THE CHEMOPREVENTIVE PROPERTY OF PARTHENOLIDE, A SESQUITERPENE LACTONE

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SUMMARY

Parthenolide (PN) is the principal sesquiterpene lactone of feverfew (*Tanacetum parthanium*), a herbal plant that has been used for the treatment of fever, migraine, and arthritis in Europe for centuries. It is well-established that PN possesses strong anti-inflammatory activity, presumably through its known inhibitory effect on the major anti-inflammatory pathways such as NF-κB and signal transducers and activators of transcription (STATs) pathways. However, little is known about the anti-cancer property of PN. Therefore, the main objective of this study is to systematically evaluate the anti-cancer property of PN using a combination of *in vivo* and *in vitro* approaches. The following studies have been conducted: (i) chemopreventive and chemotherapeutic potentials of PN using UVB-induced skin cancer model with SKH-1 hairless mice; (ii) *in vitro* investigation to elucidate the sensitization effect, and the underlining mechanisms of PN in UVB-induced apoptosis in murine epidermal cell line JB6.

We first tested the anti-cancer effect of PN in UVB-induced skin cancer model. SKH-1 hairless mice fed with PN (1 mg/day) showed a delayed onset of papilloma incidence, a significant reduction in papilloma multiplicity (papilloma/mouse) and sizes when compared to the UVB-only group. It was found that PN is as effective as Celecoxib, a specific COX-2 inhibitor with known chemopreventive property against UVB-induced skin cancer. However, our data suggested that COX-2 is unlikely to be the molecular target for PN since neither the COX-2 expression nor PGE_2 production is altered by treatment with PN. We next investigated the molecular mechanism(s) involved in the anti-cancer effects of PN using cultured JB6 murine epidermal cells. Non-cytotoxic concentrations of PN significantly sensitize cells to UVB-induced

apoptosis. Further investigations reveal that PN suppresses the UVB-induced JNK and p38 kinase activations, leading to the downstream inhibitions of c-Jun at Ser-63 and Ser-73 as well as ATF-2. Such suppressions are capable of inhibiting the pro-survival transcription factor AP-1, leading to the sensitization of cells to UVB-induced apoptosis. In addition, PN selectively promotes the pro-apoptotic PKCδ but suppress the anti-apoptotic PKCζ. More importantly, we found that PKCζ acts upstream of p38, but not JNK, to protect cell death induced by PN and UVB.

In conclusion, the overall findings suggest that PN possesses strong chemopreventive property against UVB-induced skin cancer as supported by solid *in vivo* and *in vitro* evidences. These novel findings may shed new light in understanding the anti-cancer activity of PN.

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ABBREVIATIONS

AP-1	Activator protein-1
Apaf-1	apoptosis-activating factor 1
ATP	adenosine triphosphate
Bak	Bcl-2 homologous antagonist
Bax	Bcl-2 associated X protein
BCC	basal cell carcinoma
BH3	Bcl-2 homology domain 3
Bid	BH3-interacting domain death agonist
BSA	bovine serum albumin
CDK	cyclin dependent kinase
COX	cyclooxygenase
CPD	cyclobutane-type pyrimidine dimers
Cyto c	cytochrome c
DAPI	4’,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine-tetra-acetic acid
EIA	enzyme immunoassay
EMSA	electrophoresis mobility shift assay
ERK	extracellular regulated protein kinase
FBS	fetal bovine serum

FLICE	death protease caspase-8
FLIP	FLICE inhibitory protein
GSH	reduced glutathione
IAPs	inhibitors of apoptosis
IKK	IκB kinase
IL	interleukin
IκB	NF-κB inhibitory protein
iNos	inducible isoform of nitric oxide synthase
JNK	c-Jun-N-terminal kinase
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MED	minimal erythema dose
MMP	mitochondrial membrane potential
MPT	mitochondrial permeability transition
MTT	3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide
NER	nucleotide excision repair
NF-κB	nuclear factor-Kappa B
NMSC	non-melanoma skin cancer
NO	nitric oxide
NSAIDS	non-steroidal anti-inflammatory drugs

PI	propidium iodide
PI-3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
ROS	reactive oxygen species
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulfate
STAT	signal transducers and activators of transcription
tBid	truncated Bid
TNFR1	TNF receptor 1
TNFα	tumor necrosis factor alpha
TPA	12-o-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	TdT-mediated dUTP nick end labeling
UV	ultraviolet

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Won, Y.K., Ong, C.N., Shi, X., and Shen, H.M. (2004). Chemopreventive activity of parthenolide against UVB-induced skin cancer and its mechanisms. Carcinogenesis *25*, 1449-1458.

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B-induced apoptosis via PKC but independent of AKT. Eur. J. Cancer (Suppl.) *2*, 172.

CHAPTER 1

INTRODUCTION

1.1 Parthenolide

Feverfew (*Tanacetum parthenium*) has been used as a herbal medicine for the treatment of fever, arthritis, and migraine in Europe for centuries. The crude extracts of this herb is known to have anti-microbial and anti-inflammatory properties (Brown *et al.*, 1997; Jain and Kulkarni, 1999). The principal active component in feverfew is the sesquiterpene lactone (SL) parthenolide (PN). The prefix "sesqui" indicates SLs are 15-carbon terpenoids while the suffix "olide" specifies the presence of a lactone group. Indeed, PN contains a highly electrophilic α-methylene-γ-lactone ring and an epoxide residue capable of interacting rapidly with nucleophillic sites of biological molecules (Figure 1.1) (Macias *et al.*, 1999).

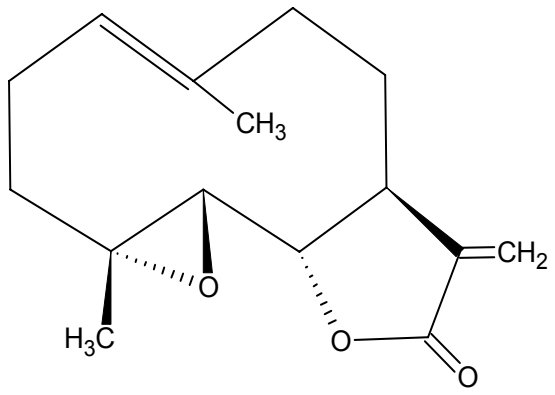


Figure 1.1 Chemical structure of PN

At present, there is some preliminary evidence showing the anti-cancer property of PN. For instance, PN is a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (Woynarowski and Konopa, 1981; Hall *et al.*, 1988; Ross *et al.*, 1999). Patel and coworkers (2000) have reported that PN sensitizes breast cancer cells to chemotherapeutic agent paclitaxel via its inhibitory effect on nuclear factor kappa

B (NF-κB). Wen and colleagues (2002) demonstrated that PN-induced apoptosis involves caspase activation and mitochondria dysfunction in hepatoma cells. Recent studies in our laboratory by another graduate student also demonstrated the anti-cancer property of PN at the cellular level (Zhang et al., 2004a, 2004b, 2004c). Here we would like to systematically discuss the bioactivity of PN, with a focus on its potential anti-cancer effect.

1.1.1 The Metabolism and bioavailability of PN

At present, the metabolism of PN has not been extensively studied. A study by Galal and coworkers (1999) illustrated that microbial transformation of PN by *Rhizopus nigricans, Streptomyces fulvissimus* and *Rhodotorula rubra* yielded a common metabolite 14-hydroxy-11 βH-dihydroparthenolide. On the other hand, an investigation of BF3-mediated rearrangements of PN provided micheliolide as a major product (Castaneda-Acosta *et al.*, 1993). Currently, there is no *in vivo* report on the bioavailability of PN. Using Caco-2 human colonic cells as *in vitro* model of the human intestinal mucosal barrier, it was found that PN is effectively absorbed through the intestinal mucosa via a passive diffusion mechanism (Khan *et al.*, 2003).

PN is a relatively safe compound with few side effects. It has been demonstrated that up to 4 mg of PN given daily as an oral tablet is well tolerated without dose-limiting toxicity (Curry *et al.*, 2004). The major side effect of PN is contact allergy. PN has been identified to be the main content in feverfew to elicit the delayed hypersensitivity effect (Hausen and Osmundsen, 1983). In recent years, PN has been used as a screening agent for Compositae allergy (Orion *et al.*, 1998).

1.1.2 The biochemical properties of PN

1.1.2.1 Reaction with thiols

Thiols (mercaptanes) comprise a class of organic compounds characterized by a sulphydryl group (C-SH). The intracellular biological thiols (or biothiols) can be classified into low molecular weight free thiols such as glutathione (GSH), and high molecular weight protein thiols (Parker, 1995). It has been shown that biothiols play a key role in (1) maintaining the spatial structure of key regulatory proteins and the bioactivity of many cellular enzymes, (2) balancing the intracellular reduction/oxidation status (redox), and (3) acting as antioxidants (Parker, 1995; Deneke, 2000; Paget and Buttner, 2003; Sen, 2000).

PN contains an α-methylene-γ-lactone moiety that is highly reactive with cellular thiols, resulting in alkylation of sulphydryl residues through Michael type addition. Under physiological condition, PN is readily released from its GSH-adduct and then react with its protein target (Schmidt *et al.*, 1999), causing the depletion of protein thiols (Zhang *et al.*, 2004a). The interaction between PN and its protein targets leads to changes in spatial structure and binding capability of proteins and thus inhibits its bioactivity (Garcia-Pineres *et al.*, 2001). Therefore it is believed that depletion of protein thiols by PN has important implications in its bioactivity.

Another important consequence of intracellular thiol depletion is the disruption of the cellular redox balance and induction of oxidative stress. In aerobic organisms, reactive oxygen species (ROS) are constantly produced during aerobic respiration. Thus, a highly regulated antioxidant defense system that consists of a range of enzymatic

proteins and non-enzymatic molecules (such as GSH) has evolved to avoid the potential oxidative damage. The intracellular reduction/oxidation status (redox) is a precise balance between levels of ROS generation and endogenous thiol buffers existing in the cell (Davis *et al.*, 2001). Enhanced ROS production and/or impaired antioxidant defense function will lead to oxidative stress, a status closely associated with many pathological processes in the cell (Halliwell, 1991). In cancer cells treated with PN, elevated levels of ROS has been observed and found to be closely associated with apoptotic cell death (Wen *et al.*, 2002; Zhang *et al.*, 2004a). It is well known that the mitochondrial respiratory chain is the major production site of intracellular ROS. Both the mitochondrial structural integrity and function are subject to a preferential redox condition. The severe oxidative stress conferred by PN-induced thiol depletion results in a disruption of the integrity of mitochondria and triggers mitochondrial permeability transition and release of mitochondrial pro-apoptotic proteins (cytochrome c, Smac, etc.) which then promote apoptosis (Wen *et al.*, 2002; Zhang *et al.*. 2004a).

1.1.2.2 Selective effects on cell proliferation and differentiation

It has been well established that tumor development is closely associated with dysregulation of the cell cycle control mechanisms through either over-expression or activation of cyclin-dependent kinases (CDK) and/or genetic loss/inhibition of CDK inhibitors (Evan and Vousden, 2001) resulting in uncontrolled cell cycling and unremitting cell proliferation. Thus targeting cell cycle regulators is an important and promising approach for cancer therapy (Carnero, 2002). PN has been reported to arrest cell cycle progression at the G2/M checkpoint, especially at low concentrations in an

invasive sarcomatoid hepatocellular carcinoma cell line (SH-J1) (Wen *et al.*, 2002). In addition, PN has been shown to work synergistically with other chemotherapeutic drugs to potentiate the differentiation of leukemia cells (Kang *et al.*, 2002; Kim *et al.*, 2002), a process known to be important in oncogenesis such as the development of leukemia (Tsiftsoglou *et al.*, 2003). This promotion of differentiation suggests the potential of PN as promising anti-cancer agents in leukemia therapy.

1.1.2.3 Induction of apoptosis

Apoptosis is a highly regulated cell death process in eukaryotic cells to eliminate unwanted or potentially harmful cells during development and tissue homeostasis (Hengartner, 2000). The detailed mechanisms of apoptosis will be discussed in later sections.

Similar to other anti-cancer agents, the ability to induce apoptosis in cancer cells is one of the important mechanisms involved in the anti-tumor property of PN. Although the detailed molecular mechanisms have not been fully elucidated, it is believed that the α-methylene-γ-lactone structure is essential for the apoptogenic activity of PN. Once inside the cells, the thiol-reactive PN quickly conjugates with GSH and depletes intracellular thiols, leading to the disruption of cellular redox status and induction of oxidative stress (Wen *et al.*, 2002; Zhang *et al.*, 2004a). The excessive amount of ROS and disrupted redox status then cause the initiation of the mitochondria-dependent apoptosis pathway. It has been shown that PN triggers mitochondrial membrane transition, loss of mitochondrial membrane potential and release of pro-apoptotic

cytochrome c, which subsequently leads to caspase activation and apoptotic cell death (Wen *et al.*, 2002).

1.1.2.4 Sensitization effect

In cancer chemotherapy, combinations of different chemotherapeutic agents are an effective approach to overcome chemo-resistance in certain cancer types. Several recent studies provide evidence that PN sensitizes human cancer cells to chemotherapeutic drugs. For instance, pretreatment with PN significantly increased the paclitaxel-induced apoptosis in breast cancer MDA-MB-231 cells (Patel *et al.*, 2000). Similar sensitization effects by PN were also observed in other cancer cell models (Nakshatri *et al.*, 2004; Zhang *et al.*, 2004b). This sensitization effect by PN is mainly due to the depletion of intracellular thiols and ROS generation or the inhibition of the anti-apoptotic NF-κB signaling pathway. In some cancer cells, the NF-κB signaling pathway is constitutively activated and consequently leads to overexpressions of a variety of NF-κB regulated anti-apoptotic proteins, such as Inhibitors of Apoptosis (IAPs), Bcl-2, and FLICE inhibitory proteins (FLIPs) (Kucharczak *et al.*, 2003). Such a mechanism well explains the sensitization effect of PN on paclitaxel-induced apoptosis in breast cancer cells with constitutively high level of NF-κB activation (Patel *et al.*, 2000). Some cell death ligands, such as TNF, are known to trigger the death receptor pathway with simultaneous activation of the anti-apoptotic NF-κB pathway (Schutze *et al.*, 1995). PN pretreatment is able to block NF-κB activation and then sensitizes TNF-mediated apoptotic cell death in human cancer cells (Zhang *et al.*, 2004b). In addition to TNF, a similar sensitization effect by PN has also been found in TRAIL-induced apoptosis, via

modulation of the c-Jun N-terminal kinase (JNK) signaling pathway (Nakshatri *et al.*, 2004). Sustained activation of JNK has also been shown to play a critical role in the sensitization effect of PN in TNFα-induced apoptosis (Zhang *et al.*, 2004b).

1.1.2.5 Anti-inflammatory effect

It is generally believed that chronic inflammation promotes cancer development in many cancers such as breast, colorectal, and skin cancer (Coussens and Werb, 2002). Many anti-inflammatory drugs such as the nonsteroidal anti-inflammatory drugs (NSAIDs) have been confirmed to reduce the risk of colon cancer formation in both animal cancer models as well as in epidemiological investigations (Coussens and Werb, 2001). Indeed, anti-inflammatory activity is a prominent bioactivity observed in PN, which is often associated with its ability to inhibit NF-κB and thus to down regulate many of the NF-κB-dependent inflammatory responsive genes such as interleukins (IL) (Mazor *et al.*, 2000), cyclooxygenase (COX) (Whan *et al.*, 2001), and inducible nitric oxide synthase (iNOS) (Wong and Menendez, 1999).

Interleukins are a big family of cytokines that play a pivotal role in inflammatory processes. As a potent inhibitor of NF-κB, PN significantly suppressed the expression and secretion of various interleukins including IL-2, IL-4, IL-8 and IL-12 (Mazor *et al.*, 2000; Kang *et al.*, 2001; Li-Weber *et al.*, 2002). In addition, PN also suppresses IL-6 secretion and signaling via the inhibition of signal transducers and activators of transcription (STATs) phosphorylation and activation (Sobota *et al.*, 2000). On the other hand, nitric oxide (NO) is another important regulatory molecule involved in the inflammatory response. Synthesis and release of NO are mediated by iNOS. It is known

that PN suppresses the gene expression of iNOS in several cell lines under stimulation by LPS, interferon-γ (INFγ) or 12-*o*-tetradecanoylphorbol-13-acetate (TPA) (Fukuda *et al.*, 2000).

Cyclooxgenase (Cox) catalyzes the metabolism of arachidonic acid to prostaglandins (PG). There are two isoforms of Cox enzyme: Cox-1 and Cox-2. Cox-1 is the constitutive isoform present in most tissues and mediates the synthesis of PGs for the normal physiological functions. Cox-2 is not detectable in most normal tissues but is induced by cytokines, growth factors, oncogenes, and tumor promoters (Kujubu *et al.*, 1991; Jones *et al.*, 1993; DuBois *et al.*, 1994; Xie and Herschman, 1995). Many studies have shown that skin cancer is one of the cancers with high expression of Cox-2 (Buckman *et al.*, 1998; Chan *et al.*, 1999; Higashi *et al.*, 2000; Kanekura *et al.*, 2000; Athar *et al.*, 2001). Furthermore, it has been demonstrated that UVB exposure induces Cox-2 expression both *in vivo* and *in vitro* (Buckman *et al.*, 1998; Isoherranen *et al.*, 1999). Pentland and co-workers (1999) illustrated that oral administration of a selective Cox-2 inhibitor, Celecoxib, reduced the tumor number and multiplicity in UVB-treated SKH-1 hairless mice. In colorectal cancer model, COX-2 was shown to promote cancer development by suppressing apoptosis, facilitating angiogenesis, and enhancing metastatic potential cancer cells (Gupta and Dubois, 2001). In summary, the ability of PN to suppress the COX-mediated pathway is either through directly inactivating COX-2 enzyme activity via interaction with sulphydryl groups on enzymes (Pugh and Sambo, 1988; Hwang *et al.*, 1996), or by indirectly inhibiting COX-2 transcription through NF-κB (Hehner *et al.*, 1998).

1.2 UVB-induced skin cancer and the molecular mechanisms

Solar ultraviolet radiation (UV) wavelength range borders at the violet end of the visible light range at 400 nm. Wavelength ranges from 400 to 320 nm is classified as UVA. UVB ranges from 320 to 280 nm, and UVC ranges from 280 to 200 nm. No radiation of wavelengths shorter than 290 nm reaches the earth surface because of the absorption in the atmosphere by oxygen and ozone. UVB is absorbed by the epidermis, resulting in epidermal cell damage, repair and hyperkeratosis (thickening of the stratum corneum). In contrast, UVA leads predominantly to dermal effects and does not cause epidermal thickening (Pearce *et al.*, 1987). In general, shorter UV wavelength tends to be stronger carcinogen when compare to the longer wavelength. UV, especially in the UVB range, has been shown epidemiologically and demonstrated experimentally to be the major cause of skin cancer in both human and animals.

Skin cancer is the most common type of cancer among Caucasians. According to the Singapore Cancer Society, skin cancer is the 7th most common cancer in both men and women in Singapore. Skin cancer can be categorized into two groups: malignant melanoma and non-melanoma skin cancer (NMSC). Malignant melanoma is relatively rare but a more severe form of skin cancer. It is derived from the melanocytes (pigment cells) in the skin. This type of tumor can grow extremely aggressive and metastasize very rapidly. NMSC is the more common type of skin cancer that includes squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). These skin cancers stem from the epithelial cells that form the epidermis. This part of the skin absorbs most of the carcinogenic UV radiation. SCC is a neoplasm of epidermal cells that differentiate toward keratin formation, and in advance stages, it will lose the structural organization

and the cells may become spindle shaped. Typically, SCC is invasive and more than 10% will metastasize (Kwa *et al.*, 1992). In contrast, basal cell carcinomas (BCC) can be locally invasive and destructive (Miller, 1991). BCC is composed of undifferentiated cells from the germinal, basal layer of the epidermis. In most cases, NMSCs are removed in an early stage of development and thus far less dangerous than malignant melanoma.

The mechanism(s) of UVB-induced skin cancer has not be fully elucidated. Many molecular cascades and targets have been proposed and are described as follow.

1.2.1 DNA damage

It is believed that DNA damage induced by UV initiates the carcinogenesis process. UV produces a number of DNA lesions, with cyclobutane-type pyrimidine dimers (CPD) being the major one. CPD may be formed by covalent interaction of two adjacent pyrimidines in the same polynucleotide chain. Another type of UV-induced DNA lesion is the pyrimidine-pyrimidone, or (6-4) lesions. The (6-4) adducts include TC, CC, and TT sequences. Generally, TC lesions occur with greater frequency than CC, and TT lesions occur only at very high UV doses. These lesions occur at a frequency that is several-fold less than CPD formation. Other types of damage may also occur, for example, single strand breaks, DNA crosslinks, and purine photoproducts (for review, see Ravanat *et al.*, 2001). Both CPD and (6-4) lesions distort the DNA helix. The ability of UV light to damage a given base is based on the flexibility of the DNA. Sequences that favor bending and unwinding are likely sites for damage formation. For instance, the possibility of CPD formation is much higher in single-stranded DNA (Becker and Wang,

1989) at the flexible ends of poly (dA) (dT) tracts, but not at their rigid center (Lyamichev, 1991).

The significance of these DNA lesions depends upon the capacity of the cell to repair the damage before it can be incorporated into the genome. Typically, DNA damage is repaired at a relatively high rate in human cells by the nucleotide excision repair (NER) mechanisms (Thoma, 1999). NER is divided into two subpathways: transcription-coupled repair (TCR) and global genome repair (GG). The TCR-NER represents the preferential repair of transcribed strands in active genes, while GG-NER refers to repair in non-transcribed parts of the genome that includes the non-transcribed strand of transcribed genes. The first step in NER is damage recognition where lesions are identified in linker DNA that is not protected by chromosomal proteins. The next step in NER is the open complex formation and damage verification. This process requires more space (about 100 bp of DNA) to exercise (Huang and Sancar, 1994), and this may be provided by chromatin remodeling. After excision of the damaged nucleotides, new DNAs are synthesized by DNA polymerases for repair purposes. Finally, DNA is completely restored by the ATP-dependent activity of DNA ligand (for review, see Thoma, 1999; Costa *et al.*, 2003).

1.2.2 NF-κB

NF-κB is a transcription factor involved in the regulation of various important cellular functions including immune response and cell growth, cell death and development. The active NF-κB complex is either a homo- or heterodimer consist of proteins from the NF-κB/RelA family. Five members of the mammalian NF-κB/RelA

proteins of two classes have been cloned and characterized (Karin and Ben-Neriah, 2000). The first class includes p65 (RelA), RelB, and c-Rel proteins that are synthesized as mature products and do not require proteolytic processing. The second class is encoded by *NFκB1* and *NFκB2* genes, and their products are first synthesized as large precursors p105 and p100. Upon further proteolytic processing, these precursors will yield the mature p50 (*NFκB1*) p52 (*NFκB2*) proteins.

As illustrated in Figure 1.2, NF-κB is bound to the inhibitory protein IκB in the cytoplasm in normal unstimulated cells. When cells are activated, the serine-specific IκB kinase (IKK) complex will phosphorylate IκB at serine residues 32 and 36 (Brown *et al.*, 1995; DiDonato *et al.*, 1996). The IKK complex consists of two catalytic subunits: IKKα and IKKβ that can phosphorylate IκB, and one regulatory subunit IKKγ. The phosphorylated IκB will be recognized by $E3RS^{I\kappa B}$, a receptor component of a SCF ligase family type E3, leading to the polyubiquitination of IκB and subsequent degradation by the 26S proteasome (Karin and Ben-Neriah, 2000). After dissociation from IκB, NF-κB is then translocated into the nucleus and binds to specific DNA binding site and consequently modulates gene expression.

The functional role of NF-κB in skin physiology and pathology has been well appreciated. In normal murine skin, the p50 subunit of NF-κB is found in the cytoplasm of basal keratinocytes. In contrast, p50 is translocated into the nucleus in the suprabasilar keratinocytes where cells undergo differentiation. This suggests that NF-κB is activated concomitant with the time at which basal epidermal cells exit cell cycle and enter terminal differentiation (Seitz *et al.*, 1998). The process allows the establishment of the epidermal barrier that is essential to keep bodily fluids in and harmful microorganisms

out. The same study showed that overexpression of p50 and p65 causes profound epidermal thinning, whereas blocking of NF-κB activity results in epidermal hyperplasia. Both conditions are associated with early death in the transgenic mice. By targeting IκB kinase, the regulator of NF-κB, similar phenotypes have been produced in which blocking of NF-κB activation was associated with pathologically thickened murine epidermis (Hu *et al.*, 1999; Li *et al.*, 1999; Takeda *et al.*, 1999). These *in vivo* results suggest that NF-κB plays an important role in modulating the phenotype of epidermal keratinocytes. In addition, NF-κB activation in the epidermis has been shown to protect against apoptosis (Chaturvedi *et al.*, 1999; Seitz *et al.*, 2000). Seitz and coworkers (2000) illustrated that blockage of NF-κB activation enhances the susceptibility of normal epithelial cells to TNF-α and Fas-induced apoptosis, presumably via suppressed expression of antiapoptotic factors such as TRAF1, TRAF2, c-IAP1, and c-IAP2.

It is believed that UV activates NF-κB (Devary *et al.*, 1993) through a distinct pathway. For instance, the UV-induced IκB degradation is not mediated through the phosphorylations of Ser-32 and Ser-36, a mechanism used by TNF-α or IL-1α. Furthermore, the activation of NF-κB induced by UV is believed to act in an IKK-independent manner (Li and Karin, 1998; Bender *et al.*, 1998). In addition, some studies have looked into the role of TNF-α receptors in UVB-induced skin tumor and NF-κB signaling pathway. Starcher (2000) showed that the absence of either TNFR1 or TNFR2 significantly reduced skin tumor in response to UVB irradiation. Tobin and colleagues (1998) demonstrated that NF-κB activation by UVB in keratinocytes causes a rapid association of TNF-α receptor 1 with its downstream partner TRAF-2. Expression of a

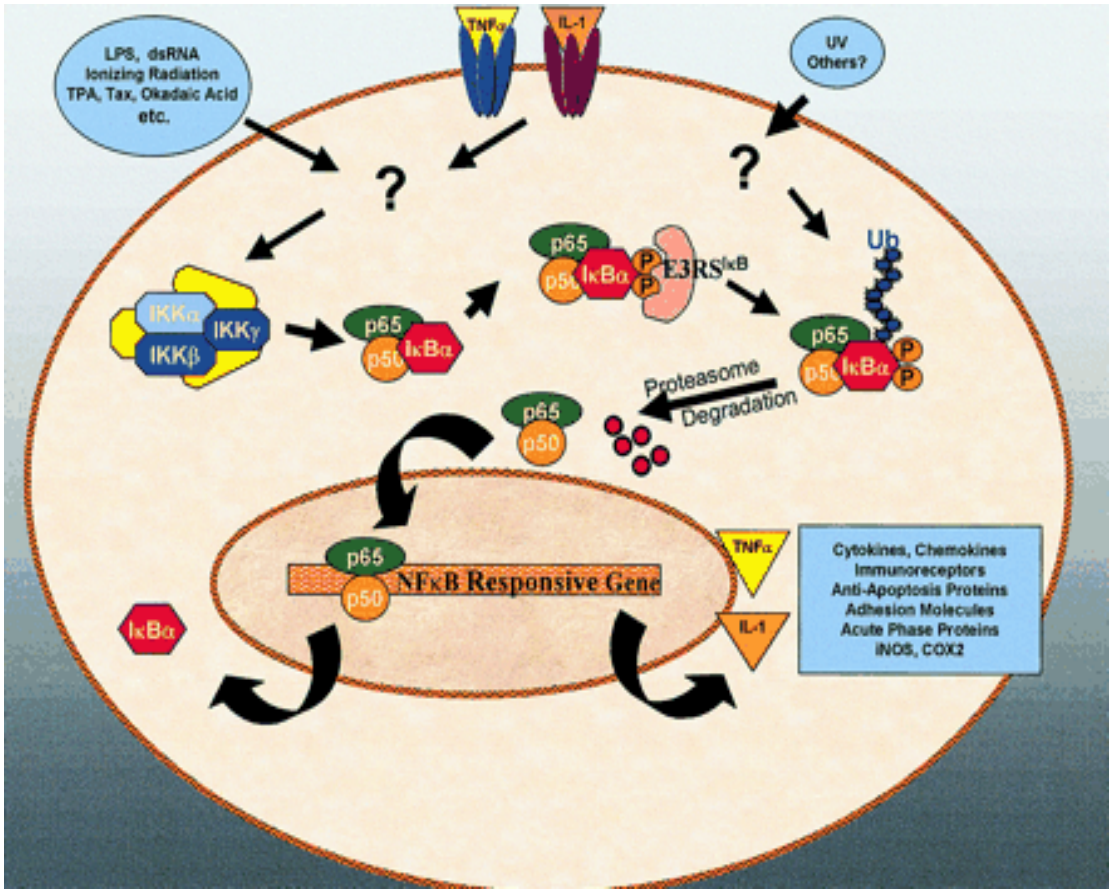


Figure 1.2 A schematic model of various stimuli including UV-induced NF-κB activation pathway (Adapted from Karin and Ben-Neriah, 2000)

dominant negative TNFR1 or TRAF-2 protein could both lead to an inhibition of UVB-induced Rel-dependent transcription. The work showed that UVB-induced activation of NF-κB via TNFR1 is a key component in the UV response in keratinocytes.

1.2.3 Activator protein (AP)-1

Activator Protein-1 (AP-1) has been shown to be an important transcription factor in UV-induced signal transduction. The mammalian AP-1 transcription factor complex consists of either homodimers or heterodimers of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Jun dimerization partners (JDP1 and JDP2), and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF). Jun proteins can form stable homodimer, while Fos forms heterodimer with Jun. The resulting AP-1 complex can then binds to and transactivates from a cis element called the TPA-response element (TRE). On the other hand, ATF proteins form homodimers as well as heterodimers with Jun that preferentially bind to cAMP responsive elements (CRE) (Karin *et al.*, 1997; Shaulian and Karin, 2001).

AP-1 activity is stimulated by many physiological stimuli such as growth factors (Wu *et al.*, 1989; Lamb *et al.*, 1997), tumor promoters (Hashimoto *et al.*, 1990; Domann *et al.*, 1994; Huang *et al.*, 1997), TNF-α (Brenner *et al.*, 1989), and interleukin-1 (Goldgaber *et al.*, 1989; Muegge *et al.*, 1989).AP-1 regulates the transcription of many genes involved in cell proliferation, apoptosis, metastasis, and cellular metabolism (Angel *et al.*, 2001; Jochum *et al.*, 2001; Ozanne *et al.*, 2000). The role of AP-1 in tumor promotion is supported by the fact that viral and cellular Jun or Fos can cause malignant transformation in fibroblasts (Lamb *et al.*, 1997; Suzuki *et al.*, 1994). Gene products

promoting invasion and metastasis are also under AP-1 regulation (Lamb *et al.*, 1997). Skin tumor cell lines that show constitutive AP-1 activity lose their ability to form tumors on s.c. injection when transfected with a dominant negative Jun mutant (Domann *et al.*, 1994). When AP-1 is blocked during tumor promotion, transformation is also inhibited (Huang *et al.*, 1997).

