科技部補助專題研究計畫成果報告
期末報告

斑蝥素及其衍生物抑制不同肝癌細胞侵襲能力及誘導細胞凋亡的機制探討

計 畫 類 別 ： 個別型計畫
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執 行 期 間 ： 103年08月01日至104年07月31日
執 行 單 位 ： 中山醫學大學醫學系

計 畫 主 持 人 ： 葉兆斌
共 同 主 持 人 ： 楊順發
計畫參與人員： 碩士級-專任助理人員：楊惟恩

報 告 附 件 ： 出席國際會議研究心得報告及發表論文

處 理 方 式 ：
1.公開資訊：本計畫涉及專利或其他智慧財產權，2年後可公開查詢
2.「本研究」是否已有嚴重損及公共利益之發現：否
3.「本報告」是否建議提供政府單位施政參考：否

中 華 民 國 104 年 10 月 22 日

中 文 摘 要 ： 斑蝥素(cantharidin)是萃取自一種名為斑蝥的昆蟲。而斑蝥素具有許多顯著的生物活性如抗癌的作用。但是由於斑蝥素的毒性太強，亦會造成正常細胞的傷害及死亡。因此，慢慢的有許多斑蝥素的衍生物被合成，例如去甲基斑蝥素(norcantharidin)及 斑蝥酸(cantharidic acid)。然而，現今的研究中對於cantharidic acid在肝癌細胞的應用上仍然未知。在本篇研究中，將要去研究cantharidic acid在肝癌細胞上所誘發細胞凋亡分子機制。結果顯示，cantharidic acid顯著地肝癌細胞的增生作用。此外，cantharidic acid所處理的肝癌細胞是經由活化caspase-3、-8、-9及裂解PARP來誘發細胞凋亡，其呈現劑量的模式。此外，西方墨點法分析結果顯示cantharidic acid以劑量依賴性的模式增加了Erk1/2, JNK1/2及P38 MAPK磷酸化。綜合歸納後，我們的結果顯示cantharidic acid是經由MAPK路徑來誘發肝癌細胞產生凋亡，這也暗示著cantharidic acid適合作另一種有效的肝癌細胞化學治療藥物。

中 文 關 鍵 詞 ： 斑蝥酸、肝癌、細胞凋亡

英 文 摘 要 ： The rate of morbidity and mortality of hepatocellular carcinoma (HCC) in Taiwan has not lessened because of difficulty in treating tumor metastasis. Cantharidic acid possesses various biological anticancer activities, including apoptosis. However, detailed effects and molecular mechanisms of cantharidic acid on HCC apoptosis are unclear. Thus, HCC cells were subjected to treatment with cantharidic acid and then analyzed to determine the effects of cantharidic acid on cell apoptosis. The results showed that cantharidic acid suppressed cell proliferation in various types of HCC cell lines. Moreover, cantharidic acid induced cell-cycle arrest of HCC cells at the G0/G1 phase. Furthermore, cantharidic acid effectively induced apoptosis of HCC cells through caspases-8, -9, and -3 activation concomitantly with a marked induction of p38 mitogen-activated protein kinase (MAPK) activation. Taken together, our results suggest that cantharidic acid inhibited HCC cell proliferation through inducing cell-cycle arrest and apoptosis and could serve as a potential additional chemotherapeutic agent for treating HCC.

英 文 關 鍵 詞 ： Cantharidic acid, Hepatocellular Carcinoma, Apoptosis

Cantharidic acid Induces Apoptosis Involving MAPKs and Caspase-8/-9/-3 Signals in Human Hepatocellular Carcinoma

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

The rate of morbidity and mortality of hepatocellular carcinoma (HCC) in Taiwan has not lessened because of difficulty in treating tumor metastasis. Cantharidic acid possesses various biological anticancer activities, including apoptosis. However, detailed effects and molecular mechanisms of cantharidic acid on HCC apoptosis are unclear. Thus, HCC cells were subjected to treatment with cantharidic acid and then analyzed to determine the effects of cantharidic acid on cell apoptosis. The results showed that cantharidic acid suppressed cell proliferation in various types of HCC cell lines. Moreover, cantharidic acid induced cell-cycle arrest of HCC cells at the G_0/G_1 phase. Furthermore, cantharidic acid effectively induced apoptosis of HCC cells through caspases-8, -9, and -3 activation concomitantly with a marked induction of p38 mitogen-activated protein kinase (MAPK) activation. Taken together, our results suggest that cantharidic acid inhibited HCC cell proliferation through inducing cell-cycle arrest and apoptosis and could serve as a potential additional chemotherapeutic agent for treating HCC.

