附件一

行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

利用蛋白質體學方法研究養殖軟珊瑚中活性成份 11-Epi-sinulariolide acetate 對肝癌細胞之抗腫瘤影響及分子機制探討

計畫類別：■個別型計畫　□整合型計畫

計畫編號：**NSC 101-2313-B-276-002**

執行期間：101 年 8 月 1 日至 102 年 7 月 31 日

執行機構及系所:美和科技大學美容系

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成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

本計畫除繳交成果報告外，另須繳交以下出國心得報告：

□赴國外出差或研習心得報告

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□出席國際學術會議心得報告

□國際合作研究計畫國外研究報告

處理方式：除列管計畫及下列情形者外，得立即公開查詢

□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

中　華　民　國 102 年 8 月 31 日

成果報告目錄

計畫中文摘要

計畫名稱：**利用蛋白質體學方法研究養殖軟珊瑚中活性成份 11-Epi-sinulariolide acetate 對肝癌細胞之抗腫瘤影響及分子機制探討**

關鍵字：

中文摘要：

目前從軟珊瑚中萃取出之有效成分進行生物醫學及治療疾病之研究逐漸增多。我們在此研究中將養殖指型軟珊瑚(*Sinularia flexibilis*)中所萃取出的11-*Epi*-sinulariolide acetate 進行對肝癌細胞HA22T 之細胞毒殺影響研究。在初步的研究中我們利用蛋白質體學的方法進行11-*Epi*-sinulariolide acetate 對HA22T 細胞的影響，此項研究目前仍未有研究發表過。目前經由二維電泳及LC/MS/MS 鑑定24 個具有差異性表現之蛋白質，這些蛋白質參與蛋白質合成及折疊過程，並且蛋白質位於粒線體及內質網內，推測粒線體及內質網在11-*Epi*-sinulariolide acetate 誘發細胞凋亡過程中可能扮演重要角色。實驗發現11-*Epi*-sinulariolide acetate 可以活化caspase 途徑所造成之細胞凋亡，以及活化Unfolded Protein Response (UPR)訊息傳遞途徑，並且經由GADD153/CHOP 表現增加，造成內質網壓力延長，使得細胞生長停滯並造成細胞凋亡。Salubrinal 是內質網壓力之抑制劑，加入salubrinal 後發現會破壞11-*Epi*-sinulariolide acetate 所造成的細胞死亡。這些結果可以發現11-*Epi*-sinulariolide acetate 造成HA22T 細胞凋亡作用可能是經由粒線體功能喪失及內質網壓力途徑所形成。此項研究將有助於海洋生物有效成分應用於肝癌研究之發展。

計畫英文摘要。（五百字以內）

Project: Proteomic investigation of anti-tumor activities and molecular mechanism of 11-Epi-sinulariolide acetate from the cultured soft coral Sinularia flexibilis on hepatoma cell carcinoma cells.

Keywords:

The extracts from soft corals have been increasingly investigated for biomedical and therapeutic purposes. The aim of this study is to examine and analyze the cytotoxicity effects of the extract 11-*Epi*-sinulariolide acetate from cultrue soft coral *Sinularia flexibilis* on HA22T hepatocellular carcinoma cell. In the present study, we used proteomics to examine the cytotoxic effects of 11-*Epi*-sinulariolide acetate on HA22T cells. This study has not been published yet. A total of 24 differential proteins have been identified by LC-MS/MS analysis after the treatment with 11-*Epi*-sinulariolide acetate.Protein profiling of 11-*Epi*-sinulariolide acetate -treated HA22T cells revealed profound protein alterations related to protein synthesis and folding, suggesting that mitochondria and endoplasmic reticulum (ER) play a role in 11-*Epi*-sinulariolide acetate -initiated apoptosis. 11-*Epi*-sinulariolide acetate leading to the activation of caspase-dependent apoptotic cell death. Unfolded Protein Response (UPR) signaling pathways are also activated after 11-*Epi*-sinulariolide acetate treatment and these changes were accompanied by increased expression of GADD153/CHOP, a transcription factor associated with growth arrest and apoptosis in the event of prolonged ER stress. Salubrinal (Sal), a selective inhibitor for ER stress, partially abrogated the 11-*Epi*-sinulariolide acetate -related cell death. These results suggest that 11-*Epi*-sinulariolide acetate exerts cytotoxicity in HA22T cells through both mitochondrial dysfunction and ER stress cell death pathways.

We will investigate the effects of 11-*Epi*-sinulariolide acetate on HA22T cells by studying the membrane proteome and phosphoproteome to understand the possible biochemical mechanism involved in the toxicology. The results of this study will beneficial to the development of marine active compounds used for cancer treatment.

報告內容

（一） 前言及研究目的

海洋藥物

將陸地上的物總與海洋的相比較之下海洋的豐富性絕對是優於陸地的，這主要原因包含了，地球上的生命起源來自於海洋，就面積來說海洋就占地球表面積約 70%，而海洋的生物物種也占地球生物總量的 87%,因此海洋可以說是地球上最大的資源寶庫。在 35 億年前最初的有機物開始出現在海洋中，而在 5.7 億年前海洋就已經發現無脊椎動物、直到 4.25 億年前陸地上才開始有生物的蹤跡。而目前生物界所歸類的 34 個動物門中，海洋就佔了 33 個，其中有 16 個門的動物只能生存於海洋的環境中。

台灣海洋資源

台灣位處亞洲中國大陸東南方,菲律賓北方,氣後橫跨了熱帶以及亞熱帶,而台灣整體面積約 36188 平方公里南北狹長，因位處大西洋西側，且四面環海因此與海有著密不可分的關係。整體而言台灣的水產及自然資源的豐沛也因此地理位置有著絕對的優勢。而這豐富的天然資源又以海洋中洋流的流向所造成，在台灣洋流主要以由菲律賓開始而上通過台灣東部海岸的黑潮暖流以及通過東部海岸的大陸沿岸洋流所構成，這兩者之中又以黑潮所帶來的資源效應最大，黑潮厚度約 200 至 1000 公尺，寬度約 200 多公里，流速約每秒 1 至 2 公尺，黑潮就好比像是大海上的高速公路，運載著大量的生物、微生物，也因此造就東南部沿岸豐富的生態系。就因如此得天獨厚的地理位置，讓更多的研究學者在對於海洋新型藥物的發掘更佳的便利，目前台灣在海洋生技方面目前為止至 2013 年 6 月約有 2399 篇 SCI 期刊被發表，且數量逐年提高，顯示目前對於藥學及化學的領域中，海洋已被視為下一個主戰場。

過去海洋藥物之應用

人類早在幾千年前就發現海洋生物中存在活性物質，對人類疾病有顯著的治療效果。我國是最早應用海洋藥物的國家之一，最早的藥物專著《神農本草經》載藥 365 種，其中海洋藥物約 10 種，歷代又繼續發展，直至明代李時珍著本草綱目已記載可供藥用的海洋生物達 90 餘種，到清代趙學敏著《本草綱目拾遺》新增海洋中藥近 10 種，總數達 100 餘種。目前的海洋藥物已經通過美國 PDA 臨床測試市售之藥品到 2012 止已多達 7 種，其中包含 4 種具有抗癌效果、1 個止痛劑、1 個抗病毒、1 個保健食品(William et al.2012)。而在抗癌方面就有 2 個出自於海綿的化學產物，及 1 個來自海鞘，顯示在海

洋中多變的生態環境確實能造能生物製造具有特殊活性與新奇的二次代謝物(Rana et al. 2011)。

美國國家癌症研究中心所進行海洋活性物質及植物活性物質分別進行癌細胞毒殺研究，發現大約1%海洋活性物質具有抗腫瘤潛力，另外植物活性物質約只有0.1%具有抗腫瘤能力，推測未來最具潛力的抗癌化合物可能來自海洋。美國每年約有 1500 個海洋活性物質被分離出來，1%具有抗癌活性 ，目前海洋抗癌藥物進入臨床或臨床前研究階段至少已有 10 個以上。 1976 年 Ruggirei 等學者於 *Science* 期刊中首次提出『drugs from the sea』之理念，並將已發現具有顯著生物活性二次代謝產物作一個系統整理。(2)而在 1980 年起美國國家癌症研究中心 (National Cancer Institute, NCI) 在印度洋與太平洋收集了超過兩萬多種的海洋天然活性物質，並且利用 60 種人類腫瘤細胞作為篩選平台，測試在臨床治療上可能具有細胞毒殺之天然二次代謝產物，做一個海洋天然物的資訊與應用資料庫之整理。(3) 目前有數個已進入抗癌、抗感染與阿茲海默症等治療評估的海洋藥物 (Table 1-2-1)。(4)未來將擴大海洋生物的活性篩選，繼續尋找高效的抗癌化合物，直接用於臨床或作為先導物進行結構改造，開發新的高效低毒的抗癌成分將成為海洋抗癌藥物研究的發展趨勢。

