EFFECTS OF ECTEINASCIDIN 770 ON LUNG CANCER NCI-H23 CELL ANOIKIS

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ผลของเอคเตนาสซิดิน 770 ต่ออะนอยคิสของเซลล์มะเร็งปอดชนิดเอ็นซีไอ–เอช 23

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การยับยั้งการแพร่กระจายของเซลล์มะเร็งไปยังบริเวณอื่นของร่างกายนั้นได้รับความสนใจเพิ่มขึ้นในงานวิจัยและงานทางด้านคลินิกที่เกี่ยวข้องกับโรคมะเร็ง ในงานวิจัยนี้ได้นำสารเอคเตนาส - ซิดิน 770 (ET-770) ซึ่งเป็นสารบริสุทธิ์ที่สกัดได้จากเพรียงหัวหอมชนิด *Ecteinascidia thurstoni* นำมาศึกษาผลของการกระตุ้นเซลล์มะเร็งปอดชนิดไม่เล็ก (H23) ให้มีความไวต่ออะนอยคิส โดยการวิเคราะห์ด้วยวิธี XTT และการวิเคราะห์ระดับของโปรตีนที่เกี่ยวข้องกับกระบวนการอะนอยคิสด้วยวิธี western blot ผลการทดลองพบว่า ET-770 สามารถเพิ่มความไวต่ออะนอยคิสในเซลล์มะเร็งปอดชนิด H23 ที่แปรผันตามความเข้มข้น โดยผ่านการกระตุ้นการทำงานของโปรตีน p53 ซึ่งมีผลทำให้ระดับของโปรตีน MCL1 ลดลง และกระตุ้นให้โปรตีน BAX ทำงานมากขึ้น แต่อย่างไรก็ตามระดับของโปรตีน BCL2 และ CAV1 ไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญ นอกจากนี้ยังพบว่าสาร ET-770 เพิ่มความไวต่ออะนอยคิสในเซลล์มะเร็งปอดชนิด H460 ได้เช่นเดียวกัน ดังนั้นสาร ET-770 นี้จึงเหมาะสมที่จะได้รับการศึกษาเพิ่มเติมและพัฒนาเป็นยารักษาโรคมะเร็งต่อไปในอนาคต

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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PHATTRAKORN POWAN: EFFECTS OF ECTEINASCIDIN 770 ON LUNG CANCER NCI-H23 CELL ANOIKIS. ADVISOR: ASST. PROF. PITHI CHANVORACHOTE, PH.D., CO-ADVISOR: KHANIT SUWANBORIRUX, PH.D., 75 pp.

Anti-metastasis have received increased research effort and clinical attention. The anoikis-sensitizing effect of ecteinascidin 770 (ET-770) was investigated in the present study in non-small cell lung cancer cells. ET-770 isolated from *Ecteinascidia thurstoni* was tested for its anoikis-sensitizing effect on H23 human lung cancer cell by XTT assay. The levels of proteins involving in anoikis of cells were determined by western blot analysis. ET-770 was shown to enhance anoikis response of human lung cancer H23 cell in a dose-dependent manner. The underlying mechanism was investigated and it was found that ET-770 sensitized the cells by activating p53 protein, which in turn down-regulated anti-apoptotic MCL1 and up-regulated BAX proteins. However, BCL2 and CAV1 proteins were not significantly affected by ET-770. Furthermore, the anoikis sensitization of ET-770 was observed in H460 lung cancer cell. The results reveal for the first time that ET-770 can sensitize anoikis through the p53 pathway and further development of this compound for therapeutic use is warranted.

Department : Pharmacology and Physiology Student's Signature .....

Field of Study : Pharmacology Advisor's Signature .....

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LIST OF ABBERVIATIONS

%	= percent
°C	= degree Celsius
Apaf-1	= apoptosis protease activating factor-1
Akt	= protein kinase B
BAD	= BCL2–associated death promoter
BAK	= BCL2 homologous antagonist/killer
BAX	= BCL2–associated X
BC	= breast cancer cells
BCL2	= B–cell lymphoma 2
BCL-xL	= B–cell lymphoma-extra large
BCL-W	= Bcl-2-like protein 2
BID	= BH3 interacting domain death agonist
BIK	= BCL2 interacting killer
BIM	= BCL2–like protein 11
BMF	= BCL2 modifying factor
BOK	= BCL2– related ovarian killer
CAS	= caspase
CAV1	= caveolin–1
Cyto C	= cytochrome *c*
QG56	= non-small lung cancer cell line: SCC
CO_2	= carbon dioxide
DMSO	= dimethyl sulfoxide
DU145	= prostate carcinoma

ECM	= extracellular matrix
ED_{50}	= effective dose for 50% of the population exposed to the drug
EGF	= epidermal growth factor receptor
eNOS	= endothelial nitric oxide synthase
ERK	= extracellular signal-regulated kinase
ET-743	= ecteinascidin 743
ET-770	= ecteinascidin 770
et al.	= et alibi, and others
EtOH	= ethanol
g	= gram
h	= hour(s)
H23, NCI-H23	= non-small lung cancer cell line: Adenocarcinoma
H 460, NCI-H 460	= non-small lung cancer cell line
HCT116	= human colon carcinoma cells
HRK	= hara-kiri
IC_{50}	= the half maximal inhibitory concentration
IGF	= insulin-like growth factor
KB	= human epidermoid carcinoma cells of the narsopharynx
KCN	= potassium cyanide
LCC	= large-cell lung cancer
MAPK	= mitogen-activated protein kinases
MCL1	= myeloid cell leukemia sequence 1
min	= minute(s)
ml	= milliliter
mM	= millimolar
nM	= nanomolar

NSCLC	= non-small cell lung cancer
NOXA or PMAIP1	= phorbol-12-myristate-13-acetate-induced protein 1
p53	= tumor suppressor protein 53
PI	= propidium iodide
PKC	= protein kinase C
PUMA	= p53 upregulated modulator of apoptosis
RPMI	= Roswell Park Memorial Institue's medium
SCC	= squamous cell carcinoma
SCLC	= small cell lung cancer
S.D.	= standard deviation
SMAC/ DIABLO	= second mitochondrial derived activator of caspases, also called DIABLO (direct IAP-binding protein with low pI)
XIAP	= X–linked lnhibitor of apoptosis
XTT	= 2, 3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt

CHAPTER I

INTRODUCTION

Background and Rationale

Lung cancer is the major cause of cancer-related mortality throughout the world. The major type of lung cancer found in patients is non-small cell lung cancer (NSCLC), which has been classified as the malignant tumor with metastasis at advanced stage by the time of diagnosis. Metastasis in patients with lung cancer has become an important hallmark of difficulty in treatment and poor prognosis because metastatic tumors cannot be completely cured by surgery and frequently exhibit resistance to anticancer drugs (Martini *et al*., 1995; Hanahan and Weinberg, 2000; Jemal *et al*., 2010).

Nowadays, anti-metastasis therapy has garnered increasing attention in cancer research field (Dollé *et al*., 2006; Stafford *et al*., 2008). The most important event during cancer metastasis is associated with the process of cancer cells escaping from apoptosis induced by cell detachment. Detachment-induced apoptosis or anoikis (Frisch and Francis, 1994) is the biological process of cells to prevent cancer cells metastasis. Therefore, survival cancer cells possessing anoikis resistance can spread from their primary sites to secondary sites (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997; Glinskii *et al.*, 2003).

Anoikis are regulated by many signaling pathways; however, intrinsic apoptosis or mitochondrial apoptosis pathway seems to be the primary machinery

which is responsible for cell anoikis and most evidences indicate proteins in the B-cell lymphoma 2 (BCL2) family as being key players (Igney and Krammer, 2002; Chiarugi and Giannoni, 2008; Simpson *et al*., 2008; Kim *et al*., 2012). Numerous studies have shown that resistance to anoikis in certain types of cancers, including lung cancer, is due to the increase of antiapoptotic proteins (Igney and Krammer, 2002; Song *et al*., 2005; Chunhacha *et al*., 2012). Among various antiapoptotic proteins in the BCL2 family, myeloid cell leukemia sequence 1 (MCL1) has garnered most attention and is believed to have a dominant effect on anoikis resistance of lung cancer cells (Song *et al*., 2005; Zhang *et al*., 2011; Chunhacha *et al*., 2012). Recently, we have shown that the overexpression of MCL1 suppressed the anoikis response in H460 human lung cancer cells, while shRNA-mediated down-regulation of this protein had an anoikis-sensitizing effect (Chunhacha *et al*., 2012). Defects in p53 activation, as well as a decrease of cellular pro-apoptotic proteins, including BCL2–associated X protein (BAX), have been shown to be involved in anoikis resistance (Lowe *et al*., 1994; Basu and haldar, 1998; Igney and Krammer, 2002; Haupt *et al*., 2003; Zhang *et al*., 2004; Hemann and Lowe, 2005; Ravid *et al*., 2005; Derksen *et al*., 2006; Jiang et al., 2010).

Moreover, CAV1, a major structural protein of caveolae in plasma membrane, plays a key role in the mediation of multiple cellular processes such as molecular transport, cell adhesion and signal transduction. CAV1 expression was shown to regulate drug resistance, confer poor prognosis and anoikis resistance (Ravid *et al*., 2005; Luanpitpong *et al*., 2010; Thompson *et al*., 2010). Taken together, agent that can enhance p53 function and/or deplete the level of proteins mediating anoikis

resistance such as BCL2, MCL1 and CAV1, may be able to induce or sensitize anoikis which are good candidate for anti-metastasis therapy.

Marine organism-derived compounds have been accepted for cancer treatment. Ecteinascidin 743 (ET-743, Yondelis^{TM}, Trabectedin (Zeltia and Johnson & Johnson)), a tetrahydroisoquinoline marine natural product, has been approved by the European Union for use in humans with soft tissue sarcoma (D'Incalci and Galmarini, 2010). Renieramycin M, another tetrahydroisoquinoline isolated from a blue sponge, *Xestospongia* sp., also possesses an ability to overcome anoikis resistance (Halim *et al*., 2011). Interestingly, ecteinascidin 770 (ET-770) is a modified product was isolated from a tunicate *Ecteinascidia thurstoni* by the KCN pretreatment. It has been also reported to possess cytotoxicity with IC_{50} values in the range of nanomolar concentrations against three human solid tumor cell lines which are HCT116 colon carcinoma, QG56 lung carcinoma, and DU145 prostate carcinoma (Saktrakulkla *et al*., 2011). As ongoing research for testing potential compounds from marine organisms for anticancer approaches, ET-770 will be investigated for its anoikis-sensitizing effect and elucidated the underlying mechanism of action of the compound focusing on the level of proteins mediating anoikis machinery of cancer cells in the present study.

Research Questions

1. Can ecteinascidin 770 (ET-770) sensitize anoikis of NSCLC NCI-H 23 cells?
2. What is the underlying mechanism of action of ET-770 on NCI-H 23 cell anoikis?

Hypothesis

ET-770 sensitizes anoikis of lung cancer NCI-H 23 cells by alteration of pro/anti-apoptotic proteins that involve in anoikis pathway.

Objectives

1. To investigate the effect of ET-770 on NSCLC NCI-H 23 cells anoikis.
2. To investigate the mechanism of action of ET-770 on NSCLC NCI-H 23 cells anoikis that may involve pro/anti-apoptotic proteins.

Expected benefits

Knowledge gained from the present study regarding anti-metastasis potential of ET-770 will benefit the development of this compound to be used in treatment to lung cancer and metastasis prevention therapy.

CHAPTER II

LITERATURE REVIEW

1. Lung Cancer

Lung cancer is the main cause of death throughout the world. In 2012, the incidence and mortality rates of the United States were both estimated 14% of all new cases diagnosed and 28% of all deaths due to lung cancer (Figure 1). The evidences indicated that a crucial risk factor of lung cancer is smoking (Barbone *et al*., 1997; Siegel *et al*., 2012).

Estimated New Cases*

Males				Females		
Prostate	241,740	29%		Breast	226,870	29%
Lung & bronchus	116,470	14%		Lung & bronchus	109,690	14%
Colon & rectum	73,420	9%		Colon & rectum	70,040	9%
Urinary bladder	55,600	7%		Uterine corpus	47,130	6%
Melanoma of the skin	44,250	5%		Thyroid	43,210	5%
Kidney & renal pelvis	40,250	5%		Melanoma of the skin	32,000	4%
Non-Hodgkin lymphoma	38,160	4%		Non-Hodgkin lymphoma	31,970	4%
Oral cavity & pharynx	28,540	3%		Kidney & renal pelvis	24,520	3%
Leukemia	26,830	3%		Ovary	22,280	3%
Pancreas	22,090	3%		Pancreas	21,830	3%
All Sites	**848,170**	**100%**		**All Sites**	**790,740**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	87,750	29%		Lung & bronchus	72,590	26%
Prostate	28,170	9%		Breast	39,510	14%
Colon & rectum	26,470	9%		Colon & rectum	25,220	9%
Pancreas	18,850	6%		Pancreas	18,540	7%
Liver & intrahepatic bile duct	13,980	5%		Ovary	15,500	6%
Leukemia	13,500	4%		Leukemia	10,040	4%
Esophagus	12,040	4%		Non-Hodgkin lymphoma	8,620	3%
Urinary bladder	10,510	3%		Uterine Corpus	8,010	3%
Non-Hodgkin lymphoma	10,320	3%		Liver & intrahepatic bile duct	6,570	2%
Kidney & renal pelvis	8,650	3%		Brain & other nervous system	5,980	2%
All Sites	**301,820**	**100%**		**All Sites**	**275,370**	**100%**

Figure 1 Estimated new cancer cases and deaths by Sex (Siegel *et al*., 2012)

2. Types of lung cancer

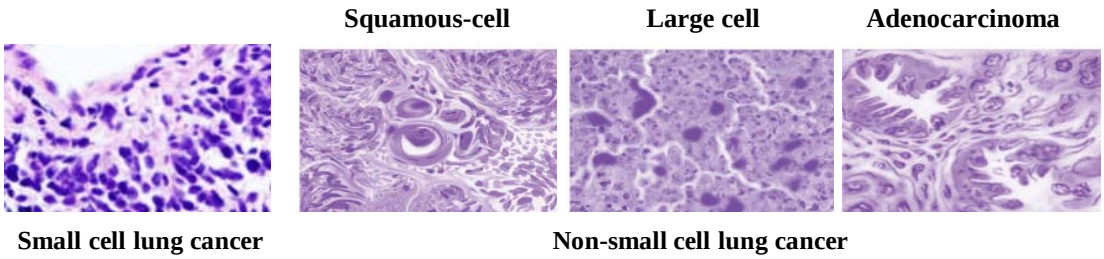


Figure 2 Microscopic views of lung cancers (www.nucleusinc.com)

Lung cancer is happened from normal lung cells continue genetic damage leading to uncontrolled cell proliferation and resulting in abnormal epithelial cells in airways of the lungs. Lung cancer can be divided into two main types based on histological characteristics under the light microscope (Figure 2): small cell lung cancer and non-small cell lung cancer, and they are treated differently (Spira and Ettinger, 2004).

