SULFORAPHANE POTENTIATES NON-MELANOMA SKIN CANCER IN UVB-TREATED NRF2 KNOCKOUT MICE

by

Marlon Taylor

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

WITH A MAJOR IN MEDICAL PHARMACOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2016

STATEMENT BY AUTHOR

The thesis titled *Sulforaphane Potentiates Non-melanoma Skin Cancer in UVB-treated Nrf2 knockout Mice* prepared by *Marlon Taylor* has been submitted in partial fulfillment of requirements for a master's degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that an accurate acknowledgement of the source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: *Marlon Taylor*

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Sally Dickinson, Ph.D.
Research Assistant Professor of Pharmacology

Defense date
12/07/2016

Patrick T Ronaldson, Ph.D.
Assistant Professor, Neuroscience - GIDP; Assistant Professor, Pharmacology; Assistant Professor, Physiological Sciences - GIDP

Acknowledgements

The author would like to acknowledge Dr. Sally Dickinson, Dr. Qin Chen, and Dr. Patrick Ronaldson for their advice and support. Most importantly, the author is indebted to his girlfriend, Teri Christina Moore, whose support helped make this endeavor successful.

TABLE OF CONTENTS

LIST OF FIGURES

Abstract

Sulforaphane is a natural product found in cruciferous vegetables which is known to have many chemopreventive properties including the induction of apoptosis and the inhibition of inflammation, cellular proliferation, and reactive oxygen species (ROS) formation. The reduction of ROS activity by sulforaphane is likely linked to the activation of NF-E2 related factor-2 (Nrf2), a transcription factor involved in cytoprotection against ROS and electrophilic stress. The skin is particularly vulnerable to oxidative stress caused by ultraviolet (UV) light, which is an established complete carcinogen. Sulforaphane has been shown to reduce both chemical and UVB-induced skin carcinogenesis in mouse models. Suppression of DMBA/TPA-induced skin tumorigenesis by sulforaphane has been shown to be dependent upon Nrf2 activity. Additional studies have shown that genetic activation of Nrf2 can protect keratinocytes against UVB-induced ROS. Nrf2 has also been implicated in regulating inflammatory responses after UVB exposure in the skin. However, the role of Nrf2 in the antitumorigenic activity of sulforaphane in the context of UVB-induced skin tumors is not well understood. We therefore performed murine experiments in order to clarify whether sulforaphane requires Nrf2 in order to block UVB-induced non-melanoma skin cancer. Consistent with the literature, we observed that wildtype (WT) mice topically treated with sulforaphane were less susceptible to UVB-induced tumor incidence and tumor burden compared to the vehicle control WT group. However, Nrf2 KO mice treated with sulforaphane presented with significantly greater UVB-induced tumor incidence and burden compared to the WT sulforaphane group, suggesting that sulforaphane may potentiate tumorigenesis in the context of UVB exposure if Nrf2 is absent. We therefore performed acute *in vivo* and *in vitro* experiments using topical sulforaphane (as per the tumor experiment) to investigate why Nrf2 KO mice developed more tumors than WT mice during UVB and sulforaphane treatment. Topical treatment of SKH-1 mice with sulforaphane did result

in slight reduction of UV-induced epidermal hyperplasia in wildtype mice which was not present in Nrf2 KO mice (trends were not significant). Surprisingly, while wildtype mice developed significantly more epidermal inflammation in our acute treatment model than did the Nrf2 KO strain (as measured by skin fold thickness), inflammation was not significantly influenced by topical sulforaphane treatment in either strain of mice. However, cell culture studies using primary mouse keratinocytes indicate that sulforaphane's ability to block UVB-induced ROS is lost in Nrf2 KO cells. Taken together, our ROS data may strengthen the hypothesis that sulforaphane increases the oxidative stress of cells during UVB treatment in the absence of Nrf2.

Introduction

Nonmelanoma skin carcinoma (NMSC), the terminology for both basal cell carcinoma and squamous cell carcinoma of the skin, leads all other cancers in incidence in the United States [1, 2]. In fact, skin cancer makes up more than a third of all cancer incidences combined [1, 2]. Ultraviolet radiation (UVR) from the sun is known to be the major risk factor associated with the development of skin cancer [1, 2]. Every nucleated cell in the skin is susceptible to carcinogenesis, giving rise to different types of skin cancer; however, it is the keratinocyte—making up over 90% of the skin cells—that is particularly vulnerable to carcinogenesis resulting in NMSC [1, 2]. BCC makes up 80% of all NMSCs, and SCC makes up the remaining 20% [1, 2]. Compared to BCC, SCC is more likely to metastasize to other tissues and cause death [1, 2]. Although the mortality for BCC is not high, it does put a huge burden on the healthcare system due to its morbidity [1, 2]. NMSCs are found in UV-exposed areas of the body such as the head, neck, and legs [1, 2].

UVR can be separated into three different categories: UVA, UVB, and UVC [reviewed in 3]. While UVC is filtered by the atmosphere, UVB and UVA contribute to skin cancer by damaging DNA and promoting inflammation [3, 4, 19]. UVA and UVB can directly damage DNA by inducing dimerization of two adjacent pyrimidines producing both cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone [5-9]. Direct DNA damage produces specific types of mutations called UV signature mutations resulting in a C to T transversion typically seen in the p53 gene in many skin cancers [9-11]. UVB can also indirectly damage DNA by activating small molecules, such as riboflavin, tryptophan, and porphyrin, into reactive oxygen species (ROS) which can then go on to damage DNA, creating DNA lesions such as 8-oxo-guanine [12-16]. In accordance with oxidative injury, heat shock, and cytokine

release, UVB can induce inflammation, which has cancer promoting effects by providing growth signals to abnormally sustain proliferative signaling [19, 20]. For example, p38 mitogen activated protein kinase (MAPK), which belongs to a family of mitogen activated protein kinases and is involved in normal cell growth and survival, is activated by stressful stimuli including UVB [20]. In addition, p38 MAPK has been shown to play a functional role in UVB-induced mouse skin carcinogenesis [21]. Inflammation may also promote carcinogenesis by providing matrix modifying enzymes which help the tumor to invade nearby tissues [20]. An increase in epidermal hyperplasia is also observed following UV exposure which is proportional to an increase in expression of proliferating cell nuclear antigen (PCNA) [22-24].

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which belongs to a family of "Cap'n'Collar" basic leucine zipper transcription factors including Nrf1, Nrf3, and p45 NFE2, is a redox sensing transcription factor involved in cytoprotection against electrophilic and oxidative stress by regulating enzymes that neutralize ROS and other electrophiles [reviewed in ref.25]. During basal conditions, Nrf2 is found in the cytosol bound to its indigenous inhibitor Keap1. While bound to Keap1, Nrf2 is kept transcriptionally inactive by a cullin 3 E3 ubiquitin ligase pathway wherein Nrf2 is constantly ubiquitinated and sent to the proteasome for degradation [reviewed in ref.25]. During periods of oxidative stress, ubiquitination of Nrf2 is disrupted, allowing for accumulation of the transcription factor in the nucleus [reviewed in ref.25]. Once in the nucleus, Nrf2 can bind to the antioxidant response element (ARE), an enhancer sequence found within the promoter regions of many antioxidant and cytoprotection related genes. This leads to the transcription of cytoprotective enzymes, such as NADPH dehydrogenase quinone 1 (NQO1), superoxide dismutase (SOD), and glutathione S-transferase (GST) that can neutralize excessive ROS [reviewed in ref.25]. Although Nrf1 and Nrf2 have overlapping functions, Nrf1

preferentially activates a subset of cytoprotective enzymes, such as metallothionein 1 and metallothionein 2, that protect the cell from heavy metal toxicity [26]. Nrf3 has also been shown to induce ARE gene products but is less transcriptionally active than either Nrf1 or Nrf2 [27]. This low transactivation activity of Nrf3 may be due to an ER-targeting sequence located in the N-terminal domain of Nrf3 [27].

Epidemiological studies have shown that consumption of broccoli, among other cruciferous vegetables, is protective against various cancers, such as those of the lung, breast, colon, and prostate. Sulforaphane (SFN) was identified as the active ingredient in broccoli's anti-carcinogenic effect through many mechanisms [reviewed in ref. 34] (Figure 1). SFN belongs to a family of organic molecules called isothiocyanates, over 120 of which have been identified, but only a few of which occur in nature. The precursor for SFN is 4-methylsulfinyl glucosinolate which is converted to active SFN by a myrosinase enzyme [reviewed in ref.34].

SFN has been shown to inhibit carcinogenesis through various mechanisms. One such mechanism of cellular protection by SFN is by regulation of the cell cycle—which is often dysregulated in cancer—at the G0/G1phase and G2/M phase [34-37] (Figure 2). The G0/G1phase of the cell cycle is regulated by cyclin dependent kinase 4 and 6 (CDK4/6), cyclin D1, and a family of cyclin dependent kinase inhibitors (CKI's) called the Cip/Kip family [reviewed in ref.38]. After the cell receives appropriate growth signals, cyclin D1 binds to CDK4, activating CDK4 and initiating the cell cycle [reviewed in ref.38]. In some cancers, such as prostate and colon, SFN has been observed to reduce the levels of cyclin D1, thus reducing in CDK4 activity and arresting the cell cycle [36]. SFN has also been shown to induce the CKI p21waf/cip1 which is a tumor suppressor that can arrest the cell cycle [36]. Another SFN chemopreventive mechanism is due to inhibition of many phase 1 enzyme activities, such as

cytochrome P450 3A4 (CYP3A4), that have the potential to metabolize small organic molecules from the diet into active carcinogens, a process called biotransformation [39-44].

