in FBS-free DMEM medium overnight. Straight wounds were created manually using a sterile pipette tip. Then fresh medium were replaced and supplemented with/without increasing dose of AE or EM. In the meantime, cell migration was stimulated by 5% FBS or 20 ng/ml EGF. Microscopic photographs were taken at 0 and 24 hr under an inverted microscope.

4.2.5 Matrigel assay

Matrigel assay was conducted to determine the invasive potential of cancer cells using BD Falcon® 8.0 μm Cell Culture Inserts (Becton Dickinson, Franklin Lakes, N.J.), as described previously (Albini *et al.*, 1987; Huang *et al.*, 2004). In brief, after starvation in FBS-free DMEM medium overnight, HepG2 and MD-MBA-231 cells were collected using cell dissociation buffer (Sigma-Aldrich, St Louis, MO) and re-suspended in FBS-free DMEM medium. Cells were then pretreated with/without increasing dose of AE or EM for 1 hr before seeding into the transwell chambers. Afterwards, cell invasion were stimulated with 5% FBS or 20 ng/ml EGF for 20 hr. At the end of treatment period, cells that migrated to the lower surfaces of the membrane were fixed with 100% methanol for 2 min and stained with 0.5% crystal violet solution (in 25% methanol) for 2 min. Cells on top of the upper surfaces of the

membrane which failed to migrate through the membrane were removed by scraping. After rinsing with distilled water, migrated cells stained with purple color were quantified in five random fields under an inverted microscope.

4.2.6 Western blotting

The Western blotting was carried out as described earlier (section 3.2.9).

4.3 Results

4.3.1 Inhibition of DNA synthesis via up-regulation of p16 by AE

Tumor suppressor protein p16 is a member of the cyclin-dependent kinase 4 (CDK4 or INK4) inhibitor protein family. It functionally competes with cyclin-D for association with cyclin-dependent kinase 4/6 (CDK4/6) and keeping CDK4/6 inactive. The cyclin-D/CDK4 complex is known to phosphorylate the retinoblastoma (Rb) protein at mid-G1 phase, which is thought to be a critical step in the progression through G1 into S-phase of the cell cycle (Sherr, 2004).

In the present study, the 2D-DIGE results in Chapter 2 (Fig. 2.5 and Table 2.2) revealed that protein p16 was up-regulated two-fold only in AE-treated cells, but not in EM-treated cells. As shown in Fig. 4.1A, 1D Western experiment confirmed that upon treatment with AE, p16 was up-regulated after 6 hr and peaked at 12 hr of treatment. This AE-induced up-regulation of p16 could also be observed in HCC-M cells (data not shown), suggesting this effect is not HepG2 cell specific. To evaluate this effect on the downstream Rb pathway, the active phosphorylation of the downstream Rb protein (ser 795, a critical phosphorylation site for CDK4 (Grafstrom *et al.*, 1999; Pan *et al.*, 1998)) was also probed.

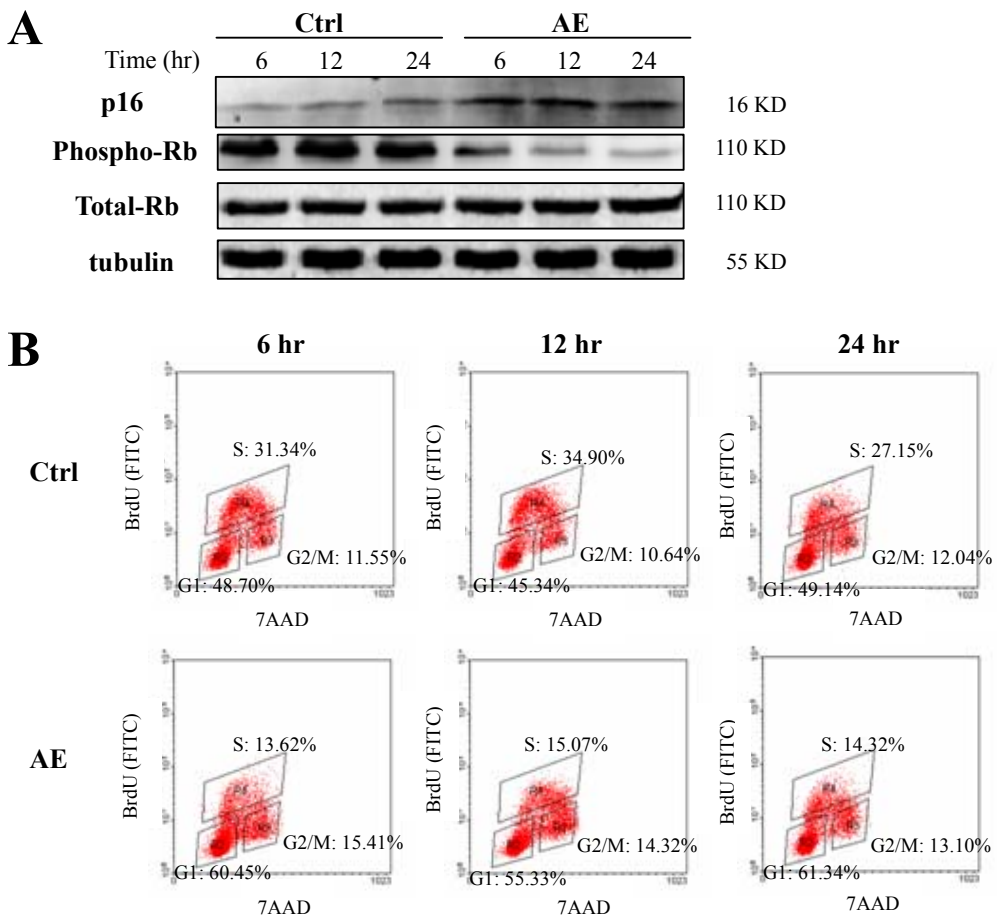


Fig. 4.1 AE inhibited DNA replication via up-regulation of p16. (A). AE affected phosphorylation of Rb protein via up-regulation of p16. HepG2 cells were treated with AE (40 μM) for the indicated time. Protein expression level of p16, phosphorylated form of Rb (ser 795, critical CDK4 phosphorylation site) and total Rb were analyzed by Western blotting. (B). AE decreased DNA synthesis. HepG2 cells were treated with 40 μM AE for the indicated time. After treatment, cells were incubated with BrdU for 30 mins, and then stained with FITC-coupled anti-BrdU and counter-stained with 7AAD. S, G1 and G2/M phase of cells are shown as indicated with the percentage of the total cell populations (n=3).

It was found that AE treatment decreased active phosphorylation of Rb protein on the CDK4 specific site Ser795 while the total Rb protein level remained unchanged (Fig 4.1A). Furthermore, the BrdU incorporation assay confirmed that there was a decrease of *de novo* DNA synthesis after treatment with AE from 6hr to 24 hr (Fig. 4.1B), which was coincident with the up-regulation of p16 and loss of Rb phosphorylation at Ser795. As shown in Fig. 2.1, AE (40 μM, 24 hr) inhibited cell growth to half the level of that of control cells, which cannot be totally explained by the marginal increase of apoptotic cell death and G2/M cell cycle arrest by AE at that time point (Fig. 2.3 and 2.4). Thus, this is the first evidence showing that the cell growth inhibition in terms of DNA synthesis inhibition, especially in the early phase, was at least partially resulted from the inactivation of pRb-E2F pathway by up-regulation of the CDK4 inhibitor p16 upon AE treatment.

4.3.2 AE inhibited cell migration via up-regulation of NDKA

Interestingly, the 2D-DIGE results revealed that AE up-regulated a metastasis suppressor, nucleoside diphosphate kinase A (NDKA, or nm23 as gene name). Low expression of NDKA had been reported to be correlated with poor patient prognosis and survival in several epidemiological cohort studies, including hepatocellular carcinoma (Boissan and Lacombe, 2006). A recent study using nm23 knockout mice demonstrated that loss of NDKA protein expression promoted lung metastasis in the SV40 mouse model of liver carcinogenesis (Boissan and Lacombe, 2006). On the contrary, forced over-expression of NDKA in highly metastatic carcinoma can significantly reduce the metastatic efficiency of the cells *in vivo*. In the present study,

NDKA expression was up-regulated by AE but not EM treatment (Fig. 2.5 and Table 2.2). As confirmed by 1D Western blotting in Fig. 4.2, AE treatment dose-dependently increased the expression level of NDKA. Whether AE could inhibit metastasis had not been reported before, although previous findings showed that its analogue EM was capable of inhibiting cancer cell invasion (Huang *et al.*, 2004), migration (Huang *et al.*, 2005) and adhesion (Huang *et al.*, 2006b). Thus the present finding of up-regulation of NDKA suggested that AE treatment may also have an anti-metastatic capability as its analogue EM.

