

Cucurbitacin D Inhibits NF- κ B Activation, Thereby Inducing Apoptosis and Suppressing Tumor Growth

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Abstract: Recently, more and more researchers are investigating the effectiveness of using traditional Chinese medicine as a treatment to treat cancer. This work investigates the Cucurbitacin D, an active component found in the traditional Chinese medicine *Trichosanthes kirilowii* tuber. Its abilities to inhibit NF- κ B activation and to induce apoptosis are studied. This work also examines the potential ability of Cucurbitacin D to inhibit TNF-induced NF- κ B activation, to inhibit TNF-dependent I κ B α degradation and TNF-dependent I κ B α phosphorylation, to repress the TNF-induced NF- κ B-dependent reporter gene expression, to potentiate the apoptosis effect, to down regulates the expression of anti-apoptotic proteins regulated by the NF- κ B pathway, and to inhibits the expression of gene products involved in cell proliferation using methods such as the EMSA, western blotting, NF- κ B-dependent reporter gene expression assay, TUNEL assay, live and dead assay, and the MTT assay. There are several possible results that can be derived from each testing. In this work, the final conclusion is that Cucurbitacin D could have the potential ability to inhibit NF- κ B activation and therefore to induce apoptosis, so it could have the potential ability to reduce cancer cell progression.

Keywords: Cucurbitacin D, NF- κ B activation, Apoptosis, *Trichosanthes kirilowii* tuber, Tumor growth

1. Introduction

Current conventional treatment of cancer mainly involves chemotherapy, radiotherapy, and surgery. However, there is an increasing evidence suggesting that these conventional treatment are ineffective, unsafe, toxic, and expensive. The major problem, especially, is associated with the resistance developed by the tumors. Therefore, more and more researchers become focusing their attention on investigating agents that can target specific tumor-related molecules. Recently, traditional Chinese medicine (TCM) are examined frequently for their potential anticancer properties. It has been suggested that these substances could lead to a development of a new therapy for treating cancer. Apoptosis, a type of programmed cell death, is one of the most widely-examined topic in cell biology. The study of apoptosis in cancer is crucial as cancer is generally described as the transformation of normal cells into malignant cells. Therefore, the defects in apoptosis resulting in evasion of cell death can play a significant role in this malignant transformation^[1]. The defects in apoptosis pathway can promote the initiation and progression of tumor, as well as the development of resistance of cancer cells to conventional treatments^[2, 3]. Recent studies also found out that the disruption of apoptosis is resulted from the mutation in cancer-related genes, which can in turn promote tumor growth^[4, 5]. Despite the fact that disruption of apoptosis have negative impact, the induction of apoptosis is found to be able to suppress tumor growth^[6, 7]. There are a number of mechanisms of how induction of apoptosis can lead to tumor regression, such as the induction of ROS generation^[8, 9], the induction of DNA double-strand breaks^[10, 11], the trigger of Ca²⁺ release from ER^[12], and other mechanisms as well. *Trichosanthes kirilowii* tuber, the tuber of the *Trichosanthes kirilowii* flowering plant in the family Cucurbitaceae, is commonly used in traditional Chinese medicine due to its ability to clear inflammation, drain lung heat, generate fluids, moisten lung dryness, clear toxicity, transform phlegm, and drain pus^[13]. In the 1980s, Trichosanthin (TCS), a type 1 ribosome-inactivating protein, is isolated from the *Trichosanthes kirilowii* tuber and is proved to be an active component of the tuber. TCS has been found to have the potential ability to participate in anti-tumor activities^[14, 15]. Recently, it is also reported that the Cucurbitacin D, a plant steroid isolated from the *Trichosanthes kirilowii* tuber, can induce apoptosis in carcinoma cells through the caspase-3 and phosphorylation of JNK in hepatocellular carcinoma cells^[16], and through the suppression of STAT3 activity in the breast cancer cells^[17]. NF- κ B is a transcription factor that affect tumorigenesis^[18].

