J. Appl. Environ. Biol. Sci., 8(8)29-34, 2018

ISSN: 2090-4274
Journal of Applied Environmental and Biological Sciences
www.textroad.com

Inhibition of Patulin-Induced Oxidative Stress in Human Esophageal Epithelial Cells by 3, 3'-Diindolylmethane

Jiawei Zhang[1,2#], Bingjie Sun[1,2#], Wenxue Li[2], Bo Zhang[1], Wei Zhu[1,2*], Min Xia[1*]

[1]School of Public Health, Sun Yat-sen University, Guangzhou, 510080, China
[2]Department of Toxicology, Guangzhou Center for Disease Control and Prevention, Guangzhou, 510440, China[1]

Received: March 2, 2018
Accepted: May 27, 2018

ABSTRACT

To understand the effects of 3,3'-diindolylmethane on oxidative stress induced by patulin in HET-1A human esophageal epithelial cells and the underlying mechanism. HET-1A cells were treated with patulin to establish an oxidative stress model. The results of the CCK-8 assay indicated that low dose of DIM shows no obviously toxic effects on the HET-1A cells. Flow cytometric analysis indicated that DIM could inhibit patulin-induced ROS production in the HET-1A cells. The Western Blotting results showed that the protein expression of phospho-p38 MAPK and phospho-SAPK in the HET-1A cells gradually decreased; In addition, phospho-NF-κB expression also decreased significantly. It can be concluded that the underlying mechanism of DIM reduced the patulin-induced oxidative stress in HET-1A cells might be the inhibition of ROS production and regulation oxidative stress-related proteins such as NF-κB and the MAPK family. These results indicated that DIM might be categorized as an effective drug for treating or delaying the injury of esophageal epithelial cells due to oxidative stress.
KEYWORDS: 3,3'-Diindolylmethane; HET-1A cells; Patulin; Oxidative stress

INTRODUCTION

For healthy humans who are not occupationally exposed to xenobiotics, the main route of exposure to chemical is mouth-digestive tract-gastrointestinal pathway. So, oral epithelial cells (OEC), esophageal epithelial cells (EEC) and gastric mucosa epithelial cells (GMEC) are often the entry of xenobiotics to the body and are often the first layer of barrier or the first target tissue of the chemicals. Exposure to chemical factors such as alcohol, drugs and even dust particles with heavy metals or organic substances, will produce toxic effects to these cells and thereby causing the development of many diseases, including epithelial cell metaplasia, abnormal apoptosis, aging, inflammation and even tumors. Recent studies have found that oxidative stress caused by exogenous factors has a key role in the process of inducing epithelial cells toxicity [1]. Oxidative stress refers to a disruption in the balance between oxidation and anti-oxidation in cells, which thus causes the generation of a large number of reactive oxygen species (ROS). On one hand, free radicals directly react with a variety of biological macromolecules to cause the degeneration of these biological macromolecules, lipid peroxidation, membrane damage, the acceleration of cell aging, and an increased risk of tumor development[2]. On the other hand, free radicals can activate many inflammatory signal moleculars, such as mitogen activated protein kinases (MAPKs) family and nuclear factor-κB (NF-κB), thus causing inflammatory responses in the skin and the degradation of the matrix. Studies have demonstrated that ROS are one of the most important factors that cause oxidative stress [3].

Patulin (PA), also known as penicillium patulum toxin and coral penicillium toxin, is a product of metabolism produced by *Penicillium* and *Aspergillus fungi*. PA shows a broad spectrum of antibiotic characteristic. However, because PA is toxic to human potentially, it is no longer used as a therapeutic drug[4]. Studies indicated that PA from apple and other fruits is mainly derived from the contamination of *Penicillium fungi*. Toxicological tests show that PA has a toxic effect on digestive and reproductive systems [5, 6]. Patulin has teratogenicity, which is harmful to the human body, resulting in the damage of respiratory and urinary system, and leading to nerve paralysis, pulmonary edema and renal failure [4, 7]. Patulin was first discovered in rotten apple and apple juice, and widely exists in many kinds of fruits and moldy silage. PA is a toxic substance, which is mainly absorbed into the digestive tract and entered the blood circulation and distributes in the organs and tissues. The toxicity of PA could be reduced after been catalyzed by the cytochrome P450 enzyme, but the metabolites are toxic, too [8, 9]. PA can lead to the damages to respiratory system, nerve system and urinary system. Many research works show that PA is a potential carcinogen, teratogen, and mutagen [10, 11]. *In vitro*,

