科技部補助專題研究計畫成果報告
期末報告

研究去甲斑蝥素調控微小核醣核酸表現與誘發前列腺癌細胞凋亡及輔助化學療法之分子機制

計 畫 類 別 ： 個別型計畫
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中 華 民 國 105 年 09 月 28 日

中 文 摘 要 ： 前列腺癌已成為全世界男性最普遍且年齡相關的癌症之一。在台灣，近年來男性罹患前列腺癌的發生率及死亡率逐年增高。許多傳統療法都被發現具有抗癌的效果，故若能有效抑制癌細胞的生長或結合臨床藥物減少副作用，將可大幅減低前列腺癌的死亡率。因此尋找天然藥物來當作癌症預防或是治療藥物是目前最重要的課題。去甲斑蝥素(Norcantharidin；NCTD)，為斑蝥素(cantharidin)的結構上去甲基化所得的衍生物。目前去甲斑蝥素被發現的生物功能中包括：抗轉移、抗癌、抗發炎、和細胞凋亡等多重功用。但是，目前去甲斑蝥素對於人類前列癌細胞的抗癌機轉，至今尚未清楚。我們實驗結果證實 NCTD誘導前列腺癌細胞凋亡因而活化caspases-3, -6, -7, -9 and -PARP，同時造成粒線體膜電位改變。此種現象主要是NCTD抑制AKT活化造成FOXO4轉位到細胞核內因而增加FOXO4結合到Mcl-1啟動子來抑制Mcl-1蛋白表現，因而誘導細胞凋亡。綜合以上實驗說明NCTD有效抑制前列腺癌細胞生長和誘導凋亡主要是透過Akt/FOXO4/Mcl-1的調控機制

中 文 關 鍵 詞 ： 前列腺癌、去甲斑蝥素、細胞凋亡

英 文 摘 要 ： Prostate cancer is an age-related cancer and the second leading of cancer death among men in United States, including Taiwan. Many traditional medicines are found to have antitumor effects on prostate cancer, and inhibition of cancer cells proliferation or combining clinical drug to decrease the side effect, and its will markly decrease the death rate. Therefore, finding of nature drug or drug therapy was important topic. Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from natural blister beetles, and display a wide range of pharmacological properties including anti-metastatic, anti-tumor, anti-inflammatory, and apoptosis effects. However, little is known about the molecular mechanism of norcantharidin on human prostate cancer cells. In our study, we found that treatment with NCTD induced apoptosis of prostate cancer cells and triggered the activations of caspases-3, -6, -7, -9 and -PARP, which was associated with mitochondria dysfunction. Mechanistic investigations suggest that NCTD inhibit Akt pathways were involved in NCTD increased the nuclear translocation of Foxo4 and its binding to the Myeloid cell leukemia-1 (Mcl-1) promoters, and resulting in an apoptotic effect. Taken together, our results provide that new insights into the critical role of NCTD effectively suppressed Mcl-1, which resulted in apoptosis induction of prostate cancer cells .

英 文 關 鍵 詞 ： prostate cancer, norcantharidin, apoptosis

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（□期中進度報告/■期末報告）

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計畫主持人：謝逸憲

共同主持人：應宗和

計畫參與人員：李宜勳

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中　華　民　國 105　年 9　月 28 日

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中文摘要

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英文摘要

Prostate cancer is an age-related cancer and the second leading of cancer death among men in United States, including Taiwan. Many traditional medicines are found to have antitumor effects on prostate cancer, and inhibition of cancer cells proliferation or combining clinical drug to decrease the side effect, and its will markly decrease the death rate. Therefore, finding of nature drug or drug therapy was important topic. Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from natural blister beetles, and display a wide range of pharmacological properties including anti-metastatic, anti-tumor, anti-inflammatory, and apoptosis effects. However, little is known about the molecular mechanism of norcantharidin on human prostate cancer cells. In our study, we found that treatment with NCTD induced apoptosis of prostate cancer cells and triggered the activations of caspases-3, -6, -7, -9 and -PARP, which was associated with mitochondria dysfunction. Mechanistic investigations suggest that NCTD inhibit Akt pathways were involved in NCTD increased the nuclear translocation of Foxo4 and its binding to the Myeloid cell leukemia-1 (Mcl-1) promoters, and resulting in an apoptotic effect. Taken together, our results provide that new insights into the critical role of NCTD effectively suppressed Mcl-1, which resulted in apoptosis induction of prostate cancer cells.

Key word: prostate cancer, norcantharidin, apoptosis

前言

Prostate cancer (CaP) is the second common cancer and the sixth leading cause of cancer death in males, one in sixth men have a lifetime risk of diagnosis and 3.4% chance of death since the prostate cancer [1,2]. Many of these genes or their protein products are under study for their value in clinical staging with the goal of more closely tailoring the selection of treatment to expected prognosis[3]. Increased concentrations of cytotoxic drugs and higher dosages of irradiation fail to improve the response to therapy and it leads to resistance to apoptosis in prostate cancer cells. Thus, it is imperative to identify anticancer agents that are nontoxic and highly effective in inducing apoptosis preferentially in tumor cells [4,5].

Norcantharidin (exo-7-oxabicylo-[2.2.1] heptane-2,3-dicarboxylic anhydride; NCTD), is a demethylated form of cantharidin, is well-known for its potential antioxidant, antitumor and anti-metastatic ability [6-9] NCTD causes less the toxicity and inflammatory side effects than cantharidin[10]. Previous studies have indicated that NCTD inhibit the proliferation and induces apoptosis of Hep3B cells via TRAIL/DR5 signal transduction [11]. NCTD mediated a Fas-dependent apoptotic cell death in human colorectal carcinoma cells [12] and induces apoptosis was increased the mitotic phase related proteins in human hepatoma cells [13]. However, several cancer cell types including prostate exert an accumulation of intercellular ROS, mitochondria dysfunction and inhibits DNA replication to NCTD treatment, majorly due to the causing DNA damage and depleting ATP[8,14]. Accumulating evidence indicated that NCTD-induced cell death was inhibits some transcriptional factors, including nuclear factor (NF)-kB, activator protein (AP)-1, and STATs, which act independently or in coordination to regulate apoptosis related gene [15,16]. FoxO transcription factors are localized in the cytoplasm, whereas under stress conditions they move to the nucleus and trigger transcriptional activities of their target genes [17]. The FoxO subfamily are involved in various signaling pathways and modulate cell cycle progression, DNA damage repair, differentiation, and promote cell death[18]. Activation of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway is a major contributor to CaP progression, and the FoxO4 which are controlled by PI3K/AKT signaling, are involved in regulating cell cycle progression and apoptosis [19]. Acetylated FoxO4 promotes the expression of a pro-apoptosis gene Bcl-2III (also known as Bim) and leads podocyte apoptosis [20]. Therefore, the potential antitumor effects of NCTD have not been clearly elucidated. In this study, we examined the antitumor role of NCTD in human prostate cancer cells using in vitro and in vivo models and explore the underlying mechanisms associated with NCTD induces apoptosis activity.