The JAK-STAT pathway, AKT-PKB pathway, and MAPK pathway are all commonly known

pathways to be associated with the Cucurbitacin family and their consequential impact on cancer [19, 20]. However, little is known about the effect of Cucurbitacin D on the NF- κ B activation pathway and the following impact on apoptosis and tumors. In addition, the NF- κ B pathway can play an important role in genes that regulate cell proliferation (Cyclin D1, c-Myc), metastasis (COX-2, MMP-9, ICAM-1), anti-apoptotic genes (Survivin, TNF, TRAF1, IL-1 β , FLIP, IAP, XIAP, Bcl-2, and Bcl-xL), drug resistance (MDR1), immunomodulation (chemokines and interleukins) and so on. This suggests a possible relationship between the inhibition of the NF- κ B and the induction of apoptosis. In general, it is possible that the inhibition of the NF- κ B activation pathway could lead to the down regulation of the NF- κ B-regulated anti-apoptotic proteins, thus encouraging programmed, apoptotic cell death [21, 22]. As a result, more and more researchers are working on showing this relationship [23].

In an attempt to show this relationship, this research report will mainly focus on the inhibition of the NF- κ B activation and the consequential down regulation of anti-apoptotic proteins gene expression resulting in the induction of apoptosis and therefore suppresses tumor proliferation. In this study, I isolated Cucurbitacin D from *Trichosanthes kirilowii* tuber and hypothesized that it will induce apoptosis to reduce tumor cell proliferation. Due to the well characterized NF- κ B activation pathway in inflammation and cell proliferation [24-26], I will hypothesize the ability of Cucurbitacin D to induce apoptosis through the inhibition of the NF- κ B pathway activation, thus leading to tumor regression.

2. Materials and Methods

2.1 Reagents

Firs of all, purified Cucurbitacin D (C₃₀H₄₄O₇) is obtained from the *Trichosanthes kirilowii* tuber and the purity of the Cucurbitacin D extracted is confirmed using liquid chromatography. Cucurbitacin D can then be dissolved in ethanol at five different concentration: 15 micro molar, 5 micro molar, 90 nano molar, 50 nano molar, and 20 nano molar. Bacteria-derived recombinant human TNF, with a specific activity of 5×10^7 units/mg a purity of homogeneity can be bought. Betulinic acid, amlexanox, parthenolide, phospho-specific anti-I κ B α (Ser32/36), an NF- κ B-containing plasmid linked to the SEAP gene, TNFR1-, TRADD-, TRAF2-, NIK-, IKK-, p65-expressing plasmids, antibodies against I κ B α , dexamethasone, antibodies against cIAP-1, cIAP-2, cFLIP, XIAP, IAP1, IAP2, Bcl-XL, Bcl-2, and antibodies against COX-2, cyclin D1 are obtained.

2.2 Cancer Cell Culture

Human cancer cell lines are also required. Human breast cancer cell MCF-7 may be started in T75 flasks at 1×10^6 cells per flask in low glucose DMEM with 10% fetal bovine serum (FBS), 2 mM glutamine, 0.01 mg/ml insulin and 1% penicillin, incubated at 37 degree Celsius in an atmosphere with 5% CO₂. Human acute leukemia T cell line TIB-152 may be obtained from the ATCC-formulated RPMI-1640 Medium, ATCC 30-2001 added with 10% FBS, incubated at 37 degree Celsius in a atmosphere with 5% CO₂. Human pancreatic cancer cell line PL45 is maintained in DMEM, incubated at 37 degree Celsius in a atmosphere with 5% CO₂. Human hepatocellular carcinoma cell line, Hep3B, was obtained and seeded in DMEM medium supplemented with 10% (v/v) fetal calf serum (FCS) 50 μ g/ml penicillin, and 50 U/ml streptomycin, incubated with 37 degree Celsius and a humidified atmosphere of 5% CO₂ in air.