2.1 Small cell lung cancer (SCLC)

The epithelial cells of SCLCs are smaller than those of lung normal cells. Cells are oat gain shaped and sometimes spindle-shaped or polygonal. It is strongly associated with a history of tobacco smoking. SCLCs are frequently found in the large airways of the lungs near the center.

2.2 Non-small cell lung cancer (NSCLC)

Non-small cell lung cancer is the majority type of lung cancer patients. NSCLC can be separated into three main subtypes: squamous-cell carcinoma,

large-cell lung cancer, and adrenocarcinoma. NSCLC are grouped together because the pattern of cell growth and maintenance are similarly.

2.2.1.1 Squamous cell carcinoma (SCC) is also called epidermoid carcinoma. This carcinoma has abnormally large and flat squamous cells. SCC often generates a substance known as keratin, which can be seen under a microscope**.** The**y** are most commonly found in men who have history of tobacco smoking.

2.2.1.2 Large-cell lung cancer (LCC) is the largest size cells of the different types of NSCLC. It is highly undiffentiated in appearance. LCC can be found in any part of lungs. Some experts believe that LCC is an immature form of adrenocarcinoma or sqaumous cell carcinoma. These tumors have poor prognosis more than other forms of NSCLC.

2.2.1.3 Adenocarcinoma is the most general types of lung cancer, accounting for approximately 40%. It occurs in women and people who have never smoked, as well as common form that associated with scarring of the lung tissue.

Most patients of either SCLC or NSCLC, have metastasis at the time of diagnosis. SCLC generally grows faster than NSCLC, however, SCLC is more responsive to chemotherapy and radiotherapy than NSCLC. We are thus focusing on NSCLC, NCI-H23 and NCI-H460 cells were chosen for represent adenocarcinoma and large cell carcinoma, respectively, in this study.

3. Stages of lung cancer

In patients with non-small cell lung cancer are classified a progression of disease by using TNM staging system (Table 1), which helps the doctor to plan an appropriate treatment. The TNM staging system is based on the size of the primary tumor (T), whether cancer cells have spreaded to nearby lymph nodes (N), and whether distant to other parts of the body termed, metastasis (M) has occurred (Table 2). The numbers of T, M, or N show the severity of the disease as follows (Spira and Ettinger, 2004).

Table 1 TNM stages of non-small cell lung cancer (Spira and Ettinger, 2004)

Non-small cell lung cancer					
Stage	**Tumor**	**Node**	**Metastasis**	**Survival Rate**	
				1 Yr	**5 Yr**
Local					
IA	T1	N0	M0	94	67
IB	T2	N0	M0	87	57
IIA	T1	N1	M0	89	55
Locally advanced					
IIB	T2	N1	M0	73	39
	T3	N0	M0		
IIA	T1	N2	M0	64	23
	T2	N2	M0		
	T3	N1	M0		
	T3	N2	M0		
IIIB	Any T	N3	M0	32	3
Advanced					
IIIB	T4	Any N	M0	37	7
IV	Any T	Any N	M1	20	1

Table 2 TMN description

TMN system description		
Primary tumor size(T)	Lymph node (N)	Metastasis (M)
T0: No evidence of primary tumor T1: Tumor that is less than 3 cm (1.5 inches) in size and is completesly surrounded by lung tissue T2: Tumor that is larger than 3 cm (1.5 inches) but is still surrounded by lung tissue and is not invading the chest wall or any of the structures in the mediastinum T3: Tumor of any size that invade the chest wall, diapharm, or the pleural of the mediastinum or heart; a T3 cancer is potentially respectable (surgically removable) T4: A tumor of any size that invade the structure of the mediastinum or the vertebral body (a backbone)	N0: No evidence of cancer in the regional lymph nodes N1: Cancer in the ipsilateral hilar lymph nodes N2: Cancer in the ipsilateral mediastinal lymph nodes N3: Cancer in the contralateral lymph nodes or in the supraclavicular area	M0: No distance metastasis found M1: Distance metastasis is present

4. Treatments of lung cancer

Normally, the standards of lung cancer therapies are close to several types of cancers. Surgery is chosen as the primary in treatment of solid tumors with or without combination therapies, including chemotherapy, radiotherapy, and targeted therapy based on the type and stage of lung cancer (Tables 1-3). However, the treatments and survival rate have dramatically dropped, because lung cancer is usually found with metastatic tumors at advanced stage by the time of diagnosis.

Since the metastatic tumor, in lung cancer patients, could not be completely cured by surgery and is frequently resistant to anticancer drugs (Freise *et al*., 1978; Spira and Ettinger, 2004; Tsuya *et al*., 2007; Komatsu *et al*., 2012), metastasis has become an important hallmark of difficulty in treatment and poor prognosis (Hanahan and Weinberg, 2000; Jemal *et al*., 2010). Because the spreading of cancer cells to secondary sites is a cause of proximately 90% of human cancer deaths, it has been well accepted that metastasis is the dominant cause of death. Nowadays, anti-metastasis therapy has thus garnered increasing attention in cancer research field (Dollé *et al*., 2006; Stafford *et al*., 2008).

Table 3 Regimen of non-small cell lung cancer treatment (Spira and Ettinger, 2004)

Stage	Primry treatment	Adjuvant therapy	Outcome
Non-small cell lung cancer			
I	Surgical resection	Chemotherapy	5-Yr survival rate, >60-70%
II	Surgical resection	Chemotherapy, with or without radiotherapy	5-Yr survival rate, >40-50%
IIIA (resectable)	Preoperative chemotherapy followed by surgical resection (preferable) or surgical resection	Radiotherapy with chemotherapy (if not given previously) or without chemotherapy	5-Yr survival rate, >15-30%
IIIA (unresectable) or IIIB (involvement of contralateral or supraclavicular lymph nodes	Chemotherapy plus concurrent radiotherapy (preferable) or chemotherapy followed by radiotherapy	None	5-Yr survival rate, >10-20%
IIIB (pleural effusion) or IV	Chemotherapy with 2 agent for 3 or 4 cycles (preferable)	None	Median survival, 8-10 mo 1-Yr survival rate, 30-35% 2-Yr survival rate, 10-15%
	Surgical resection of solitary brain metastasis and surgical resection of primary (T1) lesion		5-Yr survival rate, 10-15%

5. Metastasis

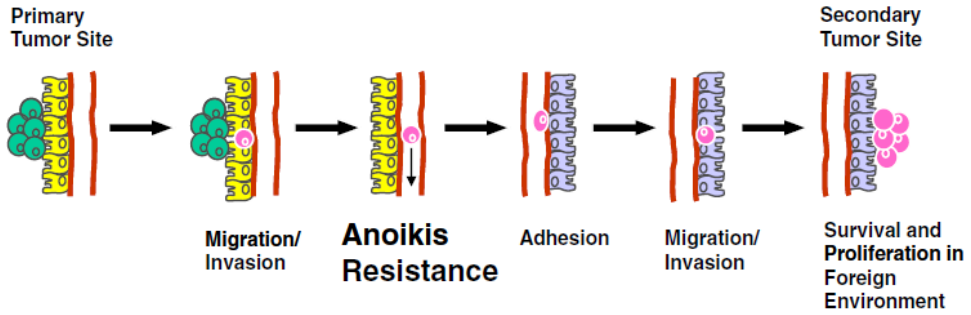


Figure 3 Step in metastasis (Simpson *et al.*, 2008)

Metastasis is the spreading of cancer disease from the primary site to the other sites. The first step, tumor cells acquire migration and invasion properties for leaving primary tumor site in order to invading through basement membrane. Normally, after epithelial cells are loss of extracellular matrix (ECM) attachment, they are triggered to apoptosis. This cell death process is named “anoikis” (Figure 3; Frisch and Francis, 1994). On the other hand, metastasized tumors have an ability to overcome apoptosis induced by loss of cell adhesion, thereby allows them to survive after detachment and travelling though the lymphatic as well as circulatory system. The cancer cells thus can spread from their primary sites to non-adjacent or adjacent parts by adhesion, invasion and migration to cell wall of either lymphatic or blood circulation (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997; Glinskii *et al.*, 2003; Simpson *et al.*, 2008).

6. Anoikis

Anoikis is one term of apoptosis which is induced by disruption of interactions between normal epithelial cells and extracellular matrix (Frisch and Francis, 1994). Loss of cell-matrix interaction could restrict inappropriate cell survival by cell cycle arrest and apoptosis (Frisch and Ruoslahti, 1997). Anoikis is a critical processing for maintaining tissue homeostasis and alteration in this apoptosis pathway is a key step in tumorigenesis. According to metastasis processes, metastasize tumors have an ability to overcome anoikis which seem to be a principle mechanism in inhibition of cancer cells expansion. Therefore, enhancing anoikis machinery would be an approach to prevent tumor metastasis. Anoikis is regulated by many signaling pathways which are mediated by intrinsic and extrinsic apoptotic pathways. However, intrinsic or mitochondrial apoptotis pathway seems to be the primary one (Haupt *et al*., 2003; Yong, *et al*., 2012).

7. Intrinsic apoptosis pathway or mitochondrial apoptosis pathway

7.1 Intrinsic apoptotic pathway and BCL2 protein classification

Intrinsic pathway has been reported in determination of cell survival which mainly regulated by the BCL2 protein family of anti-/pro-apoptosis regulators (Basu and Haldar, 1998). The members BCL2 protein family can be divided into different three subfamilies based on their homology domains and functions (Figure 4; Vachon, 2011): such as

(i) Apoptotic suppressors (anti–apoptotic proteins), including B–cell lymphoma 2 (BCL2), B–cell lymphoma-extra large (BCL-xL) and myeloid cell leukemia sequence 1 (MCL1).

(ii) Multidomain pro-apoptotic proteins, BCL2–associated X (BAX), BCL2 homologous antagonist/killer (BAK) and BCL2– related ovarian killer (BOK).

(iii) Pro-apoptotic BH3-only proteins, counting BH3 interacting domain death agonist (BID), BCL2–like protein 11 (BIM), p53 upregulated modulator of apoptosis (PUMA), BCL2–associated death promoter (BAD), BIK (BCL2 interacting killer), BCL2 modifying factor (BMF), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), and hara-kiri (HRK).

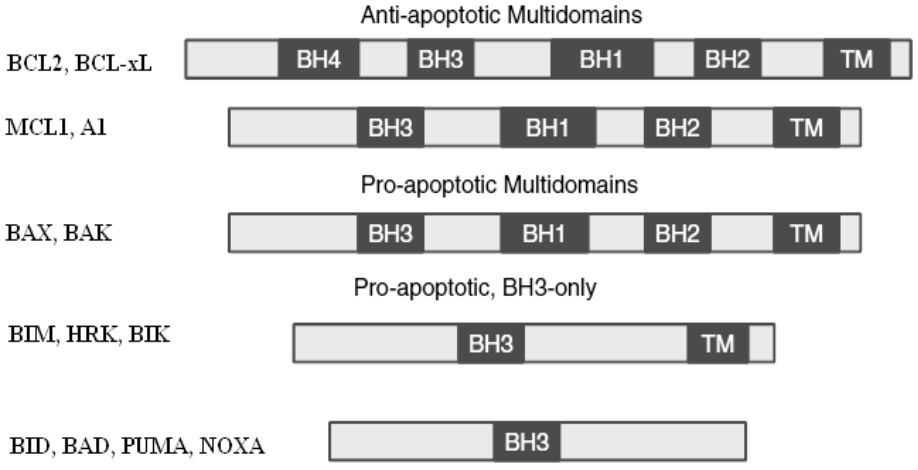
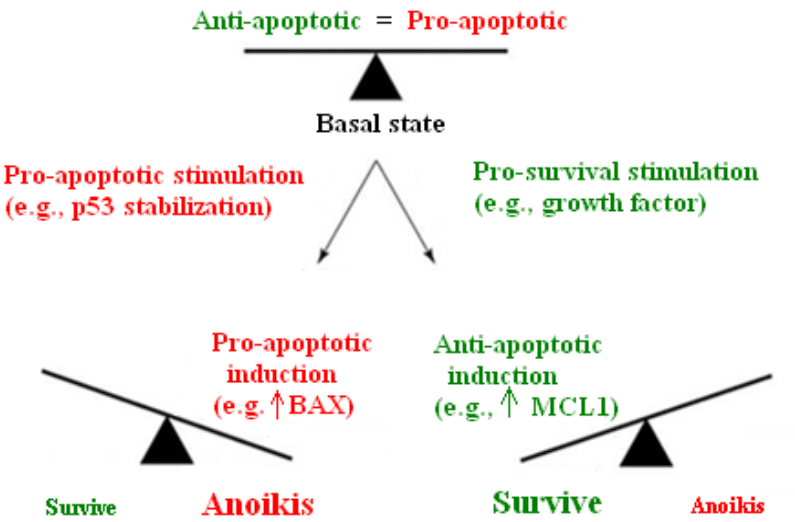


Figure 4 The composition of BCL2 homology domains (Simpson et al., 2008).

