ผลของการสัมผัสไนตริกออกไซด์ในระยะยาวต่อการดื้อยาเคมีบำบัด
ในเซลล์มะเร็งปอดชนิด เอช 292

นางปิยะพริสร ว่องวรานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON CHEMOTHERAPEUTIC RESISTANCE IN H292 LUNG CARCINOMA CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
Department of Pharmacology and Physiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2013

Thesis Title	EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON CHEMOTHERAPEUTIC RESISTANCE IN H292 LUNG CARCINOMA CELLS
By	Mrs. Piyaparisorn Wongvaranon
Field of Study	Pharmacology
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ปิยะพริสร ว่องวรานนท์ : ผลของการสัมผัสไนตริกออกไซด์ในระยะยาวต่อการดื้อยาเคมีบำบัดในเซลล์มะเร็งปอดชนิด เอช 292. (EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON CHEMOTHERAPEUTIC RESISTANCE IN H292 LUNG CARCINOMA CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ปิติ จันทร์วรโชติ, 83 หน้า.

ผลของการสัมผัสไนตริกออกไซด์ซึ่งเป็นสารที่พบว่ามักเพิ่มสูงขึ้นในบริเวณแวดล้อมของก้อนมะเร็งในระยะยาวต่อการดื้อยาเคมีบำบัดในเซลล์มะเร็งปอดยังไม่เป็นที่ทราบแน่ชัด จากการศึกษาพบว่าเมื่อเซลล์มะเร็งปอดสัมผัสไนตริกออกไซด์เป็นระยะเวลานาน 7-14 วันมีผลทำให้เซลล์มะเร็งปอดเกิดการดื้อต่อยาเคมีบำบัดชนิด ซิสพลาติน ด็อกโซรูบิซิน และอีโทโพไซด์ ในลักษณะที่ขึ้นกับความเข้มข้นและระยะเวลาที่ได้รับไนตริกออกไซด์ โดยพบว่ากลไกที่ทำให้เซลล์มะเร็งดื้อต่อยาเคมีบำบัดดังกล่าวเกี่ยวข้องกับกระบวนการปรับตัวของเซลล์โดยเพิ่มระดับของโปรตีนภายในเซลล์ที่ควบคุมกลไกการตายและการอยู่รอดของเซลล์ได้แก่ เพิ่มระดับโปรตีน caveolin-1 (Cav-1), เพิ่มระดับโปรตีน B-cell lymphoma 2 (Bcl-2) และเพิ่มระดับโปรตีน Protein kinase B (Akt) ที่อยู่ในสภาวะถูกกระตุ้น (Activated Akt) และจากการศึกษาผลของโปรตีนต่างๆที่เพิ่มขึ้นต่อการดื้อยาเคมีบำบัดโดยการทรานส์เฟกชันให้เซลล์มะเร็งปอดมีการแสดงออกของระดับโปรตีนที่ต้องการศึกษาในระดับที่แตกต่างกัน พบว่าเซลล์มะเร็งปอดที่มีการแสดงออกของระดับโปรตีน Protein kinase B (Akt) ที่อยู่ในสภาวะถูกกระตุ้นและระดับโปรตีน Bcl-2 ที่เพิ่มขึ้นมีผลต่อการดื้อยาเคมีบำบัดทั้ง 3 ชนิด คือ ซิสพลาติน ด็อกโซรูบิซิน และอีโทโพไซด์ ในขณะที่เซลล์มะเร็งปอดที่มีการแสดงออกของระดับโปรตีน Cav-1 ที่เพิ่มขึ้นมีผลต่อการดื้อยาด็อกโซรูบิซินและอีโทโพไซด์เท่านั้น และเป็นที่น่าสนใจว่าการดื้อยาที่เกิดขึ้นสามารถย้อนกลับได้เมื่อเลี้ยงเซลล์ต่อโดยไม่ได้สัมผัสไนตริกออกไซด์เป็นระยะเวลานาน 5 วัน โดยสรุปการศึกษานี้ทำให้ทราบบทบาทของไนตริกออกไซด์ที่อยู่ในบริเวณแวดล้อมของเซลล์มะเร็งในการลดการตอบสนองของเซลล์มะเร็งต่อยาเคมีบำบัดและอาจเป็นประโยชน์ในการหาแนวทางเพื่อพัฒนาวิธีการรักษาโรคได้

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556

5476213733 : MAJOR PHARMACOLOGY
KEYWORDS: CHEMOTHERAPEUTIC RESISTANCE / CISPLATIN / DOXORUBICIN / ETOPOSIDE / NITRIC OXIDE / LUNG CANCER / LONG-TERM

PIYAPARISORN WONGVARANON: EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON CHEMOTHERAPEUTIC RESISTANCE IN H292 LUNG CARCINOMA CELLS. ADVISOR: ASST. PROF. PITHI CHANVORACHOTE, Ph.D., 83 pp.

The effect of extended exposure of cancer cells to nitric oxide (NO), an endogenous mediator frequently found to be increased in a tumor area, is largely unknown. This study aimed to investigate the effect of NO treatment for 7-14 days on the susceptibility of lung cancer cells to chemotherapeutic agents, namely, cisplatin, doxorubicin, and etoposide. We report herein that long-term NO exposure for 7-14 days rendered the lung cancer cells resistant to chemotherapeutic agents, namely, cisplatin, doxorubicin, and etoposide, in dose- and time-dependent manners. The underlying mechanism was found to involve the adaptive responses of the cells, by increasing survival due to increase in the level of caveolin-1 (Cav-1) and anti-apoptotic Bcl-2, and up-regulation of activated Akt. The gene manipulation study revealed that the increase of activated Akt and Bcl-2 was responsible for the resistance to all tested drugs (cisplatin, doxorubicin, and etoposide), while the up-regulation of Cav-1 only attenuated cell death mediated by doxorubicin and etoposide. Interestingly, NO-mediated drug resistance was found to be reversible when the cells were further cultured in the absence of NO for 5 days. These findings reveal the novel role of NO presenting in the tumor environment in attenuating chemotherapeutic susceptibility and may be beneficial in contriving strategies to treat the disease.

Department: Pharmacology and Physiology
Field of Study: Pharmacology
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....

ACKNOWLEDGEMENTS

This thesis could not be successfully completed without the help and support of the kind people around me.

First of all, I would like to express my deepest gratitude to my advisor, Assistant Professor Pithi Chanvorachote, for his invaluable help, excellent advices, encouragements, guidance and supporting. Without his kindness and understanding, this thesis could not be accomplished.

Next, I would like to deeply thankful for all of staffs at the Departments of Pharmacology and Physiology, Chulalongkorn University, for their advices and helpfulness. Also, I would like to thank to the Research Instrument Center of Faculty of Pharmaceutical Sciences, Chulalongkorn University, for providing research instrument facilities.

I would like to express my sincere thanks to Dr. Varisa Pongrakhananon, Dr. Preedakorn Chunhacha, Dr. Chatchai Choatham, Mr. Wongsakorn Suchaoin, Miss Nuttida Yongsanguanchai, Miss Kanuengnit Choochuay, Mr. Ekkarat Wongpankam, Mr. Kanok Plaibua, Miss Arpasinee Sanuphan, Miss Thidarat Ruanghirun, Mr. Akkarawut Kowitdamrong, and other members in my laboratory for teaching, supporting, helping and constant encouragement throughout the course of this project.

Finally, I would like to express my truly gratitude to my parents, Mr.Sompong and Mrs.Jittawadee Magchoiy, for their best wishes and everything in my life. And the last gratefully special thanks to my husband , Kitthanaphum and my daughter, Sopitthauk, for their cheering me up and standing beside me all times even good or bad.

This work was supported by a research grant from The 90th Aniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University.

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LIST OF ABBERVIATIONS

%	= percentage
℃	= degree Celsius
µg	= microgram
µl	= microliter
µM	= micromolar
Akt	= protein kinase B (PKB)
ANOVA	= analysis of variance
Bax	= bcl-2-associated X protein
Bcl-2	= b-cell lymphoma 2
Cav-1	= caveolin-1
DMSO	= dimethyl sulfoxide
DPTA NONOate	= dipropylenetriamine NONOate
et al.	= et alibi, and others
FBS	= fetal bovine serum
g	= gram
h	= hour (s)
Mcl-1	= myeloid cell leukemia sequence 1
min	= minute (s)
ml	= milliliter
MTT	= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	= nitric oxide
NSCLC	= non-small cell lung cancer

OD	= optical density
pAkt	= phosphorylated Akt
PBS	= phosphate buffer saline
pH	= potential of hydrogen
PI	= propidium iodide
RPMI	= Roswell Park memorial institute medium
SCLC	= small cell lung cancer
shAkt	= Akt short hairpin (sh) RNA plasmid
shBcl-2	= Bcl-2 short hairpin (sh) RNA plasmid
shCav-1	= Cav-1 short hairpin (sh) RNA plasmid
TBS	= tris-buffered saline
TBST	= tris-buffered saline and tween 20
S.D.	= standard deviation
U	= unit

CHAPTER I
INTRODUCTION

Conferred resistance to chemotherapy has become a most interesting issue in the field of cancer research. As found in many types of cancer, an increase of survival pathways of cells facilitates the processes of carcinogenesis, and such pathways remain in malignant cells, potentiating their defenses against anticancer drugs, and increasing their aggressive behavior (Grille *et al.*, 2003; Brader and Eccles, 2004; McCubrey *et al.*, 2006; McKenzie and Kyprianou, 2006). Strong association was found between augmented survival signals with poor prognosis and resistance to chemotherapy (Hutchinson *et al.*, 2001; West, Sianna Castillo, and Dennis, 2002; Morishita *et al.*, 2012). In lung cancer, high mortality and low 5-year survival rates were found to be due to two key determinants; drug resistance and metastasis (Škarda, Hajdúch, and Kolek, 2008; Ray and Jablons, 2009). Most combination drugs include those which form DNA adducts, inhibit topoisomerase, and inhibit biosynthesis of macromolecules are frequently prescribed for treatment of lung cancer, however, the efficacy of such therapies was shown to be suppressed by innate (Eastman, 1991; Zeng-Rong *et al.*, 1995) or adaptive mechanisms of cancer cells (Eastman, 1991; Song *et al.*, 2006). The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway is a central mediator of cellular survival pathways and shown in a number of studies to increase drug resistance and aggressiveness of lung cancer (Matsuzaki *et al.*, 1999; Brognard *et al.*, 2001; Belyanskaya *et al.*, 2005; Tang *et al.*, 2006; Liu *et al.*, 2007). Likewise, amplification and overexpression of the B-cell lymphoma-2 (Bcl-2) proto-oncogene, or other anti-apoptotic members of the Bcl-2 family proteins, which occurs in many malignancies, including small cell lung

carcinoma, were shown to cause anticancer drug resistance by neutralizing the functions of pro-apoptotic Bcl-2 family members in apoptotic signaling (Ben-Ezra *et al.*, 1994; Ikegaki *et al.*, 1994). Recently, evidence has been reported of a significant impact of caveolin-1 (Cav-1), a main protein component of caveolae, on various lung cancer cell behaviors, including migration and invasion (Luanpitpong *et al.*, 2010), anoikis resistance (Rungtabnapa *et al.*, 2011; Chunhacha and Chanvorachote, 2012) and adhesion to endothelial surface (Chanvorachote and Chunhacha, 2013). Indeed, Cav-1 was shown to be up-regulated in many malignant tumors and was linked to poor prognosis of the diseases (Yoo *et al.*, 2003). Even though the increase of survival signals is accepted as an important mechanism by which cancer cells resist chemotherapeutic drugs, the molecular insights regarding upstream mediators of such survival augmentations are largely unknown.