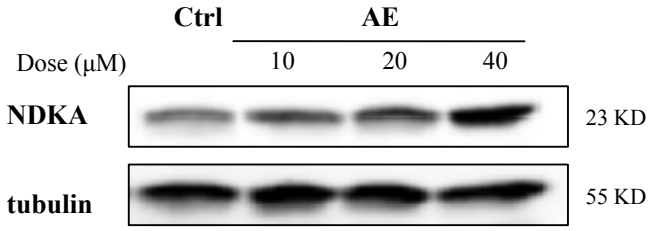


Fig. 4.2 AE increased expression level of NDKA. HepG2 cells were starved in serum-free DMEM medium overnight and then treated with increasing dose of AE from 10 to 40 μM in the fresh medium in the presence of FBS for 20 hr. At the end of treatment, cells were collected and lysed for Western-blot analysis of NDKA and tubulin.

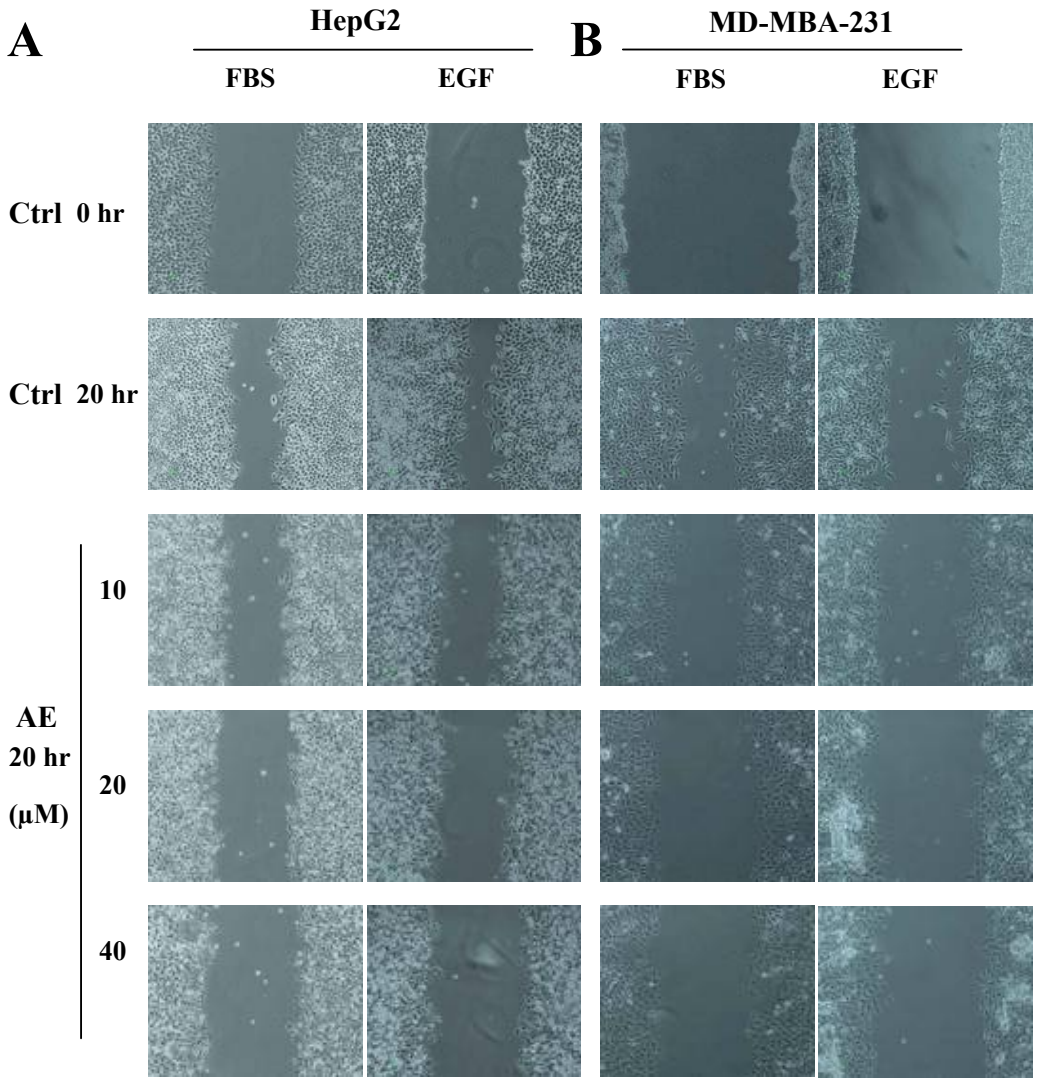


Fig. 4.3 AE inhibited cancer cell migration. Human hepatoma cell line HepG2 cells and breast cancer cell line MD-MBA-231 cells were cultured to form a confluent monolayer. Artificial straight wounds were created using a sterile pipette tip. After 3 rinses with PBS, fresh FBS-free medium was replaced. Cells were pretreated with indicated dose of AE for 1 hr and then the cell medium were supplemented with 5% FBS (or 20 ng/ml EGF) as stimulus. Microscopic photographs were taken at 0 and 24 hr.

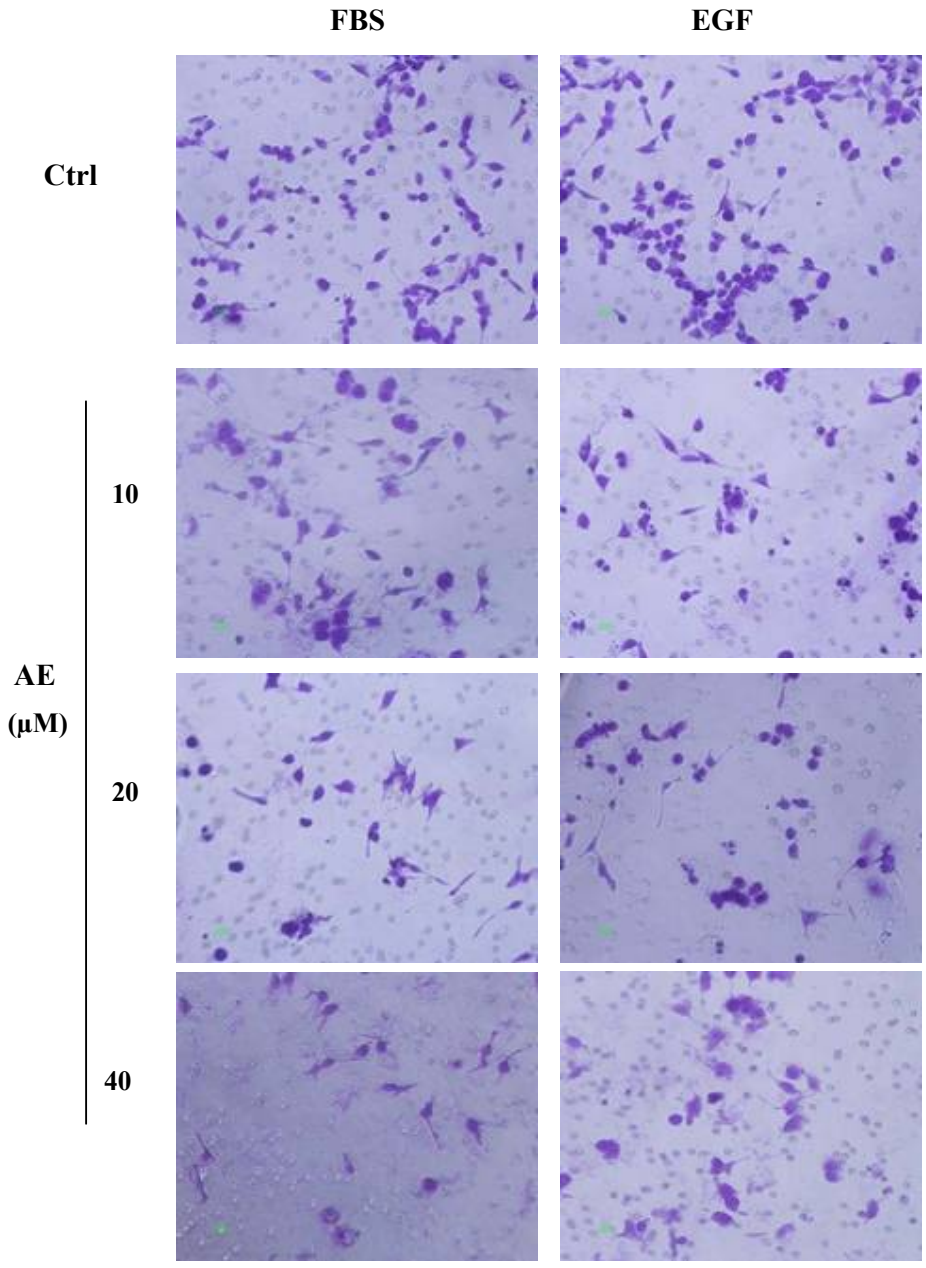


Fig. 4.4 AE inhibited cancer cell invasion. Cancer cell invasion was investigated using BD Falcon® 8.0-μm Cell Culture Inserts. After re-suspension and pretreatment of different concentrations of AE for 1 hr, HepG2 cells were seeded into the transwell chambers and supplemented in the presence or absence of 5% FBS (or 20 ng/ml EGF). After 24 hr, cells were stained with crystal violet and photographed under an inverted microscope.