Recently, considerable attention has been given to the ability of SFN to induce Nrf2 in the skin and therefore serve as a natural topical chemopreventive agent. Studies using genetic or pharmacological means to manipulate Nrf2 activity have linked the stimulation of this transcription factor to regulation of epidermal inflammation and skin cancer [46-51]. For example, in a dose dependent manor, Nrf2 knockout mice were observed to be more susceptible to two-stage DMBA/TPA-induced carcinogenesis compared to wild type mice on the C57BL/6 genetic background [49]. Nrf2 knockout mice were also observed to be more susceptible to UVB-induced inflammation than wildtype controls [50]. Using ear thickness measurements to assess skin inflammation, it was observed that the BALB/c Nrf2 knockout mice developed a longer and stronger inflammatory response compared to wildtype controls [50]. In addition, Nrf2 knockouts were more susceptible to UVB-induced oxidative stress and ROS-induced DNA lesions in BALB/c mice [50]. SFN has also been shown to inhibit DMBA/TPA-induced carcinogenesis compared to wildtype in C57BL/6 mice [51], inhibit UVB-induced inflammation in SKH-1 mice [63], and induce protective phase 2 enzymes [reviewed in 48].

However, other studies have shown SFN to promote intracellular oxidative stress. Under certain circumstances, SFN may promote oxidative stress by the induction, rather than inhibition, of certain phase 1 enzymes including CYP1A1, 1A2, 2B1/2, 3A1, 3A1/2 which may then act in a pro-oxidant manner [53]. These SFN-induced pro-oxidant responses may be both tissue specific and dose dependent [53-54]. Experiments show that Sprague-Dawley rats treated with 80µmole of SFN/animal experienced an increased in CYP activity and increase in ROS in lung microsomal preparations [53]. Another experiment showed that ROS levels doubled in

hepatocytes isolated from Sprague-Dawley rats after being exposed to 20μM SFN for 24 hours [54].

In this study, we investigated the role that Nrf2 plays in SFN's chemopreventive effect on UVB-induced skin cancer. Previous studies have found that SFN significantly inhibited UVB-induced tumorigenesis and inflammatory biomarkers in SKH-1 mice and C57BL/6 mice [49, 55]. However, we also recently observed that in the absence of Nrf2 SFN potentiates tumorigenesis compared to knockout vehicle control. Because Nrf2 is a regulator of oxidative stress and because SFN may have pro-oxidant activities, we hypothesized that SFN increases oxidative stress in skin cells in the absence of Nrf2.

Materials and Methods

Materials. Sulforaphane (SFN) was obtained from LKT Laboratories, Inc. (St. Paul, MN). All real time qPCR primers were purchased from Applied Biosystems (Branchburg, NJ) including Mouse GAPDH (Mm99999915_g1), NQO1 (Mm01253561_m1), and PCNA (Mn00448100_g1). Keratinocyte media was purchased from Lonza (Allendale, NJ). Collagen and trypsin without EDTA were purchased from Thermo Scientific (Grand Island, NY). The ROS activity assay kit was purchased from Abcam (Cambridge, MA).

Table 1
Primer sequences for cDNA amplification of selected human genes

Primer name	Interrogated Sequence	Assay Location	Amplicon Length.
GAPDH	NM_001289726.1	117	107
NQO1	NM_008706.5	545	81
PCNA	NM_011045.2	529	117

UVB Treatment. For cell culture, UVB bulbs (FS20 bulbs, National Biological Corp. Beachwood, OH) were used within a biosafety cabinet. Control cells were mock exposed in an identical cabinet. For *in vivo* treatment, six FS40T12 UVB lamps were utilized within an approved ventilated animal rack. Fluence intensity for both types of bulbs was measured using a UVX radiometer (Ultraviolet Products) as previously reported [21].

Generation of Nrf2 knockout SKH-1 hairless mice. The initial stocks of Nrf2 knockout (KO) mice were a kind gift of Dr. Jefferson Y. Chan from University of California, Irvine. The mice

were transferred from their original C57Bl/6 background onto the SKH-1 hairless genetic background, which is more susceptible to UV-induced skin tumorigenesis. To maintain the genetic diversity of the SKH-1 outbred strain, we performed an advanced intercross protocol, which incorporated new SKH-1 stocks throughout six generations of heterozygous crossing. Thus, after heterozygotes reached the sixth generation, non-related heterozygous mice were crossed to generate homozygous Nrf2KO or wildtype offspring (10 non-related pairings). These offspring were used to generate wildtype and Nrf2KO homozygous colonies, which were maintained separately. This advanced intercross protocol allowed for us to create the SKH-1 hairless colonies of wildtype/KO mice while preserving the overall genetic diversity of the strain.

UVB skin carcinogenesis. 40 wild-type SKH-1 hairless female mice and 40 Nrf2 knockout SKH-1 hairless female mice (total of 80 mice) were used as *in vivo* models to investigate the role that Nrf2 plays in SFN's ability to reduce carcinogenesis. Mice were purchased from Charles River Laboratories and housed in accordance with The University of Arizona Animal Care and Use Committee standards. Mice were split into four groups of 20 each: wild-type SKH-1 mice treated with acetone + UVB, wild-type SKH-1 mice treated with 1 μmol SFN +UVB, Nrf2 knockout SKH-1 mice treated with acetone + UVB, and Nrf2 knockout SKH-1 mice treated with 1 μmol SFN+UVB. Topical administration of 1μmole SFN (in 200 uL of acetone/back, administered between the shoulder blades and the top of the hips, and halfway down the sides) was previously shown to be effective at reducing skin cancer in SKH-1 mice in our laboratory. Mice were treated with UVB using six FS40T12 UVB lamps (National Biological Corporation) three times per week for 25 weeks as described previously [64]. Mice were pretreated with SFN or vehicle for 1 week before the start of UVB treatment and then 1 h before each irradiation.

Tumors were measured weekly, and the experiment was concluded at week 25. Tumor burden was calculated by multiplying diameter by height in millimeters. Average tumor burden was calculated by dividing the sum of individual tumor burdens each week by the number of mice in the treatment group.

Statistical analysis: Means and standard deviations summary statistics were calculated by treatment group for all outcome measurements. Primary analysis compared week 25 total tumor burden and tumor counts (multiplicity) among the 4 treatment groups. Additionally, all possible two-group comparisons were performed. These cross-sectional analyses used the Mann-Whitney or Wilcoxon Rank-sum Test. After appropriate transformations to achieve approximate normality, mixed effect models were fit for both tumor counts and tumor burdens to test for differences over time by treatment groups and to measure subject specific variability. An alternative analytical method GEE was also used. This method controls for clustering and reports robust estimates of population differences.

Harvesting Primary Keratinocytes from Newborn mice. Three–day-old mice were euthanized with CO_2 according to IACUC procedure. Killed mice were placed in a sterile beaker and immersed in betadine for 2 minutes. The betadine was discarded, and the mice were immersed again in betadine for another 2 minutes. After discarding the second betadine wash, the mice were immersed in 70% ethanol for 2 minutes, two times. After discarding the ethanol, the mice were then rinsed with sterile water and the beaker containing the mice were placed on ice in sterile hood. The limbs and tails were removed from the mice with sterile scissors. The skin was removed by cutting from tail to the nose along the ventral side and using sterile forceps to remove the skin from the body. In an 8cm cell culture dish, the skin was then floated epidermis

side up in 1X 0.25% Trypsin/EDTA free (Thermo Scientific) (5mL/mouse) at 4ºC overnight. The skins were then removed from the trypsin and placed epidermis side down on the inner lid of a fresh 8cm cell culture dish. The dermis was removed and discarded by lifting it up with forceps and discarding it while leaving the epidermis behind. The epidermis was then transferred to a small 50mL sterile beaker containing 1.3mM $CaCl_2$ Keratinocyte Basal Media (Lonza) (5mL/mouse) and minced into small pieces with sterile scissors. The small pieces of epidermis were then tritrated with a 10mL pipette 20X before being transferred to a 50mL conical tube. The tube was spun down for 5min at 8,000x*g*. The supernatant and floating pieces of stratum corneum were discarded. The pellet was resuspended in 1.3mM $CaCl_2$ cell culture media and filtered through a 100µm cell filter and spun down again at 8,000x*g*. The supernatant was discarded and the pellet resuspended in 1mL of 1.3mM $CaCl_2$media. The cells were counted, spun down and resuspended in 0.05 mM $CaCl_2$ Keratinocyte Basal Media (Lonza). The pellet was resuspended in 1.3mM $CaCl_2$ cell culture media and filtered through a 100µm cell filter, 0.05mM $CaCl_2$ Keratinocyte basal media and seeded in 6-well plates. The 6-well plates were pre-coated with collagen by adding 2mL of 10µg/mL of collagen in DPBS to each well and incubating in cell culture incubator or 60min.

***Acute UVB In vivo study*.** 20 Nrf2 knockout female SKH-1 mice (SKH1-Hr^{hr}) and 19 wild-type female SKH-1 mice were used to analyze the precancerous effect of SFN on Nrf2 knockout mouse epidermis. Mice were purchased from Charles River laboratories and housed in accordance with The University of Arizona Animal Care and Use Committee standards under an approved protocol. Mice were divided into four groups (n = 4) and either held as controls or exposed to UVB (2.5 kJ m^{-2}). Mice were pre-treated with SFN (or vehicle) 5 times over the

course of 1.5 weeks prior to UVB exposure. After SFN (or vehicle) pre-treatment, mice were treated with UVB 3 times over the course of one week (Friday, Monday, Wednesday) in order to induce a UV stress response (edema, skin thickening). During the UVB treatment period, single fold back skin thickness measurements were performed before the initial UV exposure followed by daily measurements starting 24 hr later for a total of eight days. A digital caliper was used to measure skin thickness as in indication of edema and inflammation. At the end of the study, mice were sacrificed 24 h after last UVB treatment, and their back skins were harvested and divided for snap freezing or formalin fixation for IHC.

Statistical analysis: Kruskal-Wallis and student's *t* test were used. The analysis controlled for triplicate measures per mouse per time point with random effect for mouse and controlled for baseline differences in skin thickness by adding average baseline thickness by mouse as a covariate.

***Keratinocyte cell culture*.** All HaCaT keratinocyte cell line cultures were maintained in a 37°C, 5% CO_2 incubator and checked routinely for mycoplasma contamination. All neonatal mouse primary keratinocyte cell cultures were maintained in a 36°C, 7% CO_2. HaCaT cell lines were grown in DMEM with 10% FBS and pen/strep, and neonatal mouse primary keratinocytes were grown in 0.05mM $CaCl_2$ keratinocyte media. Neonatal mouse primary keratinocyte were used within one passage as they were observed to lose viability after trying to passage more than once.