Nitric oxide (NO) is widely accepted as one of the most important mediators in cell biology (Moncada, Palmer, and Higgs, 1991; Nathan, 1992; Butler and Williams, 1993). NO is synthesized from its substrates, namely L-arginine, NADPH and oxygen, by the catalytic activity of NO synthase enzymes. Studies indicated the presence of inducible NO synthase (iNOS), producing a relatively high level of NO in the micromolar rage (Beckman *et al.*, 1990) in many kinds of cancer (Thomsen *et al.*, 1994; Thomsen *et al.*, 1995; Franchi *et al.*, 2002). The activity of iNOS in cancer cells was shown to be enhanced in response to pro-inflammatory cytokines (Tsukahara *et al.*, 1998; Kooguchi *et al.*, 2002). Together with evidence indicating that NO is released by immune cells in the tumor area (Coleman, 2001), these data support previous findings that this biologically active molecule plays a significant role in

cancer pathology (Wiseman and Halliwell, 1996; Hunt *et al.*, 1998; Annane *et al.*, 2000).

In lung cancer, NOS produces a significant amount of NO in lung neoplasia (Puhakka *et al.*, 2003) and elevated NOS activity, as well as increased cancer-related NO production, were reported in patients with lung cancer (Arias-Diaz *et al.*, 1994; Fujimoto *et al.*, 1997; Liu *et al.*, 1998). The effects of NO on lung cancer cells differ, with both anticancer and cancer-promoting effects observed (Mannick *et al.*, 1994; Chung *et al.*, 2001; Kolb *et al.*, 2001; Chen *et al.*, 2005). Previously, we found that short-term treatment with NO resulted in the inhibition of Bcl-2 degradation and thus preserved the cells from Fas ligand (FasL)- (Chanvorachote *et al.*, 2005) and cis-diamminedichloroplatinum (cisplatin)-induced apoptosis (Chanvorachote *et al.*, 2006). On the contrary, NO has been shown to possess pro-apoptotic activity, inducing oxidative stress, caspase activation, and apoptosis (Cui *et al.*, 1994; Farias-Eisner *et al.*, 1994; Chlichlia *et al.*, 1998; Gansauge *et al.*, 1998; Klein and Ackerman, 2003). These controversial findings support the principle that the effect of NO depends on the cell type and cellular redox state, as well as on its concentration and flux (Kolb, 2000; Davis *et al.*, 2001).

With regard to cancer pathology, cancer cells are surrounded by a microenvironment which is rich in NO for prolonged periods of time (Tsukahara *et al.*, 1998; Annane *et al.*, 2000; Kooguchi *et al.*, 2002). The possible adaptive responses of the cells to long-term exposure to a low level of NO were investigated in the present study. Using pharmacological approaches, we determined the effect of long-term exposure of human lung cancer cells to NO on the expression of Bcl-2 family members and survival proteins, and on susceptibility of these cells to cisplatin,

doxorubicin, and etoposide-induced apoptosis. Our findings may be important for the explanation of chemotherapeutic resistance, and might be exploited in cancer chemotherapy.

Research questions

1. Dose long-term nitric oxide exposure have any effect on chemotherapeutic resistance in H292 lung carcinoma cells?
2. What is the underlying mechanism of long-term nitric oxide exposure on chemotherapeutic resistance in H292 lung carcinoma cells?

Hypothesis

Long-term nitric oxide exposure is able to induce chemotherapeutic resistance in H292 lung carcinoma cells by interfering apoptotic pathway which may alter expression of Bcl-2 family members and increase survival proteins.

Objectives

1. To determine the effect of long-term nitric oxide exposure on susceptibility of the cells to cisplatin, doxorubicin, and etoposide-induced apoptosis in H292 lung carcinoma cells.
2. To identify underlying mechanism(s) regarding chemotherapeutic resistance-mediated by long-term nitric oxide exposure in H292 lung carcinoma cells.

CHAPTER II

LITERATURE REVIEW

Lung cancer

Lung cancer is the most common cancer and also the leading cause of cancer deaths in the United States and worldwide (Parkin *et al.*, 2005; Siegel, Naishadham, and Jemal, 2013). In 2013, the incidence and mortality rates of lung cancer in the United States are both expected, accounting for about 14% of all new cancer cases and 27% of all cancer deaths (Figure 1).

Estimated New Cases*

	Males			Females	
Prostate	238,590	28%		Breast	232,340	29%
Lung & bronchus	118,080	14%		Lung & bronchus	110,110	14%
Colorectum	73,680	9%		Colorectum	69,140	9%
Urinary bladder	54,610	6%		Uterine corpus	49,560	6%
Melanoma of the skin	45,060	5%		Thyroid	45,310	6%
Kidney & renal pelvis	40,430	5%		Non-Hodgkin's lymphoma	32,140	4%
Non-Hodgkin's lymphoma	37,600	4%		Melanoma of the skin	31,630	4%
Oral cavity & pharynx	29,620	3%		Kidney & renal pelvis	24,720	3%
Leukemia	27,880	3%		Pancreas	22,480	3%
Pancreas	22,740	3%		Ovary	22,240	3%
All Sites	**854,790**	100%		**All sites**	**805,500**	100%

Estimated Deaths

	Males			Females	
Lung & bronchus	87,260	28%		Lung & bronchus	72,220	26%
Prostate	29,720	10%		Breast	39,620	14%
Colorectum	26,300	9%		Colorectum	24,530	9%
Pancreas	19,480	6%		Pancreas	18,980	7%
Liver & intrahepatic bile duct	14,890	5%		Ovary	14,030	5%
Leukemia	13,660	4%		Leukemia	10,060	4%
Esophagus	12,220	4%		Non-Hodgkin's lymphoma	8,430	3%
Urinary bladder	10,820	4%		Uterine corpus	8,190	3%
Non-Hodgkin's lymphoma	10,590	3%		Liver & intrahepatic bile duct	6,780	2%
Kidney & renal pelvis	8,780	3%		Brain & other nervous system	6,150	2%
All Sites	**306,920**	100%		**All sites**	**273,430**	100%

*Estimates are rounded to the nearest 10 and exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder.

Figure 1 Estimated new cancer cases and deaths for most cancer sites by sex in United State 2013 (Siegel *et al.*, 2013)

Lung cancer occurred from a series of DNA mutations that can be caused by many factors, such as cigarette smoke, breathing in asbestos fibers, and exposure to radon gas leading to uncontrolled growth of abnormal cells, and resulting in abnormal of lung function. The two major subtypes of lung cancer are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which are different in their behavior and treatment. Small cell lung cancer (SCLC) is less common and frequently found in the large airways of the lungs near the center. Smoking is most strongly correlated with the SCLC type. These cells grow quickly but often respond well to chemotherapy and radiotherapy rather than NSCLC (Collins *et al.*, 2007).

NSCLC, the most common type found in lung cancer patient, can be divided into three subtypes including adenocarcinoma, squamous-cell carcinoma, and large-cell lung cancer.

1. Adenocarcinoma is the most common types of NSCLC that usually arises in peripheral areas of the lungs. Adenocarcinomas account for about 40% of all lung cancers. While this type is associated with smoking, like other types, it is also observed as well in nonsmoker patients.

2. Squamous cell carcinoma (SCC) is also known as epidermoid carcinoma. SCC most frequently arises in the central chest area in the larger bronchi. This carcinoma accounts for about 30% of all lung cancers and more commonly found in men who have history of smoking than women.

3. Large-cell lung cancer (LCLC) is the least common type of NSCLC that frequently occurs in the outer areas of the lungs and tend to grow and spread very rapidly. These cancer cells have poor prognosis more than other type of NSCLC.

Treatments of lung cancer include surgery, radiation therapy, and chemotherapy either single or in combination (Spira and Ettinger, 2004). Chemotherapy is a "systemic treatment," meaning that it works to kill cancer cells anywhere in the body. Chemotherapy is used as the primary treatment in the advanced stage that is frequently found in lung cancer patients at the time of diagnosis. Most often, treatment for advanced stage NSCLC begins with either cisplatin or carboplatin in combination with another medication such as docetaxel, doxorubicin, etoposide, gemcitabine, paclitaxel, or vinorelbine (Socinski, 2004). Single regimen is sometimes used for people who might not tolerate combination chemotherapy well, such as those in poor overall health or elderly patients. Although the use of combination chemotherapy has been found to improve response rate rather than single agents alone, about 70% of patients have poor response to combination chemotherapy treatment or relapse after the end of treatment (Socinski, 2004). Moreover studies reported that up-regulation of survival signals have been observed in lung cancer cells especially in NSCLC and revealed the strong association between the augmented survival signals with poor prognosis and chemotherapeutic resistance (Brognard *et al.*, 2001; Tang *et al.*, 2006).

We are thus focusing on the molecular insights regarding an upstream mediator of such survival augmentations. Our findings may be important for the explanation of chemotherapeutic resistance, which might be exploited in cancer chemotherapy.

Chemotherapeutic resistance in lung cancer

In lung cancer, high mortality and low 5-year survival rates were found to be caused by two determinants; metastasis and chemotherapeutic resistance (Hanahan and Weinberg, 2000; Hoffman, Mauer, and Vokes, 2000; Ray *et al.*, 2009). Intrinsic and acquired resistances to chemotherapy are fundamental problems that limit the outcome of cancer treatments. Non-small cell lung cancer (NSCLC) cells are often intrinsically resistant (evident from the first course of therapy) to certain anticancer drugs, whereas small-cell lung cancer (SCLC) cells can acquire resistance (arising during the course of treatment) with continued administration of the drug (Shanker *et al.*, 2010).

Apoptosis and chemotherapeutic resistance

Apoptosis or the process of programmed cell death is essential in homeostasis of normal tissues as well as pathological processes (Wong, 2011), whereas necrosis, another process of cell death, is accidental cell death leading to inflammation. Apoptosis is characterized by distinct morphological characteristics, such as shrinkage of cell, membrane blebbing, chromatin condensation and DNA fragmentation, and cell molecular characteristics including activation of caspases and cleavage of cytoskeletal proteins (Elmore, 2007). There are two main pathways leading to apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Apoptosis has been known to play an important role in the development of chemotherapeutic resistance (Pommier *et al.*, 2004; Wong, 2011) and nearly all chemotherapy agents ultimately induce cancer cell death by the induction of apoptosis (Bold, Termuhlen, and McConkey, 1997; Hannun, 1997; Martin and Schwartz, 1997). Chemotherapy agents may use different cytotoxic mode of

actions to induce apoptosis of cancer cells. However, the end result of those is the activation of intrinsic apoptotic pathways (Pommier *et al.*, 2004; Wang and Lippard, 2005).

Since the important part of the success of chemotherapy is due to the efficiency in induction of cancer cells apoptosis (Bold *et al.*, 1997; Hannun, 1997), decreasing of the cellular apoptotic response by many means eventually lead to cancer cell survival and progression of the cancer (Pommier *et al.*, 2004).

Intrinsic apoptotic pathway and Bcl-2 family proteins

Intrinsic pathway has been reported in determination of cell survival. Most evidence highlights the importance of Bcl-2 family proteins as key regulators of mitochondrial membrane permeability (Chao and Korsmeyer, 1998). There are two groups of these proteins which are pro-apoptotic proteins (the proteins that act to promote cell death e.g. Bax, Bak, Bad, etc.) and anti-apoptotic proteins (the proteins that act to prevent cell death e.g. Bcl-2, Mcl-1, Bcl-x_L, etc.) (Figure 2).