In order to verify the effect of NDKA by AE on cell migration, wound healing assays were conducted. As shown in Fig. 4.3, both FBS and EFG could stimulate recovery of the artificial wound through cancer cell migration. However, AE dose-dependently inhibited the wound recovery (Fig. 4.3A), which was coincident with the up-regulation of metastasis inhibitor NDKA by AE (Fig 5.2). It is noteworthy that AE, even in a lower concentration without evidence of cytotoxicity (such as 10 μM), exhibited a significant inhibitory effect, suggesting that the anti-migration effect by AE was not a result of its cytotoxicity. Similarly, this effect was consistently observed in breast MD-MBA-231 cancer cells (Fig. 4.3B), suggesting a common inhibitory effect on cancer cell migration. Thus, AE may inhibit cell migration via up-regulation of NDKA, although the mechanism has yet to be determined.

To further determine whether AE could repress cancer cell invasion, Matrigel cell invasion assay was explored as well in AE-treated HepG2 cells. As shown in Fig. 4.4, incubation of FBS (5%) or EGF (20 ng/ml) was capable of promoting cancer cell invasion through Matrigel®-coated membrane. In contrast, AE treatment could drastically reverse this action in a dose-dependent manner from 10 to 40 μM. Collectively, these findings indicated that AE, similar to its analogue EM (Huang *et al.*, 2004; , 2005), was potent in inhibiting both cancer cell migration and cell invasion. The up-regulation of metastasis inhibitor NDKA was found to be associated with the anti-invasion and anti-migration effects of AE in this study. But it is still unknown how AE regulated the expression level of NDKA and whether up-regulation of this protein is functionally essential for the anti-metastatic effect.

4.3.3 Down-regulation and dephosphorylation of cofilin by EM

As mentioned in Chapter 2, EM treatment of HepG2 cells only affected a few proteins (Fig. 2.5 and Table 2.2), which was in line with its lower cytotoxicity (Fig. 2.1). The most drastic change in protein expression was cofilin, which was 4.33-fold lower in EM-treated cells than that of control cells. It is noteworthy that this differentially-expressed protein spot exited in its acidic form because of its *pI* shift from the value of theoretical 8.2 to the experimentally observed value of 6.4. The 1D Western-blot confirmed that EM treatment decreased the cofilin phosphorylation (Ser 3), as well as its total protein expression, in a dose-dependent manner (Fig. 4.5A). These results were confirmed by 2D Western-blot using pH 3-10 non-linear IPG strips. As shown in Fig. 4.5B, the anti-cofilin IgG recognized three protein spots with different pIs at pH 6.4, 7.3 and 8.2 respectively in the DMSO-vector-treated cells. However upon EM treatment, only one spot with a pI at 8.2 and with a lower intensity could be detected. This suggested EM treatment could inhibit phosphorylation of cofilin. The down-regulation of dephosphorylated cofilin also suggested an anti-metastatic role of EM, considering that active dephosphorylated cofilin (regulated by LIM kinase) was essential for cell motility and metastasis (Ghosh *et al.*, 2004; Wang *et al.*, 2006b; Zebda *et al.*, 2000). The effect of EM on HepG2 cell migration was then evaluated as well. As expected, EM similarly inhibited cell migration (Fig. 4.6), as what was observed in AE-treated cells. These results corroborate the previous findings that EM could inhibit invasion (Huang *et al.*, 2004), migration (Huang *et al.*, 2005) and adhesion (Huang *et al.*, 2006b) in human carcinoma cells. How EM

inhibited cofilin phosphorylation is still unknown. Considering EM is a well-established kinase inhibitor, this inhibitory effect of cofilin phosphorylation could be due to a direct inhibition by EM against LIM kinase, the upstream regulatory kinase of cofilin. However, this speculation is yet to be confirmed. On the other hand, unlike AE, EM has no effect on NDKA expression (Fig. 4.5A).

In contrast, treatment of AE at the same concentration failed to decrease cofilin phosphorylation. Interestingly, AE treatment repressed the total cofilin expression (Fig. 4.5A). Comparatively, unphosphorylated cofilin, the key factor for metastasis, may be decreased by AE. This finding also suggested that AE may have a potential role in anti-metastasis through down-regulation of dephosphorylated cofilin. Because AE only repressed the active dephosphorylated form of cofilin but not cofilin phosphorylation, AE may have a specific potential in inhibiting cofilin function, although the exact mechanism is yet to be determined. Therefore, these findings indicated that EM, as well as AE, could inhibit cancer cell migration, but through a different molecular mechanism.

4.4 Discussion

The anti-cancer effects of AE were operated through different molecular pathways. Besides the redox-sensitive proteins which are responsible for AE-induced apoptosis, another two proteins, p16 and NDKA, were also validated in this Chapter. On the other hand, cofilin, the most down-regulated protein in EM-treated cells, was also studied for its possible association with cancer cell migration.

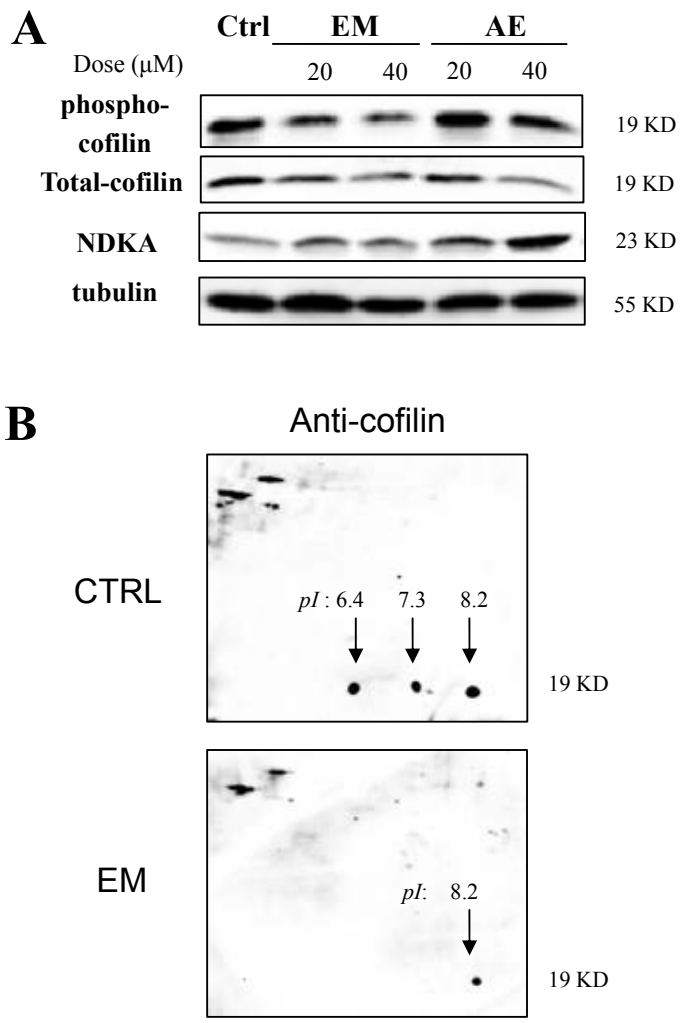


Fig. 4.5 EM inhibited both phosphorylated and dephosphorylated forms of cofilin. (A). HepG2 cells were treated with EM or AE as indicated. Then the cell lysate were subjected to Western blotting for detection of phospho-cofilin (Ser 3), total cofilin, NDKA and tubulin. (B). Cell lysate from the control cells and EM-treated cells (40 μM, 24 hr) were subjected to 2-DE and then Western-blot. Cofilin in both acdic forms and basic forms were analyzed as indicated. The pI and molecular weight of the detected cofilin spots was indicated in the 2D map.

