ENHANCEMENT OF UVB-INDUCED APOPTOSIS BY THE CHEMOPREVENTIVE BIOFLAVONOID APIGENIN IN MULTIPLE HUMAN KERATINOCYTE MODELS

By

Adnan O. Abu-Yousif

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Dissertation Committee:

_____
Curtis Klaassen, Ph.D. (Chair)

_____
Jill Pelling, Ph.D.

_____
Greg Reed, Ph.D.

_____
John Robertson, Ph.D.

_____
Leslie Heckert, Ph.D.

Date defended: _____

The Dissertation Committee for Adnan O. Abu-Yousif certifies
that this is the approved version of the following dissertation:

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Date approved: _____

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3D:	three-dimensional
AP-1:	activator protein-1
BCC:	basal cell carcinoma
BPAG1:	bullosus pemphigoid antigen 1
BrdU:	5-bromo-2-deoxyuridine
BS:	bovine serum
BSA:	bovine serum albumin
C:	cytosine
COX:	cyclooxgenenase
CPD:	cyclobutane pyrimidine dimers
DD:	death domains
DED:	death effector domains
DISC:	death-inducing signaling complex
DMEM:	Dulbecco's Modification of Eagle's Medium
DTT:	dithiothreitol
EGCG:	epigallocatechin gallate
EGF:	Epidermal Growth Factor
EIA:	Enzyme immunoassay
ELISA:	enzyme-linked immunosorbent assay
FADD:	Fas-Associated Death Domain
FADDdn:	dominant negative form of Fas-Associated Death Domain

FBS: fetal bovine serum

GA: gentamycin amphotericin

GGR: global genome repair

HKGS: human keratinocyte growth supplement

IL: interleukin

MAPK: mitogen-activated protein kinases

MOMP: mitochondrial outer membrane permeabilization

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

            sulfophenyl)-2H-tetrazolium, inner salt

NER: nucleotide excision repair

NF-κB: nuclear factor-kappa B

NHK: normal human keratinocytes

NMSC: Non-melanoma skin cancer

NSAID: nonsteroidal anti-inflammatory drugs

ODC: ornithine decarboxylase

OTKC: organotypic keratinocyte cultures

PARP: poly-ADP-ribose polymerase

PBS: phosphate buffered saline

PGE_2:   prostaglandin E_2

PI3-K: phosphotidylinositol 3-kinase

PMSF: phenylmethylsulfonyl fluoride

SCC: squamous cell carcinoma

Staur: Staurosporine

T: thymine

TCR: transcription coupled repair

TG: transglutaminase

TNF: tumor necrosis factor

TPA: 12-O-tetradecanoyl phorbol-13-acetate

TRAIL: TNF-related apoptosis-inducing ligand

UV: ultraviolet

UVB: ultraviolet-B

Abstract

Topical application of the bioflavonoid apigenin (4',5,7-trihydroxyflavone) to mouse skin effectively reduces incidence and size of skin tumors caused by UVB exposure. The ability to act as a chemopreventive compound indicates that apigenin treatment alters the molecular events initiated by UVB exposure; however, the effects of apigenin treatment on UVB-irradiated keratinocytes are still not fully understood. In the present study, we aimed to study the effect of apigenin treatment and UVB exposure on human keratinocytes and to investigate how apignein may alter the biological consequences of UVB exposure. The experiments described herein, employed three models of human keratinocytes: HaCaT human keratinocyte cells, normal human keratinocytes (NHK) cultures isolated from human neonatal foreskin, and human organotypic keratinocyte cultures (OTKC). The ability of UVB to induce cycloxygenase-2 (COX-2) was investigated due to the role it is thought to play in photocarcinogenesis. The apoptotic response of keratinocytes to UVB is thought to be critical to the development of skin cancer, and therefore was investigated in multiple human keratinocyte models. Each keratinocyte model was exposed to a moderate dose of UVB (300-1000 J/m^2), then treated with apigenin (0-50 μM) and harvested to assess apoptosis by Western blot analysis for poly-ADP-ribose polymerase (PARP) cleavage, annexin-V staining by flow cytometry, and/or the presence of sunburn cells. Apigenin treatment enhanced UVB-induced

apoptosis more than two-fold in each of the models tested. When keratinocytes were exposed to UVB, apigenin treatment stimulated changes in Bax localization, and increased the release of cytochrome c from the mitochondria compared to UVB exposure alone. Overexpression of the anti-apoptotic protein Bcl-2 and expression of a dominant negative form of Fas-Associated Death Domain (FADDdn) led to a reduction in the ability of apigenin to enhance UVB-induced apoptosis. These results suggest enhancement of UVB-induced apoptosis by apigenin treatment involves both the stress-mediated, intrinsic pathway and the receptor-mediated, extrinsic pathway of apoptosis. The ability of apigenin to enhance UVB-induced apoptosis may explain, in part, the photochemopreventive effects of apigenin.

Chapter 1: Introduction and Background

1.1 The relationship between skin cancer and UVB exposure

Skin cancer is the most common type of cancer in the United States, with over 1.3 million cases diagnosed each year (ACS 2007). Human skin cancers are divided into two sub-types that can be distinguished based on the cell of origin. Melanomas, which are derived from melanocytes, account for approximately 4% of all skin cancers. These cancers are aggressive, can metastasize, and are responsible for over 8,000 deaths annually (ACS 2007). Non-melanoma skin cancers (NMSCs) arise from epidermal keratinocytes and can be broken down into two sub-types that differ from a clinical and histological aspect: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Collectively BCC and SCC represent the most frequently diagnosed cutaneous malignancies accounting for approximately 80% and 16% of all skin cancers (Afaq *et al.* 2005). Epithelial skin cancers are typically less aggressive than melanomas because they are not as inclined to metastasize, however, NMSC has a significant impact on Americans due to the number of cases presented each year.

In order to understand the etiology of skin cancer, extensive epidemiologic, clinical, and biological studies have been conducted. These reports have concluded overexposure to solar ultraviolet (UV) radiation is responsible for the development and progression of greater than ninety percent of skin cancers (Brash *et al.* 1991; Gailani *et al.* 1996; Miller and Weinstock 1994; Urbach 1991). Primary risk factors for developing skin

cancer include: skin complexion, tan versus burning capacity, frequency of sunburns at an early age, geographic location, and occupational exposure to the sunlight (Diffey 1991).

Solar radiation consists of a continuous spectrum of electromagnetic radiation that is divided into three major components: UV light (200-400nm), visible (400-700nm), and infrared (700-5000nm). The wavelengths of UV light distinguish three sub-groups: UVA (320-400nm), UVB (280-320nm), and UVC (200-280nm) with different physiological effects. The Earth's ozone layer serves as a protective filter, preventing all UVC from reaching the Earth's surface. A significant portion of UVB is also blocked by the ozone layer, therefore 90-99% of the UV light reaching the surface of the Earth consists of UVA light (Matsui 1995), with the remainder being UVB light.

UVA rays consist of longer wavelengths, and thus are capable of penetrating deeper into the layers of the skin, however, UVA has been demonstrated to be less mutagenic than either UVB or UVC light (Phan *et al.* 2006). Exposure to UVA induces indirect damage to the basal layer of the epidermis through the formation of free radicals that exacerbate existing damage. These effects may explain the weaker initiating activity and relatively potent promoting activity of UVA exposure compared to UVB (Agar *et al.* 2004; Runger 1999). In accordance with these reports, others have demonstrated that UVA is less effective than UVB at inducing SCC (de Gruijl *et al.* 1993), and that fewer signature p53 mutations are found in UVA-

induced tumors than in those induced by overexposure to UVB (de Gruijl 2002).

Exposure to UVB radiation represents a major environmental hazard. Reports have documented that wavelengths within the UVB spectrum are the most erythemic (Anders *et al.* 1995; R. Andreichin 1987), lead to more UV-induced photoproducts (Freeman *et al.* 1989; Ley *et al.* 1983), and had the highest photocarcinogenic potential (de Gruijl and Van der Leun 1994). The molecular events listed above have been correlated to many pathologic conditions, including increased aging of the skin (Chapman *et al.* 1995), exacerbation of infections (Fisher *et al.* 1996), and induction of skin cancers (de Gruijl *et al.* 1993). Exposure to UVB initiates photocarcinogenesis by inducing damage to critical macromolecules such as DNA, protein, and lipids in the epidermis (Cole *et al.* 1986; Davies and Forbes 1986; Ichihashi *et al.* 2003; Katiyar 2003; Katiyar *et al.* 1997; Kehrer 1993; Mukhtar and Elmets 1996; Sancar 1995). Repeated exposure to UVB promotes clonal expansion of initiated cells and eventually alters signal transduction pathways and modulates tumor promoting genes independent of UVB exposure making it a complete carcinogen (Cleaver and Crowley 2002; Sarasin 1999).

The dose of UVB required to be absorbed by DNA is similar to the dose required to initiate the biological effects of UVB exposure (Petit-Frere *et al.* 1998). Mutation of the tumor suppressor p53 in keratinocytes is thought to be a key event, and may in fact be the initiating event in skin carcinogenesis

(Ananthaswamy *et al.* 1997; Brash *et al.* 1996; Ziegler *et al.* 1994). Jiang et al. have provided additional evidence supporting the role of p53 mutations in skin cancers in a report demonstrating that p53 -/- and p53 +/- mice acquire tumors of the skin more rapidly than wild type mice when exposed to UV radiation (Jiang *et al.* 1999). Collectively, these reports support the concept that DNA is the most important molecular target for UVB.

The most common DNA lesions caused by overexposure to UVB are cyclobutane pyrimidine dimers (CPD) that form between adjacent thymine (T) or cytosine (C) residues, and 6-4 photoproducts (Ichihashi *et al.* 2003; Kulms and Schwarz 2002; Matsumura and Ananthaswamy 2002; Ravanat *et al.* 2001). A majority of UV-induced DNA damage is removed by triggering cellular repair machinery. Nucleotide excision repair (NER) removes bulky DNA adducts by one of two subpathways: transcription coupled repair (TCR) or global genome repair (GGR) (Matsumura and Ananthaswamy 2002). TCR quickly removes adducts existing in actively transcribed genes, while GGR removes adducts in other parts of the genome at a much slower rate. Exposure to high doses of UVB causes extensive damage that overwhelms repair machinery, and under these circumstances apoptosis is initiated to remove potential cancer cells from the epidermis. Failure to remove the damage either by repair or apoptosis results in the retention of DNA harboring mutations in the epidermis which can lead to aberrant cell signaling involved in tumor formation (Cleaver and Crowley 2002; Sarasin 1999).

The biological effects of UVC exposure are predicted to be similar to those caused by UVB, yet may require lower dose to elicit a response (Takasawa *et al.* 2005). Fortunately, most UVC radiation is blocked from reaching the Earth's surface by the ozone layer, thereby dampening the biological significance of consequences associated with UVC exposure. Currently UVC exposure is mostly limited to occupational exposure from manmade sources of UVC, such as germicidal lamps. However, recently scientists have become increasingly more concerned about potential effects of UVC due to the depletion of the ozone layer. Mathematical models been used to predict an increase of 2-4% in the incidence of skin cancers for each 1% reduction in the ozone layer, with a larger increase in SCC (Diffey 1992; Urbach 1989).

1.2   Development of the skin, structure, and function

Skin is the largest organ in the human body. Human skin is composed of an outer epidermal layer and an underlying dermis (Mack *et al.* 2005). The epidermis is a multilayered cornified epithelim, consisting largely of keratinocytes that serve as a physical and permeability barrier between our bodies and various environmental hazards including, but not limited to UV radiation, toxic substances, dehydration and mechanical stresses (Candi *et al.* 2005; Kirfel and Herzog 2004). Terminal differentiation, or cornification of the epidermis creates four distinct layers: the basal, spinous, granular and

cornified layer.   Each layer of keratinocytes can be recognized by the position, morphology, and differentiation markers (Fig. 1).

The basal layer of keratinocytes in the epidermis is anchored to the basal lamina and can be identified by their cuboidal shape (Borradori and Sonnenberg 1999; Burgeson and Christiano 1997; Kirfel and Herzog 2004). Basal keratinocytes express keratin-5, keratin-14, transglutaminase (TG)-2, and bullosus pemphigoid antigen 1 (BPAG1). Proliferation in the epidermis is limited to the basal layer where self-renewing epidermal stem cells are found. These epidermal stem cells give rise to daughter cells that can undergo 3-5 cell divisions, before progressing upward through the epidermis into the next epidermal layer, the spinous layer (Potten 1981).

Keratinocytes within the spinous layer of the skin have lost their proliferative capacity, yet they are larger and much more metabolically active than those found in the basal layer. Expression of TG1, TG5, desmoglein-2, -3, and -4 are also characteristic of keratinocytes in the spinous layer.   As cornification continues, the keratinocytes lose their cuboidal shape, and due to reorganization of their cytoskeleton, become more squamous.   The granular layer of the epidermis can easily be identified by the presence of keratohyalin granules which contain profilaggrin. Replacement of keratin-5 and -14 with keratin-1 and -10 promotes the collapse of cells into a flattened shape, and eventually into a squame, which are found in the cornified layer (Candi *et al.* 2005). Squames are cells that lack a nucleus and cytoplasmic

organelles, but are full of keratin filaments, surrounded by an insoluble protein envelope which forms the stratum corneum. As mentioned previously the stratum corneum is the outer most layer of the epidermis, and is the first line of defense against external assault. The stratum cornem is continually shed from the skin by a form of programmed cell death (Gandarillas *et al.* 1999; Haake and Polakowska 1993; Maruoka *et al.* 1997).
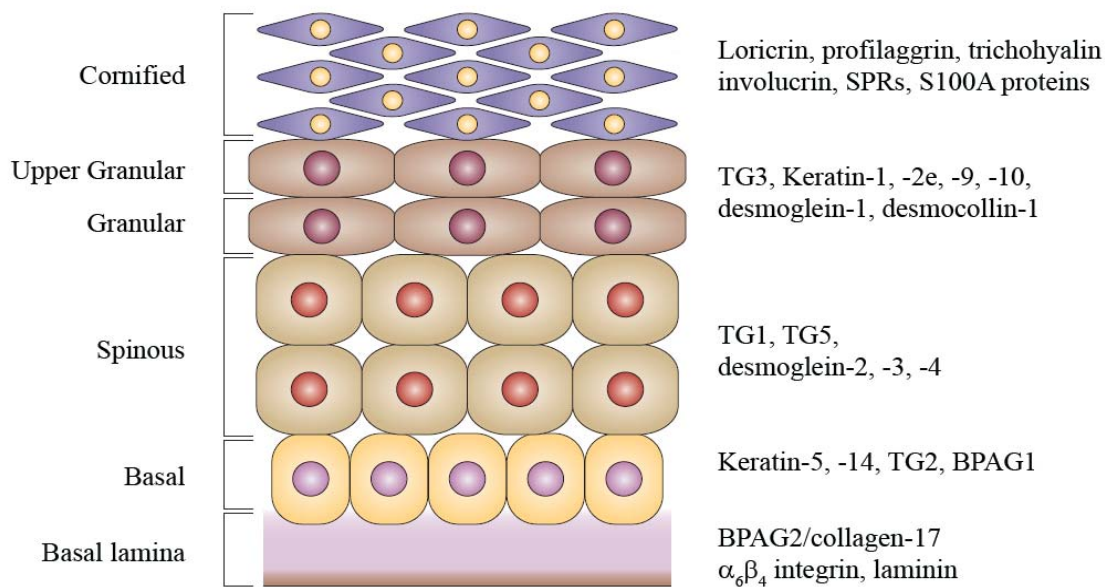
Figure 1: Terminal differentiation in the epidermis.
The proteins expressed in particular locations in the epidermis during skin differentiation are shown on the right. The layers of skin formed are indicated on the left. BPAG, bullosus pemphigoid antigen; SPR, small proline rich proteins; TG, transglutaminase.
(Adapted from Candi *et al*. 2005)

1.3   Models used to study nonmelanoma skin cancer

Keratinocytes were the first normal epidermal cell type to be grown successfully in culture (Prunieras *et al.* 1983). Our lab and others have used cell culture to investigate the biological processes associated with the development of skin cancer with the goal of determining potential targets for chemopreventive and therapeutic agents. (Lepley *et al.* 1996; Neades *et al.* 1998; Ramaswamy and Pelling 1999; Ramaswamy *et al.* 1998). The HaCaT human keratinocyte cell line is one of the most commonly used cell lines to study the molecular mechanisms responsible for tumorigenensis n in human skin.

HaCaT cells are a spontaneously immortalized, nontumorigenic cell line derived from normal human adult skin during prolonged cultivation at a reduced Ca^{2+} concentration and elevated temperature (Boukamp *et al.* 1988). An advantage to using cell lines is that many contain genetic mutations typical of those observed in human cancer cells. For example, HaCaT cells contain multiple genetic alterations including chromosomal translocations leading to the loss of one copy of chromososome 3p, 4p, and 9p (Boukamp *et al.* 1988), expression of telomerase (Fusenig and Boukamp 1998), and mutations in both alleles of the p53 tumor suppressor gene (Lehman *et al.* 1993), a characteristic often found in UV-induced skin cancers (Boukamp *et al.* 1997). The use of HaCaT cells and other established cell lines continues to provide valuable insight regarding the molecular events associated with the

development of skin cancer. However in recent years, interest has increased in using normal human keratinocytes (NHK) to study the steps of carcinogenesis.

The use of NHK cultures to study initiation of skin cancer and potential targets for chemoprevention has gained momentum due to a lack of genetic alterations in these cells compared to those present in immortalized cell lines (Galgon *et al.* 2005; Sandoval *et al.* 2007). Investigators have also taken advantage of improved access to human neonatal foreskins, which yield keratinocyte populations that have a longer half-life and less genetic mutations than those isolated from adult human skin (Barrandon and Green 1987). Genetic variability is inherent in human keratinocytes that are isolated from newborns of different backgrounds, however, this variability may lead to experimental findings that more accurately reflect the response of a representative population to environmental assaults, such as UVB exposure and the efficacy of chemopreventive compounds in normal human cells. When working with NHK, one must consider the differences in genetic background may also lead to data that appears inconsistent and thus may require testing numerous individuals for certain endpoints. For example, in the present study primary keratinocyte cultures were exposed to various doses of UVB to asses the dose of UVB required to induce modest levels of apoptosis. NHK cutures that underwent modest levels of apoptosis when exposed to 750 J/m^2 UVB were then used to investigate the ability of apigenin

to enhance UVB-induced apoptosis. It is clear that there are obvious advantages to using NHKs to study photocarcinogenesis of the skin, and those qualities can be further studied in three-dimensions by co-culturing dermal fibroblasts and NHK to generate human organotypic keratinocyte cultures (OTKC).