Key words: cantharidic acid, Apoptosis, G_0/G_1 arrest, HCC

1. Introduction

Cantharidin, norcantharidin (NCTD, exo-7-oxabicylo-[2.2.1] heptane-2,3-dicarboxylic anhydride) and cantharidic acid are known to possess anticancer activities because they suppress the activity of serine/threonine protein phosphates [1-7]. In our previous study, the structure-activity relationship (SAR) of cantharidin analogues suggested that anhydride ether oxygen in these analogues may correlate with HCC survival suppression, and the elimination of bridging ether oxygen on the ring can decrease cytotoxicity. However, cantharidin is unsuitable for cancer therapy because of its high cytotoxicity *in vitro* [IC (50) = 21 μM in primary cultured rat hepatocytes] [8]. The demethylated analogue of cantharidin is NCTD, which reduces the toxicity of cantharidin and is a potential anticancer drug for various cancer cells. A recent study showed that an NCTD-Nd3II derivative possesses anti-hepatoma activity, both *in vitro* and *in vivo*. It exerts its antiproliferative activity through apoptosis, G2/M cell-cycle arrest, and regulation of cyclin B1/cdc-2, p21, and Bcl-2/Bax [9]. Yang et al. reported that NCTD induces apoptosis of breast cancer cells through activities of mitogen-activated protein kinases and signal transducers and activators of transcription. Consequently, NCTD may disturb cell-cycle distribution of breast cancer cells through p53- and Chk-related pathways [10]. Moreover, Liao et al. reported that NCTD induces cell cycle arrest and inhibits progression of human

leukemic Jurkat T cells through mitogen-activated protein kinase-mediated regulation of interleukin-2 production [11]. Furthermore, Chang et al. showed that NCTD induced cytotoxicity in HepG2 cells by apoptosis, which is mediated through ROS generation and mitochondrial pathways [12], and a number of authors suggested that NCTD demonstrates anti-proliferative effects on human HepG2 cells in cell cultures [13]. NCTD thus inhibits the cell growth of various cancers by inducing apoptosis in cancer cells [14-22]. However, are no literature report, and these effects of cantharidic acid warrant further examination. Thus, we studied the effects, mechanisms, and pathway of cantharidic acid in HCC apoptosis.

2. Materials and Methods

2.1. Materials

Cantharidic acid purity was purchased from Sigma-Aldrich (St. Louis, MO). A 20-mM stock solution of cantharidic acid was made in dimethyl sulfoxide (DMSO; Sigma) and stored at -20 °C. The final concentration of DMSO for all treatments was < 0.5%. Antibodies specific for cleaved caspase-3, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), phosphorylated (p)-extracellular signal-regulated kinase (ERK)1/2, p-p38, p-c-Jun N-terminal kinase (JNK), ERK1/2, p38, JNK1/2, α-tubulin, and β-actin (for the Western blot analysis) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise specified, other chemicals used in this study were purchased from Sigma.

2.2. Cell Culture

HCC (SK-Hep-1) cells obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan) was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 ng/mL hydrocortisone. All cell cultures were maintained at 37 ℃ n a humidified atmosphere of 5% CO2.

2.3. In Vitro Cytotoxicity Assay

HCC (SK-Hep-1) cells were plated in 96-well microtiter plates and treated with various concentrations of cantharidic acid for 24 h, and cell viabilities were assessed using an MTS (Promega, Madison, WI) assay. The absorbance (A) was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (MQX200; Bio-Tek Instruments, Winooski, VT). The cell viability rate (multiples) was determined by: $A_{490,\ \text{cantharidic acid}} / A_{490,\ \text{vehicle}}$.

2.4. Flow Cytometric Analysis

SK-HEP-1 cells (4×10^6/ml) were treated with vehicle (0.5% DMSO) or 20 μM cantharidic acid, and the mixture was allowed to incubate for 24 h. At the end of incubation, cells were collected and fixed with 70% ethanol. Cells were stained with propidium iodide (PI) buffer (4 μg/ml PI, 1% Triton X-100, and 0.5 mg/ml RNase A in phosphate-buffered saline (PBS)) for 30 min in the dark at room temperature and then filtered through a 40-μm nylon filter (Falcon, San Jose, CA). The cell-cycle distribution was analyzed for 10^4 collected cells by a FACS Vantage flow cytometer that uses the Cellquest acquisition and analysis program (Becton-Dickinson FACS Calibur, San Jose, CA). Apoptotic cells with hypodiploid DNA content were detected

in the sub-G_1 region. All results were obtained from three independent experiments.