Real-time quantitative RT-PCR. Total RNA was extracted from either scraped frozen SKH-1 hairless mouse back skin or cultured neonatal primary keratinocytes using an RNeasy Mini Kit

(Qiagen, Frederick, MD) according to the manufacturer's protocol. Mouse GAPDH (Mm99999915_m1), NQO1 (Mm01253561_m1), and PCNA (Mn00448100_g1) primer/probes were purchased from ABI (Applied Biosystems, Branchburg, NJ). cDNAs from four individual samples for each control/treatment experiment were synthesized from 500 ng of total RNA in a 50µl reaction with master mix containing 10 9 RT buffer, 5.5 mM MgCl2, 2mM dNTPs, 2.5 µM random hexamers, 2 Units of RNase Inhibitor, and 62.5 Units of Multi Scribe Reverse Transcriptase. All MasterMix reagents were purchased from ABI (Applied Biosystems, Branchburg, NJ). Reactions were performed in a MJ Thermocycler PTC-200 (MJ Research, Inc., Watertown, MA) under the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min; 10 ng of cDNA was then used to amplify the mouse or human sequences. The conditions for quantitative PCRs were as follows: 10 min at 95°C followed by 15 s at 95°C, 1 min at 60°C for 40 cycles using 7500 Real-time PCR system from Applied Biosystems (Applied Biosystems, Foster City, CA). PCR amplification of human or mouse GAPDH was used to control quality of the cDNA. Nontemplate controls were included on each PCR plate. Amplification plots were generated and the Ct values (cycle number at which fluorescence reaches threshold) recorded. Target gene levels were normalized to the GAPDH control [$\Delta C_t = C_{t(\text{gene of interest})} - C_{t(\text{housekeeping gene})}$]. Results are representative of n = 3 for cell culture studies, three mice/group for *in vivo* studies.

Flow cytometric reactive oxygen species (ROS) assay. 6-well plates (one for No-UVB control and one for UVB treated for each genotype) were seeded with 250, 000 neonatal primary keratinocytes per well and allowed to grow for 48 hours. The media was then aspirated and the cells were washed with DPBS. The top three wells were then treated with vehicle (acetonitrile)

and the bottom three wells were treated with 10µM SFN for 16 hours. For primary keratinocytes, 10µM SFN solution was prepared in 0.05mM $CaCl_2$ Keratinocyte Basal Media (Lonza). 6-well plates were aspirated and washed with DPBS. 300µl of DPBS was left in the wells during treatment with 250 J/m^2 of UVB. For HaCaT cells, 10µM SFN solution was prepared in DMEM with pen/strep. After UVB treatment, wells were washed with DPBS. Cells were then trypsinized, transferred to 1.5 mL microcentrifuge tubes and spun down at 4,000rpms for 5min. After removing the supernatant, cells were then resuspended with 0.5mL 1X DCFDA working buffer as specified by the manufacturer. Cells were then transferred to flow cytometry tubes and read at 488/535. Results are representative of n = 3.

Statistical methods: Statistical analysis was performed as described in the respective methodology section. Student's *t*-test was performed if no other analysis method is specified.

Histology examination. SKH-1 mouse back skin samples were fixed in 10% neutral buffered formalin and paraffin embedded. 4–6 µm thick sections were stained with hemotoxylin and eosin (H&E) to allow for morphologic evaluation using a Leica DMA Multispectra Imaging System light microscope at 40X. Statistical analysis. Student's t-test was performed if no other analysis method is specified.

Results

Sulforaphane potentiated tumors in UVB treated Nrf2 null hairless mice

To assess the role that Nrf2 plays in SFN's ability to reduce UVB-induced non-melanoma skin cancer, SFN or vehicle control pretreatment occurred on two mouse genotypes (four groups total): wildtype and Nrf2-null. The four groups of mice were then treated with UVB while SFN /vehicle treatment continued for a total of 25 weeks. As expected, topical SFN did significantly reduce UVB-induced tumor incidence and tumor burden in wildtype mice compared to vehicle control ($P < 0.0001$; Fig 3a, 3b and 3c). UVB-induced tumor incidence and tumor burden were also lower in SFN treated wildtype mice compared to the vehicle control treated Nrf2-null mouse group. However, the Nrf2-null SFN treatment group developed significantly more (and larger) tumors compared to the wildtype SFN treatment group ($P<0.0002$; Fig 3a, 3b, and 3c).

Sulforaphane did not induce NQO1 mRNA gene expression in SKH-1/hr hairless mice.

To support the claim that SFN exerts its protective effect by inducing Nrf2-dependent genes, quantitative real time (RT) PCR was used to measure mRNA induction of the classical Nrf2-responsive gene NQO1 in the skin of mice treated topically with vehicle or SFN (a total of 8 treatments) (Fig 4). Not surprisingly, the baseline level of NQO1 mRNA in wildtype mice was higher than that of Nrf2-null mice (Fig 4). However, no significant difference was observed in NQO1 mRNA levels between SFN and vehicle control groups in of the either genotype.

Keratinocytes in culture require Nrf2 in order for sulforaphane to block UV-induced ROS.

Cell culture was used to determine the effect of SFN on oxidative stress in keratinocytes in culture. Although there was a positive trend in UVB induced ROS in HaCaT cell lines compared to no-UVB control, UVB did not increase ROS levels significantly in these cells ($P < 0.23$; Fig 5). However, SFN did significantly reduce UVB-induced ROS levels compared to vehicle control in HaCaT cells ($P<0.015$; Fig 5). In wildtype primary keratinocytes, UVB increased ROS level compared to non-UVB controls, although not significantly ($P<0.35$; Fig 6). SFN was able to significantly reduce UVB-induced ROS compared to vehicle control in these cells as well ($P<0.033$ Fig 6). However, in Nrf2-null primary keratinocytes, UVB treatment increased ROS levels in SFN-treated cells compared to vehicle control, although not significantly ($P<0.27$; Fig 7).

Sulforaphane did not significantly reduce UVB-Induced skin inflammation.

UVB is well known to cause cutaneous inflammation which is the result of vascular permeability and edema. Inflammation was assessed by using a vernier caliper to measure single-fold back skin thickness in wildtype and Nrf2 null mice, with and without SFN treatment. As expected, UVB did induce inflammation in both wildtype and Nrf2-null SKH-1 hairless mice ($P < 0.001$; Fig 8). Inflammation increased over time with UVB treatment in all groups. There was also reduced inflammation (skin thickness) observed in Nrf2-null mice compared to wildtype mice over 7 days of observation ($P < 0.002$; Fig 8). However, topical treatment with 1µmole of SFN did not significantly reduce inflammation compared to vehicle control using skin thickness measurements in either wildtype mice or Nrf2-null mice (Fig 8).

Sulforaphane did not significantly reduce UVB-Induced epidermal thickening.

Another well-known consequence of UVB exposure is thickening of the epidermal layer of the skin. Quantification of H&E staining confirmed that UVB significantly induced epidermal thickening compared to non-UVB treated mice. Topical SFN did reduce epidermal thickening in UVB-treated mice, although not significantly ($P< 0.138$; Fig 9a). On the other hand, topical SFN increased epidermal thickening compared to vehicle control in UVB treated Nrf2-null SKH-1 hairless mice (results not significant, $p< 0.367$; Fig 9a). In a related study, a statistically significant increase in proliferation of primary keratinocytes using qRT-PCR for PCNA expression was not observed between SFN and vehicle control in UVB treated groups (Fig 9b).

Figure 1.

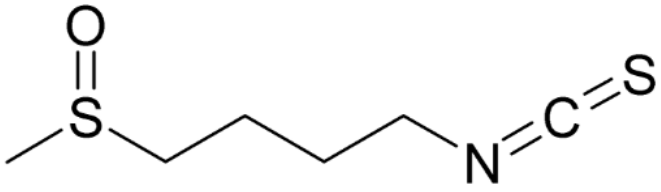


Figure 2.