Monolayer keratinocyte cultures have been a highly useful tool in basic research regarding the biology of the skin. However, since monolayer cultures fail to generate the multiple layers of the skin, in vitro studies of differentiation and pharmacokinetic studies remain limited. Pruniéras et al. recognized the limitations of studying monolayer keratinocyte cultures, and was among the first to demonstrate keratinocytes cultured at an air-liquid interface fully differentiate (Prunieras *et al.* 1983). Co-culture of keratinocytes on top of a lattice, including fibroblasts and collagen at an air-liquid interface, stimulates the synthesis of profillagrin which in turn, leads to the development of keratohyalin granules and the granular phenotype observed in cornified skin (Poumay and Coquette 2007). In collaboration with Dr. Kathleen Green, Department of Pathology, Northwestern University School of Medicine, I have developed a reproducible protocol for generating human OTKC by culturing primary keratinocyte cultures on top of a collagen-fibroblast matrix consisting of rat tail collagen I and the J2-3T3 dermal mouse fibroblast cell line. This protocol is now in regular use in the Pelling laboratory. More detail on the protocol can be found in the Materials and Methods section. Use of human

OTKC has allowed our group and others to investigate the effects of various agents that otherwise could not be studied in human cornified skin due to ethical concerns. The strength of the OTKC model is that the human keratinocytes form a thick epidermis that more closely resembles the number of cellular layers of keratinocytes found in human epidermis than are formed in models using murine keratinocytes (Fig. 2) (Burns 2006; Hengge *et al.* 1996). Whereas these cultures have proved effective, there are limitations of ex vivo culturing of human keratinocytes. OTKC can be maintained for a relatively short period of time, therefore only short-term assays can be conducted. OTKC models also lack an immune response, therefore *in vivo* studies must ultimately be conducted in order to gain a more complete understanding of events involved in the development of NMSC.

Currently, the SKH-1 hairless mouse is the most common strain of mouse used to study photocarcinogenesis because it lacks hair, making it easy to irradiate and acquires squamous cell carcinoma once subjected to UV radiation (Winkelmann *et al.* 1963). In the 1980's, initial studies using the SKH-1 hairless mouse were conducted with UVB-induced squamous cell carcinoma to determine their capacity to metabolize xenobiotics (Das *et al.* 1985). Since then, numerous studies have been conducted utilizing SKH-1 mice for the purpose of studying UVB-induced skin carcinogenesis and evaluating potential mechanisms for intervention and prevention (Ahmad and Mukhtar 2001; Kramata *et al.* 2005; Starcher 2000; Vayalil *et al.* 2004). Each

experimental model used in the study of NMSC clearly has its strengths and therefore can be used effectively; however, the limitations of each also suggest that multiple models should be used to gain a more comprehensive understanding of the molecular events responsible for the development of NMSC and the potential of compounds to effectively reverse, suppress, or prevent the process of photocarcinogenesis.
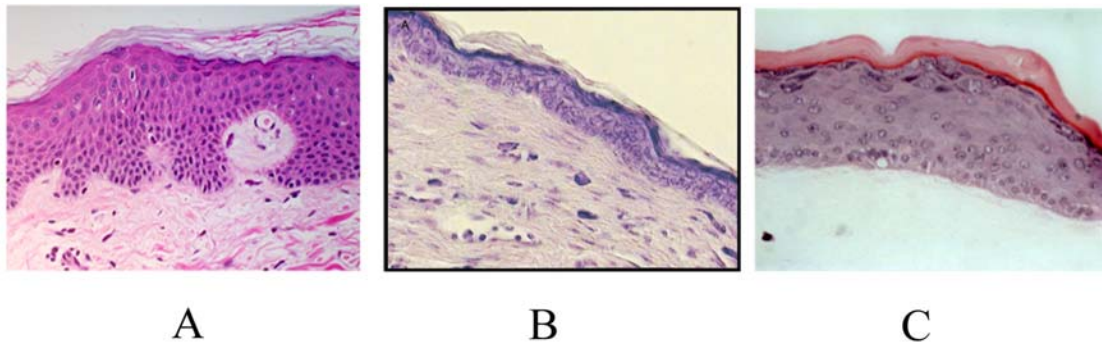
Figure 2: Histopathologic features of skin.
(A) Human skin (obtained from Northwestern University Pathology core) (B) Skh-1 hairless mouse skin (adapted from Mentor et al.) C) Human organotypic keratinocyte culture

1.4   Strategies for chemoprevention of skin cancers

Chemoprevention can most simply be defined as the use of a natural, synthetic, or biologic chemical agent used to effectively reverse, suppress, or prevent carcinogenesis. In recent years the term photochemoprevention has been coined for the study of prevention of the biological effects of UV exposure that ultimately leads to tumor development (Ahmad and Mukhtar 2001). Primary preventative measures have been used with some success against UV-induced skin cancers in humans. The public has been educated regarding the adverse effects of excessive UV exposure, the need to limit exposure by wearing protective clothing, and use of sunscreen. However these measures have had limited success at reducing the incidence of skin cancer (Bowden 2004). The following evidence supports the pressing need to develop an effective photochemopreventive regimen: 1) the incidence of skin cancers in the United States approaches or exceeds that of all other cancers combined (ACS 2007), 2) patients presenting with a NMSC lesion are at a 50% risk for developing another primary lesion in five years (Karagas *et al.* 1992), 3) organ transplant recipients and other immunosuppressed patients that develop NMSC lesions present with more aggressive forms that have a higher rate of fatality associated with the disease (Martinez *et al.* 2003; Penn 1994).   Researchers have employed numerous nontoxic compounds known to possess anti-mutagenic and anti-carcinogenic

properties in order to interfere with each stage of carcinogenesis of skin cancers.

The complex, multi-stage process of photocarcinogenesis is best characterized as the sum of multiple genetic events over time that are ultimately responsible for tumor formation. The first step in multi-stage skin carcinogenesis is 'initiation,' which involves exposure to a DNA damaging agent that causes irreversible damage to the DNA of a keratinocyte. The second stage of skin carcinogenesis, 'promotion,' involves repeated exposure to UVB, resulting in both genetic and epigenetic changes in critical genes that regulate proliferation, inflammation, differentiation, and apoptosis. Promotion results in clonal expansion of an initiated cell into a focus of cells representing a benign pre-malignant stage which may include dysplasia. Additional genetic and epigenetic events occur during the third stage of carcinogenesis, termed 'progression.' During progression, the benign intraepithelial neoplastic cells undergo additional genetic events (such as activation of an oncogene or inactivation of a tumor suppressor gene) and progressive genomic instability. Each step of carcinogenesis has been targeted by investigators that have divided the approaches into two categories: anti-mutagenic and anti-proliferative (Fig. 3).

Anti-mutagenic strategies target the initiation stage of tumorigenesis in order to alter carcinogen metabolism, limit activation of pro-carcinogens, enhance carcinogen detoxification, scavenge electrophiles, and/or enhance

DNA repair (Hursting *et al.* 1999; Steele 2003). Many investigators have attempted to identify and/or develop an agent capable of acting at the initiation stage of photocarcinogenesis. Unfortunately, these efforts have had limited success because tumor initiation is a rapid, irreversible event. Decades often pass between initiation and development of a frank tumor, providing a larger window of opportunity for intervention by an agent that can prevent, reverse, or suppress promotion and/or progression of skin tumors (Hannuksela-Svahn *et al.* 1999). Such anti-proliferative strategies target the expansion of initiated cells by limiting the many aberrant signaling pathways triggered during tumor promotion and/or progression. The early stages of tumor promotion are thought to be reversible, but at some point during carcinogenic progression, cancerous cells express or suppress genes that regulate cell growth, differentiation, inflammation, and/or apoptosis independent of exposure to tumor promoting agents. Each of these processes provide cancerous cells with a selective advantage, and thus are targeted by anti-proliferative strategies that are currently being investigated.

A number of cellular signaling molecules have been targeted by researchers interested in developing effective photochemopreventive agents. For example, nonsteroidal anti-inflammatory drugs (NSAID) and various polyphenols have been shown to inhibit the activation of carcinogens by targeting cyclooxygenase (Van Dross *et al.* 2005), and lipoxygenase enzymes (Kim *et al.* 2007). Others studies have reported that enhancement of DNA

repair reduces the adverse biological effects caused by UVB exposure (Kibitel *et al.* 1998; Kripke *et al.* 1992; Nishigori *et al.* 1996; Petit-Frere *et al.* 1998; Wolf *et al.* 1995). Katiyar et al. has shown that the anti-mutagenic green tea product epigallocatechin gallate (EGCG), prevents UVB-induced skin tumor development through modulation of interleukin (IL) -12, enhancing DNA repair, and stimulation of the immune response (Katiyar *et al.* 2007). Topical application of celecoxib (Wilgus *et al.* 2002), sulindac (Athar *et al.* 2004), and EGCG (Katiyar and Mukhtar 2001) reduce UVB-mediated inflammation, including edema, neutrophil infiltration and prostaglandin E_2 (PGE_2) production in mouse skin. Components of green tea (Lu *et al.* 2000; Lu *et al.* 2001) and other natural products including apigenin (McVean *et al.* 2002; McVean *et al.* 2000), silibinin (Dhanalakshmi *et al.* 2004), and curcumin (Bush *et al.* 2001; Jee *et al.* 1998) have been reported to increase the number of p53-positive cells, increase the number of $p21^{waf1/cip1}$-positive cells, cause delay in cell cycle progression, and/or increase in apoptotic sunburn cell formation in keratinocytes. Other targets under study include, but are not limited to the phosphotidylinositol 3-kinase (PI3-K)/Akt pathway, nuclear factor-kappa B (NF-κB) pathway, activator protein-1 (AP-1), mitogen-activated protein kinase (MAPK) pathways, cyclooxygenase (COX) enzymes, and ornithine decarboxylase (ODC). The increased number of published studies reflects an increasing interest in developing an agent that can effectively reverse, suppress, or prevent carcinogenic progression in the skin. Hopefully,

these efforts will allow such an agent to be incorporated into skin care products and sunscreens and ultimately reduce the significant economic and clinical burden placed on the millions of individuals affected by skin cancer annually.
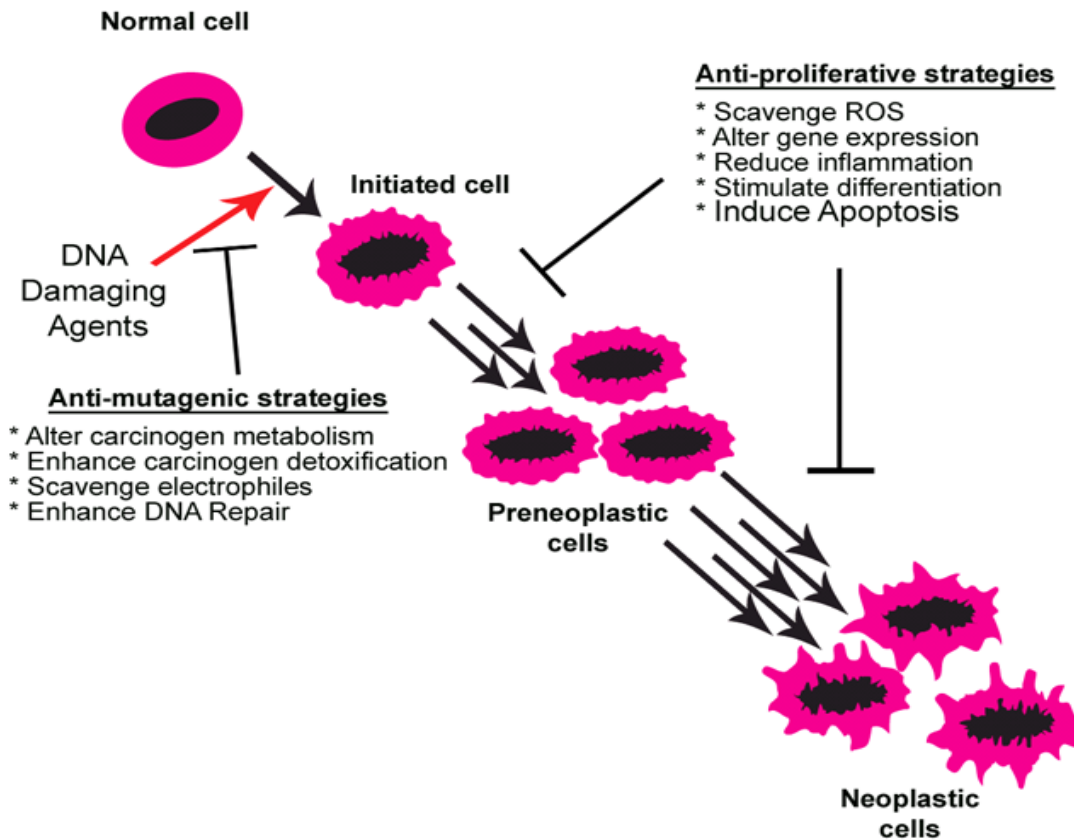
Figure 3: The process of multistage carcinogenesis in the skin and strategies for chemoprevention.
A schematic presentation of stage-specific prevention strategies. The initiation stage is characterized by the conversion of a normal keratinocyte into an initiated cell in response to DNA-damaging agents (red arrow). The promotion stage is characterized by the transformation of an initiated cell into a population of preneoplastic cells due to alterations in gene expression and cell proliferation. The progression stage is characterized by the transformation of the preneoplastic cells into a neoplastic cell population as a result of additional genetic alterations. Intervention points for strategies to prevent these processes are indicated by T-shaped lines. ROS—reactive oxygen species.

(Adapted from Hursting *et al.* 1999)

1.5 Properties of the chemopreventive bioflavonoid apigenin

As described in the previous section, many researchers are currently investigating the chemopreventive actions of plant-derived dietary agents. A major focus of research in the Pelling laboratory is the investigation of chemoprevention by a naturally occurring flavone called apigenin (4',5,7-trihydroxyflavone). Apigenin is a low molecular weight flavonoid (MW 270.4) that is practically insoluble in water, moderately soluble in hot alcohol, and is soluble in dilute KOH and DMSO (Patel *et al.* 2007). Natural apigenin is found in common fruits and vegetables such as parsley, onions, oranges, tea, chamomile, wheat sprouts and in some seasonings (Birt *et al.* 2001; Manach *et al.* 2004; Patel *et al.* 2007; Surh 2003; Yang *et al.* 2001). The natural form of apigenin is present mostly as glycoside conjugates and various acylated derivatives which increase the water solubility of apigenin (Hollman and Katan 1999; Patel *et al.* 2007; Ross and Kasum 2002). Due to its abundance in nature, many nontraditional healers have used plants containing high levels of apigenin in order to treat a variety of ailments such as indigestion, shingles, insomnia, and many more described in a review by Patel et al (Patel *et al.* 2007). In 1986 Birt and colleagues were the first to report the apigenin possessed anti-mutagenic and anti-promotion chemopreventive properties through inhibition of 12-*O*-tetradecanoyl phorbol-13-acetate- (TPA)-induced ODC activity in mouse skin (Birt *et al.* 1986).
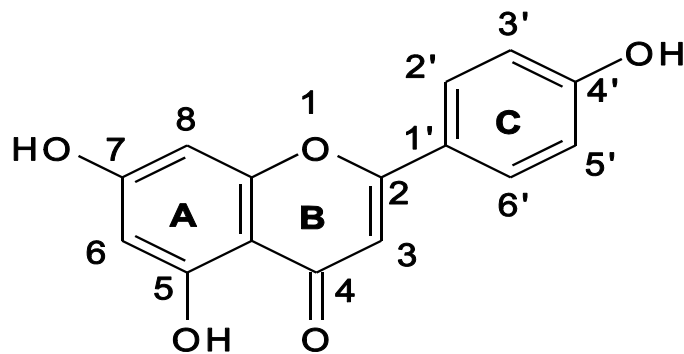
Figure 4: The structure of apigenin.

The interest in apigenin as a chemopreventive compound has steadily increased as others have confirmed that apigenin is nontoxic, and has the ability to prevent generation of ROS, genetic mutations, cell proliferation, inflammation, and progression in numerous cancerous cell lines. In order to enhance the biovavailability of apigenin, Wei et al. applied it topically to mouse skin in a study that demonstrated the ability of apigenin to inhibit skin tumor formation (Wei *et al.* 1990). Subsequent studies demonstrated topical application of apigenin to mouse skin reduced size and frequency of tumors induced by chemical carcinogens and repeated UVB exposure (Birt *et al.* 1997; Lee and Lin 1997). Our group has used these initial studies demonstrating the effects of apigenin on skin carcinogenesis as the foundation for our investigative efforts.

The Pelling laboratory and others have demonstrated that apigenin treatment of cells results in a wide variety of anti-tumorigenic and chemopreventive actions. For example, Kuo and coworkers have demonstrated the ability of apigenin to inhibit transformation (Kuo and Yang 1995), and we have reported that apigenin treatment stabilized wild-type p53 protein (McVean *et al.* 2000). Experiments by Way et al. and Yin and coworkers showed that apigenin induced growth inhibition, cell-cycle arrest and apoptosis in breast cancer cell lines (Way *et al.* 2005; Yin *et al.* 2001). Prostate cancer cell lines treated with apigenin underwent apoptosis as reported by both Gupta et al. and Shukla (Gupta *et al.* 2001; Shukla and Gupta 2004). More recently, our group has focused on the effects of apigenin on keratinocytes exposed to UVB radiation. These reports have demonstrated that apigenin treatment down-modulates both basal and UVB-induced COX-2 expression in keratinocytes (Tong *et al.* 2007; Van Dross *et al.* 2007; Van Dross *et al.* 2005). The data presented in the following report demonstrate apigenin treatment down-modulated COX-2 activity, reduced cell proliferation, stimulated apoptosis independent of PGE_2 signaling, and enhanced UVB-induced apoptosis in human keratinocytes.

1.6   Apoptosis in the skin

The similarities between apoptosis and terminal differentiation in keratinocytes at the cellular and molecular level have generated some confusion toward the classification of terminal differentiation of keratinocytes. In a report that aimed to reduce this confusion, Mitra et al. demonstrated that apoptosis can occur independently of differentiation and vice versa, suggesting there are distinct molecular events responsible for each (Mitra *et al.* 1997). For example, all of the components required for apoptosis are present in keratinocytes and need only to be activated in order to successfully complete the relatively rapid process of apoptosis. Terminal differentiation, however, is a slow coordinated process that takes about 1-2 weeks to occur, and involves altered expression of differentiation-associated proteins (Fuchs and Raghavan 2002). As described in Chapter 1.2, cornification involves the upward progression of keratinocytes from the basal layer ultimately resulting in the formation of fully differentiated squames that are sloughed off. Apoptotic keratinocytes are phagocytosed by other cells (Boehncke *et al.* 1993) as depicted in Figure 5.

Apoptosis is a controlled process that was originally defined by the observation of the following morphological features during cell death: nuclear condensation and fragmentation, membrane blebbing, cellular fragmentation into membrane-bound bodies, ultimately leading to phagocytosis of the dying cell without stimulating an inflammatory response (Wyllie *et al.* 1980). These

morphological features distinguish apoptosis from other types of cell death. They are caspase-dependent and require the cleavage of specific cellular proteins or 'death' substrates within the cell. These morphological changes have formed the basis of methods developed by others to devise assays to measure apoptosis (Samali *et al.* 1999). For example, the cytosolic protein annexin-V has a high avidity to membrane bound phosphatidylserine (PS) which translocates from the inner to the outer layer of the plasma membrane during apoptosis (Green and Steinmetz 2002). Two principal apoptotic pathways have been characterized in the literature: the death receptor-mediated, extrinsic pathway and the stress-mediated, intrinsic pathway (Fig. 6).