2.5. Annexin-V/PI Staining Assay

Apoptosis-mediated death of tumor cells was examined using a double-staining method with an FITC-labeled Annexin-V/PI Apoptosis Detection kit (BD Biosciences, San Jose, CA). For PI and Annexin-V double-staining, cells were suspended in 100 μl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, and 2.5 mM $CaCl_2$ at pH 7.4) and stained with 5 μl of FITC-conjugated Annexin-V and 5 μl of PI (50 μg/ml) for 30 min at room temperature in the dark, and then 400 μl of binding buffer was added. Apoptotic cells were analyzed via flow cytometry, by a FACScan system flow cytometric analysis. Data acquisition and analysis were performed in a Becton-Dickinson FACS Calibur flow cytometer using Cell Quest software (BD Biosciences).

2.6. Western Blot Analysis

Cell lysates were prepared as previously described [23]. Equal amounts of protein extracts (20 μg) were subjected to 10% or 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride membranes (Millipore, Belford, MA). After blocking, the membrane was incubated with primary antibodies for caspases-9, -3, and -8, PARP, ERK1/2, p-ERK1/2, p38, JNK1/2, p-

JNK1/2, α-tubulin, and β-actin. Blots were then incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody. Signals were detected via enhanced chemiluminescence using Immobilon Western HRP Substrate (Millipore, Billerica, MA).

2.7. Statistical Analysis

Values are shown as the mean ± SE. Statistical analyses were performed using the Statistical Package for Social Science software, vers. 16 (SPSS, Chicago, IL). Data comparisons were performed with Student's *t*-test when two groups were compared. A one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used when three or more groups were analyzed. Differences were considered significant at the 95% confidence level when $p < 0.05$.

3. Results

3.1. Effect of Cantharidic acid on Cell Proliferation of HCC (SK-Hep-1) cells.

To determine the efficacy of cantharidic acid against HCC cells, we first treated HCC (SK-Hep-1) cells with cantharidic acid. As shown in Figure 1, after treatment for 24 h, cantharidic acid significantly suppressed cell proliferation in concentration-dependent manners.

3.2. The Effect of Cantharidic acid on Cell-Cycle Progression and Apoptosis.

To investigate the mode of the antiproliferative effects induced by cantharidic acid, SK-HEP-1 cells were treated with 20 μM cantharidic acid for 24 h. The proportion of cells in the G_0/G_1 phase significantly increased after 24 h of treatment, with corresponding decreases of cells in the S and G_2/M phases. A representative cell-cycle distribution graph is shown in Figure 2. The internucleosomal degradation of DNA and translocation of phosphatidylserine (PS) to the extracellular membrane are two major characteristics of cell apoptosis. DNA degradation was evaluated by detecting the sub-G_1 proportion and after 24 h of treatment with cantharidic acid, percentages of the sub-G_1 cell population had also significantly increased from 2.29% to 38.4% (Figure 2). Similarly, as shown in Figure 3, we assessed the translocation of PS using Annexin-V and PI double-staining. Annexin-V-positive cells increased from

3.0% to 26.27% after treating SK-HEP-1 cells with 20 μM cantharidic acid (Figure 3).

3.3. Effects of Cantharidic acid on Activation/Expression of Pro- and Antiapoptotic Proteins.

The apoptotic process is executed by members of highly conserved caspases, and modulation of the mechanisms of caspase activation and suppression is a critical molecular target in chemoprevention, since these processes lead to apoptosis [24]. To identify the mechanisms underlying cantharidic acid-induced apoptosis in SK-HEP-1 cells, activation of caspases-3, -8, and -9 and cleavage of PARP were detected. Figure 4 shows that exposure of SK-HEP-1 cells to cantharidic acid (0~20 μM for 24 h) caused concentration-dependent degradation of procaspases-3, -8, and -9, which respectively generated fragments of caspases-3, -8, and -9. These findings suggest that activation of caspase-3/-8/-9 signals and increased expression of cleaved PARP were responsible for cantharidic acid-induced apoptotic induction in SK-HEP-1 cells.