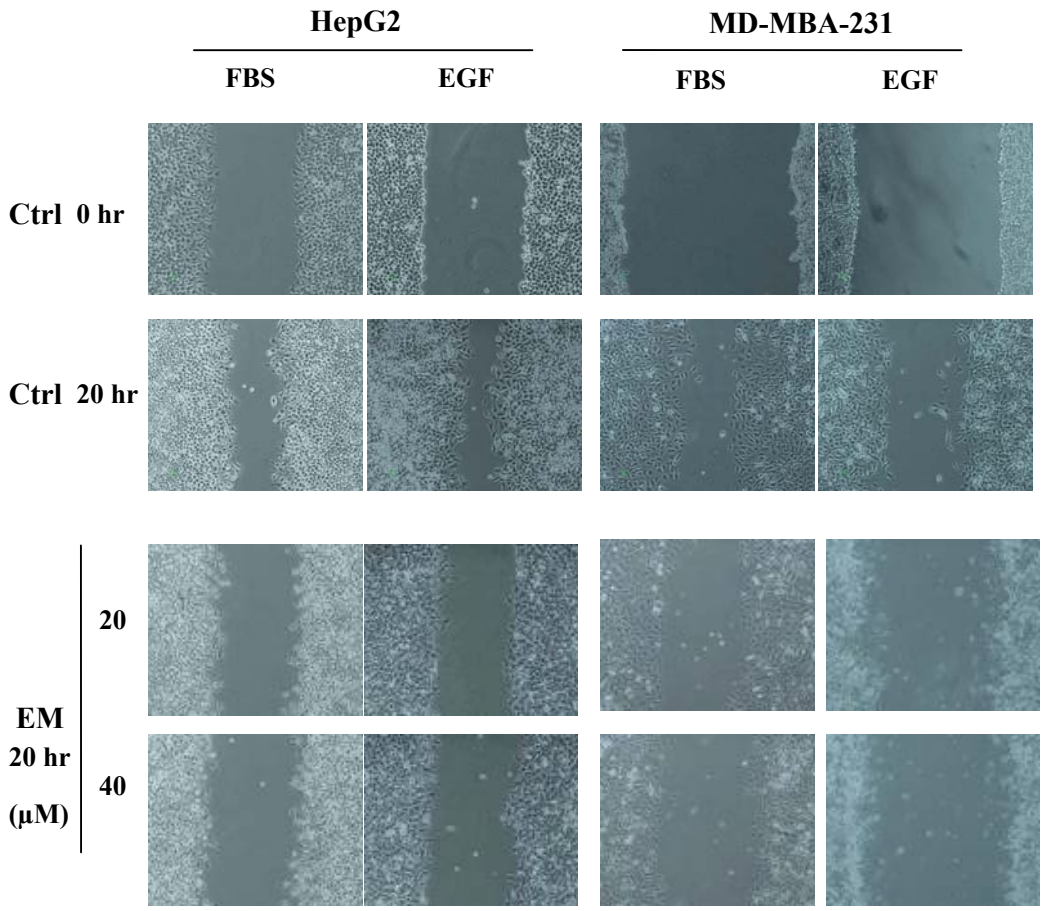


Fig. 4.6 EM inhibited cancer cell migration. Human hepatoma cell line HepG2 cells and breast cancer cell line MD-MBA-231 cells were cultured to form a confluent monolayer. Artificial straight wounds were created using a sterile pipette tip as mentioned above. After rinsing 3 times with PBS, fresh FBS-free medium were replaced. Cancer cells were pretreated with the indicated dose of EM for 1 hr. Then the cell medium was supplemented with 5 % FBS (or 20 ng/ml EGF) as stimulus. Microscopic photographs were taken at 0 and 20 hr.

AE had been shown previously to affect G1/S phase in many cancer cell lines, including hepatoma cells (Acevedo-Duncan *et al.*, 2004; Chen *et al.*, 2004a; Kuo *et al.*, 2004; Kuo *et al.*, 2002; Mijatovic *et al.*, 2005a). Some studies suggested that the p53-p21 pathway may account for G1/S cell cycle arrest induced by AE (Kuo *et al.*, 2002; Pecere *et al.*, 2003). However, these two studies focused mainly on the apoptotic role of p53 and p21 by AE. The exact molecular mechanism for AE-induced cell cycle arrest is still not clear. In the present study, the 2D-DIGE results successfully identified another G1/S cycle arrest that involved protein p16, which was responsible for the inhibition of CDK4 functions and subsequently DNA synthesis. On the contrary, p53 and p21 were not identified in the present 2D-DIGE experiment. This might be due to the narrow pH range (4-7) of IPG strips used in the present experiment, since p53 has a pI of 6.7. Subsequent 2D Western using the extended pH 3-10 IPG strips confirmed that p53 was up-regulated in AE-treated cells (data not shown).

The discovery of p16 by 2D-DIGE (Fig. 2.5 and Table 2.2), however, revealed another potential molecular pathway responsible for AE-induced G1/S cell cycle arrest. Further Western-blot validation confirmed its up-regulation and interference of down-stream Rb-E2F molecular pathway. Up-regulation of p16, the CDK4 inhibitor, was well established in blocking Rb phosphorylation in the CDK4-specific site Ser 795 (Kim and Sharpless, 2006; Sherr, 2004) and subsequently decreased DNA synthesis (Fig. 4.1A and B). On the contrary, the induction of p21 had been generally believed to preferentially inhibit CDK2 but not CDK4 activity (Bartek and Lukas,

2001; Boulaire *et al.*, 2000; Brugarolas *et al.*, 1995; Poon *et al.*, 1996). Thus, AE-induced p16, at least, could partially account for the repressed DNA synthesis, although the present study could not exclude the involvement of other CDK inhibitors.

Various stimuli had been reported to induce p16 *in vitro* and *in vivo*, especially the DNA-damaging stimuli such as UV light, oxidative stress and chemotherapeutic agents (Chen *et al.*, 2004b; Ito *et al.*, 2006; Meng *et al.*, 2003; Pavey *et al.*, 1999). Since AE was capable of inducing intracellular ROS generation, p16 was probably induced by such a mechanism in the early stage of AE treatment. But when the generated ROS reached an intolerable level, the effect by AE treatment against DNA synthesis may switch to induction of the apoptosis pathway. This speculation is still yet to be confirmed. Induction of p16 was often found to occur 2-4 weeks after DNA-damaging stress through MAPK activation (Ito *et al.*, 2006; Robles and Adami, 1998; Wang *et al.*, 2006c). It is noteworthy that in the present study p16 was induced after 6 hr and peaked at 12 hr by AE treatment of HepG2 and HCC-M cells (Fig. 4.1A). Since only a few selected cancer cells were analyzed after AE treatment, whether AE could induce p16 in other cancer cells and/or *in vivo* at such a fast rate is yet to be determined.

On the other hand, 2D-DIGE identified another up-regulated protein, nucleoside diphosphate kinase A (NDKA, gene name as nm23-H1). This protein is the first metastasis inhibitor to be identified (Steeg, 2003). Expression level of NDKA was reported in most studies to be negatively associated with some types of human solid tumors, such as liver, colon, breast and melanoma (Boissan and Lacombe, 2006;

Nakayama *et al.*, 1992; Ouatas *et al.*, 2003; Steeg, 2003), although the mechanisms responsible for the changes in NDKA expression were unclear. Experimental manipulation of NDKA consistently affected the cancer metastatic efficiency *in vivo*. Genetic nm23-null mice were susceptible to lung metastasis in the SV40 mouse model, while there was no observable effect on primary liver carcinogenesis (Boissan and Lacombe, 2006). In the present study, the up-regulation of NDKA by AE thus indicated a novel anti-metastatic effect of AE. This speculaton should not be a surprise given that its analogue EM could inhibit cancer cell migration (Huang *et al.*, 2004; , 2005; Kim *et al.*, 2005). However, the present study revealed that AE and EM may inhibit cancer cell migration through different molecular mechanisms. Up-regulation of NDKA only occurred in AE-treated cells, but not in EM-treated cells. Although the present study could not exclude other relevant pathways, up-regulation of NDKA by AE may render an inhibitory effect against cancer cell migration.

On the contrary, in EM-treated cells, 2D-DIGE detected fewer differentially-expressed proteins. Among them, cofilin at pI 6.2 was the most highly down-regulated protein spot after EM treatment. This suggested a potential role of EM in inhibiting cofilin phosphorylation (probably by inhibition of the upstream kinase LIM kinase 1 (LIMK)). The 2D Western blot revealed that the inactive phosphorylated form, together with the active dephosphorylated form of cofilin was down-regulated by EM treatment. Dephosphorylation of cofilin has been reported to an essential step for mitochondria-mediated apoptosis (Chua *et al.*, 2003), but in the present study, EM was unlikely to initiate apoptosis through this pathway. This is because the active

dephosphorylated form of cofilin was decreased rather than increased (Fig. 4.5B). More importantly, there was no translocation of cofilin from cytosol to mitochondria in the process of apoptosis and cytochrome *c* translocation (data not shown).