2.3 EMSA

Electrophoretic mobility shift assay (EMSA) is performed to assess NF- κ B activation. Nuclear and Cytoplasmic Extraction Reagents are used to extract nuclear proteins, which were then incubated with 32 P-end-labeled 45-mer double-stranded oligonucleotide (15 μ g protein with 16 f mol DNA) from the HIV (Human immunodeficiency virus) long terminal repeat, 5'-TTGTTACAA GGGACTTTC CGCTG GGGACTTTC CAGGGAGGCGTGG-3' (boldface indicates NF- κ B binding sites) for 30 minutes at 37 degree Celsius. DNA-protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels and (or) can be analyzed by non-denaturing 4% polyacrylamide gel electrophoresis. The competition with the unlabeled oligonucleotide also examines the specificity of binding. Antibodies against the p50 or p65 subunit of NF- κ B is added to nuclear extracts from TNF-treated cells for 15 min at 37 degree Celsius during the super shift assay before the complex was analyzed by EMSA. Anti-Cyclin D1 antibody can act as the negative control.

2.4 Western Blot Analysis

The impact of Cucurbitacin D on TNF-dependent I κ B α phosphorylation and I κ B α degradation can be examined using the Western Blot analysis, using antibodies against I κ B α and phosphorylated I κ B α in the same time. The expression of survival-regulating proteins that is regulated by the transcriptional activity of NF- κ B, including cIAP-1, cIAP-2, cFLIP, XIAP, IAP1, IAP2, Bcl-XL, Bcl-2, is also determined using Western Blot analysis with antibodies against these anti-apoptotic proteins. Finally, the TNF-induced expression of gene products involved in cell proliferation, which is basically cyclin D1 and COX-2, is examined using Western Blot analysis with antibodies against cyclin D1 and COX-2. To determine the level of expression of these proteins, extracts should be prepared and fractionated by SDS-PAGE. After electrophoresis, the proteins can be electro-transferred onto nitrocellulose membranes, blotted with each antibody, and detected by an ECL reagent (Amersham Biosciences). The bands can be quantified with NIH Image (National Institutes of Health, Bethesda, MD).

2.5 NF- κ B-dependent Reporter Gene Expression Assay

Secretory alkaline phosphatase (SEAP) assay is used to determine the effect of Cucurbitacin D on TNF-, TNFR-, TRADD-, TRAF2-, NIK-, IKK-, and p65-induced NF- κ B-dependent reporter gene transcription. Cells were plated in 12-well plates (2×10^5 cells/well) and transiently transfected by FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany). Cells are transfected with 0.2 μ g of the SEAP expression plasmid for 24 hours to analyze the TNF-induced reporter gene expression. The cells are then treated with 500 nM of Cucurbitacin D for 2 hours and exposed to 1 nM TNF for 24 hours. Cells can then be transfected with 0.2 μ g of various reporter gene plasmid with each 0.5 μ g of expressing plasmid and then treated with 500 nM of Cucurbitacin D for 24 hours.

2.6 TUNEL Assay

To determine apoptosis, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method is used to examine the DNA strand breaks during apoptosis with an in-situ cell death detection reagent (Roche Diagnostics, Mannheim, Germany). 5×10^5 cells were treated with 50 nM of Cucurbitacin D for 12 hours and then 10 nM TNF for 12 hours. Cells were fixed using 4% paraformaldehyde, and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate. After washing, cells are then incubated with reaction mixture for one hour at 37 degree Celsius. A flow cytometer is used to analyze stained cells.

2.7 Live and Dead Assay

The live and dead assay is used to measure apoptosis, which determines the intracellular esterase activity and plasma membrane integrity. The green fluorescing polyanionic dye calcein is used in this assay to provide green fluorescence when retaining within living cells. The red fluorescing monomer dye ethidium is also used as it can bind to nucleic acids when entering cells through damaged membranes. 1×10^5 cells were treated with 50 nM of Cucurbitacin D for 12 hours and then 10 nM of TNF for 12 hours at 37 degree Celsius. Cells were stained with the live and dead reagent (5 μ M calcein AM and 5 μ M ethidium homodimer) and incubated at 37 degree Celsius for 30 minutes. A Lapshot-2 fluorescence microscope can be used to analyze.