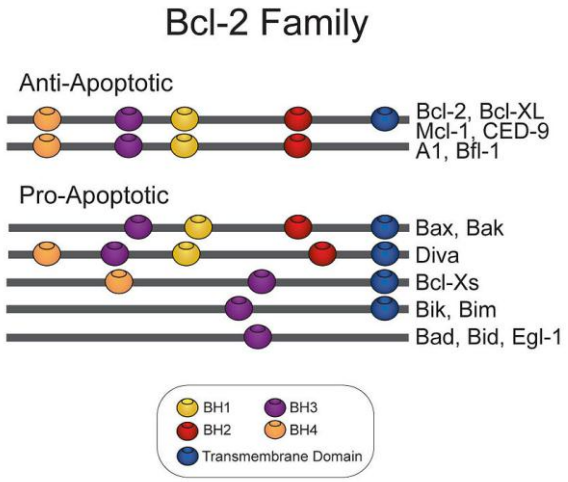


Figure 2 The members of Bcl-2 family (Chao *et al.*, 1998)

How do Bcl-2 family proteins work?

Indeed, pro- and anti-apoptotic members of the Bcl-2 family interact in order to regulate cellular apoptosis. The balance of these Bcl-2 family members determine the fate of cell whether survival or death. Shifting this balance by cellular stress (e.g. DNA damage, oxidative stress), resulting in the release of cytochrome c and other apoptotic inducing factors from mitochondria leading to the activation of the caspase cascade and the induction of apoptosis (Gross, McDonnell, and Korsmeyer, 1999). On the contrary, pro-survival stimulation would promote cellular survival by increasing the amount of anti-apoptotic proteins (Chipuk and Green, 2008) (Figure 3).

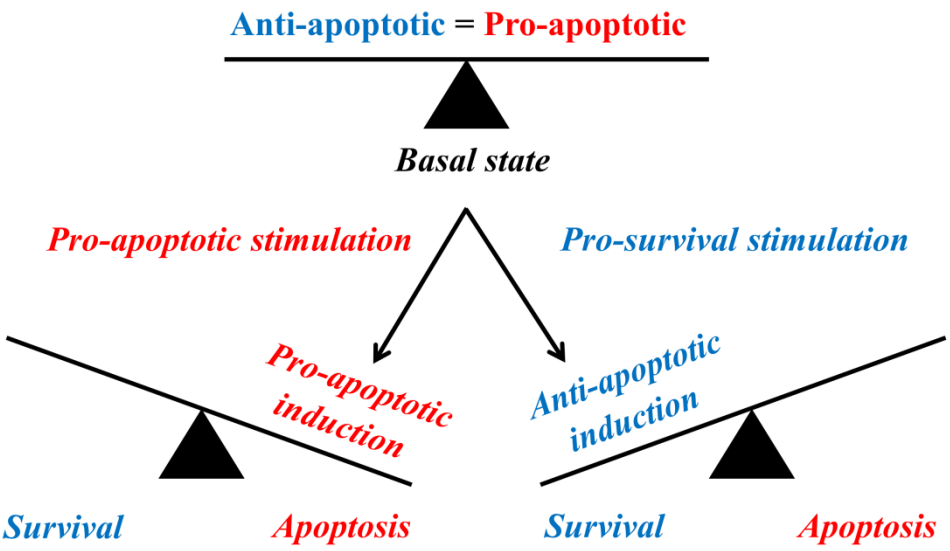


Figure 3 The balance of pro- and anti-apoptotic members of the Bcl-2 family dictates cellular survival (Chipuk *et al.*, 2008).

Molecular mechanisms of chemotherapeutic resistance

Failure to activate the process of programmed cell death or apoptosis represents an important mode of drug resistance in cancer cells. Disruption of cellular apoptotic signals is probably influenced by intrinsic properties of the cells such as an increase of anti-apoptotic proteins as well as up-regulation of survival-related cellular pathways such as Akt and caveolin-1 (Cav-1).

B-cell lymphoma-2 (Bcl-2)

Bcl-2, a member of anti-apoptotic Bcl-2 family proteins, acts to prevent apoptosis by heteromerization with Bcl-2-associated X protein (Bax). Anti-apoptotic proteins and Bax interaction prevents oligomerization of Bax and inhibits cytochrome c release (Gross *et al.*, 1999; Chipuk *et al.*, 2008; Shamas-Din *et al.*, 2013).

Overexpression of the Bcl-2 occurred in many different solid tumors including lung cancer (Ben-Ezra *et al.*, 1994; Ikegaki *et al.*, 1994). Upregulation of Bcl-2 has been found to prevent cell death induced by anticancer drugs (Miyashita and Reed, 1993; Dole *et al.*, 1994; Sartorius and Krammer, 2002; Cho *et al.*, 2006). Ectopic expression of Bcl-2 increased resistance to drug-induced apoptosis in lung cancer cell lines (Ohmori *et al.*, 1993), whereas downregulation of Bcl-2 expression by a small interfering RNA sensitized cancer cells to cisplatin (Losert *et al.*, 2007). Furthermore, inhibition of ubiquitination and degradation of Bcl-2 promoted resistance to cisplatin-induced cell death in NSCLC cells (Chanvorachote *et al.*, 2006).

These evidences have provided conclusive evidence that elevations in Bcl-2 expression may, at least partly, cause resistance to chemotherapeutic drugs, while reductions in this protein can sensitize cancer cells to apoptosis induced by chemotherapy agents.

Myeloid cell leukemia sequence 1 (Mcl-1)

Mcl-1, another anti-apoptotic member of Bcl-2 family proteins, can also inhibit Bax conformational change at mitochondrial membrane. Lung cancer cells constitutively expressing human Mcl-1 were found resistant to apoptosis induced by several chemotherapeutic drugs including cisplatin, etoposide, paclitaxel and gefitinib, whereas depletion of Mcl-1 levels by antisense Mcl-1 oligonucleotides induced apoptosis as well as sensitized such cells to apoptosis induced by chemotherapy agents (Song *et al.*, 2005).

Bcl-2-associated X protein (Bax)

Bax, a pro-apoptotic protein in Bcl-2 family members, is a crucial mediator of the mitochondrial pathway. In response to stimulation by death stimuli, Bax translocation to mitochondria is required for the release of cytochrome c and apoptosis. Blocking Bax translocation to the mitochondria contributes to apoptosis resistance.

In previous study, upregulation of Bcl-2 inhibited cisplatin-induced Bax translocation and contributed to cisplatin-resistance in human bladder cancer cells. In contrast, downregulation of Bcl-2 by siRNA potentiated the translocation of Bax and reversed the resistance to cisplatin (Cho *et al.*, 2006). In addition, downregulation of Bax significantly attenuated cytochrome c release, and decreased cisplatin-induced apoptosis in ovarian cancer cells (Yang *et al.*, 2012). In lung cancer, low expression of Bax was significantly associated with poor prognosis in NSCLC patients treated with surgical resection (Jeong *et al.*, 2008).

Protein kinase B (Akt)

Akt, a 57 kD serine/threonine kinase, is a downstream effector of PI3K via a multistep process as known as PI3K/Akt signaling pathway. Akt is shown to regulate diverse cellular processes including cell survival, proliferation, growth and migration (Osaki, Oshimura, and Ito, 2004).

Focusing on chemotherapeutic response, the PI3K/Akt acts as a central mediator of cellular survival pathways and attenuated overall response of the cells to death stimuli. Akt has been implicated as an anti-apoptotic factor in many different kinds of apoptotic stimuli, including chemotherapeutic drugs, resulting in delay of cell death or a resistance to chemotherapeutic drugs (Fresno Vara *et al.*, 2004). In many cancers, the components of PI3K/Akt signaling pathway are shown to be frequently up-regulated (Brader *et al.*, 2004) and these increasing are found to associate with poor prognosis and chemotherapeutic resistance (Morishita *et al.*, 2012). For lung cancer, overexpression of phosphorylated Akt (p-Akt) is found compared to the low level in normal lung tissue (Tang *et al.*, 2006). The up-regulation of activated Akt is demonstrated to promote resistance to chemotherapy in this type of cancer (Brognard *et al.*, 2001; Hovelmann, Beckers, and Schmidt, 2004).

Caveolin-1 (Cav-1)

Cav-1 is a main protein component of caveolae. Although role of Cav-1 in cancer is quite diverse, recent evidences have agreed that Cav-1 may play a role as cancer promoting protein. Several studies have reported that the up-regulation of Cav-1 in many malignant tumors was linked to the poor prognosis of the diseases (Yoo *et al.*, 2003).

Recently, significant impact of the Cav-1 on various lung cancer cell behaviors including migration and invasion (Luanpitpong *et al.*, 2010), anoikis resistance (Chanvorachote *et al.*, 2009; Rungtabnapa *et al.*, 2011; Chunhacha *et al.*, 2012) and adhesion to endothelial surface (Chanvorachote *et al.*, 2013) is reported. Moreover, Cav-1 is shown to involve in chemotherapeutic resistance. For example, Cav-1 expression was significantly correlated with chemotherapy response and a poor prognosis in advanced lung cancer patients treated with gemcitabine-based chemotherapy (Ho *et al.*, 2008). Furthermore, ectopic Cav-1 expression in Ewing's sarcoma cells increased resistance to doxorubicin- and cisplatin-induced apoptosis, whereas Cav-1 knockdown led to sensitized Ewing's sarcoma cells to doxorubicin and cisplatin (Tirado *et al.*, 2010).

Taken together, mediators that can up-regulate Bcl-2 or other anti-apoptosis members of Bcl-2 family proteins and/or activate survival signaling such as Akt or caveolin-1 may be able to induce chemotherapeutic resistance.

Nitric Oxide (NO)

Biochemistry of nitric oxide

Nitric oxide (NO) is a ubiquitous, water-soluble, free radical gas that exerts a wide range of biological effects. NO is generated by the oxidation of the amino acid L-Arginine under the catalytic activity of the Nitric Oxide Synthases (NOSs) isoenzymes. This reaction requires NADPH and O_2 as co-substrates and yields NO and L-citrulline as end products (Moncada *et al.*, 1991) (Figure 4).

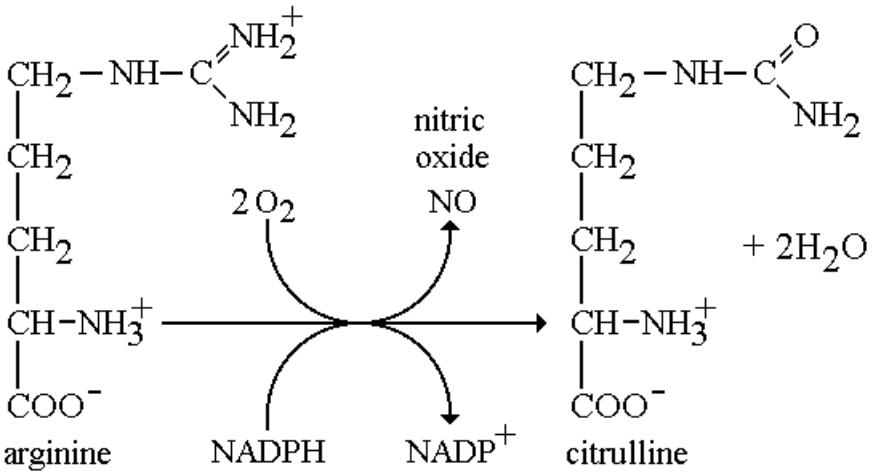


Figure 4 Nitric oxide synthesis (Nathan, 1992; Nathan and Xie, 1994)

There are three major isoforms of nitric oxide synthases (NOS), namely, neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3) and inducible NOS (iNOS or NOS2) (Nathan, 1992). All three isoforms can be found in a variety of tissues and cell types. While nNOS and eNOS are constitutively expressed in mammalian cells and Ca++/CaM-dependent, iNOS is expressed in various cell types, including macrophages, hepatocytes, osteoclasts, dendritic cells, astrocytes and epithelial cells and can be induced in cells independent of Ca^{++}/CaM when stimulated by various inflammatory ligands such as TNF-α, interferon (INF)-λ, interleukin-1 (IL-1), endotoxin, hypoxia and lipopolysaccharides. NO produced from nNOS and eNOS exerts its

biological function via the cGMP-mediated pathway. However, NO generated by the catalytic action of iNOS leads to biological and pathological functions, which are largely cGMP-independent.