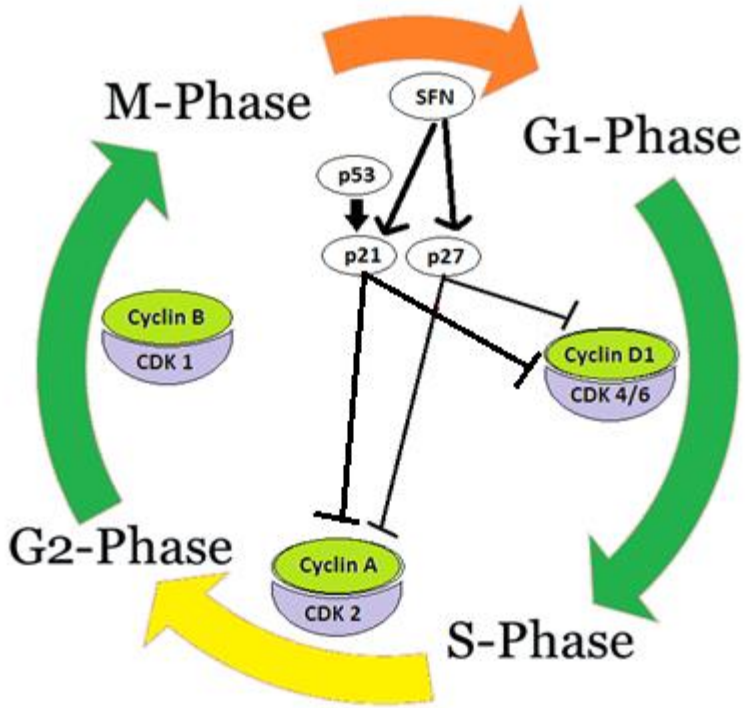


Figure 3a

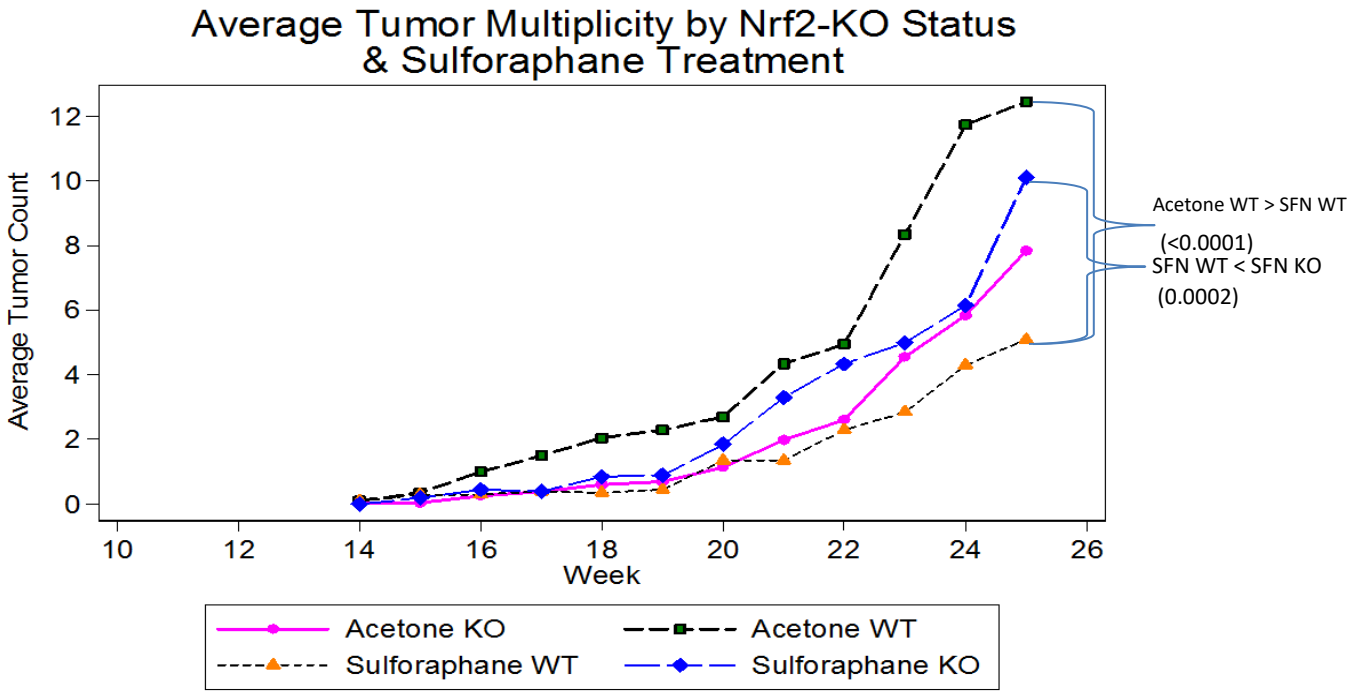


Figure 3b

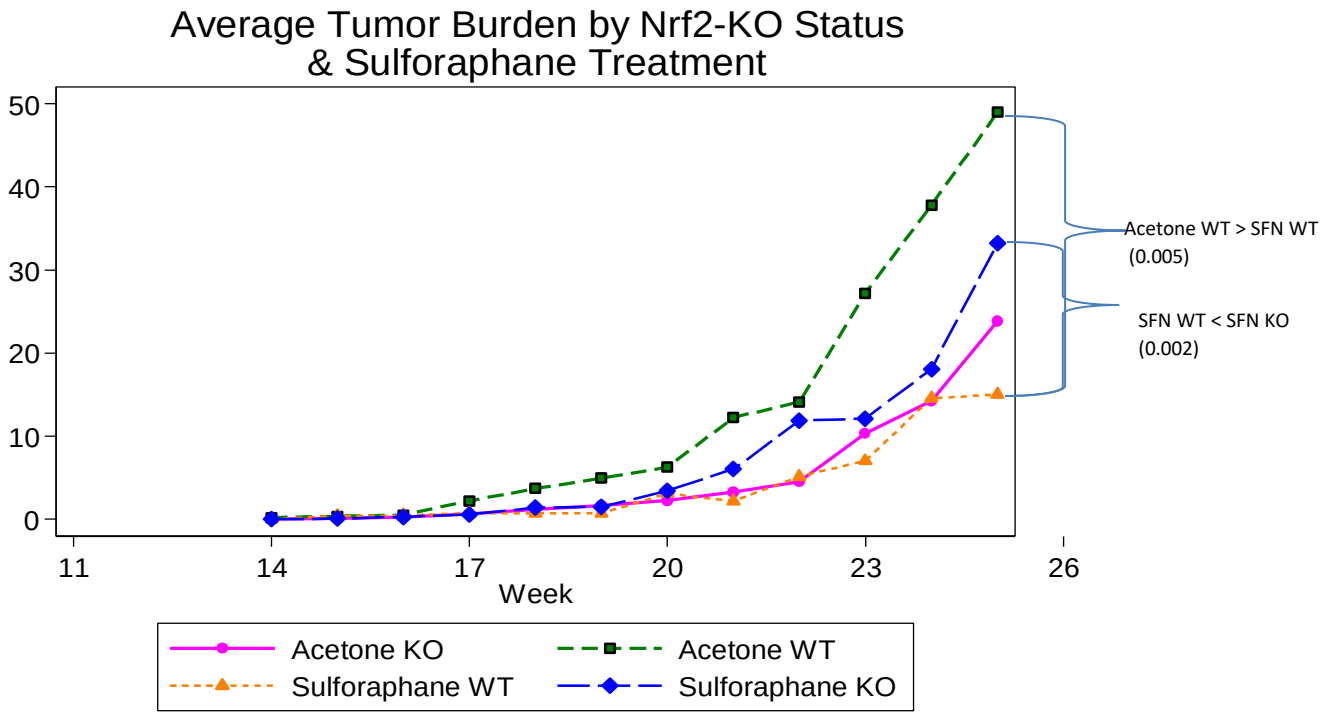


Figure 3c

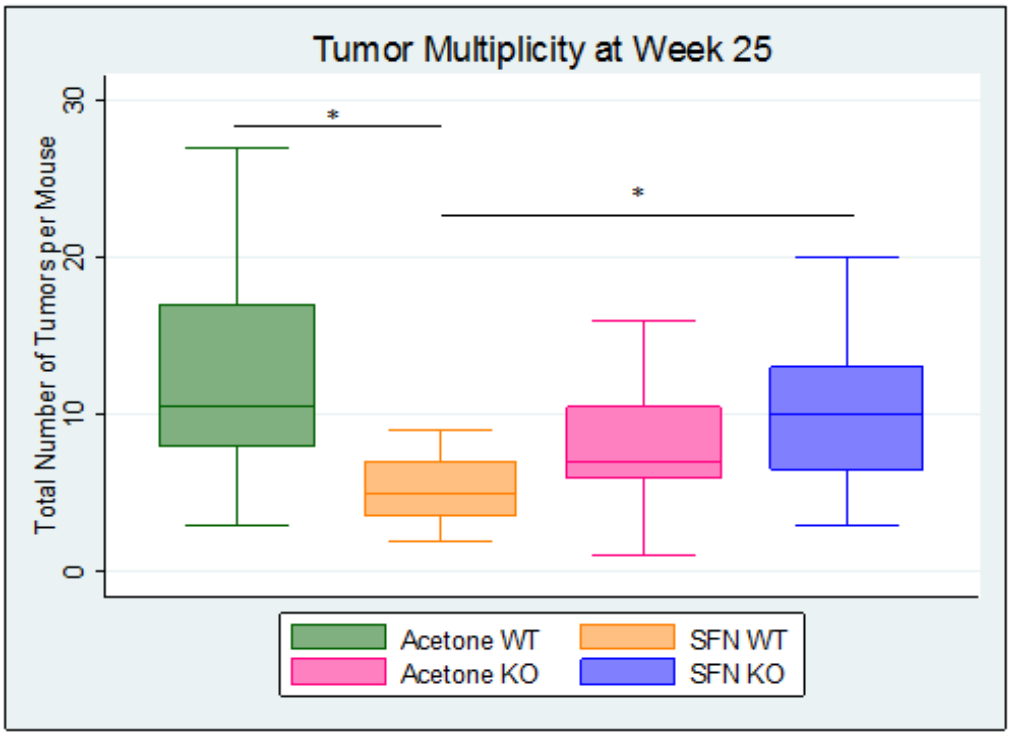


Figure 4

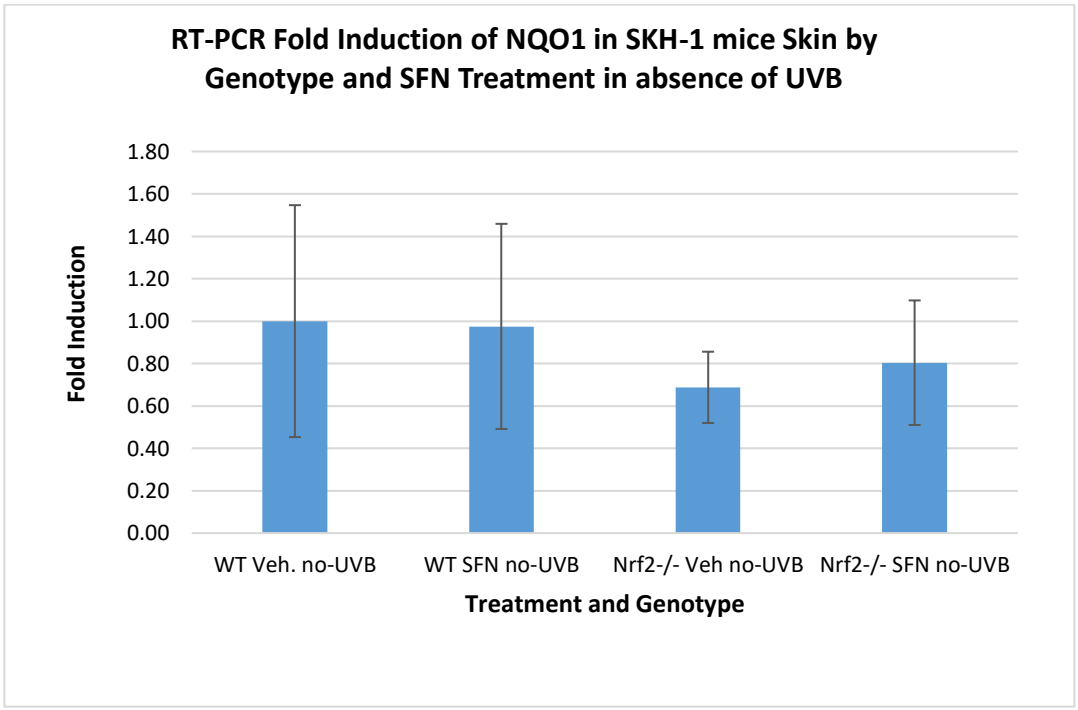


Figure 5

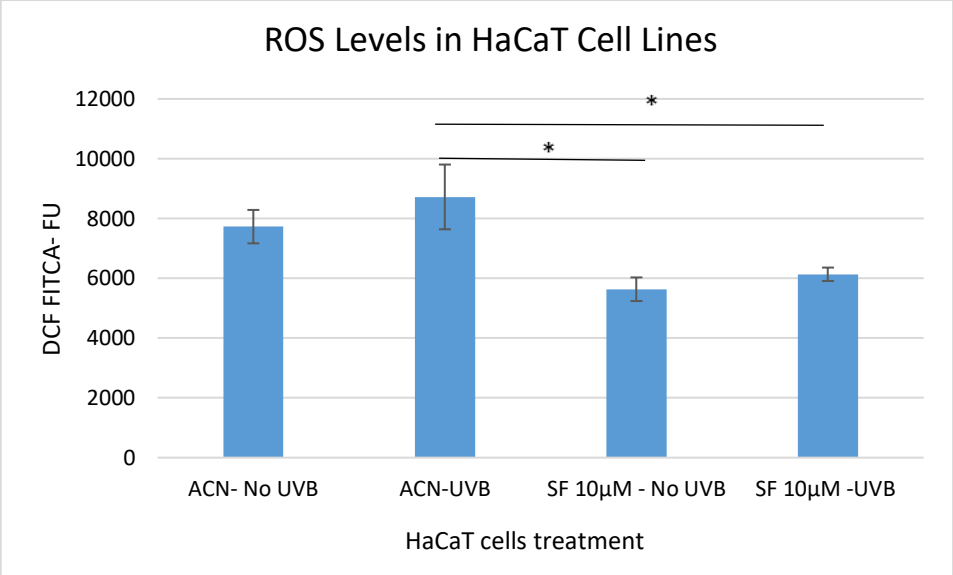


Figure 6

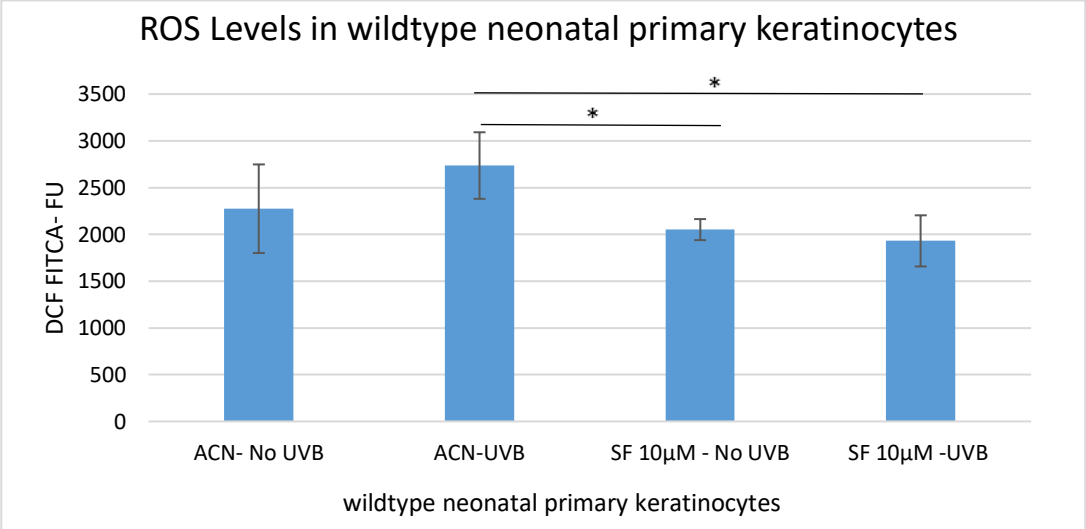


Figure 7

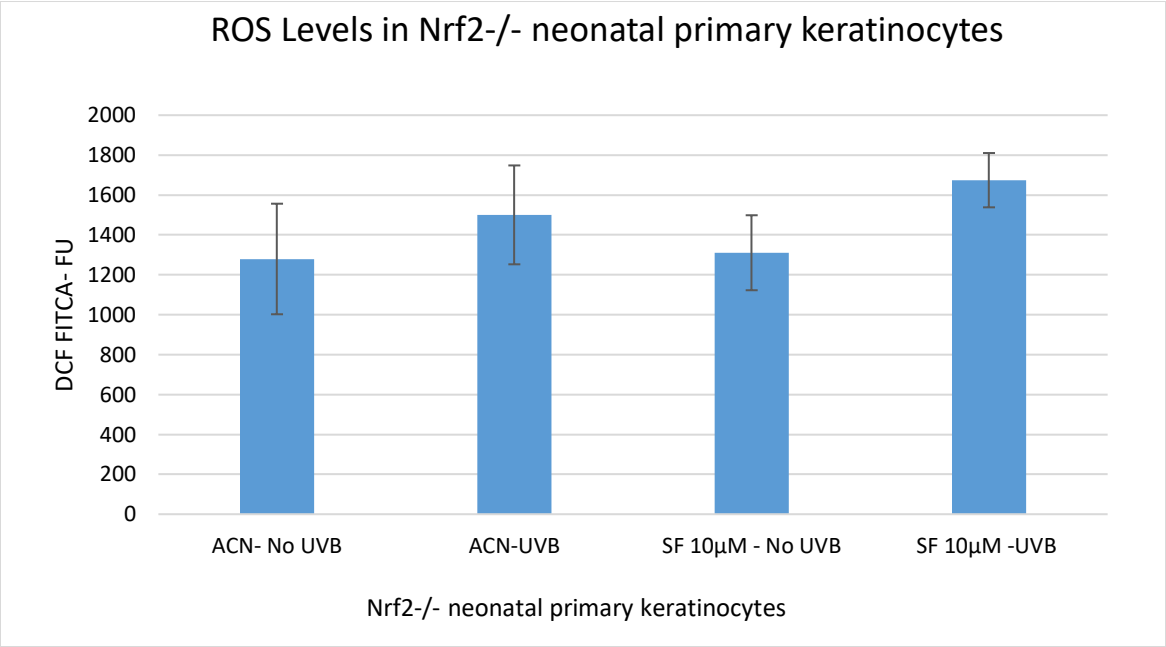


Figure 8

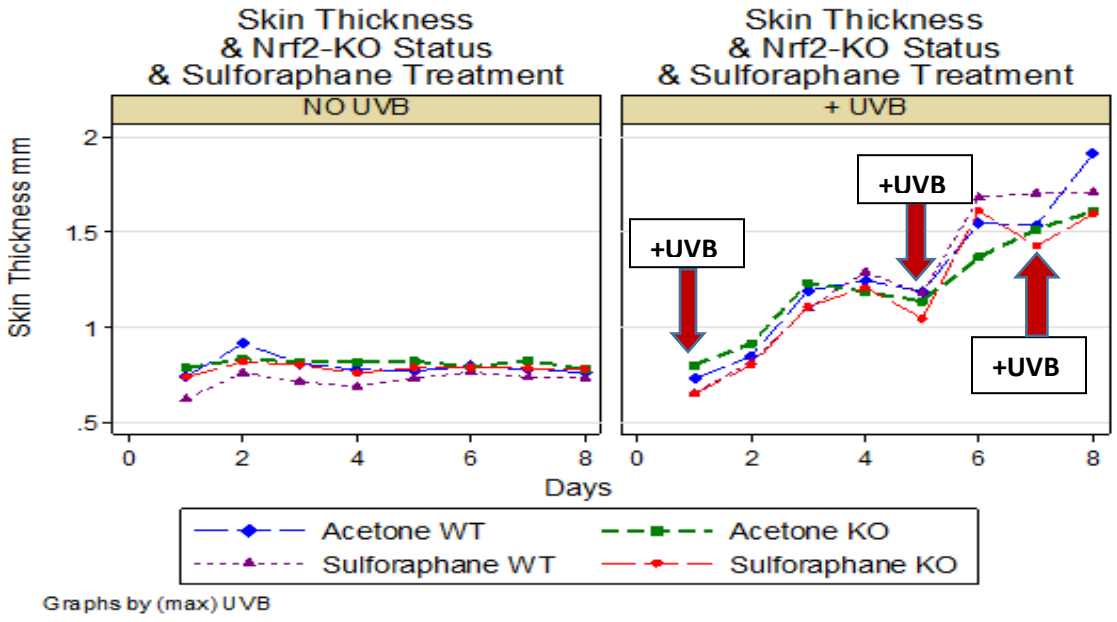


Figure 9a

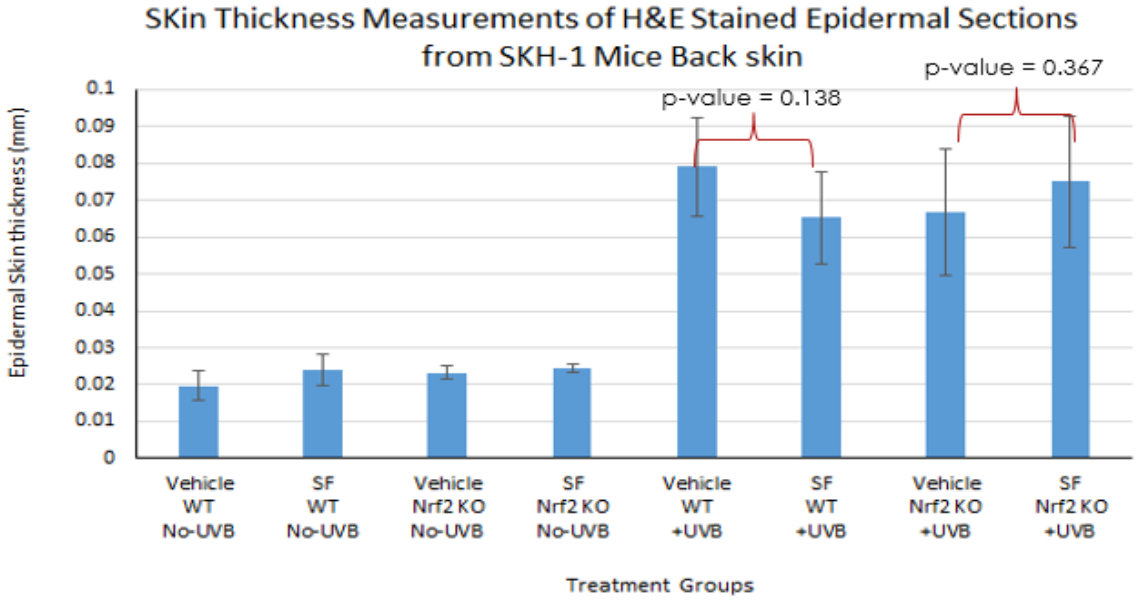
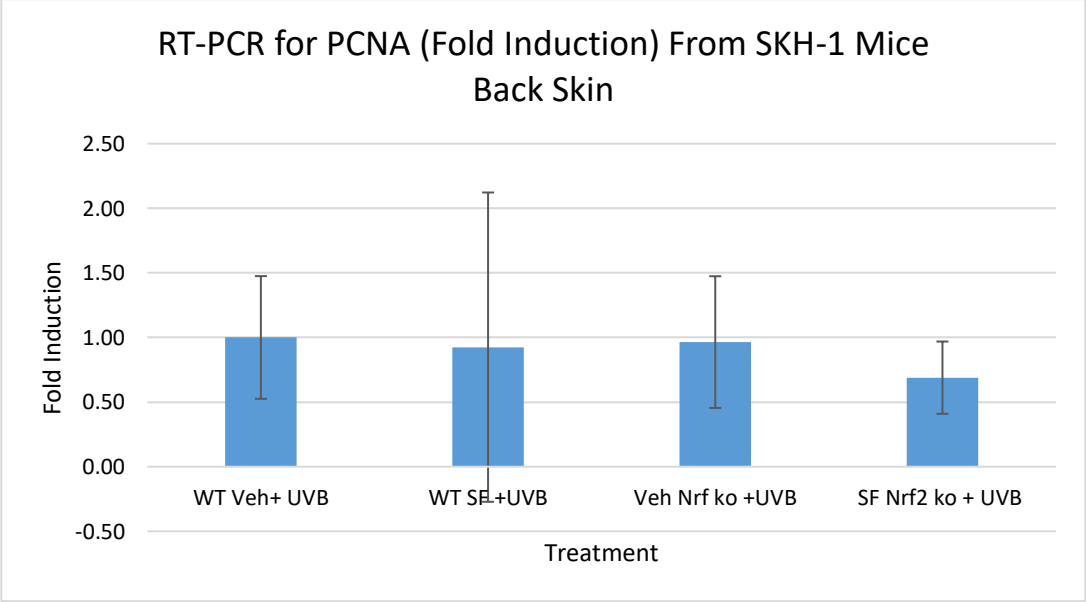


Figure 9b



Discussion

SFN, a phytochemical found in broccoli, is the active ingredient from cruciferous vegetables that was discovered to have antitumor and chemopreventive properties in epidemiological and laboratory-based studies. Many experiments have been performed with the aim of determining the mechanism of SFN's anticancer properties. Such experiments have established that SFN has the ability to regulate many cellular processes such as cell cycle progression and inflammation, a strong enabler of cancer [reviewed in ref. 34]. Considerable attention has been given to SFN's ability to induce phase II cyto-protective enzymes, which act to neutralize many carcinogens. This makes SFN an attractive chemopreventive agent. SFN was discovered to act through Nrf2 to induce these cyto-protective enzymes. In order to perform *in vivo* experiments examining the role that Nrf2 plays in SFN's ability to inhibit carcinogenesis, mouse models have been created in which Nrf2 activity is either disrupted by creating Nrf2 knockout (Nrf2 KO) mice or enhanced by various means.

Nonmelanoma skin cancer is a disease caused by ultraviolet radiation (UVR) from the sun which causes direct DNA damage and increases oxidative stress in the skin. So, with respect to skin cancer experiments, Nrf2 KO mice are treated with both UVR and drug and then compared to wild-type (WT) mice treated under the same conditions. Our lab has observed that SFN potentiates tumors in Nrf2 KO mice treated with UVB. This is an unexpected result which has not been previously reported. In some cases, pro-oxidant activity of SFN has been reported in cellular and animal models [53-54]. Therefore, we hypothesized that SFN is a pro-oxidant in the skin of Nrf2 KO SKH-1 mice treated with UVB. To investigate, we measured ROS, inflammation, and hyperplasia in our animal and cell models. We measured oxidative stress in wildtype and Nrf2 KO primary keratinocytes harvested from neonatal mice which were treated