由於目前地球暖化進而影響珊瑚生長，另外珊瑚也不能隨意採集，所以人工培育珊瑚技術對於珊瑚復育或是珊瑚活性物質未來應用於醫學研究，是一個非常重要技術。而人工養殖活珊瑚大約從50年前已開始發展，由於珊瑚皆可以利用無性生殖進行繁殖，但其在養殖過程中必須模擬珊瑚礁自然生態，例如光度、溫度、酸鹼度等，所以必須有足夠之調整系統設備加以操縱控制。雖然活珊瑚的人工養殖仍屬於難度和成本皆較高的水族技術，但已經從難以人工培育轉變成可完全長期在人工環境下養殖，目前國內海生館具備有人工養殖之技術，因此未來若是可以從軟質珊瑚中發現有效之活性物質，將可以大量生產提供穩定品質和來源的生物材料，突破在供應來源上的限制，並在未來具備了成本低、效益佳的產業商機。

肝癌的發生

肝癌的發生原因複雜，目前歸類大致上有病毒、化學致癌物質、酒精所造成肝發炎(Navas et al. 2011, Testino et al. 2010)，導致肝細胞壞死造成肝硬化，之後若無立即就醫就可能導致癌症病變，所以約80~90%的肝癌病人在肝癌產生前都有肝臟硬化的發生；因此在臨床上肝癌有所謂的三部曲。
肝炎->肝硬化->肝癌。肝癌長久以來高居台灣十大癌症死因的前兩名,在民國八十九年,位居男性癌症發生率的第一名,女性則位居第四名。肝癌的發病年齡可小至幾歲的小孩,大至 90 幾歲的老人,但大都集中於 40~50 歲以上的成年人。肝癌的患者以男性居多,男性比女性多出 3~5 倍。在台灣,發生肝癌的原因有

80%是 B 型肝炎造成,另有 10~20%則是 C 型肝炎所引起的(Gambia Hepatitis Study Group. 1987, Han et al. 2011)。台灣常見「家族性肝癌」,其原因大多起因於 B 型肝炎的母子垂直感染而造成家族中多人感染 B 型肝炎,然後再發生肝癌(Yu et al. 2000)。

（三）研究方法

3.1 養殖型脈指型軟珊瑚*Sinularia flexibilis*活性成分萃取及鑑定

脈指型軟珊瑚 *Sinularia flexibilis* 養殖工作及其活性物質分離純化及鑑定工作，皆由共同主持人海生館蘇瑞欣副研究員協助進行，分離方法步驟參考李奈倫碩士論文(2012) 。

本實驗使用之養殖型脈指形軟珊瑚 *Sinularia flexibilis*，在海洋生物博物館 0.6 噸的養殖水缸養殖。採集 *Sinularia flexibilis* 珊瑚樣品濕重約 700 公克，將珊瑚樣品進行冷凍乾燥處理，再將乾燥的珊瑚組織磨碎，秤得其乾重為 300 公克後進行萃取分離流程。

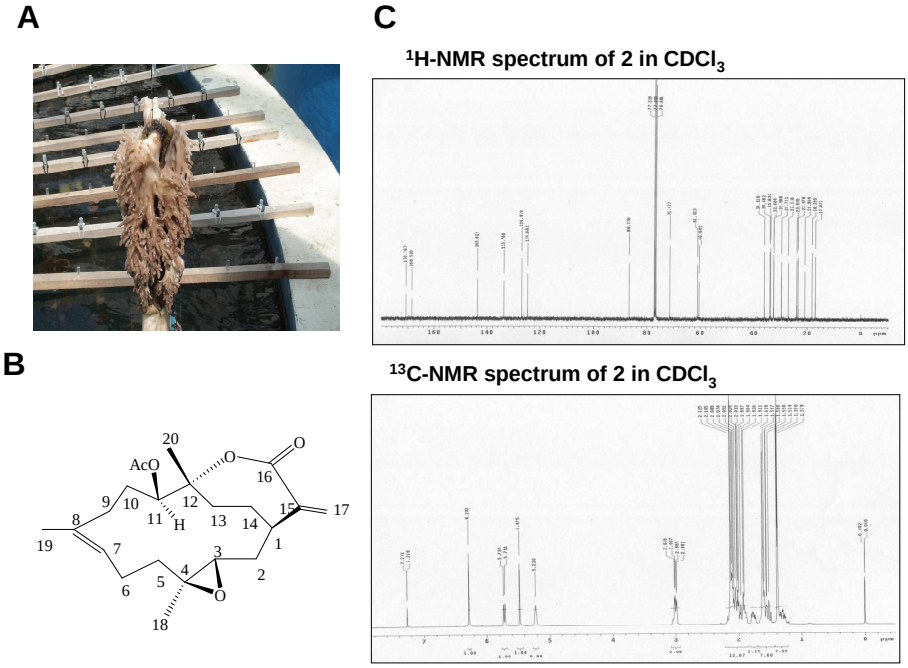

圖 1. 養殖型脈指型軟珊瑚 *Sinularia flexibilis* *即此次實驗所利用之* 11-Epi-sinulariolide acetate 化學結構

3.2 細胞培養

此次我們先選用 HepG2, HA22T, Hu7 及 Hep3B 等細胞進行培養，以進行後續研究，這些細胞分別培

養於 DMEM, RPMI 及 MEM 等培養基，並且加入 4 mM L-glutamine , 1.5g/L sodium bicarbonate 及 4.5g/L glucose, 並且添加 10%(v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin 及 1mM sodium pyruvate ，在 5% CO_2 37°C 培養箱中進行細胞培養。11-Epi-sinulariolide acetate 從養殖型脈指型軟珊瑚 *Sinularia flexibilis* 萃取，細胞分別處理不同濃度之 11-Epi-sinulariolide acetate 及控制組中加入 DMSO，並且培養 24 小時，進行後續之研究。全部實驗都進行三次以確定其再現性。

3.3 MTT 細胞活分析法

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)為一種黃色之化合物，可進入細胞粒線體內，並被 Succinate dehydrogenase 作用分解，產生非水溶性的藍紫色結晶 formazan。利用 MTT 分析方法檢測 flaccidoxide 、flaccidoxide-13-acetate 對 A2058, RT4, T24, Cal-27, HepG2, HA22T, Hu7, Hacat 及 MS1 等細胞，其抑制細胞生長效果。將 A2058, RT4, T24, Cal-27, HepG2, HA22T, Hu7, Hacat 及 MS1 細胞 ($1x10^5/cm^2$) 培養在 96-well plates 中. 加入不同濃度之 flaccidoxide 及 flaccidoxide-13-acetate 培養 24 小時後， 每一個 well 加入 50 μl MTT solution (1mg/mL in PBS) 並且在 at 37°C 反應 4 小時. 以 200μl DMSO 溶劑將藍紫色結晶溶解後，再利用 ELISA reader 595nm 吸光值來判別細胞存活的多寡，DMSO 做為控制組。

3.4 細胞移動能力及細胞刮傷試驗(Cell migration assay and wound healing assay)

細胞移動試驗，HA22T 細胞培養於 serum-free 培養基中並且將其 seed 在 polycarbonate membranes (8.0 μm, BD Biosciences, CA, USA) in the culture inserts。 HA22T 細胞加入不同濃度之 flaccidoxide 培養 24h 進行細胞 migration 測試，最後移除在上層之 non-migrating 細胞，未於下層 migrated 細胞依序以 100% methanol 固定，並且以 Giemsa (Merck, Germany)染色。 再利用 100X 顯微鏡照相。 另外細胞刮傷試驗，將 HA22T 細胞 seeded in 6-well plates，將細胞長滿後，在每個 well 利用 pipette tip 製造人工之刮傷或傷口，每 3h 觀察細胞癒合程度並以顯微鏡照相。

3.5 蛋白質萃取及蛋白質定量(Protein extraction and estimation)

細胞處理不同濃度之 11-Epi-sinulariolide acetate 後培養 24 小時，並且利用 Cell Extraction Buffer (BioSource International, Camarillo, CA, USA) 並加入 protease inhibitor cocktail (Sigma)進行細胞破碎。以 12000rpm 離心 10 分鐘後，取其上清液，再利用三倍體積之冰冷 10% TCA/Acetone 沉澱蛋白

質於 4°C 過夜。 以 8000rpm 進行離心，將上清液去除，沉澱物以 6 M urea, 2 M thiourea, 0.5% CHAPS, 0.5% IPG buffer, 20 mM DTT, and 0.002% bromophenol blue 緩衝液溶解，二維電泳樣本以 2-D Quant Kit (GE Healthcare)進行蛋白質定量。

3.6 二維電泳分析(Two-dimensional gel electrophoresis)

主要是利用 IEF 電泳將蛋白質以其等電點在膠體上分離，並利用 SDS PAGE 將蛋白質依照其分子量來分離，是目前可以觀察整體性蛋白質的主要方法。我們進行 2- DE 電泳時，每個樣本取 50μg ，利用 GE Healthcare Ettan IPGphor 3 進行分析，並且維持在 20°C，每條 strip 給予 30 A 進行 rehydration。將樣本 loading 到 IPG strip holder 中，設定以下條件進行一維電泳分析: : 200 V (2 h), 500 V (2 h), 1,000 V (2 h), 4,000 V (3 h), 8,000 V (4 h), until the total Vh reached 47,400. 當 IEF 電泳完成後，將 strip 進行 DTT 及 IAA 平衡後，進行 12.5% SDS-PAGE 分析，採用 SE 600 Ruby (Hoeffer)作為 SDS-PAGE 電泳儀器，最後 2-DE 以硝酸銀染色，並且以 PDQUest 2-DE 分析軟體，檢測蛋白質色點之差異。