2.8 MTT Assay

The modified tetrazolium salt 3-(4-(5-dimethylthiozol-2-yl) 2-5-diphenyl-tetrazolium bromide (MTT) assay is used to examine the cytotoxicity. 1×10^4 cells are treated with 50 nM Cucurbitacin D for 12 hours and then 10 nM of TNF for 24 hours at 37 degree Celsius. MTT solution is added to each well so that each well can then be incubated for 2 hours at 37 degree Celsius. 20% SDS and 50% dimethyl formamide (the extraction buffer) is added and cells were incubated for a further 24 hours at 37 degree Celsius. At 570 nm, a 96-well multi scanner is used to measure the optical density.

3. Results and Discussion

3.1 Cucurbitacin D inhibits TNF-induced NF- κ B activation in a dose- and time-dependent manner.

Five groups of cells are treated with 500 nM of Cucurbitacin D. Betulinic acid (increases NF- κ B translocation (p65) and transcriptional activity) are added as a positive control, and Amlexanox (IKK ϵ and TBK-1 inhibitor with therapeutic uses) are added to the cells as a negative control. These treatment are added to the cells for 12 hours and then the cells are then stimulated with 0.1 nM TNF for 30 minutes. The EMSA can then read the nuclear extracts to examine the TNF-induced NF- κ B activation. Then, we can investigate the band shifts shown on the EMSA to determine the level of activation.

In addition, we can investigate whether Cucurbitacin D inhibits TNF-induced NF- κ B activation in a dose- and time-dependent manner by two follow-up experiments. First, we can prepare five concentrations of Cucurbitacin D as indicated in the materials and method part for 12 hours and exposed to 0.1 nM TNF for 30 minutes. The nuclear extracts were then subjected to EMSA for reading of NF- κ B activation. Second, we can treat the cells with 250 nM of Cucurbitacin for the indicated times (1 hour, 5 hours, 8 hours, 12 hours, 17 hours and 24 hours) and exposed to 0.1 nM TNF for 30 minutes. The nuclear extracts were then subjected to EMSA for analysis of NF- κ B activation.

3.1.1 Cucurbitacin D inhibits TNF-induced NF- κ B activation in a time- and dose-dependent way

The cells are pretreated with 500 nM of Cucurbitacin D for 12 hours and then treated with 0.1 nM TNF for 30 minutes. Cells treated with positive control shows a strong and significant band shift on the EMSA, indicating a normal activation of the NF- κ B activity presented. In contrast, the cells treated with negative control and cells treated with 500 nM of Cucurbitacin D shows a weak band or no band.

Also, the EMSA shows lighter, and less significant bands when concentration increases so the TNF-induced NF- κ B activation is inhibited by the Cucurbitacin D in a dose-dependent manner. The EMSA shows lighter, and less significant bands when length of incubation increases so the TNF-induced NF- κ B activation is inhibited by the Cucurbitacin D in a time-dependent manner.

3.1.2 Cucurbitacin D inhibits TNF-induced NF- κ B activation in a non-time- and non-dose-dependent way.

The cells are pretreated with 500 nM of Cucurbitacin D for 12 hours and then treated with 0.1 nM TNF for 30 minutes. For cells treated with positive control, there is a significant band shift indicating an increase in the NF- κ B activation. The cells treated with 500 nM of Cucurbitacin D as well as the negative control all did not show any visible band shift, indicating an inhibition of the NF- κ B activation.

Also, the EMSA shows same bands when concentration increases so the TNF-induced NF- κ B activation is inhibited by the Cucurbitacin D in a non-dose-dependent manner. The EMSA shows same bands when length of incubation increases so the TNF-induced NF- κ B activation is inhibited by the Cucurbitacin D in a non-time-dependent manner.

3.1.3 Cucurbitacin D does not inhibit TNF-induced NF- κ B activation

The cells are pretreated with 500 nM of Cucurbitacin D for 12 hours and then treated with 0.1 nM TNF for 30 minutes. Cells treated with positive control and cells treated with 500 nM of Cucurbitacin D all show a visible band shift on the EMSA, indicating a normal activation of the NF- κ B activity presented even if Cucurbitacin D are incubated. In contrast, the cells treated with negative control have no visible bands.