Short wavelength ultraviolet (UV) radiation such as UVB (Ghosh *et al.*, 1993) and UVC (Adler *et al.*, 1996; Karin *et al.*, 1998) can also stimulate AP-1 activity. UVB induces c-Fos mRNA and protein levels, AP-1 binding and transactivation in HaCaT cells (Garmyn *et al.*, 1992; Ghosh *et al.*, 1993). It has been shown that UV induced JNK activation, leading to phosphorylation of c-Jun and ATF2 to enhance their transcriptional capacity (Gupta *et al.*, 1995; Karin, 1995). Wisdom and coworkers (1999) demonstrated that c-Jun protects fibroblasts from UV-induced cell death and cooperates with NF-κB to prevent apoptosis induced by TNF-α. In addition, Huang and colleagues (1999) showed that Erks is required for UV-induced AP-1 activation in mouse JB6 cells. These findings suggest AP-1 plays an important role in the UV response in addition to being involved in mitogenic and proinflammatory responses.

1.2.4 Mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinases (MAPKs) are a superfamily of enzymes that play key roles in transmitting signals from the membrane or cytoplasm to the nucleus (Seger and Krebs, 1995). Mammals express at least four distinctly regulated groups of MAPK: 1) extracellular signal-regulated kinases (ERKs); 2) c-Jun N-terminal kinases (JNKs); 3) p38; and 4) ERK5 (Chang and Karin, 2001). They are structurally related but

biochemically and functionally distinct. In Erks cascade, MAP/ERK kinase kinase kinases (MKKKs) phosphorylate and activate MAPK/ERK kinases MEK-1 and MEK-2. These in turn activate ERKs by dual phophorylation on tyrosine and threonine residues (Marshall, 1994). Growth factors and phorbol esters primarily activate ERKs, whereas stress or inflammatory cytokines are only poor activators of ERKs (Whitmarsh *et al.*, 1995; Xia *et al.*, 1995). It has been established that the ERK signaling response is critical for keratinocyte proliferation, clonal formation, and survival (Geilen *et al.*, 1996). Peus and coworkers (1999) showed that ERK 1/2 are rapidly and strongly activated within minutes of UVB exposure in human keratinocytes.

In contrast, both p38 and JNK are poorly activated by epidermal growth factor and phorbol esters, but readily activated by a wide range of cellular stress factors such as osmotic shock, heat shock, inflammatory cytokines, and UV light (Peus *et al.*, 1999; Rouse *et al.*, 1994; Raingeaud *et al.*, 1995; Derijard *et al.*, 1995; Wesselborg *et al.*, 1997). P38 can be phosphorylated and activated via MEK kinase kinase 3, 4 and 6 and small GTP-binding proteins such as Cdc42 and Rac (Lamarche *et al.*, 1996; Derijard *et al.*, 1995; Raingeaud *et al.*, 1996). MEK 4 and 7 have been shown to phosphorylate and activate JNK. Like other members of MAPK family, both p38 and JNK requires threonine and tyrosine phosphorylation for their enzymatic activities (Raingeaud *et al.*, 1995). It has been shown that UVB induces transient and rapid JNK activation within minutes of irradiation (Peus and Pittelkow, 2001). ERK5, on the other hand, is activated by MEK-5. The exact role of ERK5 signaling pathway is yet to be elucidated.

As illustrated in Figure 1.3, one or more MAPKKs catalyze the phosphorylation of MAPKs, and they are in turn activated by MAPKKKs in response to UV. Activated

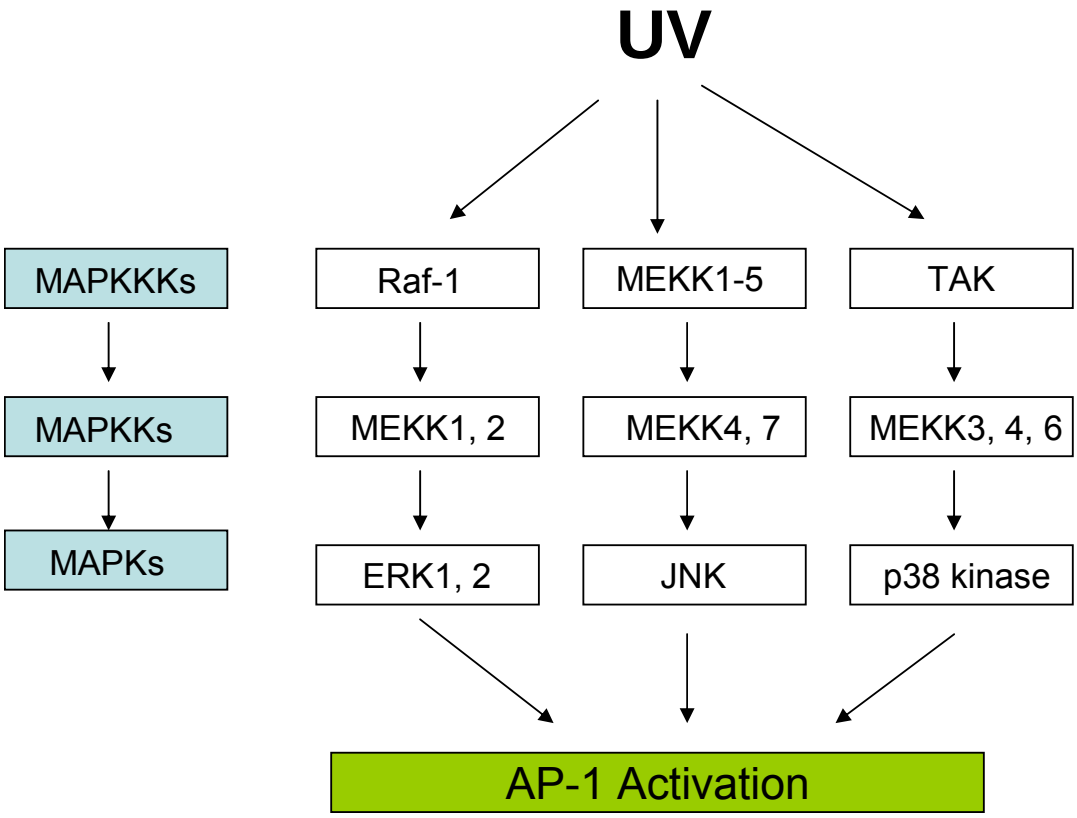


Figure 1.3 General overview of the UV-induced MAPK signaling cascade

MAPKs translocate to the nucleus where they phosphorylate target transcription factors such as AP-1.

1.2.5 Protein kinase C

Protein kinase C (PKC) is a group of serine/threonine kinases that regulate many cellular functions such as proliferation, differentiation, transformation, survival and apoptosis (Yang and Kazanietz, 2003). PKC can be classified into 3 groups based on the co-factors required for activation: 1) the Ca^{2+} and diacylglycerol (DAG)-dependent classical or conventional PKC that consists of isotypes α, β1, β2 and γ; 2) the DAG-dependent, Ca^{2+}-independent novel PKC that consist of δ, η, ε and θ, and 3) the DAG- and Ca^{2+}-independent atypical PKC that consist of ι/λ and ζ.

It has been shown that the UVB-induced AP-1 activation may involve certain types of PKCs (Huang *et al.*, 1996; 1997; 2000a). UVB induces the translocations of PKC δ and PKCε from the cytosol to membrane, an indication of their activations (Chen *et al.*, 1999). The UVB-induced activations of ERK and JNK were strongly inhibited by dominant-negative mutants of PKC δ and PKCε as well as rotterlin, a specific inhibitor of PKC δ. The result of such inhibition leads to suppressed UVB-induced apoptosis and thus suggested both PKC δ and PKCε mediate UVB-induced signal transduction and apoptosis through the activations of ERK and JNK (Chen *et al.*, 1999). Furthermore, inhibition of PKCλ/ι with a dominant negative mutant suppressed UVB-induced ERK and the subsequent AP-1 activation (Huang *et al.*, 1996). On the other hand, antisense oligonucleotide of PKCζ has been shown to inhibit the UVB-induced AP-1 activation (Huang *et al.*, 2000a).

The consequences of PKC activation by UVB is rather cell type specific and could lead to inhibition on cell proliferation or even induction of apoptosis. Among all, PKCδ seems to be the main PKC subtype with pro-apoptotic functions in response to various extracellular stimuli including UVB (Chen *et al.*, 1999; Brodie and Blumberg, 2003) whereas PKCζ has been shown to be anti-apoptotic in response to UV (Berra *et al.*, 1997; Frutos *et al.*, 1999).

1.2.6 PI3-K and AKT

Another important signaling pathway in tumorigenesis involves the phosphatidylinositol-3 kinase (PI-3K). PI-3K is known to mediate a broad range of biological effects that include cell proliferation, survival and adhesion, organization of cytoskeleton, and glucose metabolism (Hu *et al.*, 1995; Jhun *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996; Leevers *et al.*, 1999; shepherd *et al.*, 1998). PI-3 K dimer consists of a catalytic subunit P110 and a regulatory subunit P85 (Carpenter *et al.*, 1990). The P85 regulatory subunit has no catalytic activity but possesses two Src homology 2 domains and a Src homology 3 domain (Kapeller and Cantley, 1994). A region between the two Src homology 2 domains of P85 binds the NH2 terminus of P110, mediating the constitutive association of the two subunits (Kapeller and Cantley, 1994).

The role of PI metabolism in tumorigenesis was implied in observations that the regulatory subunit of PI-3K was able to interact with certain oncogenes directly. These interactions were shown to be responsible for the associated PI-3K activity (Kaplan *et al.*, 1987; Serunian *et al.*, 1990). A study by Chang and coworkers (1997) showed an oncogenic form of the catalytic subunit of PI-3K that was cloned from a retrovirus could

cause hemangiosarcomas in chickens and induce transformation of chicken embryo fibroblasts. Furthermore, Shayesteh and colleagues (1999) demonstrated the amplification of the human gene encoding the p110α catalytic subunit of PI-3K in ovarian cancer tissue samples and cell lines.

It has been shown that UVB irradiation activates PI-3K, suggesting a possible role of PI-3K in UVB-induced skin cancer (Kabuyama *et al.*, 1998; Nomura *et al.*, 2001a). Furthermore, changes in the activity of PI-3K signaling pathway due to the dysregulation of certain downstream PI-3K components such as Akt (PKB) in many cancers further elaborates the importance of PI-3K signaling pathway in tumorigenesis.

On the other hand, the serine-threonine kinase Akt (or protein kinase B) is activated by a variety of growth stimuli including epidermal growth factor and insulin through PI-3K (Burgering and Coffer, 1995; Shepherd *et al.*, 1998). In quiescent or unstimulated cells, Akt resides within the cytosol in a catalytically inactive state. Once activated, the phospholipids (PI-3,4,5-P_3 or PI-3,4-P_2) produced by PI-3K will bind to the PH domain of Akt kinases and trigger the translocation of Akt to the plasma membrane. Upon membrane localization, Akt molecules are phosphorylated at Thr-308/309 in the kinase activation loop and Ser-473/474 carboxyl-terminal tail. The phosphorylation at Thr-308/309 is necessary for Akt activation whereas Ser-473/474 phosphorylation is only required for maximal activity (Bellacosa *et al.*, 1998). PDK-1 (3-phosphoinositide dependent kinase-1), which is activated by PI-3,4,5-P_3 and PI-3,4-P_2, phosphorylates Akt at Thr-308/309. On the other hand, the putative PDK-2 phosphorylates Akt at Ser-473/474 (Alessi *et al.*, 1996; 1997; Chan and Tsichlis, 2001).

Akt has been shown to control intracellular pathways responsible for preventing cell death in response to a variety of extracellular stimuli (Marte and Downward, 1997; Dudek *et al.*, 1997; Kulik *et al.*, 1997). Notably, Akt phosphorylates Bad at Ser-136. This phosphorylation facilitates Bad binding to protein 14-3-3 and thus neutralizes the apoptotic effect of Bad (Datta *et al.*, 1997). Recent studies suggested that Akt is not only a "cell survival" kinase, but it may play an important role in other cellular functions. Akt phosphorylates and inactivates glycogen synthase kinase 3 (GSK-3) and thereby stimulates glycogen synthesis (Cross *et al.*, 1995). Akt activation also affects cell progression through regulation of cyclin D stability (Muise-Helmericks *et al.*, 1998) and inhibition of $p27^{Kip1}$ protein levels (Collado *et al.*, 2000; Medema *et al.*, 2000). In addition, Akt mediates the activation of endothelial nitric oxide synthase, an important modulator of angiogenesis and vascular tone (Fulton *et al.*, 1999; Dimmeler *et al.*, 1999). The activation of Akt also enhances telomerase activity via phosphorylation of the human telomerase reverse transcriptase subunit (Kang *et al.*, 1999).

Furthermore, identification of gene encoding Akt as a transforming oncogene that causes thymic lymphomas in mice suggests a role for Akt in tumorigenesis (Bellacosa *et al.*, 1991). Overexpression of Akt has been demonstrated in many cancers in human, for instances, ovarian (Cheng *et al.*, 1992), pancreatic (Cheng *et al.*, 1996), and breast cancers (Bellacosa *et al.*, 1995). Mutations of tumor suppressor gene PTEN, which directly antagonizes PI-3K, have been observed in some human cancers (Li and Sun, 1997; Steck *et al.*, 1997). The alteration of PTEN causes elevated phosphorylation of Akt (Dahia *et al.*, 1999; Davies *et al.*, 1999; Suzuki *et al.*, 1998). Recently, PTEN has

been shown to sensitize glioblastoma cells to chemotherapy (Mayo *et al.*, 2002). In addition, it has been shown that UVB irradiation induced Akt phosphorylation at both Thr-308 and ser-473 (Nomura *et al.*, 2001b). Their results illustrated that Erks and p38 of the MAP kinase family mediate UVB-induced Akt activation via Msk1 (mitogen and stress activated protein kinase-1). Mayo and Donner (2001) showed that phosphorylation by Akt is needed for Mdm2 (an oncoprotein) to be translocated into the nucleus to reduce p53 levels and transactivation. The study establishes a novel mitogen-regulated pathway linking the PI-3K/Akt and Mdm2/p53. Furthermore, Tang and co-workers (2001) demonstrated that overexpression of a dominant negative Akt mutant suppress UVB-mediated induction of Cox-2 promoter, implying a role of Akt in the UVB-induction of Cox-2 expression.

1.2.2.7 P53

UV radiation induces a high level of the tumor suppressor gene p53 expression in normal human skin (Healy *et al.*, 1994; Hall *et al.*, 1993; Campbell *et al.*, 1993a). In dividing cells, p53 is expressed at very low levels but is up-regulated in response to DNA damage. Up-regulation of p53 levels results in growth arrest probably via induction of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, followed by DNA repair, or apoptosis. Mutation of p53 by UV radiation would prevent G1 arrest and the initiation of apoptosis pathway and hence favor the continue division of mutant cells and the accumulation of additional mutations. Brash and co-workers (1991) demonstrated C→T and CC→TT transitions in the p53 gene in 58% of human squamous cell carcinoma. Since then, other researchers have detected p53 mutations in human basal cell carcinoma and actinic

keratoses (Ziegler *et al.*, 1993; Rady *et al.*, 1992; Moles *et al.*, 1993; Nataraj *et al.*, 1995). In animal studies, p53 mutations have been found in 20-100% of mouse skin cancers. The frequency of p53 mutations differ among mouse strains (Kress *et al.*, 1992; Kanjilal *et al.*, 1993), with CC→TT transition at dipyrimidine sites being the most detected mutation spot. Generally, mutations in p53 in UV-induced skin cancers appear to have an early onset (Campbell *et al.*, 1993b; Nakazawa *et al.*, 1994). In addition, UV signature p53 mutations were found in 60% of actinic keratoses (Hall *et al.*, 1993), an early stage of SCC. Recently, Jiang and colleagues (1999) showed that p53 knockout mice possess higher susceptibility to UVB-induced skin cancer, but these mice fail to develop spontaneous skin cancers. The results suggested that p53 plays a major role in the response of keratinocytes to UV-induced DNA damage and a less prominent role in the control of normal proliferation of skin tissue.

One of the many mechanisms that regulate p53 activity is phosphorylation at multiple sites on the N-terminal activation domain (amino acid 1-42) by several different protein kinases both in vivo and in vitro (Meek, 1998; Giaccia and Kastan, 1998). Recent studies showed that phosphorylation of p53 protein at serine 15 may play a critical role in the stabilization, up-regulation, and functional activation of p53 during cellular stress (Shieh *et al.*, 1997; Siliciano *et al.*, 1997; Unger *et al.*, 1999; Lambert *et al.*, 1998). Shieh and coworkers (1997) demonstrated that the phosphorylation of serine 15 in vitro by DNA-dependent protein kinase (DNA-PK) leads to reduced binding to its negative regulator, the oncoprotein murine double minute 2 (MDM2). This will lead to the release of p53 from the p53-MDM2 complex and stabilize p53 by preventing proteosome-mediated p53 degradation. Recently, ERKs and p38 of the MAP kinase family has been

demonstrated to phosphorylate p53 protein at serine 15 in response to UV irradiation (She *et al.*, 2000). In addition to serine 15, serine 20 and threonine 18 phosphorylation have been shown to weaken the interaction of p53 and MDM2 in vitro (Bottger *et al.*, 1999; Unger *et al.*, 1999).

In addition to the N-terminal phosphorylation, modification also occurs at the C-terminal. It has been reported that serines 376 and 378 are normally phosphorylated in unstimulated cells and ionizing radiation (IR) induces rapid dephosphorylation of serine 376 (Waterman *et al.*, 1998). The change will open up a binding site to which protein 14-3-3 can bind, leading to stimulation of the site-specific DNA binding function of p53. p53 is also acetylated at C-terminal lysine residues 320 by transcription factor PCAF and at 373 and 382 by another transcription factor p300/CBP in response to IR and UV (Gu and Roeder, 1997; Liu *et al.*, 1999; Sakaguchi *et al.*, 1998).

1.3 Apoptosis and carcinogenesis

1.3.1 General introduction

Apoptosis, or programmed cell death, was first introduced by Kerr and colleagues (1972) to describe a highly regulated and conserved cell death process in eukaryotic cells which is morphologically and biochemically distinct from necrotic cell death. Generally, the process of apoptosis can be divided into 3 distinct phases: (1) Initiation phase, which involves the activation of the central molecular machinery initiated by pro-apoptotic stimuli; (2) Effector phase, which signals the committed cells undergoing a complete activation of the apoptotic machinery; (3) Degradation phase, which is characterized by the morphological and biochemical changes associated with apoptosis (Sanfilippo and

Blaho, 2003). The morphological changes of apoptosis include cell shrinkage associated with loss of intracellular fluid and ions, plasma and nuclear membrane blebbing, chromatin condensation and DNA fragmentation, and the formation of apoptotic bodies (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Schwartzman and Cidlowski, 1993). Apoptotic bodies are removed through phagocytosis by macrophages before plasma membrane integrity is lost (Savill *et al.*, 1993). Normally, the signal for apoptotic body's clearance is triggered by the "flipping" of phosphatidylserine from the inner to the outer surface of the plasma membrane (Fadok *et al.*, 1992). The rapid clearance of apoptotic cells thus does not induce inflammatory response or tissue scarring *in vivo*.

It is generally believed that apoptosis is a genetically controlled active cell death process to eliminate unwanted or potentially harmful cells during development and tissue homeostasis (Hengartner, 2000). The machinery of apoptosis is highly conserved throughout revolution with many similarities between invertebrates and humans. It has been shown that apoptosis can be induced in many cell types by various physiological or biochemical stimuli, including hormones, toxins, chemotherapeutic agents, carcinogens, ionizing radiation, growth factor withdrawal and physical trauma (McConkey, 1998). Abnormalities in the apoptotic process can result in serious pathological conditions such as cancer (Bursch *et al.*, 1992), acute diseases (infarction, ischemia-reperfusion damage, septic shock, etc.) (Decaudin *et al.*, 1998), some auto-immune diseases (Tan, 1994), acquired immunodeficiency syndrome (AIDS) (Ameisen *et al.*, 1995) and neurodegenerative diseases (Alzheimer's disease) (Raff *et al.*, 1993). Therefore, a good understanding of the molecular processes regulating apoptosis will greatly assist in developing therapies for apoptosis-related diseases.

1.3.2 Mechanisms

Apoptosis is a genetically encoded process to removed unwanted cells during development and tissue homeostasis. Even though the mammalian signal transduction pathways affecting apoptosis is complex, substantial progress has been made in recent years to better define the nature and detail signaling pathways involved in this process. Indeed, the identities and precise substrate specificities of the "central executioner" in apoptosis, a large family of cysteine proteases or caspases, have been defined (Nicholson and Thornberry, 1997; Nicholson, 1999). In addition, a prominent role for mitochondria in apoptosis has also emerged as a critical pathway during the execution phase of apoptosis. It has been shown that mitochondria can trigger apoptosis by release of cytochrome c from intermembrane space to the cytosol (Li *et al.*, 1997). Furthermore, several protein families specifically involved in the molecular mechanism of apoptosis have been investigated, which include Bcl-2 and p53 (Fraser and Evan, 1996; Steller, 1995). Below is a brief description of the two major modulators identified in apoptosis, namely caspases and mitochondria.

1.3.2.1 Caspases

Genetic analyses in the nematode worm C *elegans* have identified two genes required for apoptosis in the developmental stage, namely *ced*-3 and *ced*-4, and one that inhibits apoptosis, *ced*-9 (Ellis *et al.*, 1991). The first human homologue of *ced-3* being identified was the interleukine-1β-converting enzyme (ICE) (Yuan *et al.*, 1993). Many subsequent studies have revealed a large family of related protease that share the property

of being cysteine protease with specificity for cleaving substrates on the carboxyl side of aspartate residues (Alnemri *et al.*, 1996). Thus, the generic term "caspase" (c-asp-ase) was adopted and the individual caspases are numbered based on their chronological order of discovery. Thus far, there are at least 14 discrete caspases have been identified in mammals (Sanfilippo and Blaho, 2003).

Two major pathways of caspase activation induced by pro-apoptotic stimuli have been characterized (Sanfilippo and Blaho, 2003). The first pathway is initiated by the extracellular binding of a death ligand to its receptor on the cell surface such as Fas/CD95 and TNFR1 (extrinsic pathway). The activated receptor complex then serves as the docking site for other adaptor proteins to recruit the initiator caspase-8 and 10, which then activate effector caspase-3. The second pathway integrates and intracellular apoptotic signal via a mitochondrial route (intrinsic pathway). It was demonstrated that cytochrome c, the electron transport chain intermediate, was released from the mitochondria at an early stage in apoptosis (Li *et al.*, 1997). Once released, cytochrome c directly binds to apoptosis protease activating factor-1 (Apaf-1) in a dATP-dependent manner and promotes a conformational change that allows caspase-9 to join the complex termed "apoptosome" (Li *et al.*, 1997). This binding results in the enzymatic activation of caspase-9, which then activate a set of effector caspases such as caspase-3, 6 and 7 (Cohen, 1997).

Although caspases have been shown to play a major role in apoptosis in various experimental systems, there are reports that show cells can also undergo caspase-independent cell death. Examples of this caspase-independent cell death can be found in glucocorticoid-induced death of thymocytes (Hirsch *et al.*, 1997), death of

haematopoietic cell lines induced by growth factor withdrawal (Ohta *et al.*, 1997), death of target cells of cytotoxic T cells (Sarin *et al.*, 1997), cell death induced by the second messenger ceramide (De Maria *et al.*, 1997), or the enforced expression of Bax (Xiang *et al.*, 1996), Bak and c-Myc (McCarthy *et al.*, 1997). Therefore, these observations argue that an alternative mechanism is able to mediate cell death in caspase-independent manner, possibly through activations of other protease such as serine protease, calpains and cathepsins (Borner and Monney, 1999). Further studies are needed to provide a better understanding for this distinct mechanism of apoptosis.

1.3.2.2 The mitochondria

It is well established that mitochondrial alterations have a major impact on apoptosis. Several independent lines of evidence have highlighted the involvement of mitochondria in apoptosis. First of all, kinetic data indicate that mitochondria undergo major changes in membrane integrity before the onset of classical signs of apoptosis. Generally, a disruption of mitochondria membrane potential (MMP) is detected early in the apoptosis process, well before all the major changes in cell morphology and biochemistry that include nuclear DNA fragmentation and the exposure of phosphotidylserine on the outer surface of plasma membrane (Susin *et al.*, 1998). Furthermore, some profound structural and functional changes in mitochondria also occur during apoptosis, leading to the release of intermembrane proteins such as cytochrome c and apoptosis inducing factor (AIF) (Liu *et al.*, 1996; Susin *et al.*, 1996; Kluck *et al.*, 1997). These mitochondria products are capable of mediating the activation of caspases

and endonucleases that cause isolated nuclei to undergo apoptosis in cell free system (Zamzami *et al.*, 1996; Susin *et al.*, 1996).

In addition, functional and pharmacological studies propose that mitochondria permeability transition (MPT) induced by the permeability transition (MT) pore opening is one of the key mediators of apoptosis. Upon activation by pro-apoptotic stimuli, a large nonselective conductance channel composed of both inner and outer membrane proteins is opened (Green and Reed, 1998). This MT pore allows ions of the matrix and intermembrane space to equilibrate and causes the mitochondrial inner transmembrane potential ($\Delta\Psi^2$m) to collapse by dispelling the H^+ gradient (Petit *et al.*, 1995; Qian *et al.*, 1997) .Opening of the PT pore also induces volume expansion of the matrix, eventually leading to the rupture of the outer membrane and the release of caspase activators such as cytochrome c, Smac/DIABLO, AIF, and EndoG nuclease into the cytosol (Green and Evan, 2002). Moreover, the importance of MPT in apoptosis is further strengthened by the fact that pharmacological inhibitors of MPT such as cyclosporine A and Bongkreikic acid are capable of preventing the loss of MMP and thus blocking apoptosis (Zamzami *et al.*, 1995; 1996).

Once released into the cytosol, cytochrome c directly binds to apoptosis protease activating factor-1 (Apaf-1) in a dATP-dependent manner and leads to its oligomerization (Zou *et al.*, 1997). This conformational change allows caspase-9 to join and forms a complex termed “apoptosome” and results in the enzymatic activation of caspase-9, which then activate a set of effector caspases such as caspase-3, 6 and 7 (Cohen, 1997). Downstream effector caspase activations can be prevented by a family of endogenous caspase inhibitors termed inhibitors of apoptosis proteins (IAPs). These cytosolic IAPs

are capable of direct binding and inhibiting the active caspase-9 as well as targeting active caspases for degradation (Suzuki *et al.*, 2001). On the other hand, an intermembrane space protein called Smac/DIABLO has been shown to disable IAPs and assist in caspase-9 activation (Du *et al.*, 2000; Verhager and Vaux, 2002). Additionally, AIF promotes apoptosis by inducing chromatin condensation (Susin *et al.*, 1996) while EndoG exerts its pro-apoptotic effect by mediating DNA fragmentation (Li *et al.*, 2001).

Finally, extensive studies have proven members of the Bcl-2 family proteins can regulate the mitochondrial apoptotic pathway (Green and Evan, 2002; Cory *et al.*, 2003; Chao and Korsmeyer, 1998). All members of Bcl-2 proteins possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4), and many of these proteins also have a conserved C-terminal transmembrane region that anchors them to the cytosolic side of the outer mitochondrial membrane (Krajewski *et al.*, 1993). Generally, these proteins can be classified into 3 categories: (1) anti-apoptotic, which include Bcl-2, Bcl-X_L, Bcl-w and Mcl-1; (2) pro-apoptotic "BH123", which share 3 of the BH domains with their anti-apoptotic counterparts, and include Bax, Bcl-XS, Bak and Bok; and (3) pro-apoptotic "BH3 only" proteins, which contain only a BH3 domain, and include Bad, Bid, Bik, Bim and Bmf (Sanfilippo and Blaho, 2002). The exact mechanisms by which Bcl-2 family members exhibit their effects are varied. For instance, Bax has been shown to create pores and can insert into the outer mitochondrial membrane to allow the passage of caspase-activating factors into the cytosol (Martinou and Green, 2001). Furthermore, Bcl-2 and Bcl-XL can bind to Apaf-1 and inhibit its association with pro-caspase-9, while the pro-apoptotic Bik may free Apaf-1 from these inhibitions (Pan *et al.*, 1998; Hu *et al.*, 1998; Chinnaiyan *et al.*, 1997; Chaudhary *et al.*, 1999). On the other hand, the BH-3

only Bid can be cleaved by activated caspases to a truncated form (tBid) that inserts into the mitochondrial membrane and promotes the release of caspase-activating factors (Zha *et al.*, 2000; Gross *et al.*, 1998; Li *et al.*, 1998; Luo *et al.*, 1998).

1.3.3 Implications of apoptosis in cancer and some therapeutic approaches

Apoptosis is an essential cell death process in maintaining development and tissue homeostasis. Each day, millions of cells undergo apoptosis in order to secure the functionality of the whole organism. Thus, it is not surprising that any defects in this important process can result in severe pathological diseases of which one of the most important is cancer (Fisher and Schulze-Osthoff, 2005). Dysregulation of apoptosis has also been shown to be crucial in the pathogenesis of cancer by: (1) allowing neoplastic cells to survive beyond their normal lifespan; (2) creating a permissive environment for genetic instability and accumulation of gene mutations; (3) facilitating growth factor- and hormone-independent cell survival; (4) promoting resistance to immune-based destruction; and (5) conferring resistance to cytotoxic anticancer agents and radiation (Reed, 1999).

Most of the key cellular members regulating apoptosis have been identified in recent years and can be targeted by therapeutic approaches, which include the death receptors, Bcl-2 proteins, tumor suppressor p53, inhibitor of apoptosis (IAP), etc. A major pathway of apoptosis is mediated by cellular death receptors from tumor necrosis factor (TNF) receptor family. Even though ligands of the death receptors can efficiently induce cell death in a variety of tumor cells, severe toxicity was observed in mice and human (Daniel *et al.*, 2001). Recently, the discovery of TNF-related apoptosis-inducing

ligand (TRAIL) has raised new hope in therapeutic approaches that target death receptors. TRAIL has been shown to specifically eliminate tumor cells while normal cells are not affected. Pre-clinical safety studies in primates demonstrated no toxicity of TRAIL treatment (Walczak *et al.*, 1999). Furthermore, it is believed that apoptosis-resistance tumors usually contain mutation in tumor suppressor p53 or overexpression of the anti-apoptotic Bcl-2 family members. TRAIL-induce apoptosis is independent of p53 or overexpression of Bcl-2 family proteins and thus able to overcome the resistance. Indeed, combinations treatments of TRAIL with conventional chemotherapeutic agents or ionizing radiation vastly enhance their therapeutic effect (Held and Schulze-Osthoff, 2001).