The down-regulation of the dephosphorylated form of cofilin was probably associated with the inhibition of cancer cell migration by EM, considering the findings that this specific cofilin isoform is required for cell motility and cancer metastasis (Ghosh *et al.*, 2004; Wang *et al.*, 2006b; Zebda *et al.*, 2000). Cofilin functioned in the regulation of actin dynamics by promoting the rate of actin depolymerization and facilitating actin filament turnover (Bailly and Jones, 2003; Chen *et al.*, 2000). This protein and its upstream kinase, LIM kinase 1, were both up-regulated in invasive cells (Wang *et al.*, 2004a). More importantly, inhibition of cofilin activity by either gene knockdown or expression of constitutively active LIMK inhibited cell motility (Hotulainen *et al.*, 2005; Yamaguchi *et al.*, 2005; Zebda *et al.*, 2000). In contrast, cofilin overexpression was found to be associated with motility of glioblastoma tumor cells in a concentration-dependent manner (Yap *et al.*, 2005). In the present study, both EM and AE treatment repressed total cofilin expression. In addition, EM inhibited cofilin phosphorylation. These results may again indicate the anti-migration role of EM and AE, which is evidenced by the impaired cell capability to recover in wound healing assay (Figs. 4.4 and 5.6).

Taken together, 2D-DIGE has proven to be an effective approach in identifying susceptible protein targets that participate in the relevant molecular pathways upon AE treatment. Besides the redox-sensitive proteins responsible for AE-induced apoptosis,

up-regulation of p16 and NDKA were found to be associated with G1/S cell cycle arrest and anti-metastatic effects, respectively. On the other hand, EM decreased both dephosphorylated and phosphorylated forms of cofilin, suggesting its role in anti-migration.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

Rhubarb root (*Rheum palmatum*), is one of the earliest and best-known Chinese herbal medicine. It has been used for centuries in China and ranked as a top medicinal herb (Li, 1982; Yang, 1997). Aloe-emodin (AE) is one of the main hydroxyanthraquinones found in Rhubarb root. This hydroxyanthaquinone exhibits multiple pharmacological effects, including anti-viral, anti-inflammatory, hepatoprotective, anti-hepatitis and anti-cancer potential (Agarwal *et al.*, 2000; Arosio *et al.*, 2000; Huang *et al.*, 2006a; Kim *et al.*, 2004; Pecere *et al.*, 2000; Woo *et al.*, 2002). Although AE has been reported to be potent in inhibiting cancer cell growth and induction of apoptotic cell death (Huang *et al.*, 2006a), the precise molecular mechanisms are yet to be determined.

Considering the structural similarity of AE to its analogue emodin (EM), it was believed that AE might share similar anti-cancer molecular pathways with that of the well-studied hydroxyanthraquinone EM. Based on literature reports, the anti-cancer potency of EM was mainly attributed to its inhibition on the pro-survival protein tyrosine kinases (e.g. $p56^{LCK}$ and HER-2/neu), and a number of other kinases (e.g. CK2, PI3K and AKT1) (Battistutta *et al.*, 2000; Frew *et al.*, 1994; Jayasuriya *et al.*, 1992; Zhang *et al.*, 1995; Zhou *et al.*, 2006). In contrast, AE was found to be a poor kinase inhibitor (Huang *et al.*, 2006a; Sarno *et al.*, 2002) and yet it exhibited similar or higher cytotoxicity against a variety of cancer cells, compared to EM (Fenig *et al.*, 2004; Lee, 2001; Shi *et al.*, 2001). Thus it might be of great interest to examine the molecular mechanism for AE-induced anti-cancer effects.

Recently, several investigations using conventional biochemical approaches had

shown that the p53-p21 molecular pathway is connected with the anti-cancer effect of AE (Kuo *et al.*, 2002; Pecere *et al.*, 2003). However, no comprehensive studies have ever been carried out to identify the molecular targets of AE. In an earlier study, Lee and his colleagues (2005) utilized 2-DE to find the proteins affected by AE in lung carcinoma H460 cells. They showed that cytosol translocation and degradation of nucleophosmin occurred during AE-induced apoptosis. However, this finding was basically descriptive. It was unable to reveal whether the expression change of nucleophosmin was functional or causative. Moreover, it was unclear whether AE only affected this specific protein but not others.

In this study, a comprehensive proteomic investigation was carried out to systematically investigate the anti-cancer targets and relevant molecular pathways of AE-treated HepG2 cells *in vitro*. The investigation consists of two major portions: (i) identification and classification of the differentially-expressed proteins in AE-treated cells by 2D-DIGE (Chapter 2); and (ii) biochemical validation and characterization of the affected proteins and the associated molecular pathways involved in AE-induced apoptosis (Chapter 3), and the other anti-cancer effects (Chapter 4). In addition, parallel experiments were also conducted in EM, to compare the molecular targets of these two structurally similar hydroxyanthraquinones.

5.1 Anticancer potential of AE: implication of proteomic findings

Proteomics is a systematic approach to determine the diverse properties of proteins in a simultaneous manner for a comprehensive understanding of the structure, function and regulation of biological systems (Patterson and Aebersold, 2003).

Proteomics and other complementary methods (e.g. genomics) have widened and deepened "the scope of biological studies from the traditional reductionist biochemical analysis of single proteins to proteome- (or genome-) wide measurement" (Patterson and Aebersold, 2003). These systematic strategies are especially important and applicable to cancer and other complex diseases, since a single molecular pathway may not be responsible for these diseases. Thus, the emerging 'system biology' approaches may aid to describe the biological systems comprehensively through integration of diverse types of data.

Different gel-dependent and –independent methods have been developed to determine the proteome expression in a complex sample mixture. Among them, 2-DE separates thousands proteins simultaneously according to two different properties, usually molecular weight and pI. The resolution and reproducibility of 2-DE have improved substantially with the introduction of commercially-available narrow pI range IPG gels and cyanine dyes (Alban *et al.*, 2003; Gorg *et al.*, 2004; Righetti *et al.*, 2004). In the present study, 2D-DIGE (2D difference gel electrophoresis) analysis using 4-7 zoom IPG strips was applied. In 2D-DIGE, proteins extracted from pooled internal standard, control and treated samples were separately labeled with different dyes (Cy2, Cy3 and Cy5), respectively. The protein mixtures were then loaded and separated on the same 2D gel and the amount of protein in each group were then quantified according to the fluorescent intensity of the dyes labeled. Differentially expressed proteins were selected by a direct comparison of the fluorescent intensity of individual protein spots (Cy5/Cy2 vs. Cy3/Cy2) and identified by MALDI TOF/TOF

MS/MS. This method has greatly increased the reproducibility, data quality and confidence of 2-DE (Alban *et al.*, 2003). Despite the maturity and unmatched performance of 2D-DIGE, this methodology is still compromised by some technical concerns, such as protein co-migration, because protein detection is based on the assumption that one protein is present in each spot. However, under stringent screening criteria based on the comparison of the theoretical with the experimental molecular weight and pI, unmatched proteins could be excluded afterwards.

A number of proteomic approaches have been conducted recently in the search for potential protein targets of some commonly-used or potential cancer therapeutic agents, including a number of phytochemical compounds extracted from medicinal herb or vegetables, such as all-trans retinoic acid, the acid form of vitamin A (Harris *et al.*, 2004; Wu *et al.*, 2004), dibenzoylmethane from licorice (Frazier *et al.*, 2004), flavone and quercetin from soy bean (Herzog *et al.*, 2004a; Herzog *et al.*, 2004b; Wenzel *et al.*, 2004), paclitaxel from the bark of *Taxus brevifolia* (MacKeigan *et al.*, 2003), *β*-phenylethyl isothiocyanate from watercress (Neo *et al.*, 2005), mushroom extract (Wang *et al.*, 2004b) and grape seed extract (Deshane *et al.*, 2004). All these studies provided a list of affected proteins, which allowed a comprehensive understanding of the induced effects after treatment by an individual agent.

However, most of the above studies only used proteomic methods to identify the up-regulated or down-regulated proteins and validate some candidate proteins using reverse transcriptase-polymerase chain reaction (RT-PCR) and/or Western blotting without detailed characterization of the functional proteins. This approach simply

reflects a descriptive change in expression level rather than functional validation. The only exception was that of Ting and her colleagues, who confirmed the functional role of the affected proteins by applying exogenous expression or siRNA knocking-down experiment (MacKeigan *et al.*, 2003). This study successfully revealed the power of integrating the proteomic profiling and functional analysis to discover novel therapeutic targets and potential cancer biomarkers.

In therapeutics studies using proteomic approaches, for example, up-regulated and/or down-regulated proteins may not be the direct protein targets of the drug. However, this information is still informative because some affected proteins could be mediators, end-products or even by-products in a series of functional changes induced by the therapeutic agents. Based on this information, a follow-up functional analysis of the affected proteins and/or associated proteins may aid to disclose the real therapeutic targets and molecular pathways responsible for the observed effects. On the other hand, proteomic approaches may detect some proteins with known pharmacological and/or toxicological functions, but are unrelated with the known therapeutic effect. This information may reveal some novel properties of the therapeutic agents. On this basis, the descriptive nature of proteomic profiling could be very informative if it is integrated with further functional biochemical validation and characterization, as shown by the study conducted in Ting's lab (MacKeigan *et al.*, 2003). In the present study, a similar approach that integrate 2D-DIGE proteomic profiling with functional cellular studies was conducted to better understand the anti-cancer effects of AE on HepG2 cells. In the present investigation, cellular

biochemical studies were firstly carried out to confirm the anti-cancer potential of AE, including inhibition of cell growth and induction of apoptosis. More importantly, it was also to determine the optimal dose and exposure time for subsequent studies.