3.7 利用 LC-MS/MS 進行蛋白質身分鑑定(Protein identification by LC-MS/MS)

膠體內水解(In-gel Digestion). 將二維電泳比對後具有差異性之蛋白質色，利用滅過菌之 tip 在 2-DE 圖譜上將其取下，並放入 microcentrifuge tube 中。 每一個加 tube 中加入 100μL of 50mM DTT in 25mM ammonium bicarbonate (pH 8.5)，並且在 37℃震盪一小時，離心去除上清液，再加入 100μL of 100mM Iodoacetamide (IAA) in 25mM ammonium bicarbonate (pH 8.5) 並且在 37℃且避光中震盪，最後在離心去除上清液，以去除 IAA 之殘留。每個 tube 中加入 0.5μg of trypsin in 50μL 25mM ammonium bicarbonate (pH 8.5)，並且放置於 37 °C 反應 16h，以進行水解作用。待最後收集 peptide solution 並且利用 SpeedVac 乾燥，以進行 LC-MS/MS 分析。

質譜分析(LC-MS/MS analysis). A peptide mixture was separated by nanoflow reversed phase C18 chromatography on nano LC using the Agilent 1200 System and PepMap100 C18, 75μmx15cm (3μm) nanoLC column or HPLC using the Agilent NanoLC 1200 System and Agilent Zobax 2.1mm x150mm C18 column. LC-MS/MS analysis employed a 10 min online trapping and desalting step followed by a 60 min 5-40 % mobile B gradient at nano flow and a 15 min 5-40% mobile B gradient at higher flow (mobile B = 98% ACN, 0.1% formic acid). 利用AB SCIEX QTRAP® 5500Q mass spectrometer (Applied Biosystems,

CA, USA) 進行分析. Mass scan range from m/z 100 to 1000 . The raw data was processed into a text file format of WIFF with Analyst 1.5.1.

3.8 免疫染色分析(Western blotting analysis)

進行免疫染色分析樣本，接經由蛋白質定量後，每一樣本取 20μg 進行分析。 當進行完 SDS-PAGE 後利用 Transphor TE 62 (Hoeffer)在 400mA 進行 1.5 h 電泳轉印,將電泳膠片中之蛋白質轉印 PVDF 膜上。轉印完後之 PVDF 膜取不同之一抗(first antibody)，依照其不同稀釋濃度在 4°C 進行反應過夜，之後利用 PBST (10 mM NaH_2PO_4, 130 mM NaCl, 0.05% Tween 20)將 PVDF 膜清洗三次，每次 10 分鐘，之後加入二抗(with horseradish peroxidase conjugate, 1:5,000 in blocking solution) 反應 1 小時後以 PBST 在清洗三次，最後以冷光呈色劑(ECL Western Blotting Reagents ;Pierce)進行檢測.

3.9 流式細胞儀分析

In order to determine the phase distribution of the cell's DNA content, propidium iodide (PI) staining was performed. HA22T 細胞以不同濃度 flaccidoxide 處理 24 小時，之後收集細胞以 PBS 清洗二次並利用 70% ethanol 固定過夜。細胞以 10 μg/ml PI (Sigma) and 10 μg/ml RNase A in PBS buffer 進行染色，在室溫避光環境中反應 15 分鐘. 實驗細胞以 FACScan flow cytometer (Becton-Dickinson, Mansfield, MA, USA) and Cell-Quest software (Becton-Dickinson) 進行分析.

3.10 粒線體膜電位分析(Mitochondrial transmembrane potential)

利用 1uM Rho123 (Molecular probes, Eugene, OR) for 30 min at 37 進行粒線體膜電位分析. Rho-123 fluorescence 利用 FACStar Plus 流式細胞儀分析且激發光源為 488 and 530 nm 波長。.

（四）結果與討論

4.1 11-Epi-sinulariolide acetate對不同肝癌細胞具有細胞毒殺之作用

首先我們利用不同肝癌細胞，包括HA22T, HepG2, Hep3B, and Huh7來研究11-Epi-sinulariolide acetate是否會造成這些癌細胞產生毒殺作用，我們以不同濃度之11-Epi-sinulariolide acetate (3, 6, 9 μg/mL)處理不同肝癌細胞後24小時，以MTT 分析方式來檢測這些細胞存活率，由結果顯示(3, 6, 9 μg/mL)對於這些肝癌細胞都具有細胞毒殺作用，但是對於HA22T肝癌姓包較為敏感其結果如圖2A所示。由於HA22T細胞對於11-Epi-sinulariolide acetate產生較高之敏感性，當9 μg/mL 11-Epi-sinulariolide acetate處理HA22T 細胞後，其細胞存活率只剩36%，並且細胞開始產生皺縮現象。

另外利用細胞刮傷試驗及垂直式cell migration 分析方式檢測細胞是否在加入11-Epi-sinulariolide acetate後會影響到細胞癒合及轉移能力，由圖3A結果可以發現，隨著11-Epi-sinulariolide acetate濃度增加，細胞癒合能力減慢，另外發現細胞在11-Epi-sinulariolide acetate濃度增加後細胞migration數目減少，其抑制細胞migration數目大約 20%, 40% and 60% for the treatment of 11-EPi-SA at the concentrations of 6, 9 and 12 μg/mL,，結果如圖3B所示。

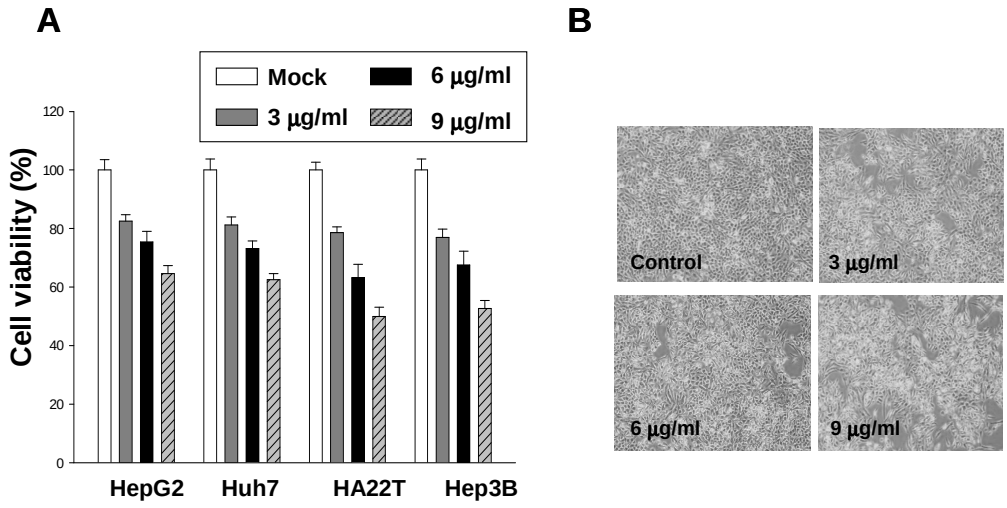

圖 2: 11-Epi-sinulariolide acetate 對不同肝癌細胞具有不同之毒殺效果 (A)以 MTT 分析不同濃度之 11-Epi-sinulariolide acetate 對 HA22T, HepG2, Hep3B, and Huh7 等細胞處理後其之細胞之存活率 (B)不同濃度之 11-Epi-sinulariolide acetate 處理 HA22T 等細胞處理細胞後之細胞型態