Members of the Bcl-2 family play an essential role in the intrinsic mitochondrial pathway, with either pro- or anti-apoptotic functions. Diverse strategies have been applied to target Bcl-2, Bcl-X_L and Mcl-1 in cancer therapy. One approach is to modulate Bcl-2 by antisense techniques to downregulate the protein. It was found that an antisense Bcl-2 construct could effectively induce apoptosis by itself or in combination with standard chemotherapeutic agents in lymphoma and leukemia cells (Campos *et al.*, 1994; Keith *et al.*, 1995). Another approach is to apply BH3 domain peptides to bind to and disrupt Bcl-2 like protein complex and thus sensitizing cells to apoptosis. Indeed, a BH3 peptide has been shown to sensitize Bcl-X_L overexpressing cells to CD-95-mediated apoptosis (Holinger *et al.*, 1999). Furthermore, several small-molecule drugs interfering with Bcl-2/Bcl-X_L function have been reported. For instance, an antibiotic from *Actinomyces*, Tetrocarcin A, was found to sensitize Bcl-2 overexpressing HeLa cells to death receptor and drug-mediated apoptosis (Nakashima *et al.*, 2000). Antimycin A3, an

inhibitor of the mitochondrial electron transport chain, has also been demonstrated to induce cell death in Bcl-2 and Bcl-X_L overexpressing cells (Tzung *et al.*, 2001).

In the past decade, substantial research have been conducted to target tumor suppressor gene p53 in cancer therapy due to the fact that inactivation of p53 is reported in more than 50% of human cancers (Wallace-Brodeur and Lowe, 1999). A promising approach is to restore the transcriptional activity of mutant p53 by introducing a small peptide to either stabilize p53 (Selivanova *et al.*, 1997; 1999) or interfere with the binding of its negative regulator Mdm2 (Chene *et al.*, 2000; Stoll *et al.*, 2001; Vassilev *et al.*, 2004). In addition, targeting heat shock protein (Hsp)-90 by geldanamycin has been reported to deplete mutant p53 by restoring the degradation of mutant p53 by proteasome in various tumor cell lines (Blagosklonny *et al.*, 1995; An *et al.*, 1997). On the other hand, inhibition of p-53 mediated transcription and apoptosis in normal tissues has been shown to relieve the genotoxic stress associated with chemo- and radiotherapy treatments. In fact, a p53 inhibitor, pifithrin-α, completely rescued mice from doses of radiation that normally killed 60% of the mice (Komarov *et al.*, 1999).

Inhibitors of apoptosis (IAPs) comprise a family of caspase-inhibiting proteins characterized by a shared conserved baculovira IAP repeat (BIR) domain (Salvesen and Duckett, 2002). In addition to their well-established caspase-inhibiting capability, growing evidence also suggests the role of IAPs in other cellular functions such as protein degradation, cell cycle control and signal transduction (Deveraux and Reed, 1999). Many studies have explored the possibility of targeting IAPs in cancer therapy with promising results accumulated through the years. For example, antisense targeting XIAP has been shown to sensitize many tumor cell types to radio- or chemotherapy

(Valentino *et al.*, 2003; Sasaki *et al.*, 2000). Furthermore, XIAP antisense therapy combined with vinorelbine are also effective in mouse xenograft model of lung cancer (Hu *et al.*, 2003). Recently, one such antisense oligonucleotide, AEG35156, has been shown to have high efficacy in multiple cancer models and has just entered phase I clinical trial (Fischer and Schulze-Osthoff, 2005).

In summary, rapid advances in the understanding of apoptosis regulation and in-depth mechanisms of action have laid a solid foundation for more exciting drugs targeting apoptosis.

1.4 Objectives of the study

At present, there is some preliminary evidence showing the anticancer property of PN. However, this property of PN has not been studied in any animal model, and the exact mechanism(s) by which PN exhibits its anticancer activity has not been fully elucidated. Thus, the main objectives of the present study are:

(1) To investigate the chemopreventive and chemotherapeutic potentials of PN against UVB-induced skin cancer in SKH-1 hairless mice

(2) To explore the molecular mechanism(s) involved in the anticancer effect of PN in cultured JB6 murine epidermal cells

CHAPTER 2

THE CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC PROPERTIES OF PARTHENOLIDE AGAINST UVB-INDUCED SKIN CANCER IN SKH-1 HAIRLESS MICE

2.1 Introduction

Traditionally, feverfew (*Tanacetum parthenium*) is used as a herbal medicine for the treatment of fever, arthritis, and migraine in Europe. The crude extracts of this herb is known to have anti-microbial and anti-inflammatory properties (Brown *et al.*, 1997; Jain *et al.*, 1999). The principal active component in feverfew is the sesquiterpene lactone PN that contains a highly electrophilic α-methylene-γ-lactone ring and an epoxide residue capable of interacting rapidly with nucleophillic sites of biological molecules (Macias *et al.*, 1999). At present, there is some preliminary evidence showing the anti-cancer property of PN. For instance, PN is a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (Woynarowski and Konopa, 1981; Hall *et al.*, 1988; Ross *et al.*, 1999). Recently, Wen and colleagues (2002) demonstrated that PN-induced apoptosis involves caspase activation and mitochondria dysfunction in hepatoma cells. To date, the anti-cancer property of PN has not been studied using *in vivo* animal models.

Skin cancer is the most common type of cancer among Caucasians (Parker *et al.*, 1997). Clinically, skin cancers are classified into two major groups: malignant melanoma (MM) and non-melanoma skin cancer (NMSC). Ultraviolet radiation (UV) wavelength range borders at the violet end of the visible light range at 400 nm. Wavelength ranges in the region of 400-320 nm is classified as UVA, while UVB ranges from 320-280 nm, and UVC ranges from 280-200 nm. Numerous studies have demonstrated UVB as the major etiological factor for NMSC (Jung, 1991; Brash, 1997; Yamawaki *et al.*, 1997), which is

derived from skin epithelial cells and can be further categorized into squamous cell carcinoma (SCC) and basal cell carcinomas (BCC) (de Grujil, 1999).

In this study, we investigated the anti-cancer property of PN using UVB-induced skin cancer model in SKH-1 hairless mice. As a positive control, we applied celecoxib, a specific COX-2 inhibitor with proven chemopreventive activity against skin cancer in a similar model (Fischer *et al.*, 1999; Pentland *et al.*, 1999). Our results demonstrated for the first time PN possesses strong cancer chemopreventive property in an animal model.

2.2 Materials and Methods

2.2.1 Chemicals and Animals

PN (97% pure) was purchased from Biomol (Plymouth Meeting, PA). Celecoxib was kindly provided by Pfizer (New York, NY). SKH-1 hairless mice were purchased from Charles River Laboratories (Wilmington, MA), and they were at 8-9 weeks of age at the beginning of the experiments. Animals were maintained at the Animal Holding Unit, NUS and all experimental protocols were approved by the University Animal Welfare Committee. All mice were housed in the Animal Holding Unit under climate-controlled environment with a 12-hour light/dark cycle. Mice were allowed free access to food pellets and water.

2.2.2 Apparatus for UVB exposure

UVB was delivered through a bank of FS24 lamps (Light Sources Inc., Orange, CT) with spectral irradiance of 280-400 nm, 80% of which in the UVB region (280-320 nm) and with a peak at around 313 nm. These UVB lamps were incorporated into a light

therapy unit similar to those in many local hospitals for the treatment of jaundice in infants (Figure 2.1a). The emitted UVB dose was quantified using a phototherapy radiometer (International Light, Newburyport, MA) equipped with IL SED 240 detector.

2.2.3 Preparation of the special food diet

Both compounds were first dissolved in DMSO, diluted with sunflower oil, and then coated onto food pellets. Food pellets were purchased from Glen Forrest Stock Feeders (Western Australia, Australia), with a fixed formula ratio using wheat, lupins, barley, soya meal, fish meal, mixed vegetable oils, canola oil, salt, calcium carbonate, dicalcium phosphate, magnesium oxide, and a vitamin and trace mineral premix. The calculated nutritional parameters are approximately 19% proteins, 4.6% total fat, and 4.5% crude fiber. Once coated, these food pellets were stored at 4°C. Fresh food pellets were supplied 3 times a week and the amount of consumption was recorded at the same time. The dosage of celecoxib is based on a published study (Fisher *et al.*, 1999) where 500 ppm of celecoxib had been shown to possess strong chemopreventive activity against UVB-induced skin cancer.

2.2.4 Determination of PN content in the prepared food pellets

To examine the stability of PN in the food pellets used in this study, the concentrations of PN in special prepared food pellets stored at 4 °C for 1, 3 and 7 days were determined using mass spectrometry. Briefly, each pellet was grinded into powder before being soaked with pure ethanol overnight. The subsequent solvents containing

PN were then subjected to MS. The mass spectra were monitored on a LCQ mass spectrometer equipped with an APCI interface (Finnigan LCQ Duo, Bremen, Germany).

2.2.5 Establishment of the UVB-induced skin cancer animal model

The *in vivo* model is based on a published method (Fisher *et al.*, 1999; Pentland *et al.*, 1999) with modifications, which includes the following procedures.

2.2.5.1 Determination of Minimal Erythema Dose (MED)

In order to establish the UVB dosage used in the current study, a short experiment to determine the minimal dose of UVB that induce erythema response (redness skin) in female SKH-1 hairless mice was conducted. Mice were divided into 6 groups (5/group) which received 0, 50, 100, 200, 300, 400, or 500 mJ/cm^2 of UVB. Erythema response was then examined 24 hours after UVB exposure. The result showed that 200 mJ/cm^2 was the MED in this study.

2.2.5.2 Induction of skin cancer by UVB

Mice were placed in a box partitioned to 6 separate compartments (Figure 2.1b) and exposed to an initial dose of 100 mJ/cm^2 of UVB (5 days/week). A weekly increment of 50 mJ/cm^2 was applied until a maximal dose of 200 mJ/cm^2 was reached. The UVB treatment was continued for 25 weeks. A negative control group (no PN treatment and no UVB exposure, n=5) was included throughout the study. Papilloma (lesion >1 mm) were examined and recorded weekly. Papilloma incidence (% of mice

with one or more papilloma) and papilloma multiplicity (# of papilloma/mouse) were determined after the termination of the UVB treatment.

2.2.6 Treatment with PN

2.2.6.1 Chemopreventive aspect

Two different PN treatment schemes were designed, aiming to examine both the chemopreventive and the chemotherapeutical value of PN against UVB-induced skin cancer. The details of both UVB irradiation and PN and celecoxib treatment schemes were summarized in Figure 2.2. In order to study the chemopreventive activity of PN, mice were fed with prepared food pellets containing DMSO, PN, or celecoxib one week prior to UVB exposure and throughout the study.

2.2.6.2 Chemotherapeutic aspect

Various treatments with PN and celecoxib and UVB irradiation schemes were summarized in Figure 2.3 to evaluate their chemotherapeutic value. Mice were exposed to an initial dose of 100 mJ/cm^2 of UVB (5 days/week). A weekly increment of 50 mJ/cm^2 was applied until a maximal dose of 200 mJ/cm^2 was reached. The UVB treatment was continued for 14 weeks. At that point, mice were given 2% DMSO, 3 mg/day or 5 mg/day PN, or 3 mg/day celecoxib mixed in the food pellets (as described above). The drug treatments lasted for 14 weeks, while body weight and papilloma number were recorded concurrently. Papilloma incidence (% of mice with 1 or more papilloma) and papilloma multiplicity (# of papilloma/surviving mice) were determined after the termination of the study.

(a)

(b)

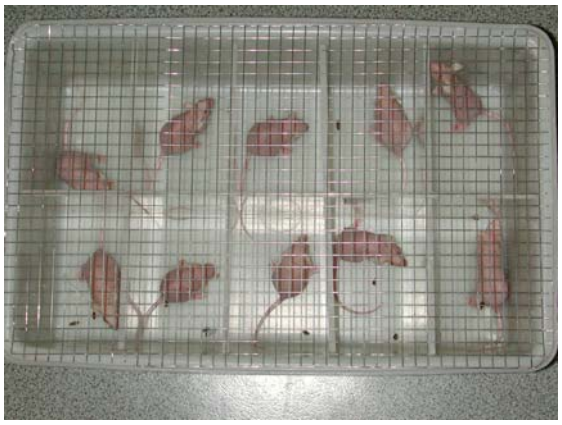


Figure 2.1 UVB exposure setup (a) UVB exposure unit with 8 UVB lamps. (b) Cage partitioned into 10 separate compartments to ensure equal exposure.

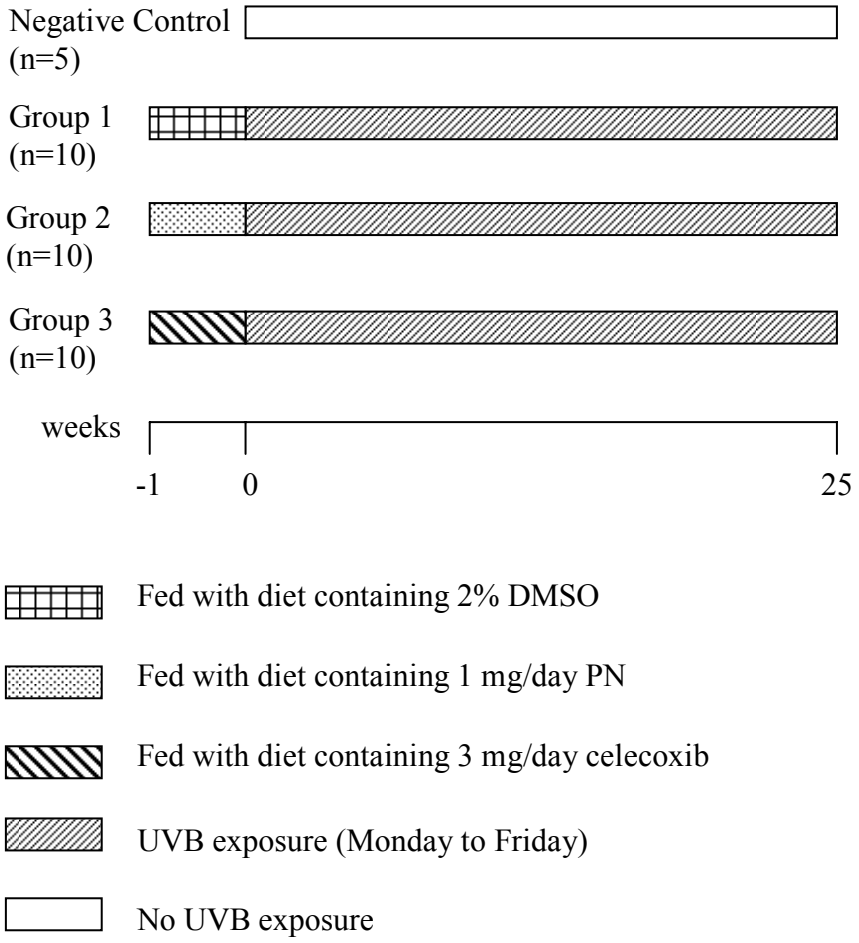
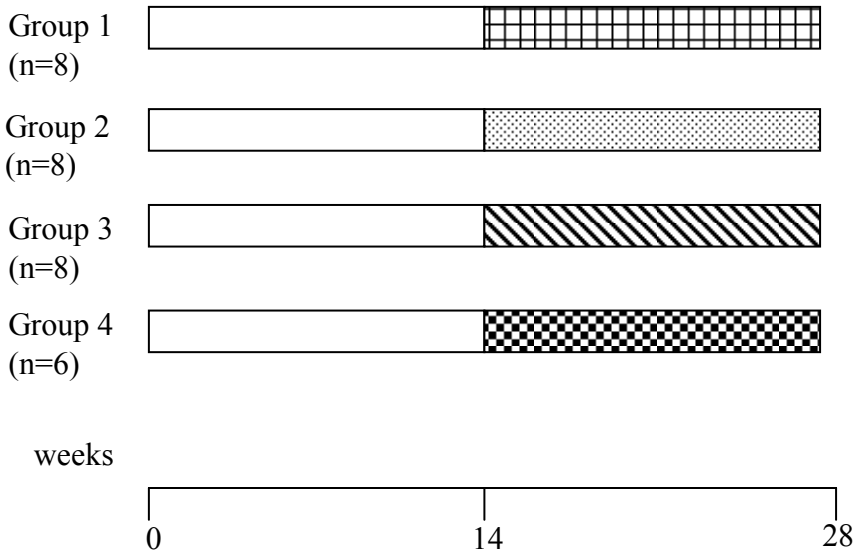


Figure 2.2 Schematic representation of the chemopreventive aspect of PN or celecoxib against UVB-induced skin cancer in SKH-1 hairless mice. Mice were placed on diet containing 2% DMSO (group 1), 1 mg/day PN (group 2), 3 mg/day celecoxib (group 3) 1 week prior to the initiation of UVB exposure and was maintained throughout the study. Mice were exposed to an initial dose of 100 mJ/cm^2 of UVB (5 days/week), with an increment of 50 mJ/cm^2 per week until a maximal dose of 200 mJ/cm^2 was reached. The UVB treatment was continued for 25 weeks. A negative control group with no UVB exposure was also included.



Fed with diet containing 2% DMSO

Fed with diet containing 3 mg/day PN

Fed with diet containing 5 mg/day PN

Fed with diet containing 3 mg/day celecoxib

UVB exposure (Monday to Friday)

Figure 2.3 Schematic representation of the chemotherapeutic aspect of PN or celecoxib against UVB-induced skin cancer in SKH-1 hairless mice. Mice were exposed to an initial dose of 100 mJ/cm^2 of UVB (5 days/week), with an increment of 50 mJ/cm^2 per week until a maximal dose of 200 mJ/cm^2 was reached. The UVB treatment was continued for 14 weeks. Mice were then placed on diet containing 2% DMSO (group 1), 3 mg/day PN (group 2), 5 mg/day PN (group 3), or 3 mg/day celecoxib (group 4) for 14 weeks.

2.2.7 Tissue collection

All mice were humanly sacrificed at the end of the 25-week or 28-week study. Papillomas were surgically removed, paraffin embedded and cut into 4 μm sections in preparation for immunohistochemistry staining. The dorsal skins of mice were also removed, snap-frozen in liquid nitrogen, and then stored at -80˚C until further analysis.

2.2.8 Immunohistochemical analysis of COX-2

All tissue sections (4 μm) fixed on microscopic slides were dewaxed via the following sequence:

1) 10 mins in xylene

2) 10 min in xylene

3) 5 min in 100% ethanol

4) 5 min in 100% ethanol

5) 5 min in 90% ethanol

6) 5 min in 70% ethanol

7) 5 min in tap water

All tissue sections were incubated in 3% hydrogen peroxide for 5 min before washing with PBS twice. Then, tissue sections were incubated in working solution of Ig blocking reagent from VECTOR® M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) for 1 hour. After washing with PBS twice, sections were incubated with working solution of MOM diluent for 5 min and proceed to incubation with anti-Cox-2 primary monoclonal antibody (Transduction Laboratories, Los Angeles, CA) for 1 hour at 37 °C. After washing with PBS twice, the MOM biotinylated anti-mouse IgG

reagent was applied to the tissue sections for 10 min, follow by 5 min incubation with Vectastain ABC reagent. Sections were then incubated with 3,3'-diaminobenzidine (DAB) substrate for 10 min. Slides were counterstained with hematoxylin-1 (Sigma-Aldrich, St Louis, MO) for 15 seconds. Finally, sections were dehydrated via the following sequence, mounted and covered with coverslips:

1) 5 min in 70% ethanol
2) 5 min in 90% ethanol
3) 5 min in 100% ethanol
4) 10 min in xylene
5) 10 min in xylene

2.2.9 PGE_2 level determination in murine skin samples

Skin samples in phosphate buffer (0.1 M, pH7.4, 1 mM EDTA, 10 μM indomethacin) were homogenized on ice for 1 min at 20,000 rpm. Equal volume of 100% ethanol was added to the homogenates, vortexed, and the mixtures were left at RT for 5 min. The mixtures were centrifuged at 3,000 g for 10 min at 4 °C, and the supernatants were transferred to other tubes and further diluted with 50 mM citrate buffer (pH4.0) until the ethanol concentration is below 15%. The samples were purified using a Sep-Pak® Vac C-18 cartridge (Waters, Milford, MA). PGE_2 in skin samples were measured using monoclonal enzyme immunoassay kit (Cayman, Ann Arbor, MI) according to manufacturer's protocol.

2.2.10 Statistical analysis

All numeric data are presented as means ± standard deviations (SD) and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons as post-hoc test (SPSS 11.5). Part of the results from the animal study was analyzed using Fisher exact probability test. A P value < 0.05 is considered as statistical significance.

2.3 Results

2.3.1 Chemopreventive property of PN against UVB-induced skin cancer in SKH-1 hairless mice

In this study, the anti-cancer property of PN was first tested in a UVB-induced mouse skin cancer model which was established based on published methods (Fischer *et al.*, 1999; Pentland *et al.*, 1999). It is noted that the special prepared food diet achieved a constant delivery of PN to the mice as shown in Figure 2.4. No papilloma was detected in the negative control group throughout the study. As shown in Figure 2.5, PN (1 mg/mouse/day) or celecoxib (3 mg/mouse/day) significantly delayed the onset of papilloma incidence comparing to the UVB-only group: the UVB-only group reached 100% tumor incidence at week 13^{th}, while PN- or celecoxib- fed groups achieved full tumor incidence at week 18^{th} and 20^{th}, respectively. Papilloma yield (number of papilloma/mouse) was also significantly reduced in mice fed with either PN or celecoxib (Figure 2.6): PN treatment decreased the papilloma multiplicity by 30%, comparable to

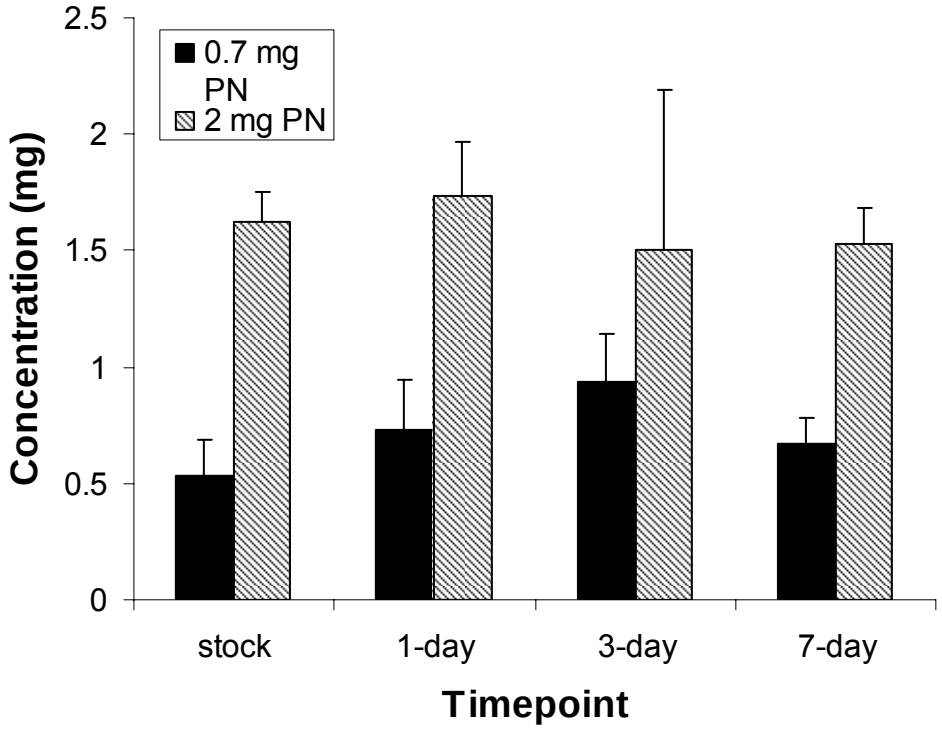


Figure 2.4 The concentrations of PN in special prepared food pellets as determined by mass spectrometry. PN was first dissolved in DMSO, diluted in sunflower oil, and then coated onto each pellet. These pellets were stored at 4 °C for 1, 3 or 7 days. Each pellet was grinded into powder form before being soaked with pure ethanol overnight. The subsequent solvents containing PN were then subjected to MS. Data were presented in means ± standard deviations from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keul multiple comparisons test.

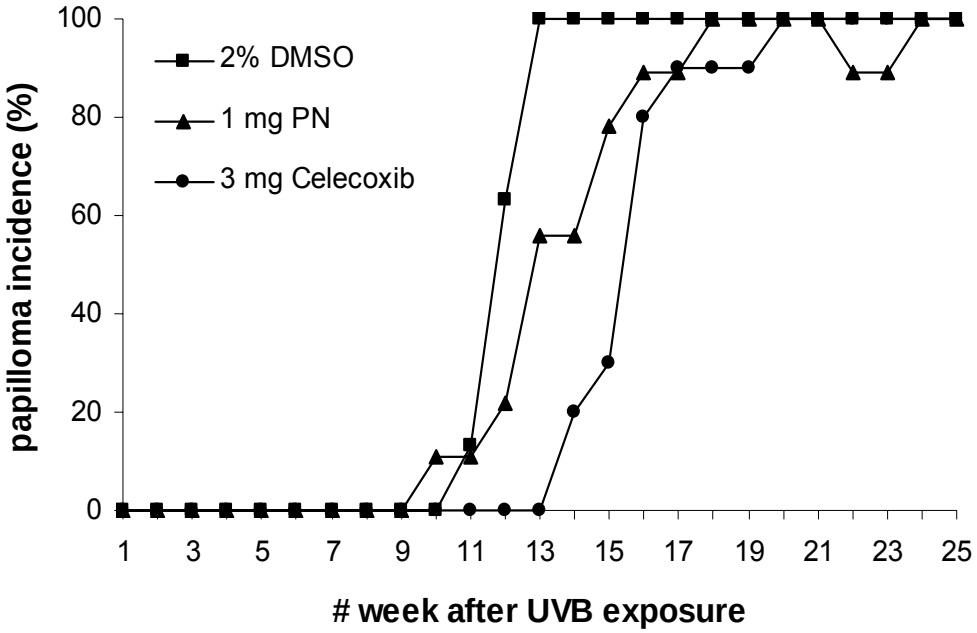


Figure 2.5 Inhibitory effects of PN and celecoxib on UVB-induced papilloma incidence in SKH-1 hairless mice. Mice were placed on diet containing PN or celecoxib (n=10) 1 week prior to the beginning of UVB irradiation (5 times/week) and the number of papillomas were counted weekly. Papilloma incidence was calculated as the percentage of mice having tumors in each treatment group and analyzed using Fisher exact probability test.

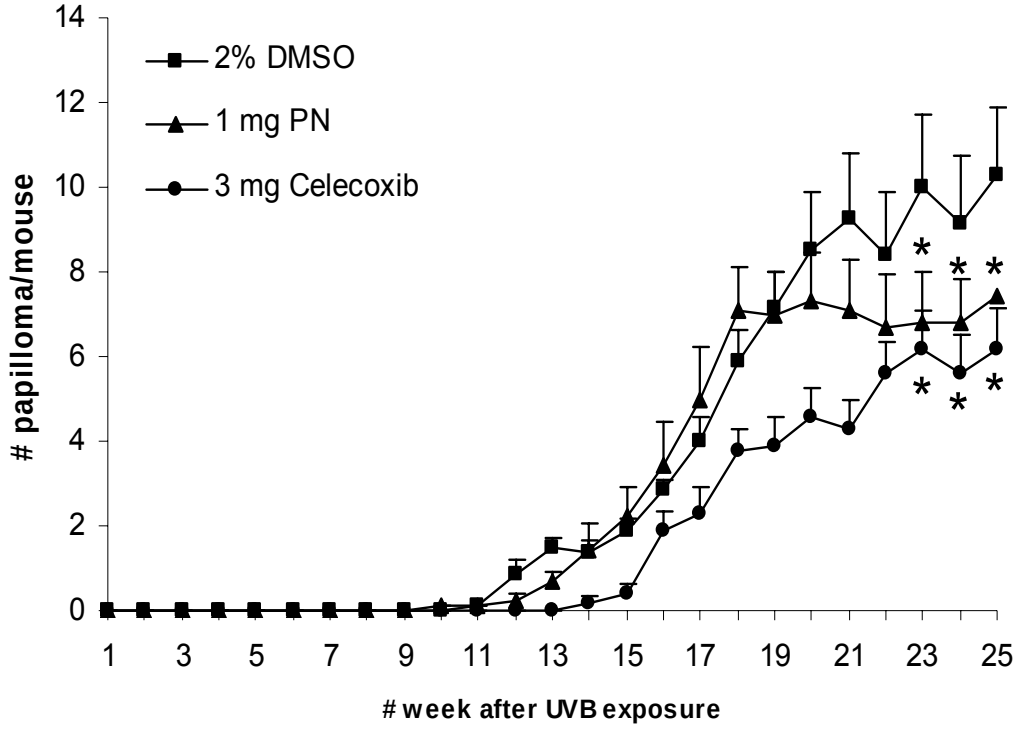


Figure 2.6 Inhibitory effects of PN and celecoxib on UVB-induced papilloma yield in SKH-1 hairless mice. Mice were placed on diet containing PN or celecoxib (n=10) 1 week prior to the beginning of UVB irradiation (5 times/week) and the number of papillomas were counted weekly. Papilloma yield was calculated as the mean values of papillomas/mouse ± SD for each treatment group, and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test on data from the last three weeks of the study. Asterisks indicate significant difference between control and treated groups ($P < 0.05$)

that of celecoxib (40%). In addition, differences in the severity of tumor growth (Figure 2.7) and the size distribution of papilloma among different groups were also noted (Table 2.1): papillomas observed in the group fed with PN were of smaller size (73% papillomas of 1-2 mm in diameter) as compared to that of the control (59%). Furthermore, immunohistochemical staining (Figure 2.8) showed both PN and celecoxib treatments markedly inhibited the UVB-induced hyperplastic response (increased thickness of the epidermis) as compared to the control group. The effect of celecoxib is more profound, and this observation is basically consistent with the data in Figure 2.5 and 2.6. Taken together, here for the first time strong evidence from this study shows that PN possesses strong chemopreventive property against UVB-induced skin cancer.