3.4. MAPKs Involved in Cantharidic acid-induced Cell-Cycle Arrest and Apoptosis

ERK was reported to be associated with the proliferation and drug resistance of hematopoietic cells [25] and be a key cellular component linking extracellular signals

to the induction and activation of cell-cycle events controlling the G_1-to-S-phase transition [26]. Upon treatment with different concentrations of cantharidic acid (0~20 μM), we found that 10 and 20 μM cantharidic acid induced dominant activation of Erk1/2, p38 MAPK, and JNK1/2 (Figure 5), suggesting that activation of MAPK might play a critical role in cantharidic acid-induced apoptosis in SK-HEP-1 cells.

Figure 1

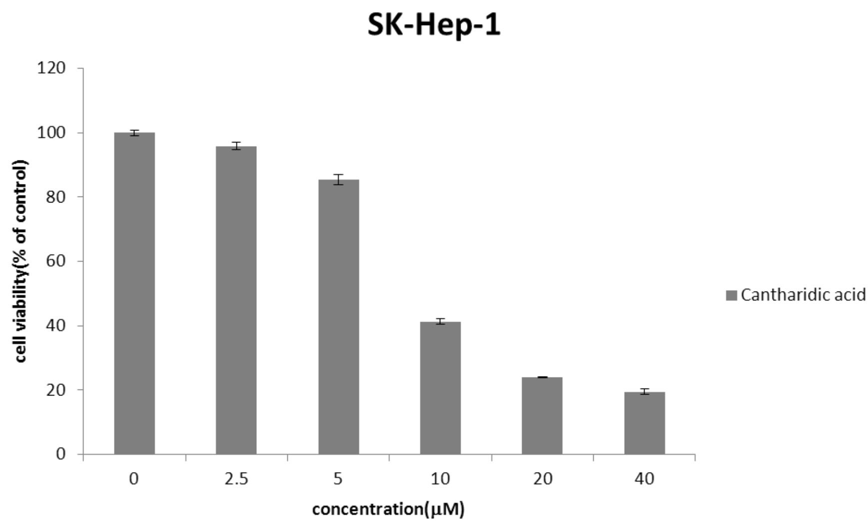

Figure 1: Effect of cantharidic acid on cell viability of HCC cell lines. SK-Hep-1 cells were treated with the vehicle (DMSO) or cantharidic acid (0~40 μM) in serum-containing medium for 24 h. Cell viability was determined by an MTS assay. Results are expressed as multiples of cell viability. Values represent the mean ± SE of 3 independent experiments.

Figure 2

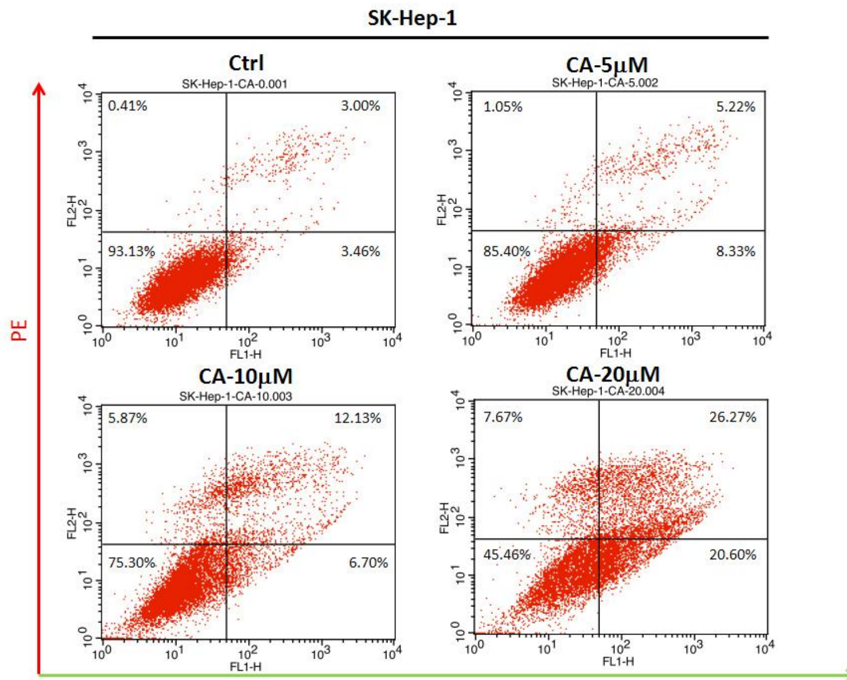

Figure 2: The effect of cantharidic acid on apoptosis as determined by flow cytometry. One representative experiment is shown. Quantitative analysis of cell apoptosis by Annexin-V and propidium iodide (PI) double-staining flow cytometry after treatment of SK-Hep-1 cells with 0-20 μM cantharidic acid or the vehicle for 24 h.