3.2 Cucurbitacin D inhibits the NF- κ B activation through inhibition of TNF-dependent I κ B α degradation

To investigate whether the inhibition of the NF- κ B activation from Cucurbitacin D was due to the inhibition of I κ B α degradation, we can treat the cells with 500 nM of Cucurbitacin D and then treat the cells with 0.1 nM TNF for regular time intervals of 10 minutes starting from zero to 2 hours. Cytoplasmic extracts are then prepared for analysis of the Western blotting using antibodies against I κ B α .

3.2.1 Cucurbitacin D inhibits the NF- κ B activation through inhibition of TNF-dependent I κ B α degradation

TNF can induce I κ B α degradation in controlled, untreated cells, which can be shown by the weakening or disappearance of blots in the Western blot. However, after the treatment with Cucurbitacin

D, all the blots are presented in the Western blot. Therefore, the TNF had no impact on I κ B α degradation, and inhibiting this degradation.

3.2.2 Cucurbitacin D does not inhibit the NF- κ B activation through inhibition of TNF-dependent I κ B α

There are significant blots presented in controlled, untreated cells as TNF can induce the I κ B α degradation. These blots remain unchanged after the treatment with Cucurbitacin D shown by the Western blotting, so there is no inhibition of the I κ B α degradation by Cucurbitacin D.

3.2.3 Cucurbitacin inhibits the NF- κ B activation through inhibition of TNF-dependent I κ B α degradation in a dose-dependent manner

We can repeat the experiment using five concentrations of Cucurbitacin D. When cells are treated with higher concentration of the Cucurbitacin D, there are more and more blots presented shown by the Western blotting. So the inhibitory effect of Cucurbitacin D on TNF-dependent I κ B α degradation is a dose-dependent manner.

3.3 Cucurbitacin D inhibits the NF- κ B activation through inhibition of TNF-dependent I κ B α phosphorylation

To examine whether the inhibition of the NF- κ B activation from Cucurbitacin D was due to the inhibition of TNF-dependent I κ B α phosphorylation, we should use parthenolide to inhibit I κ B α degradation. Cells are treated with 500 nM of Cucurbitacin D, and then with the parthenolide for one hour, then exposed to TNF for 30 minutes. We can then use an antibody that can recognize the serine-phosphorylated form of I κ B α , such as the Ser32/36, to analyze the I κ B α phosphorylation status using the Western blotting.

3.3.1 Cucurbitacin D inhibits the NF- κ B activation through inhibition of TNF-dependent I κ B α phosphorylation

There are significant blots presented in the Western blot analysis in controlled, untreated cells exposed to TNF. These blots are then weakened or disappeared when the cells are treated with Cucurbitacin D, showing that the Cucurbitacin had successfully suppressed the I κ B α phosphorylation.

3.3.2 Cucurbitacin D does not inhibit the NF- κ B activation through inhibition of TNF-dependent I κ B α phosphorylation

There are significant blots presented in the Western blot analysis in controlled, untreated cells exposed to TNF. These blots remain unchanged after the treatment of Cucurbitacin D, showing that the Cucurbitacin D has no inhibitory impact on the I κ B α phosphorylation.

3.3.3 Cucurbitacin D inhibits the NF- κ B activation through inhibition of TNF-dependent I κ B α phosphorylation in a dose-dependent manner

We can repeat the experiment using five concentrations of Cucurbitacin D. When cells are treated with higher concentration of the Cucurbitacin D, there blots presented will be more and more weak and light, shown by the Western blotting. So the inhibitory effect of Cucurbitacin D on TNF-dependent I κ B α phosphorylation is a dose-dependent manner.