NO production in lung cancer

The microenvironment of cancer cells under inflammatory conditions, all of the key cellular participants (e.g. neutrophils, macrophages, endothelial cells, and epithelial cells) can express iNOS (Nathan and Hibbs, 1991; Robbins *et al.*, 1994; Guo *et al.*, 1995). Neutrophils and macrophages express high levels of iNOS and are important sources of iNOS-derived NO. NO production from iNOS lasts much longer than from the other isoforms of NOS, and tends to produce much higher concentrations of NO in the cells. However, NO can passive diffuse through the cell membrane, thus the cancer cells are immerged in the microenvironment which is rich in NO for prolonged period of time (Hibbs *et al.*, 1988; McCall *et al.*, 1989; Munoz-Fernandez, Fernandez, and Fresno, 1992; Amin *et al.*, 1995; Evans *et al.*, 1996; Tsukahara *et al.*, 1998; Annane *et al.*, 2000; Boyle, Weissberg, and Bennett, 2002; Kooguchi *et al.*, 2002). And especially in lung cancer, several studies have documented the observation of NO production in lung neoplasia (Puhakka *et al.*, 2003) and increased cancer-related NO production were reported in lung cancer patients. Furthermore, elevated NO production has also been observed in either lung or other cancer cells, particularly in comparison to the surrounding normal cells (Arias-Diaz *et al.*, 1994; Fujimoto *et al.*, 1997; Liu *et al.*, 1998).

Role of NO in apoptosis

NO has also been demonstrated to be involved in the regulation of apoptosis. NO has been shown to be able to both induce apoptosis (known as pro apoptotic effect) and protect from apoptosis (known as anti-apoptotic effect) in different cell types. The effects of NO on apoptosis may vary depending upon the concentration of NO, type of cell used, and redox condition of cells. Endogenous NO production or exposure to appropriate amounts of NO has been reported to inhibit apoptosis in several cell types (Chung *et al.*, 2001). Likewise, NO has been shown to inhibit cell death induced by a variety of agents including chemotherapeutic agents, viral infections, and death ligands (Mannick *et al.*, 1994; Kolb, 2000; Chen *et al.*, 2005; Chanvorachote *et al.*, 2006). Thus, NO seems to play a pivotal role in the multiple cell death/survival signaling pathways and development of apoptosis resistance.

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Reagents

Cisplatin, Hoechst 33342, propidium iodide (PI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and Annexin V-FITC were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Dipropylenetriamine (DPTA) NONOate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Doxorubicin and etoposide were from Calbiochem (San Diego, CA, USA). Antibodies for Bcl-2, Cav-1, MCl-1, pAkt, Akt, ß-actin and peroxidase-labeled secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2. Instruments

- Automated cell counter (TC10TM Bio-Rad Laboratories, Inc., USA)
- Autopipette: 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl (Gilson, Inc., France)
- Cell culture plate: 6-well and 96-well (Corning, Inc., USA)
- Centrifuge (Z 383 Hermle labortechnik, Germany)
- Flow cytometer (FACSort, Becton Dickinson, Rutherford, NJ, USA)
- Fluorescence microplate reader (SpectraMax® M5, Molecular Devices)
- Fluorescence microscope (Olympus IX51 with DP70, NY)
- Humidified incubator (311 Thermo scientific, UK)
- Laminar flow cabinet (Bosstech scientific instruments, USA)
- Light microscope (Eclipse TS100 Nikon, Japan)

- Microplate reader (Wallac 1420 Perskin Elmer, USA)
- Vertex mixer (Scientific Industries, Inc. USA)
- pH meter (CG 842 Schott instruments, Germany)

3. Sample preparation

DPTA NONOate was dissolved in PBS to prepare 100 mM stock solution before it was diluted for each of working concentrations. Cisplatin and doxorubicin were prepared by diluting with deionized water to obtain the desired concentrations. Etoposide was prepared by diluting with DMSO and complete media to obtain the desired concentrations.

4. Cells culture

Human lung cancer H292 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin. Cells were trypsinized when cells reached 80–90% confluence. For long-term NO exposure experiments, cells were cultured in medium containing DPTA NONOate (non-toxic concentrations) for 7 days and 14 days. The culturing medium was replaced by freshly prepared medium containing DPTA NONOate every 3 days. All cells were grown in humidified incubators containing an atmosphere of 5% CO_2 and 95% air at 37°C. The schematic diagram of long-term nitric oxide exposure experiments is shown in figure 5

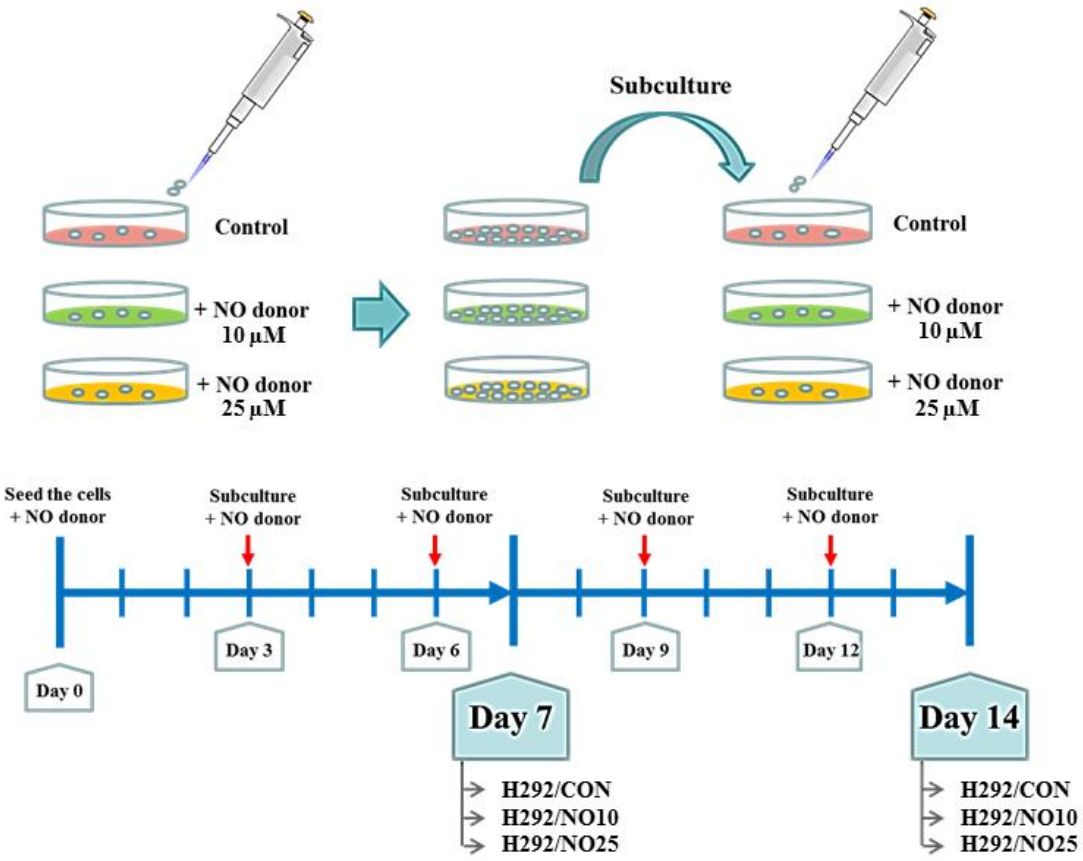


Figure 5 Long-term nitric oxide exposure experiments

5. Experimental design

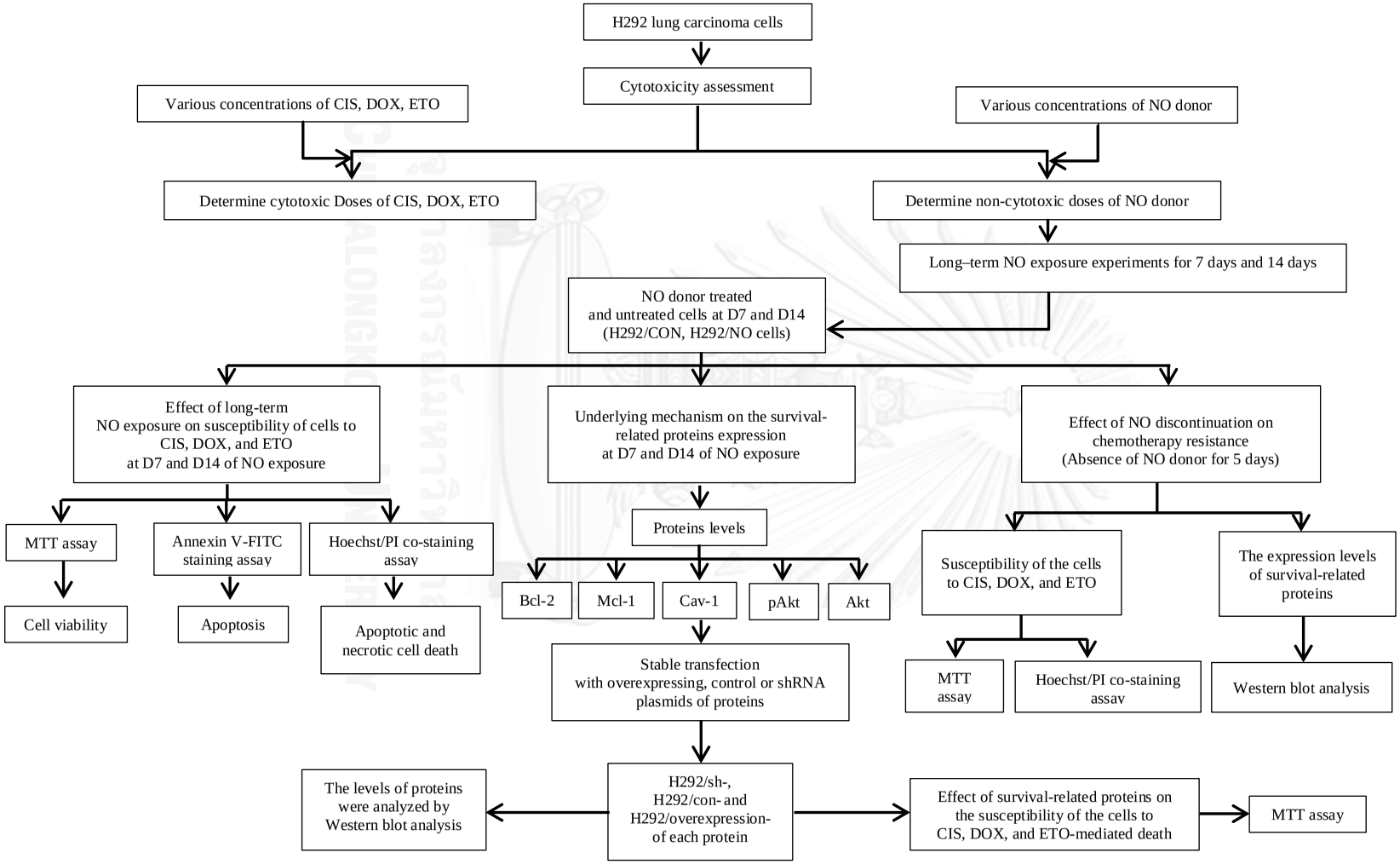


Figure 6 Experimental design of this study

5.1. Investigation on the cytotoxic effects of NO donor on cell viability in human lung cancer H292 cells

To determine non-toxic concentrations of NO donor for subsequent experiments, cell viability was analyzed by MTT colorimetric assay at 24, 48, and 72 h. MTT assay measures cellular capacity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) to purple formazan product by mitochondria dehydrogenase enzyme. Cells were seeded at 1×10^4 cells per well in 96-well plates for 12 h. Cells were treated with various concentrations of DPTA NONOate (0-200 μM) and incubated for 24, 48, and 72 h, respectively. Cells were incubated with 500 μg/ml of MTT at 37°C for 4 h. Then, MTT solution is removed and 100 μl of DMSO is added to dissolve the formazan product. The intensity of MTT product was measured at 570 nm using a microplate reader, and the percentage of viable cells was calculated relative to that of control cells.