with UVB and SFN (or vehicle). Although we were not able to observe significant induction of UVB-induced oxidative stress in cell culture, we did observe a significant reduction in UVB-induced ROS by SFN in HaCaT keratinocyte cell lines and wild-type primary keratinocytes. On the other hand, SFN seemed to slightly increase UVB-induced ROS in Nrf2-KO primary keratinocytes. We also measured UVB-induced skin inflammation in a short-term *in vivo* model. Although we were able to induce inflammation using UV, we were not able to show a significant effect of SFN on inflammation in this study, regardless of mouse genotype. Then, we measured UVB-induced epidermal hyperplasia by quantifying epidermal thickness of H&E stained skin sections. While we observed some interesting trends in the data (slight inhibition by SFN in wildtype mice, slight increase by SFN in KO mice), none of the observations were significantly different. Expression of the proliferative marker PCNA also did not change with treatment. Thus, while our hypothesis that SFN acts in a pro-oxidant role in Nrf2 KO mice seems to be generally supported by the cell culture data, there seems to be some confounding factors which prevent our other studies from achieving significant results.

Epidemiological evidence has pointed to a strong connection between inflammation and cancer. In fact, the usage of non-steroidal anti-inflammatory drugs is associated with a low risk of many cancers [4]. Nrf2 is associated with protection against inflammation [52]. BALB/c Nrf2 KO mice are more sensitive to UVB-induced inflammation compared to wild type mice [50], and induction of Nrf2 (pharmacologically or genetically) is protective against UVB-induced inflammation compared to control [56]. So, it is not surprising that others have observed SFN to have anti-inflammatory properties through the induction of Nrf2. However, SFN also has anti-inflammatory properties independent of Nrf2 [57][56]. Taken together, the anti-inflammatory properties of SFN alone make it an attractive chemopreventive agent.

However, we did not observe a difference in inflammation between SFN and vehicle treated UVB groups in our mouse skin studies.