3.4 Cucurbitacin D represses the TNF-induced NF- κ B-dependent reporter gene expression

Since DNA binding alone does not always correspond with the NF- κ B-dependent gene transcription, we must also examine the TNF-induced transcription of the NF- κ B reporter activity modulated by the Cucurbitacin D. An NF- κ B-containing plasmid linked to the SEAP gene transfect the cells transiently. The cells are then treated with 500 nM of Cucurbitacin D, and then exposed to TNF. After 24 hours, the cells are collected and measured for their SEAP activity. Also, the TNFR1-, TRADD-, TRAF2-, NIK-, IKK-, and p65-expressing plasmids are used to transfect the cells and then the cells are left untreated or treated. After 24 hours, we examine their NF- κ B-dependent SEAP expression and their NF- κ B-regulated reporter gene expression.

3.4.1 Cucurbitacin D represses the TNF-induced NF- κ B-dependent reporter gene expression

The fold activity of the NF- κ B-regulated reporter gene expression of the treated cells over the activity of the vector control (untreated cells) can be graphed. There is a clear difference with a high fold activity in untreated cells compared with a fold activity that is extremely low compared in treated cells with that

in untreated cells. This shows that the Cucurbitacin D can repress the TNF-induced NF- κ B-dependent reporter gene expression.

3.4.2 Cucurbitacin D does not repress the TNF-induced NF- κ B-dependent reporter gene expression

The fold activity of the NF- κ B-regulated reporter gene expression of the treated cells over the activity of the vector control (untreated cells) can be graphed. There is approximately no difference between the fold activity in untreated cells and treated cells, so Cucurbitacin D does not repress the TNF-induced NF- κ B-dependent reporter gene expression.

3.4.3 Cucurbitacin D represses the TNF-induced NF- κ B-dependent reporter gene expression in a dose-dependent manner

We can repeat the experiment using five concentrations of Cucurbitacin D. The difference between the fold activity in untreated and treated cells becomes greater when the concentration of Cucurbitacin D increases. This shows that the repression effect of Cucurbitacin D on TNF-induced NF- κ B-dependent reporter gene expression is in a dose-dependent manner.

3.5 Cucurbitacin D potentiates the apoptosis induced by TNF

To determine whether Cucurbitacin D can increase TNF-induced cytotoxicity, we can use the MTT assay. Cells are each seeded in a 96-well plate and are treated with 50 nM Cucurbitacin D for 12 hours, and then treated with 10 nM of TNF for another 24 hours so that cytotoxicity can be examined.

The cells can also be tested using the live and dead assay. We can first, again, treat the cells with 50 nM Cucurbitacin D for 12 hours and then with 10 nM of TNF for another 12 hours. The cells are then stained with a live and dead assay agent for 30 minutes and examined under a fluorescence microscope to measure the intracellular esterase activity and plasma membrane integrity.

In addition, the TUNEL assay can confirm the tests. Again, we can treat the cells with 50 nM Cucurbitacin D for 12 hours and then with 10 nM of TNF for another 12 hours. These cells are then stained using the TUNEL assay reagent and then examined using a flow cytometer for apoptotic effect.

3.5.1 Cucurbitacin D potentiates the apoptosis induced by TNF

Cytotoxicity is enhanced after the treatment of Cucurbitacin D. Also, the percentage of apoptosis increases, shown by the live and dead assay and the TUNEL assay. This supports that Cucurbitacin D potentiates the apoptosis induced by TNF.

3.5.2 Cucurbitacin D does not potentiate the apoptosis induced by TNF

Cytotoxicity and percentage of apoptosis remains unchanged before and after the treatment, which suggests that Cucurbitacin D does not potentiate the apoptosis induced by TNF