Caspase-8 is the apical caspase in the extrinsic apoptotic pathway, which is triggered by binding of a ligand to a death receptor such as Fas (a subset of the TNF receptor [TNFR] family). Upon binding of Fas-L to its receptor, type I cells respond by initiating oligomerization of the receptor and recruitment of the adapter molecule Fas-associated death domain (FADD), which contains both death domains (DD) and death effector domains (DED) motifs (Scaffidi *et al.* 1998). FADD binding to the death receptor stimulates binding of procaspase-8 via the DED domain in order to form the death-inducing signaling complex (DISC) (Debatin and Krammer 2004). Accumulation of procaspase-8 stimulates autocatalytic activation and processing of the downstream effector caspase, caspase-3. Another death

ligand, known as APO-2 ligand or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) initiates a similar cascade in response to binding to the TNFR (Fadeel and Orrenius 2005).

In type II cells, by comparison, not enough active caspase-8 is generated to stimulate the activation of caspase-3. Low levels of caspase-8 activation can lead to cleavage of the proapoptotic protein Bid. The truncated form of Bid translocates to the mitochondria initiating cytochrome c release creating a mitochondrial amplification loop that produces enough caspase activity to induce apoptotic cell death (Li *et al.* 1998; Luo *et al.* 1998).

In the intrinsic apoptotic pathway, caspase activation is closely linked to the integrity of the outer mitochondrial membrane. Many stress-related stimuli and proapoptotic signal transducing molecules act to disrupt the integrity of the mitochondria and induce mitochondrial outer membrane permeabilization (MOMP) (Fulda and Debatin 2006; Green and Kroemer 2004). The process of MOMP is regulated by the Bcl-2 family of proteins. BH3-only members of the Bcl-2 superfamily are believed to stimulate oligimerization of Bax and Bak at the outer mitochondrial membrane to create a pore. Formation of a pore allows for the release of proteins normally contained in between the inner and outer mitochondrial membrane, including cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G (Saelens *et al.* 2004). Release of these proapoptotic proteins into the cytosol stimulates caspase activation and/or caspase-independent death effector

molecules (Saelens *et al.* 2004). For example, the cytosolic protein Apaf-1 senses that cytochrome c has been released from the mitochondria. Cytochrome c quickly binds to Apaf-1 triggering its oligimerization and recruitment of ATP, caspase-9 to form a complex called the apoptosome (Riedl and Salvesen 2007). Activation of the initiator caspase-9 requires formation of the apoptosome, which leads to processing of the executioner caspase-3, and ultimately to apoptotic cell death.
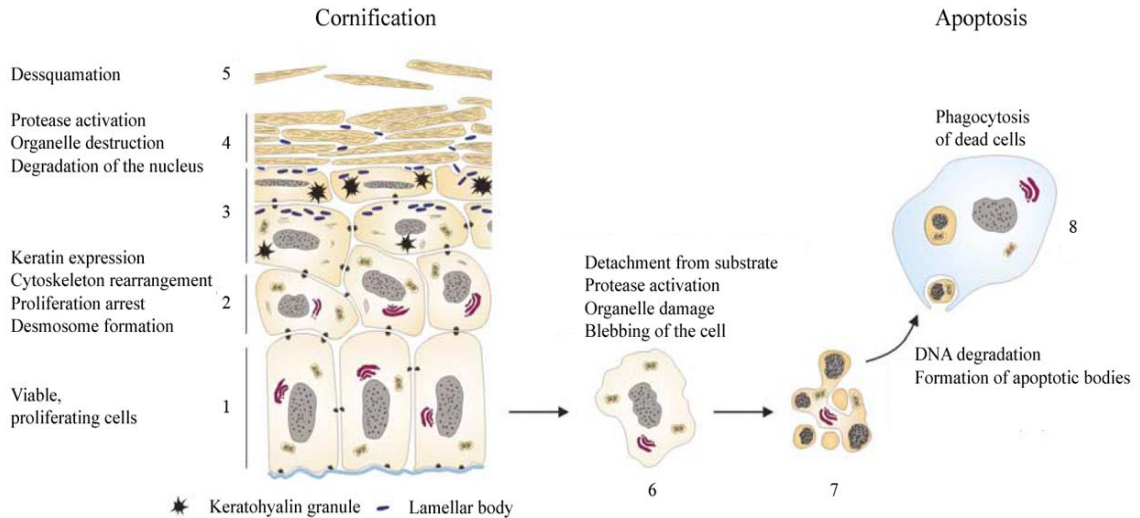
Figure 5: Morphological and biochemical changes during keratinocyte cornification and apoptosis.
(1) The basal layer of the epidermis consists of undifferentiated, mitotic keratinocytes that are attached to the basement membrane. (2) Basal keratinocytes detach from the basal membrane and start to undergo differentiation into the spinous layer. The cells begin expressing differentiation-specific proteins such as keratins 1 and 10. In addition, orchestrated cytoskeleton reorganization occurs through the epithelial sheet. (3) Keratohyalin granules are formed in the granular layer of the epidermis. DNA is degraded, organelles are destroyed, and the plasma membrane is replaced by the cornified envelope and ceramide deposition from the lamellar bodies. (4) The cornified layer consists of dead cells. (5) The dead cells are shed into the environment during desquamation. (6) Upon induction of apoptosis, the caspase cascade becomes activated and apoptotic cells start blebbing. (7) Chromatin and cytoplasm undergo condensation, internucleosomal DNA cleavage occurs, the cytoskeleton is dismantled, the cell membrane 'invaginates', and apoptotic bodies are formed. (8) Finally, apoptotic bodies are phagocytosed and degraded inside lysosomes.

(Adapted from Lippens, *et al.* 2005)
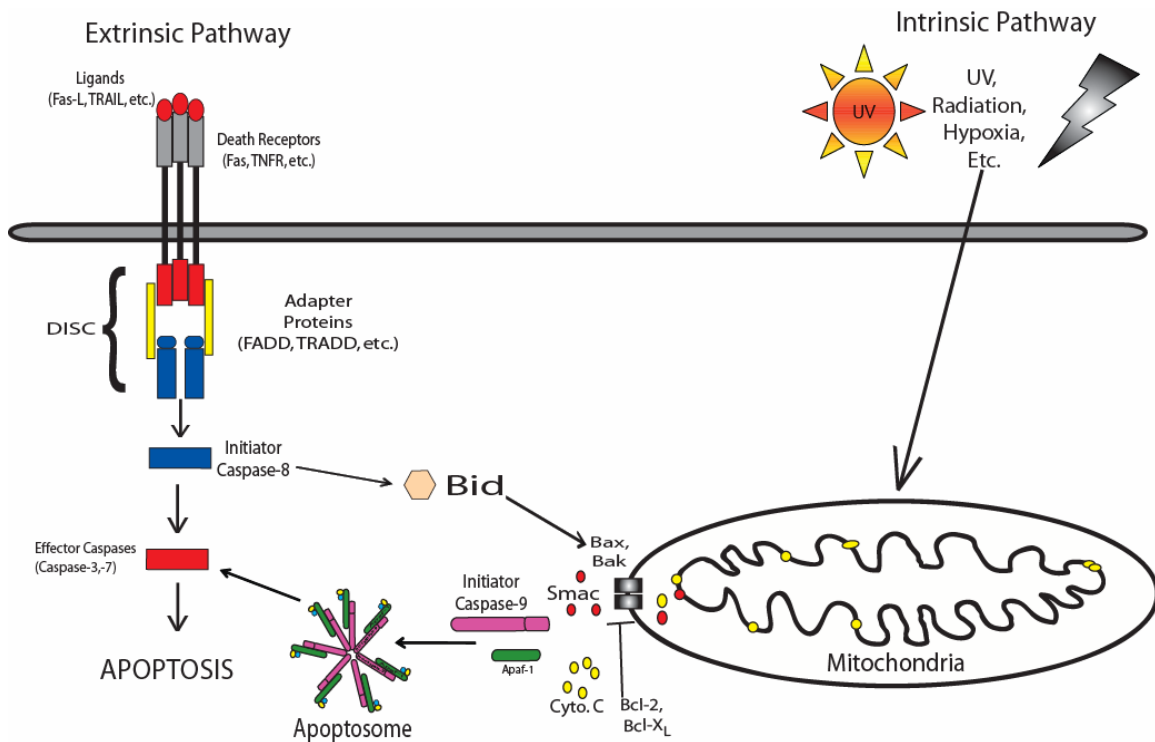
Figure 6: Apoptotic signaling pathways.
Two principal apoptotic pathways have been charcterized, the extrinsic pathway (death receptor-mediated) and the intrinsic pathway (mitochondria-mediated). The extrinsic pathway is initiated when a ligand (e.g., Fas-ligand or TNF-related apoptosis-inducing (TRAIL)) binds to its respective death receptor (e.g., Fas or TNF-R). Upon ligand binding, the receptor aggregates leading to the recruitment of the adaptor molecule Fas-associated death domain (FADD) and procaspase-8 to form the death inducing signaling complex (DISC). Once the DISC has been formed caspase-8, is activated, initiating apoptosis by direct cleavage of downstream effector caspases. Mitochondrial-mediated apoptosis is typically initiated by stress signals through the release of apoptogenic factors including cytochrome c, apoptosis inducing factor (AIF), or Smac/DIABLO from the mitochondrial intermembrane space. Release of cytochrome c into the cytosol triggers formation of a complex called the apoptosome consisting of cytochrome c, pro-caspase-9, dATP, and the scaffolding protein Apaf-1. Formation of the apoptosome leads to activation of caspase-9, which triggers activation of effector caspases leading to apoptosis. The extrinsic and intrinsic apoptotic pathways can interact under certain conditions, for example, in type II cells, weak stimulation of caspase-8 can lead to truncation of Bid, a BH3 domain-containing protein in the Bcl-2 family that can stimulate cytochrome c release from the mitochondria. See text for more details regarding each apoptotic pathway.

Chapter 2: Statement of Purpose

2.1 Significance

Skin cancer is a major public health concern. Collectively, BCC and SCC represent the most frequently diagnosed cutaneous malignancies in the United states (Afaq *et al.* 2005), with an estimated $650 million spent annually on treating patients (John Chen *et al.* 2006). According to many epidemiologic and basic studies, frequency and intensity of UVB exposure greatly increase the risk of developing skin cancer (Diffey 1991). These studies have also concluded that UVB radiation is a complete carcinogen, meaning it can induce tumor formation in the absence of any other carcinogenic agent. The latency between the rapid, irreversible process of tumor initiation and development of a frank tumor offers a large window during which a chemopreventive agent could act to prevent promotion and/or progression of a skin tumor. A number of chemopreventive strategies have been devised in hopes of identifying an effective photochemopreventive regimen that would significantly reduce the economic burden, and great discomfort associated with this malignancy. Topical application of the natural bioflavonoid apigenin has been shown to inhibit UVB-induced skin tumors in mice (Birt *et al.* 1997). Since those initial findings, the Pelling laboratory has strived to gain a better understanding of how apigenin exerts its chemopreventive properties. Two approaches currently under rigorous investigation are: the regulation of COX-2 activity and regulation of apoptosis in the skin. The data presented in the following study provide insight into the

biological effects of UVB exposure and the mechanism by which apigenin may exert its chemopreventive properties.

2.2 Overall Hypothesis

The objectives of this work were to study the molecular events triggered by UVB exposure, and the effect of apigenin treatment on these processes in three keratinocyte models: HaCaT human keratinocyte cells, NHKs isolated from human neonatal foreskin, and human OTKC. More specifically, this study focused on UVB-induced COX-2 activity and UVB-induced apoptosis in three different keratinocyte models and the effect of apigenin treatment in each model. **The hypothesis to be tested is that apigenin exerts its chemopreventive properties in part by reducing COX-2 activity; thereby enhancing UVB-induced apoptosis in keratinocytes.** It is further hypothesized that apigenin enhances UVB-induced apoptosis by modulating both the intrinsic and extrinsic apoptotic pathways.

2.3 Purpose of Specific Aims

2.3.1 Specific Aim 1: Characterization of the individual effects of UVB exposure and apigenin treatment on human keratinocytes.

Human keratinocytes were exposed to various doses of UVB radiation and incubated to study the effect of UVB on COX-2 protein expression levels

and the dose necessary to induce apoptosis in each particular model. This aim also determined the ability of apigenin treatment to alter cell viability and induce apoptosis in human keratinocytes. Whereas UVB has been well documented as an apoptosis inducing agent, and the effect of apigenin treatment has been evaluated in many cell lines (Fotsis *et al.* 1997; Kuo and Yang 1995; Lee and Lin 1997; Shukla and Gupta 2004; Yin *et al.* 2001), many of the effects of UVB exposure and apigenin treatment on HaCaT keratinocytes and NHKs remain largely unknown. The data accumulated in this aim provided the framework for subsequent biological assays that were performed on keratinocytes treated with apigenin following UVB exposure.

2.3.2 Specific Aim 2: Investigate the combined effects of apigenin treatment on molecular events initiated by UVB exposure in human keratinocytes.

The ability to act as a complete carcinogen has led many to study the various biological effects of UVB exposure. Among the processes triggered by UVB exposure, two of the most actively investigated as potential chemo-preventive strategies are the induction of COX-2 activity and regulation of apoptosis in the skin (Akunda *et al.* 2007). Studies in our lab and others have demonstrated that exposure to UVB radiation increases the expression of COX-2 in mouse keratinocytes (Tong *et al.* 2007; Van Dross *et al.* 2007).

There is also clear evidence that inhibition of COX-2 enzymatic activity and reducing the formation of the downstream product PGE_2 provides protection from tumor development (Akunda *et al.* 2007; Tripp *et al.* 2003; Wilgus *et al.* 2000). Therefore, a better understanding of the ability of apigenin to modulate COX-2 protein expression and its enzymatic activity is needed to further explain the chemopreventive properties of apigenin.

Apoptosis of UV-damaged keratinocytes is critical to preventing skin cancer (Ziegler *et al.* 1994). Currently many are investigating the ability of various natural products including: sanguinarine (Reagan-Shaw *et al.* 2006), silibinin (Dhanalakshmi *et al.* 2005), curcumin (Park and Lee 2007), and caffeine (Koo *et al.* 2007; Lu *et al.* 2004) to prevent UVB-induced carcinogenesis by enhancing UVB-induced apoptosis. However, it is unknown whether the chemopreventive properties of apigenin can be explained by the ability to enhance UVB-induced apoptosis. In the current study, multiple biological assays, including Western blot analysis, annexin-V staining, and immunohistochemical staining were employed to evaluate the ability of apigenin to enhance UVB-induced apoptosis. The data obtained from these studies suggest that apigenin can exert its chemopreventive properties through enhancement of UVB-induced apoptosis in human keratinocytes. In addition these studies provided a foundation for the investigating the involvement of the extrinsic and intrinsic pathways of apoptosis (aim 3).

2.3.3 Specific Aim 3: Investigate the involvement of the intrinsic and extrinsic apoptotic pathways in the enhancement of UVB-induced apoptosis by apigenin treatment in human keratinocytes.

Studies conducted in the previous aim demonstrate that apigenin, like many other natural products, can enhance UVB-induced apoptosis in human keratinocytes. However, very little is known about which molecular events in particular are involved in the enhancement of UVB-induced apoptosis. Other researchers have attempted to explain the phenomenon of enhancement of UVB-induced apoptosis by the modulation of stress proteins such as ATM (ataxia talengectasia mutated), ATR (ATM and rad3-related) and DNA-PK (DNA-dependent protein kinase). Previous studies have concluded that apigenin activated ATM/ATR and the p38 stress kinase (O'Prey *et al.* 2003), however, the effect of apigenin treatment on mediators of the intrinsic and extrinsic pathway remain largely unknown. Immunofluorescence, Western blot analysis, and annexin-V staining were employed to further investigate the role of each apoptotic pathway in normal human keratinocytes, and genetically modified human keratinocytes. HaCaT keratinocytes were infected with retroviral vectors encoding genes for overexpression of Bcl-2, expression of FADDdn, or control vector (pBABE). Overexpression of Bcl-2 has been documented to protect against intrinsic apoptosis and thus provides insight regarding the ability of apigenin to enhance UVB-induced apoptosis by

potentiating the intrinsic apoptotic pathway. Expression of FADDdn limits the efficiency of the receptor-mediated, extrinsic pathway by reducing the functionality of the DISC, which is critical to activation of caspase-8. The studies conducted in this aim provide a possible explanation for the biological effects that are altered by apigenin treatment in human keratinocytes exposed to UVB radiation.

Chapter 3: Materials and Methods

3.1 Reagents and plasmids.

DMSO, apigenin, and staurosporine were purchased from Sigma Chemical Co., St. Louis, MO. TRAIL (TNF-Related Apoptosis Inducing Ligand, Apo2L, TL2, TNFSF10) was obtained from United States Biological (Swampscott, MA). Hexadimethrine bromide (polybrene) was purchased from Fisher Scientific (Hanover Park., IL). Epidermal Growth Factor (EGF) was purchased from Biomedical Technologies, Inc (Stoughton, MA). pBABE vector was purchased from Addgene, and the plasmid 1764 was donated by Dr. Robert Weinberg (M.I.T. Cambridge, MA). Human Bcl-2 overexpression vector in pBABE was a generous gift from Dr. Vincent Cryns (Northwestern University, Chicago, IL). The FADDdn—LZRS expression vector was a kind gift from Dr. Mitch Denning (Loyola University, Maywood, IL).

3.2 Cell Culture.

HaCaT cells, a spontaneously immortalized human keratinocyte cell line, were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) from GIBCO (Carlsbad, Ca) containing 10% heat-inactivated fetal bovine serum (GIBCO), supplemented with penicillin/streptomycin/L-glutamine (Invitrogen, Carlsbad, Ca). HaCaT cells were 85-90% confluent at the time of exposure to UVB or "sham" irradiation, followed by treatment with 0, 10, or 20 μM apigenin. HaCaT/pBABE-U6 and HaCaT/p53shRNA cells were kindly provided by Dr. Sam Lee (Massachusetts General Hospital and Harvard

Medical School, Charlestown, MA). The Lee laboratory created the HaCaT/pBABE-U6 and HaCaT/p53shRNA cell lines by stable transfection with empty pBabe-U6 vector or pBabe-U6 encoding p53 shRNA as described by Boswell et. al (Boswell *et al.* 2007). These cell lines were maintained in 1 μg/ml puromycin.

The Phoenix-Ampho retroviral packaging cells used to transduce HaCaT cells with retrovirus encoding FADDdn or overexpression of Bcl-2 were obtained from American Type Culture Collection (Manassas, VA) with permission from Dr. Gary P. Nolan (Stanford University Medical Center, Stanford, CA). The packaging cells were cultured in DMEM (GIBCO) supplemented with penicillin/streptomycin/L-glutamine (Invitrogen) and 10% heat-inactivated FBS (GIBCO). J2-3T3 fibroblasts were cultured in DMEM containing 10% heat-inactivated bovine serum (GIBCO) and gentamicin/amphotericin solution (Cascade Biologics, Portland, OR).