As shown in Fig. 2.1, AE induced higher cytotoxicity than its analogue EM, suggesting that AE had a higher potential in induction of apoptotic cell death and cell cycle arrest. This finding confirmed several earlier studies on Merkel cell carcinoma (Fenig *et al.*, 2004), lung carcinoma CH27 (Lee, 2001), human oral squamous cell carcinoma (HSC-2) and salivary gland tumor (HSG) cell lines (Shi *et al.*, 2001). It is also worth noting that AE also exhibited a higher cytotoxic effect against cancer cells than non-cancerous immortal cells (Fig. 2.2).

2D-DIGE successfully separated around 1600 proteins spots (Fig. 2.5) and 40 of these spots were either up- or down-regulated by more than 2-fold in the Cy5-labeled treated samples versus the Cy3-labeled control samples. These results were summarized in Table 2.2. These differentially-expressed proteins were found to be distributed mostly in cytoplasm, followed by in the mitochondria and nucleus, respectively. These affected proteins were functionally classified and found to be associated with oxidative stress, cell cycle arrest, anti-metastasis and anti-hepatitis (Fig. 2.7). For example, the redox-sensitive proteins formed the largest protein cluster upon AE treatment; suggesting AE's effect in inducing oxidative stress. Among them, the antioxidant protein, peroxiredoxin was most highly up-regulated (Table 2.2). To assess whether these redox-sensitive proteins play a proapoptotic or anti-apoptotic role in AE-induced apoptosis, some of these redox-sensitive proteins and their associated

pathways were then investigated (Chapter 3). In addition, AE up-regulated p16 (Fig. 3.5), a crucial CDK4 inhibitor responsible for 1/S cycle arrest. Although AE had been reported to affect the p21 pathway and in turn initiated G1/S arrest (Kuo *et al*., 2002; Pecere *et al*., 2003), its association with up-regulation of p16 and induction of G1/S arrest had never been reported before. Here, the up-regulation of p16 and the relevant pathway were also validated and characterized using a biochemical approach (Chapter 4). It was also noted that in hepatoma cells, AE also increased the expression level of NDKA (Fig. 2.5). This protein is known to be a cancer metastasis inhibitor (Boissan and Lacombe, 2006). Its up-regulation suggested a novel potential anti-metastatic function of AE. To validate this potential functional role of NDKA up-regulation, biochemical assays for cell migration and invasion were conducted (see Chapter 4). Taken together, the 2D-DIGE proteomic analysis successfully provided some new candidate protein targets for the known anti-cancer effects of AE.

Our results also revealed that EM, an analogue of AE, had a lower cytotoxicity (see Fig. 2.1) and affected a narrower spectrum of protein targets than AE in hepatoma cells (Table 2.5). In EM-treated cells, for example, the redox-sensitive proteins PARK7, PDIA3 and ACTG were the most highly up-regulated proteins, but their levels of differential expression were lower than that for AE (Table 2.5). On the other hand, acid form of cofilin was found to be down-regulated by EM but not AE (Fig. 2.5 and Table 2.2), although the molecular mechanism is yet unknown. The decreased phosphorylation of cofilin may be a result of an inhibition of its up-stream kinase by EM, a well-reported kinase inhibitor (Huang *et al*., 2006a; Sarno *et al*., 2002).

It is worth noting that the above mentioned proteins have been functionally correlated with AE- or EM-induced anti-cancer effects, although the other differentially-expressed proteins may also play a role in the regulation of cell fate. Extensive experiments, such as exogenous gene over-expression or knock-down of the affected protein will aid to explore its functional role in AE treatment in HepG2 cells. Using pH range of 6-10 IPG strips in a future study may help to identify some other important basic proteins responsible for AE's anti-cancer effects. For example, p53 was reported to be associated with apoptosis induced by AE (Kuo *et al*., 2002; Pecere *et al*., 2003), but it was not identified in the present study. However, the 2D-Western-blot analysis using the wide-range pH 3-10 IPG strips revealed the up-regulation of p53 upon AE treatment (data not shown). Similarly, other proteomic approaches such as HPLC-based fractionation of proteins/peptides may help to find some important hydrophobic and low abundant proteins (Alban *et al*., 2003; Gorg *et al*., 2004), which were not detected in the present gel-based experiment.

5.2 Anticancer potential of AE: induction of apoptosis through ROS generation and ASK1/JNK pathway

Redox radicals are found to be important signal transduction molecules involved in cell proliferation, senescence and apoptosis (necrosis). These actions are generally mediated by ROS through reversible or irreversible oxidative modification of key cellular macromolecules (DNA, protein and lipids). It has long been believed that redox radicals and oxidative stress are associated with cancer and other diseases (Jacobson *et al*., 1997; Kerr *et al*., 1972; Okada and Mak, 2004).

It seemed to be contradictory at first sight that ROS can both promote cell malignant transformation and kill cancer cells. But increasing evidence favors the idea that ROS exhibited such a 'double-edged sword' effect depending on its strength and duration. On the one hand, persistent oxidative stress was found in cancer cells (Toyokuni *et al*., 1995). This increased ROS generation has been proposed to be derived from mutations that occurred in nuclear or mitochondrial genes encoding the components of the mitochondrial electron transport chain (Wallace, 2005); and result in oncogenic transformation and higher proliferative potentials in these mutated cells as compared to their normal parental cells (Klaunig and Kamendulis, 2004; Loo, 2003). Nuclear factor-κB (NF-κB) and AP-1 are the most important transcription factors reported to be affected by free redox radicals in these processes (Bubici *et al*., 2006; Clive and Greene, 1996; Gloire *et al*., 2006; Loo, 2003).

On the other hand, when ROS generation reaches an intolerable level, suicidal or therapeutic pro-apoptotic pathways, such as the well-established redox-sensitive ASK1-JNK pathways, are ready to be activated (Benhar *et al*., 2002; Trachootham *et al*., 2006). The already elevated level of ROS thus makes cancer cells more vulnerable than normal cells comparatively. This assumption was mostly supported by a variety of cellular studies using cancer therapeutic drugs to induce senescence and/or apoptotic cell death in cancer cells (Dolado *et al.*, 2007; Muller *et al.*, 1998; Rosato *et al.*, 2003; Takahashi *et al.*, 2006). Taken together, a low level of ROS generation may help oncogenic transformation but a higher level of ROS makes cancer cells susceptible to the induced cell death and cell growth arrest. If this 'double-edged

sword' effect of ROS works under *in vivo* conditions in human, therapies could be developed accordingly through delicate modulation of redox balance: either to keep a relatively reducing environment in normal cells to protect oncogenic transformation, or to strengthen ROS generation beyond the tolerable limit to induce apoptotic cell death in vulnerable cancer cells.

Interestingly, many phytochemicals with polyphenol structure (such as epigallocatechin gallate extracted from tea, resveratrol from grape and apigenin from soy bean) exhibit a dual-role in the production of radical species and subsequent oxidative stress (Loo, 2003). On the one hand, the phenolic structure enables phytochemicals to counteract the harmful oxidative injury induced by other strong oxidants (Shahidi and Wanasundara, 1992), whilst the therapeutic dose of phenolic phytochemicals can selectively induce oxidative stress in cancer cells (Loo, 2003). The option to be an antioxidant or oxidant for phenolic phytochemicals thus depends on the basal cellular oxidant/antioxidant level, the dose of phenolic chemicals, and other crucial factors.

Similar to these phytochemicals, AE has been reported to be capable of reducing excessive ROS generation induced by other strong oxidants both *in vitro* and *in vivo* (Arosio *et al*., 2000; Malterud *et al*., 1993). In the present study, this compound was also found to up-regulate a cluster of redox-sensitive proteins (e.g. peroxiredoxin, DJ-1, PDIA3, etc.), as revealed in the 2D-DIGE study (Fig. 2.7). This finding suggested that AE, similar to other so-called "antioxidant" phytochemicals, can induce excessive ROS generation. Further biochemical validation using the ROS indicator

CM-H_2DCFDA, and Western blotting, confirmed that AE can induce oxidative stress (Fig. 3.2) and in turn initiate ROS-dependent mitochondria-mediated intrinsic apoptotic cell death in hepatoma cells (Fig. 3.1).