[1] # contributed equally to this work.
* To whom the correspondence should be address

***Corresponding Author:** **Wei Zhu,** School of Public Health, Sun Yat-sen University, Guangzhou, 510080, China & Department of Toxicology, Guangzhou Center for Disease Control and Prevention, Guangzhou, 510440, China. E-mail:dlk@gzcdc.org.cn
Min Xia, School of Public Health, Sun Yat-sen University, Guangzhou, 510080, China
E-mail: xiamin@mail.sysu.edu.cn

PA could induce DNA damage and gene mutation in various cell strains [12-14]. *In vivo*, hypodermic injection of PA has a carcinogenic effect on mice [14]. The chemical mutagenesis committee of food, consumer goods and the environment of the UK has classified PA as mutagenic substance. So, it is essential to inhibit or prevent the toxicity of PA.

Indole-3-carbinol (I3C) is a confirmed cancer chemopreventive substance and can be extracted from cruciferous vegetables including radish, celery, and cauliflower. 3,3'-diindolylmethane (DIM) is a dimer obtained from the oligomerization of IC3 under acidic conditions, such as in gastric acid. The anticancer and anti-oxidant abilities of DIM have been confirmed in many cell models [15, 16]. However, whether DIM can inhibit patulin-induced toxicity in human HET-1A esophageal epithelial cells as well as the mechanism underlies the toxicity has not been reported. Therefore, in this study, patulin was used as a xenobiotic to observe the antagonistic effect of DIM on patulin-induced toxicities in HET-1A cells and the underlying mechanism of DIM was investigated so as to offer a novel theoretical principal for the prevention and disposal method of toxicity-induced by PA to esophageal epithelial cells.

MATERIAL AND METHODS

Materials

Human esophageal epithelial cell HET-1A was got from China Center for Type Culture Collection. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin and trypsin were purchased from Gibco (USA). DIM, PA, dimethyl sulfoxide (DMSO) and dichlorodihydrofluorescein diacetate (DCFH-DA) were supplied by Sigma (USA). The rabbit anti-phospho-NF-κB, anti-phospho-p38 MAPK, and anti-phospho-SAPK/JNK antibodies and mouse anti-β-actin monoclonal antibodies were all bought from Cell Signaling Technology (CST,USA). Flasks, culture dishes, and plates were all obtained from Corning (USA).

Cell culture

HET-1A cells were cultured with DMEM containing FBS (10%), penicillin (100 IU/mL), and streptomycin (0.1 mg/mL) at 37℃ in an atmosphere with 95 % humidity and a 5 % volume fraction of CO_2. The cell was tested or passaged when the cells reached 80 % - 90 % confluency.

Inhibition of cell proliferation experiment

The Cell Counting Kit-8 (CCK-8) (Beyotime) was used to determine cell survival rates. HET-1A cells at a concentration of 5×10^4 cell/mL were cultured in DMEM for 24 h, and then were treated with DIM for 24, 48, and 72 h. The medium was changed with serum-free medium prior to analyzing the inhibitory effect of PA. After a 2-h treatment with PA, the cells were cultured for another 24 h. After treatment, 10 μL of the CCK-8 reagent was added into the well, and then incubated at 37℃ for 1 h. The Optic Density values (OD) at 450 nm were measured with Microplate Reader (Thermo, USA). The cell survival rates (CSR) were calculated: $CSR\ (\%)=[(OD_{\text{treatment group}}-OD_{\text{blank control}})/(OD_{\text{control}}-OD_{\text{blank control}})]\times100$.

Detection of intracellular ROS content via flow cytometry

HET-1A cells were cultured into 6-well plates for 24 h. The experiment group was treated with DIM (10 μM) for 24 h and then treat PA for 24 h. After treatment, 10 μL DCFH-DA was added into each well, and then cultured for 0.5 h. The cellular ROS content was then detected via flow cytometry (Becton, USA).