7.2 How does BCL2 family work?

Dimerization between anti-apoptotic and pro-apoptotic proteins play an essential role to program cells die or live (Figure 5a). In a hypothetical a basal state, the levels of anti-apoptotic and pro-apoptotic proteins are equal, shifting this balance changes cellular fate. For example, a stress (e.g. DNA damage or chemotherapy) leads to induction of pro-apoptotic proteins and results in cell death.

a)



b)

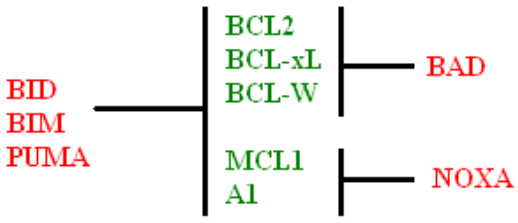


Figure 5 The balance of anti-apoptotic and pro-apoptotic BCL2 proteins predicts cellular survival. a) The rheostat model. b) The anti-apoptotic proteins neutralization model (adapted from Chipuk and Green, 2008).

So far, biochemical and genetic evidences suggest that pro-apoptotic BH3-only proteins act only in a specific way to neutralize the pro-survival function (Figure 5b). For instance, BAD and BMF can only counteract the functions of BCL2, BCL-xL or BCL-W, whereas NOXA exclusively counteracts MCL1 and A1. On the other hand, a potent killer, for example, BIM and PUMA can engage all pro-survival BCL2 proteins with comparable binding affinities (Kuwana *et al*., 2005; Labi *et al*., 2008).

8. BCL2 family proteins and anoikis processing

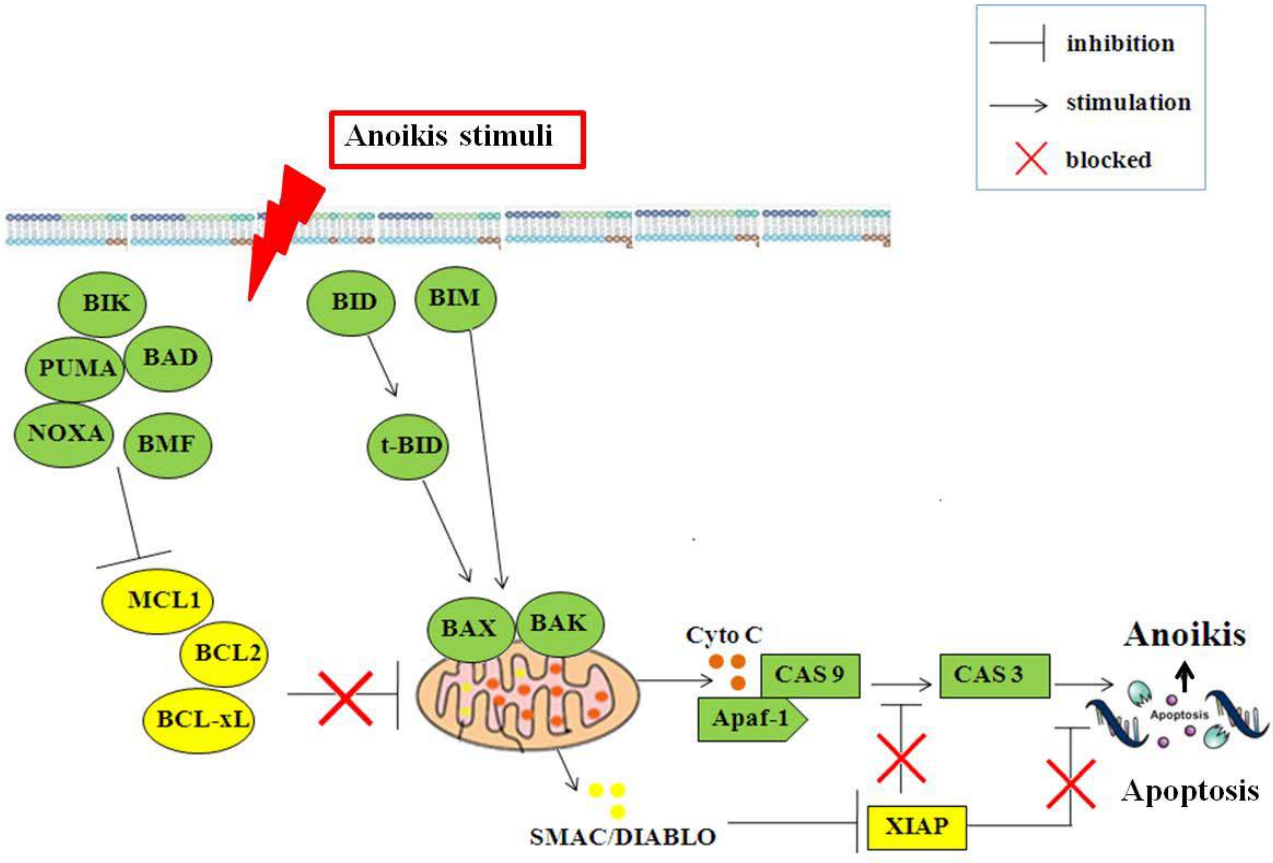


Figure 6 Intrinsic apoptosis pathways (adapted from Simpson *et al*., 2008)

Intrinsic apoptotic pathway is activated during anoikis (Figure 6). BID is required for anoikis of mammary epithelial cells. BIM is sequestered with cytoskeletal complexes when cells attach to extracellular matrix (Wang, 2001). BMF is sequestered to myosin V motor complexes. Upon loss of cell attachment, they are released and translocated to mitochorndia for neutralizing the pro-survival (e.g. BCL2, MCL1, BCL-xL) functions. Thereby BAX-BAK oligomer or BAX-BAX oligomer formations on outer mitochondria membrane (OMM) result in formation of pore. Integrity on mitochondrial membranes is then lost, because cytochrome *c* (Cyto C) and second mitochondrial derived activator of caspases/ direct IAP-binding protein with low pI (SMAC/DIABLO) from inter membrane of mitochondria are released to cytosol (Wang, 2001). Cytochrome *c* acts as a cofactor with Apaf-1 (Apoptosis protease activating factor-1) in recruiting the precursor/inactive form of the initiator caspase 9 (CAS 9). Among these, CAS 9 is activated which in turn to initiate execution caspases, such as CAS 3 and CAS 7. Whereas SMAC/DIABLO is released for blocking an inhibitor of apoptosis proteins, X–linked lnhibitor of apoptosis (XIAP), leading to trigger CAS 9 and CAS 3, confer sequential DNA degradation and cell death. (Bouillet and Strasser, 2002; Martin and Vuori *et al*., 2004; Kuwana, *et al*., 2005; Willis *et al*., 2007; Vachon, 2011).

9. Anoikis resistance and molecular proteins

For metastasis to occur, cancer cells have to escape detachment-induced apoptosis, termed anoikis (Frisch and Francis, 1994), thereby spreading to secondary sites (Hanahan and Weinberg, 2000; Simpson *et al*., 2008). Intrinsic apoptosis or

mitochondrial apoptosis pathway seems to be the primary machinery which is responsible for cell anoikis and most evidence indicates proteins in the B-cell lymphoma 2 (BCL2) family as being key players (Igney and Krammer, 2002; Chiarugi and Giannoni, 2008; Simpson *et al*., 2008; Kim *et al*., 2012). Numerous studies have shown that resistance to anoikis in certain types of cancers, including lung cancer, is due to the increase of antiapoptotic proteins such as BCL2 and MCL1 proteins (Igney and Krammer, 2002; Song *et al*., 2005; Chunhacha *et al*., 2012).

9.1 The BCL2 family

Among various antiapoptotic proteins in the BCL2 family, myeloid cell leukemia sequence 1 (MCL1) has garnered most attention and is believed to have a dominant effect on anoikis resistance of lung cancer cells (Song *et al*., 2005; Zhang *et al*., 2011; Chunhacha *et al*., 2012). MCL1 is important for maintainace of anoikis sensitivity (Wood *et al*., 2007), anoikis initiation is also dependent on the post-translational regulation of MCL1 by ubiquitin-ligase E3 (Zhong *et al*., 2005). Under cell-detachment condition, the degradation of MCL1 and contemporary transcription up-regulation of BIM are necessary for initiating anoikis. MCL1 inhibits BIM at the mitochondrial membrane, thereby preventing activation of related BH3-only factors to assembly of BAX-BAK oligomers-induced apoptosis (Opferman *et al*., 2007). Remarkable, BAX regulation occurs at multiple levels and activation of either BAX or BAK plays the major initiators apoptosis in intrinsic pathway (Wei *et al*., 2001). Recently, we have shown that overexpression of MCL1 suppresses the anoikis response in H460 human lung

cancer cells, while shRNA-mediated down-regulation of this protein has an anoikis-sensitizing effect (Chunhacha *et al*., 2012).

9.2 The p53

Human p53 gene resides at chromosome 17p13.1, encoding a 393 amino acid protein of 53 kDa (Jiang *et al*., 2010). The p53 functions originally as a transcription factor to activate or repress a large number of some target genes involved in the three major DNA damage responses including DNA damage repair, cell cycle arrest and apoptosis. Loss of p53 could lead to the genomic instability and tend to escape from apoptosis, resulting in the development of malignancy (Basu and Haldar, 1998). The absence of tumor suppressor, p53 in somatic mouse hepatocellular carcinoma has shown to promote the metastasis (Lewis *et al.*, 2005). So far, p53 transcription is important to regulate intrinsic apoptosis pathways (Haupt *et al*., 2003).

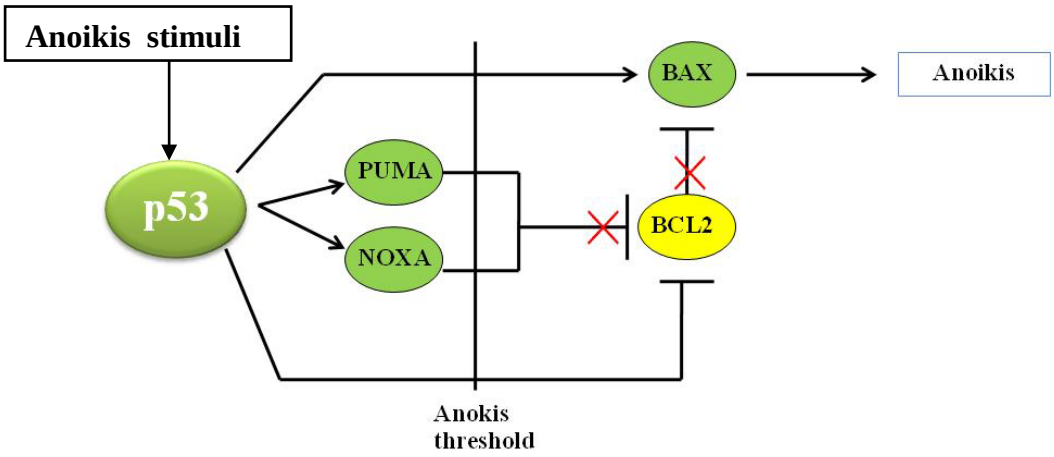


Figure 7 p53 and BCL2 family connection (adapted from Hemann and Lowe, 2006)

In case of intrinsic pathway, BCL2 family members are the down-stream regulation of p53, the apoptotic activator (Figure 7). Its targets are subsets of the BCL2 family genes which are BAX, NOXA, PUMA as well as the most recently identified, BID. DNA damage can sensitize p53 up-regulation involved BCL2 family members that are the down-stream target regulation such as BAX, NOXA, PUMA, resulting in apoptosis. Defects in p53 activation leads to induce BCL2 expression (Hemann and Lowe, 2006) and up-regulates expression of the anti-apoptotic MCL-1 protein (Pietrzak and Puzianowska-Kuznicka, 2008), as well as a decrease of cellular pro-apoptotic protein, including BAX, were shown to be involved in anoikis resistance (Lowe *et al*., 1994; Basu and haldar, 1998; Igney and Krammer, 2002; Haupt *et al*., 2003; Mihara *et al*., 2003; Zhang *et al*., 2004; Ravid *et al*., 2005; Derksen *et al*., 2006; Jiang *et al*., 2010).

9.3 The CAV1

Caveolae are characterized by specific scaffolding proteins, called caveolins which play an important role in vascular trafficking and signal transduction (Carver and Schnizer, 2003). Majority structure of caveolae is cholesterol, glycol-sphingolipid and caveolin (Parton and simons, 2007). Recently, there are three members of caveolins which are caveolin-1 (CAV1), caveolin-2 (CAV2) and caveolin-3 (CAV3). CAV1 has two isoforms that are CAV1α and CAV1β (Williams and Lisanti, 2004).

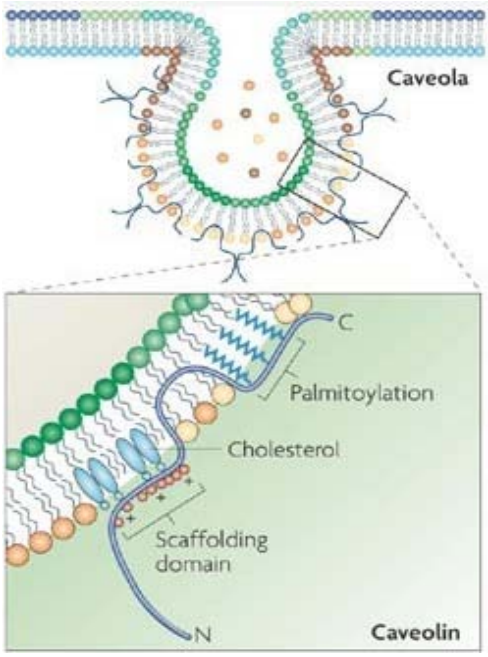


Figure 8 The structures of caveola and caveolin (Parton and simons, 2007)

CAV1 is the most common protein in research, since it is a major structural protein of caveolae in plasma membrane invaginations which are mediated in multiple cellular processes such as molecular transportation, cell adhesion and signal transduction. CAV1 acts as a scaffolding protein via its binding to signaling molecules such as epidermal growth factor (EGF) receptor, Src family members, mitogen-activated protein kinases (MAPK), protein kinase C (PKC), endothelial nitric oxide synthase (eNOS), and G- protein α subunit though its scaffolding domain by negative regulation (Staubach and Hanisch, 2011; Yong *et al.,* 2012). Moreover it has been regarded as a tumor suppressor because it is down-regulated in transformed cells and re-expression of CAV1 inhibits colony formation and induces apoptosis in transformed and breast cancer cells (Fiucci *et al*., 2002). Conversely, previous studies have been shown that

over-expression of CAV1 increases anoikis resistance, and cancer cell survival and its expression also related to increased metastatic and poor prognosis in lung cancer (Luanpitpong *et al*., 2010) as well as prostate cancer (Thompson *et al*., 2010). Additionally, CAV1 expression inhibits anoikis by inhibiting p53 activation and activating insulin-like growth factor (IGF) receptor-mediated extracellular Signal-regulated Kinase (ERK) and protein kinase B (Akt) signaling pathway upon cell detachment (Ravid *et al*., 2006), as well as overexpression of CAV1 suppresses the anoikis response in H460 human lung cancer cells by up-regulation of MCL1 protein (Chunhacha *et al*., 2012). As reported above, CAV1 appears to have dual functions depending on cell types. Thus, CAV1 has also attention in this research.