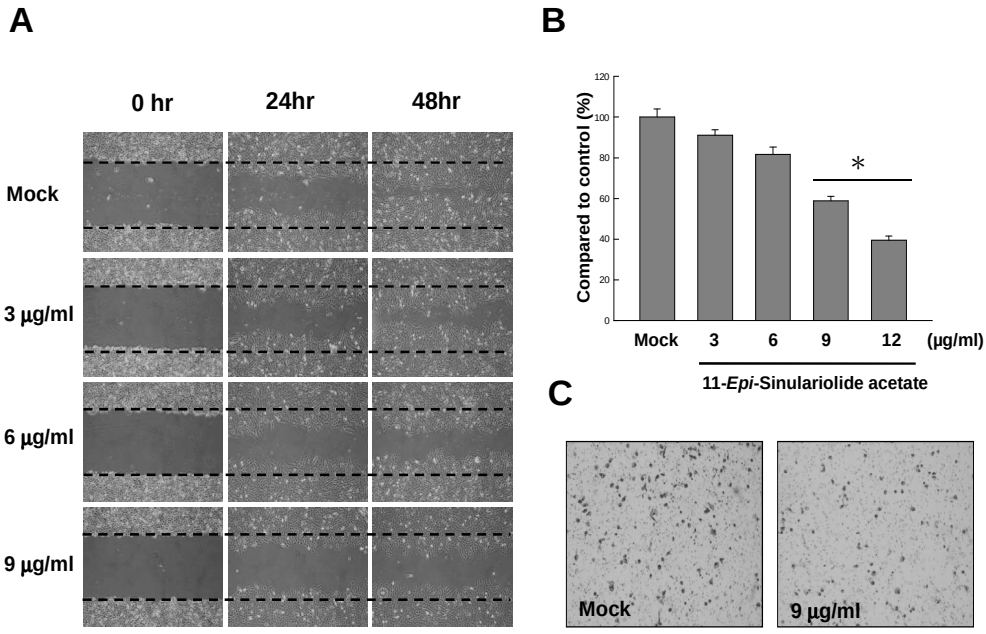

Figure 3. 11-EPi-SA inhibits the cell migration and induces the appearance of apopotic characteristics in the HA22T hepatoma cells. (A) Mock- or 11-EPi-SA-treated HA22T cells in the areas between two solid lines were migrated cells in the gaps during the indicated time periods. Images represented apparent reduction of the HA22T cell migration upon treated with 3, 6 and 9 μg/mL of 11-EPi-SA for 24 h and 48 h, respectively. (B) The quantitative measurement of the cell migration was shown and compared with the cells treated with DMSO. The result showed dose-dependently suppressed HA22T cell migration (* P< 0.001). Data shown here were the representatives of three independent experiments. (C) The enlarged view of migrated HA22T cells were clearly reduced (upon treatment with 9 mg/ml of 11-EPi-SA) compared with cells treated with DMSO at the 100X magnification.

4.2 HA22T細胞經由11-Epi-sinulariolide acetate處理後產生細胞凋亡現象

目前許多從海洋珊瑚中萃取出來之化學分子，如 diterphenoids, diterpenes以及 prostanoids已經被研究發現其具有許多生物活性，包括幼發如前列腺癌細胞、肝癌、乳癌、大腸癌、子宮頸癌以及口腔上皮癌等癌細胞產生細胞凋亡作用及細胞毒殺作用(10-14)。誘發癌細胞產生細胞凋亡作用是目前預測腫瘤反應及抗癌治療中之重要指標，細胞凋亡會造成細胞型態改變、細胞產生皺縮現象、核染色體凝集，細胞核內產生DNA斷裂以及細胞凋亡複合體形成。

由於11-Epi-sinulariolide acetate抑制肝癌細胞生長及是否誘發細胞凋亡之機制仍未被研究過，因此我們利用流式細胞儀以及細胞螢光染色方法，研究11-Epi-sinulariolide acetate是否會造成 HA22T

細胞產生細胞凋亡現象，由目前實驗結果可以發現在不同濃度11-Epi-sinulariolide acetate處理下(3, 6, 9 g/mL)，以Hoechst and Mitotracker followed by monitoring the morphological changes under the microscopy. chromatin condensation and mitochorial swelling so as to detect the Mitotracker.，HA22T細胞在11-Epi-sinulariolide acetate濃度9 g/mL 處理下，，結果如圖6-A 所示。另外利用Annexin V-FITC/PI 染色進行流式細胞儀分析，此種分析方法可以清楚分析三種型態之細胞:分別為凋亡細胞(Annexin V-FITC positive, PI negative)，壞死細胞 (Annexin V-FITC positive, PI positive) 及存活細胞(Annexin V-FITC negative, PI negative). 由圖6-B結果可以發現在處理 10μg/mL濃度後，細胞早期凋亡細胞從9.3%增加至23.7%，由這些結果可以發現11-Epi-sinulariolide acetate確實會造成HA22T細胞產生細胞凋亡現象。

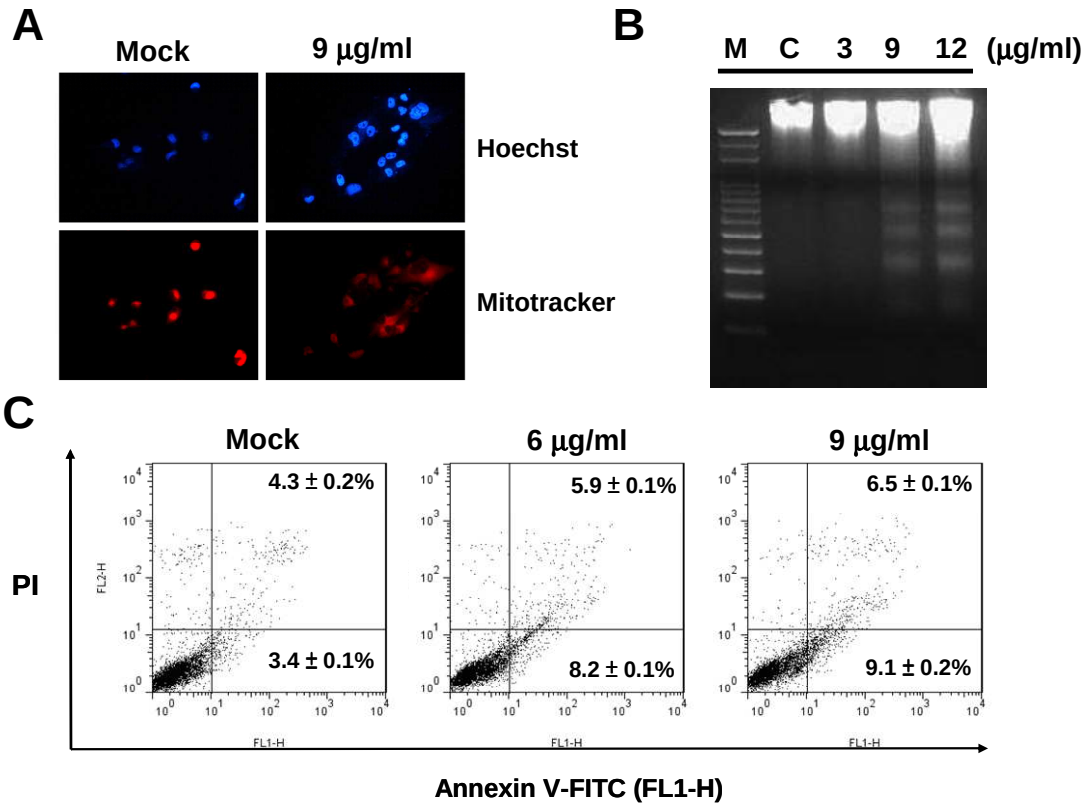

Figure 4. The appearance of apoptotic characteristics in the 11-EPi-SA treated HA22T cells. (A) HA22T cells were treated with DMSO or 11-EPi-SA at the final concentrations of 9.0 μg/mL for 24 h. The cells are then harvested, and fixed with paraformaldehyde for immunofluorescent staining. After permeabilization, the cells were then stained with Mitotracker Red or Hocest and visualized by a fluorescent microscope. (B) Detection of DNA degradation fragments after 11-EPi-SA treatment for 12 and 24 h at indicated

concentrations. (C) Detection of apoptotic HA22T cells after 11-EPi-SA treatment by Annexin V-FITC/PI analysis.

HA22T 細胞經 11-EPi-SA 處理後蛋白質體之研究

從以上之結果可以發現 11-Epi-SA 會誘發 HA22T 細胞產生細胞凋亡現象，如細胞皺縮、DNA 產生片段以及粒線體失活(as shown in Fig 3B to Fig. 4C)。

我們利用蛋白質研究方法進行 HA22T 細胞經 11-Epi-SA 作用後蛋白質體之變化，將實驗分別進行控制組及 11-Epi-SA 處理組，培養 24 小時後破細胞取出其蛋白質，再經由 TCA/Aceton 沉澱後進行二維電泳分析，每一樣本取 50 g，進行 2-DE (pI 4-7)分析，經由硝酸銀染色後(Fig. 5A)，利用 PDQuest image analysis software (Bio-Rad) 分析表現量差異在 1.5 倍以上之蛋白質色點，這些蛋白質色點在經由 in-gel digestion 後，經 LC-MS-MS 鑑定其蛋白質身分，經過鑑定後，總共有 15 個表現量增高蛋白質，及 9 個蛋白質表現量下降 。

第一組蛋白質與 stress response proteins相關, 包括Peroxiredoxin-2, Heat-shock protein 27, Thioredoxin-dependent peroxide reductase, Protein disulfide-isomerase, Protein DJ-1, Calreticulin and 78kDa glucose-regulated protein precursor. 第二組與蛋白質摺疊相關蛋白質，包括Stess-70, 60kDa heat shock protein and T-complex protein 1 subunit zeta. 第三組與細胞訊息傳遞有關之蛋白質包括cytoplasmic protein NCK1, Guanine nucleotide-binding protein subunit beta 2 and Rho GDP-dissociation inhibitor 1. 其他相關蛋白質如mRNA processing proteins, heterogeneous nuclear ribonucleoprotein A/B and H3; cell differentiation protein, vimentin.