2.3.2 Effect of PN on UVB-induced COX-2 activity in murine skin

It has been well known that COX-2 over-expression is implicated in UVB-induced skin cancer (Buckman *et al.*, 1998; Chan *et al.*, 1999; Athar *et al.*, 2001), and COX-2 inhibitors are known to be effective in preventing UVB-induced skin cancer (Fischer *et al.*, 1999; Pentland *et al.*, 1999). Surprisingly, in the present study COX-2 is unlikely to be the direct molecular target of either PN or celecoxib. As shown in Figure 2.9, UVB exposure led to COX-2 over-expression in skin tissue determined by immunohistochemistry, while either PN or celecoxib failed to suppress this reaction (Figure 2.9). A consistent pattern of changes was also found for the PGE_2 level in the skin tissue: UVB exposure markedly enhanced the PGE_2 level, while no reduction was found with either PN or celecoxib treatment (Figure 2.10). Therefore, it is believed that

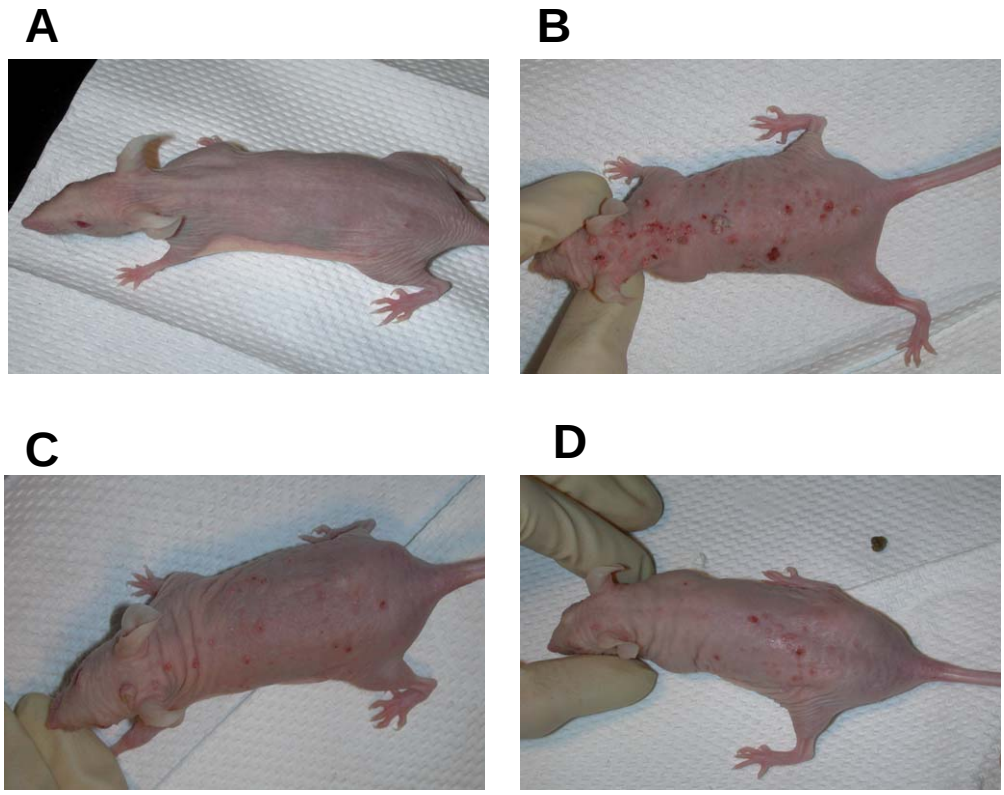


Figure 2.7 Tumor growth in UVB-treated mice fed with diet containing (B) DMSO, (C) 1 mg/day PN and (D) 3 mg/day celecoxib. A negative control group on normal diet with no UVB exposure was also included (A). Mice were placed on these diet one week prior to and were continued throughout the 25 weeks of UVB exposure (5 times/week). Pictures were taken at the end of the 25-week study.

Table 2.1 Size distribution of papillomas

Treatment Group	n	% small papillomas (1-2 mm)#	% big papillomas (≥ 3 mm)
Control (UVB only)	10	59.00 ± 10.52	41.0 ± 9.32
PN + UVB	10	73.67 ± 6.54*	26.33 ± 5.34*
Celecoxib + UVB	10	65.90 ± 6.15	34.10 ± 11.1

Means ± SD

* $P<0.05$ comparing to the UVB-only control group using one-way ANOVA with Student-Newman-Keuls multiple comparisons test.

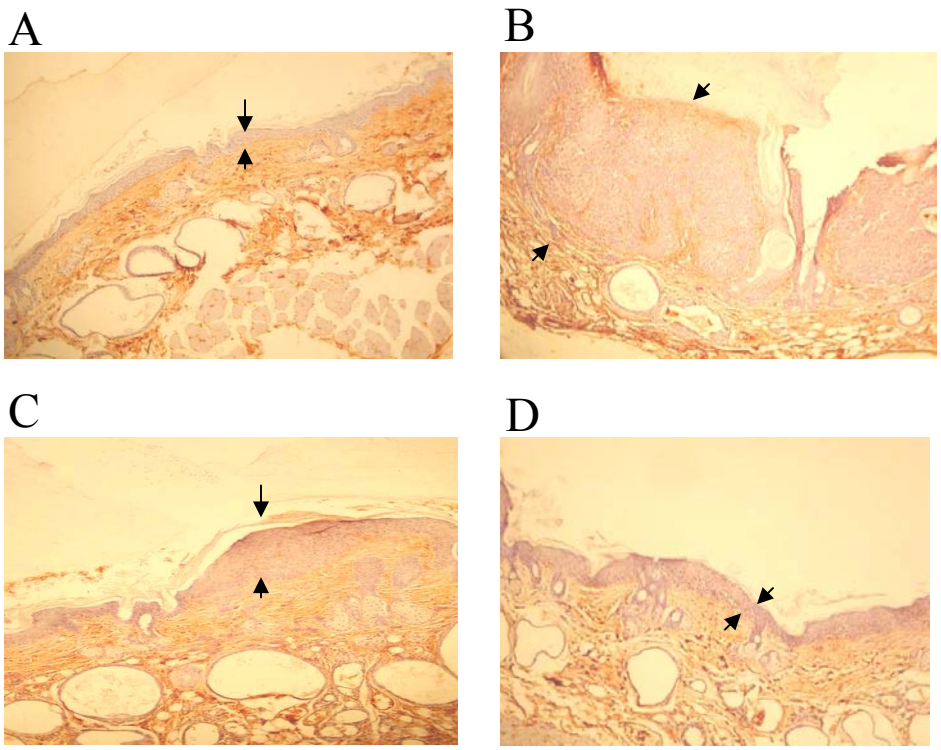


Figure 2.8 Effects of PN and celecoxib on UVB-induced hyperplasia (area between the arrows). Mice were placed on diet containing (B) DMSO, (C) 1 mg/day PN and (D) 3 mg/day celecoxib. A negative control group on normal diet with no UVB exposure was also included (A). Mice were placed on these diet one week prior to and were continued throughout the 25 weeks of UVB exposure (5 times/week). At the end of the study, dorsal skins of the mice were removed and formalin fixed. Cut sections (4μm) were processed for hematoxylin and eosin staining, and photomicrographed at 400x.

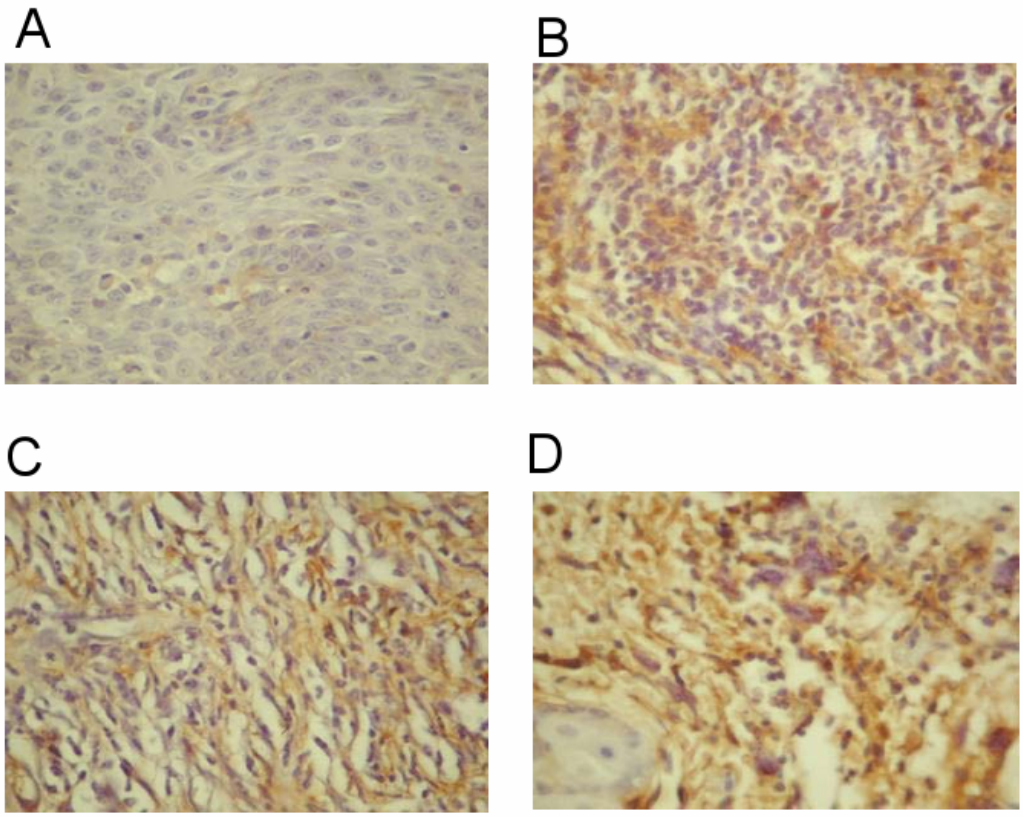


Figure 2.9 Immunohistochemistry staining of COX-2 in mouse skin samples. Skin samples were stained for COX-2 at the end of the study protocol. Images of (A) negative control, (B) DMSO, (C) PN and (D) celecoxib-treated groups were photomicrographed at 1000x. COX-2 protein was stained brown mainly in the cytoplasm.

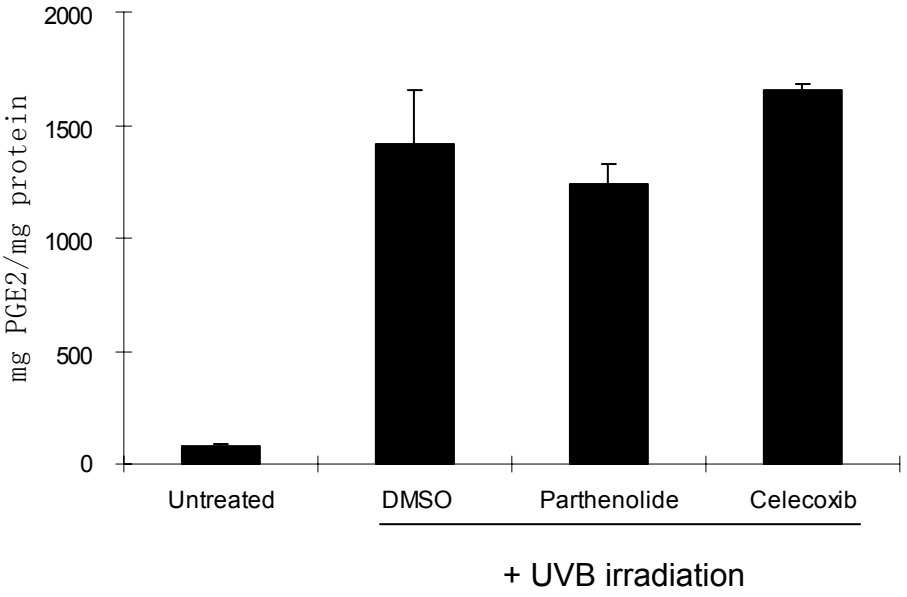


Figure 2.10 PGE_2 levels in untreated or UVB-irradiated mice fed with DMSO-, PN- or celecoxib-containing diets. PGE_2 was extracted from the skin samples at the end of the study protocol and analyzed by enzyme immunoassay described in materials and methods. Data were presented in means ± SD.

the chemopreventive activity of PN or celecoxib might be independent of COX-2 in the present experimental system.

2.3.3 Chemotherapeutic property of PN against UVB-induced skin cancer in SKH-1 hairless mice

Generally when one is diagnosed with cancer, all risk factors associated are advisedly avoided. Thus, this account has been incorporated into the present experimental design: UVB exposure for 14 weeks to induce skin cancer, at which UVB exposure was stopped and the treatment initiated. However, the results showed that PN is not an effective chemotherapeutic agent: Papilloma yield (number of papilloma/mouse) was not significantly different between in mice treated with DMSO control and either dosages of PN (3 or 5 mg/day) (Figure 2.11). Differences in the severity of tumor growth (Figure 2.12) and the size distribution of papilloma among different groups were not detected (Table 2.2). Thus, it is concluded that PN is not an effective single chemotherapeutic agent against UVB-induced skin cancer.

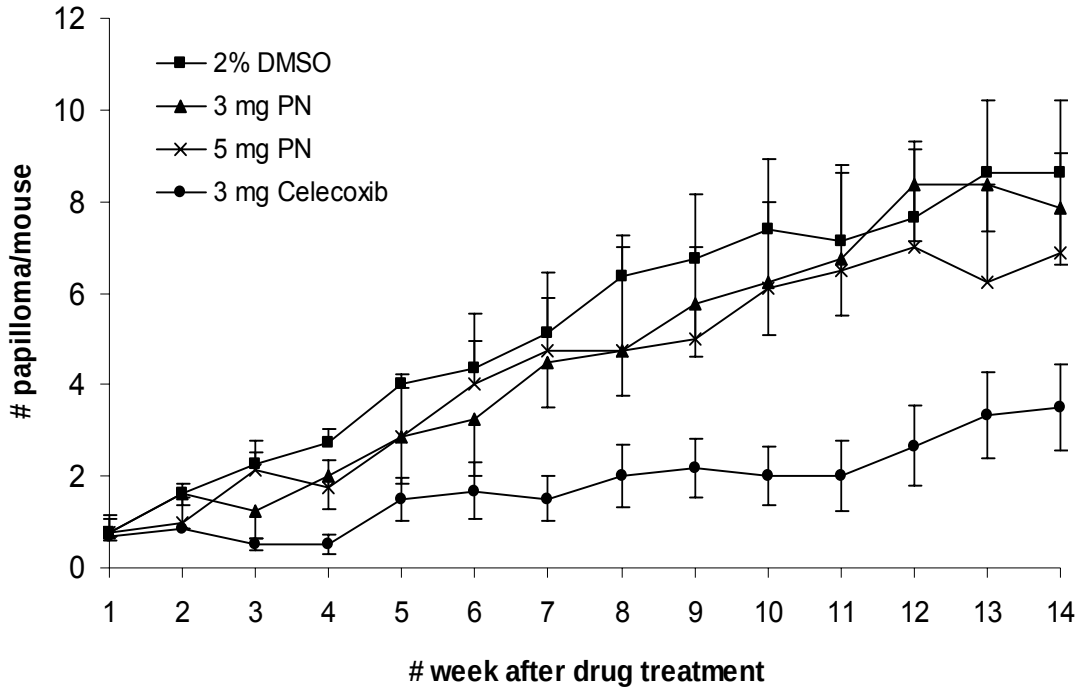


Figure 2.11 PN has no effect on papilloma yield after 14 weeks of UVB exposure. Mice were placed on diets containing (A) DMSO, (B) 3 mg/day PN (C) 5 mg/day PN, and (D) 3 mg/day celecoxib after 14 weeks of UVB exposure (5 times/week) and the number of papillomas were counted weekly. Papilloma yield was calculated as the mean values of papillomas/mouse ± SD for each treatment group, and analyzed using one-way ANOVA with student-Newman-Keuls multiple comparisons.

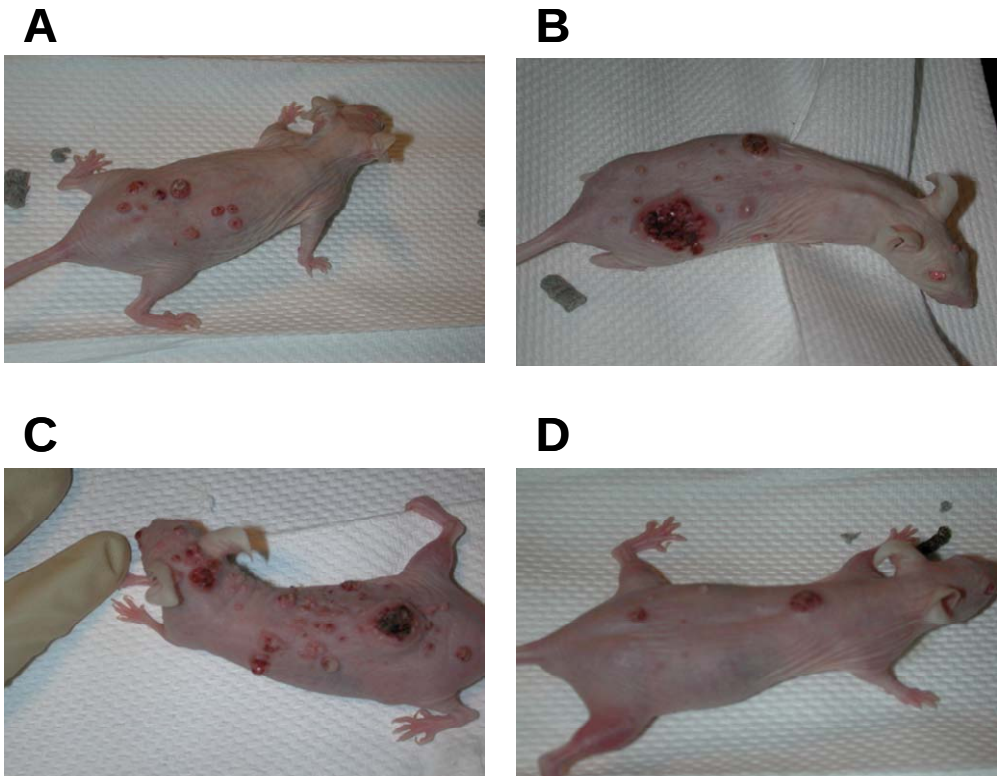


Figure 2.12 Tumor growth in UVB-treated mice fed with diet containing (A) DMSO, (B) 3 mg/day PN (C) 5 mg/day PN, and (D) 3 mg/day celecoxib. Mice were placed on these diets after 14 weeks of UVB exposure (5 times/week) and continued for 14 weeks. Pictures were taken at the end of the 28-week study.

Table 2.2 Size distribution of papillomas

Treatment Group	n	% small papillomas (1-2 mm)#	% big papillomas (≥ 3 mm)
Control (UVB only)	8	68.12± 7.41	31.88 ± 7.97
3 mg/day PN + UVB	8	65.08 ± 5.89	34.92 ± 7.59
5 mg/day PN + UVB	8	58.93 ± 4.42	41.07± 11.01
Celecoxib + UVB	6	80.95 ± 11.42	19.05 ± 10.41

Means ± SD

2.4 Discussion:

Previous studies have demonstrated PN as a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (Woynarowski and Konopa, 1981; Hall *et al.*, 1988; Ross *et al.*, 1999). More recently, PN has been reported to be a potent apoptosis inducer in human cancer cells (Wen *et al.*, 2002). Furthermore, it has been shown that PN sensitizes breast cancer cells to chemotherapeutic agent paclitaxel (Patel *et al.*, 2000). However, the anti-cancer potential of PN has not been tested on animal models. Here for the first time clear evidence has shown that PN possesses chemopreventive property against UVB-induced skin cancer in female SKH-1 hairless mice: 1) PN treatment delayed the onset of tumor incidence from week 13th to week 18th (Figure 2.5); 2) PN reduced tumor multiplicity by 30% (Figure 2.6); 3) smaller papilloma sizes were found in mice treated with PN (Table 2.1); and 4) PN treatment reduced the hyperplastic response of the epidermis induced by UVB irradiation (Figure 2.8). The chemopreventive capability of PN is found to be as effective as the pharmaceutical COX-2 inhibitor celecoxib in preventing UVB-induced photocarcinogenesis (Fischer *et al.*, 1999; Pentland *et al.*, 1999).

COX-2 is the inducible subtype of the enzyme cyclooxygenase (COX) which converts free arachidonic acid to prostaglandins (Marnett, 2000). The fact that UVB upregulates COX-2 and prostaglandins production suggested COX-2 may contribute to photocarcinogenesis (Buckman *et al.*, 1998; Grewe *et al.*, 1993). Preliminary findings from our laboratory revealed a positive correlation between cytotoxic response to PN and COX-2 expression (unpublished data), which leads us to hypothesize that PN may exert its effect in a COX-2-dependent manner. However, the results showed that neither

treatment of PN nor the specific COX-2 inhibitor celecoxib alter COX-2 expression or prostaglandins production induced by UVB (Figure 2.9 and 2.10), suggesting that COX-2 is unlikely to be the direct molecular target for PN and celecoxib. This finding is in concordance with previous report in which long term oral treatment of celecoxib has no effect on UVB-induced expression of COX-2 (Fischer *et al.*, 1999). Furthermore, *in vitro* studies also demonstrated that celecoxib induces apoptosis and cell cycle arrest in a COX-2 independent manner (Elder *et al.*, 1997; Grosch *et al.*, 2001; Waskewich *et al.*, 2002). In contrast, a recent study reported that topical treatment of celecoxib is as effective as oral route in inhibiting UVB-induced skin cancer while also suppressed PGE_2 production induced by UVB (Wilgus *et al.*, 2003). It remains to be determined whether such a difference in PGE_2 suppression by celecoxib is due to the different routes of administration applied.

Initially, several studies have been planned to identify the possible molecular targets responsible for the chemopreventive activity of PN against UVB-induced skin cancer by examining the changes in mRNA and protein expression levels of certain important proteins being implicated in UVB-induced skin cancer. However, it was later found that the mRNA prepared from the murine skins was severely degraded when the qualities of RNA were tested by electrophoresis through agarose gels containing formaldehyde. Moreover, the qualities of proteins extracted from the same batch of murine skin samples were also questionable and thus not suited for further analysis. Thus, further identifications for the possible molecular targets were unsuccessful. In summary, strong evidence from this study has demonstrated the chemopreventive property of PN against

UVB-induced skin cancer in SKH-1 hairless mice. The results also suggested that such chemopreventive activity is probably mediated in a COX-2-independent manner.

CHAPTER 3

PARTHENOLIDE SENSITIZES CELLS TO UVB-INDUCED APOPTOSIS BY TARGETING THE AP-1 MAPK PATHWAY

3.1 Introduction:

The mechanism(s) of UVB-induced skin cancer have not been fully understood. Several transcriptional factors including activator protein-1 (AP-1), NF-κB, nuclear factor of activated T cells (NF-AT), and signal transducers and activators of transcription (STATs) have been linked to the tumor-promoting ability of UVB (Angel *et al.*, 2001; Siebenlist *et al.*, 1995; Huang *et al.*, 2000; Zhang *et al.*, 2001). AP-1 is an important nuclear transcription factor involved in many cellular functions such as cell proliferation, cell death, cell survival and differentiation (Shaulian and Karin, 2002). The mammalian AP-1 complex consists of either homodimers or heterodimers of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Jun dimerization partners (JDP1 and JDP2), and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) (Karin *et al.*, 1997). Depend on the composite, the resulting AP-1 complex can then binds to and transactivates either the TPA-response element (TRE) or the cAMP responsive elements (CRE) to regulate the transcription of many genes involved in cell proliferation, apoptosis, metastasis, and cellular metabolism (Angel *et al.*, 2001; Jochum *et al.*, 2001; Ozanne *et al.*, 2000). One important aspect of AP-1 function is its role in tumor promotion based on the fact that viral and cellular Jun or Fos can cause malignant transformation in fibroblasts (Lamb *et al.*, 1997; Suzuki *et al.*, 1994). Gene products promoting invasion and metastasis are also under AP-1 regulation (Lamb *et al.*, 1997). Furthermore, inhibition of UVB-induced skin cancer has been shown to be mediated through suppression of AP-1 transactivation (Barthelman *et al.*, 1998).

It has been well documented that PN is a potent inhibitor of NF-κB signaling pathway which contributes to its anti-inflammatory activity (Bork *et al.*, 1997; Hehner *et*

al., 1998; 1999). In contrast, relatively little is known with regards to the effects of PN on other signaling molecules such as AP-1 and their involvements in its anti-cancer activity. The main objective of this study is to determine the mechanism(s) involved in the cancer chemopreventive property of PN. The main focus is on its effect on AP-1 and mitogen-activated protein kinases (MAPKs) signaling cascade using JB6 murine epidermal cells. The results provide evidence that the inhibitory effects on AP-1 and MAPK serve as one of the underlying mechanisms for the cancer chemopreventive property of PN.

3.2 Materials and Methods

3.2.1 Cell line and Chemicals

Murine epidermal cell line JB6 was obtained from Dr. Shi XL from NIOSH, USA. JB6 murine epidermal cells stably transfected with an AP-1 luciferase reporter plasmid have been reported previously (Ding *et al.*, 1999; 2001).PN (97% pure) was purchased from Biomol (Plymouth Meeting, PA). Celecoxib was kindly provided by Pfizer (New York, NY). Anti p-JNK, JNK, p-p38, p38, p-Erk, Erk, p-c-Jun, c-Jun, p-ATF-2 and ATF-2 polyclonal antibodies were purchased from Cell Signaling (Beverly, MA). Secondary antibodies (horseradish peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG) and enhanced chemiluminescence substrate were from Pierce (Rockford, IL). γ-p32 ATP was obtained from Perkin-Elmer (Boston, MA). SP600125 was purchased from Calbiochem (San Diego, CA). Other common chemicals were from Sigma-Aldrich (St. Louis, MO).

3.2.2 Cell culture and treatment

All cells were cultured in MEM supplemented with 5% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2/air atmosphere. Cells were seeded in 6-well plates or 60 mm cultural dishes and starved with MEM containing 0.5% FBS for 24 hrs after reaching 80% confluence. After pre-treatment with medium containing various chemicals at designated concentrations, cells were washed with PBS once and then exposed to UVB in fresh PBS. Cells were returned to incubator with the addition of the previous culture medium until time of collection.

3.2.3 Detection of cell viability—LDH leakage

Activity of lactate dehydrogenase (LDH) in the medium was measured using a Cobas Mira S Analyser with the test kit (Abbott Laboratories, Irving, TX, USA). The total LDH activity was determined by ultrasonication and assessed by expressing as percentage LDH leakage (LDH in medium/total LDH activity x 100).

3.2.4 Determination of sub-G1 cells

It is well established that DNA fragmentation during apoptosis may lead to extensive loss of DNA content and result in a distinct sub-G1 peak when analyzed using flow cytometry (Nicoletti *et al.* 1991). Hence, sub-G1 assay was used to quantify the apoptotic effect of UVB and PN-UVB. At the end of various designated treatments, cells were scrapped, washed, fixed and permeabilized with 70% ice-cold ethanol at 4 ^{o}C for 2 hours. Cells were then incubated at 37 ^{o}C for 15 min with freshly prepared PI solution (0.1 % Triton X-100, 200 μg / ml RNase A, and 20 μg / ml PI in PBS). 10,000 cells

from each group were analyzed using flow cytometry (Coulter Epics Elite ESP, Miami, USA), and data obtained were analyzed using WinMDI2.7 software (Scripps Institute, La Jolla, USA).

3.2.5 DNA gel electrophoresis

The DNA fragmentation pattern (DNA ladder), which is a hallmark of apoptosis, was assessed by agarose gel electrophoresis. DNA was extracted using a DNA extraction kit from PureGene according to manufacturer's protocol. Briefly, cells were collected after designated treatment. After a short centrifugation at 13,000 g for 15 seconds, Cell Lysis Solution was added to the cell pellet, followed by RNase A solution and 10 min incubation at 37 ^{o}C. The cell lysates were then cooled for 1 min before adding Protein Precipitation Solution, followed by vigorous vortexing for 20 seconds. After centrifugation at 13,000 g for 5 min at 4 ^{o}C, the supernatants containing the DNA were then precipitated with 100% isopropanol. The samples were mixed by gentle inversion for 50 times and followed by centrifugation at 13,000 g for 5 min at 4 ^{o}C. The DNA pellets were then washed with 70% ethanol and allowed to dry for 10-15 min. DNA was then rehydrated in DNA Hydration Solution. Five μg of DNA were then electrophoresed in 1.5 % agarose gel stained with EB (1 μg/ml) at 50 V in 0.5 X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH8.0), visualized under UV and photographed.

3.2.6 Western blot analysis

Cells were scrapped after the designated treatment and washed with ice-cold PBS twice. Pellets were resuspended in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS,

10% glycerol, protease inhibitor cocktail), sonicated on ice for 15 seconds, and centrifuged at 10,000 g for 10 minutes at 15^{o}C. 30 μg of proteins were separated on 10% SDS-polyacrylamide gel in Mini-Protein II system (Bio-Rad). Following electrophoresis the protein was transferred to a PVDF membrane (Millipore, Bedford, MA, USA) and subsequently hybridized with various antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station.

3.2.7 Electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared according to a published method with modifications (Hehner *et al.*, 1998). JB6 cells were collected after the designated treatment, washed with ice-cold PBS twice and then resuspended in 120 μl of Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protenase inhibitor cocktail). After 15 minutes of incubation on ice, Nonidet P-40 was added (final concentration 0.3%) and tubes were vortex vigorously. The lysates were centrifuged at 2,000 g for 10 min at 4^{o}C. The resulting nuclear pellet was resuspended in 40 μl of Buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail) and incubated on ice for 30 minutes. The nuclear extracts were collected after 15 minutes of centrifugation at 20,000 g at 4^{o}C. Protein concentration was quantified using Bio-Rad protein assay kit (Hercules, CA). All samples were kept at -70^{o}C until assay. The DNA binding reaction mixture contained 5 μg of nuclear extract, 5 x Buffer C (100 mM HEPES, pH7.9, 20% glycerol, 1 mM DTT and 300 mM KCl), 2 μg poly (dI-dC), 2 μg BSA and incubate for 30 min on ice. ^{32}P labeled NF-κB oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3', 3'-

TCAACTCCCCTGAAAGGGTCCG-5') or AP-1 oligonucleotide (5'-CGCTTGATGAGTCGACCGGAA-3', 3'-GCGAACTACTCAGTCGGCCTT-5') was then added in a total volume of 20 μl. The DNA-protein complexes were resolved in 5% polyacrylamide gel using a vertical gel electrophoresis apparatus (Gibco BRL). Gels were then dried and exposed to an X-ray film (Kodak) at -70^{o}C overnight.

3.2.8 AP-1 transactivation assay

JB6 cells stably transfected with an AP-1 luciferase reporter plasmid were subjected to designated treatments. At 6 hours post UVB irradiation, the cell lysates were collected after the addition of cell lysis buffer (Promega). Luciferase activity was measured using a luciferase assay kit (Promega). The relative light units (RLU) were then determined in a luminometer (Lumi-one, Trans Orchid, Tampa, FL) for 15 seconds after a 5 second delay time.

3.2.9 Statistical analysis

All numeric data are presented as means ± standard deviations (SD) and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons as post-hoc test (SPSS 11.5). A P value < 0.05 is considered as statistical significance.

3.3 Results

3.3.1 PN sensitizes cells to UVB-induced apoptosis in a dose-dependent manner

In order to elucidate the mechanism(s) involved in the chemopreventive property of PN against UVB-induced skin cancer, a mouse epidermal cell line JB6 was used in the

subsequent *in vitro* study. The cytotoxicity of PN was first tested on this cell line using LDH leakage (Figure 3.1) and DNA content analysis of sub-G1 cells (Figure 3.2). The LD_{50} was estimated at 8.5 μM. Next, a series of investigations were conducted to determine if PN has any synergistic or antagonistic effect on UVB-induced apoptosis. High dosage of UVB is capable of inducing apoptosis in JB6 cells and the preliminary experiment showed that 50 mJ/cm^2 UVB was the lowest observed effect level (LOEL) with marginal cytotoxic effect to cells. Pre-treating cells with non-cytotoxic concentrations of PN (2.5 and 5 μM) greatly enhanced UVB (50 mJ/cm^2)-induced cell death as measured by LDH leakage (Figure 3.3) and percentage of sub-G1 cells determined by DNA content analysis (Figure 3.4). Similar results were also found in DNA gel electrophoresis showing DNA fragmentation, a hallmark of apoptosis (Figure 3.5). Based on the common understanding that removal of damaged cells via apoptosis is an important anti-oncogenic process (Hildesheim *et al.*, 2002; Kulms and Schwarz, 2002; D'Errico *et al.*, 2003), it is thus believed that such sensitization by PN may serve as one of the underlying mechanisms for its chemopreventive property against UVB-induced skin cancer.