9.4 The anoikis resistance model

From previous studies as mentioned above, our study suggests the model of anoikis resistance as shown in Figure 9. Among these, agents that can enhance p53 function and/or deplete the level of proteins mediating anoikis resistance such as BCL2, MCL1 and CAV1, may be able to induce or sensitize anoikis and good candidates for anti-metastasis therapy.

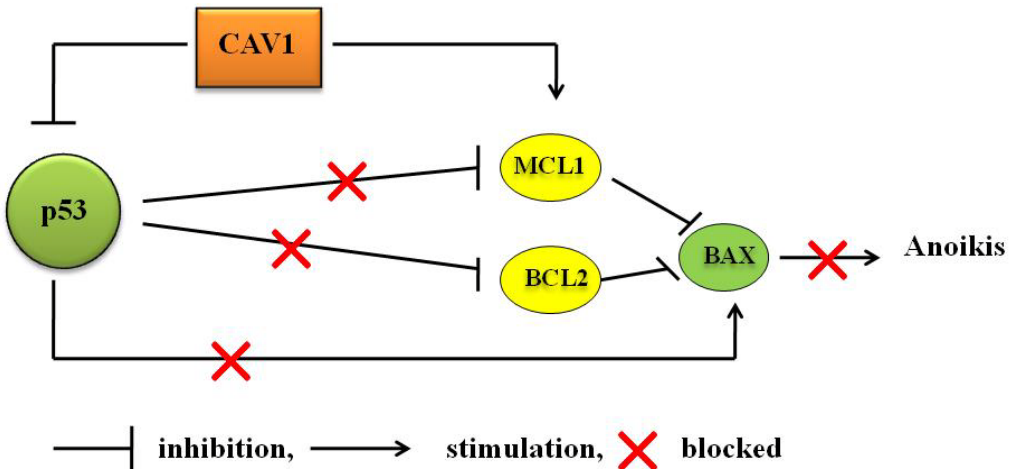


Figure 9 The anoikis resistance model. Overexpression of CAV1 suppresses the anoikis response by up-regulation of MCL1 protein and inhibiting p53 activation. Depletion of p53 leads to induce anti-apoptotic BCL2 and MCL1 proteins expression, which turn into inactivates pro-apoptotic BAX function at mitochondria, resulting in anoikis resistance.

10. Ecteinascidin770 (ET-770)

a)

b)

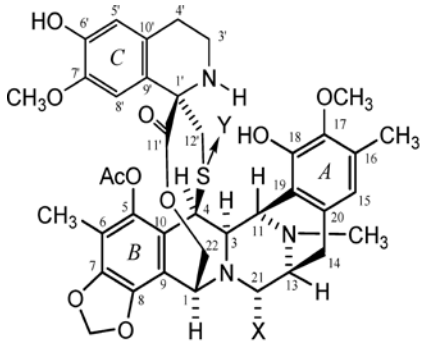


Figure 10 Ecteinascidin **a)** *Ecteinascidia thurstoni* (Chavanich *et al*., 2009), **b)** Structures of Ecteinascidins marine natural products (Puthongking *et al*., 2006).

Recently, marine-derived compounds have garnered increased attention to be used for cancer therapy since ecteinascidin 743 (ET-743, Yondelis**™,** Trabectedin (Zeltia and Johnson & Johnson)), a tetrahydroisoquinoline marine product, has been approved by European Union for use in humans with soft tissue sarcoma (D'Incalci and Galmarini, 2010). ET-743 has a unique mechanism of action based on its binding to the minor groove of DNA to interfere with cell division, activate transcription, and DNA repair (Cuevas and Francesch, 2009). Moreover, ET-743 has been reported to exhibit its function through p53 activation (Takebayashi *et al*., 2001). Also, renieramycin M which is another related tetrahydroisoquinoline isolated from a blue sponge, *Xestospongia* sp. has been shown to possess ability to down-regulate MCL1 and BCL2 proteins in lung cancer cells and overcome anoikis resistance (Halim *et al*., 2011). As an ongoing research in searching novel activity and potential compounds from marine organisms, ecteinascidin 770 (ET-770), a stablized ecteinascidin derivative is investigated for anti-metastasis potential in the present study.

Interestingly, ET-770 is a modified product isolated from a tunicate *Ecteinascidia thurstoni* by the KCN pretreatment. The tunicate was collected by scuba divers at Phuket Island at a depth range of 1-5 m. ET-770 has been also reported to posses cytotoxicity with IC_{50} values in the range of nanomolar concentrations against three human solid tumor cell lines which are HCT116 colon carcinoma, QG56 lung carcinoma, and DU145 prostate carcinoma (Table 4; Saktrakulkla *et al*., 2011). Moreover it also has cytotoxicity on human epidermoid carcinoma of the narsopharynx (KB) and breast cancer cells (BC) with ED_{50} values 3.4×10^{-2} and 3.2×10^{-3} µM, respectively (Charupant, 2000).

Table 4 Antitumor activity of ET-743 and ET-770

In vitro IC50 (nM)		
Tumor type	**ET-743[a]**	**ET-770[b]**
P388 (mouse leukemia)	0.34	-
L1210 (mouse leukemia)	0.66	-
A549 (lung cancer)	0.26	-
QG56(lung cancer)	-	2.40
HT29 (colon cancer)	0.46	-
HTC116 (colon cancer)	-	0.60
MEL-28 (melanoma)	0.5	-
DU145 (prostate cancer)	-	0.81

a, Rinehart, 2000; b, Saktrakulkla et al., 2011

Even though ET-770 has been previously reported to have potent antitumor activity (Saktrakulkla *et al*., 2011), effect of such a compound in the regulation of lung cancer cell anoikis has not been investigated. The present study thus aims to investigate the possible anoikis sensitizing activity of ET-770 and elucidate the underlying mechanism of action of the compound focusing on the level of proteins mediating anoikis machinery of cancer cells.

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Reagents

Ecteinascidin 770 (ET-770) was provided by Dr. Khanit Suwanborirux. Trypsin, Hoechst 33342, propidium iodide (PI), dimethysulfoxide (DMSO) and 2, 3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt (XTT) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies for MCL1, BCL2, rabbit pAb and CAV1; mouse pAb were obtained from Abcam Ltd. (Cambridge, USA). p53-HRP, mouse monoclonal IgG2a, β-Actin; mouse monoclonal IgG, and BAX-HRP, rabbit polyclonal IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA).

2. Cell culture

Human non-small cell lung cancer H 23 and H 460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA.). Cells were cultured in RPMI 1640 medium containing 5% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml penicillin/streptomycin (Gibco, Gaithersburg, MA, USA) in a 5% CO_2 environment at 37°C.

3. Equipments

Laminar flow cabinet, carbon dioxide incubator, autopipettes for 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl, pipette tips for 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl, cell culture plate: 96-well and 6-well normal (Nunc), conical tube: 15 ml and 50 ml (Neptune), bottle: 100 ml, 250 ml, 500 ml and 1,000 ml (Duran) disposable pipette: 1ml and 5ml, hemocytometer, pH meter, vertex mixer, balance, microplate reader (Perskin Elmer, USA) and fluorescence microscope by Olympus IX51 with DP70 camera (Olympus, Tokyo, Japan), Flow cytometer (FACSort, Becton Dickinson, Rutherford, NJ, USA)**.**

4. Sample preparation

ET-770 was dissolved in dimethyl sulfoxide (DMSO) and distilled water to achieve indicated concentrations containing less than 0.1% DMSO.

5. Cytotoxicity Assay

Cells were seeded into 96-well plates 1×10^5 cells/ml for 24 h and then treated with various concentrations of ET-770 for 24 h. Cells were incubated with 20 µM of 2,3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt (XTT) reagent for a further 4 h at 37°C. The intensity of the formazan product was measured at 450 nm using a microplate reader. All analyses were established in at least three independent replicate cultures. The cell viability was calculated from

optical density (OD) ratio of treated to non-treated control cells and is presented as a percentage of the non-treated control.

$$\% \text{ Cell viability} = \left(\frac{OD_{450} \text{ of treatment}}{OD_{450} \text{ of control}} \right) \times 100$$

6. Apoptosis and necrosis assays

Hoechst 33342 and propidium ioide (PI) staining assay. Cells were stained with 10 μM Hoechst 33342 and 5 μg/ml PI. Cells that had intensely condensed and/or fragment nuclei stained by Hoechst 33342 were considered as apoptotic cells, those staining only with PI were considered as necrotic cells. The fluorescent dye stained in cells was visualized under a fluorescence microscope by Olympus IX51with DP70 camera (Olympus, Tokyo, Japan).

7. Anoikis assay

NCI-H 23 or NCI-H 460 cells in the culture plate was detached and made into a single-cell suspension in RPMI-serum free medium and then seeded into an ultra-low attachment plate (Cornig, Acton, MA, USA) at a density of 1.5×10^5 cells/ml. Cells were then harvested at 0, 3, 6, 9, 12, and 24 h. Cell viability was measured by XTT assay as mentioned above.

8. Cell cycle analysis

After specific treatment for 12 h, detached cells were harvested by centrifugation at 4,500 rpm for 5 min at 4 °C in a 15-ml polyethylene tube. Consequently, the pellet was gently re-suspended in 1 ml of cold 10% FBS/ PBS, then gently dropwised cold absolute EtOH (2.5 ml), while vertexing and kept it overnight at -20°C for 24 h. Afterward, cells were centrifuged as above, washed 1 time with cold 10% FBS/ PBS and re-centrifuged. The supernatant was subsequently removed and the pellet was pre-incubated with propidium iodide buffer containing 10% PBS 10x, 10% RNaseA 1 mg/ml, 0.1% Triton-X100, 0.1% 0.5M EDTA, 2% PI 10 mg/ml at 37°c for 30 min. Apoptotic DNA fragmentation is determined by sub G_0 fraction of cell cycle analysis. Cell cycle profiles were analzyed by FACScan flow cytometer (Becton Dickinson, Rutherford, NJ, USA) using a 488 nm excitation beam and a 630 nm band-pass filter with CellQuest software (Becton Dickinson).

9. Western blot method

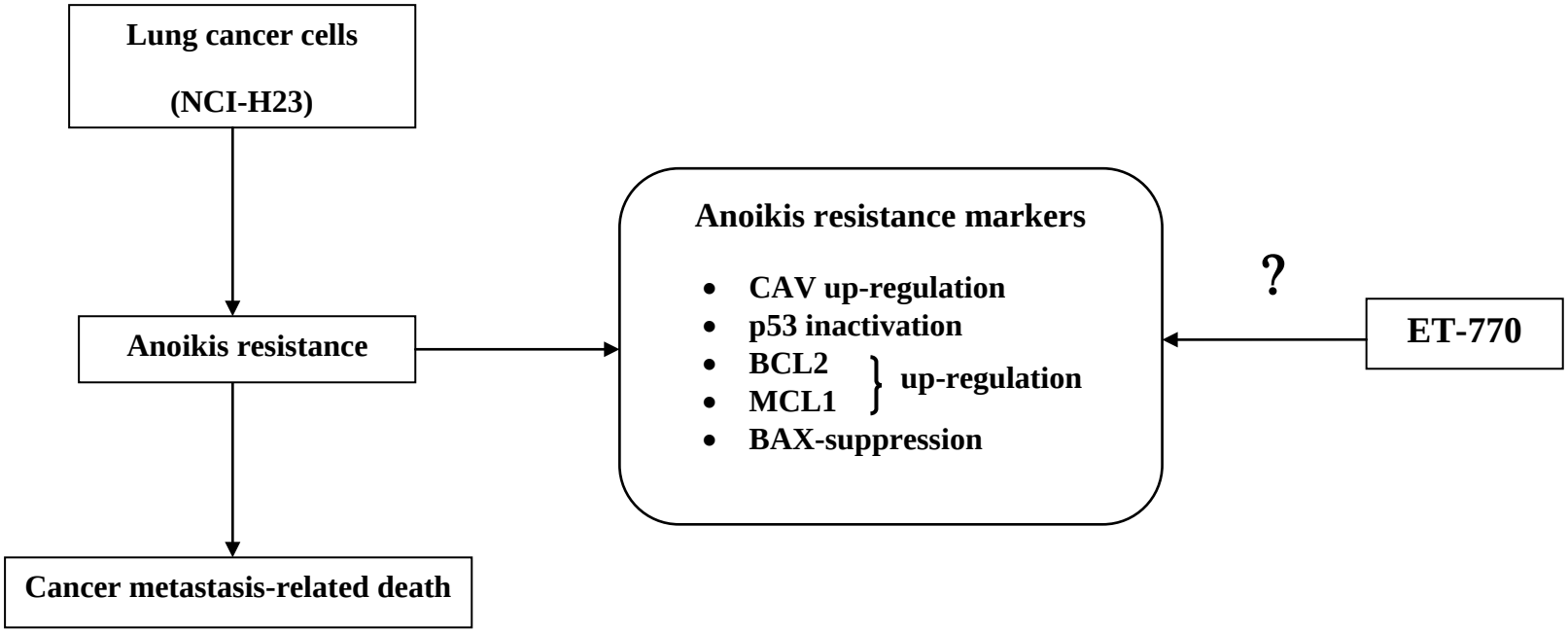
To determine proteins, after specific treatment, cells were incubated with lysis buffer containg 20 mM Tris-HCl (pH 7.5), 1% triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor cocktail (Roche, Indianapolis, IN, USA) for 30 min on ice. The supernatants were collected after centrifuging at 12000×g for 15 min at 4°C. The protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL, USA). Each sample was