To validate the differential expression pattern, which was obtained from the 2-DE maps (Fig. 5A), several identified proteins were investigated by western blotting using anti-prohibitin, CALR, GRP78, Hsp60, stress-70, Hsp27 and PDI specific antibodies, respectively. There were five up-regulated proteins (prohibitin, CAL, GRP78, stress-70 and PDI) and two down-regulated proteins (Hsp60 and Hsp27) observed in the 11-EPi-SA-treated HA22T cells, which is consistent to the exhibited expression pattern from the 2-DE maps (Fig. 5B).

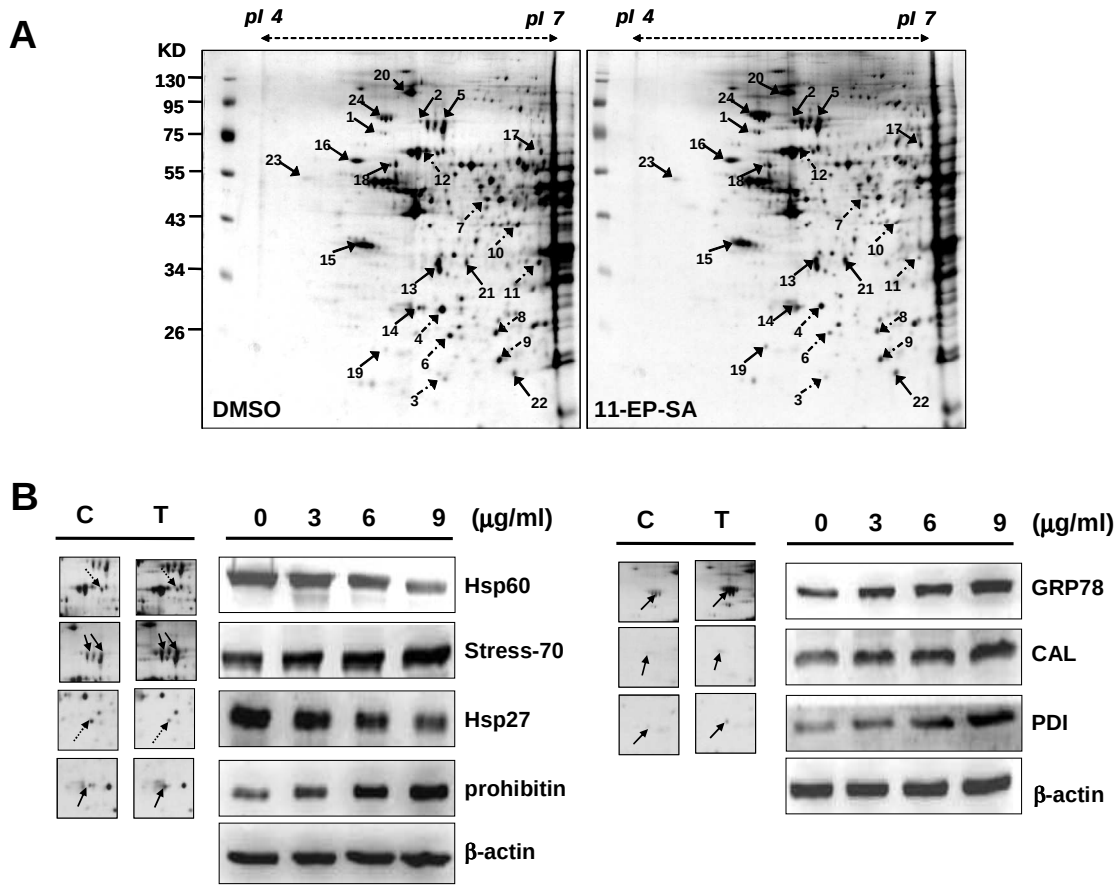

Figure 5. Identification of the differential expressed proteins from mock- and 11-EPi-SA-treated HA22T hepatoma cells by two-dimensional gel electrophoresis (*2-DE*). (A) HA22T cells treated with DMSO (control) or with 11-EPi-SA at the concentration of 9.0μg/mL (treatment) for 24 h, followed by harvesting the cells and cell lysates were prepared as described in the Materials and Methods. A total amount of 100 μg proteins were subjected to 2-DE and proteins visualized by silver staining. PDQuest image analysis software (Bio-Rad) was employed for detecting the differential protein spots. (B) Validation of identified selected proteins from 2-DE. The cell lysates prepared from mock- and 11-EPi-SA-treated cells were subjected to SDS-PAGE for protein separation and the detection of some identified proteins including GRP78, CAL, PDI, Hsp60, Stress-70, Hsp27 and prohibitin, respectively, by western blotting using specific antibodies as indicated. C: control, the DMSO-treated cells; T: 11-EPi-SA treated cells. β-actin was used as the loading control.

11-EPi-SA causes mitochondrial dysfunction

細胞凋亡是已知的細胞程序是死亡。其不僅僅是可以調節細胞之生理過程，並且去除 DNA 傷害避免對於周遭正常細胞場生傷害，並且去除許多有毒之化學物質(Thompson, 1995; Zi and Simoneau, 2005). 檢測粒線體失活是一種細胞凋亡現象，在此研究中利用 JC-1 來檢測粒線體膜電位(ΔΨm)之改變。由實驗結果可以發現在 11-EPi-SA 處理 HA22T 細胞可以發現明顯的降低紅色螢光，並且增加綠色螢光，這些結果可以明顯觀察到處理 11-EPi-SA 會造成細胞膜電位改變。(Fig. 6A & 6B).根據之前研究指出，細胞膜電位改變會造成粒線體失活現象產生 (Imberti et al., 1993). 也有研究指出 It has aristolochic acid (ArA), cyclosporine A (CyA) 及 trifluoperazine (TFZ), 為可以抑制粒線體膜電位改變。在一些肝癌細胞，CyA 加上 TFZ 可以避免粒線體去極化，並且保護並防止粒線體失活。 為了要證實 11-EPi-SA 會誘導 HA22T 細胞產生粒線體膜去極化，進而造成細胞凋亡，在處理 11-EPi-SA 前先加入 trifluoperazine (TFZ), aristolochic acid (ArA), and cyclosporine A (CyA). 其結果如 Fig. 6B, 全不知抑制劑皆可以避免 11-EPi-SA 造成粒線體產生膜電位改變，使細胞存活率上升 (Fig. 6B). 除此之外，許多粒線體失活相關蛋白質，包括 Bax, cytochrome *c* , Bcl-2 及 Bcl-xl 蛋白質，皆利用免疫染色分析法進行驗證(Fig. 6C). 最後抑制劑處理 HA22T 細胞後利用 JC-1 進行染色，並利用螢光顯微鏡檢測其細胞膜之變化，.由結果可以發現這些抑制劑會減低綠色螢光，推測其會抑制粒線體膜改變 (Fig. 6D). 統合這些結果可以得知，11-EPi-SA-會誘導 HA22T 細胞經由粒線體相關途徑產生細胞凋亡現象。