3.3.2 PN inhibits NF-κB and AP-1 DNA binding activity as well as transcriptional activity of AP-1 induced by UVB

PN is a potent inhibitor of NF-κB signaling pathway. Indeed, pretreatment of 1-5 μM PN significantly inhibited the UVB-induced DNA binding ability of NF-κB (Figure 3.6). In addition, it is well documented that AP-1 is an important transcription factor mainly involved in cell survival and proliferation (Shaulian and Karin, 2002). UVB has

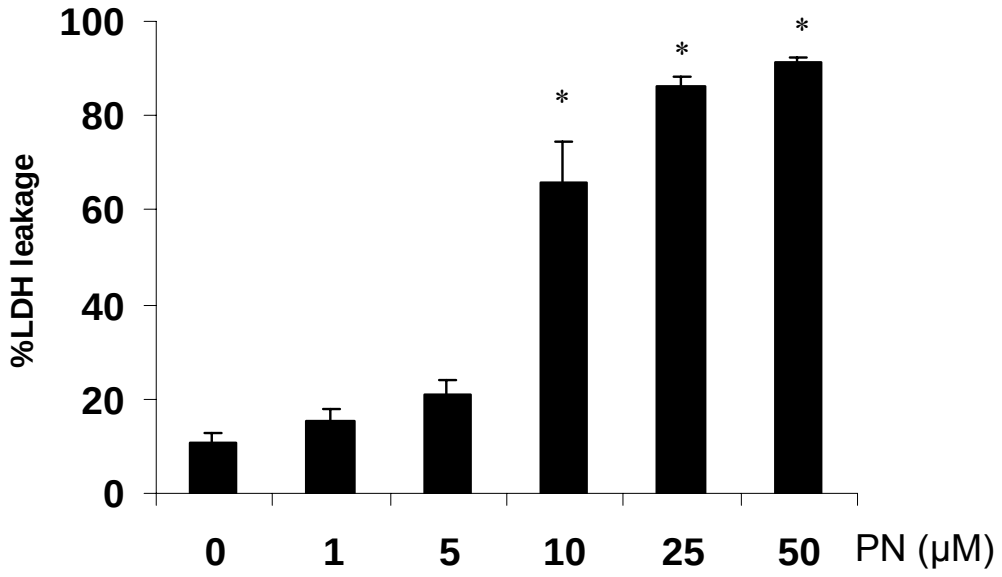


Figure 3.1 PN dose-dependently induced apoptosis. JB6 cells were treated with various concentrations of PN and the viability of cells was determined by LDH leakage 24 hours later as described in materials and methods. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. An asterisk indicates statistical significance with a *P*-value < 0.05.

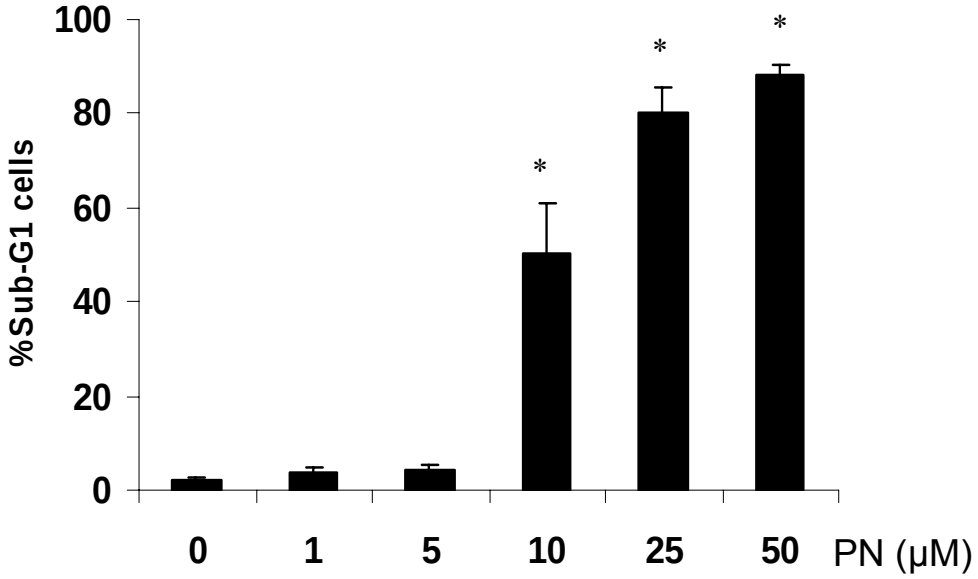


Figure 3.2 PN dose-dependently induced apoptosis. JB6 cells were treated with various concentrations of PN and the viability of cells was determined by DNA content analysis for sub-G1 cells 24 hours later as described in materials and methods. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. An asterisk indicates statistical significance with a *P*-value < 0.05.

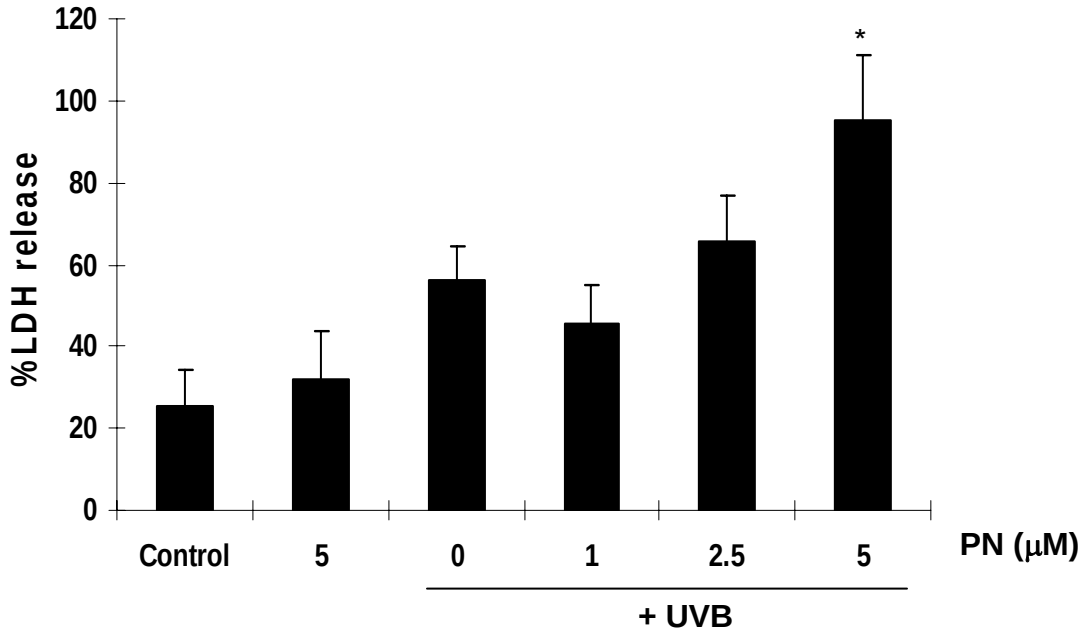


Figure 3.3 PN sensitizes cells to UVB-induced apoptosis. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 hours and subjected to UVB irradiation of 50 mJ/cm^2. The viability of cells was determined by LDH leakage 24 hours after UVB irradiation as described in materials and methods. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. An asterisk indicates statistical significance with a *P*-value < 0.05.

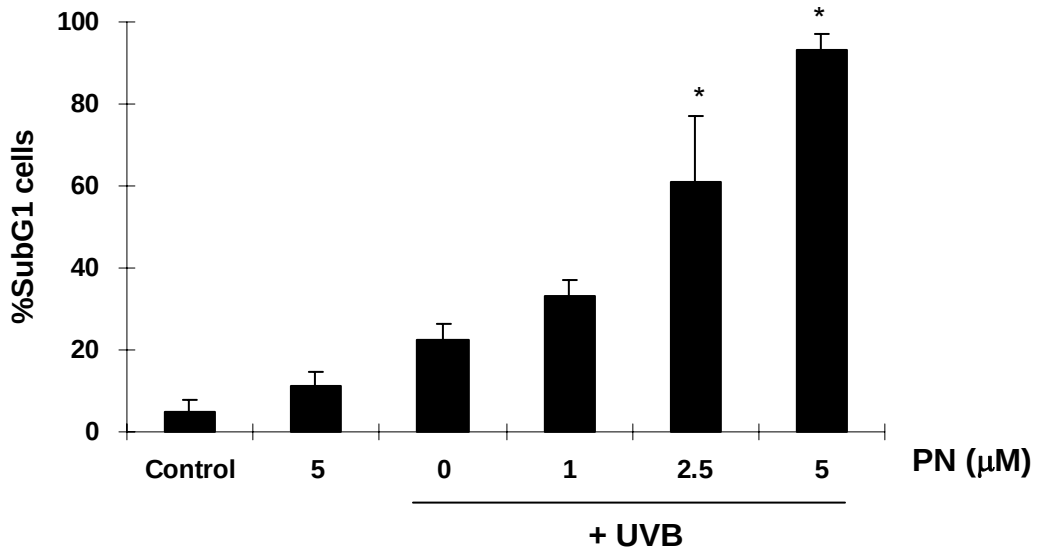


Figure 3.4 PN sensitizes cells to UVB-induced apoptosis. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 hours and subjected to UVB irradiation of 50 mJ/cm^2. The viability of cells was determined by DNA content analysis for sub-G1 cells 24 hours after UVB irradiation as described in materials and methods. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. An asterisk indicates statistical significance with a *P*-value < 0.05.

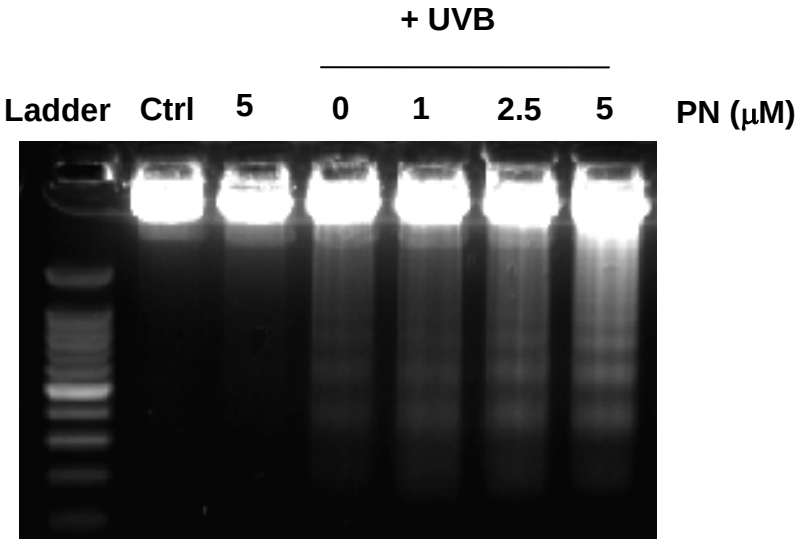


Figure 3.5 PN sensitizes cells to UVB-induced apoptosis. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 hours and subjected to UVB irradiation of 50 mJ/cm^2. The viability of cells was determined by DNA fragmentation assay 24 hours after UVB irradiation as described in materials and methods. DNA fragments were resolved by electrophoresis at 50 V on 1.5% agarose gels impregnated with ethidium bromide, detected by UV transillumination and analyzed using Kodak Image Station 440.

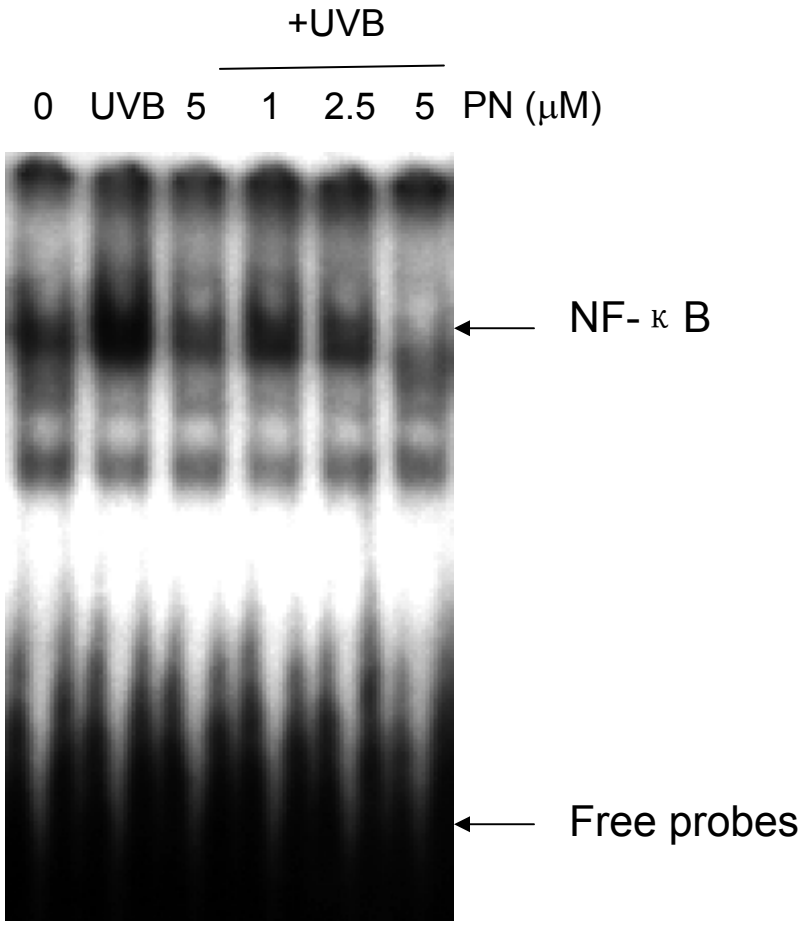


Figure 3.6 PN inhibits the UVB-induced DNA binding activity of NF-κB. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 hours, followed by UVB irradiation with 50 mJ/cm^2. Control cells were mock irradiated. Cells were harvested 6 hours after UVB irradiation. Five μg of nuclear extracts were subjected to EMSA as described in Materials and Methods.

been reported to induce DNA binding activity of AP-1 in JB6 cells (Huang *et al.*, 1997). As shown in Figure 3.7, 50 mJ/cm^2 UVB vastly induced the AP-1 binding activity in JB6 cells. Pretreatment with PN (2.5-5 μM) effectively suppressed UVB-induced DNA binding capability of AP-1. The effect of PN on AP-1 transcriptional activity was also examined in JB6 cells stably transfected with an AP-1 luciferase reporter plasmid. Cells were pre-treated with 1-5 μM of PN for 2 hours and followed by UVB irradiation (50 mJ/cm^2). Being consistent with the findings in EMSA, significant reduction of AP-1 luciferase activity was observed with higher concentrations of PN (2.5-5 μM) (Figure 3.8).

3.3.3 PN inhibits UVB-induced phosphorylations of c-Jun and ATF-2

Both c-Jun and ATF-2 are important components of AP-1 complex (Karin *et al.*, 1997). It has been reported previously that UV-induced AP-1 DNA binding is independent of new protein synthesis, and both Jun and ATF-2 activation occur as a result of post-translational modification involving changes in the phosphorylation states (Buscher *et al.*, 1988; Devary *et al.*, 1991; van Dam *et al.*, 1995). Thus, further investigations were conducted to test if PN suppresses UVB-induced AP-1 activation via inhibition of the phosphorylation states of its key components. As shown in Figure 3.9A, pre-treatment with 5 μM of PN completely blocked UVB-induced c-Jun phosphorylation at both Ser63 and Ser73 sites. No changes in total protein level for c-Jun were detected in cells treated with either UVB and/or PN. Similar inhibition on UVB-induced ATF-2 phosphorylation was also observed with PN pre-treatment (Figure 3.9B). Therefore, it is

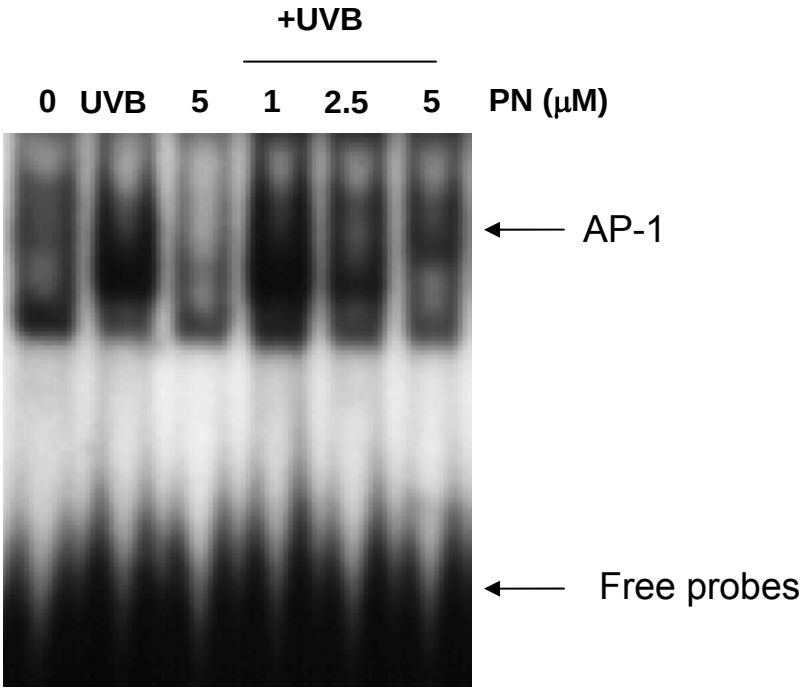


Figure 3.7 PN inhibits the UVB-induced DNA binding activity of AP-1. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 hours, followed by UVB irradiation with 50 mJ/cm^2. Control cells were mock irradiated. Cells were harvested 6 hours after UVB irradiation. Five μg of nuclear extracts were subjected to EMSA as described in Materials and Methods

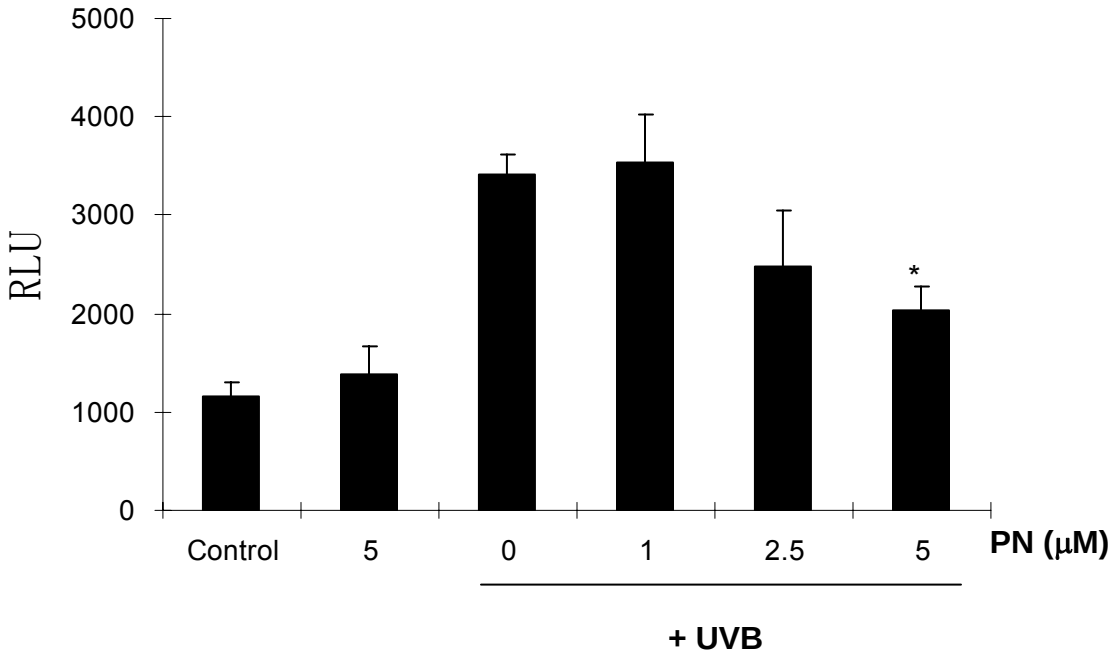
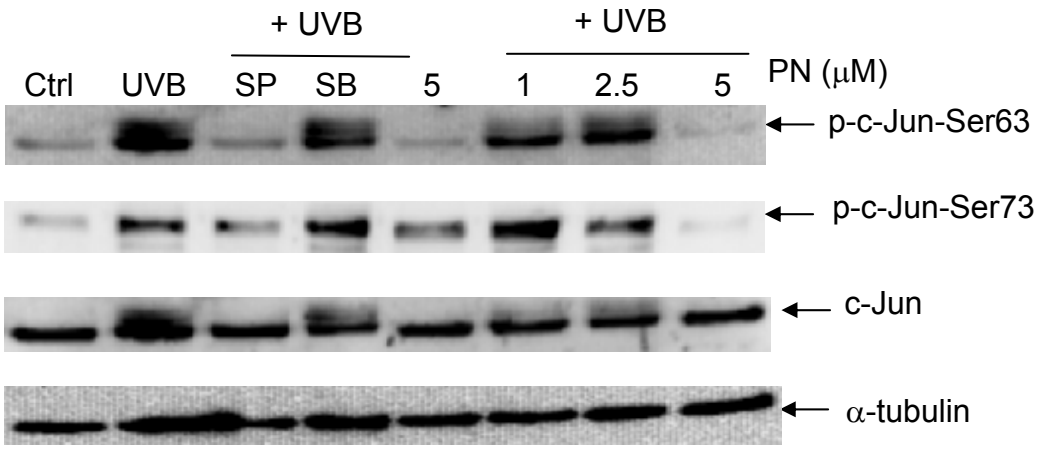


Figure 3.8 PN inhibits the UVB-induced transcriptional activity of AP-1. JB6 cells stably transfected with an AP-1 luciferase reporter plasmid were pre-treated with 1, 2.5 or 5 μM of PN for 2 hours, followed by UVB irradiation with 50 mJ/cm^2. Control cells were mock irradiated. Cells were harvested 6 hours after UVB irradiation. Luciferase activity was determined as described in Materials and Methods. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. An asterisk indicates statistical significance with a *P*-value < 0.05.

(A)



(B)

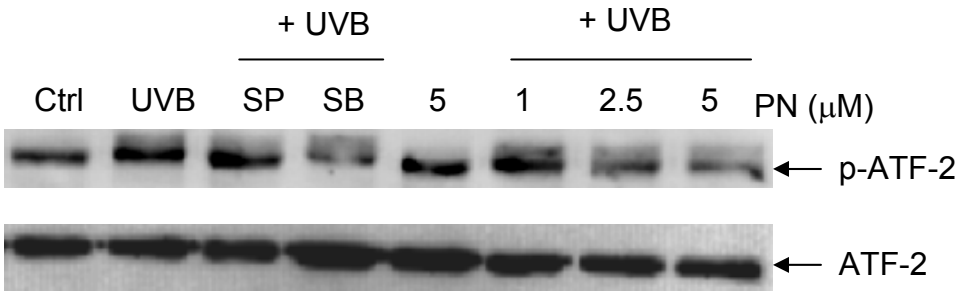


Figure 3.9 PN inhibits the UVB-induced phosphorylations of (A) c-Jun and (B) ATF-2. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN, 20 μM of SP600125, or 10 μM of SB203580 for 2 hours and then subjected to UVB irradiation of 50 mJ/cm^2. Cells were harvested 2 hours after UVB irradiation. Thirty μg of proteins were separated on 10% SDS-polyacrylamide gels and the subsequent membranes were hybridized with anti-p-c-Jun, c-Jun, p-ATF-2, ATF-2 antibodies. α-Tubulin was blotted as loading control. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station 440.

believed that PN suppresses UVB-elicited AP-1 activation via reduced phosphorylation levels of its key components such as c-Jun and ATF-2.

3.3.4 PN blocks UVB-induced MAPK pathways

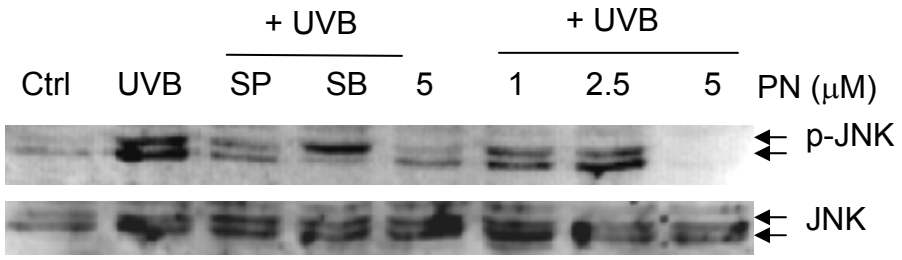
JNK and p38 are two main groups of MAPKs that are directly involved in UVB-elicited AP-1 activation (Adler *et al.*, 1996; Chen and Bowden, 2002). c-Jun and ATF-2 serve as direct downstream targets for JNK and p38, respectively. In order to further understand the mechanisms involved in the inhibitory effect of PN on UVB-induced AP-1 activation, a series of experiments were conducted to study if PN acts on UVB-elicited JNK and p38 activation. Pre-treatment with 5 μM of PN completely blocked UVB-induced JNK phosphorylation without affecting the total JNK protein level (Figure 3.10A). Similar inhibitory effect on UVB-induced p38 phosphorylation was also observed in cells pre-treated with PN (Figure 3.10B). Therefore, it is believed that PN suppresses UVB-mediated AP-1 activation through its inhibitory effects on JNK and p38.

3.3.5 PN sensitizes UVB-induced apoptosis via JNK and p38

It is still controversial with regards to the exact role of JNK and p38 in UV-induced apoptosis. In mouse embryonic fibroblasts, JNK has been shown to be required for UV-induced cell death (Tournier *et al.*, 2000). On the contrary, there is evidence indicating that JNK and p38 act as cell survival/anti-apoptotic mechanism in UV-treated cells (Wisdom *et al.*, 1999; Ivanov and Ronai, 2000; Chouinard *et al.*, 2002). As shown above (Figure 3.4), PN effectively sensitizes UVB-induced apoptosis in JB6 cells. Notably, similar sensitization was also found in cells pre-treated with SP600125 (a

specific inhibitor of JNK) or SB203580 (a specific inhibitor of p38), although to a less extent (Figure 3.11). The effectiveness and specificity of these two inhibitors were confirmed by the significant reduction in phosphorylation of their respective targets (Figure 3.9 and 10). Data from this experiment thus provide indirect evidence that PN may sensitize UVB-induced apoptosis via its inhibitory effect on JNK and p38. It appears that both JNK and p38 contribute to the cell survival mechanisms in UVB-treated JB6 cells, based on the observation that PN as a dual-inhibitor of JNK and p38 are much more effective than the individual inhibitor for sensitizing UVB-induced apoptotic cell death.

(A)



(B)

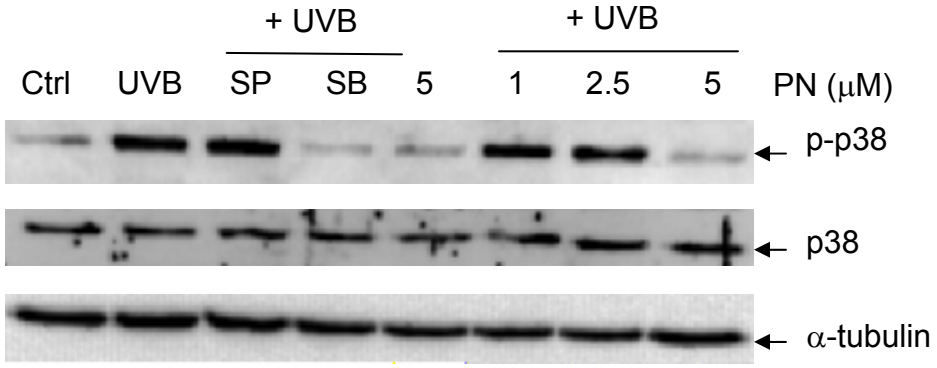


Figure 3.10 PN inhibits the UVB-induced phosphorylations of (A) JNK and (B) p38. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN, 20 μM of SP600125, or 10 μM of SB203580 for 2 hours and then subjected to UVB irradiation of 50 mJ/cm^2. Cells were harvested 2 hours after UVB irradiation. Thirty μg of proteins were separated on 10% SDS-polyacrylamide gels and the subsequent membranes were hybridized with anti-p-JNK, JNK, p-p38, p38 antibodies. α-tubulin was blotted as loading control. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station 440.

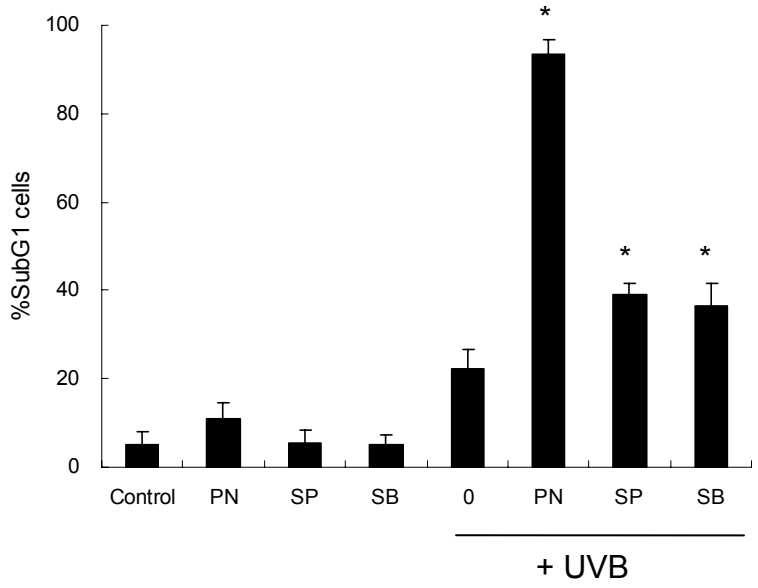


Figure 3.11 PN, SP600125, and SB203580 sensitize cells to UVB-induced apoptosis. JB6 cells were pretreated with 5 μM of PN, 20 μM of SP600125, or 10 μM of SB203580 for 2 hours and then subjected to UVB irradiation of 50 mJ/cm^2. The viability of cells was determined using PI staining in sub-G1 assay at 24 hours post-UVB treatment. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. An asterisk indicates statistical significance with a *P*-value < 0.05.

3.4 Discussion

Nuclear transcriptional factors such as AP-1, NF-κB, NF-AT and STATs have been reported to be involved in the UVB-induced signaling pathway (Angel *et al.*, 2001; Siebenlist *et al.*, 1995; Huang *et al.*, 2000; Zhang *et al.*, 2001). In order to further understand the possible mechanism(s) contributing to the observed chemopreventive property of PN, we investigated the effects of PN on AP-1 and the related MAPKs signaling cascade using JB6 murine epidermal cells. AP-1 plays a key role in pre-neoplastic to neoplastic transformation in cell culture and the blockage of AP-1 activities has been shown to inhibit cell transformation (Dong *et al.*, 1994). Moreover, AP-1 has been shown to regulate apoptosis, with both pro-apoptotic and anti-apoptotic function (Shaulian and Karin, 2002). It appears that the exact outcome of AP-1 manipulations is highly tissue- and developmental stage-specific. Results from the present study demonstrated that PN pre-treatment inhibits the UVB-induced DNA binding (Figure 3.7) and transcriptional activity of AP-1 (Figure 3.8) in addition to its well known suppressive effect on NF-κB (Figure 3.6). Two of the major components of AP-1 complex, c-Jun and ATF-2, can activate AP-1 via post-translational modification involving changes in the phosphorylation states (Buscher *et al.*, 1988; Devary *et al.*, 1991; van Dam *et al.*, 1995). In response to UV, c-Jun is rapidly phosphorylated at both Ser63 and Ser73 sites along with ATF-2. The data showed that PN subdues UVB-induced AP-1 transactivation via blockage of c-Jun and ATF-2 phosphorylations (Figure 3.9A and B). The outcome of these series of inhibitions seem to lead to massive sensitization to UVB-induced apoptosis (Figure 3.4), suggesting an anti-apoptotic role of AP-1. Such anti-apoptotic function of AP-1 and c-Jun has been reported previously. Wisdom and co-workers (1999)

noted that c-Jun protects cells from UV-induced apoptosis and phosphorylation of c-Jun on Ser63 and Ser73 is required for such protection. Moreover, Ivanov and colleagues (2001) demonstrated that c-Jun protects cells against UV-induced cell death via co-operation with STAT3 to suppress transcription of Fas.