To confirm non-toxic effect of NO donor, apoptotic and necrotic cell death were detected by Hoechst 33342 and PI co-staining assay. NO donor-treated cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml of PI for 30 min at 37°C. Nuclei condensation and/or DNA fragmentation of apoptotic cells and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70) after 24 h of treatment.

5.2. Characterization of H292 cell death induced by cisplatin, doxorubicin, or etoposide

Cell death induced by cisplatin, doxorubicin, or etoposide was characterized in H292 cells. The lethal concentrations (LC) of each drug were determined in order to obtain the optimum concentrations used for further experiments. Cells were seeded at 1 x 10^4 cells per well in 96-well plates for 12 h. Cells were treated with various concentrations of cisplatin (0-200 μM), doxorubicin (0-10 μM), or etoposide (0-100 μM), and incubated for 24 h. Then, cell viability was investigated by MTT assay. Apoptotic and necrotic cell death were detected by Hoechst 33342 and PI co-staining assay.

5.3. Investigation on chemotherapeutic resistance induced by long-term NO exposure in human lung cancer H292 cells

To investigate the effect of long-term exposure to NO on chemotherapeutic resistance, the susceptibility of the cells to cisplatin, doxorubicin, and etoposide-induced apoptosis after long-term NO exposure experiments were determined. At day 7 and day 14 after long-term NO exposure experiments, DPTA NONOate-treated and untreated cells were seeded at 1 x 10^4 cells per well in 96-well plates for 12 h. Cells were treated with various concentrations of cisplatin, doxorubicin, and etoposide and incubated for 24 h. Then, cell viability was investigated by MTT assay. Apoptotic and necrotic cell death were determined by Hoechst 33342 and PI co-staining assay. Annexin V-FITC staining assay was also used to detect apoptosis. Briefly, after specific treatments, cells were collected, re-suspended, and incubated with Annexin V-FITC for 30 min at room temperature, and cells were scored as apoptosis by flow cytometry using a 488-nm excitation beam and a 538-nm

band-pass filter (FACSort; Becton Dickinson, Rutherford, NJ, USA). The mean fluorescence intensity was quantified by CellQuest software (Becton Dickinson).

5.4. Investigation on underlying mechanism regarding chemotherapeutic resistance mediated by long-term NO exposure in human lung cancer H292 cells

To identify the possible underlying mechanism regarding chemotherapeutic resistance mediated by long-term NO exposure in H292 cells, the expression levels of survival-related proteins that play the prominent role on chemoresistance were focused. Western blot analysis was performed to evaluate p-Akt, Akt, Mcl-1, Bcl-2, BAX and Cav-1 expression. At day 7 and day 14 after long-term NO donor exposure experiments, 1 x 10^6 DPTA NONOate treated and untreated cells were collected and incubated in lysis buffer containing 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 protease inhibitor cocktail (Roche Molecular Biochemicals) for 40 minutes on ice. The cell lysates were collected by centrifugation at 12000 rpm for 15 minutes at 4 ℃ and the protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins from each sample (60 µg) were denatured by heating at 95℃ for 5 min with Laemmli loading buffer and subsequently loaded onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis. After separation, proteins were transferred onto 0.45 µM nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST [25 mM Tris-HCl (pH 7.5), 125 mM NaCl, and 0.05% Tween 20] and incubated with the appropriate primary antibodies at 4℃ overnight. The membranes were then

washed twice with TBST for 10 min and incubated with horseradish peroxidase-labeled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by chemiluminescence substrate (Supersignal West Pico; Pierce, Rockfore, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad). The ß-actin was used as a loading of controls each treatments.

To test whether the increasing of survival-related proteins mediated by long-term NO exposure are involved in resistance to apoptosis induced by cisplatin, doxorubicin, and etoposide, stable transfections of overexpression, control, or knockdown plasmids of the survival-related proteins were generated and the effect of each protein on the susceptibility of the cells to chemotherapeutic drugs-mediated death were determined. Cav-1 expression plasmid was obtained from the American Type Culture Collection (ATCC). Cav-1, Bcl-2, and Akt knockdown plasmids (shRNA) were obtained from Santa Cruz Biotechnology. The Akt expression plasmid was obtained from Addgene (Cambridge, MA, USA). The Bcl-2 plasmid was generously provided by Dr. C. Stehlik (West Virginia University Cancer Center, Morgantown, WV, USA). Stably transfected cells were generated by culturing H292 cells in a six-well plate until they reached 60% confluence. Fifteen microliters of Lipofectamine 2000 reagent and 2 µg of overexpression, control, or shRNA plasmids of each protein were used to transfect the cells in the absence of serum. After 12 h, the medium was replaced with culture medium containing 5% FBS. Approximately 36 h after the beginning of transfection, the cells were digested with 0.03% trypsin, plated onto 75-cm^2 culture flasks, and cultured for 24 to 28 days with antibiotic selection. The stable transfectants were pooled, and the expressions of the survival-related proteins in the transfectants were confirmed by western blotting. The cells were cultured in

antibiotic-free RPMI 1640 medium for at least two passages before use in each experiment. Then, stably transfected cells were left untreated or treated with cisplatin, doxorubicin, or etoposide, and the cell viability at 24 h was measured by MTT assay.

5.5. Investigation on reversible effect of NO-mediated chemoresistance in the condition of NO discontinuation

To examine whether the effect of NO-mediated chemoresistance could be reversible in the absence of NO donor, chemotherapeutic susceptibility and the expression levels of the survival-related proteins were determined. H292 cells after 14 days of NO exposure were cultured in the absence of NO donor for 5 days. The cells were treated with cisplatin, doxorubicin, or etoposide for 24 h. Cell survival was determined by MTT assay and apoptosis cells were evaluated by Hoechst 33342 staining. The expression levels of the survival-related proteins were determined by western blotting as described previously.

6. Statistical analysis

Mean data from at least three independent experiments were normalized to the results for the control cells. Statistical differences between means were determined using an analysis of variance (ANOVA) and *post hoc* test at a significance level of $p < 0.05$, and data are presented as the mean ± SD.