3.6 Cucurbitacin D down-regulates the expression of anti-apoptotic proteins regulated by the NF- κ B pathway

Since the treatment of Cucurbitacin D to the cells can have the potential impact of inducing apoptosis, the relationship of this effect with the expression survival-regulating proteins that is regulated by the transcriptional activity of NF- κ B can be evaluated to find the relationship between the inhibition of the NF- κ B pathway and the induction of apoptosis. Anti-apoptotic proteins including cIAP-1, cIAP-2, cFLIP, XIAP, IAP1, IAP2, Bcl-XL, Bcl-2 are used for the testing. One group of the cells are left untreated. One group of the cells are treated with 500 nM dexamethasone for 6 hours to act as another control group. Another group of the cells are incubated with 500 nM Cucurbitacin D for 6 hours, and then treated with 200 nM TNF for another 6 hours. The whole-cell extracts were prepared, and 30 μ g of the whole-cell lysate was analyzed by Western blotting using antibodies corresponding with these proteins. In a follow-up experiment, the level of the anti-apoptotic proteins including cIAP-1, cIAP-2, cFLIP, XIAP, IAP1, IAP2, Bcl-XL, Bcl-2 is determined at different times after the treatment with Cucurbitacin D and TNF, including 0 hour after, 1 hour after, 2 hours after, 4 hours after, and 8 hours after.

3.6.1 Cucurbitacin D down-regulates the expression anti-apoptotic proteins regulated by the NF- κ B pathway

The two groups of cells that are left untreated or that are treated with dexamethasone would show strong, dark and significant blots on the Western blotting analysis. The one group of cells treated with

Cucurbitacin D would show a much weaker blots when compared with the other two groups. This suggests that the expression of the anti-apoptotic proteins are down regulated after the treatment with Cucurbitacin D.

3.6.2 Cucurbitacin D does not down-regulates the expression anti-apoptotic proteins regulated by the NF- κ B

The two groups of cells that are left untreated or that are treated with dexamethasone would show strong, dark and significant blots on the Western blotting analysis. The one group of cells treated Cucurbitacin D would show the same dark and significant blots. This suggests that the expression the anti-apoptotic proteins are not down regulated by the treatment of Cucurbitacin D.

3.6.3 Cucurbitacin D down-regulates the expression anti-apoptotic proteins regulated by the NF- κ B pathway in a time-dependent manner

In the follow-up time-course modulation experiment, the level of anti-apoptotic proteins is determined at different time after the treatment. The group of cells that are left untreated and the groups of cells treated with dexamethasone would show the constant dark and significant blots continuously from 0 hour after the treatment to 8 hours after the treatment. The group of cells treated with Cucurbitacin D would show a trend with weaker blots as the length of time after the treatment increases. This suggests that the Cucurbitacin D down-regulates the expression anti-apoptotic proteins regulated by the NF- κ B pathway in a time-dependent manner as the expression of these anti-apoptotic proteins decreases as time increases.

3.7 Cucurbitacin D inhibits TNF-induced expression of gene products involved in cell proliferation

To determine the impact of treatment of Cucurbitacin D on the TNF-induced expression of gene products involved in cell proliferation, Western blots analysis is used. It is commonly know that both cyclin D1 and COX-2 are required for proliferation of different cell types[36][37]. Therefore, the impact of Cucurbitacin D on the level of expression of COX-2 and cyclin D1 gene products is determined to examine the potential ability of Cucurbitacin D to reduce cancer cell proliferation. One group of cells are left untreated. Another group of cells are treated with 500 nM of Cucurbitacin D for 6 hours and then 200 nM of TNF for another 6 hours. The whole cell extract is prepared and then analyzed by Western blot analysis using antibodies against the two proteins.

3.7.1 Cucurbitacin D inhibits TNF-induced expression of gene products involved in cell proliferation

On the group of cells that are untreated, there are dark, significant blots shown on the Western blot analysis. On the other group of cells treated with Cucurbitacin D, there are weak and even absent blots. This suggests that Cucurbitacin D had successfully inhibits the expression of cyclin D1 and COX-2, and therefore has the potential ability to control and reduce cancer cell proliferation.

3.7.2 Cucurbitacin D does not inhibit TNF-induced expression of gene products involved in cell proliferation

On the group of cells that are untreated, there are dark, significant blots shown on the Western blot analysis. On the other group of cells treated with Cucurbitacin D, there are the same dark and significant blots when compared with the untreated cells. This suggests that Cucurbitacin does not inhibit TNF-induced expression of cyclin D1 and COX-2, and therefore does not have the potential ability to control and reduce cancer cell proliferation.