In the present study, higher level of apoptosis was induced in AE-treated cells compared with that of EM (Figs. 2.3 and 2.4). This action may be derived from AE's potency in inducing a higher level of ROS production and sustained JNK activation. Aloe-EM treatment caused marked oxidative stress and depletion of intracellular glutathione (GSH) in hepatoma cells (Figs. 3.2 and 3.5). This redox-inducing action in turn caused deleterious protein oxidations (Figs. 3.3 and 3.4). It has been well established protein oxidations are essential for the regulation of apoptosis (Benhar *et al*., 2002; Trachootham *et al*., 2006).

In this study, oxidation of protein peroxiredoxins, GST-π and Trx occurred upon AE treatment (Figs 3.4, 3.12 and 3.13). Peroxiredoxin functioned as antioxidant protein (Wood *et al*., 2003) and as shown in this study, knocking-down of this protein by siRNA sensitized AE-induced apoptosis (Fig. 3.4). In contrast, exogenous expression of antioxidant Cu/Zn-SOD or pretreatment of GSH donor (GSH-MEE) protected cells from excessive ROS generation and apoptosis (Figs. 3.5 and 3.9). On the other hand, redox-sensitive GST-π and Trx are inhibitory binding subunit of JNK and ASK1 (JNK upstream kinase) respectively (Adler *et al*., 1999; Saitoh *et al*., 1998). Oxidation of these two proteins activated JNK, and in turn initiated mitochondria-mediated apoptosis (Figs. 3.12 and 3.13). The essential pro-apoptotic role of sustained JNK activation was then validated by both pharmacological

inhibition and genetic over-expression of dominant negative JNK (Figs. 3.10 and 3.11). These results, which have not been reported hitherto, revealed that AE induced apoptosis via oxidative stress and constitutive JNK activation. This ROS-dependent action by AE is comparable to that of other phenolic phytochemicals, such as pathenolide, sulforaphane, diallyl disulfide, phenethyl isothiocyanate, curcumin and epigallocatechin-3-gallate (Collett and Campbell, 2004; Filomeni *et al*., 2003; Hu *et al*., 2003; Qanungo *et al*., 2005; Singh *et al*., 2005; Zhang *et al*., 2004a), thus suggesting a common ROS-dependent cell death pathway.

In the present study the redox-sensitive MAPK p38 was found to be unaffected (Fig. 3.6), although AE has been shown to induce p38-invovled apoptosis in lung carcinoma cells (Yeh *et al*., 2003). This inconsistency might be due to different characteristics of the cell lines. On the other hand, MAPK ERK activation was generally believed to be pro-survival (Reddy *et al*., 2003; Torres, 2003). ERK inhibition was previously found to be accompanied with induction of differentiation and apoptosis after AE treatment in rat C6 glioma cells (Mijatovic *et al*., 2005a). In the present study, ERK was constitutively deactivated by AE (Fig. 3.6). However, the ERK inhibition was found to be ROS-independent and dispensable for apoptosis (Fig. 3.9). The reason how AE inhibit ERK phosphorylation is yet to be determined. Considering that its analogue EM is an ERK inhibitor, AE may also exhibit direct inhibition on ERK or upstream kinases.

Taken together, the results of this study have provided clear evidence that conventional “antioxidant” phytochemicals like AE can have therapeutic potential

through induction of oxidative stress and ROS-dependent apoptotic pathway.

5.3 Biochemical validation of other affected proteins that involved in G1/S arrest and anti-migration effect

Besides the redox-sensitive proteins which are responsible for the apoptosis-inducing effect, further biochemical validation and characterization of p16 and NDKA established two novel anti-cancer properties of AE. It was found that AE induced cell growth inhibition via up-regulation of p16 and anti-metastasis via up-regulation of NDKA. These two effects have not been reported thus far, but were implicated by the result of 2D-DIGE (Fig. 2.7).

The G1/S growth inhibition by AE was previously suggested to be initiated by activation of the p53-p21 pathway (Kuo *et al*., 2002; Pecere *et al*., 2003). The discovery of p16 by 2D-DIGE in the present study (Table 2.2), however, revealed another potential molecular pathway responsible for AE-induced G1/S cell cycle arrest. Further Western-blot validation confirmed its up-regulation and interference of downstream Rb-E2F molecular pathway (Fig 4.1). Tumor suppressor protein p16 is a CDK4 inhibitor. This protein functions in deactivating CDK4/6 and its downstream protein, Rb, and in turn represses DNA synthesis through G1 into S-phase of the cell cycle (Sherr, 2004). The role of the up-regulated p16 in G1/S arrest was confirmed in the present study, by blocking the Rb phosphorylation at the CDK4 specific site Ser795 (Fig 4.1). On the contrary, the induction of p21 has been generally believed to preferentially inhibit CDK2 but not CDK4 activity (Bartek and Lukas, 2001; Boulaire *et al*., 2000; Brugarolas *et al*., 1995; Poon *et al*., 1996). Thus, up-regulation of p16 by

AE, at least, partially accounted for the repression of DNA synthesis, although the present study can not exclude the involvement of other CDK inhibitors. More importantly, inhibition of this pathway happened early in the treatment after 6 hr, which may explain how AE inhibited cell growth at early stage before induction of apoptosis after 24 hr.

On the other hand, up-regulation of the metastasis inhibitor NDKA by AE but not EM (Fig. 2.7) may enable the anti-metastatic effect of AE. Expressional level of NDKA was reported in most studies to be negatively associated with cancer metastatic potentials (Boissan and Lacombe, 2006; Nakayama *et al*., 1992; Ouatas *et al*., 2003; Steeg, 2003). Recently, the essential anti-metastatic role of NDKA was demonstrated by the increased lung metastasis in NDKA knockout mice of liver carcinogenesis (Boissan and Lacombe, 2006). Although the anti-metastatic effect of AE has never been reported before, its analogue EM was found to be capable of inhibiting cancer cell invasion (Huang *et al*., 2004), migration (Huang *et al*., 2005) and adhesion (Huang *et al*., 2006b). The finding of NDKA up-regulation by AE was confirmed by conventional Western blotting (Fig. 4.2). Furthermore, the anti-metastatic function of NDKA up-regulation was confirmed using the cell migration and invasion assay (Figs. 4.3 and 4.4). AE treatment dose-dependently inhibited cell migration (delayed recovery of wound healing) and invasion stimulated by either FBS or EGF in hepatoma HepG2 cells and breast carcinoma MD-MBA-231 cells.

The exact mechanisms of how AE up-regulates p16 and NDKA are still unknown. The present study could only provide a functional association between the

expressional regulation of these two proteins (p16 and NDKA) and the observed anti-cancer effects (G1/S arrest and anti-migration), respectively. Further experiments directed to the transcriptional regulation and/or protein turnover of these two proteins, are needed to obtain a better understanding of the mechanisms.

The proteome changes in EM-treated cells were also investigated in parallel with AE. The results suggested that EM induced fewer protein changes. Besides the redox-sensitive proteins, EM decreased significantly cofilin phosphorylated form as well as its dephosphorylated form (Fig. 4.5). Based on the literature, the down-regulation of the dephosphorylated form of cofilin by EM was probably associated with two different known functions: pro-apoptosis (Chua *et al*., 2003) or anti-metastasis (Ghosh *et al*., 2004; Wang *et al*., 2006b; Zebda *et al*., 2000). However, in the present study, EM is unlikely to initiate apoptosis through down-regulation of cofilin. This is because the level of active pro-apoptotic dephosphorylated form of cofilin was decreased rather than increased (Fig. 4.5B). More importantly there is no translocation of cofilin from cytosol to mitochondria in the process of apoptosis induction and cytochrome *c* translocation as reported previously (Chua *et al*., 2003). Instead, the anti-metastatic effect of EM was confirmed by the cell migration assay (Fig. 4.6). Thus, the decreased expression of cofilin may account for the anti-migration effect of EM. How EM inhibits the phosphorylation of cofilin is yet unknown. This action is possibly through inhibition of its up-stream kinase, LIMK by EM, a well-known kinase inhibitor. Similarly, AE also decreased the expression level of dephosphorylated cofilin but it did not affect its phosphorylated form. This again

reflects the difference between AE and EM in inhibiting protein kinases (see Chapter 2). The decreased level of cofilin may be a possible reason for AE-induced anti-migration effect, although the mechanisms are yet to be determined.

5.4 Summary and conclusions

In the present study, the anti-cancer properties of AE have been systematically investigated by an integrated proteomics and biochemical approach. The investigation comprised of three main parts: (i) 2D-DIGE analysis of the proteome change in AE-treated HepG2 cells; (ii) biochemical validation and characterization of proteins associated with apoptosis; (iii) biochemical validation and characterization of other anti-cancer proteins. The major findings and the pathways involved are summarized in Fig. 5.1. One of the main findings is that AE induced apoptosis through oxidative stress (protein oxidation of peroxiredoxins, GST-π and Trx) and ASK1/JNK activation. Furthermore, the regulation of tumor suppressor p16 and metastasis suppressor NDKA induced by AE may account for the G1/S arrest and anti-metastatic effects, respectively.