Detect the expression of proteins

Cells were lysed with lysis buffer. Total cellular proteins were then extracted, and the concentrations of protein were determined with the BCA method. The amount of protein in each well was 40 μg. Samples were separated using 4 - 12 % NuPAGE precast gels (Invitrogen) and were transferred onto a methanol-immersed polyvinylidene fluoride membrane. The membrane was blocked with skimed milk at room temperature for 1 h and was incubated with primary antibodies (antibodies to phospho-NF-κb, phospho-p38 MAPK, and phospho-SAPK/JNK antibodies at 1:2,000 dilution, the mouse anti-β-actin antibody at 1:1,000 dilution) at 4℃ overnight. After washing with phosphate-buffered saline (PBS)-Tween (PBST) buffer 3 times for 10 min each, the membrane was incubated with goat anti-rabbit secondary antibodies or goat anti-mouse secondary antibodies at room temperature for 2 h and then washed with PBST. The protein bands were developed with chemiluminescence reagents, and the film was developed and fixed. The gray densities of the Western blot results were scanned using the Quantity One software. The relative O.D. value of each band was measured, and the results were compared for the semi-quantitative analysis.

Statistical analysis

The data were statistically analyzed with the SPSS software. All the data were presented as $\bar{x} \pm s$. One-way analysis of variance (ANOVA) and Dunnett's t test were performed. The examination level was α=0.05. The protein expression levels were examined using Pearson's correlation analysis. The difference of statistically significant was defined as P value less than 0.05.

RESULTS

The inhibitory effect of DIM on the proliferation of HET-1A cells

The effect of DIM on the proliferation HET-1A cells was measured to confirm the maximum dose of DIM used in these research works had no toxic effect. Cells were treated with different levels of DIM, and the results indicated that the DIM concentrations and cell survival rates had a dose-response relationship. With 48 h of treatment at concentrations lower than 10 μM, DIM had no obvious toxic effects on the HET-1A cells (P>0.05) (Table 1). Therefore, concentrations of 5 μM and 10 μM were chosen for subsequent experiments.

Table 1. Effects of 1-10 μM DIM on the survival rate of HET-1A cells (%)

group	24 h	48 h	72 h
Control group	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1 μM	103.41 ± 2,83	102.11 ± 0.99	103.02 ± 3.85
2.5 μM	100.38 ± 2.06	101.65 ± 0.91	104.01 ± 2.87
5 μM	102.29 ± 2.71	99.81 ± 0.91	103.89 ± 3.95
7.5 μM	100.22 ± 2.71	101.31 ± 2.20	96.64 ± 2.29*
10 μM	101.42 ± 4.25	99.37 ± 0.45	86.98 ± 3.57**
F value	1.434	2.958	27.453
P value	0.241	0.057	0.000

Note: * P<0.05, ** P<0.01 compared to the control group.

The inhibitory effect of PA on the proliferation of HET-1A cells

As shown in Table 2, treatment with 50 μM PA for 2 h had significant inhibitory effects on the cells, and the cell survival rate was reduced to 82.25%. With increasing PA concentrations, the inhibitory effect on the HET-1A cell growth significantly increased; after treatment with 200 μM PA for 2 h, the cell survival rate was 71.93%, the difference between 50 and 200 μM PA was statistically significant (F=104.836, P<0.05). Therefore, the exposure condition for the PA treatment was between 50-200 μM for 2 h.

Table 2. Effects of 50-200 μM PA on the survival rate of HET-1A cells (%)

group	2 h
Control group	100.00 ± 0.00
50 μM	82.25 ± 2.80**
100 μM	78.65 ± 2.02**
150 μM	76.63 ± 1.50**
200 μM	71.93 ± 1.58**
F value	104.836
P value	0.000

Note: * P<0.05, ** P<0.01 compared to the control group.

Inhibition by DIM of PA-induced ROS production

As shown in Table 3, with increasing PA concentrations, the concentrations of cellular ROS in the treatment group also gradually increased. After the treatment of HET-1A cells with PA for 2 h, the cellular ROS level was approximately 3.6 times that in the control group. After 24 h of DIM pretreatment, the cellular ROS level distinctly decreased as compared with those in the group treated with PA alone (F=61998.17, P<0.05). This result indicated that DIM could effectively inhibit PA-induced ROS production in the HET-1A cells.