3.8 Summary of possible results

Table 1 below summarizes all the possible results that may have been shown during the experiments.

Table 1: this table shows the summary of the combination of the possible results.

	Inhibition of NF- κ B activation	Inhibition of I κ B α degradation	Inhibition of I κ B α phosphorylation	Repression of the NF- κ B-dependent reporter gene expression	Induction of apoptosis	Down regulation of the expression of anti-apoptotic proteins regulated by the NF- κ B pathway	Inhibition of the expression of gene products involved in cell proliferation
Result 1	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Phenotype 1	Weaker band or no band	All blots present	Weaker and lighter blots	Clear difference in graph of fold activity	Cytotoxicity increases; percentage of apoptosis increases	Weaker blots	Weaker blots
Result 2	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Phenotype 2	Strong band just as control	Blots disappear	Dark blot just as control	No difference in graph of fold activity	Cytotoxicity remains unchanged; percentage of apoptosis decreases	Dark blot just as control	Dark blots just as usual
Result 3	Positive in time-dependent manner	Positive in dose-dependent manner	Positive in dose-dependent manner	Positive in dose-dependent manner		Positive in time-dependent manner	
Phenotype 3	Weaker band when increasing time	Number of blots present increases with increasing concentration	Weaker and lighter blots as concentration increases	Larger and clearer difference in graph of fold activity when concentration increases		Weaker blots as time increases	
Result 4	Positive in dose-dependent manner						
Phenotype 4	Weaker band when increasing concentration						

4. Conclusion

Previous studies have examined the impact of Cucurbitacin D on the JAK-STAT pathway, the AKT-PKB pathway and the MAPK pathway, as well as its relationship with the treatment of cancer and other diseases. However, little is known about the impact of Cucurbitacin D on the NF- κ B activation pathway and this connection with apoptosis and cancer. Therefore, this research report hypothesizing that Cucurbitacin D may inhibit the NF- κ B activation pathway and may induce the apoptosis is developed. There are several possible results in this research. First of all, the inhibition of TNF-induced NF- κ B activation is examined. Secondly, since I κ B α degradation and I κ B α phosphorylation are two essential factors for NF- κ B pathway to be activated, the mechanism resulting in the inhibition of the TNF-induced NF- κ B activation pathway is examined through these two aspects: the inhibition of TNF-dependent I κ B α degradation and the inhibition of the TNF-dependent I κ B α phosphorylation. In addition, this research report also examines the ability of Cucurbitacin D to represses the TNF-induced NF- κ B-dependent reporter gene expression. This is important because DNA binding is not the only factor corresponding with NF- κ B-dependent gene transcription, suggesting that additional regulatory steps must be involved. Then, the potential ability of Cucurbitacin D to induce apoptosis is determined. To examine the mechanism and relationship between the inhibition of NF- κ B pathway, the expression of anti-apoptotic proteins regulated by the NF- κ B pathway is examined. And finally, the resulting cancer cell proliferation was investigated to see if the treatment of Cucurbitacin D can reduce the cancer cell proliferation, and the two possible results are either the inhibition of cancer cell proliferation, or unchanged cancer cell proliferation. There are further studies that can be developed from this research report. Further study of this topic could include the test to show whether the ability of Cucurbitacin D to suppress TNF-induced I κ B α degradation is mediated through the inhibition of I κ B α phosphorylation. Similarly, we can test whether Cucurbitacin D acts like a proteasome inhibitor like ALLN. Also, further study of this topic could include the ability of Cucurbitacin D to suppress TNF-induced tumor-cell invasion activity, such as the down regulation of tumor metastasis-related genes, including MMP9, COX2, and ICAM1. All in

all, NF- κ B activation can regulate several chemokines, interleukins, and hematopoietic growth factors. Therefore, the results of this research report might indicate that Cucurbitacin D is a potent inhibitor of NF- κ B activation, which could be a responsible question allowing cells to be pro-apoptotic, anti-metastatic, anti-inflammatory, and anti-proliferative.

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