Figure 3

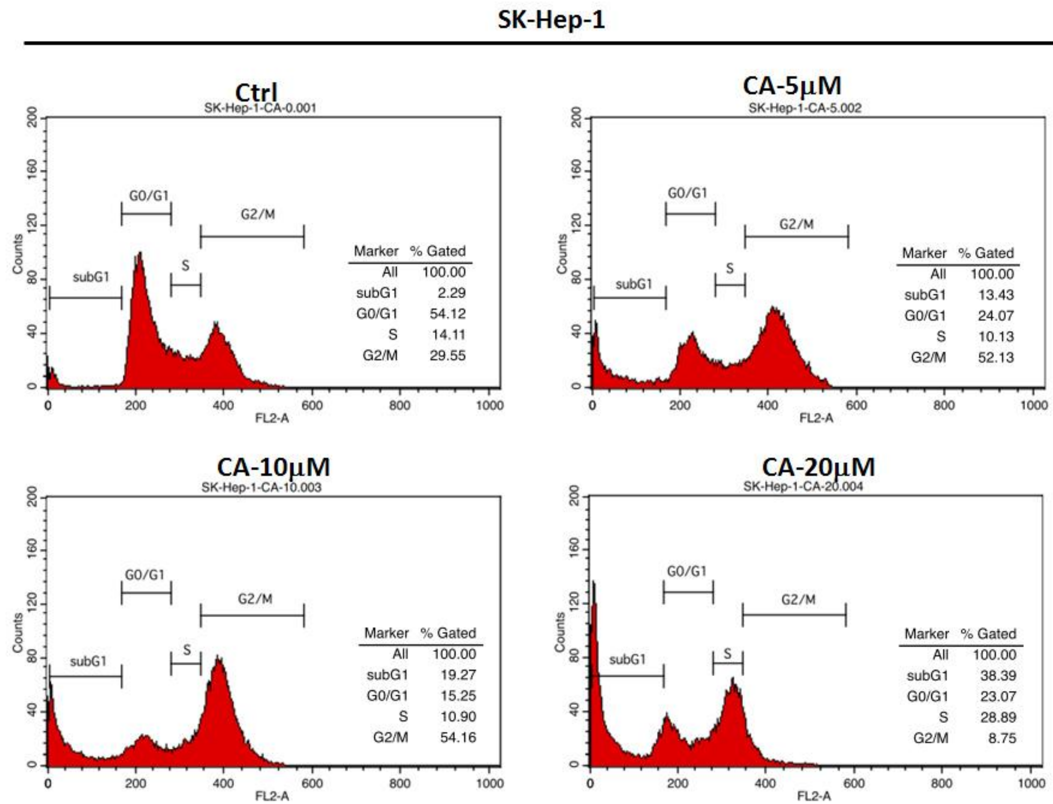

Figure 3: The effect of cantharidic acid on cell-cycle progression as determined by flow cytometry. One representative experiment is shown.