In summary, the findings from this study suggest that such an integrated approach has led to a better understanding of the anti-cancer effects of AE. The findings also demonstrated that AE exhibited potent anti-cancer properties through multiple molecular pathways.

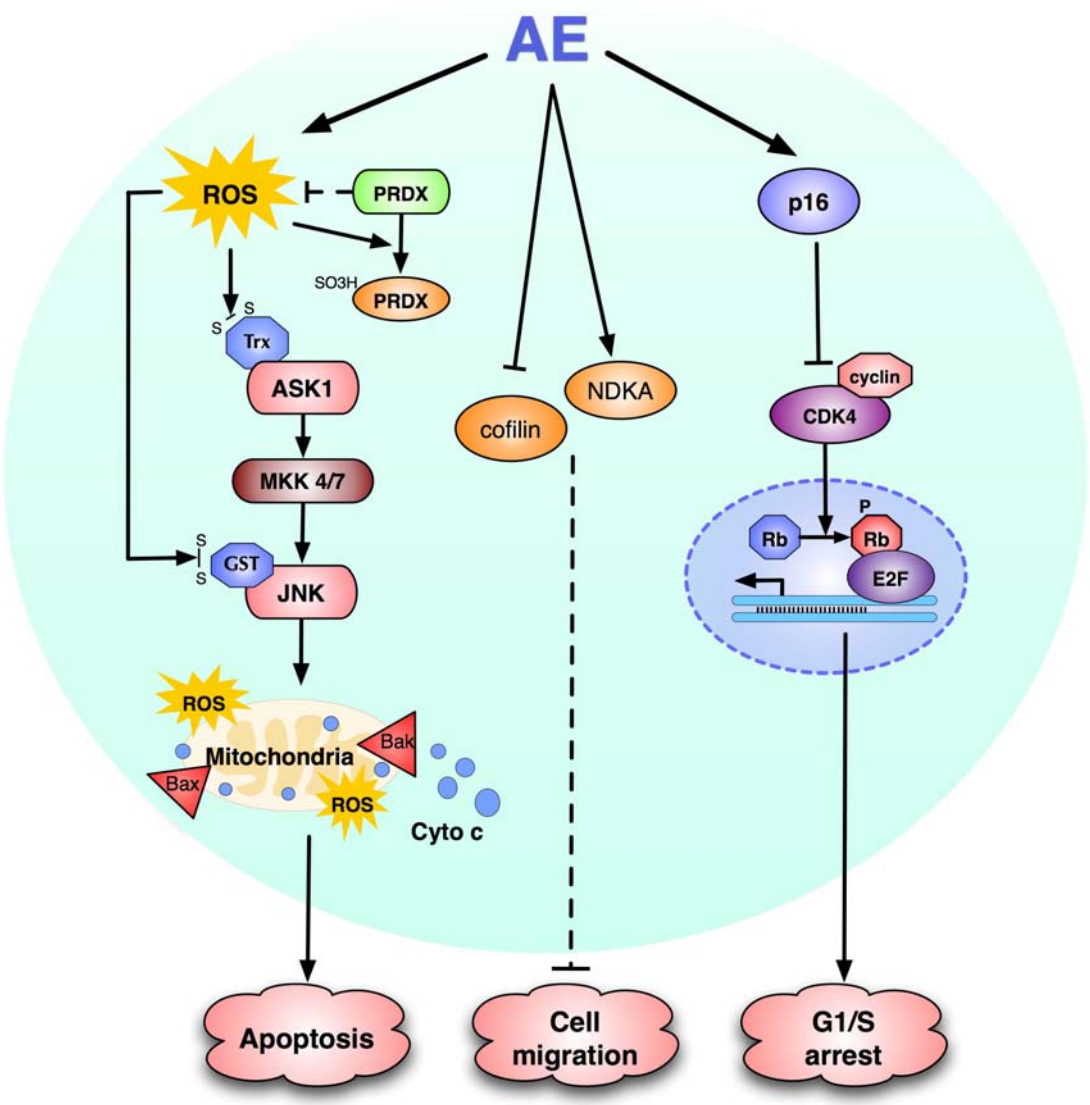


Fig.5.1 Mechanisms involved in the anti-cancer effects of AE.

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Anticancer effects of aloe-emodin on HepG2 cells: cellular and proteomic studies.

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Abstract: Aloe-emodin (AE) is one of the main bioactive anthraquinones of Rheum palmatum, a widely used herbal medicine. Several recent studies suggested that AE possesses potent anticancer properties, although the mechanisms are yet to be fully elucidated. The present study aimed to identify the molecular targets of AE in a human hepatocellular carcinoma cell line, HepG2. We first found that AE was more cytotoxic and effective in inducing apoptosis and cell cycle arrest than its analog emodin (EM). Proteomic study using 2-D DIGE revealed that AE affected multiple proteins associated with oxidative stress, cell cycle arrest, antimetastasis, and hepatitis C virus replication. For example, peroxiredoxins (PRDX) and DJ-1, both of which are redox-sensitive proteins, were among those markedly up-regulated, suggesting the presence of oxidative stress in AE-treated cells. Further biochemical studies demonstrated that AE enhanced the intracellular level of reactive oxygen species and

oxidation of PRDX-2, -4, and DJ-1. In addition, AE inhibited DNA synthesis via up-regulation of the CDK4 inhibitor p16 and inhibition of Rb phosphorylation. Furthermore, AE was able to decrease cell migration via up-regulation of the metastasis inhibitor, nm23. Taken together, AE induced anticancer effects in HepG2 cells via multiple pathways by affecting different protein targets.

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Critical role of oxidative stress and sustained JNK activation in aloe-emodin-mediated apoptotic cell death in human hepatoma cells.

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Abstract: Aloe-emodin (AE), one of the main bioactive anthraquinones of Rheum palmatum, possesses potent antitumor properties. Our previous proteomic study revealed that AE-induced apoptosis was associated with oxidative stress and oxidation of many redox-sensitive proteins. In this study, we aimed to further dissect the cell death-signaling pathways in AE-induced apoptosis. AE was found to cause redox imbalance and deplete the intracellular-reduced glutathione (GSH). Manipulation of the intracellular GSH with buthionine-L-sulfoximine (a GSH synthesis inhibitor) sensitized, and with glutathione monomethyl ester (a GSH donor) protected the AE-induced apoptosis, respectively. More importantly, AE treatment led to evident and sustained activation of c-Jun N-terminal kinase (JNK), an important stress-responsive mitogen-activated protein kinase (MAPK). Over-expression of antioxidant gene sod1 significantly reduced AE-induced JNK activation and cell death,

suggesting that oxidative stress-mediated JNK is the effector molecule in AE-induced apoptosis. Such a notion was clearly supported by subsequent studies in which JNK activation was inhibited by JNK inhibitor, JNK small interfering RNA knockdown or over-expression of dominant-negative JNK. In addition, we provided evidence demonstrating the critical role of apoptosis signal-regulating kinase 1, a well-established MAPK kinase kinase, in AE-induced JNK activation and apoptotic cell death. Finally, we showed that dissociation of inactive JNK–Glutathione S-transferase pi (GST-pi) complex was also involved in JNK activation through GST-pi oxidation. Taken together, these results suggest that AE-induced apoptotic cell death is mediated via oxidative stress and sustained JNK activation.

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Anti-cancer properties of anthraquinones from Rhubarb.

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Abstract: Rhubarb has been used as a traditional Chinese medicine since ancient times and today it is still present in various herbal preparations. In this review the toxicological and anti-neoplastic potentials of the main anthraquinones from Rhubarb, Rheum palmatum, will be highlighted. It is interesting to note that although the chemical structures of various anthraquinones in this plant are similar, their bioactivities are rather different. The most abundant anthraquinone of rhubarb, emodin, was capable of inhibiting cellular proliferation, induction of apoptosis, and prevention of metastasis. These capabilities are reported to act through tyrosine kinases, phosphoinositol 3-kinase (PI3K), protein kinase C (PKC), NF-kappa B (NF-kB), and mitogen-activated protein kinase (MAPK) signaling cascades. Aloe-emodin is another major component in rhubarb found to have anti-tumor properties. Its anti-proliferative property has been demonstrated to be through the p53 and its downstream p21 pathway. Our recent proteomic study also suggests that the molecular targets of these

two anthraquinones are different. However, both components were found to be able to potentiate the anti-proliferation of various chemotherapeutic agents. Rhein is the other major rhubarb anthraquinone, although less well studied. This compound could effectively inhibit the uptake of glucose in tumor cells, caused changes in membrane-associated functions and led to cell death. Interestingly, all three major rhubarb anthraquinones were reported to have in vitro phototoxic. This re-evaluation of an old remedy suggests that several bioactive anthraquinones of rhubarb possess promising anti-cancer properties and could have a broad therapeutic potential.