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研究方法

Reagents and antibodies

Norcantharidin (NCTD) MTT, DAPI, JC-1 reagents and Flag antibody were purchased from Sigma (Louis, MO, USA). A 100-mM solution of NCTD was prepared in dimethyl sulfoxide (DMSO), stored at 20°C and protected from light, and diluted to needed concentrations for studies. Caspase-3/7 inhibitor Z-DEVE-FMK, caspase-6 inhibitor Z-VEID- FMK, caspase-9 inhibitor Z-LEHD- FMK, and Pan caspase inhibitor Z-VAD-FMK were purchased from BioVision (Mountain View, CA). The Akt inhibitor LY294002 was purchased from Calbiochem (San Diego, CA). Flag-FOXO4 were purchased from Sigma (Louis, MO, USA). Western blotting antibodies against cleaved-caspase-3, -caspase-6, -caspase-7, -caspase-8, -caspase-9, -PARP, p-ERK1/2, p-p38, p-JNK, p-Akt, ERK1/2, p38, JNK1/2, Akt, Mcl-1, Bcl-2, Bax, Cytochrome C, Lamin B, COXIV, -tubulin, -actin and si-FOXO4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Bak, Bim and Bcl-xL were purchased from Epitomics, Inc. (Burlingame, CA, USA). Antibodies against FOXO4 (AFX-1) were purchased from Novus Biologicals (Littleton, CO, USA). FLAG-Foxo4 was purchased from Addgene (Cambridge, MA, USA). Horseradish peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Promega (Madison, WI, USA).

Cell culture

The human PC3 (BCRC. 60112), DU145 (BCRC. 60348), 22Rv1 (BCRC. 60545) and LNCap (BCRC. 60088) cell lines were purchase from Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan). These cell lines were maintained in DMEM, RPMI 1640, or MEM with 10% FBS. The human prostatic epithelial cell lines RWPE-1 cell (ATCC® CRL-11609) was purchased from the American Type Culture Collection (ATCC). RWPE-1 cells were maintained in the complete K-SFM medium with contains 50 mg/ml of BPE and 5 ng/ml EGF. All cell lines mediums were containing 100 U/ml penicillin, 10 mM HEPES, 0.1 mM NEAA and 1 mM sodium pyruvate, the cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2. Cells were passaged every 2–3 days to obtain an exponential growth.

Measurement of cell viability with the MTT assay

The effect of NCTD on the cytotoxicity of cells were determined by the MTT assay, cells were plated into 24-well plates at 4 × 104 cells/well, grown for 24 h, and treated with 0.1% DMSO or different concentrations of NCTD (0, 20, 40, and 80 μM). After 24 h and 48 h incubation, MTT was added to each well (0.5 mg/ml final concentration), mixture of MTT and cells was further incubated for 4 h at 37°C. The viable cell number was directly proportional to the production of formazan following the solubilization with isopropanol. The color intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The experiments were performed in triplicate.

Annexin-V/PI staining assay

PC3 and 22Rv1 cell were cultured on 6-cm dishes and treated with various concentrations of NCTD for 24 h, then for FITC labeled Annexin V/PI Apoptosis Detection kit (BD Biosciences, CA, USA), cells were suspended with 100 μl of binding buffer and stained with 5μl of FITC-conjugated Annexin-V and 5 μl of PI

for 30 min at room temperature in dark place and then added 400 μl of binding buffer were analyzed via flow cytometry (FACSCalibur, BD Biosciences).

Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)

PC3 and 22Rv1 cells were plated on 6-cm dishes after treatment and were incubated until 60% to 80% confluences was reached, cells treated with various concentrations of NCTD for 24 h, and then cells were loaded with 3 μg/ml JC-1 dye for 30 min at 37 °C and washed for 5 min by PBS buffer. After incubation, samples were excited simultaneously by 488 nm argon-ion laser sources, the JC-1 monomer and JC-1 aggregate can be detected separately by the flow cytometry FL1 and FL2 channels respectively.

Plasmids, Small Interfering RNA and miRNA Transfection

Transfections were done using Lipofectamine 2000 Transfection Reagent (Invitrogen). PC3 or 22Rv1 cells were cultured on 6-cm or 24-well dishes at 37 ˚C for 24 h. Added lipofectamine and plasmid DNA (1 or 3 g), siRNA (200 nM) in 100 μl of absence of serum medium followed by equilibration at room temperature for 5 min after mixing. The complex was added to cells and incubated for 6 h. At 6 h after transfection, remove complexes and change/add fresh mediums containing 10% FBS for 24 h, then after 24 h, treated with 40 μM NCTD for 24 h, the cells were harvested and subjected to Western blot analysis and luciferase assay.

Western blot analysis

Equal amounts of protein extracts (25 μg) were subjected to either 10 or 12 % SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA, USA). The membrane was blocked with 5% non-fat milk in TBST buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1h, and then incubated with primary antibodies against in TBST overnight at 4 °C, then detected by enhanced chemiluminescence using the Immobilon western-HRP substrate (Millipore, Billerica, MA, USA).

Preparation of nuclear, mitochondrial and cytosolic fractions

Mitochondrial, nuclear and cytosolic subcellular fractions were isolated by cell disruption followed by differential centrifugation and washing steps, as previously described [30]. Briefly, lysis homogenates were centrifuged at 1,000 g for 20 min at 4°C, and supernatants were then centrifuged at 12,000 g for 20 min at 4°C. Pellets contained the enriched mitochondrial and nucleus fraction, whereas supernatants contained the cytoplasmic fraction, equal amounts of proteins were analyzed by western blotting.