Table 3. Effects of DIM (10 μM) on PA-induced ROS production

group	PA concentration				
	0 μM	1.25 μM	2.5 μM	5 μM	10 μM
Model group	2,459 ± 9.61	4,704 ± 16.44	5,016 ± 12.23	5,780 ± 32.19	11,348 ± 17.52
Treatment group (DIM, 10 μM)	2,039 ± 19.50	3,300 ± 19.08	3,541 ± 14.27	4,467 ± 10.83	7,274 ± 25.63
t value	33.407	96.577	134.429	66.975	227.271
P value	0.000	0.000	0.000	0.000	0.000

DIM inhibit the activation of MAPK and NF-κB induced by PA

Two hours after treating HET-1A cells with PA, the levels of phospho-p38 MAPK, phospho-NF-κB, and phospho-SAPK/JNK were assayed. The results indicated that with increasing PA concentrations, the gray value ratios of the roteins showed an increasing trend (Fig. 1). The Pearson correlation analysis resulted in values of $r>0.946$ and $P<0.05$ for the PA concentrations and expression levels of the oxidative stress-related proteins. This result indicated that PA could induce the increase of the proteins expression. The data presented in Fig. 2, in which cells were pretreated with DIM for 24 h followed by exposure to PA, reveal that with an increase in the DIM concentration and the extension of the PA treatment time (>30 min) the relative O.D. ratio of each protein band exhibited a decreasing trend (statistical values between groups, $P<0.05$). The Pearson correlation analysis between the DIM concentration and the levels of proteins, between the PA treatment time and the levels of the proteins resulted in r values that were lower than -0.742 and -0.936, respectively. Therefore, DIM could significantly inhibit PA-induced phospho-NF-κB, phospho-p38 MAPK, and phospho-SAPK/JNK expression. In addition, when the PA treatment time was longer, the inhibitory effect was more evident.

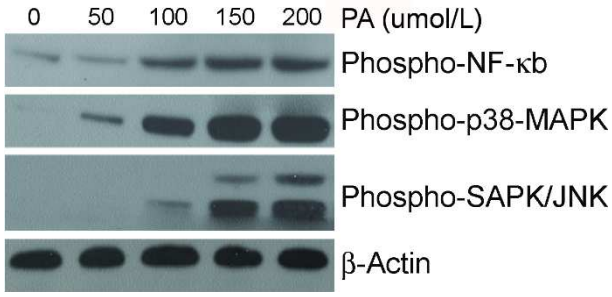

Fig 1. PA increased phosphorylation levels of phospho-NF-κB, phospho-p38 MAPK, and phospho-SAPK/JNK

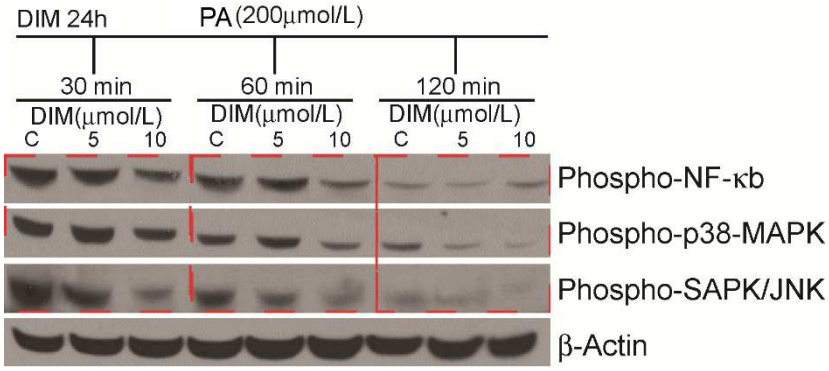

Fig 2. DIM had inhibitory effects on PA-induced activation of phospho-NF-κB, phospho-p38 MAPK, and phospho-SAPK/JNK

DISCUSSION

Many studies showed that cell injury could be caused by ROS-induced oxidative stress [17]. ROS can damage nuclear DNA and mitochondrial DNA. In addition, ROS can activate matrix metalloproteinases in HET-1A cells to damage the extracellular matrix and degrade dermal collagen and elastin, thus causing cell injury [18].

Increasing evidence indicates that PA is one of the important factors causing the amplification of oxidative stress in malignancies [19]. This study successfully established an oxidative stress model with HET-1A cells treated by PA. In the HET-1A cells, PA significantly increased cellular ROS concentrations and activated the expression of oxidative stress proteins.