Figure 4

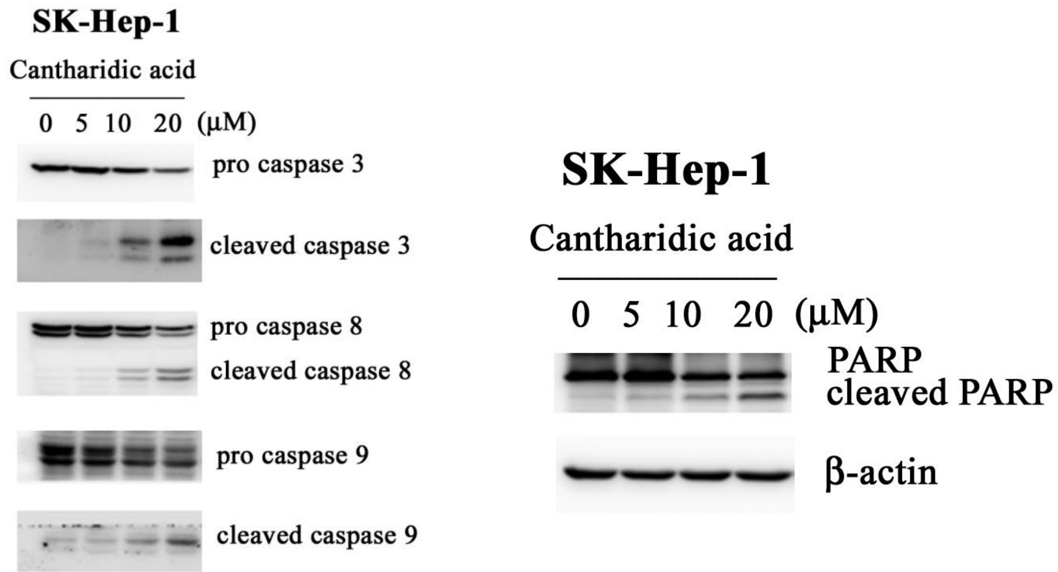

Figure 4: Effects of cantharidic acid on activation/expression of pro- and antiapoptotic proteins. Expression levels of caspase-3, -8, and -9, and PARP were assessed by a Western blot analysis after treatment with various concentrations of cantharidic acid (0~40 μM) for 24 h.

Figure 5

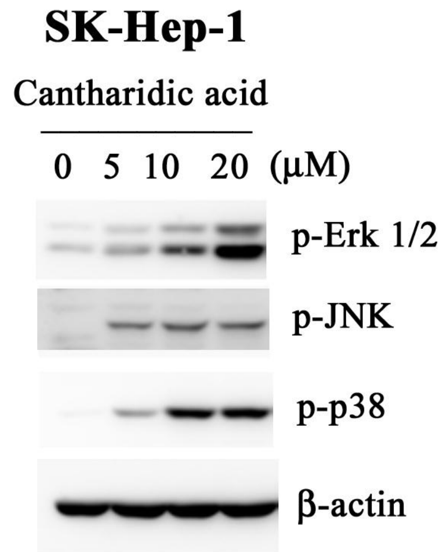

Figure 5: Effect of cantharidic acid on the mitogen-activated protein kinase (MAPK) pathway. Phosphorylation levels of extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK)1/2, and p38 were assessed by a Western blot analysis after treatment with various concentrations of cantharidic acid (0~20 μM) for 24 h.

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行政院國家科學委員會補助國內專家學者出席國際學術會議報告

104 年 10 月 22 日

<table>
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及職稱</td><td>中山醫學大學醫學系副教授</td></tr>
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名稱</td><td colspan="3">(中文) 2015 歐洲暨美國癌症大會聯合會議
(英文) EACR-AACR-SIC Special Conference 2015</td></tr>
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題目</td><td colspan="3">(中文) Cantharidic acid 抑制肝癌細胞的增生及誘導細胞凋亡的機制探討
(英文) Cantharidic acid suppresses the proliferation and induces apoptosis involving MAPKs and caspase-8/-9/-3 signals in human hepatocellular carcinoma cells</td></tr>
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一、參加會議經過

於 6/19 抵達義大利佛羅倫斯。6/20 前往會議地點 (Firenze Fiera) 報到及領取大會議程及摘要手冊，並聆聽演講。6/21 將準備好的論文海報張貼於指定位置。並於會議尚未開始的時間參觀會場內其他相關發表。

二、與會心得

此次會議為美國癌症學會及歐洲癌症學會共同舉辦的會議，主題主要為抗癌物的作用標的，因此非常符合我們的科技部計畫內容。希望國內也可以多多舉辦類似的研討會。

三、建議

國內也應增加舉辦如此大型會議、增加補助出國額度、或盡量補助教師與學生出國開會或短期研究之經費，讓我們有機會與國際經驗豐富學識淵博的學者交流。

四、攜回資料名稱及內容

會議議程手冊

科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/22

<table>
<tr><td rowspan="3">科技部補助計畫</td><td>計畫名稱: 斑蝥素及其衍生物抑制不同肝癌細胞侵襲能力及誘導細胞凋亡的機制探討</td></tr>
<tr><td>計畫主持人: 葉兆斌</td></tr>
<tr><td>計畫編號: 103-2314-B-040-021-　　　　學門領域: 一般外科</td></tr>
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103年度專題研究計畫研究成果彙整表