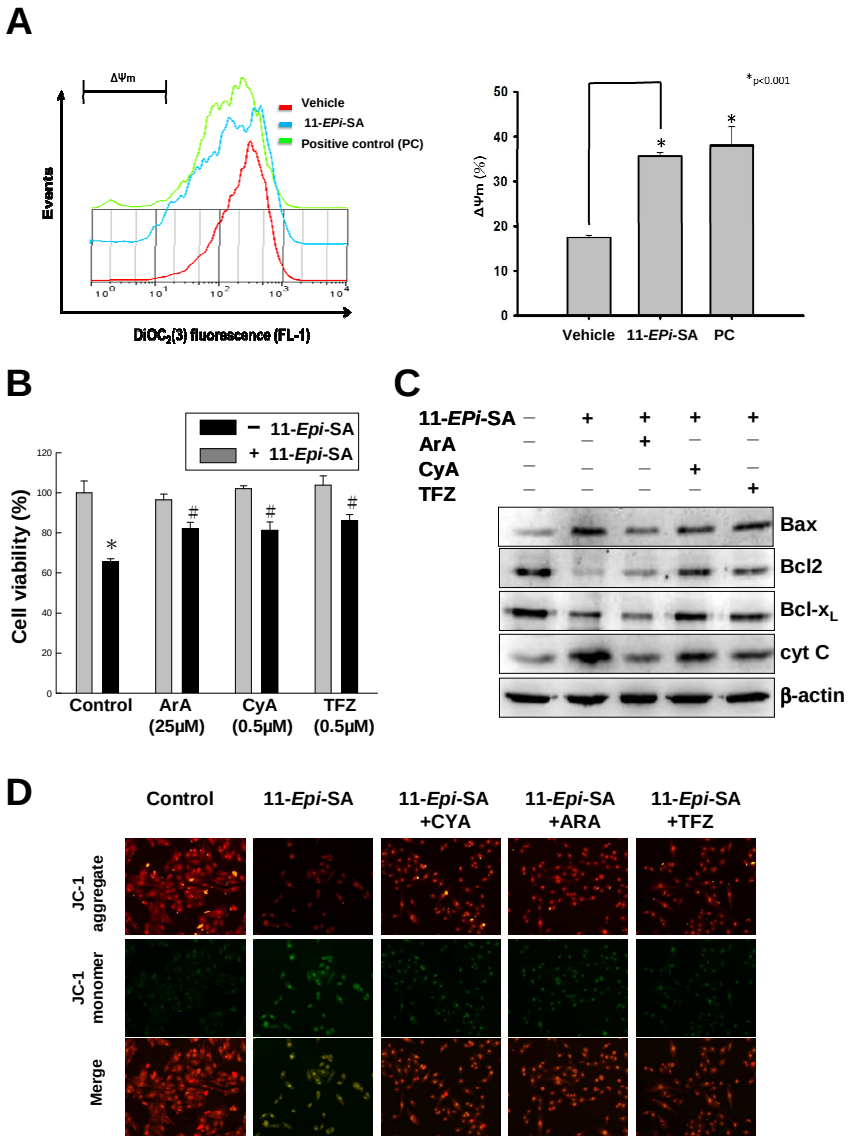

Figure 6. 11-dehydrosinulariolide induced apoptosis through the mitochondrial related pathway. (A) Measurement of the mitochondrial membrane permeability transition (MMP) in the HA22T cells upon 11-EPi-SA treatment. Left panel, the quantitation of mitochondrial membrane potential from mock-, 11-EPi-SA treated and CCCP positive control . (B) Effect of different mitochondrial permeability transition inhibitors on the cell viability upon treatment with 11-EPi-SA. The mock- and 11-EPi-SA treated cells were added with different inhibitors as indicated for 24h, followed by examination the cell viability by MTT assay. The data shown the representative of three independent experiments. (p statistically significant difference compared with the control). (C) Examination of the differentiated expression level of some mitochondrial-related apoptosis pathway proteins response to the different inhibitors as indicated on the 11-EPi-SA treated cells. (D) HA22T cells were treated as indicated, stained with JC-1 dye, incubated with cells for 20 min at 37 °C, 5% CO_2 and imaged under fluorescence microscope at the emission wavelength of

580 nm (red, upper panels, JC-1 aggregate) and 530 nm (green, middle panels, JC-1 monomer).

11-EPi-SA activates the mitochondrial related pathway in dose- and time-dependent manners

caspase-dependent pathway 是一個已知的細胞凋亡相關途徑。Bax 蛋白質, a 為一個 pro-apoptotic 家族蛋白質, 其被認為在粒線體失活所造成之細胞凋亡扮演一個重要角色。實際上, Bax 與 Bcl-2 (anti-apoptotic member) 之比值,會影響到粒細體膜電位改變造成 cytochrome c 釋放,並且造成 caspase 被活化。(Green et al., 2011; Putchala et al., 2013; Seya et al., 2012). 為了得到更詳細之證據來證明 11-EPi-SA 誘導細胞凋亡是藉由 caspase-dependent pathway, 許多粒線體相關蛋白質, 包括 Bax, Bcl2, Bcl-xL, Bad, p-Bad, Bid, PUMA, and p53 進行免疫染色分析. 其結果如圖 7A 所示， Bax, Bad, Bid, PUMA, and p53 表現量會隨 11-EPi-SA 處理濃度增加及時間增長其表現量增加。許多之報告指出粒線體細胞凋亡主要是藉由 Bcl-2 家族所影響(Leanza et al., 2012). BH3-only proteins, 包括 Bad, Bim, Bid, Bik, and PUMA 在 Bax 被活化後其隨即會被活化(Green and Reed, 1998). Bax 活化是藉由 p53 transcription-dependent and/or transcription-independent mechanism (Chipuk et al., 2004). 我們的結果呈現不同之細胞凋亡相關蛋白質在 11-EPi-SA-處理下有不同之表現量，由此可以知道 11-EPi-SA 可以藉由粒線體失活途徑產生細胞凋亡現象。 (Fig 7A).

The caspase-dependent pathway is activated in the 11-EPi-SA-treated HA22T cells

The casepase-9 and caspase-3 蛋白質可以藉由活化粒線體死亡路徑(Bratton and Salvesen, 2010; Reubold and Eschenburg, 2012; Smith and Schnellmann, 2012; Snigdha et al., 2012; Wurstle et al., 2012). 我們利用免疫染色分析來研究 caspase-3 and caspase-9 是否會參與在 11-EPi-SA 誘發之細胞凋亡途徑之中，由結果可以發現在 11-EPi-SA 處理後， pro-caspase-9 及 pro-caspase-3 表現量會隨濃度增加及處理時間增加其表現量減少，而 caspase-9 cleaved polypeptide fragments (37kDa and 35kDa 表現量增加(7B).除此之外，一些研究提及 PARP-1 (116kDa)在細胞凋亡過程中會被 caspase 所切除 (Chaitanya et al., 2010). 我們的研究可以發現 11-EPi-SA 處理後 PARP-1 cleavage fragment (89 kDa) 表現量上升. 綜和這些研究, 這些研究可以發現 mitochondrial-related apoptosis 蛋白質在 11-EPi-SA 處理後會被活化。

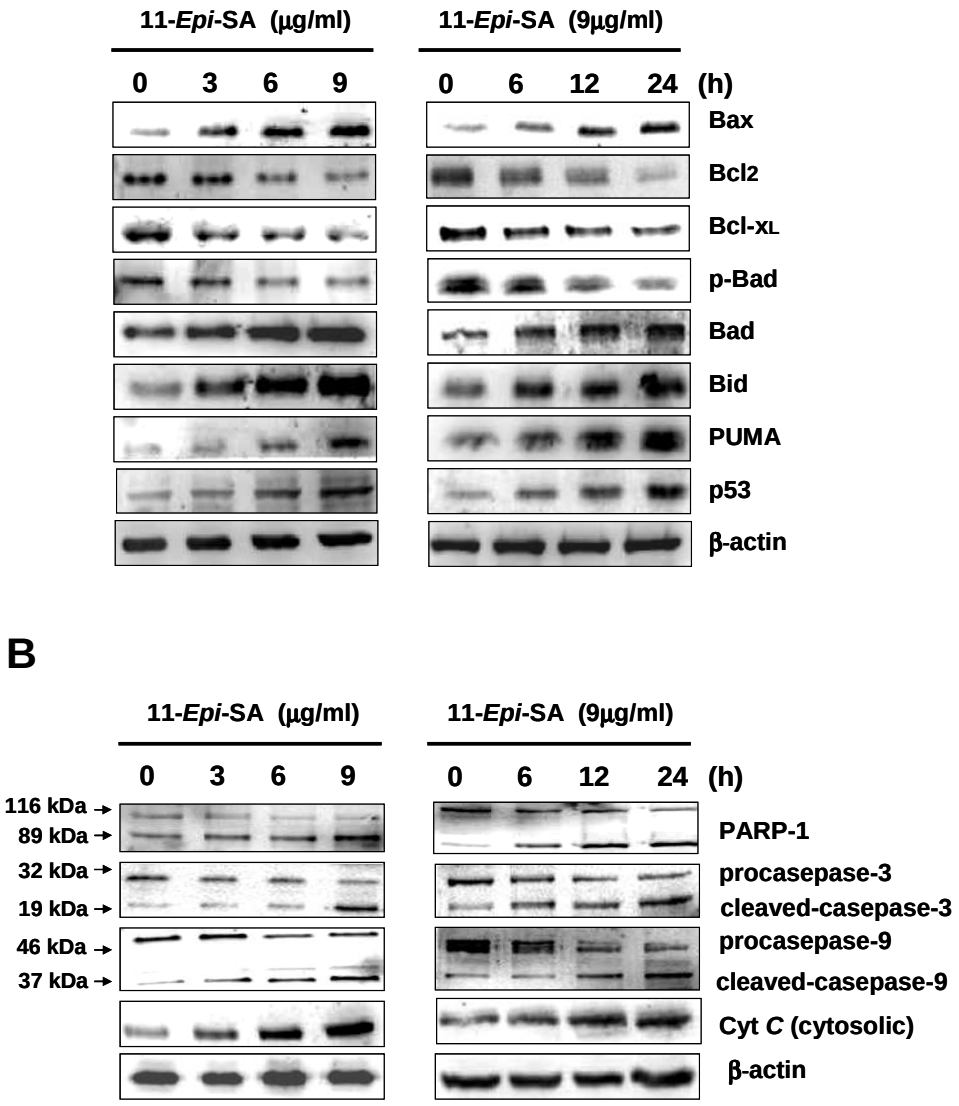

Figure 7. 11-EPi-SA activates the mitochondrial related-apoptotic pathway proteins. (A) HA22T cells were treated without or with 11-EPi-SA at different concentrations (right panel) and different time points (right panel) as indicated. The cells were then harvested for the detection of protein expression level by western blot analysis using the specific antibodies as indicated. β-actin was used as the loading control. (B) Caspase-dependent pathway proteins were activated in the 11-EPi-SA treated cells. The cell lysates were harvested at 11-EPi-SA treatment as indicated above, followed by the examination of PARP-, caspase-3, caspase-9 and cytochrome c proteins using the specific antibodies as indicated. β-actin was used as the loading control.