RNA extraction and qRT-PCR

Total RNA was extracted from isolated cells using either TRIzol reagent (Thermo Fisher Scientific Inc.). cDNA were reverse transcribed from total RNA using ReverTra Ace qPCR RT Master Mix buffer (Toyobo, Japan), The PCR of FOXO1, FOXO3a and FOXO4 mRNA was determined using SYBR Green PCR Master Mix (Promega) as described by the manufacturer. Primers for polymerase chain reaction (PCR) were designed as follows: FoxO1-F, 5 ´ -GCCATGTAAGTCCCATCAGGA-3 ´ , FOXO1-R, 5 ´ -ATCGGAACAAGAACGTGGAATC-3 ´ ; FOXO3a-F, 5 ´ -CTTCAAGGATAAGGGCGACA-3 ´ , FOXO3a-R, 5 ´ -TCTTGCCAGTTCCCTCATT-3 ´ ; FOXO4-F, 5 ´ -TCATCAAGGTTCACAACGAGGC-3 ´ ;FOXO4-R, 5 ´ -AGGACAGACGGCTTCTTCTTGG-3 ´ . The PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and evaluated with UV light. In

the qRT-PCR step, cDNA was amplified with SYBR Green PCR Master Mix (Applied Biosystems) using primers specific for FOXO1, FOXO3a, FOXO4, and glyceraldehyde-3- phosphate dehydrogenase (GAPDH). GAPDH was used as internal control to normalize differences in total RNA levels in each sample. A threshold cycle (Ct) was observed in the exponential phase of amplification, the relative mRNA expression level was determined by calculating the ΔΔCt values and the fold change was expressed as $2^{-\Delta\Delta Ct}$.

Immunofluorescence Staining

Cells were grown onto Lab-Tek 12-well chamber slides (Thermo Fisher Scientific, USA) and fixed with 3 % paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS for 15 min. Then, the cells were incubated with primary antibodies against Mcl-1 (dilution 1:300, Abcam) and FOXO4 (dilution 1:300, Abcam) incubated overnight at 4°C. The cells were also incubated with Cy3-conjugated anti-rabbit or anti-mouse IgG (dilution 1:200) for 1 h at room temperature in the dark. The cell nucleus was counterstained using DAPI for 10 min. Fluorescence was monitored and photographed with a confocal microscope.

Chromatinimmunoprecipitation Assay

PC3 cells were cross-linked with 1% formaldehyde for 10 min at room temperature and add with 125 mM glycine for 5 min at room temperature, then washed with cold phosphate-buffered saline. The cell pellet was resuspended in lysis buffer, chromatin was extracted, then sonicated using a Misonix 3000 Sonicator with a microtip (16 cycles at power 6 with 20s of sonication and 60 s of rest) an average DNA length of 500 to 1000 bp. Antibodies were added to each of the samples, which were then rotated at 4°C overnight. After interaction with protein A beads and incubation overnight at 65°C to reverse the cross-links, the DNA was dissolved in Tris-EDTA buffer and analyzed by PCR. The anti-FoxO1 antibodies were added separately into the reaction solutions. Primers used for PCR were as follows: Mcl-1 (-1053/-1046): 5'-AACCTGTAAGAACTAATGAT-3'-3’ (sense) and 5'-GATTACAGCTGTGAGCCACC-3’ (antisense); Mcl-1 (-1911/-1904): 5'-CAGAGCCCGGTCTGTAATCC-3' (sense) and 5'-AGCCTCGCGAGTAGCGAGGAA-3' (antisense). Enrichment was calculated as the ratio between the net intensity of each bound sample divided by the input and the vehicle control sample divided by the input (bound/input)/(control/input).

Statistical analysis

All experiments were repeated at least three times independently. Data are expressed as mean ± S.D and analyzed with SPSS 10.0 and Sigma plot statistical software (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed using Student’s t-test or analysis of variance (ANOVA) were used for statistical analysis. P values of 0.05 or less were considered statistically significant

實驗結果

【1】**NCTD induces apoptosis in prostate cancer cell lines.**The chemical structure of NCTD was shown in Fig. 1a. To investigate the inhibitory effect of NCTD on cell viability in four prostate cancer cells (PC3, DU145, LNCap and 22Rv) and one normal prostate cells (RWPE-1). These cells were treated to various concentrations of NCTD (0~120 μM) for 24 and 48 h, and the cell viability was measured by MTT assay. NCTD inhibited the growth of four prostate cancer cells in both a dose- and time-dependent manner (Fig 1B-1E). Low cytoxicity of normal prostate RWPE-1 cells (Fig-1F). The concentrations of 20, 40, and 80 μM incubating for 24 h were chosen for the subsequent studies. To determine whether the growth inhibitory effect of NCTD was through the induction of apoptosis. We found that NCTD significant increase in apoptosis populations by Annexin V/PI staining by Flow cytometry in dose-dependent manner (Fig 1G). Western blot showed that PC3 and 22Rv cells treated with NCTD had significantly increased levels of the cleaved active forms of caspase-6, -7, -9, -3 and -PARP, but no change on cleaved caspase-8 protein levels (Fig. 1H). To confirm the contribution of caspase activation in NCTD-induced apoptosis, PC3 and 22Rv cell were pretreated with various the specific inhibitors for caspase-6 (Z-VEID-FMK), caspase-9 (Z-LEHD-FMK), caspase-3/-7 (Z-DEVD-FMK), and pan caspase inhibitor (Z-VAD-FMK) could significantly attenuated NCTD-induced apoptosis (Fig. 1I). These finding provide that NCTD induces apoptosis in prostate cancer cells.