3.3 Isolation and culture of normal human keratinocytes (NHK)

Primary keratinocytes were isolated from normal human neonatal foreskin obtained from the Pathology Core Facility (Robert H. Lurie Comprehensive Center of Northwestern University) and stored in transfer medium Hank's Balanced Salt Solution (HBSS) without calcium and magnesium, containing FBS. Primary keratinocyte isolation was carried out within 48 hours of circumcision. Briefly, the foreskin was washed with PBS

and excess fat tissue and blood vessels were removed from the foreskin. The tissue was then floated in Dispase II (Roche Diagnostics, Chicago, IL), epidermal side up overnight at 4°C. The following morning, the epidermal layer of skin was separated from the dermis with forceps and placed in 0.25% trypsin/1mM EDTA (Invitrogen) for 10 minutes at 37°C 5% CO_2. Next, the tissue was mechanically agitated to dislodge cells followed by trypsin neutralization using serum. The tissue/cell suspension was then strained through a sterile 40 μm nylon cell strainer (BD Biosciences, San Diego, CA) and pelleted by centrifugation. The isolated primary keratinocytes were then washed with M154CF keratinocyte media containing 0.07mM $CaCl_2$, gentamicin/amphotericin solution, and human keratinocyte growth supplement (Cascade Biologics). Experiments on primary keratinocytes were conducted on cells at passage 3 or 4.

3.4   Preparation of human organotypic keratinocyte cultures.

Aliquots of 1 x 10^6 J2-3T3 fibroblasts were resuspended in 10 x reconstitution buffer and 10 x DMEM (Sigma) followed by addition of rat tail collagen I (BD Biosciences, Bedford, MA). Sodium hydroxide was added to the mixture until a pH of 7.4 was achieved. The collagen gel mixture containing fibroblasts was then dispensed into Falcon Cell Culture Inserts (pore size 3.0 μM), placed in BD Companion Plates (BD Biosciences), and allowed to polymerize by incubating at 37°C for 45 minutes. The

collagen/fibroblast gels were then cultured for 24 hours submerged in E-Media as described by Wu et al (Wu *et al.* 1982). Subconfluent monolayer cultures of primary keratinocytes were trypsinized and 1 x10^6 keratinocytes were resuspended in E-media containing 5ng/ml EGF and seeded on top of each collagen gel.   Keratinocytes form a monlayer sheet on top of the collagen/fibroblast matrix while cultured submerged in E-media for 2 days containing 5 ng/ml EGF with daily media changes. Media was then aspirated carefully, the Falcon inserts were placed into a BD BioCoat™ Deep-well Plate designed for use with 6-well size BD Falcon™ Cell Culture Inserts, and cultured at an "air/liquid interface" with daily media changes.

3.5   UVB exposure and apigenin treatment of keratinocytes.

Prior to UVB exposure, the culture medium was removed and reserved. Cells were washed with phosphate-buffered saline (PBS) twice, followed by either exposure to the dose of UVB radiation indicated, apigenin treatment or a combination of the UVB exposure followed by apigenin treatment.. UVB was provided by FS40T12-UVB lamps (National Biological Corporation, Twinsburg, OH) with peak emission at 313 nm. A Kodacel K6808 filter (Eastman Kodak, Rochester, NY) was used to filter out wavelengths of UVC (wavelengths below 295 nm). The reserved cell culture medium was then returned to the cell culture dish containing either apigenin, dimethyl sulfoxide (DMSO), or no compound followed by incubation at 37°C for the period of

time indicated. Apigenin (Sigma) stock solutions were prepared in DMSO and added to the reserved culture medium to achieve the desired final concentration. The concentration of DMSO in cell cultures was less than 0.1%. Experiments on human OTKC were optimized so that after 6 days of culturing at the air/liquid interface, the OTKC were exposed to UVB or "sham" irradiation, then incubated with 0, 25, or 50 μM apigenin for 24 hours, and cultures were harvested on day 7.

3.6   Western blot analysis

Cells were harvested, and lysed in Triton Lysis Buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 150 mM NaCl) containing protease and phosphatase inhibitor cocktails (Sigma). Whole cell lysates were collected and then sonicated 3 times for 3 seconds followed by centrifugation. Aliquots containing equal amounts of protein from samples were resolved by SDS-PAGE, transferred onto a PVDF membrane and blocked for 1 hour using 5% nonfat dry milk. The membranes were probed with primary antibodies for COX-2 (Santa Cruz Biotechnlology, Inc., Santa Cruz, CA), p53-FL-393 (Santa Cruz Biotechnology), phospho-Ser15-p53 (Cell Signaling, Danvers, MA), Caspase-3 (Cell Signaling Technology), PARP (Cell Signaling), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Chemicon (Temecula, CA) overnight at 4°C in 5% nonfat dry milk. Following incubation with appropriate horseradish peroxidase-conjugated secondary

antibodies, signals were detected with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

3.7   Histological analysis and immunohistochemistry

Organotypic human keratinocyte cultures and samples were fixed in 10% neutral-buffered formalin and were cut in three sections. The middle section was processed for histology and embedded lengthwise in paraffin to ensure a cross-section cut. Each 5 micron section was dewaxed and stained with hemotoxylin and eosin to evaluate tissue architecture and formation of sunburn cells, which are a hallmark of apoptotsis in the epidermis as described previously by Brash et al (Brash 1997). Sunburn cells can be identified in the epidermis as cells staining with a small, condensed pyknotic nucleus and highly eosinophilic (pink) cytoplasm. Quantitative assessment of keratinocytes staining positive for sunburn cell formation from each sample was performed by counting the number of positive cells per mm of tissue while blinded from the identity of the sample. Statistical significance was determined by paired t-test.

3.8   Cell Viability Assay

Cell viability was assessed in cells using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) in accordance with the manufacturer's instructions. Briefly cells were seeded in

sterile 96-well cell culture dishes for 24 hours, and then serum-starved overnight. The following day cells were treated with apigenin or DMSO (vehicle control) as indicated. Just before use, 100 μl of PMS solution was added to 2.0 ml of MTS reagent and mixed. Next, 20 μl of the PMS/MTS solution was added to each test well and incubated for 4 hours at 37°C, 5% CO_2. Absorbance was measured on a plate reader at 490 nm. Cell viability was then determined by comparing cell viability in the treated cultures to control (untreated) cultures. The untreated control samples were set at 100% viable.

3.9 Bromodeoxyuridine (BrdU) incorporation assay

NHKs were seeded in 96-well plates. The keratinocytes were then pre-treated with 0, 0.01, 0.1, 1.0, 10, 100, or 100 ng/ml PGE_2 for 1 hour, then incubated with medium containing PGE_2 and either 10ng/ml indomethacin or 15 μM apigenin for 18 hours. BrdU label was added during the last six hours of incubation to allow for incorporation into the cells. After 18 hours, the cells were fixed and permeabilized as directed by the manufacturer (Calbiochem, San Diego, CA), and assayed for BrdU incorporation.

3.10 COX-2 enzyme immunoassay (EIA)

Cox-2 enzyme activity was quantified using the COX-2 inhibitor screening assay as recommended by the manufacturer (Cayman Chemical,

Ann Arbor, MI). Briefly, assay buffer, heme, and COX-2 enzyme were added to each well, followed by the addition of DuP-697, apigenin or DMSO (solvent control) at the concentrations indicated. Next, a colorimetric substrate solution was added to each well, followed by the addition of arachidonic acid. The plate was incubated for 5 minutes at 25°C and then absorbance was read at 590 nm using a plate reader.

3.11   Prostaglandin E_2 (PGE_2)   enzyme linked immunosorbent assay (ELISA)

Keratinocytes were grown in 12-well plates to near confluence. After treatment, cell-free culture media were collected and prostaglandin E_2 (PGE_2) levels were determined by competitive enzyme-linked immunosorbent assay (ELISA), as directed by the manufacturer (Cayman Chemical Co., Ann Arbor, MI). The production of PGE_2 was normalized to protein concentrations.

3.12   Annexin-V apoptotic assay

Cells were exposed to UVB or "sham" irradiation followed by incubation with medium containing either apigenin or vehicle control (DMSO) as indicated. Apoptotic assays were then conducted per manufacturer's instructions. Briefly, cells were harvested by trypsinization and washed using PBS, followed by two washes in Annexin wash buffer (BD Biosciences). Apoptotic cells were labeled using Annexin-V-APC, and counter stained using

DAPI. Annexin-V-positive cells were measured using a Dako Cytomation CyAn flow cytometer (Flow Cytometry Core Facility, Northwestern University). The average of replicate experiments was used to determine statistical significance by paired t-test using Prism 3.0 software.

3.13 Immunofluorescence

To observe cytochrome c and Bax, keratinocytes were adhered to glass cover slips, exposed to 0 or 750 J/m^2 UVB, and incubated with 0, 10, or 20 μM apigenin for 16 hours. During the last 30 minutes of incubation, Mitotracker Red CMXRos (Invitrogen) was diluted in cell culture media to a final concentration of 500 nM. NHKs were fixed in 2% paraformaldehyde for 10 minutes at room temperature and then ice cold 90% methanol for 5 minutes. Cells were blocked in 5% goat serum (GIBCO) in PBS containing 0.02% saponin (Sigma) for Bax staining and 5% goat serum in PBS containing 0.01% Triton-X for cytochrome c staining. Cells were then incubated with primary antibody to cytochrome c (BD Biosciences) and Bax (Upstate Cell Signaling) at 1:100 dilution in blocking buffer. The appropriate Alexa Fluor-conjugated secondary antibody (Invitrogen) was used at 1:100 dilution in blocking buffer followed by mounting the cells on slides using Vectashield containing DAPI (Vector Laboratories, Burlingame, Ca). Cell staining was observed by fluorescence microscopy using a ZEISS axiovert

200man fluorescence microscope and photographed using an AxioCam HR camera.

3.14 Transduction of keratinocytes

Phoenix cells were transfected with 6 μg of either Bcl-2 overexpressing vector, FADDdn expressing vector, or control vector (pBABE vector) using lipofectamine 2000 (Invitrogen) in Opti-MEM media for 6 hours as directed by manufacturer. Cells were then incubated in DMEM overnight at 37°C. Phoenix cells were then selected for 2-3 days in media containing 1 μg/ml puromycin. Upon completion of selection, Phoenix cells were placed at 32°C overnight for optimal virus production and the supernatant was collected for subsequent infection. Keratinocytes were seeded at 100,000 cells/ well of a six-well dish. On the following day the retroviral supernatant was collected from the dishes and centrifuged to remove cell debris. Hexadimethrine bromide (polybrene; Sigma H-9268) was added to the viral supernatant to a final concentration of 4 μg/ml. Keratinocyte media was aspirated and 1.5 ml of viral supernatant was placed in each well being infected. The 6-well plates were wrapped in parafilm and centrifuged at 1300 rpm for 1 hour at 32°C. The viral supernatant was discarded post infection and fresh DMEM with FBS was added to the keratinocytes.

Chapter 4: Experimental Results

4.1   Characterization of the individual effects of UVB exposure and apigenin treatment on human keratinocytes.

4.1.1 Exposure to UVB radiation induces COX-2 activity in human keratinocytes

Previous studies in the Pelling lab demonstrated that UVB exposure induced COX-2 protein expression in 308 mouse keratinocytes (Tong *et al.* 2007; Van Dross *et al.* 2007). Induction of COX-2 protein expression was optimal 8 hours post UVB exposure. In order to examine the effects of UVB on a human keratinocyte cell line, HaCaT cells were exposed to 500 J/m^2 UVB or sham radiation, and then incubated for 8 hours at 37°C.   Cell lysates were collected and harvested for Western blot analysis. The membrane was probed using a primary antibody to COX-2. In agreement with previous findings, COX-2 protein expression was induced in HaCaT cells 8 hours post UVB exposure (Fig. 7).

4.1.2 Exposure to UVB radiation induces apoptosis in human keratinocytes

UVB is a known inducer of apoptosis in many cell lines, however we wanted to establish the UVB dose capable of producing moderate (but not

complete) levels of apoptosis in each of the three model systems used in this study. Each of the human keratinocyte models tested was susceptible to UVB-induced apoptosis, with sensitivity varying depending on the model. The dose of UVB required to induce intermediate levels of apoptosis in HaCaT cells was demonstrated by Western blot analysis to be 300 J/m^2 UVB. This dose produced modest levels of apoptosis in HaCaT cells 8 hours post-irradiation, as indicated by cleavage of PARP, a repair enzyme known to be cleaved during apoptosis (Fig. 8A).

Next, human primary keratinocyte cultures were exposed to various doses of UVB to establish the dose required to induce a moderate level of apoptosis in this system. Interestingly, testing of primary keratinocyte cultures isolated from different donors revealed varying levels of sensitivity to UVB radiation. A representative Western blot of whole cell lysates from primary human keratinocytes irradiated with 0, 250, 500, 750, 1000, 1250, or 1500 J/m^2 UVB is shown in Figure 8B. In the majority of experiments with primary human keratinocyte cultures, we observed PARP cleavage at moderate levels 24 hours after exposure to 750 J/m^2 UVB.

Lastly, we exposed human organotypic keratinocyte cultures (OTKC) to different doses of UVB (0-2000 J/m^2) to determine the dose required to induce moderate levels of apoptosis in this third model system. Our results of Western blot analysis for PARP cleavage (Fig. 9A) show that a slightly higher dose of UVB (1000 J/m^2) was required to induce moderate levels of apoptosis

in human OTKC compared to the dose capable of inducing moderate levels of apoptosis in the monolayer primary keratinocyte cultures (750 J/m^2, Fig. 8B). UVB-induced apoptosis can also be confirmed by the observation of sunburn cells and the presence of the active form of caspase-3 in the epidermis detected by immunohistochemical staining (Fig. 9B).

Keratinocytes cultured in an organotypic setting are often referred to as epidermal equivalents because they differentiate, thus mimicking the formation of basal, spinosum, granular and the stratum corneum layers that are observed in human skin. The stratum corneum formed during terminal differentiation serves to protect the skin from external assault. The barrier provided by the presence of multiple differentiated epidermal layers in this experimental model may account for the slightly higher dose of UVB necessary to induce moderate levels of apoptosis. It is of note that sensitivity to UVB-induced apoptosis in human OTKC derived from a specific human foreskin was correlated to the sensitivity of keratinocytes when they were exposed to UVB in monolayer culture (data not shown). Intermediate levels of apoptosis were observed in most human OTKC in response to 1000 J/m^2 UVB radiation (Fig. 9A). The results presented in this section provided us with a specific dose of UVB radiation capable of inducing a moderate level of apoptosis in each experimental model. The dose of UVB identified above for each model system was used in subsequent experiments to determine the ability of apigenin to enhance UVB-induced apoptosis.

4.1.3 *Apigenin treatment decreases cell viability in human keratinocytes by inducing apoptosis.*

The natural bioflavonoid apigenin is an anti-mutagenic, anti-carcinogenic compound that has been documented to trigger apoptosis in many cell lines. In order to test the effects of apigenin on cell viability MTS assays were conducted on HaCaT cells. Incubation of HaCaT keratinocytes with 5, 10, 15, 20, or 25 μM apigenin for 24 hours reduced cell viability by 21%, 35%, 42%, 51%, and 57 % respectively (Fig. 10A). This decrease in cell viability led us to explore the possibility that apigenin decreased cell viability by induction of apoptosis.

To assess the ability of apigenin to induce apoptosis we incubated HaCaT keratinocytes with 0, 5, 12.5, 25, 37.5, or 50 μM apigenin for 24 hours. The cells were then harvested and analyzed by Western blot analysis for cleavage of poly-ADP-ribose polymerase (PARP) and caspase-3 (Fig. 10B). The membrane was also probed for actin as a loading control. Apoptosis, as indicated by PARP cleavage and decrease in pro-caspase-3, was observed at concentrations of apigenin as low as 12.5 μM. Simultaneously, the cleaved product of PARP and caspase-3 appeared, indicating that apigenin induced apoptosis in HaCaT keratinocytes. Apigenin induced apoptosis in a concentration-dependent manner as evidenced by the progressive increase in

PARP and caspase cleavage in HaCaT cells treated with 25, 37.5 or 50 μM apigenin (Fig 10B).

4.1.4 Apigenin reduces cell proliferation and stimulates apoptosis independent of prostaglandin E$_2$ (PGE$_2$) signaling.

In order to gain a better understanding of the mechanism by which apigenin reduced cell viability and induced apoptosis, I explored the possibility that apigenin was altering PGE$_2$ signaling pathways. PGE$_2$ stimulates cell proliferation. Konger et al. has demonstrated that pre-treatment with PGE$_2$ protected human keratinocytes from indomethacin-induced reduction in cell proliferation (Konger *et al.* 1998). Here, NHKs were pre-treated with 0, 0.1, 1, 10, 100, or 1000 ng/ml PGE$_2$ for 1 hour and then incubated with either 10 ng/ml indomethacin or 15 μM apigenin for 18 hours as described in the Materials and Methods section. Indomethacin was used as control, which has been shown to reduce cell proliferation in a PGE$_2$-dependent fashion (Konger *et al.* 1998). PGE$_2$ was able to restore proliferation in keratinocytes treated with 10 ng/ml indomethacin, but was unable to restore proliferation in keratinocytes treated with 15 μM apigenin (Fig. 11A). In agreement with the above findings, pre-incubation with 0.1, 1, or 10 ng/ml PGE$_2$ was not able to protect against apigenin-induced apoptosis in primary human keratinocyte cultures (Figure 11B). Collectively, these

findings demonstrate that apigenin reduces cell proliferation independent of PGE_2 signaling, and is consistent with our conclusion that apigenin-induced apoptosis also occurs independent of PGE_2 signaling.

4.1.5   Apigenin is not an enzymatic inhibitor of COX-2 activity

Previous work by Dr. Van Dross in the Pelling laboratory has demonstrated that apigenin treatment down-modulated UVB-induced COX-2 protein expression in mouse keratinocytes (Van Dross *et al.* 2007). In order to further investigate the possibility that apigenin acts as an enzymatic inhibitor of the COX-2 enzyme itself, a COX inhibitor screening assay was used which measures COX-2 dependent production of prostaglandins. The ability to inhibit prostaglandin production was assessed by adding apigenin or the known COX-2 inhibitor DuP-697 to a test tube. The reaction was initiated by the addition of the enzymatic target of COX-2, arachidonic acid. DuP-697 clearly inhibited COX-2 enzymatic activity in a concentration-dependent manner. Whereas apigenin has been shown to reduce both basal and induced COX-2 protein expression, incubation with apigenin had little effect on the production of prostaglandins (Fig. 12), demonstrating that apigenin is not a traditional enzymatic inhibitor of COX-2 and that apigenin has other molecular targets that lead to reduced COX-2 protein expression.
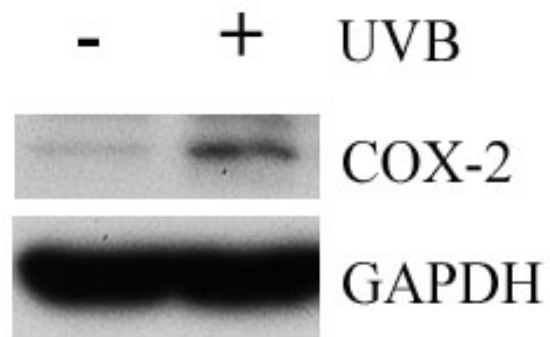
Figure 7: UVB induces COX-2 protein in HaCaT Keratinocytes.
HaCaT keratinocytes were irradiated with 500 J/m^2 UVB and then incubated for 8 hours. The cells were harvested and probed with primary antibodies to COX-2 and GAPDH for Western blot analysis.