The UV-activated signal transduction pathway is primarily mediated by mitogen-activated protein kinases (MAPKs). Mammals express at least four distinctly regulated groups of MAPK: Erks, JNK, p38, and Erk5 (Chang and Karin, 2001). All MAPKs are activated by dual phosphorylation on threonine and tyrosine at T-X-Y motifs within the activation loop. Once activated, they translocate to the nucleus and phosphorylate target transcription factors. In response to UV, key MAPKs such as JNK and p38 are activated, which in turn phosphorylate c-Jun at Ser-63 and Ser-73 sites and ATF-2, respectively (Devary *et al.*, 1991; van Dam *et al.*, 1995). It is rather controversial in regards to the exact role of JNK in UV-mediated apoptosis. For instance, JNK has been shown to be required for UV-induced apoptotic cell death in mouse embryonic fibroblasts (Tournier *et al.*, 2000), while other studies demonstrated the anti-apoptotic role of JNK in UV-induced apoptosis (Wisdom *et al.*, 1999; Ivanov *et al.*, 2001). Results from the present study showed that the inhibition of JNK leads to sensitization of JB6 cells to UVB-induced apoptosis, suggesting an anti-apoptotic role of JNK in our system (Figure 3.11). In general, the exact role of JNK in apoptosis varies depending on a number of factors that include the nature of the stimuli, cell type, the duration of activation and more importantly, the interaction of other signaling pathway such as NF-κB and the PI3K-AKT cascades (Davis, 2000; Lamb *et al.*, 2003; Lin, 2003). The involvement of those

cell survival signaling pathways in PN-mediated cell death sensitization remains to be further investigated.

In the present study, the potent inhibitory effect of PN on UVB-induced p38 activation (Figure 3.10) was also noted, and p38 inhibition leads to sensitization of cells to UVB-induced apoptosis (Figure 3.11), implying an anti-apoptotic role of p38 in UVB-induced apoptotic cell death. Although it is still controversial regarding the exact role of p38 in apoptosis, p38 has been reported to protect cells from UV-induced apoptosis through down regulation of NF-κB activity and Fas expression (Ivanov and Ronai, 2000). Some other mechanisms have also been proposed to be involved in the anti-apoptotic function of p38. For instance, the p38 MAPK inhibitor SB203580 can trigger a significant, Ras-independent activation of c-Raf in certain cell lines in the concentration range of 8-25 μM (Hall-Jackson *et al.*, 1999; Kalmes *et al.*, 1999). Activated Raf may phosphorylate and inactivate Bad, a pro-apoptotic member of the Bcl-2 family of proteins, or up-regulate the transcription of pro-survival genes to prevent cytochrome *c* release and subsequently apoptosis (Bonni *et al.*, 1999). Furthermore, Raf-1 has been shown to promote cell survival by antagonizing apoptosis signal-regulating kinase 1 (ASK1), an important mediator of apoptotic signaling (Chen *et al.*, 2001). In the present study, PN has been shown to be a dual inhibitor of both JNK and p38 in UVB-treated cells (Figure 3.10). Incidentally, the PN-elicited sensitization of JB6 cells to UVB-induced apoptosis is twice as strong as with the individual JNK and p38 inhibitor (Figure 3.11). These findings suggest that both JNK and p38 contribute to the cell survival mechanisms in UVB-treated cells.

UV exposure could result in direct or indirect DNA damage, and the damage is normally repaired by a nucleotide excision repair (NER) mechanism (Thoma, 1999). Most of the irreparable DNA-damaged cells will be eliminated through apoptosis, as evident in skin with the appearance of sunburn cells (Kulms and Schwarz, 2002). However, not all cells with irreparable DNA damage will undergo apoptosis. DNA lesions that are not repaired or incorrectly repaired may lead to mutations and subsequently carcinogenesis. Therefore, the removal of damaged cells through sensitizing cells to apoptosis denotes an effective mean in preventing carcinogenesis process (Hildesheim *et al.*, 2002; Kulms and Schwarz, 2002; D'Errico *et al.*, 2003).

In summary, the data from this study for the first time demonstrated the synergistic effect of PN and UVB in sensitizing cells to apoptosis. Such sensitization appears to be mediated through inhibition on AP-1, JNK and p38 signaling pathways and may contribute to the cancer chemopreventive activity of PN.

CHAPTER 4

PARTHENOLIDE SENSITIZES CELLS TO UVB-INDUCED APOPTOSIS VIA PKC DEPENDENT PATHWAYS

4.1 Introduction:

Feverfew (*Tanacetum parthenium*) has been used as a herbal plant for centuries in Europe, with known anti-microbial and anti-inflammatory properties (Brown *et al.*, 1997; Jain and Kulkarni, 1999). PN, a sesquiterpene lactone, is one of principal bioactive components of this plant. It is believed that the bioactivity of PN is mediated via the highly electrophilic α-methylene-γ-lactone ring and an epoxide residue that are capable of interacting rapidly with nucleophillic sites of biological molecules (Macias *et al.*, 1997; Zhang *et al.*, 2005). PN is a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (Woynarowski and Konopa, 1981; Hall *et al.*, 1988; Ross *et al.*, 1999). In addition, PN has been reported to induce apoptosis via caspase activation and mitochondria dysfunction (Wen *et al.*, 2002), disruption in intracellular thiols and calcium equilibrium (Zhang *et al.*, 2004a), as well as activation of pro-apoptotic Bcl-2 family proteins (Zhang *et al.*, 2004c). On the other hand, PN also has been shown to sensitize cells to apoptosis induced by various stimuli such as TNF-α and TRAIL, presumably through its action on activator protein-1 (AP-1) signaling pathway (Zhang *et al.*, 2004b; Nakshatri *et al.*, 2004). The results from chapter 3 have also shown the sensitization activity of PN on UVB-induced apoptosis in JB6 cells. The *in vivo* anti-cancer activity of PN has been studied recently. For instance, the results from chapter 2 demonstrated that PN possesses strong chemopreventive property against UVB-induced skin cancer in SKH-1 hairless mice, whereas Sweeney and co-workers (2005) revealed that PN in combination with docetaxel is capable of reducing metastasis and improving survival in xenograft model of breast cancer.

The electromagnetic spectrum of ultraviolet (UV) can be grouped into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). UVB exposure is the main etiological factor for non-melanoma skin cancer in human (Fry and Ley, 1989). The mechanism(s) of UVB-induced skin cancer have not been fully understood. Several transcriptional factors including AP-1, nuclear transcription factor-kappa B (NF-κB), nuclear factor of activated T cells (NF-AT), and signal transducers and activators of transcription have been linked to the tumor-promoting ability of UVB (Angel *et al.*, 2001; Siebenlist *et al.*, 1995; Huang *et al.*, 2000; Zhang *et al.*, 2001).

Protein kinase C (PKC) is a group of serine/threonine kinases that regulate many cellular functions such as proliferation, differentiation, transformation, survival and apoptosis (Yang and Kazanietz, 2003). PKC can be classified into 3 groups based on the co-factors required for activation: 1) the Ca^{2+} and diacylglycerol (DAG)-dependent classical or conventional PKC that consists of isotypes α, β1, β2 and γ; 2) the DAG-dependent, Ca^{2+}-independent novel PKC that consist of δ, η, ε and θ, and 3) the DAG- and Ca^{2+}-independent atypical PKC that consist of ι/λ and ζ. The consequences of PKC activation by UVB is rather cell type specific and could lead to inhibition on cell proliferation or even induction of apoptosis. Among all, PKCδ seems to be the main PKC subtype with pro-apoptotic functions in response to various extracellular stimuli including UVB (Chen *et al.*, 1999; Brodie and Blumberg, 2003) whereas PKCζ has been shown to be anti-apoptotic in response to UV (Berra *et al.*, 1997; Frutos *et al.*, 1999).

As shown in chapter 3, suppression of mitogen activated protein kinase and AP-1 signaling cascade by PN contributes to its sensitization effect on UVB-induced apoptosis in JB6 cells. It is also known that certain subtypes of PKC regulate the MAPK-AP-1

pathway in UVB-treated cells (Chen *et al.*, 1999; Huang *et al.*, 2000). Hence, the main objective of this study is to explore the involvement of PKC so as to further understand the underline mechanism(s) of the chemopreventive property of PN. The data demonstrate for the first time that PN selectively inhibits the UVB-induced PKCζ activation while further enhances that of PKCδ, both contribute to the sensitization effect of PN on UVB-induced apoptosis in murine epidermal JB6 cells.

4.2 Materials and Methods

4.2.1 Cell line and chemicals

Murine epidermal cell line JB6 was obtained from Dr. Shi XL from NIOSH, USA. PN (97% pure) was purchased from Biomol (Plymouth Meeting, PA). Anti p-JNK, JNK, p-p38, and p38 polyclonal antibodies were purchased from Cell Signaling (Beverly, MA), while anti HA, PKC-δ and ζ polyclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Secondary antibodies (horseradish peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG) and enhanced chemiluminescence substrate were from Pierce (Rockford, IL). γ-p32 ATP was obtained from Perkin-Elmer (Boston, MA). SP600125 was purchased from Calbiochem (San Diego, CA). PKC inhibitors GF109203X and rotterlin were purchased from Calbiochem (San Diego, CA). Other common chemicals were from Sigma-Aldrich (St. Louis, MO). Other common chemicals were from Sigma-Aldrich (St. Louis, MO).

4.2.2 Cell culture and treatment

JB6 murine epidermal cells were cultured in MEM supplemented with 5% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2/air atmosphere as described earlier. Cells were seeded in 60 mm cultural dishes and starved with MEM containing 0.5% FBS for 24 hrs after reaching 80% confluence. After pre-treated with various reagents at designated conditions, cells were washed with PBS once and then exposed to UVB in fresh PBS. UVB was delivered through a bank of FS24 lamps (Light Sources Inc., Orange, CT) with spectral irradiance of 280-400 nm, 80% of which in the UVB region (280-320 nm) and with a peak around 313 nm. The emitted UVB dose was quantified using a phototherapy radiometer (International Light, Newburyport, MA) equipped with IL SED 240 detector. Cells were returned to incubator with the addition of the previous culture medium until time of collection.

4.2.3 Transient transfection

Wild-type and dominant negative (DN) PKCδ and ζ plasmids were kindly provided by Dr. JW Soh from Inha University, Incheon, Korea. As described previously (Soh and Weinstein, 2003), pHACE-PKC-WT expression plasmids were generated by ligating full-length open reading frames of different PKC isoforms into pHACE digested with *Eco*RI. On the other hand, pHACE-PKC-DN expression plasmids were generated by ligating full-length open reading frames of PKC isoforms with a dominant negative (DN) (K to R or K to M) point mutation at the ATP binding site into pHACE digested with *Eco*RI. DN-p38α and DN-p38β_2 plasmids were gifts from Dr. J Han (Scripps Research Institute, La Jolla, CA, USA). As described before, p38β double mutant (p38β

(AF)) was created by substituting Thr^{188} with Ala and Tyr^{190} with Phe using a PCR-based procedure(Jiang *et al.*, 1996) , whereas the plasmid DN-Flag-p38α MAP kinase was prepared using the expression vector pCMV5 and the p38 cDNA (Zhang *et al.*, 1995). The flag epitope tag DYKDDDDK was added to the amino-terminal region by PCR recombination. In the present study, cells were co-transfected with designated PKC plasmids or and a transfection marker pDsRed (Clontech, Palo Alto, CA) using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Cells were subjected to various treatment 24 hours after transfection.

4.2.4 Determination of apoptotic cell death

In this series of experiments, cells were first transiently transfected with various PKC plasmids and pDsRed fluorescence protein. Apoptotic cell death was then determined using DNA content analysis coupled with flow cytometry after 4',6-diamidino-2-phenylindole (DAPI) staining. Briefly, cells were scrapped, washed, incubated with 0.5% para-formaldehyde for 1 hour at room temperature at the end of various designated treatments. Cells were then fixed and permeabilized with 70% ice-cold ethanol at 4 ^{o}C for 2 hours. Cells were then incubated for 15 min at room temperature with freshly prepared DAPI solution (100 mM Tris, pH7.4, 150 mM NaCl, 1 mM CaCl, 0.5 mM MgCl, 0.1 % Nonidet P-40). 20,000 cells from each group were analyzed by flow cytometry using Becton Dickinson FACSVantage SE system (Franklin Lakes, NJ) (Benhar *et al.*, 2001). Only those transfected cells with expression of the red fluorescence protein were then gated for analyzing the percentage of sub-G1 cells using WinMDI 2.7 software (Scripps Institute, La Jolla, USA).

4.2.5 PKC translocation assay

PKC translocation assay was performed based on a published method (Chen *et al.*, 1999) with modifications. In brief, , 15 minutes after UVB irradiation cells were washed once with ice-cold PBS and then sonicated in homogenization buffer A (20 mM Tris-HCl, pH8.0, 10 mM EGTA, 2mM EDTA, 2 mM DTT, 1 mM PMSF and protease inhibitor cocktail) for 10 seconds on ice. The lysate was then centrifuged at 100,000 g for 1 hour at 4 ^{o}C. The supernatant was collected as the cytosolic fraction. The pellet was then resuspended in homogenization buffer B (with 1% Triton X-100 in buffer A) and sonicated for another10 seconds on ice. The suspension was centrifuged at 15,000 g for 15 min at 4 ^{o}C. The supernatant was collected as the membrane fraction. Thirty μg of proteins were separated on 8% SDS-polyacrylamide gel in Mini-Protein II system (Bio-Rad). Following electrophoresis the protein was transferred to a PVDF membrane (Millipore, Bedford, MA, USA) and subsequently hybridized with anti-PKCδ and ζ antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station.

4.2.6 PKC kinase assay

PKC kinase assay was performed according to a published method (Soh and Weinstein, 2003) with modifications. Briefly, cells were harvested 30 min after UVB irradiation in PKC lysis buffer (50 mM HEPES, pH7.5, 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1 mM PMSF, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate, protease inhibitor cocktail). The cell lysate was then centrifuged at 12,000 g for 15 min at 4 ^{o}C and the supernatant was collected as

cellular protein. 400 μg of protein was immunoprecipitated with anti-PKC antibodies (4 μg) overnight and followed by incubation with protein G-sepharose for 1 hour. The immunoprecipitates were washed 5 times with ice-cold kinase buffer (50 mM HEPES, pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate). The kinase assay was initiated by adding 30 μl of kinase buffer containing 10 μg of GST-MARCKS substrate, 0.5 μCi of [γ-^{32}P] ATP and protease inhibitor cocktail. The reaction was performed for 30 min at 30 ^{o}C and was terminated by adding 3x sampler buffer. All reaction mixes were then boiled for 5 min before being separated on a 10% SDS-polyacrylamide gel in a Mini-Protein II system (Bio-Rad). Gels were then dried and exposed to an X-ray film (Kodak) at room temperature.

4.2.7 PKC activity assay

The activity of PKC kinase was studied using another published method with modification (Sando, 2003). Briefly, cells were harvested 30 min after UVB irradiation in PKC lysis buffer (50 mM HEPES, pH7.5, 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1 mM PMSF, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate, protease inhibitor cocktail). The cell lysate was then centrifuged at 12,000 g for 15 min at 4 ^{o}C and the supernatant was collected as cellular protein. 400 μg of protein was immunoprecipitated with anti-PKC antibodies (4 μg) overnight and followed by incubation with protein G-sepharose for 1 hour. The IP complexes were then washed twice with Buffer A (1 x PBS, 1% NP-40, and 1 mM PMSF), twice with Buffer B (10 mM Tris, pH7.5, and 0.5 M LiCl), twice with Buffer C (10 mM Tris, pH7.5, 1 mM EDTA, and 50 mM NaCl), and once with kinase buffer (10

mM MOPS, 5 mM $MgCl_2$, and 0.3 mM $CaCl_2$). The kinase activity was initiated by adding 30 µl of kinase buffer containing 10 µg of MARCKS substrate, 0.5 µCi of [γ-^{32}P] ATP and protease inhibitor cocktail. The reaction was performed for 6 min at 30 ^{o}C and was terminated by adding 3 µl of stop solution (2 M ATP and 0.5 M EDTA, pH8.0). The reaction mixture was then transferred to a P81 ion-exchange paper square (2 x 2 cm, Whatman International, Maidstone, UK). These P81 ion-exchange papers were then washed thrice with 75 mM orthophosphoric acid and once with 95% ethanol. The papers were allowed to dry before subjected to scintillation counting in 2 ml of scintillation fluid using Berkman LS 6000 Liquid scintillation counting system (Berkman Coulter Inc., Fullerton, CA, USA).

4.2.8 Western blot analysis

Cells were scrapped after the designated treatment and washed with ice-cold PBS twice. Pellets were resuspended in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, protease inhibitor cocktail), sonicated on ice for 15 seconds, and centrifuged at 10,000 g for 10 minutes at 15^{o}C. 30 µg of proteins were separated on 10% SDS-polyacrylamide gel in Mini-Protein II system (Bio-Rad). Following electrophoresis the protein was transferred to a PVDF membrane (Millipore, Bedford, MA, USA) and subsequently hybridized with various antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station.

4.2.9 Statistical analysis

All numeric data are presented as means ± standard deviations (SD) from at least 3 independent experiments and analyzed using one-way ANOVA with student-Newman-Keul as post-hoc comparison. A P value < 0.05 is considered as statistically significance.

4.3 Results

4.3.1 PN sensitizes UVB-induced apoptosis via PKC-dependent pathways

The findings from chapter 3 demonstrated that PN is capable of sensitizing JB6 cells to UVB-induced apoptosis via MAPK-AP-1 signaling pathway. It is known that UVB activates PKC and PKC regulates the MAPK-AP-1 signaling pathway (Bode and Dong, 2003). The involvement of PKC in PN-sensitized UVB-induced apoptosis was first tested using 2 PKC inhibitors. As shown in Fig. 4.1, pre-treatment with GF109203X, a pan-PKC inhibitor also sensitizes cells to UVB-induced apoptosis, although to a lesser extent than PN. In contrast, pre-treatment with a specific PKCδ inhibitor rotterlin is capable of completely protecting cells from apoptosis induced by UVB alone or PN plus UVB (Fig. 4.2). Data from this study thus suggested that PKCδ is critical in both UVB and PN-UVB induced apoptosis, consistent with some of the earlier reports that PKCδ possesses mainly pro-apoptotic function in response to various extracellular stimuli (Chen *et al.*, 1999; Brodie and Blumberg, 2003).

4.3.2 PN selectively regulates different isoforms of PKC in UVB-induced activations

UVB is known to activate certain subtypes of PKC such as PKCδ and PKCζ (Chen *et al.*, 1999; Berra *et al.*, 1997). One of the critical events of PKC activation is the

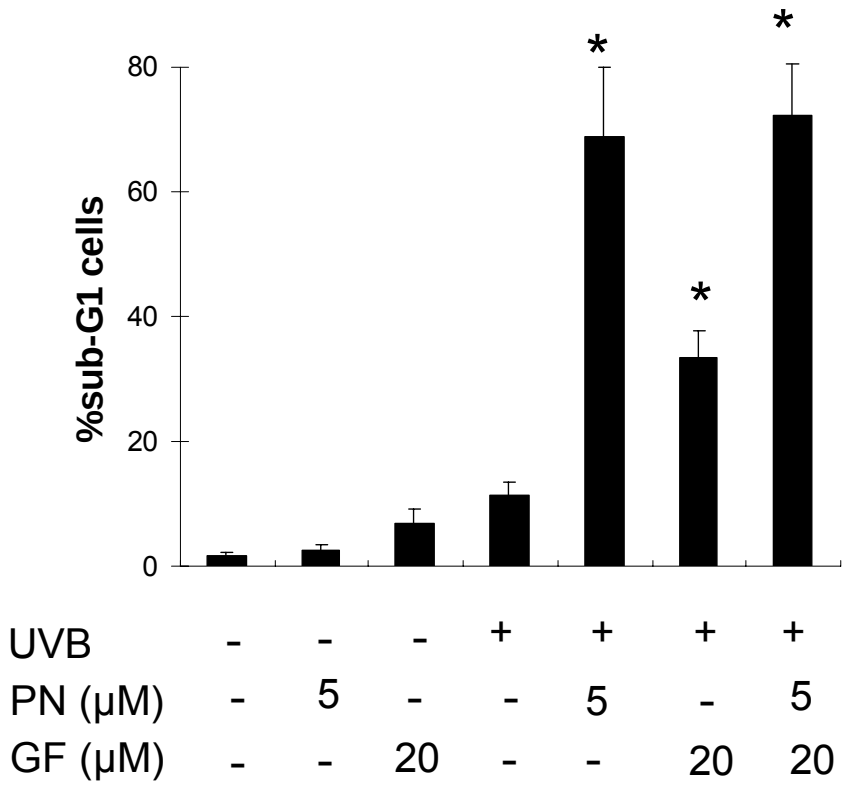


Figure 4.1 Involvement of PKC in cell death induced by PN+UVB. JB6 cells were pre-treated with 5 μM PN for 2 hours or 20 μM GF109203X (a pan-specific PKC inhibitor) for 1 hour and then subjected to 50 mJ/cm^2 of UVB. In some groups, cells were first pre-treated with GF109203X for 1 hour, followed by PN and UVB. Apoptosis was quantified with DNA content/sub-G1 analysis 24 hours after UVB irradiation. Data were presented in means ± SD from 3 independent experiments. An asterisk indicates statistical significance comparing to the untreated control group ($P < 0.05$).

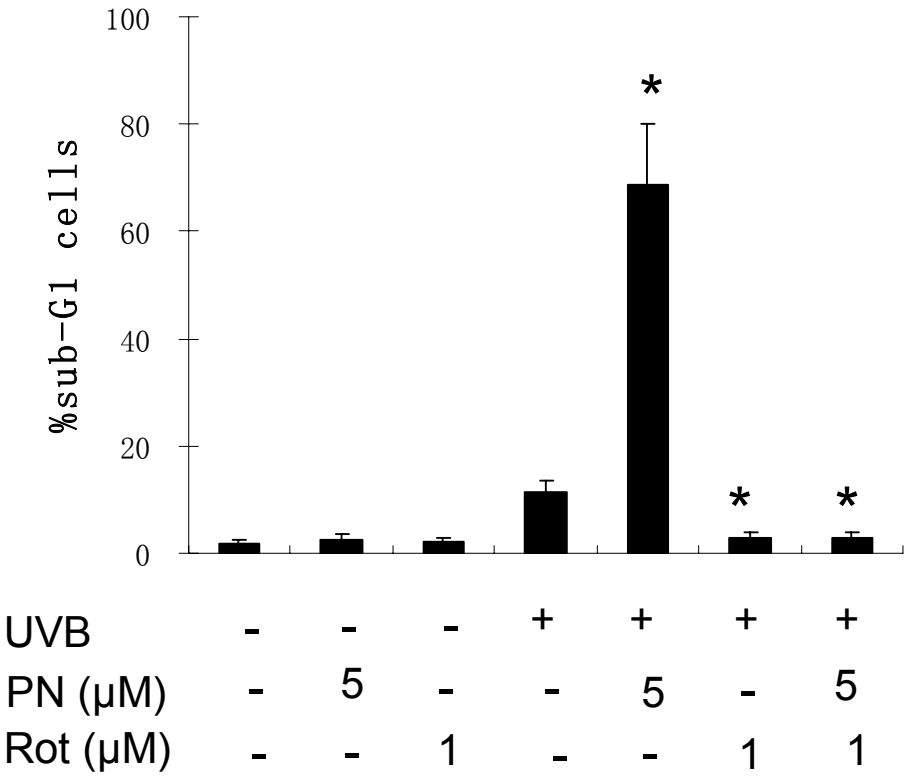


Figure 4.2 Involvement of PKCδ in cell death induced by PN+UVB. JB6 cells were pre-treated with 5 μM PN for 2 hours or 1 μM rotterlin (a specific PKCδ inhibitor) for 1 hour and then subjected to 50 mJ/cm^2 of UVB. In some groups, cells were first pre-treated with rotterlin for 1 hour, followed by PN and UVB. Apoptosis was quantified with DNA content/sub-G1 analysis 24 hours after UVB irradiation. Data were presented in means ± SD from 3 independent experiments. An asterisk indicates statistical significance comparing to the untreated control group ($P < 0.05$).

translocation from the cytosol to membrane (Sando, 2003). In this study, the UVB-induced PKC activation was first measured by determining PKC membrane translocation. Figure 4.3 provides convincing evidence that UVB induced membrane translocations of PKCδ and ζ 15 min post-irradiation in JB6 cells. Similar changes were also observed in other PKC isoforms such as PKCη and PKCλ (data not shown). When cells were pre-treated with 5 μM of PN, the UVB-induced translocation of PKCζ was significantly inhibited (Fig. 4.3b) while the translocation of PKCδ was further enhanced (Fig. 4.3a). A consistent pattern of changes was also detected when the activation of PKC was measured using the *in vitro* PKC kinase assay as well as PKC kinase activity assay: PN inhibited the UVB-induced activation of PKCζ (Fig. 4.4b and Figure 4.5) while further enhanced that of PKCδ (Fig. 4.4a and Figure 4.6).

In order to further understand the differential roles of PKC isoforms on the sensitization effect of PN on UVB-induced apoptosis, cells were transiently transfected with wild-type or DN forms of PKCδ or ζ plasmids, together with pDsRed as the transfection marker, followed by PN-UVB treatment. When the morphological changes of apoptotic cell death were examined under an inverted fluorescence microscope, it was found that the DN-PKCδ transfected cells became rather resistant to PN-UVB-induced apoptosis while the wild-type PKCδ transfected cells underwent massive apoptosis upon PN-UVB treatment (Fig. 4.7). On the contrary, over-expression of wild-type PKCζ offered significant protection against PN and UVB-induced apoptosis (Fig. 4.7). Such findings are basically consistent with the effect of PKC inhibitors as shown earlier (Fig. 4. 1 and 4.2).

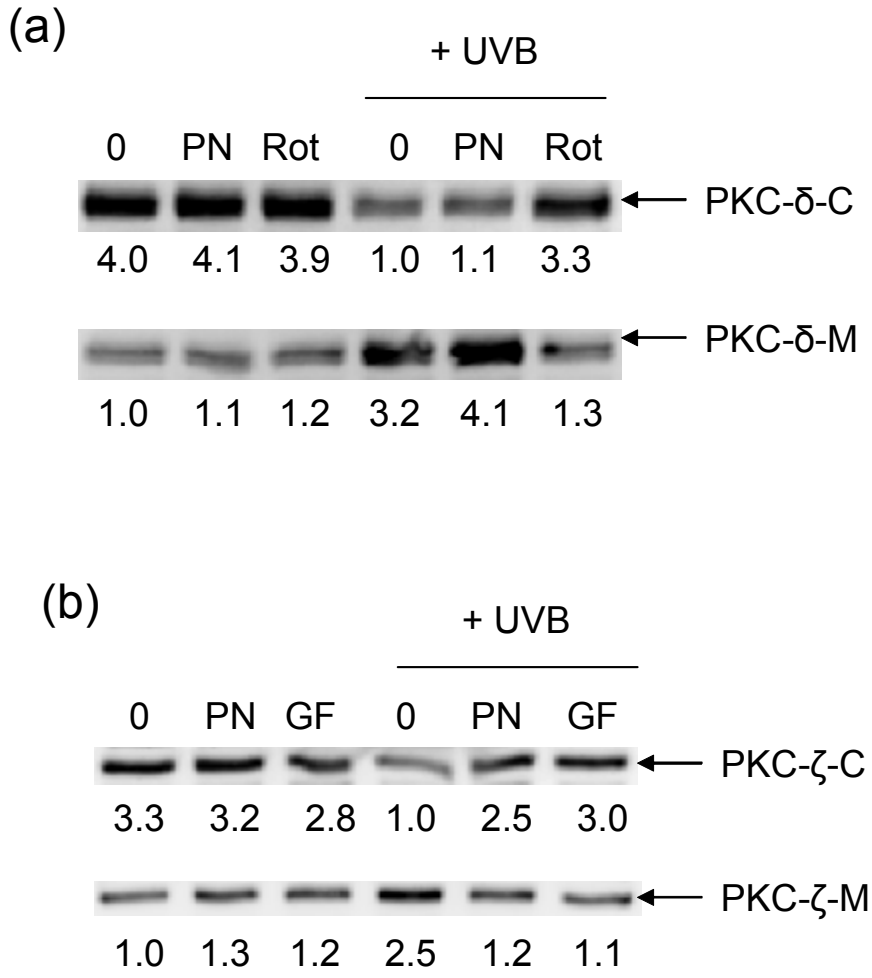


Figure 4.3 PKC activation in cells treated with PN and UVB measured by PKC membrane translocation. (a) PN enhanced UVB-induced PKC δ membrane translocation; (b) PN inhibited UVB-induced PKC ζ membrane translocation. Cells were pre-treated with 5 μM PN for 2 hours, 20 μM GF109203X or 1 μM rotterlin for 1 hour and then subjected to 50 mJ/cm^2 of UVB. Cells were harvested 15 min after UVB irradiation. Thirty μg of cytosolic or membrane proteins were separated on 8% SDS-polyacrylamide gels and blotted with respective anti-PKC antibodies. Data were quantified using Kodak Image Station 440.