UVB has also been shown to induce epidermal hyperplasia which has the ability to develop into cancer. Hyperplasia is the result of increased cell proliferation and/or decreased apoptosis [22-24]. It has been shown that UVB increases cell proliferation through EGFR, destroying EGFR phosphatases and deactivating them [59]. This, in turn, activates the MAPK pathway resulting in the expression of cyclin D1 which is needed to push the cell through the cell cycle [59]. SFN has been shown to inhibit the induction of cyclin D1 [36] and to sensitize cells to apoptosis [66-67]. Thus, one would expect that SFN would also show protection against hyperplasia. Based upon our tumor results from the SKH-1 model, we were surprised to see no significant difference in hyperplasia between SFN and vehicle treated UVB groups when mice were treated short-term with SFN and UVB.

There could be many reasons for the lack of congruity between our data and that reported by others. For example, there is a plethora of biochemical changes observed in Nrf2-null mice even in the absence of stress. Nrf2-null mice have been reported by others to have non-stress-induced levels of ROS 1.6 to 4.0 times higher than wild-type mice C57BL/6 immortalized mouse embryonic fibroblast cells (MEF) [62]. This increase in ROS levels has been shown to affect cell cycle progression. More Nrf2-null cells were observed to be stuck in the G2/M-phase of the cell cycle when compared to wild-type cells [63]. Also, this abnormality was corrected when exogenous glutathione was added to the Nrf2-null cells. Other examples include changes in insulin signaling and cellular unfolded protein response in the liver of C57B/SV129 mice [65]. Also, differences in global gene expression are observed between pharmacologically induced and

genetically induced Nrf2 [66]. Taken together with the fact SKH-1 mice are outbred strains, these biochemical changes could be the source of the data incongruity.

Future Directions

It should be noted that SFN has also been shown to induce DNA damage at dietary concentrations [67]. SFN-induced DNA damage was observed to be both tissue and dose dependent which also was correlated with apoptosis and cell cycle arrest. Therefore, future research should investigate if UVB treated Nrf2 knockout SKH-1 hairless mice are more susceptible to SFN-induced DNA damage compared vehicle wild-type control and if SFN-induced DNA damage leads to carcinogenesis. In addition, SFN has been shown to inhibit the transcription factor activator protein-1 (AP-1), a well-known facilitator in UVB-induced NMSC [64]. Future research should also investigate if SFN inhibition of AP-1 is altered in the absence of Nrf2.