Many phytochemicals have anti-oxidant functions. I3C is an active ingredient in cruciferous vegetables that has been confirmed to be an effective ROS scavenger [20]. As an important derivative of I3C under acidic conditions, such as in gastric acid, DIM is the major bioactive component that enables I3C to exert anti-oxidant activity. This study found that low doses of DIM could effectively reduce PA-induced ROS production. DIM may inhibit ROS production through a variety of mechanisms [21, 22]. It is known that the anti-oxidant mechanisms of DIM include the inhibition of ROS accumulation to inhibit the expression of the NF-κB signals[23, 24] and the inhibition of oxidative stress through the BRCA1-dependent anti-oxidant signaling pathway[25]. The family of MAPKs, which includes MAPK, ERK and JNK[26], is a cluster of threonine / serine kinases in cells. These family members can transduce extracellular stimulatory signals into cells; therefore, the MAPKs play very important roles in many processes of cell biological reactions. Among these processes, as an important member of the MAPK family, p38 MAPK participates not only in the process of cell growth and development but also in the regulation of cell proliferation; therefore, p38 MAPK is considered to be a hub of a variety of signal transduction pathways[27]. Currently, many studies indicate that the MAPK signaling pathway is associated with oxidative stress. In addition, during oxidative stress, the expression levels of some of the proteins in the MAPK family also increase. However, it has not been reported whether the mechanism by which DIM exerts an anti-oxidant function is mediated by the inhibition of the MAPK signals.

The NF-κB signaling pathway is closely associated with oxidative stress in cells. NF-κB is an induced transformation factor; this factor is very sensitive to ROS produced by oxidation. ROS can directly activate NF-κB in some cells[28]; activated NF-κB can induce cells to express nitric oxide synthase (NOS), different adhesion molecules, and other cytokines. These components act cooperatively together to aggravate the damage produced by oxidative stress. Previous literature has shown that DIM inhibits the accumulation of ROS and inhibits the activation of NF-κB signals, thus exerting anti-oxidant activities. Similarly, the MAPK signaling pathway is also closely associated with oxidative stress. However, whether DIM can inhibit oxidative stress through the MAPK signaling pathway is still unknown. Based on the HET-1A oxidative stress model that was established, this study found that with increasing DIM concentrations, the expression levels of PA-activated oxidative stress-related proteins decreased significantly; this inhibitory function gradually became significant as the PA treatment time increased. Given the combination of results in this study, it is suggested that, in HET-1A cells, DIM could exert anti-oxidative stress functions, the mechanisms underlies which might be attributed to inhibit ROS and activate the NF-κB and MAPKs signals.

In summary, we speculate that DIM could play a specific role in inhibiting the oxidative injury in esophageal epithelial cells, suggesting that DIM might be used as an effective drug for treating or delaying oxidative-stress types of digestive tract injury.

Financial disclosure

The authors are funded by the project of Guangzhou Science Technology and Innovation Commission (No.1563000502, W.Z.), The National Natural Science Foundation of China (81402717,WX.L., 81773403, B.Z., 81741101, W.Z.); 4.The Key Project of Medicine Discipline of Guangzhou (2017-2019-07, W.Z.).

The funders had no role in designing, data collecting and analyzing, deciding to publish, or editing of the manuscript.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

REFERENCES

1. Abida, O., et al., *Biomarkers of oxidative stress in epidermis of Tunisian pemphigus foliaceus patients.* J Eur Acad Dermatol Venereol, 2013. **27**(3): p. e271-5.
2. Verma, A.K. and S.B. Prasad, *Changes in glutathione, oxidative stress and mitochondrial membrane potential in apoptosis involving the anticancer activity of cantharidin isolated from redheaded blister beetles, epicauta hirticornis.* Anticancer Agents Med Chem, 2013. **13**(7): p. 1096-114.
3. Ines, D., et al., *Date seed oil inhibits hydrogen peroxide-induced oxidative stress in human epidermal keratinocytes.* Int J Dermatol, 2010. **49**(3): p. 262-8.
4. Ciegler, A., A.C. Beckwith, and L.K. Jackson, *Teratogenicity of patulin and patulin adducts formed with cysteine.* Appl Environ Microbiol, 1976. **31**(5): p. 664-7.