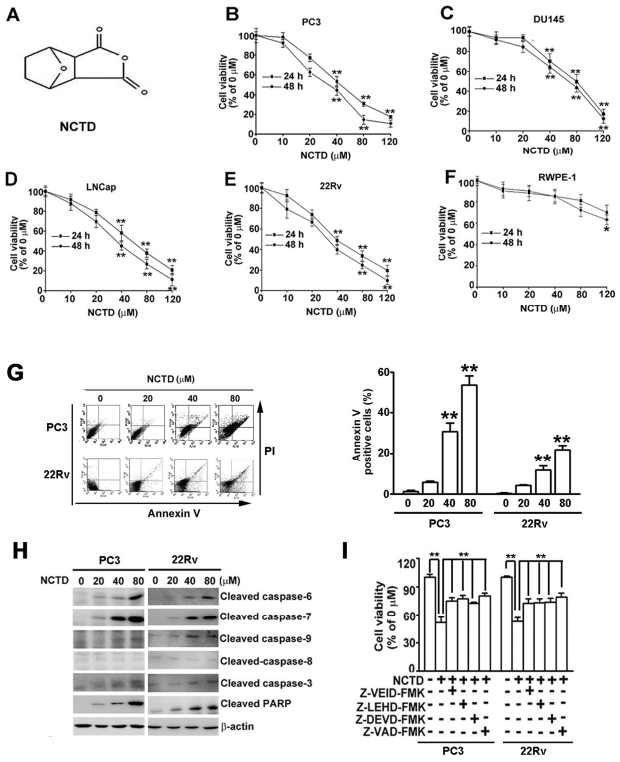

Fig-1. Effects of NCTD on cell growth and induces apoptosis in prostate cancer cell lines. (A) The chemical formula of NCTD. (B-E) The cell viability of four human prostate cancer cell lines (PC3, DU145, LNCap, 22Rv1) and (F) human normal prostatic epithelial cell lines RWAE-1 cells were treated with NCTD

(0–120 μM) for 24 and 48 h. Cell viability was determined by the MTT assay. (G) Cell apoptosis of PC3 and 22Rv1 cells were measured with Annexin-V and PI double-stained flow cytometry after treated with NCTD for 24 h. (H) The protein expression levels of caspase-6, -7, -9, -8, -3, and PARP were assessed by western blot analysiss. β-actin is shown as a protein loading control. (I) Cells were either pretreated with specific inhibitors for Z-VEID-FMK (caspase-6 inhibitor), Z-VEID-FMK inhibitor (caspase-9 inhibitor), Z-DEVD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK(caspase-3/-7 inhibitor), and Z-VAD-FMK (pan caspase inhibitor) for 2 h, followed by incubation with 40 μM NCTD for 24 h. Cell viability was evaluated by the MTT assay. **P<0.01 compared with control.

【2】**NCTD induces mitochondria dysfunction and regulates Bcl-2 family protein expression.** In our previously results suggested that NCTD induced cleaved active forms of caspase-9 in PC3 and 22Rv1 cells, we next investigated whether NCTD was capable of inducing mitochondrial membrane potential (ΔΨm) depolarization by using JC-1 assay. As shown in Fig. 2A, treatment of PC3 and 22Rv1 cells with 40 or 80 μM of NCTD were increased JC-1 green fluorescence can be seen in both the PC3 and 22Rv1 cells (Fig. 2A). To investigate the mechanism underlying NCTD induced ΔΨm depolarization in PC3 and 22Rv1 cells, a marked decreased the expression of Bcl-2, Bcl-xL, Mcl-1 and Bim, and increased the expression of Bak and Bax in both the PC3 and 22Rv1 cells treated with NCTD in a dose dependent manner (Fig. 2B). In addition, treatment with NCTD decreased the mitochondrial levels of cytochrome C, but increased the cytosolic levels of cytochrome C (Fig. 2C). These results suggest that NCTD induced apoptosis through induces mitochondria dysfunction and regulation of Bcl-2 family proteins in PC3 and 22Rv1 cells.

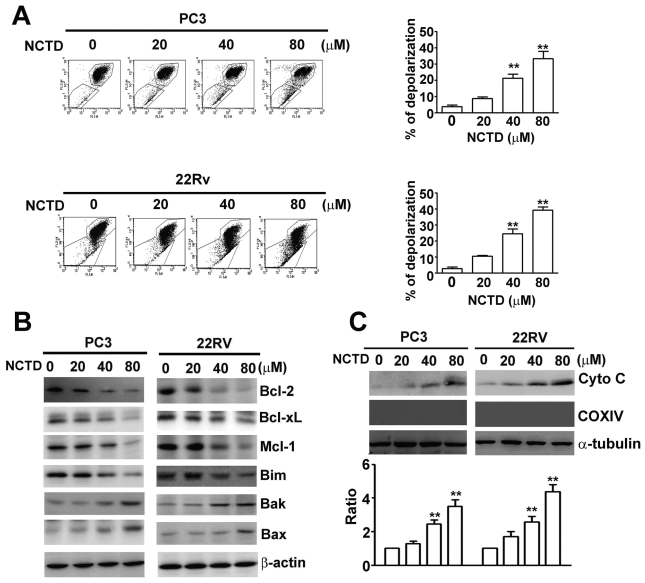

Fig-2. NCTD induces loss of mitochondrial membrane potential and cytochrome C release as well as regulates Bcl-2 family protein expression. Cells were treated with various concentrations of NCTD (0, 20, 40, 80 μM) for 24 h. (A) Mitochondrial membrane potential assay by flow cytometry following JC-1 reagents (B) Western blot analysis with antibodies specific for Bcl-2, Bcl-xL, Mcl-1, Bim, Bak and Bax. (C) The level of cytochrome c in cytosolic fractions was measured by western blotting analysis. α-tubulin is shown as the

cytosol protein control, whereas COXIV is shown as mitochondrial protein control. Results shown are the mean ± standard deviation (SD). **P<0.01 compared with control.

【3】**NCTD induced apoptosis via the inactivation of Akt-dependent pathway.** Several studies have indicated the NCTD induces apoptosis involved in MAPKs and Akt pathways of various cancer cells. Western blotting analysis showed that NCTD only inhibited the phosphorylation of Akt in a dose-dependent manner. No significant changes in phosphorylation of either ERK1/2 or JNK1/2 and p38 levels were observed in NCTD treated cells (Fig-3), suggesting that NCTD-induced apoptotic through inactivation of Akt expression in prostate cancer cells.