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<tr><td colspan="3">計畫主持人：葉兆斌</td><td colspan="5">計畫編號：103-2314-B-040-021-</td></tr>
<tr><td colspan="8">計畫名稱：斑螯素及其衍生物抑制不同肝癌細胞侵襲能力及誘導細胞凋亡的機制探討</td></tr>
<tr><td colspan="3" rowspan="2">成果項目</td><td colspan="3">量化</td><td rowspan="2">單位</td><td rowspan="2">備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）</td></tr>
<tr><td>實際已達成數（被接受或已發表）</td><td>預期總達成數（含實際已達成數）</td><td>本計畫實際貢獻百分比</td></tr>
<tr><td rowspan="12">國內</td><td rowspan="4">論文著作</td><td>期刊論文</td><td>0</td><td>0</td><td>100%</td><td rowspan="3">篇</td><td></td></tr>
<tr><td>研究報告/技術報告</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>研討會論文</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專書</td><td>0</td><td>0</td><td>100%</td><td>章/本</td><td></td></tr>
<tr><td rowspan="2">專利</td><td>申請中件數</td><td>0</td><td>0</td><td>100%</td><td rowspan="2">件</td><td></td></tr>
<tr><td>已獲得件數</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td rowspan="2">技術移轉</td><td>件數</td><td>0</td><td>0</td><td>100%</td><td>件</td><td></td></tr>
<tr><td>權利金</td><td>0</td><td>0</td><td>100%</td><td>千元</td><td></td></tr>
<tr><td rowspan="4">參與計畫人力（本國籍）</td><td>碩士生</td><td>0</td><td>0</td><td>100%</td><td rowspan="4">人次</td><td></td></tr>
<tr><td>博士生</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>博士後研究員</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專任助理</td><td>1</td><td>1</td><td>100%</td><td></td></tr>
<tr><td rowspan="12">國外</td><td rowspan="4">論文著作</td><td>期刊論文</td><td>0</td><td>0</td><td>100%</td><td rowspan="3">篇</td><td></td></tr>
<tr><td>研究報告/技術報告</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>研討會論文</td><td>1</td><td>1</td><td>100%</td><td>EACR-AACR-SIC Special Conference 2015</td></tr>
<tr><td>專書</td><td>0</td><td>0</td><td>100%</td><td>章/本</td><td></td></tr>
<tr><td rowspan="2">專利</td><td>申請中件數</td><td>0</td><td>0</td><td>100%</td><td rowspan="2">件</td><td></td></tr>
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<tr><td rowspan="4">參與計畫人力（外國籍）</td><td>碩士生</td><td>0</td><td>0</td><td>100%</td><td rowspan="4">人次</td><td></td></tr>
<tr><td>博士生</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
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<tr><td>專任助理</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td colspan="2">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</td><td colspan="6">無</td></tr>
</table>

產業技術發展之具體效益事項等，請以文字敘述填列。）			
	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性）	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

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： 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100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） 斑蝥素(cantharidin)是萃取自一種名為斑蝥的昆蟲。而斑蝥素具有許多顯著的生物活性如抗癌的作用。但是由於斑蝥素的毒性太強，亦會造成正常細胞的傷害及死亡。因此，慢慢的有許多斑蝥素的衍生物被合成，例如去甲基斑蝥素(norcantharidin)及 斑蝥酸(cantharidic acid)。然而，現今的研究中對於cantharidic acid在肝癌細胞的應用上仍然未知。在本篇研究中，將要去研究cantharidic acid在肝癌細胞上所誘發細胞凋亡分子機制。結果顯示，cantharidic acid顯著地肝癌細胞的增生作用。此外，cantharidic acid所處理的肝癌細胞是經由活化caspase-3、-8、-9及裂解PARP來誘發細胞凋亡，其呈現劑量的模式。此外，西方墨點法分析結果顯示cantharidic acid以劑量依賴性的模式增加了Erk1/2, JNK1/2及P38 MAPK磷酸化。綜合歸納後，我們的結果顯示cantharidic acid是經由MAPK路徑來誘發肝癌細胞產生凋亡，這也暗示著cantharidic acid適合作另一種有效的肝癌細胞化學治療藥物。