Figure 8: UVB-induced apoptosis in human keratinocytes.

Cell lysates were harvested and PARP cleavage was detected using a monoclonal PARP antibody to assess apoptosis induced by UVB exposure. Membranes were stripped and reprobed for GAPDH as a loading control. (A) HaCaT cells were grown to 80-90% confluence and then exposed to 0, 300, 500, 750, or 1000 J/m^2 UVB. The cells were incubated for 8 hours. (B) Normal human keratinocyte cultures were grown to 60% confluence, exposed to 0, 250, 500, 750, 1000, 1250, or 1500 J/m^2 UVB and then incubated for 24 hours.

A
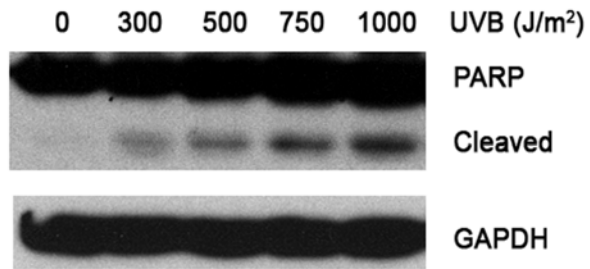
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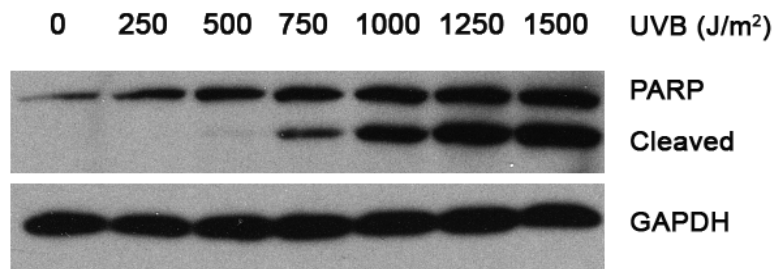
Figure 9: UVB-induced apoptosis in human organotypic keratinocyte cultures (OTKC).

(A) Human OTKC were cultured at the air/liquid interface for 6 days and then exposed to 0, 1000, 1500, or 2000 J/m^2 UVB and incubated for 24 hours. The epidermis was removed from the dermis with forceps and was lysed in urea sample buffer. (B) Human OTKC were cultured for 6 days at the air/liquid interface then exposed to 1000 J/m^2 UVB or sham radiation and incubated for 24 hours at 37°C. The OTKC tissue was then fixed and processed for histopathologic staining with hematoxylin and eosin or an antibody to active caspase-3. Arrows indicate the presence of apoptotic cells.

A
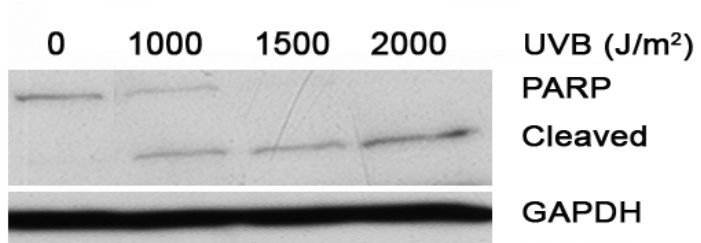
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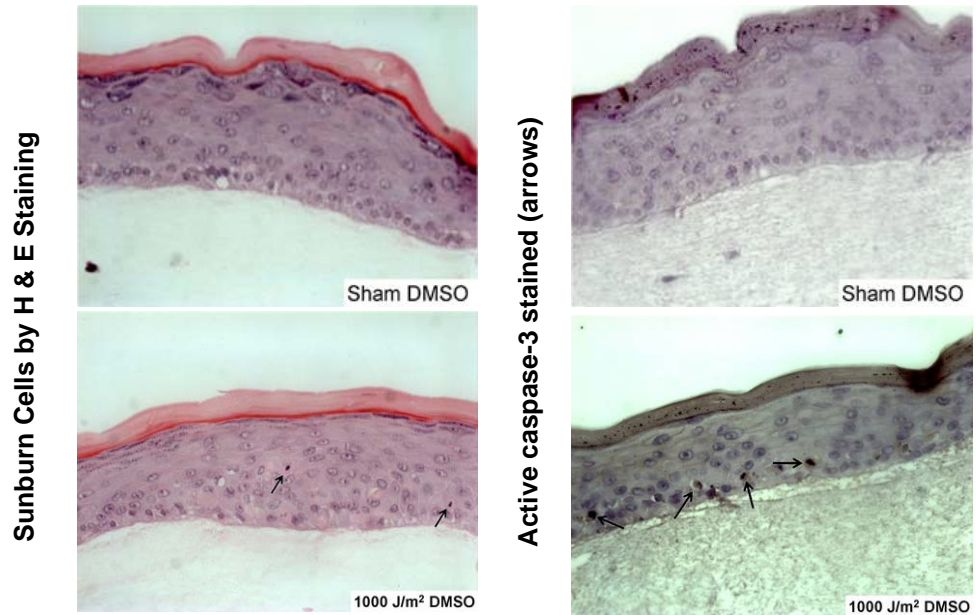
Figure 10: Apigenin decreases cell viability by inducing apoptosis.

(A) HaCaT cells were plated at 15,000 cells per well in a 96 well dish and treated with 0, 5, 10, 15, 20, or 25 μM apigenin for 24 hours. Cell viability was evaluated by MTS assay and plotted against control (0μM Apigenin) treatment. (B) Apigenin induced apoptosis in a concentration-dependent manner. HaCaT cells were incubated with 0, 5, 12.5, 25, 37.5, or 50 μM apigenin for 24 hours and harvested for Western Blot analysis using primary antibodies to PARP, Pro-caspase-3, Cleaved caspase-3 and Actin.

A
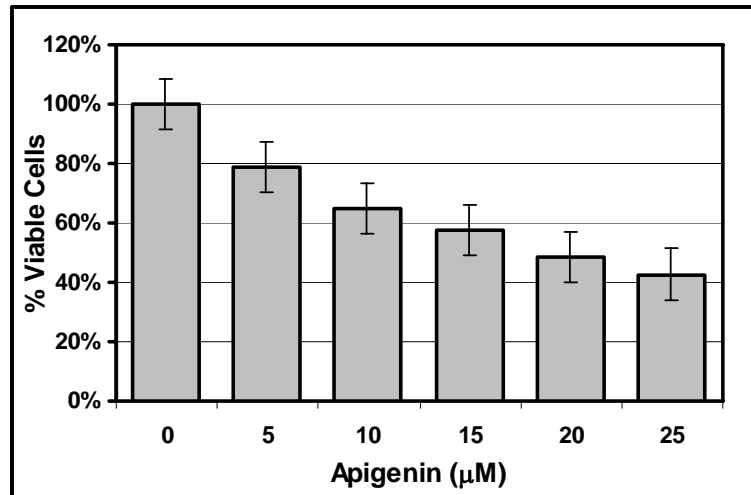
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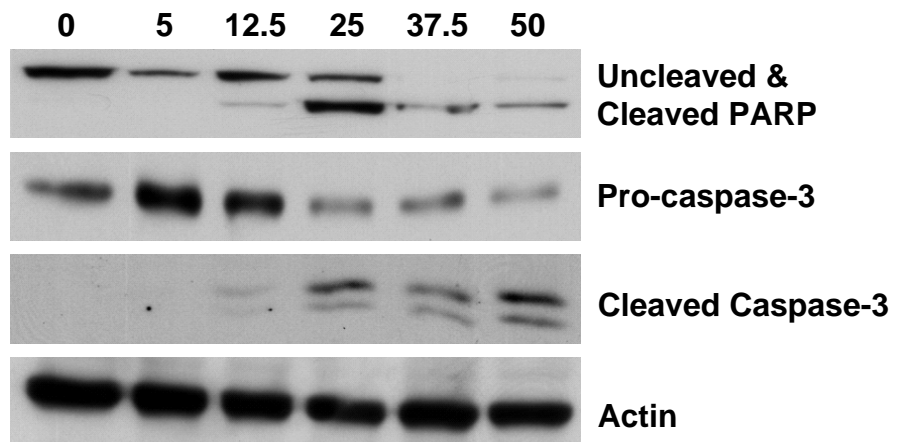
Figure 11: Apigenin reduced cell proliferation and induced apoptosis independent of PGE$_2$ signaling.

(A) NHKs were pre-treated with 0, 0.01, 0.1, 1.0, 10, 100, or 100 ng/ml PGE$_2$ and then incubated with either 10ng/ml indomethacin or 15 μM apigenin for 18 hours and assayed for BrdU incorporation. (B) Keratinocytes were pre-treated with either 0, 0.1, 1.0, or 10 ng/ml PGE$_2$ and then treated for 8 hours with 15 μM apigenin. Cell lysates were collected and analyzed by Western blot using antibodies to PARP and GAPDH.

A
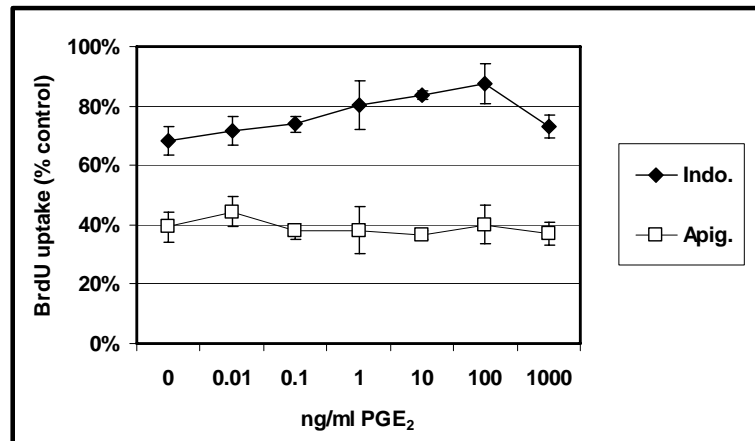
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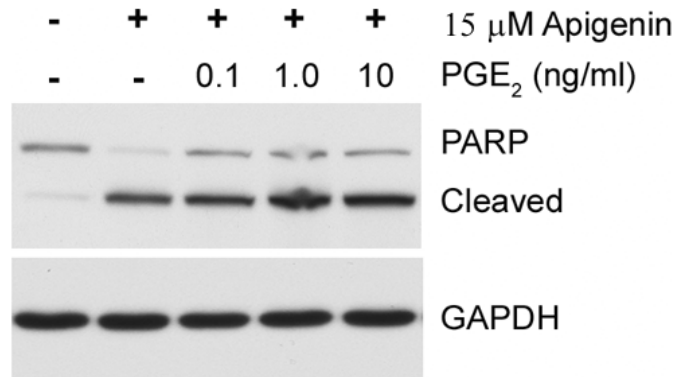
Figure 12: Apigenin is not an enzymatic COX-2 inhibitor.

The ability of apigenin to inhibit COX-2 enzymatic activity was measured by determining the colorimetric change correlated to conversion of arachidonic acid into prostaglandins by COX-2. Samples were read on a plate reader at 590 nm. The average of three independent experiments is shown.
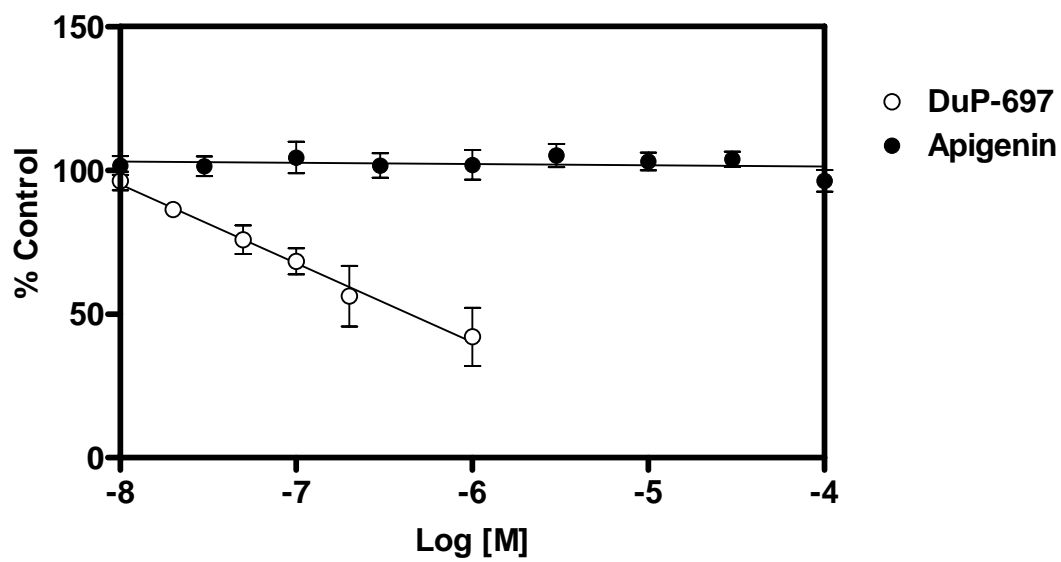
4.2 Investigate the combined effects of apigenin treatment on molecular events initiated by UVB exposure in human keratinocytes.

4.2.1 Apigenin treatment of UVB-irradiated keratinocytes down-modulates UVB-induced COX-2 activity.

Experiments conducted in the previous aim demonstrated that UVB induced COX-2 protein expression, as shown by Western blot analysis, in HaCaT cells. Next, HaCaT cells were exposed to either 500 J/m^2 or sham radiation, then incubated with 0, 15, or 50 μM apigenin for 8 hours at 37°C. Upon completion of incubation, the cells were harvested and equal amounts of protein from each sample were analyzed by Western blot using primary antibodies to COX-2 and GAPDH (Fig. 13A). Apigenin treatment inhibited UVB-induction of COX-2 protein expression, with 50μM apigenin reducing the COX-2 protein level to that in cells exposed to "sham" radiation. These findings, taken together with those in the previous aim demonstrate that apigenin modulates COX-2 protein expression.

Induction of COX-2 signaling and subsequent conversion of arachidonic acid into prostaglandins has been shown to be involved in carcinogenesis of many cancers (Chun *et al.* 2007; Han *et al.* 2007; Yiu and Toker 2006). The most abundantly produced prostaglandin produced in the

skin is prostaglandin E_2 (PGE_2) (Chun *et al.* 2007). To investigate the affect of UVB on PGE_2 production and the ability of apigenin to alter the production of PGE_2, keratinocytes were exposed to UVB or sham radiation and then incubated in cell culture medium containing 0 or 50 μM apigenin for 8 hours at 37°C. Cell culture medium was then collected and used to detect PGE_2 production by ELISA. The ability of UVB to induce COX-2 protein expression suggests that an increase in the production of PGE_2 would occur post exposure to UVB. The results presented in Figure 13B show that PGE_2 production increased nearly 10-fold in keratinocytes exposed to UVB. Furthermore, a decrease in the production of PGE_2 was observed when keratinocytes were incubated with 50 μM apigenin compared to cells exposed to keratinocytes vehicle control (Fig. 13B). Whereas apigenin is not an enzymatic inhibitor of COX-2, the results presented herein are consistent evidence that apigenin inhibits COX-2 protein expression, with subsequent reduction in the production PGE_2.

The effect of apigenin treatment on signaling molecules that target COX-2 expression at the transcriptional and post-translational level are the focus of other investigators in the Pelling lab. Briefly, studies by Dr. Van Dross demonstrate that one pathway by which apigenin inhibits COX-2 expression is through modulation of upstream stimulatory factor (USF) transcriptional activity (Van Dross *et al.* 2007). Further studies by Dr. Xin Tong in the Pelling lab demonstrated that in addition to transcriptional

regulation, apigenin also prevents COX-2 expression through mediation of T-cell-restricted intracellular antigen 1-related protein (TIAR) suppression of translation (Tong *et al.* 2007).

4.2.2 UVB-induced apoptosis is enhanced by apigenin treatment in HaCaT keratinocytes.

The ability of apigenin treatment to enhance UVB-induced apoptosis was first examined in human HaCaT cells. HaCaT cells were exposed to 0 or 300 J/m^2 UVB radiation followed by incubation with 0, 10, or 20 μM apigenin for 8 hours. Whole cell lysates were collected and analyzed by Western blot analysis for extent of PARP cleavage. Apigenin treatment enhanced the extent of UVB-induced apoptosis in HaCaT keratinocytes as quantified by PARP cleavage (Fig. 14A) compared to UVB exposure alone. Because Western blot analysis for PARP cleavage is difficult to quantify, flow cytometry was used to determine the percentage of annexin-V positive staining cells per 10,000 events as a second method to measure apoptosis. HaCaT cells were exposed to UVB or sham radiation and then incubated with 0, 10, or 20 μM apigenin. The cells were then harvested and stained with annexin-V to assess the percent of apoptotic cells. Apoptosis increased 1.8- and 2.2-fold in UVB-irradiated HaCaT cells treated with 10 and 20 μM apigenin ($P < 0.05$),

respectively, compared to UVB exposure alone, demonstrating the ability of apigenin to enhance UVB-induced apoptosis in HaCaT cells.

4.2.3 Enhancement of UVB-induced apoptosis by apigenin treatment occurs independent of p53 status.

HaCaT cells possess a mutant, low-functioning p53 with an increased p53 protein half-life (Boswell *et al.* 2007 and references therein). Therefore, we investigated whether the mutant p53 protein present in HaCaT cells modulated the enhancement of UVB-induced apoptosis by apigenin treatment. We used a stable HaCaT cell line in which p53 expression was ablated with shRNA targeting p53 [kindly provided by Dr. Sam Lee (Boswell *et al.* 2007)], to investigate whether or not the increase in apoptosis in apigenin-treated, UVB-irradiated HaCaT cells was p53-dependent. HaCaT/p53shRNA cells were exposed to UVB or sham radiation, incubated in medium containing 0 or 20 μM apigenin for 8 hours, and then harvested for Western blot analysis. The results shown in Figure 15A confirm that p53 has been ablated in two different HaCaT clones (p53shRNA clone 15 and p53shRNA clone 22). As expected, Western blot analysis of HaCaT/pBABE-U6 (vector control) cells demonstrated the presence of total p53 protein (Fig. 15A). Cell lysates from UVB-irradiated, apigenin-treated, and UVB + apigenin-treated HaCaT/pBABE-U6 cells, exhibited elevated levels of

phospho-ser15 p53 compared to untreated HaCaT/pBABE-U6 cells (Fig. 15A). In both HaCaT/p53shRNA clones (see lanes 8 and 12) and in HaCaT/pBABE-U6 cells (see lane 4) we observed enhancement of UVB-induced apoptosis by apigenin treatment as evidenced by increased PARP cleavage (Fig. 15A). Apoptosis was also assessed by measuring the percent of annexin-V positive cells by flow cytometry (Fig. 15B). Apigenin treatment enhanced UVB-induced apoptosis in both clone 15 and clone 22 of HaCaT/p53shRNA cells, as well as in HaCaT/pBABE-U6 cells. These findings demonstrate that enhancement of UVB-induced apoptosis by apigenin treatment occurs via a p53-independent pathway.