5. Frizzell, C., C.T. Elliott, and L. Connolly, *Effects of the mycotoxin patulin at the level of nuclear receptor transcriptional activity and steroidogenesis in vitro.* Toxicol Lett, 2014. **229**(2): p. 366-73.
6. Maidana, L., et al., *Effects of patulin and ascladiol on porcine intestinal mucosa: An ex vivo approach.* Food Chem Toxicol, 2016. **98**(Pt B): p. 189-194.
7. Reddy, C.S., P.K. Chan, and A.W. Hayes, *Teratogenic and dominant lethal studies of patulin in mice.* Toxicology, 1978. **11**(3): p. 219-23.
8. Marin, S., et al., *Mycotoxins: occurrence, toxicology, and exposure assessment.* Food Chem Toxicol, 2013. **60**: p. 218-37.
9. Siraj, M.Y., et al., *Effect of patulin on hepatic monooxygenase in male mice.* J Environ Pathol Toxicol, 1980. **4**(2-3): p. 545-53.
10. Degen, G.H., et al., *Daily uptake of mycotoxins - TDI might not be protective for nursed infants.* Toxicol Lett, 2017. **277**: p. 69-75.
11. Oh, S.Y., et al., *Assessing interactions of binary mixtures of Penicillium mycotoxins (PMs) by using a bovine macrophage cell line (BoMacs).* Toxicol Appl Pharmacol, 2017. **318**: p. 33-40.
12. Boussabbeh, M., et al., *Crocin and quercetin prevent PAT-induced apoptosis in mammalian cells: Involvement of ROS-mediated ER stress pathway.* Environ Toxicol, 2016. **31**(12): p. 1851-1858.
13. Pfenning, C., et al., *The mycotoxin patulin reacts with DNA bases with and without previous conjugation to GSH: implication for related alpha,beta-unsaturated carbonyl compounds?* Arch Toxicol, 2016. **90**(2): p. 433-48.
14. Saxena, N., et al., *Role of mitogen activated protein kinases in skin tumorigenicity of patulin.* Toxicol Appl Pharmacol, 2011. **257**(2): p. 264-71.
15. Banerjee, S., et al., *Attenuation of multi-targeted proliferation-linked signaling by 3,3'-diindolylmethane (DIM): from bench to clinic.* Mutat Res, 2011. **728**(1-2): p. 47-66.
16. Beaver, L.M., et al., *3,3'-Diindolylmethane, but not indole-3-carbinol, inhibits histone deacetylase activity in prostate cancer cells.* Toxicol Appl Pharmacol, 2012. **263**(3): p. 345-51.
17. Luo, H., et al., *Resveratrol enhances ionizing radiation-induced premature senescence in lung cancer cells.* Int J Oncol, 2013. **43**(6): p. 1999-2006.
18. Vayalil, P.K., et al., *Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin.* J Invest Dermatol, 2004. **122**(6): p. 1480-7.
19. Rojkind, M., et al., *Role of hydrogen peroxide and oxidative stress in healing responses.* Cell Mol Life Sci, 2002. **59**(11): p. 1872-91.
20. Shimamoto, K., et al., *Antioxidant N-acetyl-L-cysteine (NAC) supplementation reduces reactive oxygen species (ROS)-mediated hepatocellular tumor promotion of indole-3-carbinol (I3C) in rats.* J Toxicol Sci, 2011. **36**(6): p. 775-86.
21. Benabadji, S.H., et al., *Anticarcinogenic and antioxidant activity of diindolylmethane derivatives.* Acta Pharmacol Sin, 2004. **25**(5): p. 666-71.
22. Nho, C.W. and E. Jeffery, *Crambene, a bioactive nitrile derived from glucosinolate hydrolysis, acts via the antioxidant response element to upregulate quinone reductase alone or synergistically with indole-3-carbinol.* Toxicol Appl Pharmacol, 2004. **198**(1): p. 40-8.
23. Bowie, A. and L.A. O'Neill, *Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries.* Biochem Pharmacol, 2000. **59**(1): p. 13-23.
24. Dudek, E.J., F. Shang, and A. Taylor, *H(2)O(2)-mediated oxidative stress activates NF-kappa B in lens epithelial cells.* Free Radic Biol Med, 2001. **31**(5): p. 651-8.
25. Yao, Z., et al., *3,3'-Diindolymethane ameliorates adriamycin-induced cardiac fibrosis via activation of a BRCA1-dependent anti-oxidant pathway.* Pharmacol Res, 2013. **70**(1): p. 139-46.
26. Fecher, L.A., R.K. Amaravadi, and K.T. Flaherty, *The MAPK pathway in melanoma.* Curr Opin Oncol, 2008. **20**(2): p. 183-9.
27. Sakai, N., et al., *Involvement of extracellular signal-regulated kinase and p38 in human diabetic nephropathy.* Am J Kidney Dis, 2005. **45**(1): p. 54-65.
28. Baeuerle, P.A., *The inducible transcription activator NF-kappa B: regulation by distinct protein subunits.* Biochim Biophys Acta, 1991. **1072**(1): p. 63-80.