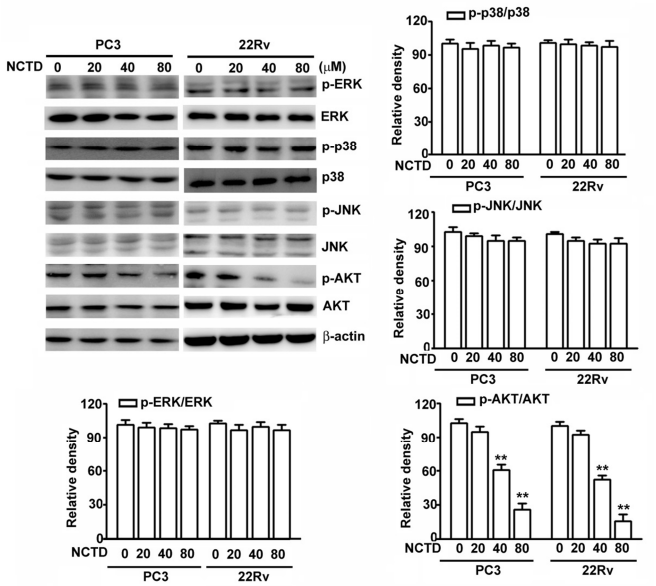

Fig. 3 Effect of NCTD on AKT and MAPKs associated proteins in PC3 and 22Rv1 cells. Total as well as phosphorylated ERK 1/2, p38, JNK 1/2, AKT and b-actin (loading control) levels were measured by Western blot in PC3 and 22Rv1 cells. Results shown are the mean ± standard deviation (SD). **P<0.01 compared with control.

【4】We investigated whether the NCTD was able to modulate Akt signaling pathways. As shown as Figure 4A, pretreated with LY294002 (PI3K inhibitor) prior to the addition of NCTD (40 μM) for 24 h. LY294002 significantly suppressed AKT phosphorylation, Bcl-2 and Mcl-1 expression and increased the activation of caspase-3/-9, and -PARP by NCTD treatment (Fig. 4A). The MTT assay results showed that the significantly enhanced cytotoxicity effects of NCTD on PC3 cells were enhanced when treatment with NCTD was combined with LY294002 in comparison to NCTD treated cells alone (Fig. 4C). In addition, Annexin V-FITC/PI double stained assays and JC-1 assay revealed that NCTD combined with LY294002 resulted in a significantly greater number of apoptotic cells (Fig. 4D) and loss of MMP (Fig. 4G and 4H) than NCTD treated cells alone. In addition, we examined whether NCTD mediated apoptosis by inhibiting Akt activation, we transiently transfected constitutive-Akt (HA-Akt) plasmids to PC3 cells were incubated for 24 h with or without NCTD (40 μM). The result showed that overexpression of Akt significantly reduced the expression of

cleaved-caspase-3, -6, -7, -9, -PARP and downregulation of Mcl-1 and Bcl-2 by NCTD treatment, compared with the NCTD treatment cells alone (Fig 4B). Moreover, overexpression of Akt also showed a reversal effects on NCTD-induced cytotoxicity (Fig-4E), cell death (Fig. 4F) and loss of MMP (Figure 4I, 4J). Together, these data demonstrate that Akt pathway plays an important role in NCTD-induced cell apoptosis.

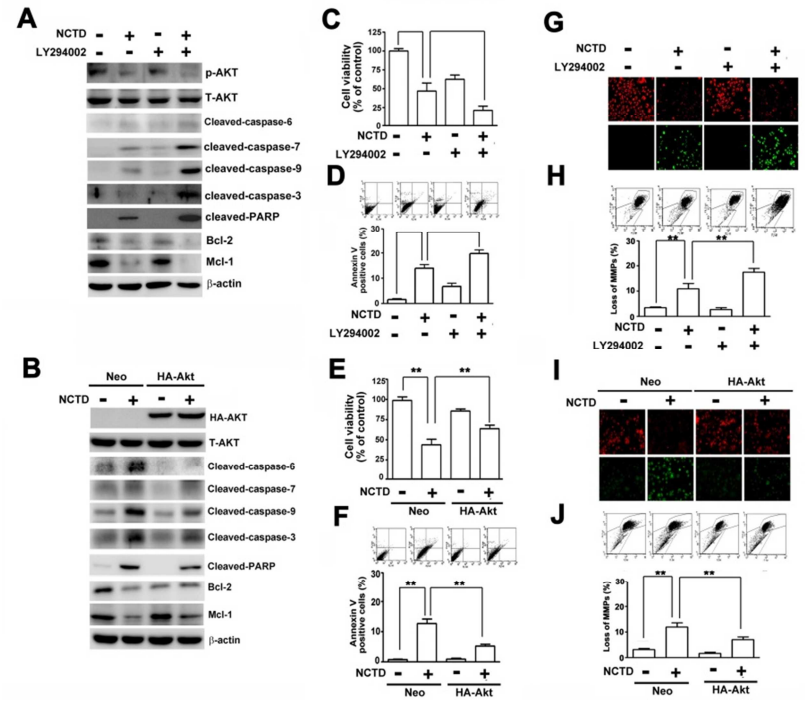

Fig. 4 Involvement of AKT inactivation in NCTD induces cell apoptosis. (A) PC3 cells pre-treated with or without LY294002 (30 uM) for 1h, and (B) transfection of Neo or HA-AKT plasmid in PC3 cells for 24 h followed by NCTD (40uM) for 24h. The p-AKT, AKT, cleaved-caspase-6, -7, -9, -3, -PARP, Bcl-2 and Mcl-1 were assessed by western blot. (C, E) Cell viability was measured by MTT assay. (D, F) Cell apoptosis were measured with Annexin-V and PI double-stained by flow cytometry (G, I) Cells were stained with JC-1 reagents and incubated at 37C for 30 min. The mean JC-1 fluorescence intensity was detected using immunofluorescence assay, and (H. J) quantified by the density of mitochondrial depolarization were measured with JC-1 stained by flow cytometry. Results shown are the mean ± standard deviation (SD). **P<0.01 compared with control