As noted above, SFN has been shown to inhibit UVB-induced inflammation [52]. However, our results show no difference (in either wildtype or Nrf2 knockout mice) between vehicle or SFN treated skins. This result may suggest a compensation mechanism for the loss of Nrf2. Nrf1, Nrf2, and Nrf3 can all bind to the ARE and induce expression of cytoprotective genes to varying degrees, and several groups have suggested that the effects of Nrf2 loss may be dampened by the presence of the other Cap 'n' Collar transcription factors [27, reviewed in ref. 28]. Nrf3 has been shown to play a role in inflammation in 129S6/SvEvTac mouse lung [28]. It has been observed in BALB/c mouse keratinocytes that the Nrf3 (but not Nrf2) mRNA level is induced in Nrf2 knockout mice compared to wild-type controls during wound healing [68].

Thus, future research should investigate whether SFN can alter Nrf3 levels in Nrf2 knockout mice during UVB treatment compared to vehicle control.

The application of SFN as a chemopreventive agent suggests the need for long-term usage of SFN on the skin. Therefore, future research should investigate long-term consequences for topical administration of SFN given the balance of positive and negative effects that have been noted in animal models. In addition, it should also be determined if SFN applied topically reaches the blood plasma and affects systemic Nrf2 activity in a significant manner. This may be important in light of potential drug interactions in individuals administering SFN to the skin. We look forward to continuing this research in order to verify whether topical application of this natural product would be beneficial for individuals at risk for NMSC.

Figure Legend:

Figure 1. Chemical structure of sulforaphane.

Figure 2. Sulforaphane inhibits different phases of the cell cycle. SFN can induce the tumor suppressor proteins p21 and p27. P21 and p27 may then arrest the cell in G_1 or G_2-phase.

Figure 3. SFN potentiates carcinogenesis in Nrf2 knockout mice. SKH-1 hairless wildtype and Nrf2-null mice were topically pre-treated for 1 week with 1µmol SFN /back (or vehicle) before initiation of 25 weeks of thrice weekly treatments with UVB. Mice continued pretreatments with SFN or vehicle 1hr before each UV exposure. Micrometer measurements were used to assess: (a) tumor multiplicity and (b) tumor burden.

Figure 4. Measurement of NQO1 mRNA induction. SKH-1 wild type and Nrf2-null mice were pre-treated with 1µmol SFN (or vehicle) 5 times over the course of 1.5 weeks (n=3). NQO1 mRNA induction was assessed by RT-PCR. Results are means ± SD.

Figure 5. Measurement of UVB-induced ROS in HaCaT cell lines. Cells (n=3) were pre-treated with 10µM SFN for 16 hours then treated with 250 J/m^2 of UVB to induce ROS. Dichlorofluorescin diacetate (DCFDA) fluorogenic dye was used to assay for Reactive Oxygen Species (ROS). $P< 0.015$ for group comparisons between SFN and vehicle in UVB treated HaCaT cells. Results are means ± SD.

Figure 6. Measurement of UVB-induced ROS wildtype SKH-1 hairless neonatal primary keratinocytes. Cells (n=3) were pre-treated with 10µM sulforaphane for 16 hours and then treated with 250 J/m^2 of UVB to induce ROS. Dichlorofluorescin diacetate (DCFDA) fluorogenic dye was used to assay for Reactive Oxygen Species (ROS) using flow cytometry.

P< 0.03 for group comparisons between SFN and vehicle in UVB treated neonatal primary keratinocytes. Results are means ± SD.

Figure 7. Measurement of UVB-induced ROS in Nrf2-null SKH-1/hr neonatal primary keratinocytes. Cells (n=3) were pre-treated with 10μM SFN for 16 hours then treated with 250 J/m^2 of UVB to induce ROS. Dichlorofluorescin diacetate (DCFDA) fluorogenic dye was used to assay for Reactive Oxygen Species (ROS) using flow cytometry; P< 0.35 for group comparisons between SFN and vehicle in UVB treated Nrf2-null SKH-1 neonatal primary keratinocytes. Results are means ± SD.

Figure 8. UVB-induces inflammation in SKH-1 wild type and Nrf2-null mice. SKH-1 wildtype or Nrf2-null mice were pre-treated with 1μmol SFN (or vehicle) 5 times over the course of 1.5 weeks (n=3 for non-UVB treated groups and n=4 for UVB treated groups). The mice were then treated with 2.5 kJ/m^2 of UVB (5 times) over the course of one week while continuing SFN and vehicle treatment. Inflammation assessed by single fold back skin thickness measurements.

Figure 9. UVB induces epidermal hyperplasia in SKH1 wild type and Nrf2-null mice. SKH-1 wildtype mice pre-treated with 1μmol SFN (or vehicle) 5 times over the course of 1.5 weeks. The mice were then treated with 2.5 kJ/m^2 of UVB (as described in Figure 6a) over the course of one week while continuing SFN and vehicle treatment. (a) Epidermal hyperplasia (thickness) assessed by microscopy of hematoxylin and eosin stained back skin sections. (b) Epidermal proliferation assessed by quantitative real-time PCR of mRNA for the marker PCNA from SKH-1 adult mouse epidermal lysates. Results are means ± SD.

References

1. Rubin AI, Chen EH, Ratner D. Basal cell carcinoma. (2005) New England Journal of Medicine 353:2262–2269
2. Alam M, Ratner D. Cutaneous squamous cell carcinoma (2001) New England Journal of Medicine 344: 975–983.
3. N De Gruijl FR (2000) Photocarcinogenesis: UVA vs UVB. Methods Enzymol 319: 359–366.
4. Colotta, F., Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30, 1073–1081
5. De Gruijl FR (2000) Photocarcinogenesis: UVA vs UVB. Methods Enzymol 319: 359–366.
6. Markovitsi D, Gustavsson T and Banyasz A (2010) Absorption of UV radiation by DNA: spatial and temporal features. Mutat Res 704: 21–28.
7. Johns HE, et al (1964) The ultraviolet photochemistry of thymidylyl-(3'→5')-thymidine. J Mol Biol 9: 503–524.
8. Taylor J-S and Cohrs MP (1987) DNA, light, and Dewar pyrimidones: the structure and biological significance of TpT3. J Am Chem Soc 109: 2834–2835.
9. Matsunaga T, Kotaro H and Nikaido O (1991) Wavelength dependent formation of thymine dimers and (6-4) photoproducts in DNA by monochromatic ultraviolet light ranging from 150 to 365 nm. Photochem Photobiol 54: 403–410.
10. Sekiguchi M and Tsuzuki T (2002) Oxidative nucleotide damage: consequences and prevention. Oncogene 21: 8895–8904.
11. Kino K and Sugiyama H (2005) UVR-induced G-C to C-G transversions from oxidative DNA damage. Mutat Res 571: 33–42.
12. Tessman I and Kennedy MA (1991) The two-step model of UV mutagenesis reassessed: deamination of cytosine in cyclobutane dimers as the likely source of the mutations associated with photoreactivation. Mol Gen Genet 277: 144–148.
13. Tessman I, Liu S and Kennedy MA (1992) Mechanism of SOS mutagenesis of UV-irradiated DNA: mostly error-free processing of deaminated cytosine. Proc Natl Acad Sci USA 89: 1159–1163.
14. Peak JG, Peak MJ and MacCoss M (1984) DNA breakage caused by 334-nm ultraviolet light is enhanced by naturally occurring nucleic acid components and nucleotide coenzymes. Photochem Photobiol 39: 713–716.
15. Walrant P and Santus R (1974) n-Formyl-kynurenine, a tryptophan photooxidation product, as a photodynamic sensitizer. Photochem Photobiol 19: 411–417.
16. MacCormick JP, et al (1976) Characterization of a cell-lethal product from the photooxidation of tryptophan: hydrogen peroxide. Science 191: 468–469.
17. Krasnovsky Jr AA (1979) Photoluminescence of singlet oxygen in pigment solutions. Photochem Photobiol 29: 29–36.

18. Sekiguchi M and Tsuzuki T (2002) Oxidative nucleotide damage: consequences and prevention. Oncogene 21: 8895–8904.
19. Clydesdale GJ, Dandie GW, Muller HK. (2001) Ultraviolet light induced injury: immunological and inflammatory effects. Immunol Cell Biol. 79(6):547-68.
20. Kim AL, Labasi JM, Zhu Y, Tang X, McClure K, Gabel CA, Athar M, Bickers DR.(2005) Role of p38 MAPK in UVB-induced inflammatory responses in the skin of SKH-1 hairless mice. J Invest Dermatol.;124(6):1318-25.
21. Dickinson, S. E., Olson, E. R., Zhang, J., Cooper, S. J., Melton, T., Criswell, P. J., et al. (2011). P38 MAP kinase plays a functional role in UVB-induced mouse skin carcinogenesis. Molecular Carcinogenesis, 50, 469–478.
22. Pearse AD, Gaskell SA, Marks R. (1987) Epidermal changes in human skin following irradiation with either UVB or UVA. J. Invest. Dermatol 88: 83–7.
23. Ouhtit A, Muller HK, Davis DW, Ullrich SE, McConkey D, Ananthaswamy HN. (2000) Temporal events in skin injury and the early adaptive responses in ultraviolet-irradiated mouse skin. Am. J. Pathol. 156: 201–7.
24. Wedemeyer N. , Gohde W., and Potter T. (2000) Flow Cytometric Analysis of Reverse Transcription-PCR Products: Quantification of p21WAF1/CIP1 and Proliferating Cell Nuclear Antigen mRNA Clinical Chemistry 46:8 1057–1064.
25. Ma Q (2013) Role of Nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. Toxicol. 53:401–26.
26. Ohtsuji M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD, Yamamoto M. (2008) Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. J Biol Chem.;283(48):33554-62.
27. Zhang Y, Kobayashi A, Yamamoto M, Hayes JD. (2009) The Nrf3 transcription factor is a membrane-bound glycoprotein targeted to the endoplasmic reticulum through its N-terminal homology box 1 sequence. J Biol Chem.284(5):3195-210.
28. Chevillard G1, Blank V. (2011) NFE2L3 (NRF3): the Cinderella of the Cap'n'Collar transcription factors. Cell Mol Life Sci.;68(20):3337-48.
29. Ambrosone C. B., McCann, S. E., Freudenheim, J. L., Marshall, J. R., Zhang, Y. and Shields, P. G. (2004) Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. J. Nutr. 134, 1134 – 1138.
30. Joseph, M. A.,Moysich, K. B., Freudenheim, J. L., Shields, P.G., Bowman, E. D., Zhang, Y., Marshall, J. R. and Ambrosone, C. B. (2004) Cruciferous vegetables, genetic polymorphisms in glutathione S-transferasesM1 and T1, and prostate cancer risk. Nutr. Cancer 50, 206 – 213.
31. Lin, H. J., Probst-Hensch, N. M., Louie, A. D., Kau, I. H., Witte, J. S., Ingles, S.A., Frankl, H.D.,Lee,E.R. and Haile,R. W. (1998) Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas. Cancer Epidemiol. Biomarkers Prev. 7, 647 – 652.