7. Conceptual framework

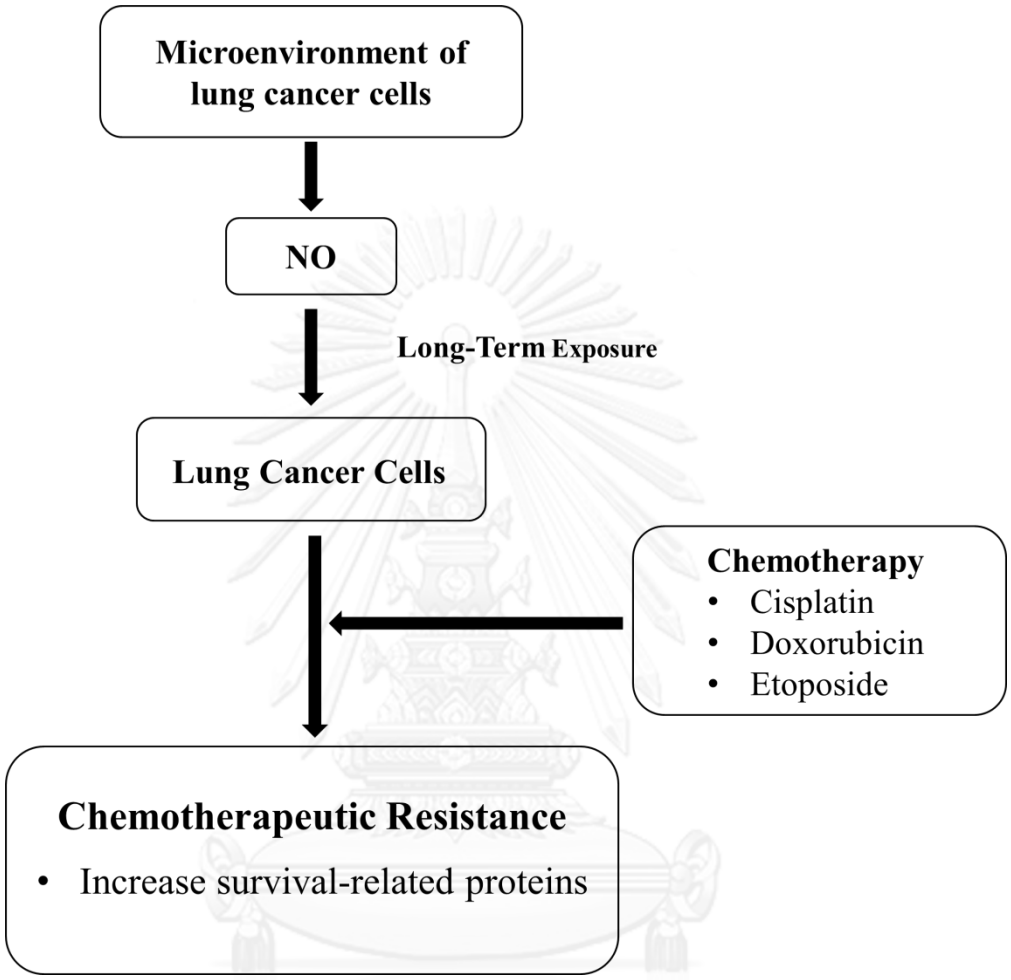


Figure 7 Conceptual framework of this study

CHAPTER IV

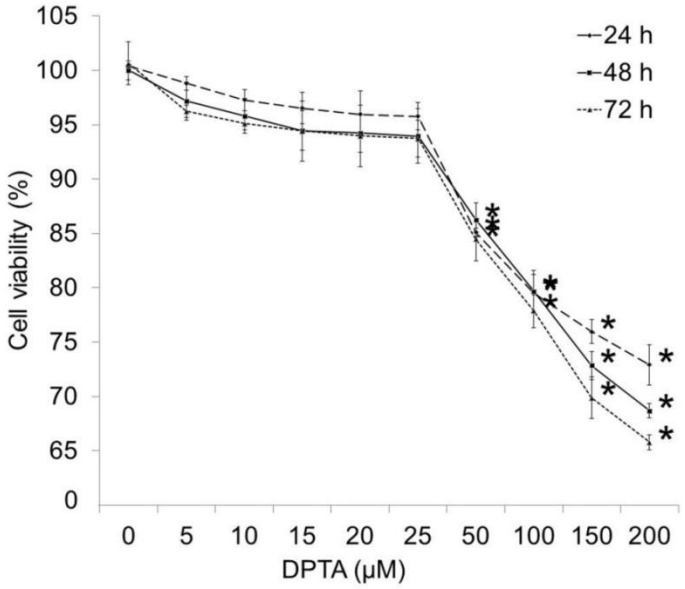
RESULTS

1. Effect of nitric oxide donor on cell viability of human lung cancer H292 cells.

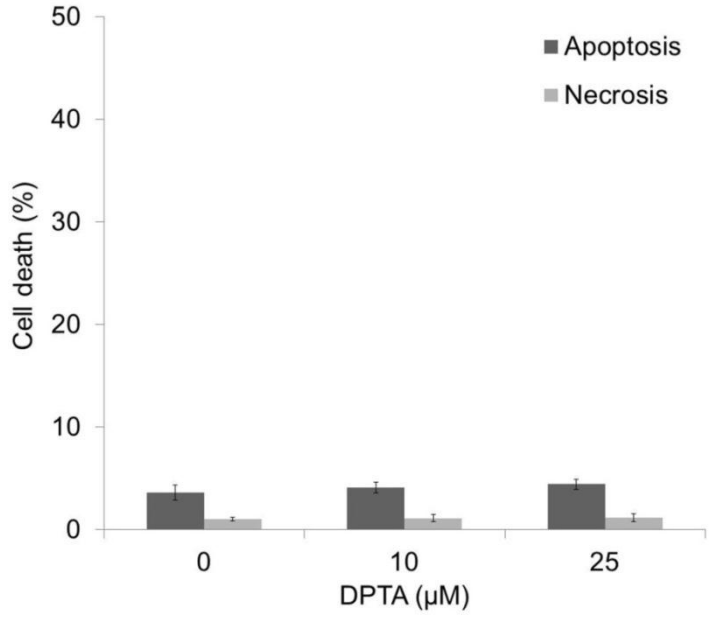
We first characterized the effects of NO donor on cell viability and selected the concentrations that caused no significant effect on viability of the cells for further experiments. Cells were cultured in the presence or absence of DPTA NONOate (5-200 μM), a slow-releasing NO donor, and cell viability was determined after 24-72 h.

The results showed that treatment of the cells with DPTA NONOate at the concentrations 5-25 μM caused neither cytotoxicity nor proliferative effects on the cells (Figure 8A). A significant decrease of cell viability was first detected in cells treated with 50 μM for 24 h, when approximately 85% of the cells remained alive. The concentrations of NO donor at 10 and 25 μM were selected and their non-toxic effect was confirmed by apoptosis and necrosis detection. Figures 8B and C show that treatment of the cells with 10 and 25 μM caused no significant alteration in terms of apoptosis and necrosis in comparison to those of the non-treated control.

A


105
100
95
90
85
80
75
70
65
0
Cell viability (%)
24 h
48 h
72 h
0
5
10
15
20
25
50
100
150
200
DPTA (μM)

B


50
40
30
20
10
0
Cell death (%)
Apoptosis
Necrosis
0
10
25
DPTA (μM)

C

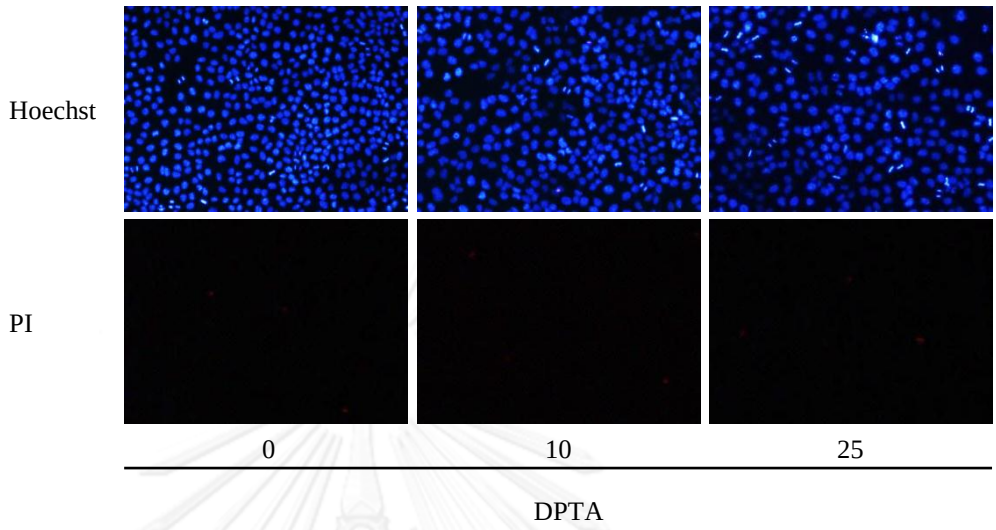


Figure 8 Effect of NO donor on cell viability of human lung cancer H292 cells. Cells were treated with various concentrations of DPTA NONOate (0-200 μM) for 24, 48 and 72 h. (A) Cell viability was determined by MTT assay. (B) Apoptotic and necrotic cell death were determined by Hoechst 33342 and PI co-staining assay. (C) Apoptotic and necrotic cells were visualized under a fluorescence microscope. Values are means ± SD (n = 3); *$p < 0.05$ versus non-treated control cells.

2. Effect of chemotherapeutic agents on cell death in H292 lung cancer cells

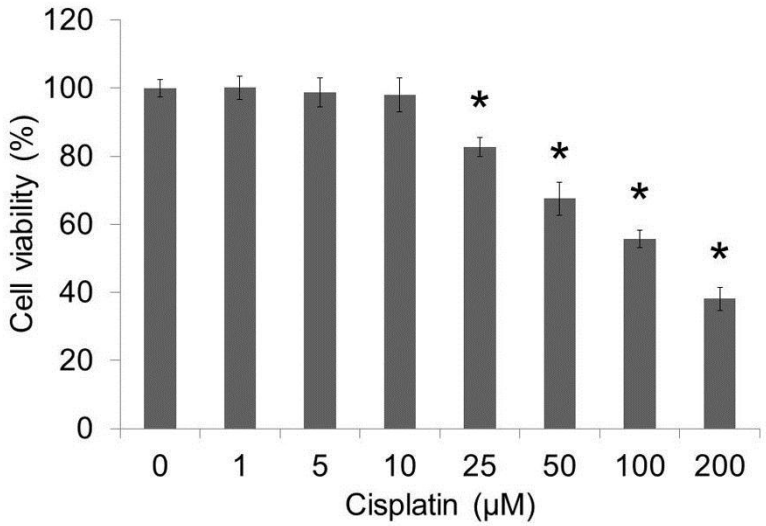
To characterize cell death induced by cisplatin, doxorubicin, and etoposide, the effect of these chemotherapeutic agents on cell viability in H292 cells was examined by MTT assay. Apoptotic and necrotic cell death were also detected by Hoechst 33342 and PI co-staining assay. H292 cells were treated with various concentrations of cisplatin (0-200 μM), doxorubicin (0-10 μM), or etoposide (0-100 μM), and incubated for 24 h. Then, MTT assay and Hoechst 33342 and PI co-staining assay were performed.

2.1. Effect of cisplatin on cell death in H292 lung cancer cells

At 24 h after treatment, cell viability was significantly decreased in response to cisplatin at the concentrations of 25, 50, and 100 μM with approximately 40% reduction in cell viability was observed in the 100 μM cisplatin-treated group (Figure 9A). Also, treatment of the cells with cisplatin showed that the main mode of cell death was apoptosis, as indicated by chromatin condensation and/or nuclear fragmentation detected with Hoechst 33342. While the PI-positive necrotic cell death was undetected (Figure 9B). The results indicated that cisplatin reduced the viability of H292 cells in a dose-dependent manner.

Since 25-100 μM of cisplatin significantly decreased cell viability of H292 cells, these doses would be used for further experiments to determine the effect of long-term NO exposure on susceptibility of the cells to this drug.

A



B

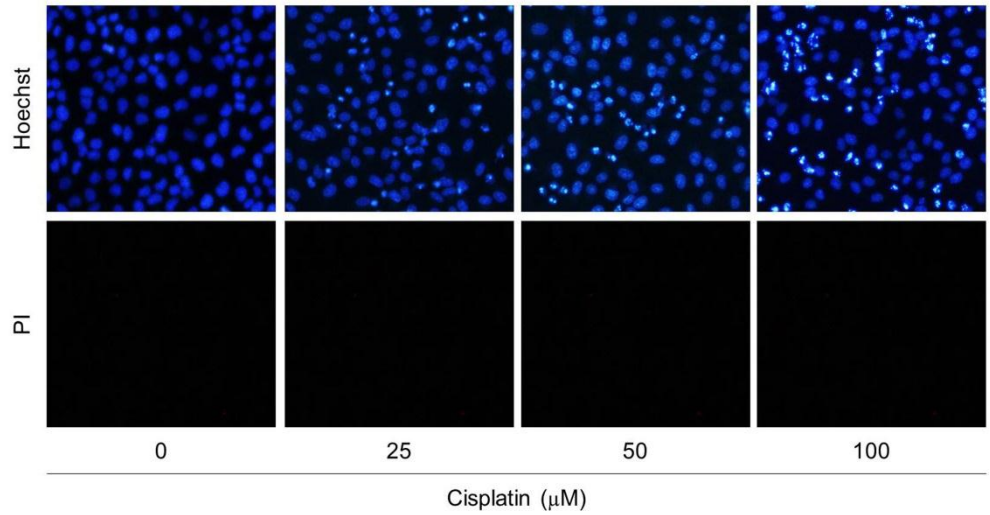
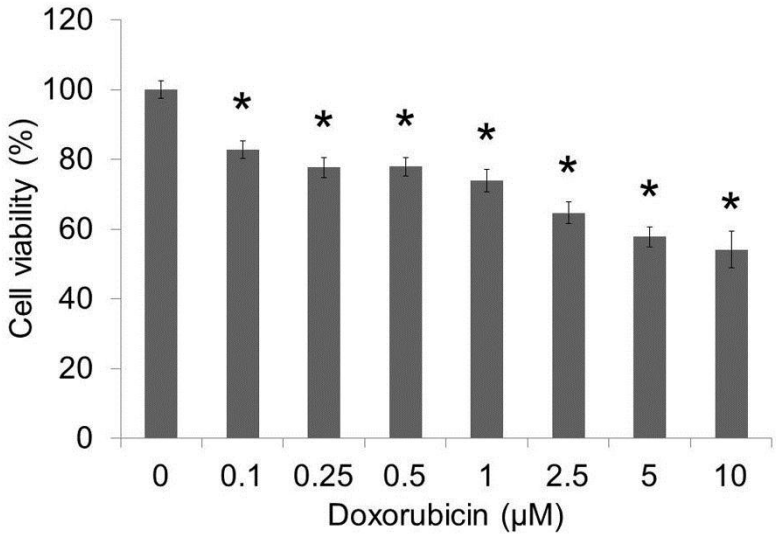


Figure 9 Effect of various concentrations of cisplatin on cell death in H292 cells. (A) Cells were treated with various concentrations of cisplatin (0-200 μM). After 24 h, cell viability was determined by MTT assay. Values are means ± SD (n = 3); *$p < 0.05$ versus non-treated control cells. (B) Apoptotic and necrotic cell death were observed under a fluorescence microscope by Hoechst 33342 and PI co-staining assay.

2.2. Effect of doxorubicin on cell death in H292 lung cancer cells

At 24 h after treatment, cell viability was significantly decreased in response to doxorubicin at all concentrations range from 0.1-10 μM with approximately 40% reduction in cell viability was observed in the 5 μM doxorubicin-treated group (Figure 10A). Doxorubicin at the concentrations of 1, 2.5, and 5 μM would be used for further experiments. The results showed that doxorubicin reduced the viability of H292 cells in a dose-dependent manner. In addition, doxorubicin-mediated apoptosis was observed by Hoechst 33342 staining (Figure 10B).