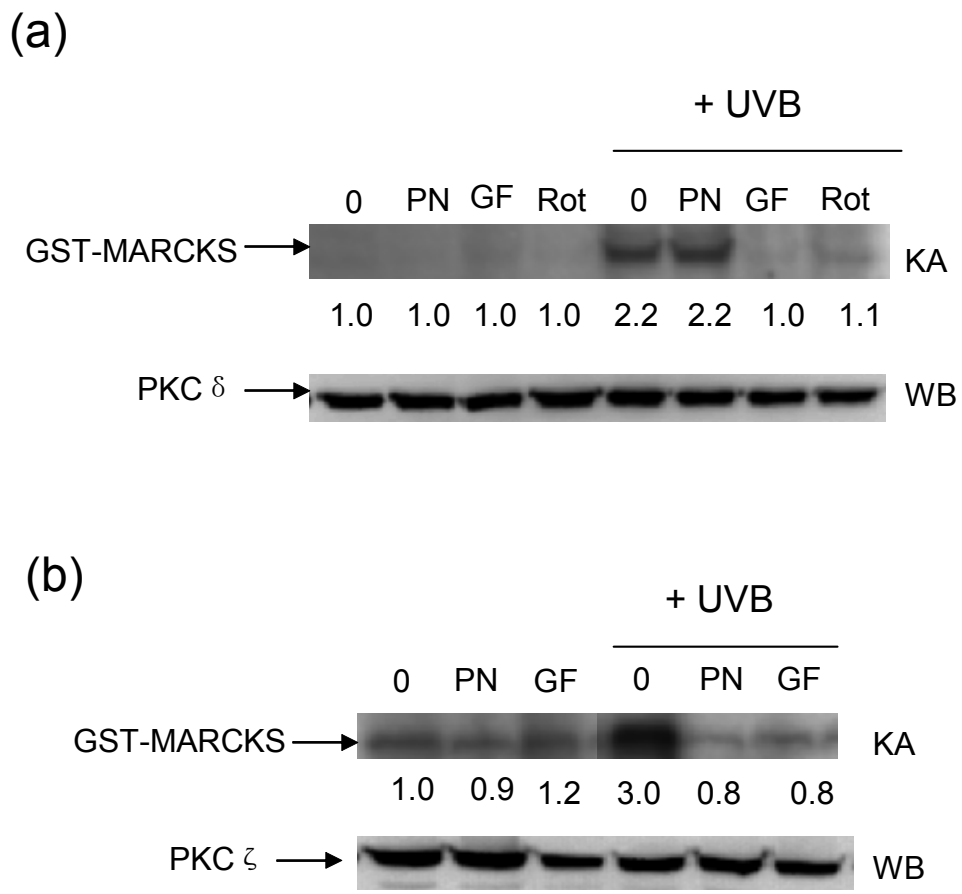


Figure 4.4 PKC activation in cells treated with PN and UVB measured by PKC kinase assay. (a) PN enhanced UVB-induced PKC δ kinase activity; (b) PN inhibited UVB-induced PKC ζ kinase activity. Cells were pre-treated with 5 μM PN for 2 hours, 20 μM GF109203X or 1 μM rotterlin for 1 hour and then subjected to 50 mJ/cm^2 of UVB. Cells were harvested 30 min after UVB irradiation. Cell lysate was immunoprecipitated with anti-PKCs antibodies and then subjected to PKC kinase assay as described in Materials and Methods. Data were quantified using Kodak Image Station 440.

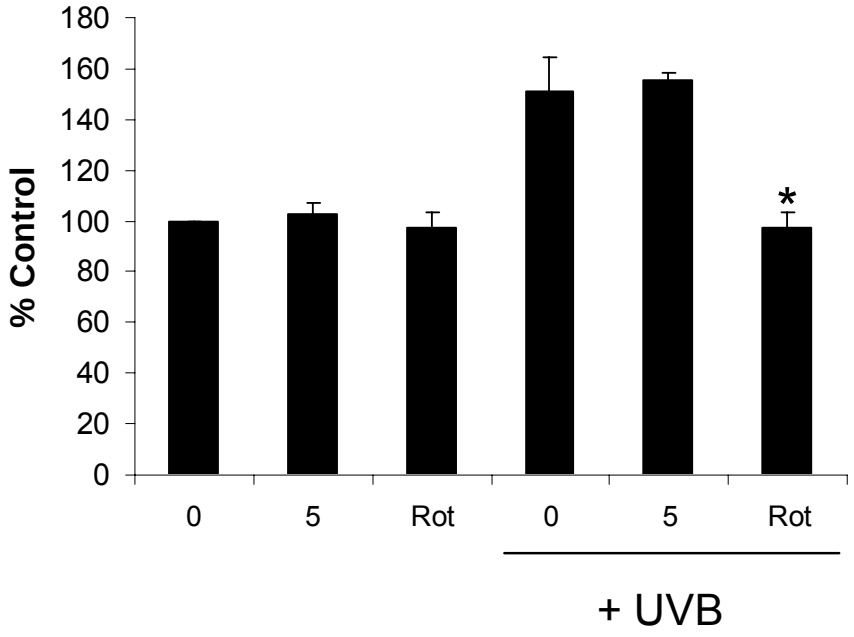


Figure 4.5 PN sustained UVB-induced PKC δ kinase activity measured by PKC activity assay. Cells were pre-treated with 5 μM of PN for 2 hours or 1 μM of rotterlin for 1 hour before subjected to 50 mJ/cm^2 of UVB. Cells were harvested 30 min after UVB irradiation. Cell lysate was immunoprecipitated with anti-PKCs antibodies and then subjected to PKC activity assay as described in Materials and Methods. An asterisk indicates statistical significance comparing to the UVB-treated only group ($P < 0.05$).

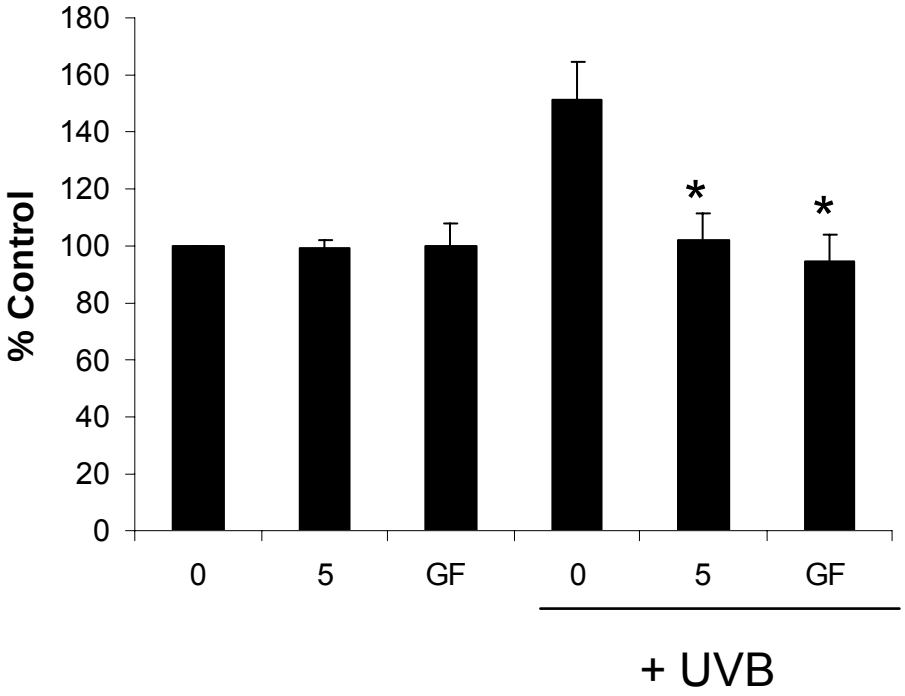


Figure 4.6 PN inhibited UVB-induced PKCζ kinase activity measured by PKC activity assay. Cells were pre-treated with 5 μM of PN for 2 hours or 20 μM of GF109203X for 1 hour before subjected to 50 mJ/cm^2 of UVB. Cells were harvested 30 min after UVB irradiation. Cell lysate was immunoprecipitated with anti-PKCs antibodies and then subjected to PKC activity assay as described in Materials and Methods. An asterisk indicates statistical significance comparing to the UVB-treated only group ($P < 0.05$).

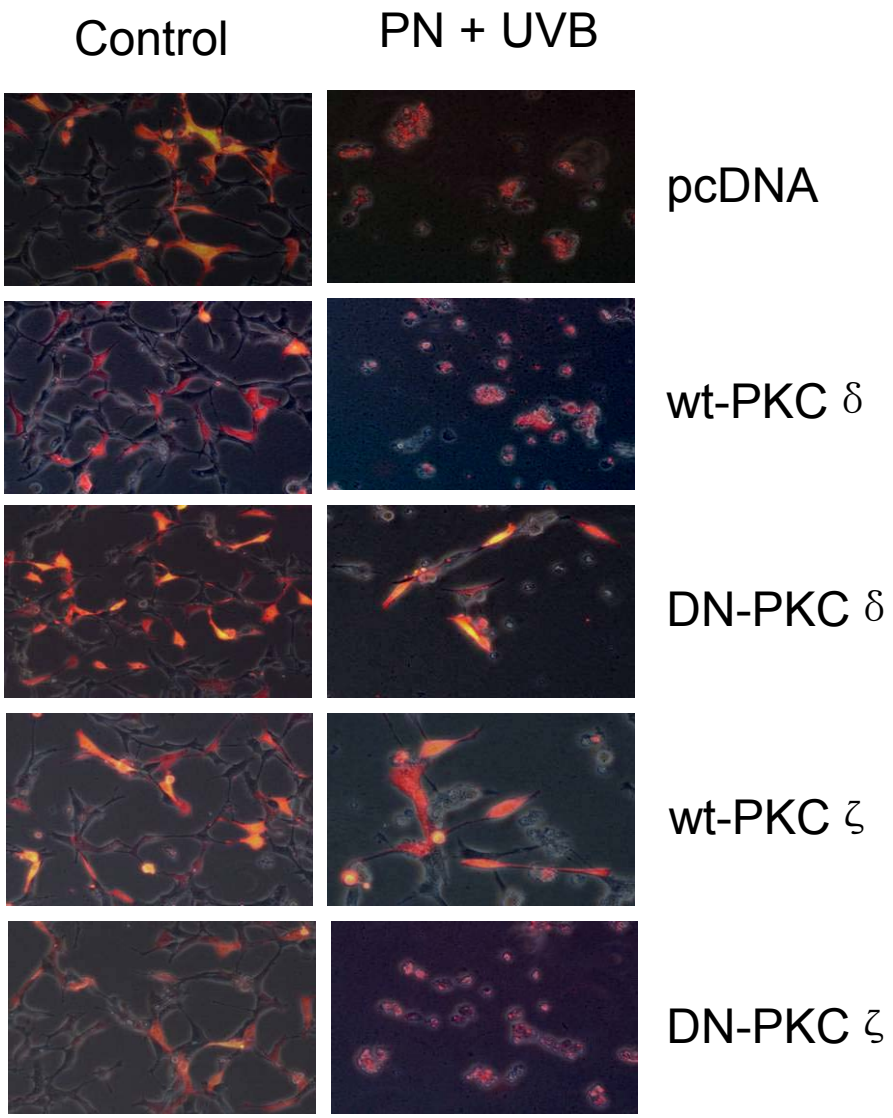


Figure 4.7 Impact of PKC over-expressions on apoptosis induced by PN+UVB. JB6 cells were transiently transfected with pcDNA, wild-type or DN forms of PKCδ or ζ and a transfection marker pDsRed as described in Materials and Methods. Twenty-four hours after transfection, cells were pre-treated with 5 μM of PN for 2 hours and then subjected to 50 mJ/cm^2 of UVB. The morphological changes of apoptotic cell death were examined under an inverted fluorescence microscope 24 hours after UVB irradiation. Cells with successful transfection were marked in red.

In order to obtain more quantitative data, another approach was used by analyzing DNA content/sub-G1 cells among those transfected cells (Fig. 4.8). Being consistent with the morphological changes, cells transfected with DN-PKCδ or wild-type PKCζ were resistant to PN and UVB-induced apoptotic cell death (Fig. 4.9 and 4.10). In contrast, over-expression of wild-type PKCδ or DN-PKCζ significantly enhanced cell death induced by PN and UVB treatment (Fig. 4.9 and 4.10).

4.3.3 PKCζ acts upstream of p38 MAPK but not JNK

Previous findings from chapter 3 illustrated that the MAPK-AP-1 pathway is one of the molecular targets of PN. Certain PKC isoforms have been shown to regulate the MAPK signaling cascade. For example, PKCδ has been reported to affect the UVB- induced phosphorylations of Erk 1/2 and JNK (Chen *et al.*, 1999) while PKCζ primarily targets Erk 1/2 (Huang *et al.*, 2000). Since PKCs are known to response differently depending on cell type and stimuli, the effect of PKCδ and ζ on MAPK activation was first verified in our experimental system. The results confirmed that PN pre-treatment is capable of blocking UVB-induced p38 activation, similar to the effect of a specific p38 inhibitor SB203580 (Fig. 4.11a). Interestingly, pre-treatment with a pan-specific PKC inhibitor, GF109203X, inhibited the UVB-induced p38 activation to certain extent, while the PKCδ specific inhibitor, rotterlin, has no effect on p38 activation (Fig. 4.11a). Next the relationship between PKC and p38 activation was examined using genetic approaches. When cells were transfected with wild-type or DN-PKCδ, neither UVB-induced p38 activation nor the inhibitory effect of PN was affected as compared to the pcDNA-transfected control (Fig. 4.11b). Intriguingly, over-expression of wild-type PKCζ

abolished the inhibitory effect of PN on UVB-induced p38 phosphorylation, while the over-expression of DN-PKCδ even completely blocked UVB-induced p38 activation (Fig. 4.11c). Therefore, these data clearly suggest that it is PKCζ, but not PKCδ, that is responsible for UVB-induced p38 activation.

Since PN is able to inhibit the phosphorylation of JNK induced by UVB as shown previously (Chapter 3), the functional role of PKC in UVB-induced JNK activation was also examined. As shown in Fig. 4.12a, the two PKC inhibitors had no effect on UVB-induced JNK activation. Furthermore, over-expressions of the two DN forms of PKCδ or PKCζ had no effect on either UVB-induced JNK activation or the inhibitory effect of PN (Fig. 4.12b). In contrary to the previously findings (Chen *et al.*, 1999; Huang *et al.*, 2000), the total levels of MAPKs are not affected by the over-expressions of either wild-type or DN forms of PKCδ and ζ. It is thus believed that UVB-induced JNK activation is independent of PKC activation.

In order to further confirm the functional linkage between PKCζ and p38 activation in protection of apoptosis induced by PN and UVB, JB6 cells were transiently transfected with wild-type PKCζ together with DN-p38α and DN-p38β_2 plasmids. As shown in Fig. 4.13a, over-expression of DN-p38α and DN-p38β_2 completely inhibited UVB-induced p38 activation. More importantly, while the over-expression of wild-type PKCζ offered significant protection against apoptosis induced by PN+UVB, the co-transfection of the DN forms of p38 protein abolished the protective effect of wild-type PKCζ and greatly sensitized cells to PN-UVB-induced apoptosis (Fig. 4.13b). Such observations thus provide strong evidence that anti-apoptotic function of PKCζ is achieved via p38 activation and PN is likely to act through PKCζ to suppress p38 activation and then enhance apoptosis in UVB-treated JB6 cells.

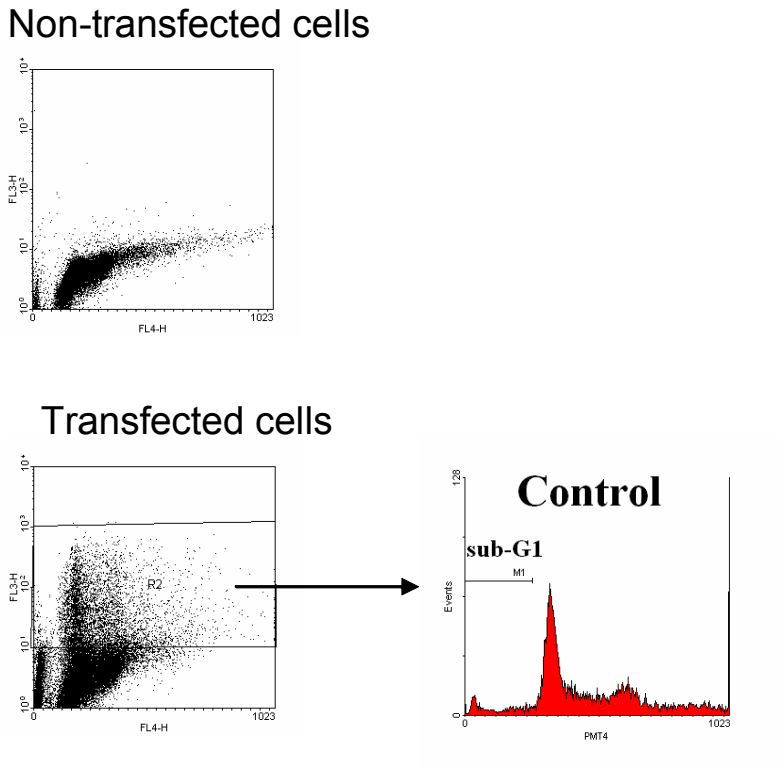


Figure 4.8 Determination of apoptosis in pDsRed transfected cells. Apoptotic cell death was determined with DNA content/sub-G1 analysis coupled with flow cytometry after DAPI staining. JB6 cells were first transiently transfected with various PKC plasmids and pDsRed as a transfection marker, followed by treated with PN and UVB. In total of 20,000 cells from each group analyzed using flow cytometry, only those transfected cells with expression of the red fluorescence protein were then selected for further analysis for percentage of sub-G1 cells.

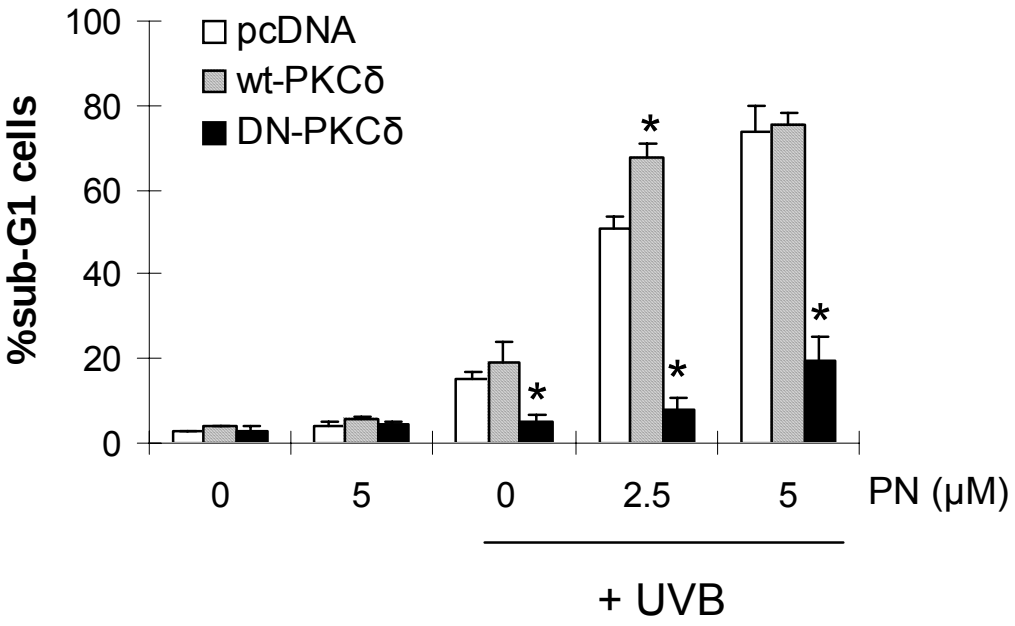


Figure 4.9 Effect of wt- and DN- PKCδ expression on PN-UVB-induced apoptosis in JB6 cells. JB6 cells were first transfected for 24 hours, followed by treatment with PN and UVB. Cells were collected for DNA content analysis 24 hours after UVB irradiation. Data were presented in means ± SD from 4 independent experiments. An asterisk indicates statistical significance comparing to the group transfected with pcDNA only ($P < 0.05$).

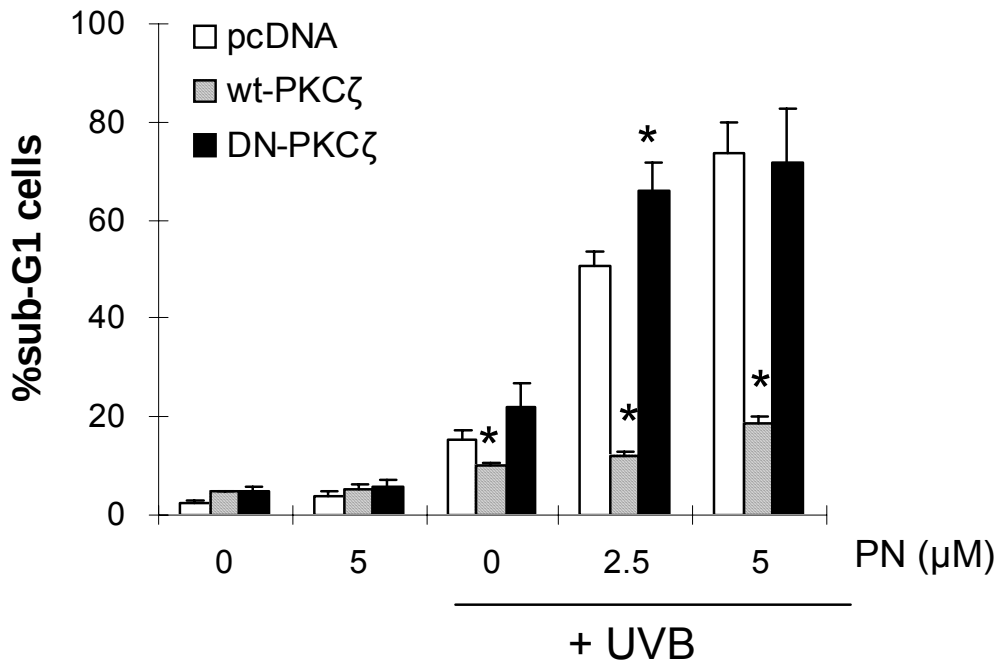


Figure 4.10 Effect of wt- and DN- PKC ζ expression on PN-UVB-induced apoptosis in JB6 cells. JB6 cells were first transfected for 24 hours, followed by treatment with PN and UVB. Cells were collected for DNA content analysis 24 hours after UVB irradiation. Data were presented in means ± SD from 4 independent experiments. An asterisk indicates statistical significance comparing to the group transfected with pcDNA only ($P < 0.05$).

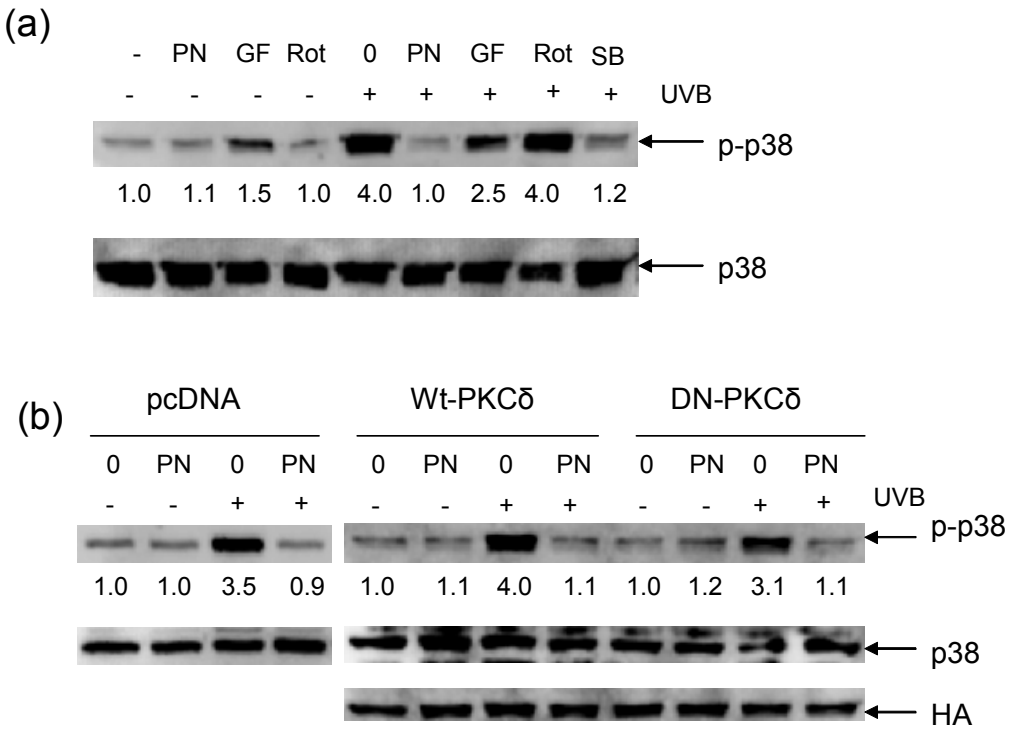

(a)
- PN GF Rot 0 PN GF Rot SB
- - - - + + + + + UVB
p-p38
1.0 1.1 1.5 1.0 4.0 1.0 2.5 4.0 1.2
p38
(b)
pcDNA
Wt-PKCδ
DN-PKCδ
0 PN 0 PN 0 PN 0 PN 0 PN 0 PN
- - + + - - + + - - + + UVB
p-p38
1.0 1.0 3.5 0.9 1.0 1.1 4.0 1.1 1.0 1.2 3.1 1.1
p38
HA
(c)
Wt-PKCζ
DN-PKCζ
0 PN 0 PN 0 PN 0 PN
- - + + - - + + UVB
p-p38
1.0 1.0 3.0 3.0 1.0 1.1 1.2 1.1
p38
HA

Figure 4.11 Effect of PKC on p38 activation in UVB-treated cells. (a) PN and pan-specific PKC inhibitor GF109203X, but not the specific PKCδ inhibitor rotterlin, inhibited UVB-induced p38 phosphorylation. Cells were pre-treated with 5 μM PN for 2 hours, 20 μM GF109203X or 1 μM rotterlin for 1 hour and then subjected to 50 mJ/cm^2 of UVB. (b) Over-expressions of wt- or DN forms of PKCδ did not affect p38 activation induced by either UVB alone or PN plus UVB. (c) Over-expression of DN- PKCζ blocked UVB-induced p38 activation and over-expression of wt-PKCζ reversed the inhibitory effect of PN on UVB-induced p38 activation. JB6 cells were first transiently transfected with pcDNA, wt- or DN forms of PKCδ or ζ for 24 hours, followed by treatment with PN and UVB. Cells were harvested 30 min after UVB irradiation. Thirty μg of cellular proteins were separated on 10% SDS-polyacrylamide gels and the subsequent membranes were hybridized with anti- p-p38, p38 and HA antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station 440.

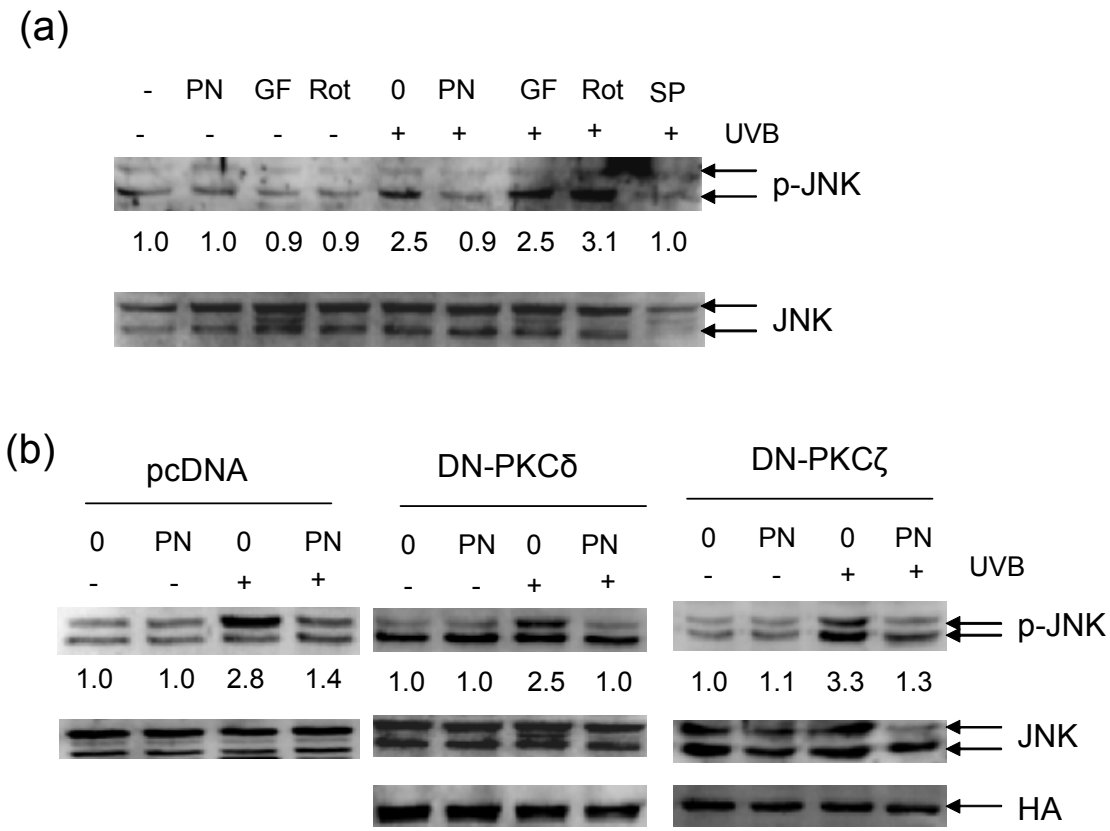


Figure 4.12 No evident effect of PKC on JNK activation in UVB-treated cells. (a) PKC inhibitor GF109203X and rotterlin has no evident effect on UVB-induced JNK activation. JB6 cells were treated as described in Fig. 6a. (b) Over-expressions of DN forms of PKCδ and PKCζ failed to affect JNK activation induced by UVB alone or PN plus UVB. JB6 cells were transiently transfected with pcDNA, DN forms of PKCδ or ζ for 24 hours, followed by treatment with PN and UVB. Cells were harvested 30 min after UVB irradiation. Thirty μg of cellular proteins were separated on 10% SDS-polyacrylamide gels and the subsequent membranes were hybridized with anti- p-p38, p38 and HA antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station 440.