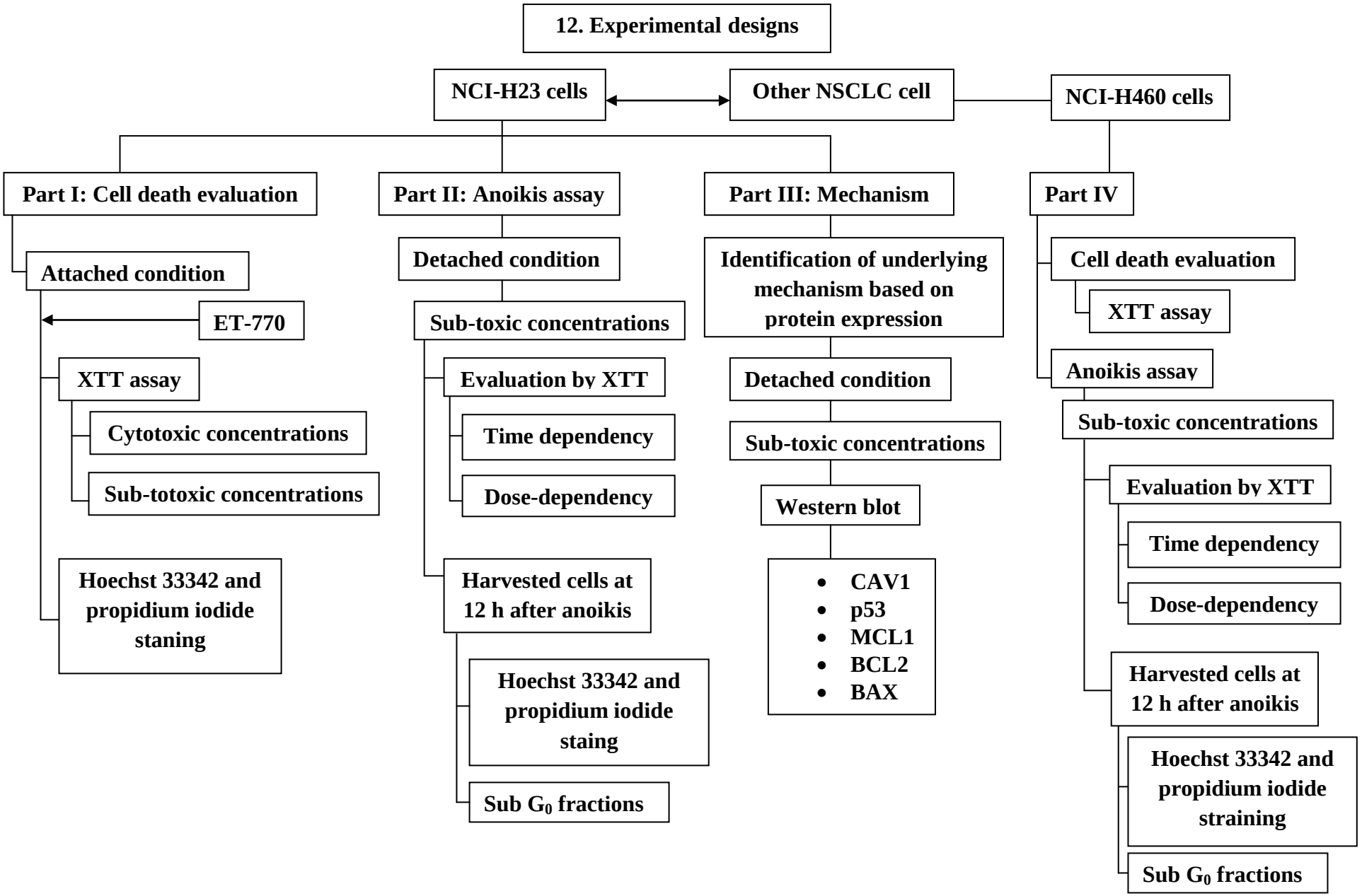
denaturized by heating at 95°C for 5 min with Laemmli loading buffer. Subsequently, the sample was loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as equal amount of proteins (40 μg/lane) and transferred to polyvinylidene difluoride (PVDF) membranes. The transferred membranes were blocked for 1 h in 5% skin milk in TBST [25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20] and incubated with MCL1, BCL2, and CAV1 (Abcam, Cambridge, MA,USA), as well as p53, β-Actin, and BAX (Santa Cruz, CA, USA) at 4°C overnight. Membranes were washed three times with TBST for 8 min, followed by incubation with horseradish peroxidase-conjugated specific secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. The immune complexes were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified by imaging densitometry using analyst/PC densitometry software (Bio-Rad). Mean densitometric data from independent experiments were normalized with β-Actin protein.

10. Statistical Analysis

The data were presented as the mean ± standard deviation (S.D.). Mean data from at least three independent experiments were normalized to result in the non-treated control. Values were analyzed by one-way ANOVA and significance levels were determined by post hoc analysis with Tukey's test at $p < 0.05$ for between group comparisons.

11. Conceptual framework




12. Experimental designs
NCI-H23 cells
Other NSCLC cell
NCI-H460 cells
Part I: Cell death evaluation
Part II: Anoikis assay
Part III: Mechanism
Part IV
Attached condition
ET-770
XTT assay
Cytotoxic concentrations
Sub-totoxic concentrations
Hoechst 33342 and propidium iodide staning
Detached condition
Sub-toxic concentrations
Evaluation by XTT
Time dependency
Dose-dependency
Harvested cells at 12 h after anoikis
Hoechst 33342 and propidium iodide staing
Sub G0 fractions
Identification of underlying mechanism based on protein expression
Detached condition
Sub-toxic concentrations
Western blot
CAV1
p53
MCL1
BCL2
BAX
Cell death evaluation
XTT assay
Anoikis assay
Sub-toxic concentrations
Evaluation by XTT
Time dependency
Dose-dependency
Harvested cells at 12 h after anoikis
Hoechst 33342 and propidium iodide straining
Sub G0 fractions

Part I. ET-770 induces human non-small cell lung cancer H23 cells apoptosis

1.1 Cytotoxicity of ET-770 on NSCLC, H23 cells

To determine effect of ET-770 on cell viability in attached cell condition, H23 cells were seeded into 96-well plates and treated with various concentrations of ET-770 (0.5, 1, 5, 10, and 50 nM) for 24 h then cell viability was analyzed by XTT assay.

1.2 Mode of cell death in response to ET-770 treatment on H23 cells

To determine mode of cell death in response to ET-770 treatment in cell attached condition, H23 cells were seeded into 96-well plates and treated with various concentrations of ET-770 (0.5, 1, 5, 10, and 50 nM) for 24 h then cell viability was analyzed by Hoechst 33342 and propidium iodide staining.

Part II. ET-770 sensitizes human non-small cell lung cancer H23 cells to anoikis

2.1 Effect of sub-toxic concentrations of ET-770 on H23 cell anoikis

To determine characteristic of detachment-induced apoptosis by dose and time-dependent manner, sub-toxic concentrations of ET-770 obtained from attached condition assay were chosen. Attached H23 cells in the culture plate were tripsinized to a single cell suspension in RPMI-serum free medium and seeded into 6-well ultra-low attachment plates (Cornig, Acton, MA) at the density of 1×10^5 cells/ml. Suspended cells were treated with various sub-toxic

doses (0.5, 1, 5, 10, and 50 nM) and incubated at 37 °C. Cells were harvested at time 0, 3, 6, 9, 12 and 24 h. After that, cell viability was determined by XTT assay as mentioned above.

2.2 Mode of cell death in response to ET-770 treatment on H23 cell anoikis

To determine mode of cell death in response to ET-770 treatment, suspended cells were treated with 0.5, 1, 5, 10, and 50 nM of ET770, harvested at 12 h, and seeded into 96-well plate. Apoptotic and necrotic cell deaths were determined by Hoechst 33342 and propidium iodide straining. Cells that performed intensely condensed and/or fragment nuclei stained by Hoechst 33342 were considered as apoptotic cells, whereas PI stained only the DNA of cell membrane-damaged cell that are considered as necrotic cells. The fluorescent dye stained in cells was visualized under a fluorescence microscope (Olympus IX51 with DP70).

2.3 Effect of ET-770 in sub G_0 fraction of detached H23 cell anoikis

To confirm the effect of ET-770 on H23 cell anoikis, suspended cells were treated with the absence or the presence of 0.5, 1, 5, 10, and 50 nM of ET-770 for 12 h. Cells were harvested by centrifugation at 4,500 rpm for 5 min at 4 °C in a 15 ml-polyethylene tube. Consequently, pellets were gently re-suspended in 1 ml of cold 10% FBS/ PBS, gently dropwised cold absolute EtOH (2.5 ml), while the tube was vortexed. It was kept overnight at -20°C for 24 h. Afterward, Cells were centrifuged cells as above, washed 1 time with

cold 10% FBS/ PBS and re-centrifuged. The supernatant was subsequently removed and the pellets were pre-incubated with propidium iodide buffer containing 10% PBS 10x, 10% RNaseA 1 mg/ml, 0.1% Triton-X100, 0.1% 0.5M EDTA, 2% PI 10 mg/ml at 37°c for 30 min. Sub G_0 fraction was analzyed by FACScan flow cytometer (Becton Dickinson, Rutherford, NJ, USA) using a 488 nm excitation beam and a 630 nm band-pass filter with CellQuest software (Becton Dickinson).

Part III. Mechanism of action of ET-770 - induced cell death

To determine the mechanism of action of ET-770 that may involve protein expressions, after treatment of ET-770 for 12 h, cells were harvested and proteins were collected. Expression of anti-apoptotic proteins (BCL2, MCL1), pro-apoptotic protein (BAX) and other association proteins (CAV1, p53) were investigated. The levels of proteins involving in anoikis of cells were determined by Western blot analysis.

Part IV. Anoikis sensitizing effect of ET-770 in other NSCLC

4.1 Cytotoxic effect of ET-770 on H460 cells

Cells were seeded into 96-well plates at a density of 1×10^5 cells/ml and treated with various concentrations of ET-770 (0.5, 1, 5, 10, and 50 nM) for 24 h. After specific treatments, cells were incubated with 20 μM of XTT reagent

for 4 h at 37 °C. The intensity of the formazan product was measured at 450 nm using a microplate reader. All analyses were established in at least three independent replicate cultures. The cell viability was calculated from optical density (OD) ratio of treated to non-treated control cells and presented as percentage to the non-treated control value.

4.2 Effect of sub-toxic concentrations of ET-770 on H460 cell anoikis

To determine characteristic of detachment-induced apoptosis by dose and time-dependent manners, sub-toxic concentrations of ET-770 obtained from attached condition assay were chosen. Attached H460 cells in the culture plate were tripsinized to a single cell suspension in RPMI-serum free medium and seeded into 6-well ultra-low attachment plates (Cornig, Acton, MA) at the density of 1×10^5 cells/ml. Suspended cells were treated with various sub-toxic doses (5, 10, and 50 nM) and incubated at 37 °C. Cells were harvested at time 0, 3, 6, 9, 12 and 24 h. After that, cell viability was determined by XTT assay as mentioned above.

4.3 Mode of cell death in response to ET-770 treatment on H460 cell anoikis

To confirmed characteristic of detachment-induced apoptosis and the effect of sub-toxic ET-770 (5 and 10 nM) on anoikis, apoptotic and necrotic cell deaths were determined by Hoechst 33342 and propidium iodide straining on anoikis cells harvested at 12 h.

4.4. Effect of ET-770 in sub G_0 fraction of detached H460 cell anoikis

To confirm the effect of ET-770 on H460 cell anoikis, suspended cells were absent or treated with 5 and 10 nM of ET770 for 12 h. Cells were harvested by centrifugation at 4,500 rpm for 5 min at 4 °C in a 15 ml-polyethylene tube. Consequently, pellets were gently re-suspended in 1 ml of cold 10% FBS/ PBS, gently dropwised cold absolute EtOH (2.5 ml), while the tube was vortexed. It was kept overnight at -20°C for 24 h. Afterward, Cells were centrifuged cells as above, washed 1 time with cold 10% FBS/ PBS and re-centrifuged. The supernatant was subsequently removed and the pellets were pre-incubated with propidium iodide buffer containing 10% PBS 10x, 10% RNaseA 1 mg/ml, 0.1% Triton-X100, 0.1% 0.5M EDTA, 2% PI 10 mg/ml at 37°c for 30 min. Sub G_0 fraction was analzyed by FACScan flow cytometer (Becton Dickinson, Rutherford, NJ, USA) using a 488 nm excitation beam and a 630 nm band-pass filter with CellQuest software (Becton Dickinson).

CHAPTER IV

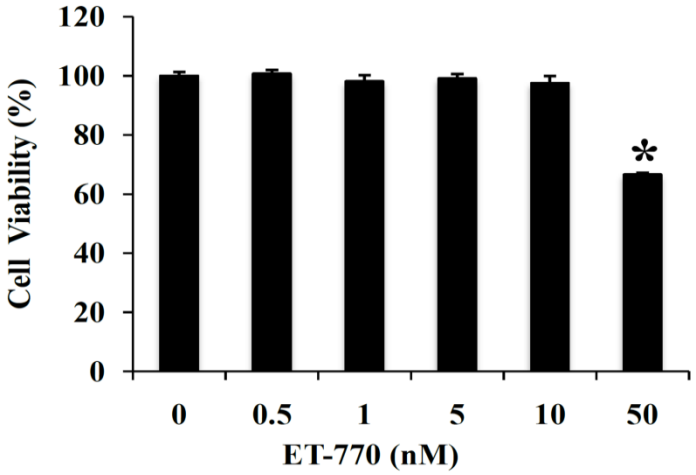
RESULTS

Part I. ET-770 induces human non small cell lung cancer H23 cells apoptosis.