4.2.4 UVB-induced apoptosis is enhanced by apigenin treatment in normal human keratinocytes (NHKs).

The ability of apigenin to enhance UVB-induced apoptosis in multiple preparations of primary human keratinocyte cultures was tested in order to rule out the possibility that apigenin treatment enhanced UVB-induced apoptosis only in HaCaT keratinocytes. Primary keratinocyte cultures demonstrating moderate levels of apoptosis when irradiated with 750 J/m^2 UVB were used to evaluate the ability of apigenin to enhance UVB-induced apoptosis. Primary human keratinocytes were exposed to 0 or 750 J/m^2 UVB then incubated with medium containing 0, 10, or 20 μM apigenin for 24 hours,

and harvested for Western blot analysis. Apigenin treatment of UVB-irradiated keratinocytes resulted in increased apoptosis as measured by PARP cleavage (Fig. 16A), compared to UVB alone. To generate quantitative values for the extent of enhancement of UVB-induced apoptosis in primary keratinocytes treated with apigenin, the cells were harvested and stained with annexin-V and quantified the percentage of apoptotic events by flow cytometry. UVB-irradiated cells incubated with 10 and 20 μM apigenin exhibited a respective 1.9- and 2.7-fold increase in apoptosis (P < 0.05), compared to UVB exposure alone (Fig. 16B). The results presented in this section demonstrate that apigenin treatment leads to enhancement of the apoptotic response initiated by UVB exposure in primary human keratinocyte monolayer cultures.

4.2.5 UVB-induced apoptosis is enhanced by apigenin treatment in human organotypic keratinocyte cultures.

Numerous studies have been conducted on the effectiveness of chemopreventive compounds in monolayer cell culture. Here, we cultured primary human keratinocytes isolated from neonatal foreskin in an organotypic setting to more accurately predict the effect of apigenin treatment on human skin exposed to UVB. Human organotypic keratinocyte cultures (OTKC) were exposed to 0 or 1000 J/m^2 UVB, then incubated with 0, 25, or

50 μM apigenin for 24 hours. The epidermal layer was then separated from the dermal fibroblast collagen matrix, and tissue lysates were analyzed by Western blot. The data presented in Figure 17A show that human OTKC irradiated with 1000 J/m^2 UVB followed by treatment with 50 μM apigenin exhibited an increased level of cleaved PARP compared to UVB alone. Treatment of human OTKC with 25 μM apigenin had a negligible effect on UVB-induced apoptosis.

To further evaluate the extent of apoptosis in organotypic human keratinocyte cultures, samples were exposed to 0 or 1000 J/m^2 UVB followed by treatment with 0, 25, or 50 μM apigenin. OTKC samples were fixed in 10% neutral buffered formalin, sectioned and stained using hematoxylin and eosin to evaluate architecture of the human OTKC and the presence of apoptotic sunburn cells, which are characterized by a pyknotic nucleus and highly eosinophilic (pink) cytoplasm. Representative sections of human OTKC tissue exposed to 0 or 1000 J/m^2 in combination with 0, 25, or 50 μM apigenin are shown in Figure 17B with sunburn cells indicated by arrows. Apoptotic sunburn cells in human OTKC were counted and then averaged as described in the Materials and Methods section to assess the level of enhancement of UVB-induced apoptosis in human OTKC (Fig. 17C). In agreement with the Western blot data (Fig. 16A), quantitative analysis of the data collected showed that treatment with 50 μM apigenin after UVB exposure resulted in a 2.4-fold (P < 0.01) increase in apoptosis. Treatment with 25 μM apigenin

treatment had a negligible effect on UVB-induced apoptosis (1.1-fold change, P value > 0.5). These findings are the first to demonstrate enhancement of UVB-induced apoptosis by apigenin treatment of human OTKC.

Figure 13: Apigenin down-modulates UVB-induced COX-2 acitivity.

(A) Keratinocytes were exposed to UVB or sham radiation and then treated with 0, 15, or 50 μM apigenin. Samples were analyzed by Western blot using primary antibodies to COX-2 and GAPDH. (B) Keratinocytes were exposed to sham or UVB radiation then treated with 0 or 50 μM apigenin and incubated for 8 hours. PGE_2 levels in the culture medium were measured by competitive ELISA.

A

−	+	−	+	−	+	500 J/m²
0	0	15	15	50	50	Apigenin (µM)

COX-2

GAPDH

B

75
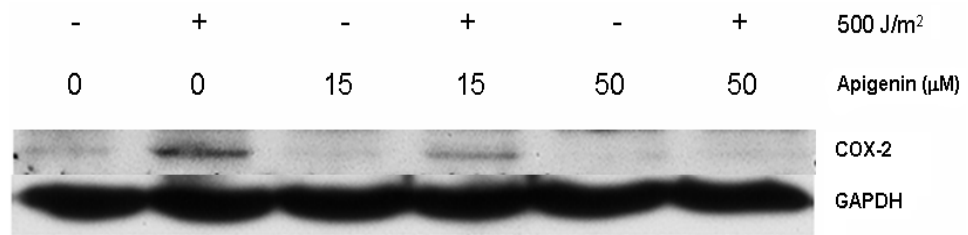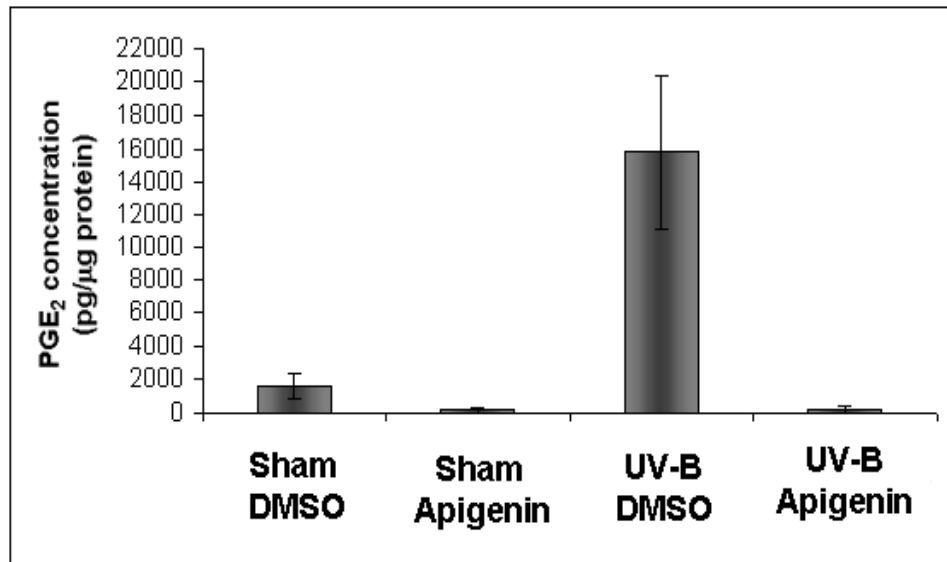
Figure 14: Apigenin treatment enhances UVB-induced apoptosis in HaCaT cells.

(A) HaCaT cells were exposed to 0 or 300 J/m^2 UVB, and incubated with 0, 10, or 20 μM apigenin for 8 hours. A representative Western blot measuring apoptosis by PARP cleavage is shown. An antibody to GAPDH was used as a loading control. (B) HaCaT cells were exposed to 300 J/m^2 UVB or sham radiation followed by incubation with 0, 10, or 20 μM apigenin for 8 hours. Cells (5 x 10^5) were stained with annexin-V and 10,000 events were counted by flow cytometry. An increase in the percent of annexin-V positive cells indicate increased apoptosis. Columns represent mean values (n=3); Bars represent ± SE. * represents P < 0.05, compared to UVB exposure alone.

A

B

HaCaT Keratinocytes

77
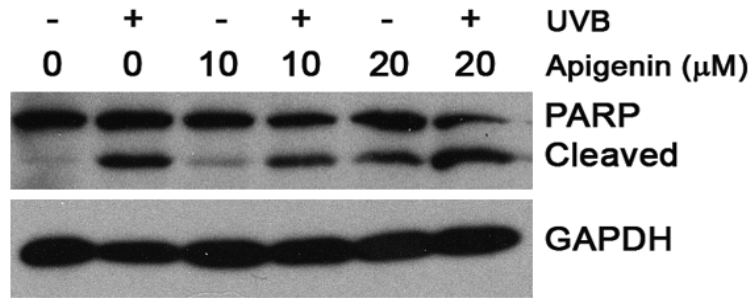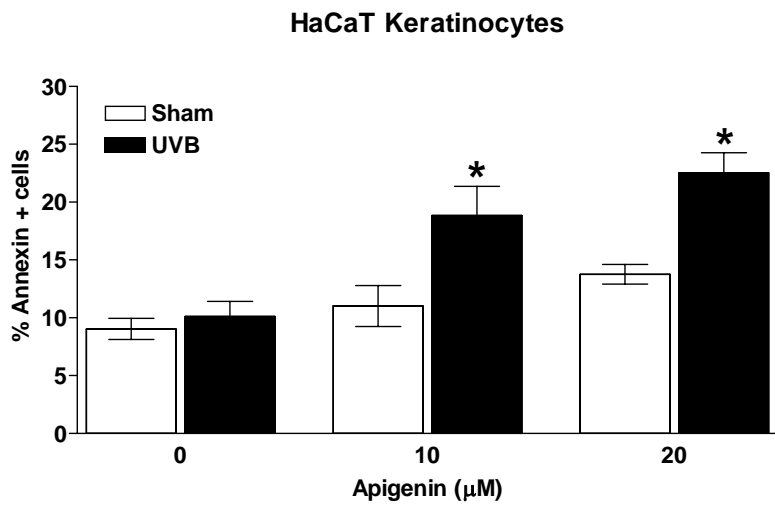
Figure 15: Apigenin treatment enhances UVB-induced apoptosis independent of p53 status.

(A) HaCaT cells stably transfected with either a pBabe-U6 vector expressing p53 shRNA (clones 15 and 22 are shown) or empty control vector were exposed to 0 or 300 J/m^2 UVB, and incubated with 0 or 20 μM apigenin for 8 hours. A representative Western blot measuring apoptosis by PARP cleavage, phosphorylated serine-15 p53 (p-Ser15 p53), total p53, and GAPDH (loading control) is shown. (B) The percentage of apoptosis in HaCaT cells stably transfected with either a pBabe-U6 vector expressing p53 shRNA (clones 15 and 22 are shown) or empty control vector exposed to 300 J /m^2 UVB or sham radiation followed by incubation with 0 or 20 μM apigenin for 8 hours was quantified by annexin-v staining. Cells (5 x 10^5) were stained with annexin-V and 10,000 events were counted by flow cytometry. An increase in the percent of annexin-V positive cells indicates increased apoptosis. Columns represent mean values (n=4); Bars represent ± SE. * represents P < 0.05, compared to UVB exposure alone.

A
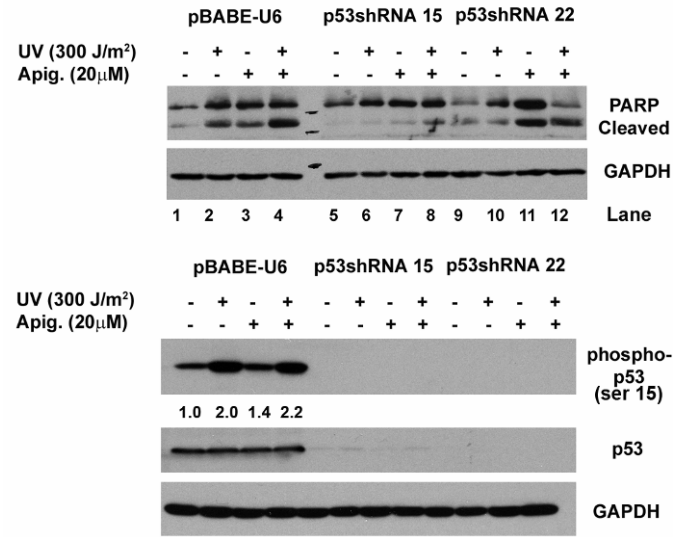
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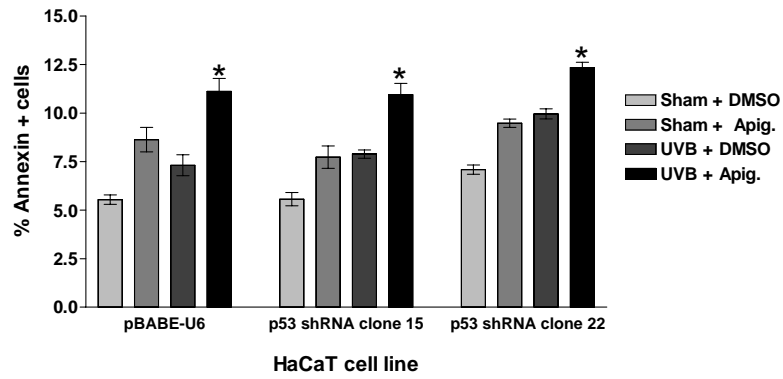
Figure 16: Apigenin enhances UVB-induced apoptosis in NHKs

(A) NHKs were exposed to 0 or 750 J/m^2 UVB and then incubated for 24 hours with 0, 10, or 20 μM apigenin. Apoptosis was evaluated by assessing PARP cleavage by Western blot analysis using a monoclonal antibody to PARP. The membranes were stripped and reprobed for GAPDH as a loading control. (B) NHKs were exposed to 750 J/m^2 UVB or sham radiation followed by incubation with 0, 10, or 20 μM apigenin for 24 hours. Cells (5 x 10^5) were stained with annexin-V and 10,000 events were counted by flow cytometry. Increased annexin-V positive cells indicate increased apoptosis. Columns, mean (n=3, using keratinocytes isolated from 3 different donors); bars, SE. *, $P < 0.05$, compared to UVB exposure alone.

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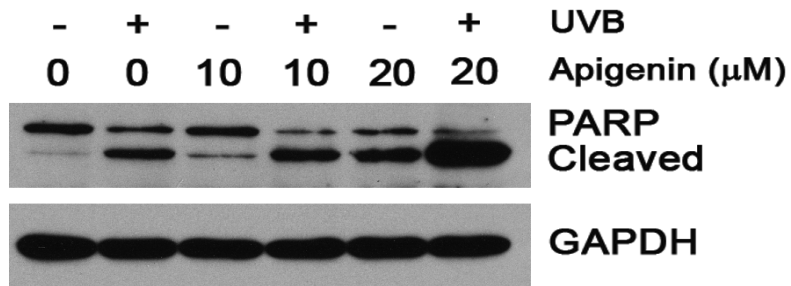
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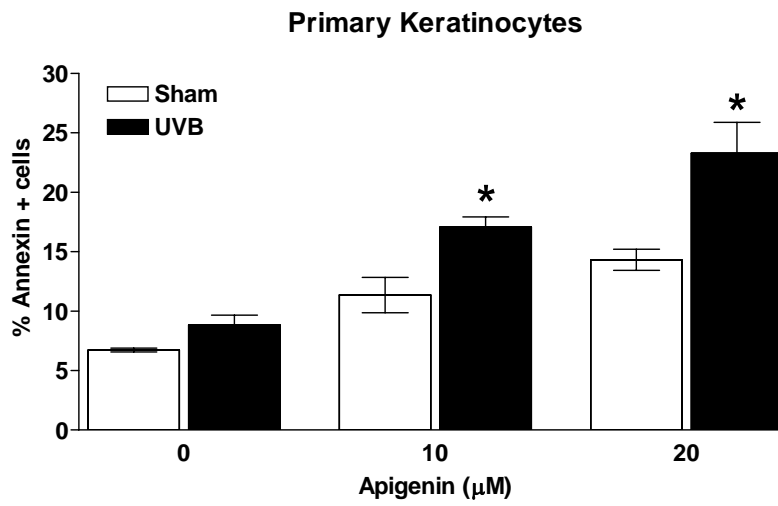
Figure 17: Apigenin enhances UVB-induced apoptosis in human organotypic keratinocyte cultures (OTKC).

(A) Human OTKC were exposed to 0 or 1000 J/m^2 UVB, incubated with 0, 25, or 50 μM apigenin, and harvested after 24 hours. A Western blot for PARP cleavage and GAPDH is shown. (B) Human OTKC were exposed to 1000 J/m^2 UVB or sham radiation, treated with 0, 25, 50 μM apigenin for 24 hours, and then fixed and processed for histopathologic staining with hematoxylin and eosin. Arrows indicate the presence of apoptotic sunburn cells. Bars, 50 μm. (C) The average number of sunburn cells counted per mm of tissue is depicted in the graph. Columns represent mean (n=3, using keratinocytes isolated from 3 different donors); bars, SE. *, P < 0.05, compared to UVB exposure alone.