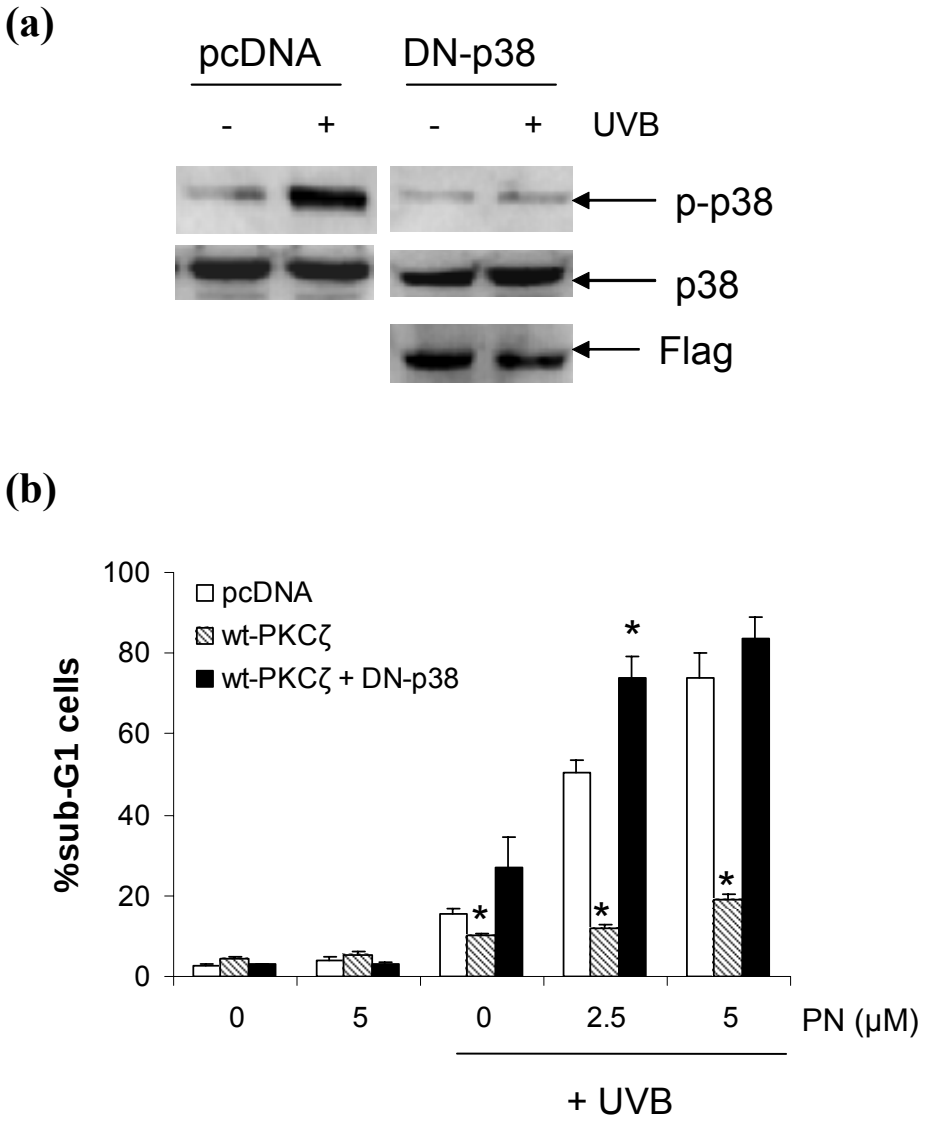


Figure 4.13 functional relationships between PKCζ and p38 in UVB-induced apoptosis. (a) Over-expressions of DN-p38α and DN-p38β_2 completely blocked the UVB-induced p38 phosphorylation. JB6 cells were transiently transfected with DN-p38α and DN-p38β_2 for 24 hours, followed by UVB exposure (50 mJ/cm^2). Cells were harvested 30 min after UVB irradiation. Thirty μg of cellular proteins were separated on 10% SDS-polyacrylamide gels and the subsequent membranes were hybridized with anti-p-p38, p38 and Flag antibodies. (b) Over-expressions of DN-p38α and DN-p38β_2 completely abolished the protective effect of wt-PKCζ on apoptosis induced by PN and UVB. Cells were transfected with wt-PKCδ, with or without DN-p38α andDN-p38β_2 for 24 hours, followed by treatment with PN and UVB. Cells were collected for DNA content analysis for sub-G1 cells 24 hours after UVB irradiation. Data were presented in means ± SD from 3 independent experiments. An asterisk indicates statistical significance comparing to the group transfected with pcDNA only ($P < 0.05$).

4.4 Discussion

Even though the underlying mechanism(s) of the carcinogenic ability of UVB have not been fully understood, a number of molecular targets have been identified to be involved in UVB carcinogenesis, which include AP-1, NF-κB, NF-AT, and STATs (Angel *et al.*, 2001; Siebenlist *et al.*, 1995; Huang *et al.*, 2000; Zhang *et al.*, 2001). Results from chapter 2 and 3 have shown that PN possesses strong chemopreventive property against UVB-induced skin cancer via its potent inhibitory effect on the MAPK-AP-1 signaling pathway. In this study, PN has been demonstrated to selectively regulate the activities of PKC: promotion of the pro-apoptotic PKCδ and suppression of the pro-survival PKCζ. Moreover, strong evidence indicates that PN acts on PKCζ upstream of p38 to sensitize UVB-induced apoptotic cell death. As demonstrated in this part of the study, the results reveal a new mechanism involved in the anti-cancer function of PN.

PKC can be classified into 11 isoforms based on the co-factors required for activation. UVB is known to activate certain PKC isoforms such as PKCδ, ε, ζ, λ/ι, and η, leading to apoptosis or cell survival (Chen *et al.*, 1999; Berra *et al.*, 1997; Huang *et al.*, 2000; Matsumura *et al.*, 2003). It has been reported that UVB induces phospholipase A_2 activation and arachidonic acid release and activates PKC (Blackshear *et al.*, 1988). Furthermore, UVB irradiation of cultured cells is also known to elevate levels of DAG (Punnonen and Yuspa, 1992) as well as to generate reactive oxygen species that may activate PKC (Sun *et al.*, 2000). Among all the UVB-activated PCK isoforms, PKCδ seems to be the main subtype involved in apoptotic signaling induced by various stimuli including UVB (Brodie and Blumberg, 2003). It is generally believed that activated PKCδ decreases mitochondrial membrane potential, resulting in cytochrome c release, caspases activations and apoptosis (Brodie and

Blumberg, 2003). On the other hand, PKCζ has been shown to promote cell survival by either stimulating the nucleotide excision repair activity (Louat *et al.*, 2004) or phosphorylating Rel A and subsequently activate the NF-κB survival pathway (Duran *et al.*, 2003).

One important observation of this study is the differential effect by PN on different PKC isoforms in cells treated with UVB: PN selectively enhances the pro-apoptotic PKCδ and suppresses the anti-apoptotic PKCζ (Fig. 4.3- 4.6). Indeed, selective effects on different PKC isoforms have been reported previously. Sodium butyrate has been shown to upregulate PKC-ε while downregulate PKC-β during erythroid differentiation (Rivero and Adunyah, 1998). UCN-01, a staurosporine analogue, has been illustrated to have selective effect on different PKCs isoforms using *in vitro* kinase assay (Seynaeve *et al.*, 1994). In addition, a diazene carbonyl derivative diamide, which oxidizes thiols to disulfides through addition/displacement reactions at the diazene bond, stimulates the pro-apoptotic PKCδ while inactivates the oncogenic PKCε (Chu *et al.*, 2003).

The differential regulation on PKCδ and ζ is also appreciated by the difference in their molecular structures. Although both PKCs contain an N-terminal regulatory domain and a C-terminal catalytic kinase domain, PKCδ contains two cysteine-rich C1 subdomains (Jaken, 1996) while PKCζ has only one zinc finger binding region (Ono *et al.*, 1989). DAG is known to bind to one of the cysteine-rich C1 subdomains and activate PKCδ. In contrast, PKCζ is regulated by other lipid cofactors such as phosphatidylinositol (PI) 3,4,5-P_3 and ceramide (Muller *et al.*, 1995). At present, the exact mechanisms responsible for the differential regulation of PN on UVB-induced PKC activation are not known. One possible explanation is the likely involvement of the caspase cascade in PKC activation. For

instance, PKCδ is cleaved by caspase-3 during apoptosis to a more catalytically active fragment (Emoto *et al.*, 1995). In contrast, caspase-3 dependent cleavage of PKCζ generates a fragment that corresponds to its catalytic domain and is enzymatically inactive (Frutos *et al.*, 1999). Since PN is a potent activator of caspase-3 (Wen *et al.*, 2002; Zhang *et al.*, 2004a), it is possible that both PKCδ and ζ were cleaved by caspase-3 in response to PN-UVB treatment. As a result, the catalytically active PKCδ and the inactive fragment of PKCζ are generated, leading to more profound cell death induced by PN-UVB.

Currently, it is still controversial with regards to the exact role of JNK and p38 in UV-induced apoptosis, with both pro- and anti-apoptotic activities being reported (Ivanov and Ronai, 2000; Tournier *et al.*, 2000; Wisdom *et al.*, 1999). Previous findings in chapter 3 indicated that PN sensitizes UVB-induced apoptosis by inhibiting both JNK and p38, leading to blockade in the pro-survival AP-1 pathway. The link between MAPK and PKC has been reported previously. Chen and co-workers (1999) showed that PKCδ and PKCε mediate UVB-induced signal transduction and apoptosis through the activation of ERK and JNK. Furthermore, inhibition of PKCλ/ι with a dominant negative mutant suppressed UVB-induced ERK and the subsequent AP-1 activation (Huang *et al.*, 1996). On the other hand, antisense oligonucleotide of PKCζ has been shown to inhibit the UVB-induced AP-1 activation (Huang *et al.*, 2000). In this study, convincing evidence suggests that PKCζ acts upstream of p38, but not JNK, to protect cell death induced by PN-UVB, based on experimental data using both pharmacological and genetic approaches. For instance, over-expression of wide-type PKCζ reverses the inhibitory effect of PN on UVB-induced p38 phosphorylation (Fig. 4.11c), and transfections with wild-type PKCζ together with DN-p38α and DN-p38β_2 plasmids completely abolished the protective effect of wild-type PKCζ

and greatly sensitized cells to PN-UVB-induced apoptosis (Fig. 4.13b). Therefore, it is clear that the sensitization activity of PN on UVB-induced apoptosis is likely achieved through its inhibitory effect on PKCζ and p38 signaling pathway.

In summary, the results show for the first time PN sensitizes JB6 cells to UVB-induced apoptosis through selective regulation on the pro-apoptotic PKCδ and the pro-survival PKCζ functions. Furthermore, the data also suggest that the UVB-induced p38 MAPK activation is regulated via a PKCζ dependent mechanism. These findings may shed new light in understanding the anti-cancer activity of PN.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

PN is the major sesquiterpene lactone in the traditional herbal medicine feverfew (*Tanacetum parthenium*), a plant that has been used for the treatment of fever, migraine, and arthritis for centuries (Knight, 1995). It is believed that PN mediates its effect through the highly electrophilic α-methylene-γ-lactone ring and an epoxide residue that are capable of interacting rapidly with nucleophillic sites of biological molecules (Macias *et al.*, 1997; Zhang *et al.*, 2005). The anti-microbial and anti-inflammatory properties of PN are well studied (Brown *et al.*, 1997; Jain *et al.*, 1999). However, the anti-cancer property of PN has not been fully explored. Some preliminary studies have shown that PN is a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (Woynarowski and Konopa, 1981; Hall *et al.*, 1988; Ross *et al.*, 1999). In addition, PN has been shown to induce apoptosis in certain cancer cells via caspase activation and mitochondria dysfunction (Wen *et al.*, 2002), disruption in intracellular thiols and calcium equilibrium (Zhang *et al.*, 2004a), as well as activation of pro-apoptotic Bcl-2 family proteins (Zhang *et al.*, 2004c). More recently, PN has been shown to reduce metastasis and improve survival in xenograft model of breast cancer when applied in combination with docetaxel (Sweeney *et al.*, 2005), as well as preventing UVB-induced skin photoaging (Tanaka *et al.*, 2005).

The objective of this study was to systematically investigate the anti-cancer potential of PN using both *in vivo* animal model and *in vitro* cell culture. The following studies were conducted: (i) chemopreventive and chemotherapeutic potentials of PN using UVB-induced skin cancer model with SKH-1 hairless mice (Chapter 2); (ii) *in vitro* investigation to elucidate the sensitization effect, and the underlining mechanisms of PN in UVB-induced apoptosis, in murine epidermal cell line JB6. (Chapter 3 and 4).

5.1 Chemopreventive property of PN

Prior to this study, the anti-cancer property of PN has never been studied using *in vivo* animal models. The results from this sudy provide clear evidence that PN possesses strong chemopreventive property against UVB-induced skin cancer in female SKH-1 hairless mice: 1) PN treatment delayed the onset of tumor incidence from week 13th to week 18th (Figure 2.5); 2) PN reduced tumor multiplicity by 30% (Figure 2.6); 3) smaller papilloma sizes were found in mice treated with PN (Table 2.1); and 4) PN treatment reduced the hyperplastic response of the epidermis induced by UVB irradiation (Figure 2.8). The chemopreventive capability of PN is found to be as effective as the pharmaceutical COX-2 inhibitor celecoxib in preventing UVB-induced photocarcinogenesis (Fischer *et al.*, 1999; Pentland *et al.*, 1999).

5.2 Chemotherapeutic property of PN

On the other hand, the chemotherapeutic activity of parthenolide was also evaluated in this model in which parthenolide was applied after the onset of photocarcinogenesis. In contrast to its potent chemopreventive activity, parthenolide is not an effective chemotherapeutic agent against UVB-induced skin cancer since no significant difference in papilloma multiplicity (Figure 2.13) and the size distribution of papilloma among different treatment groups (Table 2.2) was detected.

The exact reason(s) behind PN's failure as a single chemotherapeutic agent is not known. One of the possibilities could be due to insufficient dosing of PN and a higher dosage of PN may be needed in order to achieve any therapeutic effect. To date, the biosafty and bioavailability of PN have not been studied in SKH-1 hairless mice model.

Unfortunately, such studies were unable to be conducted due to budgetary constrain. Moreover, the route of administration of PN could be a factor that affects our observation. It has been shown that celecoxib applied topically results in suppression on UVB-induced PGE_2 production (Wilgus *et al.*, 2003), while oral route exerts no effect (Fisher *et al.*, 1999).

5.3 Role of COX-2 in the anti-cancer activity of PN

COX-2 is the inducible subtype of the enzyme cyclooxygenase (COX) which converts free arachidonic acid to prostaglandins (Marnett, 2000). The fact that UVB upregulates COX-2 and prostaglandins production suggests that COX-2 may contribute to photocarcinogenesis (Buckman *et al.*, 1998; Grewe *et al.*, 1993). Preliminary findings from our laboratory revealed a positive correlation between cytotoxic response to PN and COX-2 expression (unpublished data), which leads us to hypothesize that PN may exert its effect in a COX-2-dependent manner. Previous studies have also demonstrated that parthenolide is able to suppress COX-mediated pathway either through directly inactivating COX-2 enzyme activity (Pugh and Sambo, 1988; Hwang *et al.*, 1996), or by indirectly inhibiting COX-2 transcription through NF-κB (Hehner *et al.*, 1998). However, the results from chapter 2 showed that neither treatment of parthenolide nor the specific COX-2 inhibitor celecoxib alter COX-2 expression or prostaglandins production induced by UVB (Figure 2.9 and 2.10), suggesting that COX-2 is unlikely to be the direct molecular target for parthenolide and celecoxib. This finding is in concordance with an earlier report in which long term oral treatment of celecoxib has no effect on UVB-induced expression of COX-2 (Fischer *et al.*, 1999). Furthermore, *in vitro* studies also

demonstrated that celecoxib induces apoptosis and cell cycle arrest in a COX-2 independent manner (Elder *et al.*, 1997; Grosch *et al.*, 2001; Waskewich *et al.*, 2002). In contrary, a recent study reported that topical treatment of celecoxib is as effective as oral route in inhibiting UVB-induced skin cancer while also suppressed PGE_2 production induced by UVB (Wilgus *et al.*, 2003). It is thus believed parthenolide and celecoxib can act via either COX-2 dependent or independent pathways, although the exact molecular mechanisms responsible for such effect remain to be further determined.

In summary, the findings from chapter 2 have demonstrated the chemopreventive property of parthenolide against UVB-induced skin cancer in SKH-1 hairless mice. The results also suggested that such chemopreventive activity is probably mediated in a COX-2-independent manner.

5.4 Anti-cancer potential of PN- sensitization to UVB-induced apoptosis

The second part of this thesis is to study the underlining mechanisms responsible for the chemopreventive property of PN using murine epidermal cell line JB6 with focuses on the following two main cell signaling patheways i) AP-1 and the related MAPKs signaling cascade, and ii) protein kinase C.

Apoptosis is an essential cell death process in maintaining development and tissue homeostasis. It is believed that any defects in this important process can result in severe pathological diseases and one of the most important outcomes is cancer (Fisher and Schulze-Osthoff, 2005). Dysregulation of apoptosis has also been shown to be crucial in the pathogenesis of cancer in many ways such as: (1) allowing neoplastic cells to survive beyond their normal lifespan; (2) creating a permissive environment for genetic

instability and accumulation of gene mutations; (3) facilitating growth factor- and hormone-independent cell survival; (4) promoting resistance to immune-based destruction; and (5) conferring resistance to cytotoxic anticancer agents and radiation (Reed, 1999).

Indeed, UV exposure could result in direct or indirect DNA damage, and the damage is normally repaired by a nucleotide excision repair (NER) mechanism (Thoma, 1999). Most of the irreparable DNA-damaged cells will be eliminated through apoptosis, as evident in skin with the appearance of sunburn cells (Kulms and Schwarz, 2002). However, not all cells with irreparable DNA damage will undergo apoptosis. DNA lesions that are not repaired or incorrectly repaired may lead to mutations and subsequently carcinogenesis. Therefore, the removal of damaged cells through sensitizing cells to apoptosis denotes an effective mean in preventing carcinogenesis (Hildesheim *et al.*, 2002; Kulms and Schwarz, 2002; D'Errico *et al.*, 2003).

5.5 Involvement of AP-1 and MAPK in PN-induced sensitization to UVB-induced apoptosis

AP-1 plays a key role in pre-neoplastic to neoplastic transformation in cell culture and the blockage of AP-1 activities has been shown to inhibit cell transformation (Dong *et al.*, 1994). Moreover, AP-1 has been shown to regulate apoptosis, with both pro-apoptotic and anti-apoptotic function (Shaulian and Karin, 2002). It appears that the exact outcome of AP-1 manipulations is highly tissue- and developmental stage-specific. In the present study, it has been demonstrated that, in addition to its well known suppressive effect on NF-κB (Figure 3.6), PN pre-treatment inhibits the UVB-induced

DNA binding (Figure 3.7) and transcriptional activity of AP-1 (Figure 3.8). Two of the major components of AP-1 complex, c-Jun and ATF-2, can activate AP-1 via post-translational modification involving changes in the phosphorylation states (Buscher *et al.*, 1988; Devary *et al.*, 1991; van Dam *et al.*, 1995). In response to UV, c-Jun is rapidly phosphorylated at both Ser63 and Ser73 sites along with ATF-2. The data showed that PN subdues UVB-induced AP-1 transactivation via blockage of c-Jun and ATF-2 phosphorylations (Figure 3.9A and B). The outcome of these series of inhibitions seem to lead to significant sensitization to UVB-induced apoptosis (Figure 3.4), suggesting an anti-apoptotic role of AP-1 in UVB-treated cells.

Similar observations regarding the anti-apoptotic function of AP-1 and c-Jun have been reported previously. Wisdom and co-workers (1999) noted that c-Jun protects cells from UV-induced apoptosis and phosphorylation of c-Jun on Ser63 and Ser73 is required for such protection. Moreover, Ivanov and colleagues (2001) demonstrated that c-Jun protects cells against UV-induced cell death via co-operation with STAT3 to suppress transcription of Fas.

The UV-activated signal transduction pathway is primarily mediated by mitogen-activated protein kinases (MAPKs). Once activated, they translocate to the nucleus and phosphorylate target transcription factors. In response to UV, key MAPKs such as JNK and p38 are activated, which in turn phosphorylate c-Jun at Ser-63 and Ser-73 sites and ATF-2, respectively (Devary *et al.*, 1991; van Dam *et al.*, 1995). It is rather controversial with regards to the exact role of JNK in UV-mediated apoptosis. For instance, JNK has been shown to be required for UV-induced apoptotic cell death in mouse embryonic fibroblasts (Tournier *et al.*, 2000), while other studies demonstrated the anti-apoptotic

role of JNK in UV-induced apoptosis (Wisdom *et al.*, 1999; Ivanov *et al.*, 2001). The results from chapter 3 showed that the inhibition of JNK leads to sensitization of JB6 cells to UVB-induced apoptosis, suggesting an anti-apoptotic role of JNK in the present system (Figure 3.11). In general, the exact role of JNK in apoptosis varies depending on a number of factors that include the nature of the stimuli, cell type, the duration of activation and more importantly, the interaction of other signaling pathway such as NF-κB and the PI3K-AKT cascades (Davis, 2000; Lamb *et al.*, 2003; Lin, 2003). The involvement of those cell survival signaling pathways in PN-mediated cell death sensitization remains to be further investigated.

In the present study, the potent inhibitory effect of PN on UVB-induced p38 activation was also noted (Figure 3.10). This p38 inhibition leads to sensitization of cells to UVB-induced apoptosis (Figure 3.11), implying an anti-apoptotic role of p38 in UVB-induced apoptotic cell death. Although it is still controversial regarding the exact role of p38 in apoptosis, p38 has been reported to protect cells from UV-induced apoptosis through down regulation of NF-κB activity and Fas expression (Ivanov and Ronai, 2000). Some other mechanisms have also been proposed to be involved in the anti-apoptotic function of p38. For instance, the p38 MAPK inhibitor SB203580 can trigger a significant, Ras-independent activation of c-Raf in certain cell lines in the concentration range of 8-25 μM (Hall-Jackson *et al.*, 1999; Kalmes *et al.*, 1999). Activated Raf may phosphorylate and inactivate Bad, a pro-apoptotic member of the Bcl-2 family of proteins, or up-regulate the transcription of pro-survival genes to prevent cytochrome *c* release and subsequently apoptosis (Bonni *et al.*, 1999). Furthermore, Raf-1 has been shown to promote cell survival by antagonizing apoptosis signal-regulating kinase 1 (ASK1), an

important mediator of apoptotic signaling (Chen *et al.*, 2001). In the present study, PN has been demonstrated to be a dual inhibitor of both JNK and p38 in UVB-treated cells (Figure 3.10). Incidentally, PN-elicited sensitization of JB6 cells to UVB-induced apoptosis is twice as strong as with the individual JNK and p38 inhibitor (Figure 3.11). These findings suggest that both JNK and p38 contribute to the cell survival mechanisms in UVB-treated cells.

5.6 Involvement of PKC in PN-induced sensitization to UVB-induced apoptosis

Another important molecular target in UVB-mediated signal transduction is protein kinase C (PKC). UVB is known to activate certain PKC isoforms such as PKCδ, ε, ζ, λ/ι, and η, leading to apoptosis or cell survival (Chen *et al.*, 1999; Berra *et al.*, 1997; Huang *et al.*, 2000; Matsumura *et al.*, 2003). It has been reported that UVB induces phospholipase A_2 activation and arachidonic acid release and activates PKC (Blackshear *et al.*, 1988). Furthermore, UVB irradiation of cultured cells is also known to elevate levels of DAG (Punnonen and Yuspa, 1992) as well as to generate reactive oxygen species that may activate PKC (Sun *et al.*, 2000). Among all the UVB-activated PKC isoforms, PKCδ seems to be the main subtype involved in apoptotic signaling induced by various stimuli including UVB (Brodie and Blumberg, 2003). It is generally believed that activated PKCδ decreases mitochondrial membrane potential, resulting in cytochrome c release, caspases activations and apoptosis (Brodie and Blumberg, 2003). On the other hand, PKCζ has been shown to promote cell survival by either stimulating the nucleotide excision repair activity (Louat *et al.*, 2004) or phosphorylating Rel A and subsequently activate the NF-κB survival pathway (Duran *et al.*, 2003).

One important observation of this study is the differential effect by PN on different PKC isoforms in cells treated with UVB: PN selectively enhances the pro-apoptotic PKCδ and suppresses the anti-apoptotic PKCζ (Fig. 4.3- 4.6). Indeed, selective effects on different PKC isoforms have been reported previously. Sodium butyrate has been shown to upregulate PKC-ε while downregulate PKC-β during erythroid differentiation (Rivero and Adunyah, 1998). UCN-01, a staurosporine analogue, has been illustrated to have selective effect on different PKCs isoforms using *in vitro* kinase assay (Seynaeve *et al.*, 1994). In addition, a diazene carbonyl derivative diamide, which oxidizes thiols to disulfides through addition/displacement reactions at the diazene bond, stimulates the pro-apoptotic PKCδ while inactivates the oncogenic PKCε (Chu *et al.*, 2003).

The differential regulation on PKCδ and ζ is also appreciated by the difference in their molecular structures. Although both PKCs contain an N-terminal regulatory domain and a C-terminal catalytic kinase domain, PKCδ contains two cysteine-rich C1 subdomains (Jaken, 1996) while PKCζ has only one zinc finger binding region (Ono *et al.*, 1989). DAG is known to bind to one of the cysteine-rich C1 subdomains and activate PKCδ. In contrast, PKCζ is regulated by other lipid cofactors such as phosphatidylinositol (PI) 3,4,5-P_3 and ceramide (Muller *et al.*, 1995). At present, the exact mechanisms responsible for the differential regulation of PN on UVB-induced PKC activation are not known. One possible explanation is the likely involvement of the caspase cascade in PKC activation. For instance, PKCδ is cleaved by caspase-3 during apoptosis to a more catalytically active fragment (Emoto *et al.*, 1995). In contrast, caspase-3 dependent cleavage of PKCζ generates a fragment that corresponds to its catalytic domain and is enzymatically inactive (Frutos *et al.*, 1999). Since PN is a

potent activator of caspase-3 (Wen *et al.*, 2002; Zhang *et al.*, 2004a), it is possible that both PKCδ and ζ were cleaved by caspase-3 in response to PN-UVB treatment. As a result, the catalytically active PKCδ and the inactive fragment of PKCζ are generated, leading to more profound cell death induced by PN-UVB.

Currently, it is still controversial with regards to the exact role of JNK and p38 in UV-induced apoptosis, with both pro- and anti-apoptotic activities being reported (Ivanov and Ronai, 2000; Tournier *et al.*, 2000; Wisdom *et al.*, 1999). The findings from Chpater 3 indicated that PN sensitizes UVB-induced apoptosis by inhibiting both JNK and p38, leading to blockade in the pro-survival AP-1 pathway. The link between MAPK and PKC has been reported previously. Chen and co-workers (1999) showed that PKCδ and PKCε mediate UVB-induced signal transduction and apoptosis through the activation of ERK and JNK. Furthermore, inhibition of PKCλ/ι with a dominant negative mutant suppressed UVB-induced ERK and the subsequent AP-1 activation (Huang *et al.*, 1996). On the other hand, antisense oligonucleotide of PKCζ has been shown to inhibit the UVB-induced AP-1 activation (Huang *et al.*, 2000). In this study, convincing evidence suggest that PKCζ acts upstream of p38, but not JNK, to protect cell death induced by PN-UVB, based on experimental data using both pharmacological and genetic approaches. For instance, over-expression of wide-type PKCζ reverses the inhibitory effect of PN on UVB-induced p38 phosphorylation (Fig. 4.11c), and transfections with wild-type PKCζ together with DN-p38α and DN-p38β_2 plasmids completely abolished the protective effect of wild-type PKCζ and greatly sensitized cells to PN-UVB-induced apoptosis (Fig. 4.13b). Therefore, it is clear that the sensitization activity of PN on UVB-induced apoptosis is likely achieved through its inhibitory effect on PKCζ and p38

signaling pathway.

5.7 Conclusion

In this study, the anti-cancer property of PN has been systematically investigated in both *in vivo* animal model and *in vitro* cell culture. The main experiments conducted were: i) evaluation of the chemopreventive and chemotherapeutic potentials of PN against UVB-induced skin cancer in SKH-1 hairless mice model; ii) the PN-sensitized UVB-induced apoptosis and the underlining mechanisms. The major findings of this study are:

1) PN possesses strong chemopreventive property against UVB-induced skin cancer in SKH-1 hairless mice. This chemopreventive property is probably mediated in a COX-2 independent manner.
2) PN is not effective as a single chemotherapeutic agent against UVB-induced skin cancer in SKH-1 hairless mice
3) PN sensitizes murine epidermal cells JB6 to UVB-induced apoptosis via the following mechanisms as outlined in Figure 6.1:
    a) PN suppressed the UVB-induced JNK and p38 kinase activations, leading to the downstream inhibitions of c-Jun at Ser-63 and Ser-73 as well as ATF-2. Such suppressions are capable of inhibiting the pro-survival transcription factor AP-1, leading to sensitization of cells to UVB-induced apoptosis.
    b) PN selectively enhances the pro-apoptotic PKCδ and suppresses the anti-apoptotic PKCζ. Furthermore, PKCζ acts upstream of p38, but

not JNK, to protect cell death induced by PN and UVB

In summary, the new findings in this study demonstrate the potent anti-cancer property of PN in addition to its well-known anti-inflammatory action. The experimental evidences suggest that PN, the major sesquiterpene lactone in feverfew, is a promising chemopreventive agent.

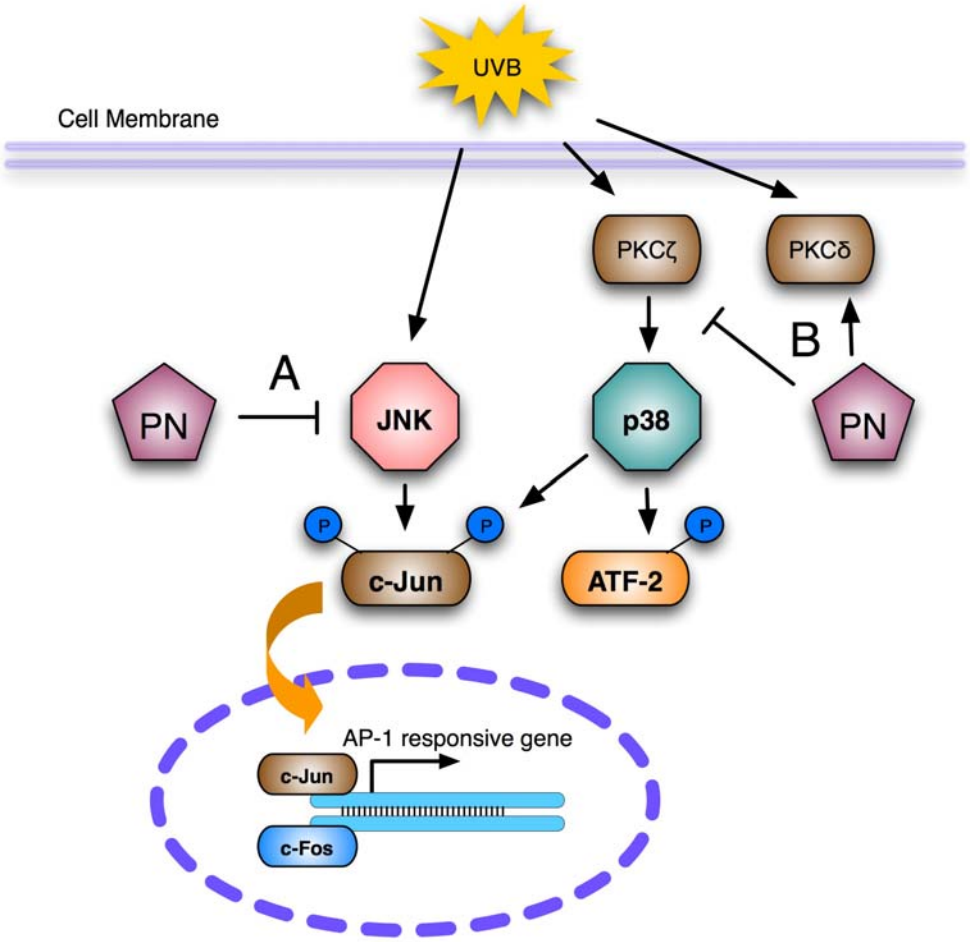


Figure 5.1 Mechanisms involved in PN-sensitized UVB-induced apoptosis. (A) PN is capable of sensitizing cells to UVB-induced cell death by inhibiting JNK and p38 kinase activation, blocking the downstream phosphorylations of c-Jun and ATF-2, and thus suppressing the pro-survival transcription factor AP-1. (B) PN selectively enhances the pro-apoptotic PKCδ while suppresses the anti-apoptotic PKCζ. The suppression on PKCζ will then lead to inhibition on p38 kinase and shuts down AP-1.

CHAPTER 6

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