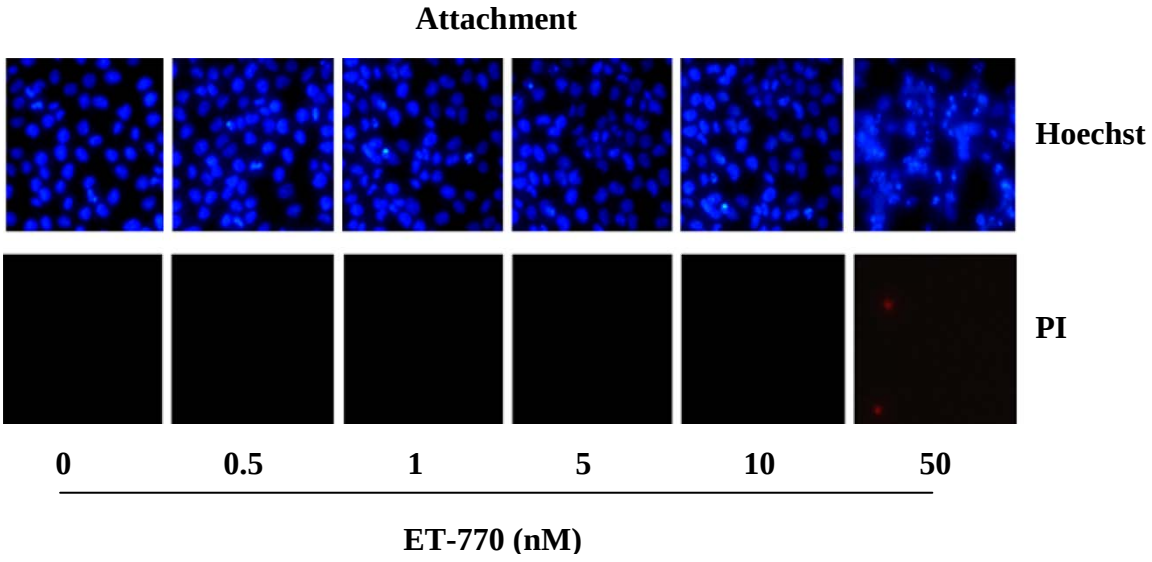
The cytotoxic effect of ET-770 was first characterized. H23 lung cancer cells were incubated with different concentrations of ET-770 (0-50 nM) for 24 h, and cell viability was analyzed. Figure 10A shows that ET-770 at the concentration of 50 nM significantly reduced the viability of H23 cells, while at lower concentrations (0.5-10 nM), it exhibited neither toxic nor proliferative effects. Therefore, concentrations of 0.5-10 nM were further used for anoikis experiments.

Furthermore, Hoechst 33342/PI nuclear staining was performed to elucidate the mode of cell death induced by ET-770. The results revealed that only a minimal number of cells exhibited apoptotic (~5%) and necrotic (<1%) characteristics when the cells were treated with 0.5-10 nM of ET-770 (Figures 10B and C). As shown in Figure 10A, ET-770 at 50 nM significantly reduced cell viability to ~66%. Consistently, such a concentration of ET-770 induced approximately 40% apoptosis and 3.9% necrosis of H23 human lung cancer cells (Figures 10B and C).

A


120
100
80
60
40
20
0
Cell Viability (%)
*
0
0.5
1
5
10
50
ET-770 (nM)

B


Attachment
Hoechst
PI
0
0.5
1
5
10
50
ET-770 (nM)

C

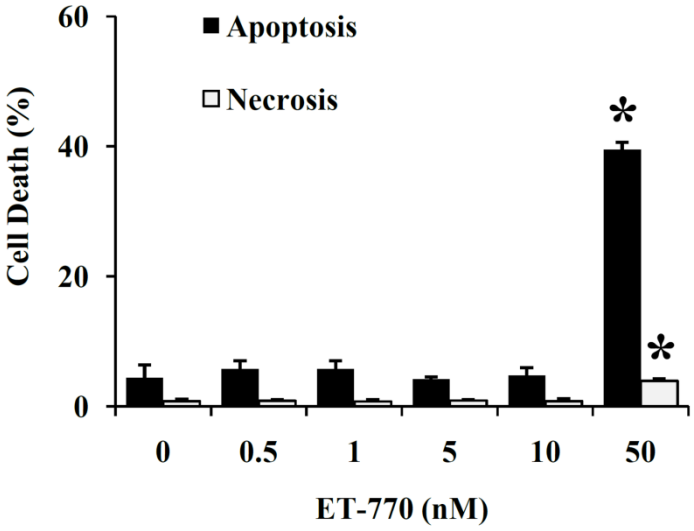


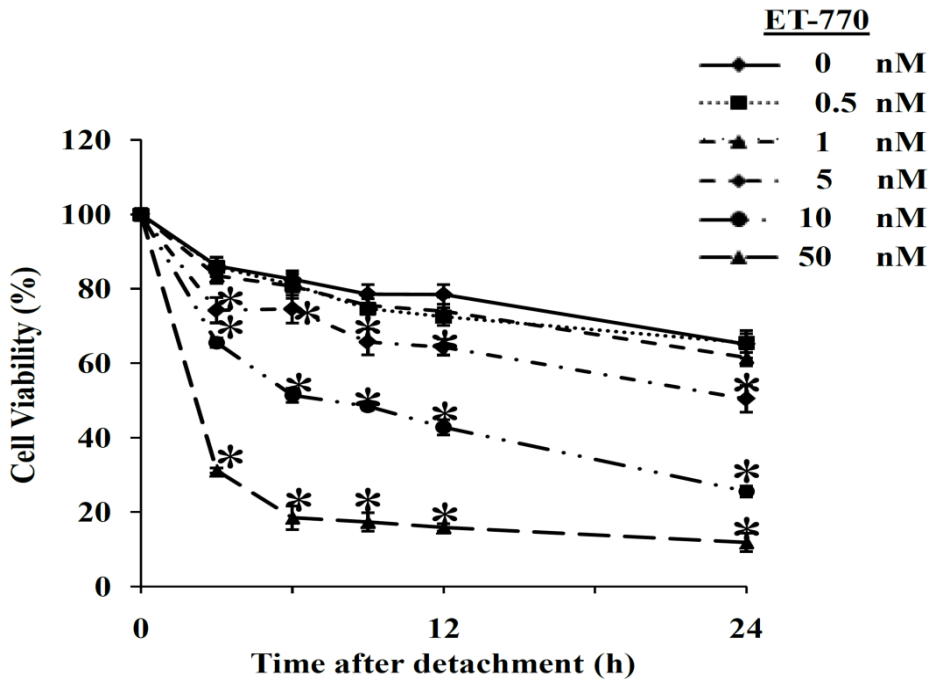
Figure 11 Cytotoxic effect of ecteinascidin 770 (ET-770) on H23 human lung cancer cells. H23 cells were treated with different concentrations of ET-770 (0–50 nM) for 24 h. **A)** Cell viability was determined by 2, 3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt (XTT) assay. Values are means ± S.D. (n=5), **p*<0.05 *versus* non-treated controls. **B)** Nuclear morphology of Hoechst33342/PI staining of cells was captured under fluorescence microscopy. **C)** Percentages of cell apoptosis and necrosis were obtained from Hoechst 33342/PI assays.

Part II. ET-770 sensitizes human non-small cell lung cancer H23 cells to anoikis

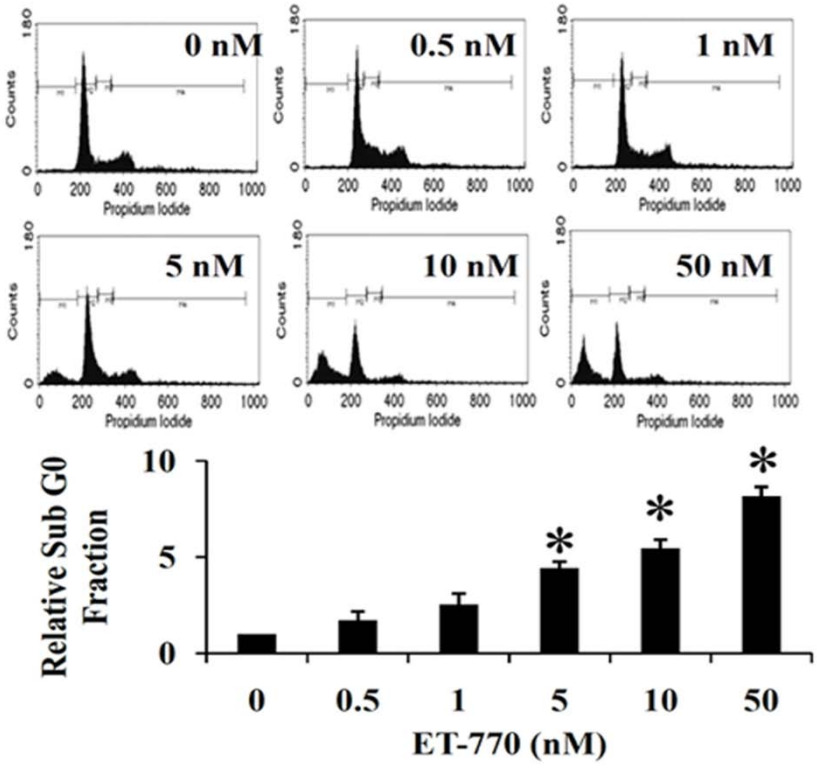
To study the effect of ET-770 on cell anoikis, cells were detached and suspended in the presence or absence of ET-770 (0-50 nM) and cell viability was analyzed at various times (0-24 h). Figure 11A shows that ET-770 at concentrations of 5-50 nM significantly reduced cell viability of the detached cells as compared to non-treated controls. A significant decrease in cell viability was observed as early as 3 h after cell detachment, with approximately 75%, 65% and 30% of cells remaining viable in response to 5, 10 and 50 nM of ET-770, respectively.

To determine whether the observed reduction in cell viability was due to apoptotic cell death, we analyzed the sub G_0 fraction by flow cytometry and the profile of nuclear morphology of the cells by Hoechst 33342/PI staining assay. Cell cycle analysis showed that the sub G_0 fraction was significantly increased in the ET-770-treated cells, suggesting that apoptosis was indeed the main mechanism of death under these conditions (Figure 11B). Hoechst 33342/PI staining assay also indicated condensed and/or fragmented nuclear morphology of apoptotic cells in response to ET-770, while PI-positive cells were only detected in the cells treated with 50 nM of ET-770 (Figure 11C).

A


ET-770
0 nM
0.5 nM
1 nM
5 nM
10 nM
50 nM
Cell Viability (%)
120
100
80
60
40
20
0
0
12
24
Time after detachment (h)

B


0 nM
0.5 nM
1 nM
5 nM
10 nM
50 nM
Counts
Propidium Iodide
Relative Sub G0 Fraction
10
5
0
0
0.5
1
5
10
50
ET-770 (nM)

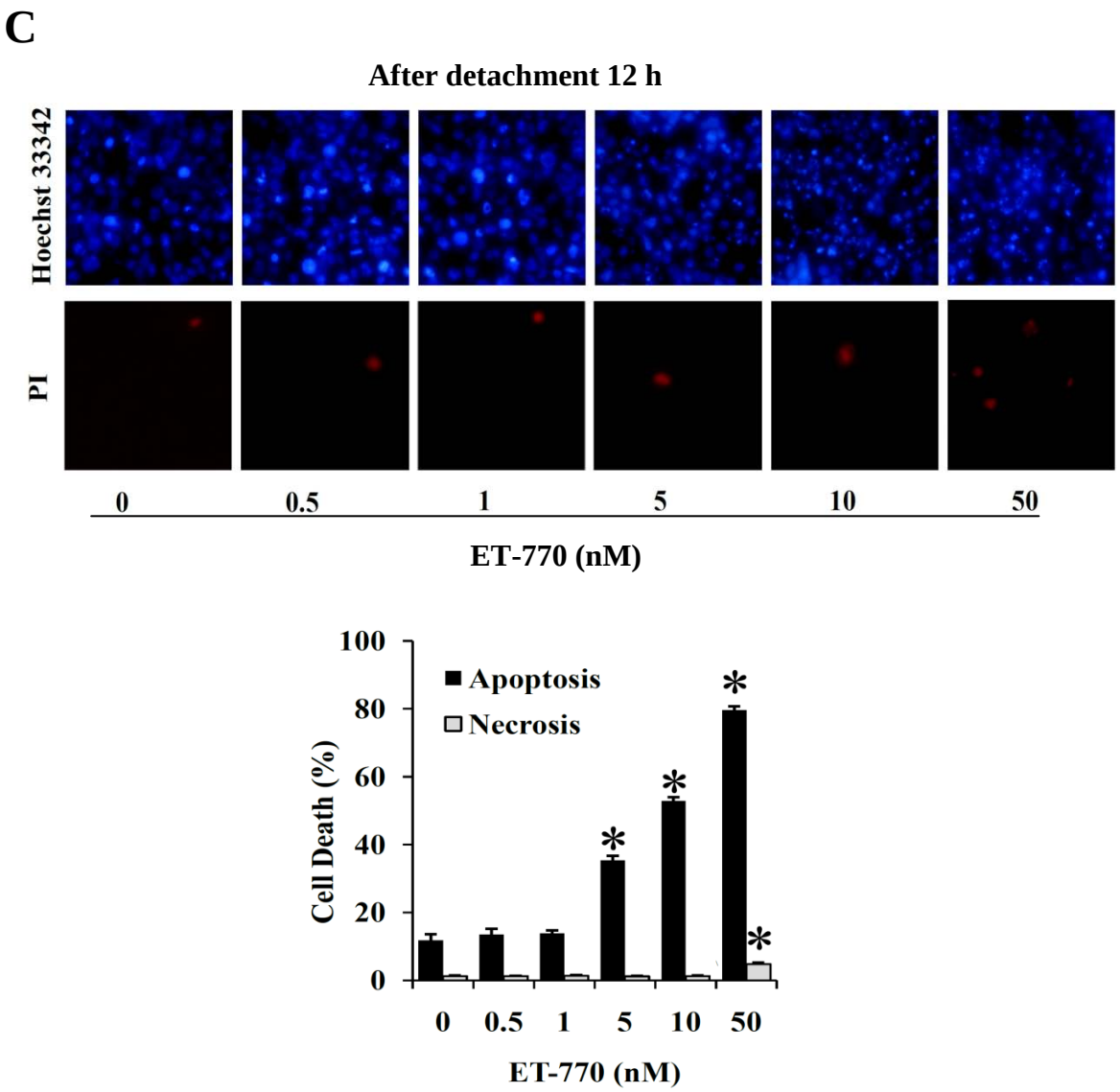
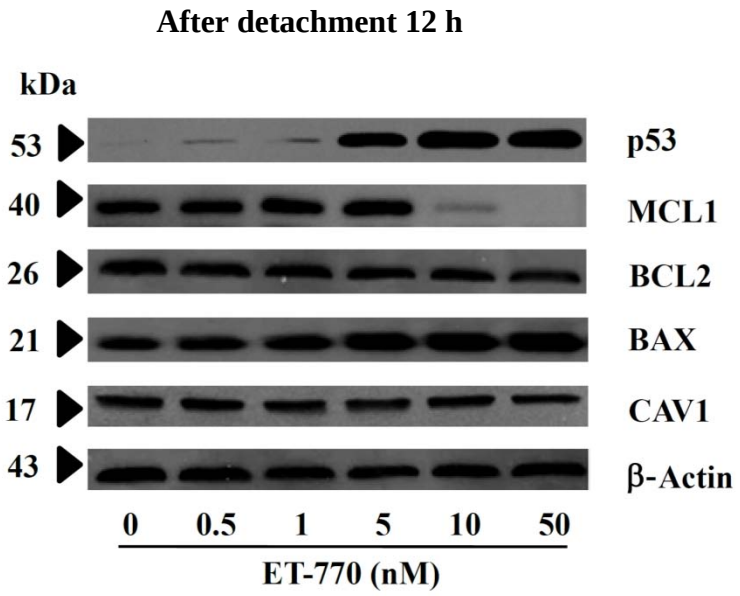