A



B

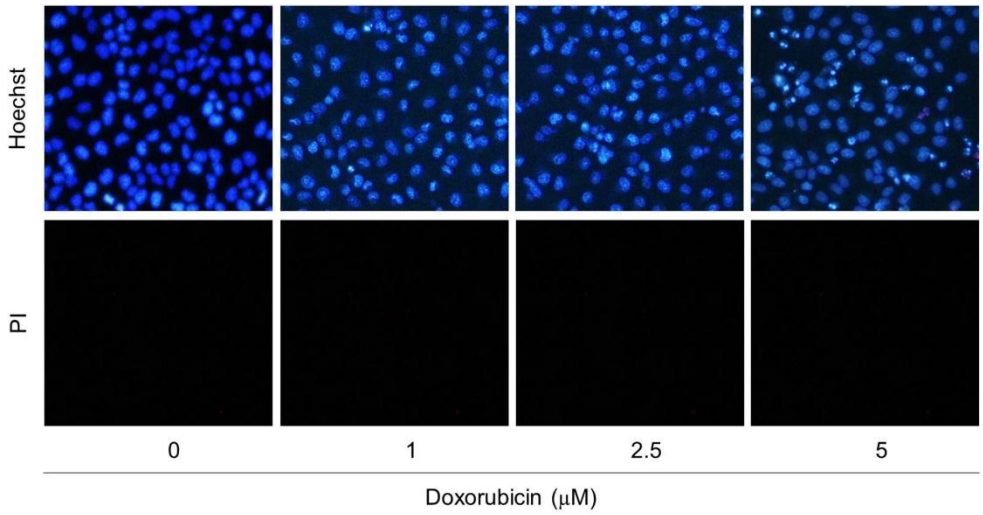
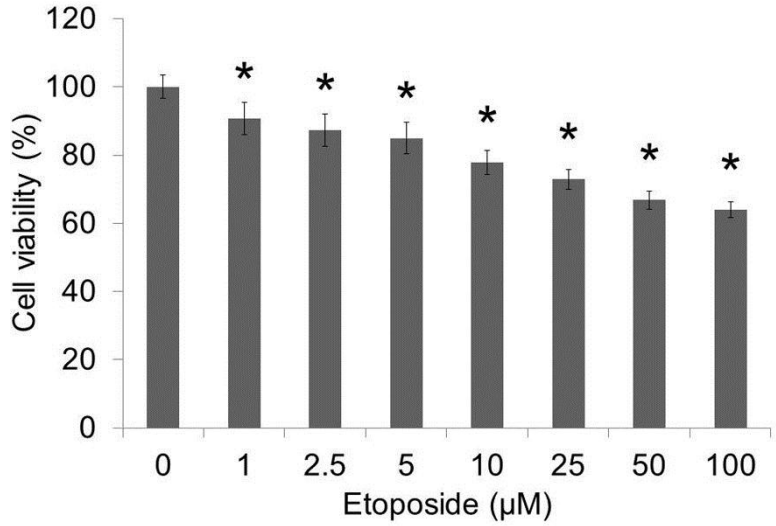


Figure 10 Effect of various concentrations of doxorubicin on cell death in H292 cells. (A) Cells were treated with various concentrations of doxorubicin (0-10 μM). After 24 h, cell viability was determined by MTT assay. Values are means ± SD (n = 3); *$p < 0.05$ versus non-treated control cells. (B) Apoptotic and necrotic cell death were observed under a fluorescence microscope by Hoechst 33342 and PI co-staining assay.

2.3. Effect of etoposide on cell death in H292 lung cancer cells

At 24 h after treatment, cell viability was significantly decreased in response to etoposide at all concentrations range from 1-100 μM with approximately 40% reduction in cell viability was observed in the 50 μM etoposide-treated group (Figure 11A). Etoposide at the concentrations of 10, 25, and 50 μM would be used for further experiments. The results showed that etoposide reduced the viability of H292 cells in a dose-dependent manner. The same as cisplatin and doxorubicin, etoposide-mediated apoptosis was observed by Hoechst 33342 staining (Figure 11B).

A



B

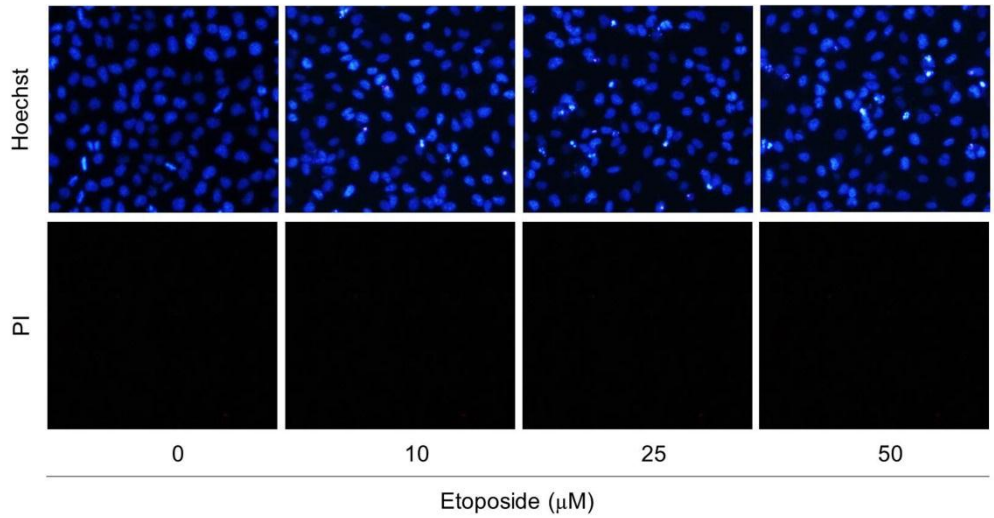


Figure 11 Effect of various concentrations of doxorubicin on cell death in H292 cells. (A) Cells were treated with various concentrations of etoposide (0-100 μM). After 24 h, cell viability was determined by MTT assay. Values are means ± SD (n = 3); *p < 0.05 versus non-treated control cells. (B) Apoptotic and necrotic cell death were observed under a fluorescence microscope by Hoechst 33342 and PI co-staining assay.

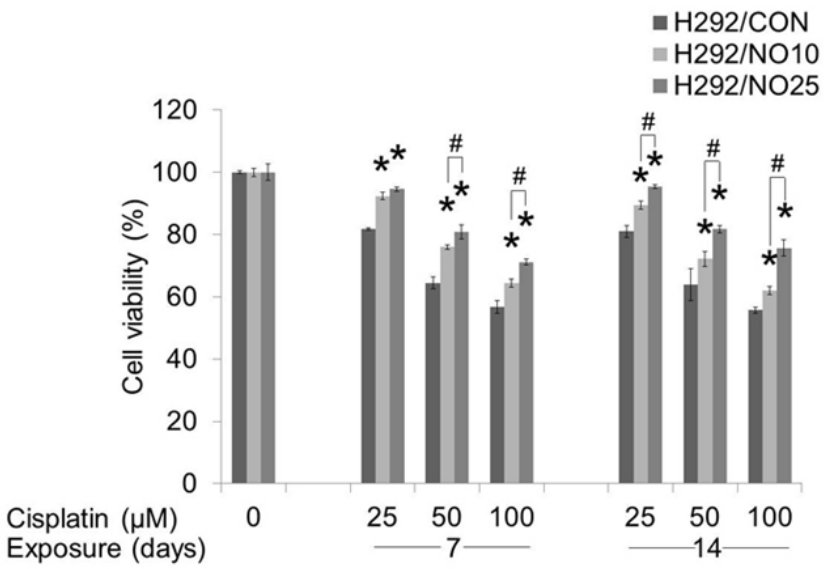
3. Long-term nitric oxide exposure potentiates cell resistance to chemotherapeutic agents.

To investigate the effect of long-term exposure to NO on chemotherapeutic resistance, lung cancer H292 cells were cultured in the presence or absence of DPTA NONOate (0-25 μM) for either 7 or 14 days, prior to treatment with various concentrations of cisplatin, doxorubicin, or etoposide. After 24 h, cell viability, apoptosis, and necrosis were determined.

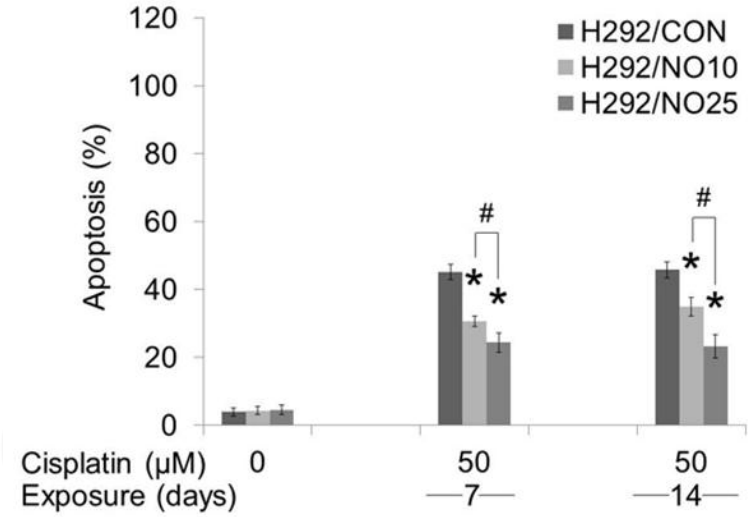
Figure 12A indicates that cisplatin at the concentrations of 25-100 μM significantly reduced cell viability of H292 cells in a dose-dependent manner with approximately 40% reduction in cell viability was observed in the 100 μM cisplatin-

treated group. Interestingly, the cells exposed to DPTA NONOate at the concentrations of 10 and 25 μM exhibited significant resistance to such cisplatin-mediated death (Figure 12A). It is worth noting that the NO-treated cells in both 7-day and 14-day experiments exhibited similar drug-resistance profiles. Treatment of the cells with cisplatin also caused a dose-dependent increase in cell apoptosis over that of the controls, as indicated by increased nuclear fluorescence and chromatin condensation detected by Hoechst 33342 and PI co-staining. At 50 μM cisplatin, approximately 40% of H292 cells exhibited apoptotic morphology, whereas the PI-positive necrosis cell was undetected. Interestingly, NO treatment significantly reduced the number of apoptotic cells induced by cisplatin treatments (Figure 12B and C). To confirm the apoptosis-inducing effect of cisplatin, cells were similarly treated with cisplatin, and apoptotic cells were determined by annexin V-based assay and flow cytometry. Consistently, our results showed that cisplatin-induced apoptosis in NO-treated H292 cells was significantly reduced in comparison to that of the control H292 cells (Figure 12D).