【5】NCTD Suppression of Mcl-1 Promoter Requires FoxO4. Some reports showed that FOXO transcription factors are direct downstream targets of Akt and can bind to the promoters of Bcl-2 family gene to promote their transcription and lead to cell apoptosis. Therefore, we measured the mRNA expression of FOXO family genes (FOXO1, FOXO3a and FOXO4) on NCTD-induced apoptosis in prostate cancer cells by RT-PCR and qRT-PCR assay. We found that NCTD induces FOXO4 mRNA expression in both PC3 and 22Rv1 cells. No significant changes in either FOXO1 or FOXO3a levels were observed in NCTD treated cells (Fig. 5A). We next examined NCTD whether induces FOXO4 binds to the Mcl-1 promoter by ChIP analysis. The promoter sequence of Mcl-1 genes was analyzed using the TRANSFAC 4.0 database to identify the

putative FoxO4 binding sequence (-1053/-1046 and -1911/-1904) (Fig. 5B). Results of ChIP analysis indicated that NCTD only increased FOXO4 binds over the promoter proximal -1053 to -1046 regions of the Mcl-1 promoter (Fig 5C). We next examined the effects of AKT on NCTD-induced FOXO4 binding to Mcl-1 promoter. PC3 cells were transiently transfected with siAkt or ectopically expressing flag-FOXO4 and treated with or without NCTD (40 μM), knockdown Akt enhanced NCTD-induced FOXO4 binding to Mcl-1 promoter (Fig 5D). Interestingly, overexpression of Akt inhibited NCTD-induced FOXO4 binding to Mcl-1 promoter (Fig 5E). Together, these results suggest that suppression of AKT activity enhances NCTD induces FOXO4 inhibited Mcl-1 transcriptional activity in prostate cancer cells.

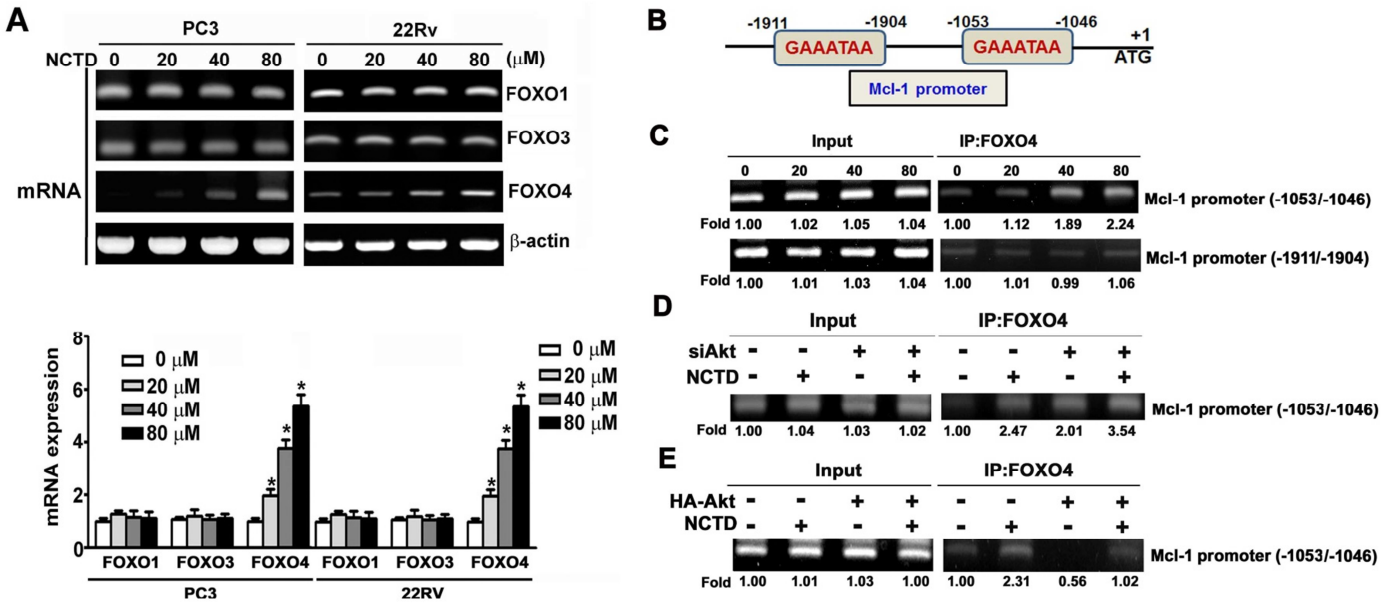

Fig. 5 Regulation of FOXO4 by NCTD. (A) PC3 cells were treated with NCTD (0~80 μM) for 24 h, the mRNA of FOXO1, FOXO3a and FOXO4 levels were then determined by RT-PCR and qRT-PCR. (B) The binding consensus sites of FOXO4 transcription factors on Mcl-1 promoter are labeled. (C) Chromatin immunoprecipitation using anti-FOXO4 antibodies was performed on chromatin extracted from various concentration of NCTD treated PC3 cells, (D) transfection with oligonucleotides of AKT siRNA, (E) transfection with Neo or HA-AKT plasmid, then treated with NCTD (40 mM) for 24 h, and specific Mcl-1 promoter regions were amplified by PCR. Input samples were used as positive control. (E) Nuclear extracts were prepared and mean nucleus of FOXO4 expression was detected using western blot. (F) Cells were fixed, permeabilized, and stained with FOXO4 and DAPI, and visualized under a fluorescence microscope.