Figure 12 Ecteinascidin 770 (ET-770) sensitizes H23 lung cancer cells to anoikis. **A)** Detached cells were treated with 0–50 nM of ET-770 and cell viability was determined by XTT assay at the indicated times. Values are means ± S.D. (n=5), **p*<0.05 *versus* non-treated controls. **B)** Sub G_0 fraction of the cells was evaluated by flow cytometry and propidium iodide. **C)** Apoptosis and necrosis were detected by Hoechst 33342/PI staining assay. The percentages of cell apoptosis and necrosis were obtained. Values are means ± S.D. (n=3), **p*<0.05 *versus* non-treated controls at 12 h.

Part III. ET-770 sensitizes H23 cell anoikis by inducing cell death through a p53 dependent mechanism.

In order to clarify the mechanism behind ET-770 in sensitizing anoikis, effects of ET-770 on anoikis regulatory proteins including p53, MCL1, BCL2, BAX, and CAV1 were evaluated by western blot analysis. Detached H 23 cells were treated with different concentrations of ET-770 (0-50 nM) for 12 h and the expression of the indicated proteins was analyzed. Figures 12A and B show that ET-770 significantly increased p53 and BAX levels, while reducing that of MCL1. However, no significant alteration of BCL2 and CAV1 was observed in response to ET-770. Together, these results revealed that ET-770 sensitizes H23 cells to anoikis through a p53-dependent mechanism.

A



B

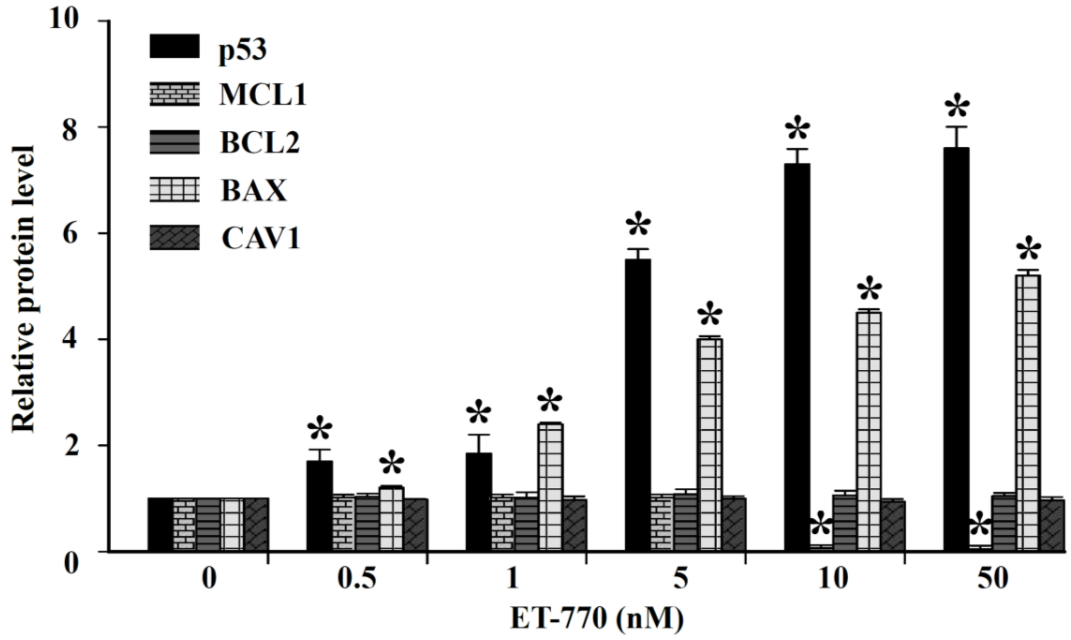


Figure 13 Ecteinascidin 770 (ET-770) sensitizes H23 cell to anoikis through a p53-dependent mechanism. Cells were detached and incubated with 0–50 nM of ET-770 for 12 h. **A)** Caveolin 1 (CAV1), p53, myeloid cell leukemia sequence 1 (MCL1), B-cell lymphoma 2 (BCL2) and BCL2–associated X protein (BAX) expressions were determined by western blotting. Blots were also carried out with β-Actin antibody to confirm equal loading of the samples. **B)** Plot shows values relative to controls at 12 h. Values are means ± S.D. (n=3), * $p<0.05$ *versus* non-treated controls.

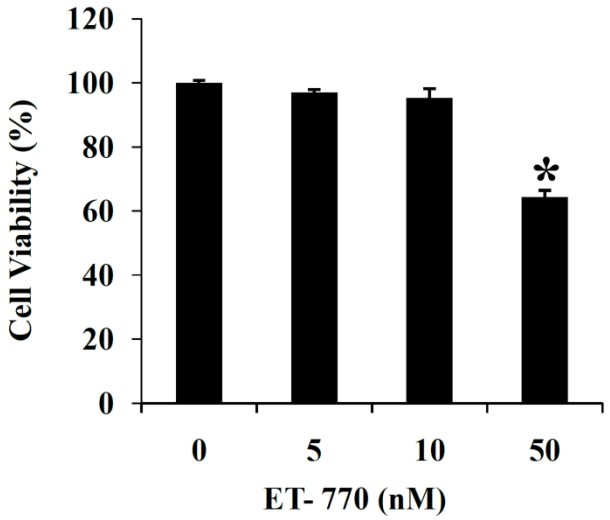
Part IV. Anoikis sensitizing effect of ET-770 on H460 cells

Having shown that ET-770 sensitized H23 cells to anoikis, we provided further information of such a sensitizing effect on other lung cancer cells. Sub-toxic concentrations of ET-770 were determined and used for anoikis assay of H460 human lung cancer cells. Cytotoxic evaluation indicated that ET-770 at 5 and 10 nM caused no significant toxic effects on H460 cells (Figure 13A).

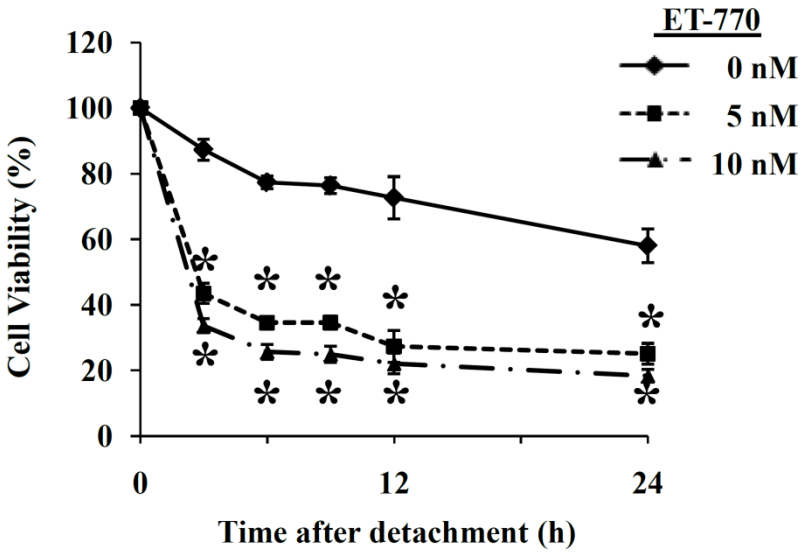
Anoikis of H460 cells in response to ET-770 was similarly determined as described above. Consistent with H23 cells, H460 exhibited anoikis resistance, with approximately 86% and 60% of the cells remaining viable after 3-h and 24-h detachment, respectively (Figure 13B). Figure 13B shows that a significant decrease in cell viability was observed as early as 3 h, with approximately 43% and 33% of cells remaining viable in response to 5 and 10 nM of ET-770, respectively.

Moreover, we analyzed the sub G_0 fraction by flow cytometry and the profile of chromosomal DNA of the cells by Hoechst 33342/PI staining assay at 12 h after detachment. Hoechst 33342/PI staining assay indicated an intense signal of nuclear fluorescence, chromatin condensation and DNA fragmentation of apoptotic cells in response to ET-770 in a dose-dependent manner (Figure 13C). Flow cytometric analysis of the sub G_0 fraction also indicated a similar increase of the sub G_0 fraction in response to ET-770 as found in the experiment for H23 cells (Figure 13D). Together, our findings indicate that ET-770 sensitizes lung cancer cells to anoikis.

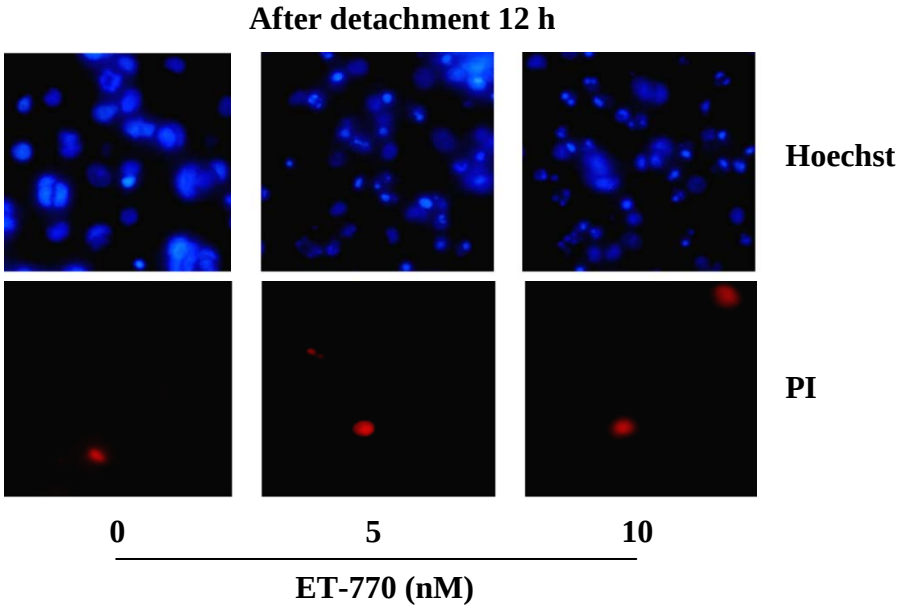
A

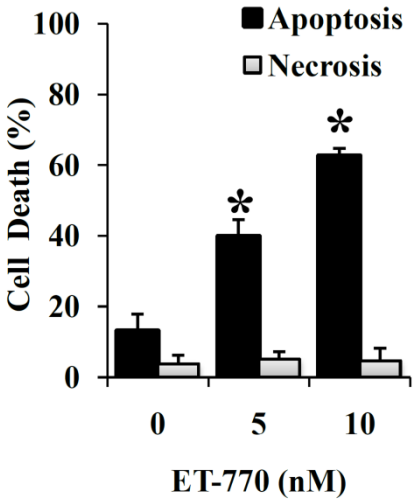

120
100
80
60
40
20
0
Cell Viability (%)
*
0
5
10
50
ET- 770 (nM)

B


ET-770
0 nM
5 nM
10 nM
120
100
80
60
40
20
0
Cell Viability (%)
0
12
24
Time after detachment (h)

C


After detachment 12 h
Hoechst
PI
0
5
10
ET-770 (nM)


Apoptosis
Necrosis
Cell Death (%)
100
80
60
40
20
0
*
*
0
5
10
ET-770 (nM)

D

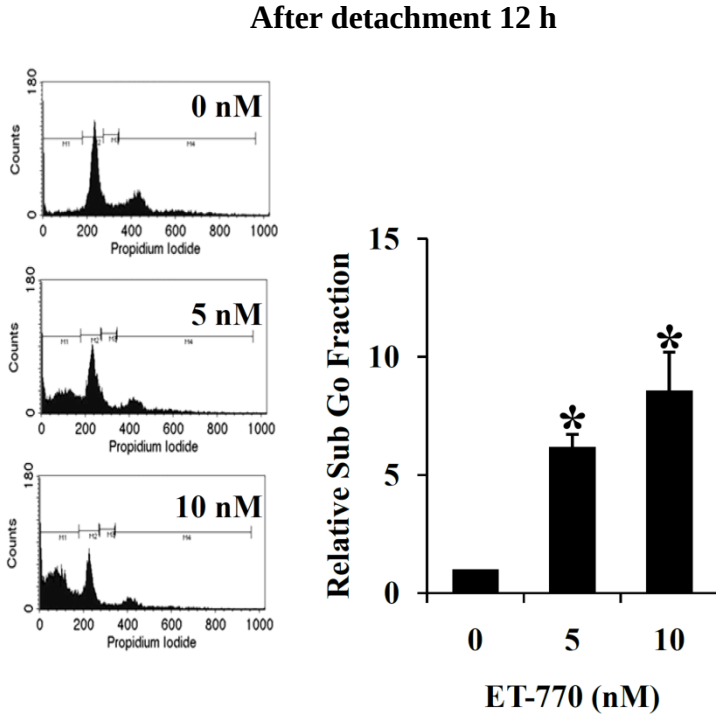


Figure 14 Ecteinascidin 770 (ET-770) induces anoikis sensitization of H460 human lung cancer cells. H460 cells were treated with 0–50 nM of ET-770 for 24 h. **A)** Cell viability was determined by 2, 3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt (XTT) assay. Values are means ± S.D. (n=3), **p*<0.05 *versus* non-treated controls. **B)** Detached cells were treated with 5 and 10 nM of ET-770 and cell viability was determined by 2,3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt (XTT) assay at the indicated times. Values are means ± S.D. (n=4), **p*<0.05 *versus* non-treated controls. **C)** Apoptosis and necrosis were detected by Hoechst 33342/PI staining assay at 12 h. **D)** Cells undergoing anoikis were evaluated in terms of the sub G_0 fraction. Values are means ± S.D. (n=3), **p*<0.05 *versus* non-treated controls at 12 h.