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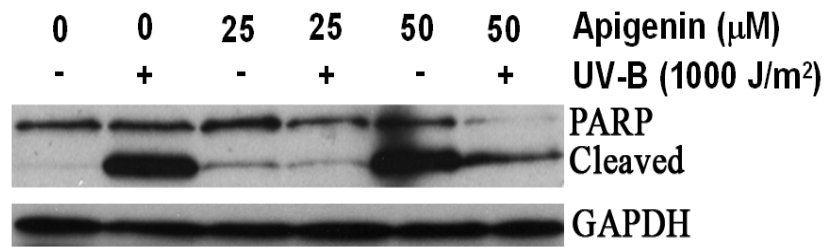
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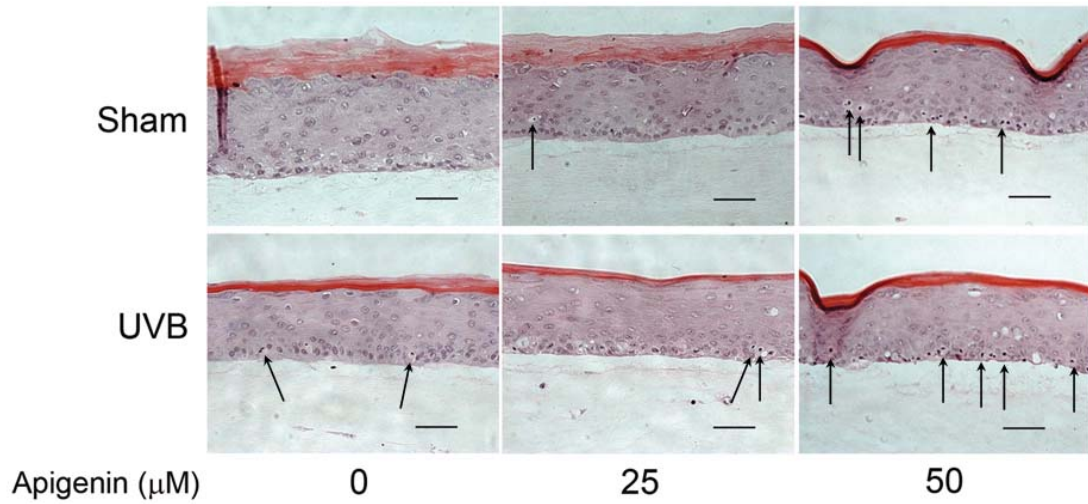
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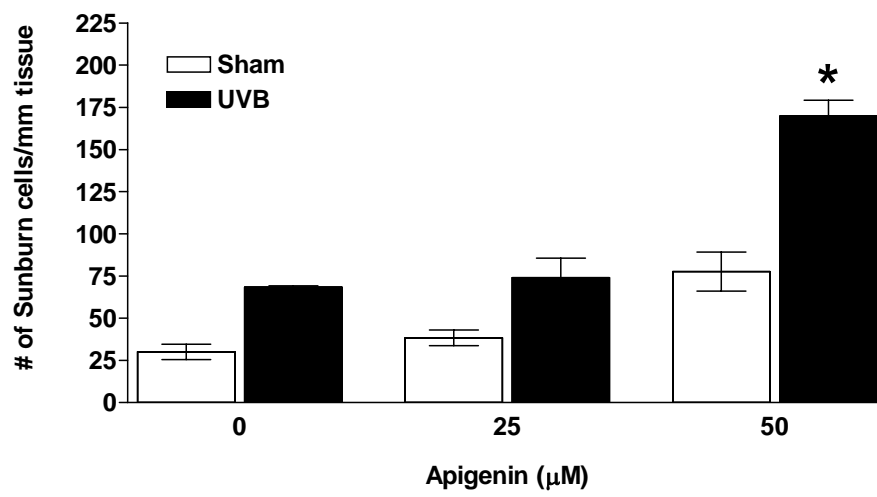
4.3 Investigate the involvement of the intrinsic and extrinsic apoptotic pathways in the enhancement of UVB-induced apoptosis by apigenin treatment in human keratinocytes.

4.3.1 Apigenin treatment increases Bax localization in human keratinocytes that have been exposed to UVB radiation

In the present report, we have employed a number of approaches to delineate whether apigenin enhancement of UVB-induced apoptosis in keratinocytes is occurring through the intrinsic pathway, extrinsic pathway, or both. Indirect immunofluorescence was used to define the intracellular events altered by apigenin treatment of UVB-irradiated keratinocytes. First, the effect of UVB and/or apigenin treatment on the localization of Bax in primary keratinocytes was assessed. As seen in Figure 18, Bax is uniformly distributed in keratinocytes exposed to "sham" radiation, followed by treatment with 0, 10, or 20 μM apigenin. Bax staining was more distinct and appeared more punctuate in keratinocytes exposed to UVB compared to sham-irradiated keratinocytes. A marked increase in the appearance of punctate, localized staining of Bax was observed in Primary keratinocytes exposed to UVB, then incubated with 10 or 20 μM apigenin compared to

primary keratinocytes exposed to UVB then incubated with medium containing DMSO (vehicle control).

4.3.2 Apigenin treatment of human keratinocytes exposed to UVB radiation facilitates release of cytochrome c from the mitochondria.

To evaluate whether the alterations in Bax localization lead to the release of cytochrome c, we exposed primary human keratinocytes to 0 or 750 J/m^2 UVB radiation, then treated with 0, 10, or 20 μM apigenin, and stained the cells with an anti-cytochrome c antibody. When primary keratinocytes were exposed to 750 J/m^2 UVB, followed by incubation with 0, 10, or 20 μM apigenin, cytochrome c staining appeared more diffuse in a concentration-dependent manner, suggesting cytochrome c was being released from the mitochondria (Fig. 19). No change in cytochrome c staining was observed in the "sham"-irradiated keratinocytes treated with 0, 10, or 20 μM apigenin. The above findings suggest that apigenin treatment of UVB-irradiated cells enhanced localization of Bax to the mitochondria, which stimulated cytochrome c release and initiation of the intrinsic pathway of apoptosis.

4.3.3 Overexpression of the anti-apoptotic protein Bcl-2 in human keratinocytes protects human keratinocytes from the ability of apigenin to enhance UVB-induced apoptosis.

To investigate further how apigenin enhances UVB-induced apoptosis, keratinocytes were infected with retrovirus encoding an overexpressing form of the anti-apoptotic protein Bcl-2. Transduced keratinocytes were exposed to UVB or "sham radiation" followed by treatment with 0, 10, or 20 μM apigenin, then harvested for annexin-V staining by flow cytometry. Overexpression of Bcl-2 significantly reduced the level of apoptosis in each treatment group compared to cells transduced with control vector (Fig. 20). When Bcl-2 overexpressing keratinocytes were exposed to UVB radiation and treated with 0, 10, or 20 μM apigenin, we observed a 42, 52, and 42% reduction in apoptosis, respectively, compared to vector controls. Therefore, our results support our hypothesis that apigenin is enhanceing UVB-induced apoptosis by targeting the intrinsic apoptotic pathway.

4.3.4 Expression of a dominant negative FADD (FADDdn) in human keratinocytes protects from the ability of apigenin to enhance UVB-induced apoptosis.

The above studies have demonstrated that the intrinsic pathway of apoptosis mediates the ability of apigenin to enhance UVB induced apoptosis. In order to explore the possibility that the extrinsic apoptotic pathway is also stimulated by apigenin treatment of UVB-irradiated keratinocytes, we infected keratinocytes with a retroviral construct coding for FADDdn. The level of apoptosis in FADDdn keractinocytes exposed to UVB- or sham-irradiation followed by incubation with 0, 10, or 20 μM apigenin was measured by quantifying the percentage of cells staining positive for annexin-V by flow cytometry. The percent of annexin-V positive cells was lower in FADDdn keratinocytes than in vector controls for all treatment groups (Fig. 21). FADDdn keratinocytes were more resistant to enhancement of UVB-induced apoptosis when treated with 0, 10, or 20 μM apignein. The percentage of apoptotic cells changed very little when exposed to UVB followed by 20 μM apigenin treatment compared to "sham"-irradiated DMSO-treated FADDdn keratinocytes. Collectively, our results imply that enhancement of UVB-induced apoptosis by apigenin treatment involves activation of the intrinsic pathway, and potentiation of the receptor-mediated extrinsic apoptotic pathway.

Figure 18: Apigenin stimulates Bax oligomerization in keratinocytes exposed to UVB radiation.

NHKs were adhered to glass coverslips overnight, then exposed to 750 J/m^2 UVB or sham radiation followed by treatment with 0, 10, or 20 μM apigenin for 16 hours. Cells were fixed and immunostained for Bax. Arrows point to apoptotic cells. Bars, 50 μm.

Sham

UVB

Apigenin(μM)   0   10   20
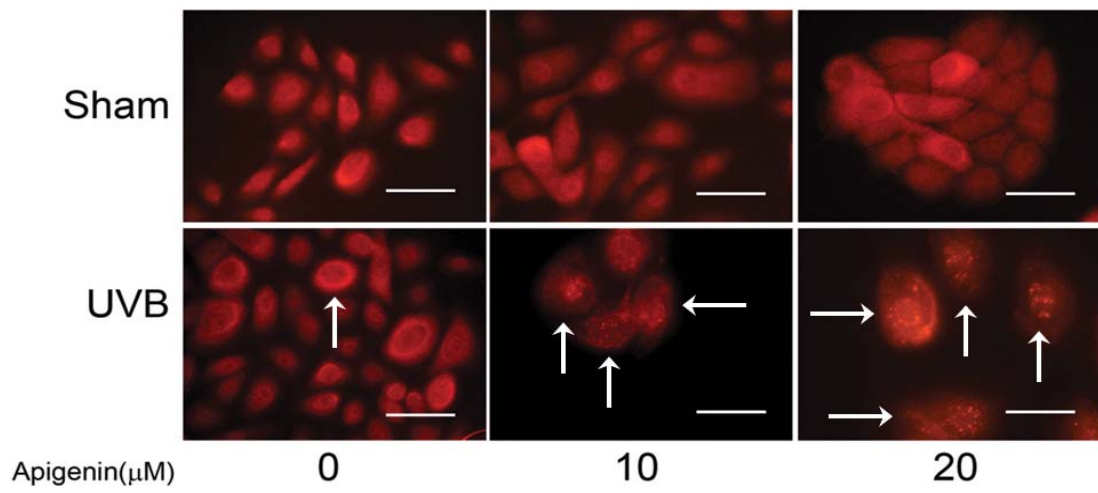
Figure 19: Apigenin stimulates cytochrome c release from the mitochondria in keratinocytes exposed to UVB radiation.

Cytochrome c release from the mitochondria is detected in NHKs using antibody to cytochrome c. Cells were adhered to glass coverslips, fixed, then immunostained. Cells were exposed to 750 J/m^2 UVB or sham radiation followed by incubation in media containing 0, 10, or 20 μM apigenin for 16 hours. Cytochrome c release from the mitochondria is indicated by arrows. Bars, 50 μm.

Sham

UVB

Apigenin(μM)   0   10   20
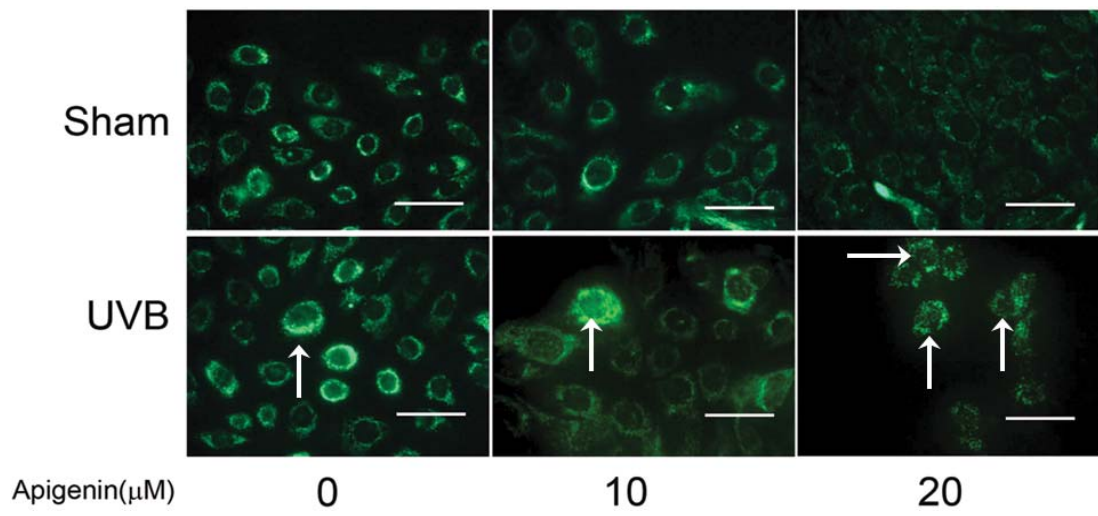
Figure 20: Enhancement of UVB-induced apoptosis by apigenin treatment involves the intrinsic pathway of apoptosis.

HaCaT human keratinocytes overexpressing Bcl-2 or expressing control vector (pBABE) were exposed to 0 or 300 J/m^2 UVB followed by incubation with 0, 10, or 20 μM apigenin for 8 hours. Cells (5 x 10^5) were harvested and stained with annexin-V and 10,000 events were counted by flow cytometry. Columns, mean (n=3); bars, SE. *, P < 0.05, compared to control vector.
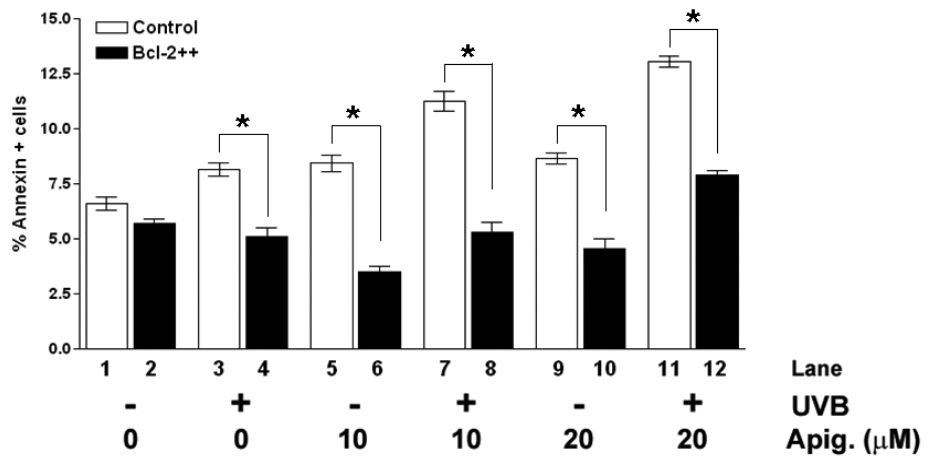
Figure 21: Enhancement of UVB-induced apoptosis by apigenin treatment involves the extrinsic apoptotic pathway.

HaCaT keratinocytes expressing control vector (pBABE) or FADDdn-LZRS were exposed to 0 or 300 J/m^2 UVB and then incubated in media containing 0, 10, or 20 μM apigenin. Cells (5 x 10^5) were harvested and stained with annexin-V and 10,000 events were counted by flow cytometry. Columns, mean (n=3); bars, SE. *, P < 0.05, compared to control vector.
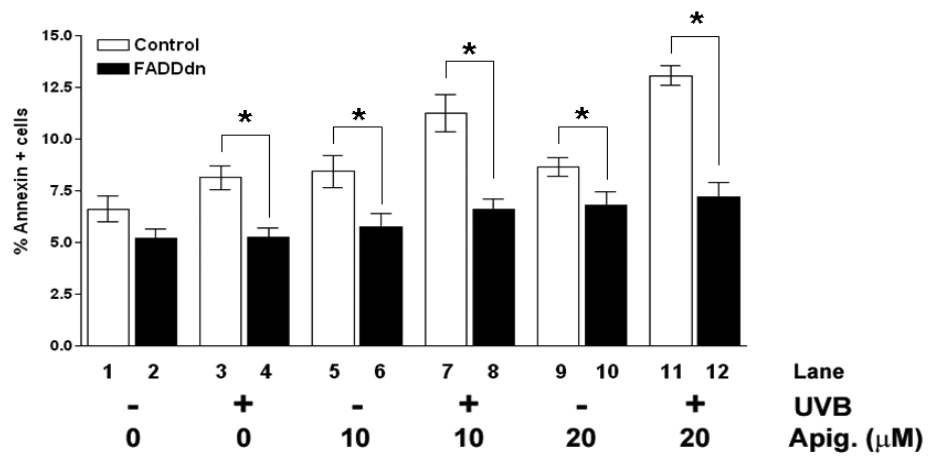
Chapter 5: Discussion

5.1   General discussion

There are currently two major strategies being employed for the prevention of UVB-induced skin cancer. The first approach involves use of sun screens and other agents to prevent UVB-induced damage, thus reducing the formation of "initiated" cells. Tumor initiation is a rapid, irreversible process in which mutations alter the response of initiated basal stem cells of the epidermis, making anti-initiation strategies difficult to envision (Afaq *et al.* 2005). However, the stages of UVB-induced tumor promotion and progression in skin cancer occur over a period of years to decades, thus providing a larger window for chemopreventive intervention. Therefore, the second chemoprevention strategy aims to eliminate initiated cells with carcinogenic potential. This strategy is attractive clinically, because it allows intervention during the longer period of time between development of preneoplastic foci and appearance of the frank tumor.

Interest in the use of apigenin as a chemopreventive agent began more than fifteen years ago when Wei and coworkers showed that topical application of apigenin effectively inhibited chemical carcinogenesis in mouse skin (Wei *et al.* 1990). Subsequent studies by Birt and coworkers revealed that apigenin applied topically also inhibited UVB-induced photocarcinogenesis in SKH-1 hairless mice (Birt *et al.* 1997). At the time, little was known about how apigenin exerted its chemopreventive actions.

Apigenin is a yellow compound, and therefore early theories on apigenin's mode of action were attributed to merely providing a "sunscreen effect." However, the Pelling laboratory demonstrated effects of apigenin exceed that of a sunscreen by showing topical application of apigenin to SKH-1 mice weeks after UVB exposure still reduced tumor size and multiplicity (unpublished results).

Further studies by others have increased our understanding of molecular targets of apigenin and further demonstrated that apigenin is an effective chemopreventive agent. For example, Kuo and coworkers reported that apigenin treatment induced the reversion of transformed phenotypes of v-H-ras transformed NIH 3T3 cells by inhibiting MAPK activity and its downstream oncogenes (Kuo and Yang 1995). Experiments by Way et al. and Yin and coworkers showed that apigenin induced growth inhibition, cell-cycle arrest and apoptosis in breast cancer cell lines (Way *et al.* 2005; Yin *et al.* 2001). Similiarly, Wang and coworkers reported that apigenin induced G_2/M arrest in human colon cancer cell lines (Wang *et al.* 2000). Previously, our lab demonstrated that apigenin treatment increased wild-type p53 protein stability in keratinocytes (McVean *et al.* 2000). We also demonstrated that apigenin can induce reversible G_2/M cell cycle arrest (Lepley *et al.* 1996), and that G_2/M arrest was accompanied by inhibition of the p34(cdc2) cyclin-dependent kinase protein level and activity in a p21(waf1)-independent manner (McVean *et al.* 2002). Van Dross et al. demonstrated apigenin

treatment can activate ERK1/2, p38 MAPK pathways, and downstream transcriptional activators in 308 keratinocytes and human colon carcinoma cell line HCT116 (Van Dross *et al.* 2003).

More recently, our group has investigated the combined effects of apigenin treatment on keratinocytes exposed to UVB radiation (Tong *et al.* 2007; Van Dross *et al.* 2007; Van Dross *et al.* 2005). These reports have demonstrated that apigenin treatment down-modulates both basal and UVB-induced COX-2 expression in mouse keratinocytes. A number of other laboratories have shown selective COX-2 inhibition can reduce tumor formation using agents such as celecoxib and SC-791 (Akunda *et al.* 2007; Fischer *et al.* 1999; Tripp *et al.* 2003; Wilgus *et al.* 2003; Wilgus *et al.* 2000), presumably by increasing apoptosis (Orengo *et al.* 2002; Raj *et al.* 2006) in cells that have carcinogenic potential. A recent study by Akunda et al. reported COX-2 deficiency significantly increased UVB-induced epidermal apoptosis, in agreement with previous findings by Tripp et al. (Akunda *et al.* 2007; Tripp *et al.* 2003). In the present study we demonstrate that apigenin's efficacy as a chemopreventive agent may be explained by its ability to down-modulate COX-2 activity and enhance UVB-induced apoptosis in human keratinocytes.

While numerous studies have evaluated the effects of apigenin on various cell types, we believe this is the first study to report the ability of apigenin to enhance UVB-induced apoptosis in human keratinocyte cultures.