【6】**NCTD enhances translocation of FOXO4 to nucleus and induces cell apoptosis**. Given that the FOXO4 function as a transcriptional repressors and induced apoptosis in prostate cancer cells . Thus we further investigated whether FOXO4 involved in NCTD-induced apoptosis. We suggested that NCTD induces nucleus expression of FOXO4 in PC3 and 22Rv1 cells in a dose dependent manner (Fig. 6A), and treatment of PC3 cells with NCTD were enhanced the nuclear translocation of FOXO4 and inhibited Mcl-1 protein

expression was observed by fluorescence microscopy (Fig. 6B). To demonstrate the FOXO4 activation plays a role in NCTD induced apoptosis, PC3 cells were transfected with FOXO4 siRNA or ectopically expressing flag-FOXO4, and then treated with or without NCTD (40 μM) for 24 h by the western blotting, combined treatment with siFOXO4 (200 nM) and NCTD (40 μM) in PC3 cells caused significantly decrease in expression of cleavage form of caspase-3, -9, -PARP and upregulation of Mcl-1 expression, compared with NCTD treatment alone (Fig. 6C. left). However, combined treatment with HA-FOXO4 and NCTD has opposite effect (Fig. 6C. right). To further assess the functional significance of FOXO4 activation in NCTD-mediated cell apoptosis. Combined treatment with siAKT and NCTD were markedly inhibits the growth (Fig. 6D, upper) and induces the apoptotic cells (Fig. 6E, upper), compared with the NCTD treatment cells alone. In addition, combined treatment with flag-FOXO4 and NCTD has opposite effect (Fig. 6D, 6E down). These results demonstrate NCTD enhanced the nucleus location of FOXO4 to induce apoptosis

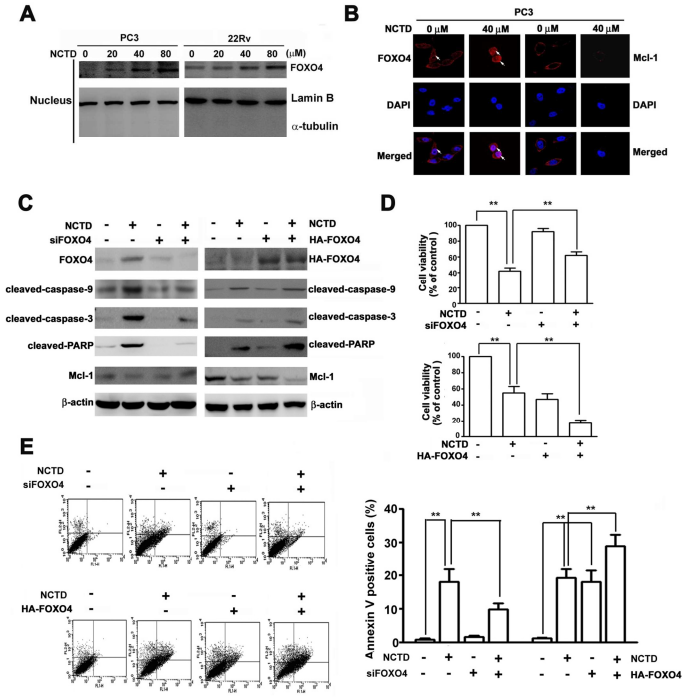

Fig.6. FOXO4 involved NCTD-induced apoptosis. (A) PC3 cells were transiently transfected of siFOXO4 or HA-FOXO4 plasmid for 24 h. After transfection, cells were treated with NCTD (40 μM) for 24 h. The FOXO4, cleaved-caspase-9, -3, -PARP and Mcl-1 were measured by western blot. (B) Cell viability was measured by MTT assay. (C) Cell apoptosis were measured with Annexin-V and PI double-stained by flow cytometry. Results shown are the mean ± standard deviation (SD). **P<0.01 compared with control

討論

This study demonstrated the NCTD was induced on apoptosis in prostate cancer cells, either by direct induction of apoptosis or by regulating the FOXO4 target Mcl-1. These actions were produced, through the phosphorylation of Akt/FoxO4/Mcl-1 and/or the induction of miR-320d targeted genes Mcl-1. There is accumulating evidence that the efficacy of antitumor agents-NCTD is related to the target tumor cells to respond of the apoptosis. NCTD has been demonstrated to be effective as a potential cancer chemoprevention agent against and caused induce apoptosis in numerous types of cancer cell lines in vitro and in vivo, [21-23]. The present study was designed to determine whether NCTD exerts cytotoxic activity against prostate cancer by inducing apoptosis, in our results, we found that NCTD induced apoptosis of prostate cancer cells in a dose- and time-dependent manner, and triggered the activations of caspases-3, -6, -7, -9 and the cleavages of PARP, which was associated with mitochondria dysfunction downregulation of Mcl-1. In addition, we discovered treatment of PC3 cells with NCTD not only inhibited activation of the phosphorylation of Akt and enhanced the FoxO4 expressio was translocated to the nucleus, but also through increasing miR-320d and reducing the expression of Mcl-1.

In the present study, to investigate the effect of NCTD supplementation on cytotoxicity and apoptosis in four prostate cancer cells was evaluated, and our results showed the NCTD inhibited the growth of prostate cancer cells. NCTD inhibits LNCap and 22RV cells which have low metastatic potentia, and inhibits PC3 and DU145 cells the metastatic and malignant tumors. In addition, non-tumorigenic cell line RWPE-1 demonstrated greater resistance to the cytotoxic effect of NCTD. These results indicated that NCTD was able to specifically anti-tumor activity in prostate cancer cells, but possesses with a low toxicity in on the non-tumorigenic cell line. Caspases have been known to play pivotal role implementation of apoptosis, have two major caspases activation pathways, mitochondrial and death receptor pathway, the mitochondrial t-Bid leads to the release of cytochrome c (cyt C) and caspase-9 is activated by cyt C and activates caspase-3, another pathway partial activation of caspase 8 is mediated by death receptor stimulation[24-25]. That NCTD caused accumulation of cytosolic cytochrome c and activation of caspase-9 but not Fas/ FasL/ caspase-8 pathway in several cancer cells, include human oral cancer cells, leukemic cells and melanoma cells[22,24,29]. In our study, similar to the previous the NCTD induced caspase-dependent cell death by the appearance of several apoptotic features, including the loss of plasma membrane integrity, activation of the caspase-3, -6, -7 and -9, and PARP cleavage, but not activation of the caspase-8. These results indicate that NCTD-induced cell death is through the mitochondrial pathway of apoptosis was not involved the Fas/ FasL/ caspase-8 death receptor pathway.