CHAPTER V

DISCUSSION AND CONCLUSION

So far, it is well accepted that metastasis is the dominant cause of death in many types of cancer, including lung cancer (Hanahan and Weinberg, 2000). Among multiple processes of cancer metastasis, the survival of cancer cells after detachment has dramatically gained attentions in cancer research (Igney and Krammer, 2002; Shore and Viallet, 2005; Dollé *et al*., 2006; Chiarugi and Giannoni, 2008; Labi *et al*., 2008; Simpson *et al*., 2008; Kim *et al*., 2012). Cell loss of attachment or inappropriate contact with the extracellular matrix or surrounding cells induces apoptosis termed anoikis, which is the primary biological process that prevents cancer metastasis (Frisch and Francis, 1994). As a hallmark of successful metastasis, anoikis resistance is considered an important capability of cancer cells required for spreading (Hanahan and Weinberg, 2000; Igney and Krammer, 2002; Dollé *et al*., 2006; Chiarugi and Giannoni, 2008; Simpson *et al*., 2008; Kim *et al*., 2012). Therefore, anoikis sensitization is interesting for cancer prevention as well as therapy.

The main machinery of anoikis involves intrinsic apoptosis signaling pathway in which the interaction between anti-apoptotic and pro-apoptotic proteins plays an essential role (Igney and Krammer, 2002; Chiarugi and Giannoni, 2008; Simpson *et al*., 2008; Kim *et al*., 2012). Among anti-apoptotic proteins in such a pathway, MCL1 has been shown to be the major protein in inhibition of anoikis in many types of cancer, and to be an important cause of anoikis resistance (Opferman *et al*., 2003; Zhong *et al*., 2005; Wacheck *et al*., 2006; Woods *et al*., 2007; Boisvert-Adamo *et al*.,

2009). Overexpression of CAV1 suppresses the anoikis response in H460 human lung cancer cells by up-regulation of MCL1 protein (Chunhacha *et al*., 2012), as well as CAV1 expression inhibits anoikis by inhibiting p53 activation and activating IGF receptor-mediated ERK and Akt signaling pathway upon cell detachment (Ravid *et al*., 2006). In terms of anoikis, cancer cell suppression of p53 activation, as well as a decrease of cellular pro-apoptotic protein, including BAX, was shown to be involved in anoikis resistance (Zhang et al., 2004; Ravid *et al*., 2005; Derksen *et al*., 2006). Consistent with, induction of p53 leads to reduce BCL2 expression (Hemann and Lowe, 2006) and down-regulates expression of the anti-apoptotic MCL1 protein induce apoptosis (Pietrzak and Puzianowska-Kuznicka, 2008), which turn into activates BAX function at mitochondria, inducing apoptosis. Taken together, our study suggests the model of anoikis resistance as shown in Figure 9. Thereby, agents that can enhance p53 function and/or deplete the level of proteins mediating anoikis resistance such as BCL2, MCL1 and CAV1, may be able to induce or sensitize anoikis and good candidates for anti-metastasis therapy.

ET-770, an ecteinascidin stabilized derivative isolated from a Thai tunicate, *E. thurstoni*, was demonstrated herein, to our knowledge for the first time to sensitize anoikis in human non-small lung cancer cells. Interestingly, H23 cells used in the present study exhibited characteristics of anoikis-resistance, with more than 60% of cell surviving after 24-h detachment. This response of the cells may be explained by the low level of p53 in cells under the detached condition (Figure 12A). ET-770 at non-toxic concentrations was able to increase its used at concentrations of 5-10 nM (Figure 12A) and such an increase of p53 was accompanied by the increase of BAX

and the decrease of MCL1, resulting in anoikis of these cells. Further, the anoikis sensitization of ET-770 was observed in H460 lung cancer cell.

Likely to ET-770, ET-743, a newly approved anti-cancer drugs, has been also reported to exhibit its function through p53 activation (Takebayashi *et al*., 2001). Nevertheless, p53 status does not appear to correlate to sensitivity to ET-743 because the anti-proliferative activity of ET-743 appears to be unalterated to either cell lines having wild type p53 or mutations of p53 (D'Incalci *et al*., 2002). Although ET-743 has more potent antitumor activity than ET-770 (Table 4), after treatment cells with 1 nM ET-743 for 24 h in attached condition, ET-743 does not alter an expression of BAX protein on human fibrosacroma cell HT-1080, liposacroma cell HS-18, and human colon cancer cell HT-29 (Li *et al*., 2001). These data probably suggest that ET-743 may sensitize H23 and H460 cells anoikis via p53 pathway with or without BAX protein alteration. However, only some compounds or drugs are used at sub-toxic concentrations can sensitize tumor cell anoikis (Songserm *et al*., 2012).

In conclusion, we thus provided insight of the molecular mechanism indicating that ET-770 sensitized H23 cell anoikis through a p53-dependent pathway, which in turn down-regulated anti-apoptotic MCL1, and increased pro-apoptotic BAX protein expression (Figure 15).

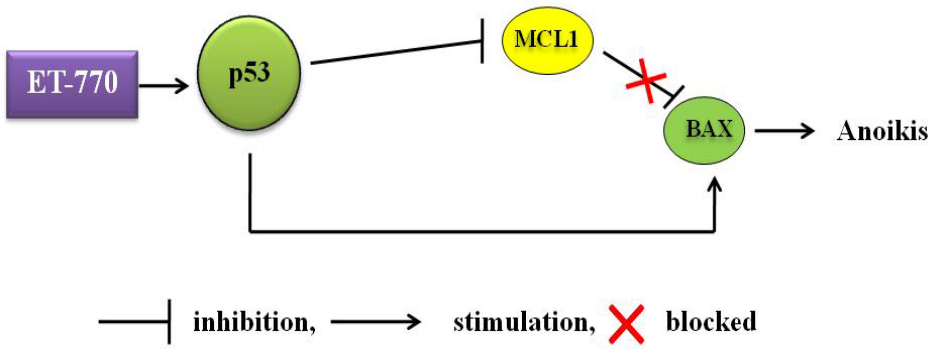


Figure 15 ET-770 sensitizes H23 cell anoikis through a p53-dependent pathway.

ET-770 may be a good candidate for anti-metastasis therapy. These findings may be beneficial for development of new approaches for cancer therapy to improve a patient's quality of life.

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Herdman, 1891 and *Clavelina cyclus* Tokioka & Nishikawa, 1975, in Thailand. Aquatic Invasions 4(2): 349-351.

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APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 5 The percentage of H23 cell viability was determined by XTT assay after treatment with various concentrations of ET-770 in dose-dependent manner.

ET-770 (nM)	Cell viability (%)
0	100.00 ± 1.23
0.5	100.66 ± 1.27
1	98.05 ± 2.14
5	99.03 ± 1.59
10	97.51 ± 2.35
50	66.52 ± 0.65*

Values are means ± S.D. (n=5); *$p < 0.05$ versus non-treated control.

Table 6 The percentage of apoptotic and necrotic cell deaths in attachment condition was determined by Hoechst 33342 and propidium iodide straining.

ET-770 (nM)	**Apoptotic cells (%)**	**Necrotic cells (%)**
0	4.37 ± 2.00	0.78 ± 0.30
0.5	5.69 ± 1.34	0.86 ± 0.20
1	5.77 ± 1.26	0.73 ± 0.33
5	4.22 ± 0.30	0.93 ± 0.12
10	4.77 ± 1.20	0.80 ± 0.36
50	39.52 ± 1.13*	3.92 ± 0.35*

Values are means ± S.D. (n=3); *$p < 0.05$ versus non-treated control.

Table 7 The percentage of H23 viability was determined by anoikis assay. Suspended cells were treated with various sub-toxic doses of ET-770 at various time points in a dose and time-dependent manner.

Conc. (nM)	**Cell viability (%)**					
	Time (h)					
	0	**3**	**6**	**9**	**12**	**24**
0	100±0.47	86.11±2.26	82.56±2.06	78.57±2.60	78.50±2.61	65.09±3.67
0.5	100±0.41	85.88±2.63	81.10±3.67	74.70±1.39	72.50±2.37	65.44±2.51
1	100±1.39	83.50±2.02	80.69±3.27	75.53±1.85	73.96±3.78	61.54±2.27
5	100±1.63	74.25±3.39*	74.57±3.76*	65.73±3.50*	64.34±2.14*	50.40±3.57*
10	100±1.11	65.57±1.25*	51.38±1.93*	48.47±1.13*	42.80±2.04*	25.56±1.50*
50	100±1.18	31.20±0.70*	18.49±3.15*	17.38±2.49*	15.86±1.01*	11.87±2.52*

Values are means ± S.D. (n=5); *$p < 0.05$ versus non-treated control.

Table 8 The percentage of apoptotic and necrotic cell death in detachment condition at time points 12 h was determined by Hoechst 33342 and propidium iodide straining on H23 cells.

ET-770 (nM)	**Apoptotic cells (%)**	**Necrotic cells (%)**
0	11.73 ± 1.79	1.22 ± 0.24
0.5	13.38 ± 1.77	1.25 ± 0.09
1	13.82 ± 0.93	1.29 ± 0.20
5	35.33 ± 1.38*	1.19 ± 0.17
10	52.83 ± 1.10*	1.24 ± 0.24
50	79.62 ± 1.08*	4.73 ± 0.46*

Values are means ± S.D. (n=3); *$p < 0.05$ versus non-treated control at 12 h.

Table 9 The percentage of sub G_0 fraction of apoptotic cells was evaluated by flow cytometry and propidium iodide in detachment condition at a time point 12 h.

ET-770 (nM)	**Relative Sub G_0 fraction**
0	1.00 ± 0.00
0.5	1.72 ± 0.46
1	2.54 ± 0.57
5	4.43 ± 0.34*
10	5.46 ± 0.44*
50	8.16 ± 0.49*

Values are means ± S.D. (n=3); *$p < 0.05$ versus non-treated control at 12 h.

Table 10 The relative protein level values over controls at 12 h after detachment on H23 cells.

Conc. (nM)	Relative protein levels					
	Types of protein					
	p53	**Mcl-1**	**Bcl-2**	**Bax**	**Cav-1**	**β-actin**
0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
0.5	1.70±0.22*	1.02±0.06	1.03±0.06	1.21±0.03*	0.98±0.01	1.02±0.03
1	1.85±0.35*	1.02±0.06	1.02±0.10	2.40±0.03*	0.97±0.06	1.00±0.22
5	5.50±0.20*	1.03±0.06	1.08±0.10	4.00±0.06*	1.00±0.05	0.99±0.04
10	7.30±0.29*	0.07±0.05*	1.06±0.09	4.50±0.07*	0.94±0.05	1.01±0.03
50	7.60±0.42*	0.05±0.06*	1.05±0.06	5.20±0.11*	0.96±0.07	0.98±0.06

Values are means ± S.D. (n=3); *$p < 0.05$ versus non-treated control at 12 h.

Table 11 The percentage of H460 cell viability was determined by XTT assay after treatment with various concentrations of ET-770 in a dose-dependent manner.

ET-770 (nM)	**Cell viability (%)**
0	100.00 ± 0.67
5	96.97 ± 0.88
10	95.23 ± 2.90
50	64.36 ± 2.45*

Values are means ± S.D. (n=5); *$p < 0.05$ versus non-treated control.

Table 12 The percentage of H460 viability was determined by anoikis assay. Suspended cells were treated with various sub-toxic doses of ET-770 at various times in a dose and time dependent manner.

Conc. (nM)	**Cell viability (%)**					
	Time (h)					
	0	**3**	**6**	**9**	**12**	**24**
0	100±0.89	87.29±3.22	77.36±1.94	76.35±2.35	72.67±6.42	57.98±5.12
5	100±0.73	43.55±3.11*	34.38±1.53*	34.60±1.06*	27.36±4.88*	25.11±3.18*
10	100±1.63	33.68±2.12*	25.69±2.28*	24.94±2.47*	22.11±3.10*	18.40±1.92*

Values are means ± S.D. (n=5); *$p < 0.05$ versus non-treated control.

Table 13 The percentage of apoptotic and necrotic cell deaths in detachment condition at a time point 12 h was determined by Hoechst 33342 and propidium iodide straining on H460 cells.

ET-770 (nM)	**Apoptotic cells (%)**	**Necrotic cells (%)**
0	13.00 ± 2.16	3.75 ± 3.55
5	39.76 ± 4.77*	5.08 ± 2.14
10	62.53 ± 4.85*	4.67 ± 2.41

Values are means ± S.D. (n=3); *$p < 0.05$ versus non-treated control at 12 h.

Table14 The percentage of sub G_0 fraction of apoptotic cells was evaluated by flow cytometry and propidium iodide in detachment condition on H460 cells at a time point 12 h.

ET-770 (nM)	**Relative Sub G_0 fraction**
0	1.00 ± 0.00
5	6.20 ± 0.52*
10	8.56± 1.64*

Values are means ± S.D. (n=3); *$p < 0.05$ versus non-treated control at 12 h.

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