32. Spitz, M. R., Duphorne, C. M., Detry, M. A., Pillow, P. C., Amos, C. I., Lei, L., deAndrade,M., Gu,X., Hong,W.K. and Wu,X. (2000) Dietary intake of isothiocyanates: evidence of a joint effect with glutathione S-transferase polymorphisms in lung cancer risk. Cancer Epidemiol. Biomarkers Prev. 9, 1017 – 1020.
33. Wang, L. I., Giovannucci, E. L., Hunter, D., Neuberg, D., Su, L. and Christiani, D. C. (2004) Dietary intake of cruciferous vegetables, glutathione S-transferase (GST) polymorphisms and lung cancer risk in a Caucasian population. Cancer Causes Control 15, 977 – 985.
34. N. Jugea,b R. F. Mithen and M. Traka, (2007) Molecular basis for chemoprevention by sulforaphane: a comprehensive review Cell. Mol. Life Sci. 64 1105 – 1127
35. Bryant, C. S., Kumar, S., Chamala, S., Shah, J., Pal, J., Haider, M., et al. (2010). Sulforaphane induces cell cycle arrest by protecting RB-E2F-1 complex in epithelial ovarian cancer cells. Molecular Cancer, 9, 47.
36. Shen,G., Xu,C., Chen,C., Hebbar,V. and Kong,A.H. (2005) p53-independentG1 cell cycle arrest of human colon carcinoma cells HT-29 by sulforaphane is associated with induction of p21CIP1 and inhibition of expression of cyclin D1. Cancer Chemother Pharmacol, Sep 17, 1–11.
37. Hee-Jeong Jeong1, Seon-Mi yu1, Jae-Chang Jung2 and Song-Ja Kim1 (2012) Sulforaphane inhibits proliferation by causing cell cycle arrest at the G2/M phase in rabbit articular chondrocytes Molecular Medicine Reports 6: 1199-1203
38. Vermeulen et al., 2003 K. Vermeulen, D.R. Van Bockstaele, Z.N. Berneman The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer Cell Prolif., 36 (2003), pp. 131–149.
39. Maheo, K., Morel, F., Langouet, S., Kramer, H., Le Ferrec, E., Ketterer, B. and Guillouzo, A. (1997) Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. Cancer Res. 57, 3649 – 3652.
40. Barcelo, S., Gardiner, J. M., Gescher, A. and Chipman, J. K. (1996) CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. Carcinogenesis 17, 277 – 282.
41. Barcelo, S., Mace,K., Pfeifer,A.M. and Chipman, J. K. (1998) Production of DNA strand breaks by N-nitrosodimethylamine and 2-amino-3-methylimidazo[4,5-f]quinoline in THLE cells expressing human CYP isoenzymes and inhibition by sulforaphane. Mutat. Res. 402, 111 – 120.
42. Gross-Steinmeyer, K., Stapleton, P. L., Tracy, J. H., Bammler, T. K., Lehman, T., Strom, S. C. and Eaton, D. L. (2005) Influence of Matrigel-overlay on constitutive and inducible expression of nine genes encoding drug-metabolizing enzymes in primary human hepatocytes. Xenobiotica 35, 419 –438.

43. Zhou, C., Poulton, E. J.,Grun, F., Bammler, T. K., Blumberg, B., Thummel, K. E. and Eaton, D. L. (2006) The dietary isothiocyanate, sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor (SXR). Mol. Pharmacol.71, 220 – 229.
44. Yoxall,V., Kentish, P., Coldham,N., Kuhnert,N., Sauer,M. J. and Ioannides, C. (2005) Modulation of hepatic cytochromes P450 and phase II enzymes by dietary doses of sulforaphane in rats: implications for its chemopreventive activity. Int. J. Cancer 117, 356 – 362.
45. Talalay, P. (2000) Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors 12, 5 – 11.
46. Prochaska, H. J., Santamaria, A. B. and Talalay, P. (1992) Rapid detection of inducers of enzymes that protect against carcinogens. Proc. Natl. Acad. Sci. USA 89, 2394 – 2398.
47. Fahey, J.W., Zhang,Y. and Talalay, P. (1997) Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc. Natl. Acad. Sci. USA 94, 10367 – 10372.
48. Zhang Y, Li J, Tang L. 2005. Cancer-preventive isothiocyanates: Dichotomous modulators of oxidative stress. Free Radic Biol Med 38:70–77.
49. Saw CL, Huang MT, Liu Y, Khor TO, Conney AH, Kong AN. (2011) Impact of Nrf2 on UVB-induced skin inflammation/photoprotection and photoprotective effect of sulforaphane. Mol Carcinog.50(6):479-86.
50. Kawachi Y, Xu X, Taguchi S, et al. Attenuation of UVB-induced sunburn reaction and oxidative DNA damage with no alterations in UVB-induced skin carcinogenesis in Nrf2 gene-deficient mice. J Invest Dermatol 2008;128:1773–1779.
51. Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, Conney AH, Kong AN. (2006) Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer Res.;66(16):8293-6.
52. Shibata A, Nakagawa K, Yamanoi H, Tsuduki T, Sookwong P, Higuchi O, Kimura F, Miyazawa T. (2010) Sulforaphane suppresses ultraviolet B-induced inflammation in HaCaT keratinocytes and HR-1 hairless mice. J Nutr Biochem.;21(8):702-9
53. Paolini M, Perocco P, Canistro D, Valgimigli L, Pedulli GF, Iori R, Della Croce C, Cantelli-Forti G, Legator MS, Abdel-Rahman SZ. 2004. Induction of cytochrome P450, generation of oxidative stress and in vitro cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. Carcinogenesis 25:61–67.
54. Payen L, Courtois A, Loewert M, Guillouzo A, Fardel O. 2001. Reactive oxygen species-related induction of multidrug resistance-associated protein 2 expression in primary hepatocytes exposed to sulforaphane. Biochem Biophys Res Commun 282:257–263.
55. Dinkova-Kostova AT, Jenkins SN, Fahey JW, Ye L, Wehage SL, Liby KT, Stephenson KK, Wade KL, Talalay P. (2006) Protection against UV-light-induced skin

carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. Cancer Lett.;240(2):243-52.
56. E.V. Knatko, S.H.Ibbotson, Y.Zhang, M.Higgins, J.W.Fahey, P.Talalay, etal. Nrf2 activation protects against solar-simulated ultraviolet radiation in mice and humans. Cancer Prev.Res8(2015)475–486.
57. Martinon F., Mayor A.,and Tschopp J. (2009) The Inflammasomes: Guardians of the Body Annu. Rev. Immunol. 27:229–265
58. Greaney A. J., Maier N. K., Leppla S. H. and Moayeri M. (2016) Sulforaphane inhibits multiple inflammasomes through an Nrf2-independent mechanism. J Leukoc Biol. 99(1):189-99.
59. Xu Y, Shao Y, Voorhees JJ et al. (2006) Oxidative inhibition of receptortype protein-tyrosine phosphatase kappa by ultraviolet irradiation activates epidermal growth factor receptor in human keratinocytes. J Biol Chem; 281:27389–97.
60. Singh, A. V., Xiao, D., Lew, K. L., Dhir, R. and Singh, S. V. (2004) Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts in vivo. Carcinogenesis 25, 83 – 90.
61. Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M. A., Chevolleau, S., Gasc, N., Tulliez, J. and Terce, F. (2000) Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res. 60, 1426 – 1433.
62. J.T. McDonald, K. Kim, A.J. Norris, E. Vlashi, T.M. Phillips, C. Lagadec, etal., (2010). Ionizing radiation activates the Nrf2 antioxidant response, Cancer Res.70: 8886–8895.

63. N M Reddy, S R Kleeberger, J H Bream3, P G Fallon, T W Kensler1, M Yamamoto and S P Reddy (2008) Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling Oncogene. 27, 5821–5832.
64. Dickinson SE, Melton TF, Olson ER, Zhang J, Saboda K, Bowden GT.(2009) Inhibition of activator protein-1 by sulforaphane involves interaction with cysteine in the cFos DNA-binding domain: implications for chemoprevention of UVB-induced skin cancer. Cancer Res.;69(17):7103-10.
65. Beyer TA, Xu W, Teupser D, auf dem Keller U, Bugnon P, Hildt E, Thiery J, Kan YW, Werner S. (2008) Impaired liver regeneration in Nrf2 knockout mice: role of ROS-mediated insulin/IGF-1 resistance. EMBO J.;27(1):212-23.
66. Yates, M.S., Tran, Q.T., Dolan, P.M., Osburn, W.O., Shin, S., McCulloch, C.C., Silkworth, J.B., Taguchi, K., Yamamoto, M., Williams, C.R., Liby, K.T., Sporn, M.B., Sutter, T.R. & Kensler, T.W. (2009) Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid-treated mice. Carcinogenesis 30, 1024–1031.
67. Ferreira de Oliveira JM1, Remédios C, Oliveira H, Pinto P, Pinho F, Pinho S, Costa M, Santos C. (2014) Sulforaphane induces DNA damage and mitotic abnormalities in human

osteosarcoma MG-63 cells: correlation with cell cycle arrest and apoptosis. Nutr Cancer.;66(2):325-34.

68. Braun S, Hanselmann C, Gassmann MG, auf dem Keller U, Born-Berclaz C, Chan K, Kan YW, Werner S. (2002) Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. Mol Cell Biol; 22(15):5492-505.