11-EPi-SA induces the activation of ER stress-related pathway.

11-EPi-SA 影發之細胞凋亡也可能經由ER stress所影發。ER 是主要細胞訊息傳遞之胞器，並且可以偵測及反應細胞目前之狀況，調節細胞平衡。若在ER 中累積太多未摺疊蛋白質，會打亂細胞平衡狀態。在ER中具有三個內質網壓力感應器，包括including unfolded protein response (UPR), ER associated degradation (ERAD) and apoptosis (Kim et al., 2008; Xu et al., 2005). 許多未折疊蛋白質累積於內質網中，the chaperones such as GRP78 參與在蛋白質摺疊，造成釋放出transmembrane proteins PERK, IRE1 ，and ATF6 並且在ER-stress 下活化 UPR. 在此 elicited signaling pathway, UPR can be a demolition response or self-rescuing. 舉例來說，For example, PERK-mediated signaling pathway 可以經由細胞吞噬作用有利於細胞存活(Kouroku et al., 2007) 或是經由提升 ATF4 and pro-apoptotic transcription factor CHOP造成細胞凋亡現象 (Oyadomari and Mori, 2004). 在此研究中ER chaperones: GRP78, GRP94, calreticulin (CAL) and calnexin 在11-EPi-SA 處理後表現量, 會隨著實驗及濃度而增加(Fig. 8A). PERK, ATF6 and IRE1 是屬於 ER-associated sensor proteins, 參與在活化調控GRP78 promoter (Ma and Hendershot, 2004). 在ER stress 下 , phosphorylated PERK 可以phosphorylate eIF2 造成降低轉錄作用及蛋白質合成; IRE1 和 ATF6 可以促進ER chaperone 之表現量(Araki and Nagata, 2011; Saito et al., 2011; Yan et al., 2002).

在目前研究中這些ER stress sensors proteins可以作為驗證ER stress是否參與11-EPi-SA促進細胞凋亡之作用。首先我們發現phosphorylated PERK and eIF2 表現量增加，但是 PERK eIF2 則沒有影響(Fig. 8B). ATF6-f ATF4及 CHOP 在11-EPi-SA 處理後表現量增加(Fig. 8B)由此結果可以推測 PERK signaling transduction pathways 可以活化phospho-eIF2 造成ATF4表現量增加，進而結合到 GRP78 promoter 上，造成GRP78表現量增加 (Lenna et al., 2013; Luo et al., 2003). ATF6 是依個basic leucine zipper protein 可以持續誘導GRP78表現. 在ER stress下， endogenous ATF6 (p90 ATF6) 可以被斷裂成 50 KDa fragment (p50 ATF6) 可以作為transcription factor 進入 nucleus中，進而活化 UPR genes, 包括 GRP78 calreticulin. (Haze et al., 1999). 活化之CHOP 造成down regulate Bcl-2 protein 而且增加Bim 蛋白質表現量(Puthalakath et al., 2007). 由上述之研究可以推測11-EPi-SA 會引發 ER stress 造成 HA22T 細胞死亡.

許多調節蛋白質會參與在細胞凋亡過程中，在此我們研究其他蛋白質是否也參與細胞凋亡途徑中: (1) 在11-EPi-SA 處理後，IRE1a 及 p-ASK1表現量增加; (2) the phosphorylated form of JNK (p-JNK) 及

c-Jun (p-c-Jun) 表現量增加，但是 JNK 及 c-Jun 則沒有改變 (Fig. 8C). The IRE1- protein具有 serine/threonine kinase 及 endoribonuclease功能. 活化IRE1- 可以接附在 TNF receptor-associated factor 2 (TRAF2) and recruit the apoptosis signal-regulating kinase 1 (ASK1). 在許多壓力或是刺激下 ASK1 可以活化c-Jun N-terminal kinase (JNK) 及 p38 ，JNK a及 p38具有多方面功能調節細胞，包括細胞凋亡，細胞分化，細胞增生及細胞轉移之功能 (Wagner and Nebreda, 2009). ASK1也是廣為被接受是一個重要調節 JNK and p38活化調節細胞死亡蛋白質 (Ichijo et al., 1997). 這些結果可以呈現推測至少有二個pathway PERK-ATF6-CHOP 及 IRE1- -JNK-cJun, 參與在11-EPi-SA-誘導之細胞凋亡.

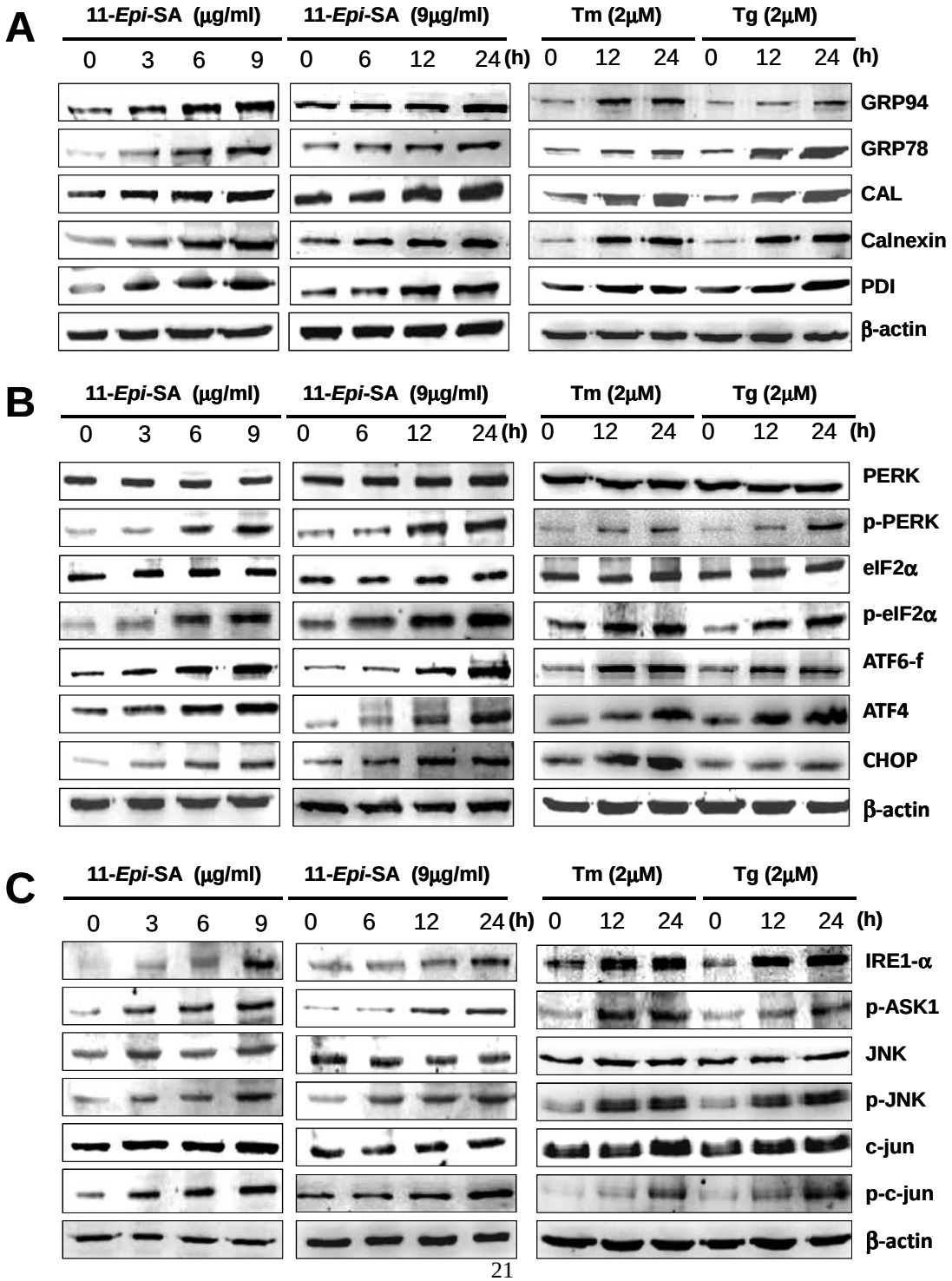

Figure 8. 11-EPi-SA stimulates the ER stress-mediated apoptotic pathway. HA22T cells were treated without or with 11-EPi-SA at different concentrations (right panel) and different time points (middle panel) as indicated, followed by harvesting the cells for the detection of ER stress-related apoptotic pathway proteins as indicated. Please note that, on the right pane, cells treated with either Tm or Tg were considered as the indication of ER stress response.

Inhibition of ER-stress-related pathways rescues the cell cytotoxicity of HA22T cells by 11-EPi-SA.