We observed this phenomenon in HaCaT cells, in primary human keratinocyte cultures, and in organotypic human keratinocyte cultures. These results eliminate the possibility that our observations are limited to HaCaT cells. Collectively, the observations made in the present study show that apigenin enhances UVB-induced apoptosis in human keratinocytes through both the extrinsic, receptor-mediated apoptotic pathway, and the intrinsic apoptotic pathway.

5.2   Overall conclusions

5.2.1 Characterization of the individual effects of UVB exposure and apigenin treatment on human keratinocytes.

The correlation between UVB exposure and skin cancer incidence was observed over a century ago (1894) when Paul Gerson Unna first described pre-cancerous histopathologic changes in the skin of sailors (Albert and Ostheimer 2002). Since those observations, numerous studies have been conducted to elucidate the mechanism by which UVB stimulates carcinogenesis of skin cancers. UVB is a complete carcinogen that can induce irreversible damage to cells and then upon repeated exposure promotes the clonal expansion of damaged cells. Moreover, exposure to UVB light is thought to be responsible for greater than 90% of skin cancers (Brash *et al.* 1991). Risk factors for developing skin cancer are primarily

associated with the frequency and intensity of one's exposure to UVB (Diffey 1991).

To evaluate the ability of apigenin to modulate the molecular events induced by UVB exposure that are thought to be responsible for photocarcinogenesis, we needed to determine the individual effects of UVB exposure and apigenin treatment on human keratinocytes. There is active interest in gaining a better understanding of the role of UVB-induced COX-2 activity and the regulation of apoptosis in human keratinocytes during photocarcinogenesis. We first investigated the effects of UVB exposure on COX-2 expression and the dose of UVB required to induce apoptosis in human keratinocytes.

HaCaT human keratinocyte cells were exposed to 500 J/m^2 UVB or sham radiation, then incubated for 8 hours based on previous studies on 308 mouse keratinocytes in the Pelling laboratory. The cells were then harvested and assayed for COX-2 protein expression by Western blot. The level of COX-2 protein significantly increased in response to this dose of UVB (Fig. 7). Recent reports have shown that COX-2 is critical during photocarcinogenesis and that COX-2 deficiency increased epidermal apoptosis and impairs recovery following acute UVB exposure (Akunda *et al.* 2007).

UVB is a known inducer of apoptosis, however, in order to study the effect of apigenin treatment on UVB-induced apoptosis we needed to establish a dose of UVB capable of inducing moderate, but not complete

apoptosis. Three human keratinocyte models were irradiated with different doses of UVB and then assayed for apoptosis. HaCaT cells possess a mutant, low-functioning p53 with an increased p53 protein half-life (Boswell *et al.* 2007) and are the most sensitive to UVB radiation. Lower doses of UVB radiation required to induce apoptosis (300 J/m^2) in HaCaT keratinocytes than is required to induce apoptosis in primary keratinocyte cell cultures (750 J/m^2) or human OTKC (1000 J/m^2). The higher dose of UVB required to induce apoptosis in primary keratinocyte cell cultures compared to HaCaT cells is explained largely due to the wt-p53 present in NHKs (Boswell *et al.* 2007). Human OTKCs were likely more resistance to UVB-induced apoptosis than NHKs because protective stratum corneum that is formed during terminal differentiation of the NHKs in this model. The dose of UVB radiation that induced moderate levels of apoptosis identified above for each model system was used in subsequent experiments to determine the ability of apigenin to enhance UVB-induced apoptosis.

Previous studies have indicated that apigenin is also capable of inducing apoptosis in various cancer cell lines (Gupta *et al.* 2001; Shukla and Gupta 2004; Way *et al.* 2005). A concentration-dependent decrease in cell viability was observed in HaCaT cells incubated in medium containing 0, 5, 10, 15, 20, or 25 μM apigenin by the MTS cell viability assay (Fig. 10A). Western blot analysis revealed that apigenin treatment also led to a concentration-dependent increase in the cleavage of PARP and procaspase-

3, indicating that apigenin treatment was inducing apoptosis in HaCaT cells (Fig. 10B). The ability of apigenin to induce apoptosis in HaCaT cells explains the decrease in cell viability observed in the previous experiments. These studies allowed us to determine the concentration of apigenin to be used in later experiments conducted on keratinocytes exposed to UVB followed by apigenin treatment.

A report by Konger et al. demonstrated that the enzymatic COX-2 inhibitor, indomethacin reduced cell proliferation in human keratinocytes by preventing PGE_2 signaling (Konger *et al.* 1998). The results presented herein show that while PGE_2 can protect NHKs from indomethacin-induced growth inhibition, it can not protect from apigenin-induced growth inhibition and apoptosis (Fig. 11). In the present study, we demonstrate that even though apigenin decreased UVB-induced COX-2 protein expression, it does not do so by directly inhibiting COX-2 enzymatic activity (Fig. 12). Taken together, these results distinguish apigenin's mode of action from other inhibitors of COX-2 protein expression.

5.2.2 Investigate the combined effects of apigenin treatment on molecular events initiated by UVB exposure in human keratinocytes.

The expression of COX-2 increases during inflammation and has been documented to increase in most cancers (Ding *et al.* 2000; Fischer *et al.* 1999; Ohshima *et al.* 2005). It follows that many investigators have used various approaches to reduce COX-2 expression and activity. The Pelling lab has shown that the natural bioflavonoid apigenin can inhibit inducible COX-2 expression by either modulating transcription or suppressing protein translation (Tong *et al.* 2007; Van Dross *et al.* 2007). Exposure to UVB led to a 10-fold increase in the production of PGE_2, indicating the increase in COX-2 protein expression correlates to an increase in the ability of COX-2 to convert arachidonic acid into prostaglandins. PGE_2 is the most abundantly produced prostaglandin in the skin (Chun *et al.* 2007). Apigenin treatment reduced PGE_2 production compared to vehicle control in both sham- and UVB-irradiated samples (Fig. 13). The ability of apigenin to reduce COX-2 protein expression may lead to increased apoptosis in human keratinocytes exposed to UVB, as COX-2 deficiency has been reported to increase UVB-induced epidermal apoptosis (Akunda *et al.* 2007).

Currently, many natural products known to possess chemopreventive properties are being evaluated for the ability to enhance UVB-induced apoptosis (Dhanalakshmi *et al.* 2005; Park and Lee 2007; Reagan-Shaw *et al.* 2006) because apoptosis of keratinocytes damaged by UVB exposure is critical to prevention of skin cancers (Ziegler *et al.* 1994). In the present report we evaluated the ability of apigenin to enhance UVB-induced apoptosis

based on the finding that apigenin induces apoptosis in human keratinocytes. HaCaT cells with a mutant p53, appear the most sensitive to enhancement of UVB-induced apoptosis (Fig. 14). Interestingly, the ability of apigenin to enhance UVB-induced apoptosis was found to be a p53-independent process by using p53shRNA/HaCaT cells (kindly provided by Dr. Sam W. Lee). Apigenin treatment also enhanced UVB-induced apoptosis in both human primary keratinocyte cultures and human OTKCs (Fig. 15 and 16). We believe this is the first report of an agent that can enhance UVB-induced apoptosis in primary keratinocyte cultures or human OTKCs.

5.2.3 Investigate the involvement of the intrinsic and extrinsic apoptotic pathways in the enhancement of UVB-induced apoptosis in human keratinocytes by apigenin treatment.

UVB radiation induces both intrinsic and extrinsic pathways of apoptosis. The intrinsic pathway is characterized by increased mitochondrial permeability, subsequent cytochrome c release, formation of the apoptosome, and processing of initiator and effector caspases, ultimately leading to apoptosis (Gogvadze *et al.* 2006; Martinou and Green 2001; Sitailo *et al.* 2002). Sitailo et al. demonstrated the critical involvement of the intrinsic, mitochondria-mediated apoptotic pathway in response to UVB radiation in keratinocytes by showing expression of dominant negative caspase-9 led to

almost complete inhibition of caspase-3, -8, and -9 activation typically induced by UVB (Sitailo *et al.* 2002).

In the present report apigenin treatment enhanced the localization of Bax to the mitochondria in NHKs exposed to UVB radiation. As described previously, Bax is thought to oligomerize at the mitochondrial membrane, allowing for release of cytochrome c and loss of mitochondrial membrane potential. These findings support the hypothesis that apigenin enhances UVB-induced apoptosis through the intrinsic, stress-mediated apoptotic pathway. HaCaT cells that overexpress the anti-apoptotic protein Bcl-2 were generated to further investigate the role of the intrinsic apoptotic pathway. The results presented herein, demonstrate that apigenin treatment of Bcl-2 overexpressing HaCaT cells resulted in reduced apoptosis providing supportive evidence that apigenin is working at least in part through the intrinsic apoptotic pathway.   However, incomplete protection from enhancement of UVB-induced apoptosis in keratinocytes overexpressing the anti-apoptotic protein Bcl-2 allows for the possibility that apigenin may enhance UVB-induced apoptosis by potentiating receptor-mediated apoptosis as well.

A recent report by Sakai et al. has shown apigenin can sensitize cells to receptor-mediated cell death in various tumor cell lines if used in combination with TRAIL (Horinaka *et al.* 2006). Therefore HaCaT keratinocytes expressing a dominant negative form of FADD (FADDdn) were

generated to assess the involvement of the extrinsic, receptor-mediated apoptotic pathway. The results shown in Fig 21 demonstrate FADDdn expression in keratinocytes significantly reduced enhancement of UVB-induced apoptosis by apigenin treatment compared to vector control. The inability of apigenin to enhance UVB-induced apoptosis in keratinocytes expressing FADDdn suggests that the extrinsic apoptotic pathway plays a critical role in apigenin's enhancement of UVB-induced apoptosis. It is possible that direct activation of death receptors, such as Fas and TNFR1, caused by UVB radiation (Aragane *et al.* 1998; Bang *et al.* 2002; Sheikh *et al.* 1998) could be involved in enhancement of UVB-induced apoptosis observed in irradiated keratinocytes treated with apigenin.

In the present study the following observations were made: 1) Apigenin treatment enhanced UVB-induced apoptosis in each model of human keratinocytes as evaluated by Western blot analysis, annexin-V staining by flow cytometry and/or quantification of sunburn cells. 2) Apigenin treatment of human keratinocytes facilitates changes in Bax localization and cytochrome c release when exposed to UVB radiation, 3) Overexpression of Bcl-2 incompletely protects keratinocytes from enhancement of UVB-induced apoptosis when treated with apigenin, and 4) FADDdn expression partially blocked apigenin enhancement of UVB-induced apoptosis. The findings presented here support the hypothesis that apigenin treatment enhances

UVB-induced apoptosis by modulating both the intrinsic and extrinsic apoptotic pathways.

5.3   Future Directions

The precise mechanisms by which apigenin enhances UVB-induced apoptosis are still unclear. The data presented herein indicate that both the intrinsic and extrinsic apoptotic pathways of apoptosis mediate the enhacement of UVB-induced apoptosis by apigenin treatment. However, in order to fully understand the role of each apoptotic pathway and the target of apigenin more studies must be conducted.

Induction of apoptosis in cells repeatedly exposed to UVB exposure is thought to be an important protective mechanism aimed at removing irreversibly damaged and potentially carcinogenic keratinocytes from the epidermis. Future areas of interest include elucidation of the molecular targets of apigenin responsible for enhancing UVB-induced apoptosis. For example, it would be exciting to use shRNA that targets apoptotic proteins such as caspase-8 and bid for the extrinsic pathway, and caspase-9, Bax, and Bak for the intrinsic pathway of apoptosis and asses the ability of apigenin to enhance UVB-induced apoptosis (see Figure 23).

Also of great interest would be to identify whether apigenin is inducing apoptosis in cells that have DNA damage as hypothesized in Figure 24. It is anticipated that apigenin is targeting cells with DNA damage because many have reported that apigenin is nonmutagenic, noncarcinogenic, and anti-carcinogenic flavonoid at concentrations above those used in the present study (Birt *et al.* 1986; Czeczot *et al.* 1990). These studies would alleviate concerns regarding induction of apoptosis in healthy keratinocytes, and strengthen rationale for the use of apigenin or a derivative of apigenin as a photochemopreventive agent.

Figure 22: Enhancement of UVB-induced apoptosis by apigenin treatment involves both intrinsic and extrinsic apoptotic pathways.

Use of FADDdn HaCaT keratinocytes demonstrated that enhancement of UVB-induced apoptosis is mediated in part by the extrinsic pathway. Use of Bcl-2 overexpressing HaCaT keratinocytes demonstrated that enhancement of UVB-induced apoptosis involves the intrinsic apoptotic pathway. Therefore use of more genetically modified keratinocytes would assist in gaining a better understanding of the effects of apigenin treatment of keratinocytes exposed to UVB radiation. Arrows indicate possible targets of apigenin treatment that act to enhance UVB-induced apoptosis
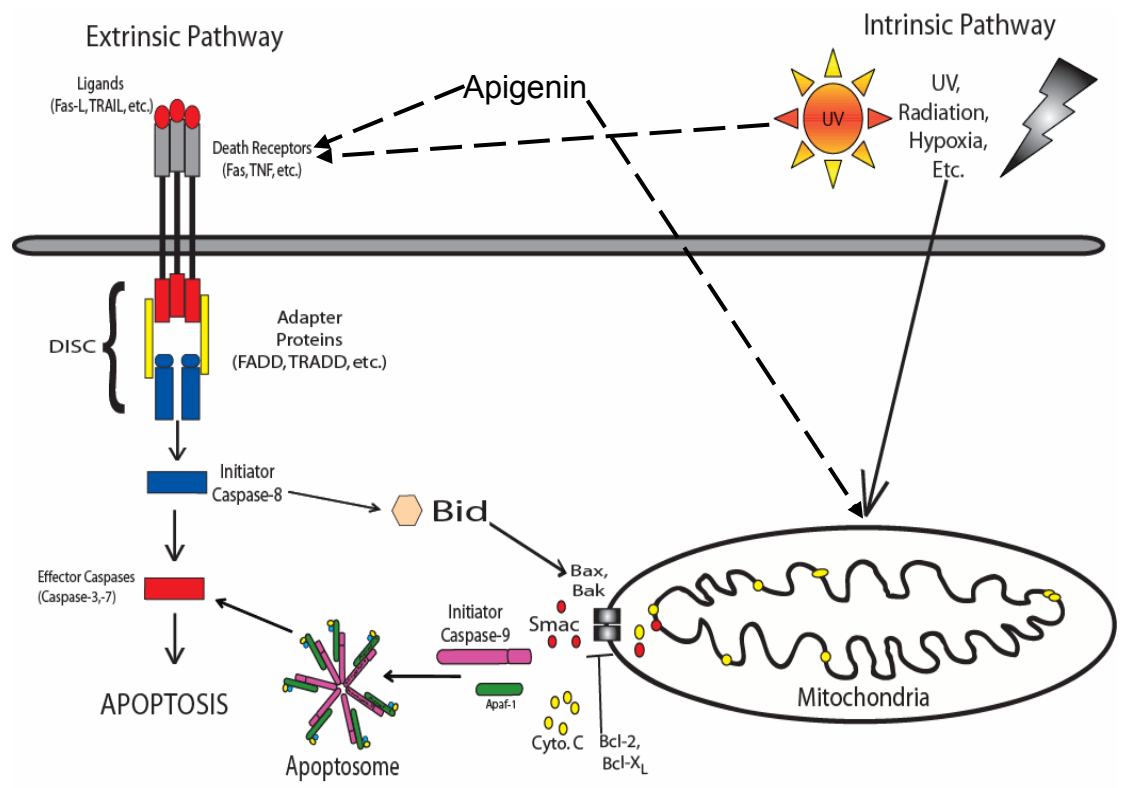
Figure 23: Proposed mode of action for enhancement of UVB-induced apoptosis by apigenin treatment.

The non-mutagenic, antimutagenic flavonoid apigenin has been shown to reduce skin tumor formation in SKH-1 mice. Shown is a depiction of the proposed response of keratinocytes to both low and high doses of UVB radiation. Also indicated is a proposed model for how apigenin may exerts its chemopreventive effects in the skin. Black X—UVB-induced damage, Yellow X—Successful DNA repair, Red X—Apoptotic cells.

Low Dose of UVB

Low Dose of UVB

Low Dose of UVB   Apigenin Treatment

DNA Repair

DNA repair initiated,
Apigenin induces
apoptosis

High Dose of UVB

High Dose of UVB

High Dose of UVB   Apigenin Treatment

DNA repair overwhelmed,
UVB-induced apoptosis
occurs

Apigenin enhances
UVB-induced
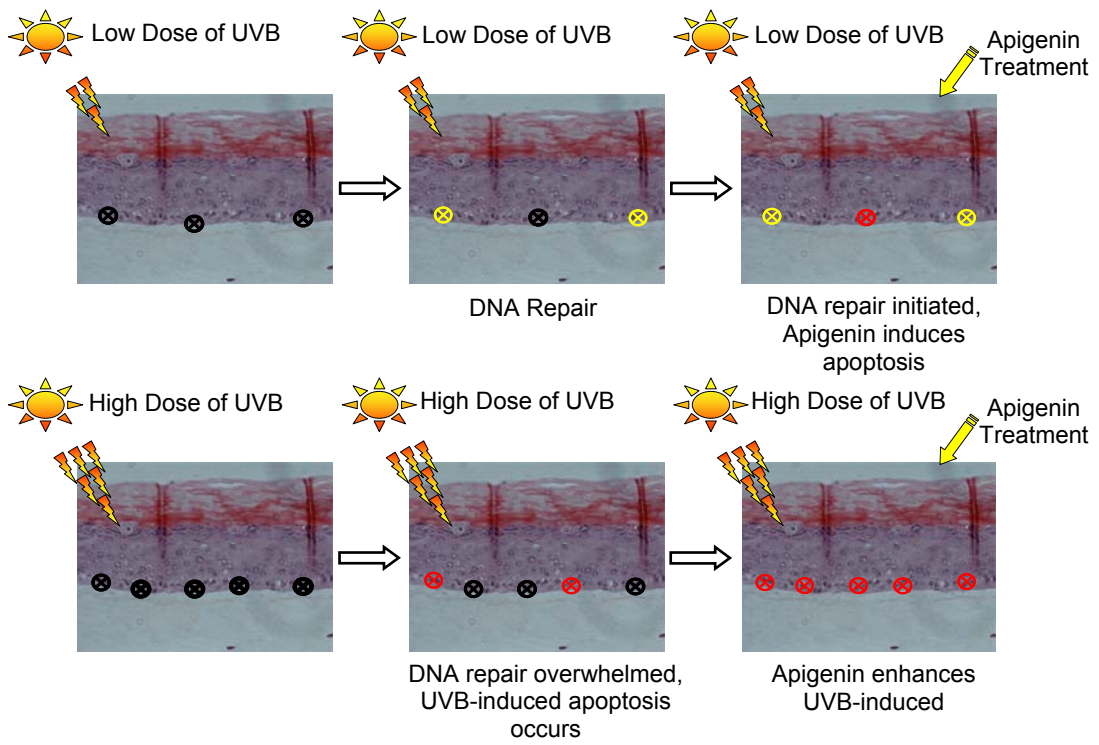
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