Mitochondria play important role in death signal transduction and amplification of the apoptotic response. Mitochondrial changes, including permeability transition pore opening and the collapse of $\Delta\Psi m$, induces apoptosis by activation of caspases [26]. Their damage during the cell suicide process of apoptosis is essentially responsible for cellular demise in most cells. A key of the B-cell lymphoma-2 (BCL-2) family, determines the integrity of mitochondria in the face of apoptotic insult, these organelles release proteins into

the cytosol which trigger caspase activation or perform other functions relevant to apoptosis, including cytochrome c [27]. The Bcl-2 family of proteins regulate apoptosis by controlling mitochondrial permeability. The anti-apoptotic proteins Bcl-2 and Mcl-1 reside in the outer mitochondrial wall and inhibit cytochrome c release[28]. Myeloid cell leukemia 1 (Mcl-1), is a pro-survival member of the Bcl-2 family of proteins, is overexpressed and the Mcl-1 gene is amplified in many tumor types, Mcl-1 expression can be induced by PI3K/ AKT signaling pathway[29.30]. Previous studies discovered the hepatocellular carcinoma mediated apoptosis by NCTD was associated with activation of mitochondrial apoptosis signaling pathway, and markedly decrease in expression of Mcl-1[31]. In the present study, the treatment of PC3 and DU145 cells with NCTD resulted in the loss of $\Delta\Psi m$ in a time-dependent manner, and western blot results showed a significantly decreased the anti-apoptotic proteins Mcl-1 expression and release cytochrome c into the cytosol which trigger apoptosis. The results indicate that NCTD induced apoptosis of prostate cancer cells, through decreased Mcl-1 expression make the mitochondrial damage- mediated caspase pathway.

The PI3K/AKT signal transduction cascade has been investigated extensively for its roles in oncogenic transformation, several studies implicated the PI3K and Akt in prevention of apoptosis [32,33]. AKT is a serine threonine kinase, which has been shown to be activated in various cancers and AKT is activated by PI3K. A specific inhibitor of PI3K, LY294002 initiated the blockade of AKT signaling, thus inhibited AKT phosphorylation [34]. The present findings show that NCTD treatment resulted in diminished AKT phosphorylation in Fig. 4. Additional combination with LY294002 potentiated the ability of NCTD to induce apoptosis of PC3 cells and significantly downregulated AKT phosphorylation, indicating that inactivation of the PI3K/AKT pathway played a critical role in NCTD-induced PC3 cells apoptosis. AKT plays an important role in cell survival through multiple downstream targets of apoptosis, the target include FoxO, mTOR, NF-κB. Previous studies have shown NCTD facilitates LPS-mediated immune responses by upregulation of AKT/NF-kB signaling in macrophages [35]. Farther, the NCTD induced apoptosis in Mantle cell lymphoma through the PI3K/Akt/ NF-κB signaling pathway[36]. Based on the above background, to clarify the downstream targets expression in PC3 cancer cell by NCTD treatment, we examined the corresponding downstream targets gene. Western blotting analysis showed that no significant changes in NF-κB or mTOR levels were observed in any of the NCTD treated cells, data not to shown. This demonstrated NCTD induced apoptosis not through the PI3K/Akt/NF-κB or mTOR signaling pathway. Interestingly, the PC3 cells treated with NCTD induced of the FoxO family-FoxO4 expression, and translocation to nuclear.

The forkhead box transcription factor, class O (FoxO), is a mammalian homolog of DAF-16, which is known to regulate life span in *Caenorhabditis elegans*. FoxO proteins are transcription factors that function downstream of the PI3K/AKT and directly control the expression of genes involved in apoptosis, cell cycle progression, and stress responses[37]. In our study, overexpress the FoxO4 cells were markedly sensitive to NCTD inhibited the growth of four PC3 cells and enhance cell apoptosis, in Fig. 6. But especially, the previous studies have shown a genomic shRNA screen the PI3K/AKT-inactivating downstream target, FoxO4, in LNCap cells line, and knockdown of FoxO4 in human LNCaP cells causes increased invasion in vitro and lymph node (LN) metastasis in vivo. This may be associated with that NCTD participate induces apoptosis in

diverse prostate cancer cell. Many studies have shown that induced apoptosis by regulating the AKT/FoxO/Bim signaling pathway, the FoxO family proteins regulate cell survival by transcriptionally modulating the expression of death receptor ligands and Bim, a pro-apoptotic protein belongs to the BH3-only subgroup of BCL-2 family [38.39]. In this present findings demonstrate the PC3 cells with NCTD induced of the FoxO4, and overexpression of FoxO4 significantly enhance the NCTD-induced apoptosis through activation of caspase and mitochondrion-related protein (i.e Bcl-2, Mcl-1) pathway, our finding provides a new insight into a possible mechanism that AKT/FoxO4/Mcl-1 signaling pathway induced apoptosis by NCTD.

科技部補助計畫衍生研發成果推廣資料表

日期:2016/09/28

科技部補助計畫	計畫名稱: 研究去甲斑蝥素調控微小核醣核酸表現與誘發前列腺癌細胞凋亡及輔助化學療法之分子機制
	計畫主持人: 謝逸憲
	計畫編號: 104-2320-B-040-001- 學門領域: 中醫藥學

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：謝逸憲	**計畫編號：**104-2320-B-040-001-
計畫名稱：研究去甲斑蝥素調控微小核醣核酸表現與誘發前列腺癌細胞凋亡及輔助化學療法之分子機制	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			1		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			1		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		

		其他	0		
	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	1		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   本次研究利用證實去甲斑螯素抑制四株人類前列腺癌細胞（PC3、DU145、LNCap和22Rv)生長且呈現劑量和時間的線性關係。同時證實去甲斑螯素也證實誘導細胞週期停留、DNA斷裂和caspase是透過抑制AKT活化,因而誘導FOXO4蛋白表現來抑制Mcl-1的轉錄和轉譯作用.因此在學術發展方面利用基礎研究與臨床應用方式證實去甲斑螯素在前列腺癌方面當作抗癌藥物的發展上深具潛力。此外在社會方面的研究成果以基礎與專業領域的結合，可以對社會大眾提供新的研發成果及治療成效做出貢獻，讓民眾了解使用NCTD安全性及毒性的評估方式，保障民眾使用NCTD的用藥安全.希望未來可以利用基礎研究與臨床應用方式證實去甲斑螯素在前列腺癌方面當作抗癌藥物及輔助性化療藥物的發展上深具潛力。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）