為了更加證實11-EPi-SA 誘導細胞凋亡式經由ER-stress pathway，我們利用二個不同之抑制劑 (Salubrinal and SP600125) 進行檢測，在11-EPi-SA處理後是否是經由 PERK- and IRE1- 活化細胞凋亡途徑。 由實驗結果可以發現在加入Salubrinal 抑制劑後，細胞存活率從62% 提升至 80%，另外加入 SP600125後細胞存活率從 62% 提升至90% (Fig. 9A). 另外利用TUNEL 螢光分析方式檢測DNA fragmentation ，如結果所示 Fig. 9B,在11-EPi-SA 處理過後之 HA22T 細胞具有有許多Tunel stain. 相反的在加入Salubrinal or SP600125抑制劑後，其Tunel stain 明顯減少，顯示其可以抑制經由, indicating that both of the inhibitors block the PERK- and IRE1- -活化之細胞凋亡途徑。而且在加入saulubrinal 後 p-elF2 , ATF4, ATF6-fragment and CHOP 表現量明顯下降(Fig. 9C, left panel). 相同之現象在加入 SP600125 抑制劑後， p-c-Jun 表現量明顯下降(Fig. 9C, right panel). Salubrinal為 ER stress 抑制劑，可以抑制eIF2 磷酸化並且保護細胞抵抗ER stress, SP600125為 JNK-specific抑制劑可以阻斷 IRE1- -JNK-cJun pathway 避免細胞凋亡產生，由結果可知11-EPi-SA 在HA22T 細胞產生細胞凋亡現象是經由PERK-ATF6-CHOP or the IRE1- -JNK-cJun pathways.

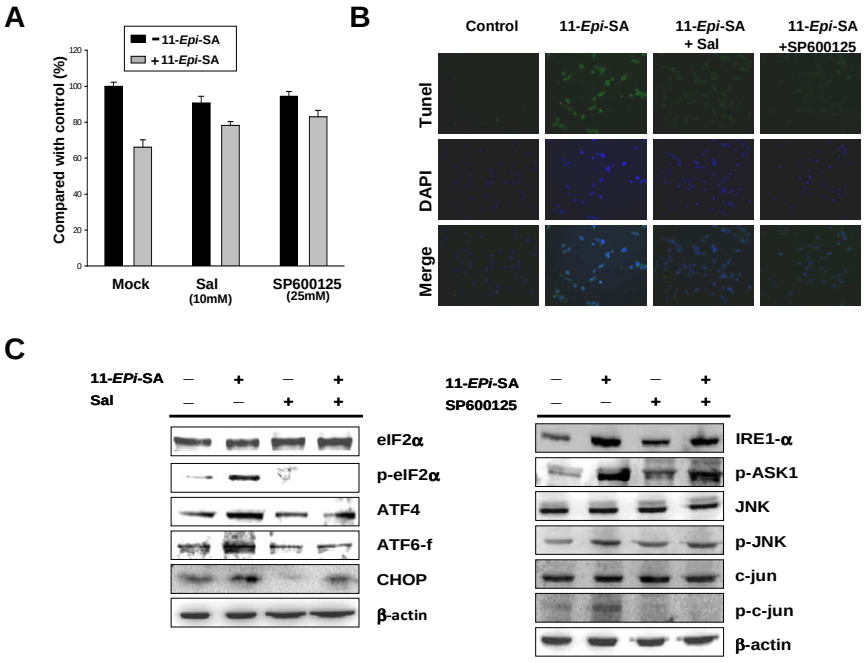

Figure 9. 11-EPi-SA induces HA22T cell apoptosis through two distinct pathways. (A) Two inhibitors, Salubrinal and SP600125, rescue the cell cytotoxicity on the HA22T cells by 11-EPi-SA. (B) The decrease of 11-EPi-SA-induced apoptotic signaling cascades as assessed by the Tunel staining method. (C) Validation of altered protein expression caused by two inhibitors. HA22T cells were treated with 11-EPi-SA in the presence or absence of Salubrinal (left panel) or SP600125 (right panel) for 24h, followed by harvesting the cell lysates for the detection of the ER stress mediated pathways proteins as indicated. Western blotting was performed by standard procedure as described elsewhere using the specific antibodies as indicated.

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附件二

國科會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：並將此研發成果發表於國際期刊，符合原計畫設定之預期目標。

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）

目前利用此研究計畫經費已經研發成果已有 **6** 篇發表在 SCI 學術期刊、**1** 篇正在審稿中。

已接受 paper

1.Neoh CA*, Wang YL*, Din ZH, Su JH, Chen YK, Tsai FJ, Weng SH and **Wu YJ** (2012) Induction of apoptosis by sinulariolide from the Soft Coral through mitochondrial-related and p38 MAPK pathway on human bladder carcinoma cells. ***Marine drugs*** (10), 2893-2911 (SCI: 3.978) **(Correspondence Author)**

2. Tsai TC *, **Wu YJ** *, Su JH, Lin WT, Lin YS (2013) A New Spatane Diterpenoid from the Cultured Soft Coral Sinularia leptoclados. ***Marine drugs*** (11), 114-123 (SCI:3.978) **(First Author)**

3. Su CC, Wong BS, Chih C, **Wu YJ***, Su JH* (2013) Oxygenated Cembranoids from the Soft Coral Sinularia flexibilis. ***International journal of molecular sciences*** **(14) 4317-4325** (SCI:2.464) **(Correspondence Author)**

4. Li HH, Su JH*, Chiu CC*, Lin JJ, Yang ZY, Hwang WI, Chen YK, Lo YH, **Wu YJ.** Proteomic investigation of the sinulariolide-treated melanoma cells A375: effects on the cell apoptosis through mitochondrial-relate pathway and activation of caspase cascade. ***Marine drugs***. (2013) ***Marine drugs*** **(**11), 2625-2642; **(**SCI:3.978**)** (Correspondence Author)

5.Chen YJ, Su JH*, Tsao CY*, Hung CT, Chao HH, Lin JJ, Liao MH, Yang ZY, Hung HH, Tsai FJ, Weng SH, **Wu YJ (2013**) Sinulariolide induced hepatocellullar carcinoma apoptosis through activation of mitochondrial-related apoptotic and PERK/eIF2α/ATF4/CHOP pathway. ***Molecules*** (18), 10146-10161 (SCI:2.48, accepted) **(Correspondence Author)**

投稿中 paper

Jen-Jie Lin[1], Robert YL Wang [2], Jui-Hsin Su[3], Jiing-Chuan Chen[4], Chien-Chih Chiu[5], Chi-Ruei Huang [2], Ming-Hui Liao[1†], Yu-Jen Wu[5†] (2013)11-*Epi*-sinulariolide acetate isolated from cultured soft corals exerts cytotoxicity on HA22T cells through endoplasmic reticulum stress pathway mitochondria dysfunction. (submitted BMC cancer journal) .

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）

(1).學術成就：

在此研究計畫支持下我們發現 11-EPi-sinulariolide acetate 確實具有誘導肝癌細胞產生細胞凋亡之潛力，未來將利用此化合物進行動物實驗，觀察此化合物可以抑制動物腫瘤之生長，未來將有助於開發一個新的海洋抗腫瘤化合物。

附件三

國科會補助計畫衍生研發成果推廣資料表

日期：___年__月__日

<table>
<tr><td>國科會補助計畫</td><td colspan="3">計畫名稱：
計畫主持人：
計畫編號：　　領域：</td></tr>
<tr><td rowspan="2">研發成果名稱</td><td colspan="3">（中文）</td></tr>
<tr><td colspan="3">（英文）</td></tr>
<tr><td>成果歸屬機構</td><td></td><td>發明人
（創作人）</td><td></td></tr>
<tr><td rowspan="2">技術說明</td><td colspan="3">（中文）

（200-500 字）</td></tr>
<tr><td colspan="3">（英文）</td></tr>
<tr><td>產業別</td><td colspan="3"></td></tr>
<tr><td>技術/產品應用範圍</td><td colspan="3"></td></tr>
<tr><td>技術移轉可行性及預期效益</td><td colspan="3"></td></tr>
</table>

註：**本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。**

附件四

國科會補助專題研究計畫項下出席國際學術會議心得報告

日期：___年___月___日

計畫編號	NSC　　—　　—　　—　　—　　—		
計畫名稱			
出國人員 姓名		服務機構 及職稱	
會議時間	年　月　日至 年　月　日	會議地點	
會議名稱	(中文) (英文)		
發表論文 題目	(中文) (英文)		

一、參加會議經過

二、與會心得

三、考察參觀活動(無是項活動者略)

四、建議

五、攜回資料名稱及內容

六、其他

附件五

國科會補助專題研究計畫項下赴國外(或大陸地區)出差或研習心得報告

日期：___年___月___日

計畫編號	NSC　　—　　—　　—　　—　　—		
計畫名稱			
出國人員 姓名		服務機構 及職稱	
出國時間	年　月　日至 年　月　日	出國地點	

一、國外(大陸)研究過程

二、研究成果

三、建議

四、其他

附件六

國科會補助專題研究計畫項下國際合作研究計畫國外研究報告

日期：___年___月___日

計畫編號	NSC　　—　　—　　—　　—　　—		
計畫名稱			
出國人員 姓名		服務機構 及職稱	
合作國家		合作機構	
出國時間	年　月　日至 年　月　日	出國地點	

一、國際合作研究過程

二、研究成果

三、建議

四、其他