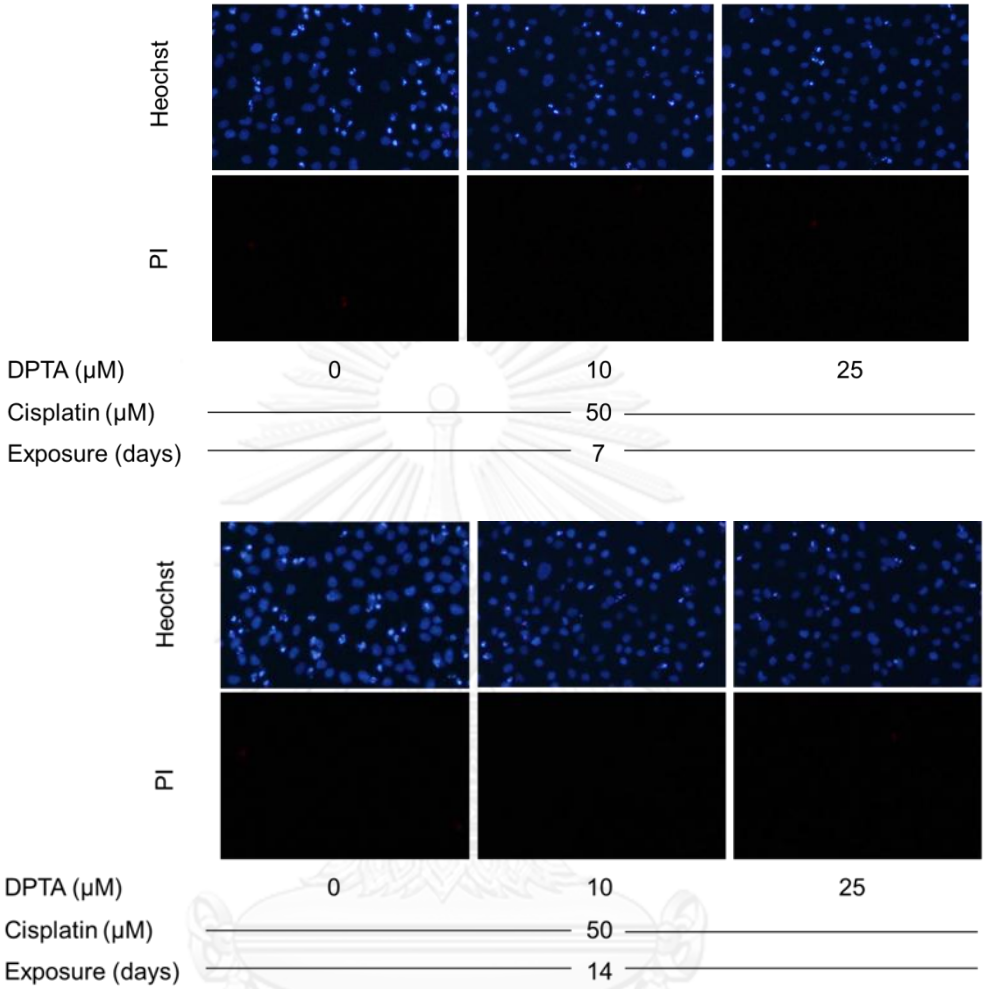
A


H292/CON
H292/NO10
H292/NO25
Cell viability (%)
120
100
80
60
40
20
0
Cisplatin (μM) 0 25 50 100 25 50 100
Exposure (days) 7 14

B


H292/CON
H292/NO10
H292/NO25
Apoptosis (%)
120
100
80
60
40
20
0
Cisplatin (μM) 0 50 50
Exposure (days) 7 14

C



D

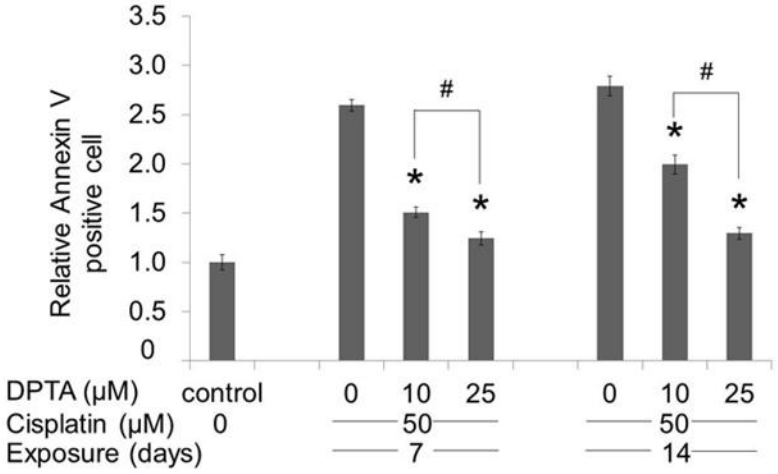
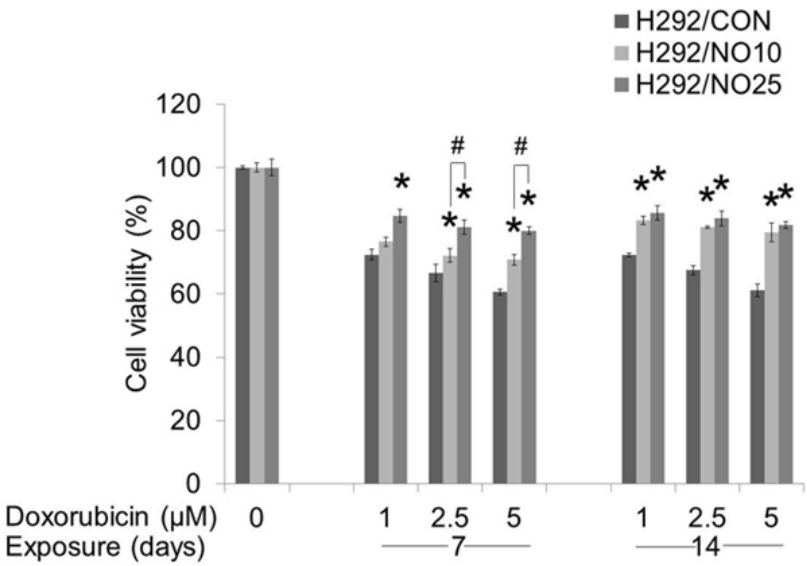


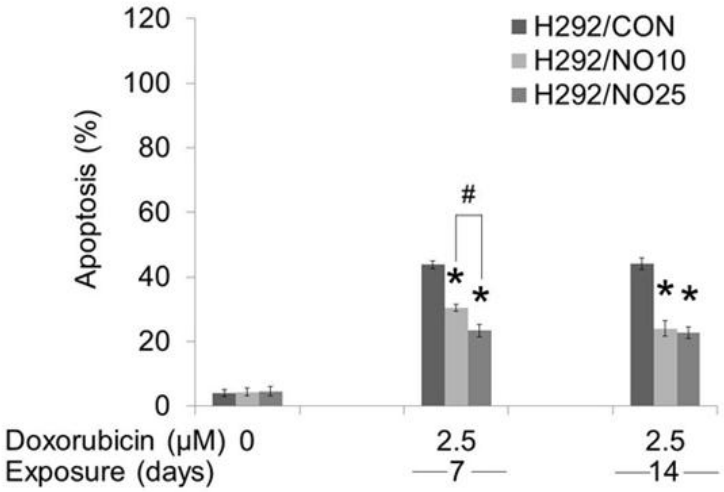
Figure 12 Long-term NO exposure mediates cell resistance to cisplatin. H292 cells were incubated with DPTA NONOate (0-25 μM) for either 7 or 14 days, prior to treatment with various concentrations of cisplatin (0-100 μM) for 24 h. (A) Cell survival was determined by MTT assay. (B) Apoptotic cell death was determined by Hoechst 33342 and PI co-staining assay. (C) Morphology of cell nuclei was visualized under a fluorescence microscope after Hoechst 33342/PI co-staining. (D) Apoptosis was evaluated by flow cytometry using Annexin V-FITC. Values are means ± SD (n = 3); $^{*}p < 0.05$ versus DPTA non-treated cells, $^{\#}p < 0.05$ versus 10 μM DPTA-treated cells.

The similar treatments as well as cell viability, apoptosis, and necrosis evaluations were applied for investigating the effects of long-term NO treatment on sensitivity to doxorubicin and etoposide. For doxorubicin, treatment of the H292 cells with 1-5 μM of the drug resulted in a significant decrease of cell viability (Figure 13A). As expected, exposure to NO donor for 7 and 14 days at the concentrations of 10 and 25 μM significantly reduced the response of the cells to doxorubicin. The dose-dependent effect of NO treatment on doxorubicin could only be detected in the cells that were treated with NO for 7 days, whereas at 14 days, similar resistance was observed at both concentrations of NO. Likewise, apoptosis assays (Hoechst 33342 and annexin-V) confirmed that the NO-treated cells exhibited a significant reduction of apoptosis in response to doxorubicin treatment compared to that of the control cells (Figure 13B and D).

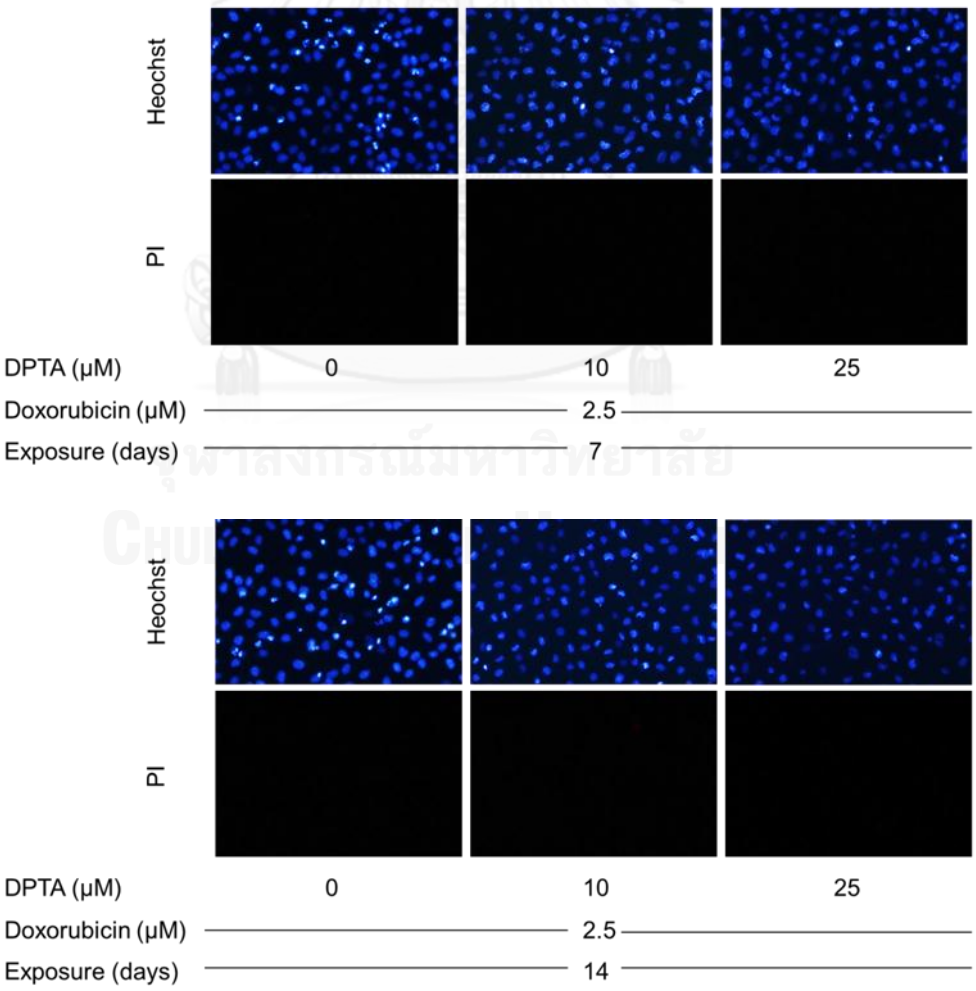
A



B


120
100
80
60
40
20
0
Apoptosis (%)
H292/CON
H292/NO10
H292/NO25
#
*
*
*
*
Doxorubicin (μM) 0
2.5
2.5
Exposure (days)
7
14

C


Heochst
PI
DPTA (μM)
0
10
25
Doxorubicin (μM)
2.5
Exposure (days)
7
Heochst
PI
DPTA (μM)
0
10
25
Doxorubicin (μM)
2.5
Exposure (days)
14

D

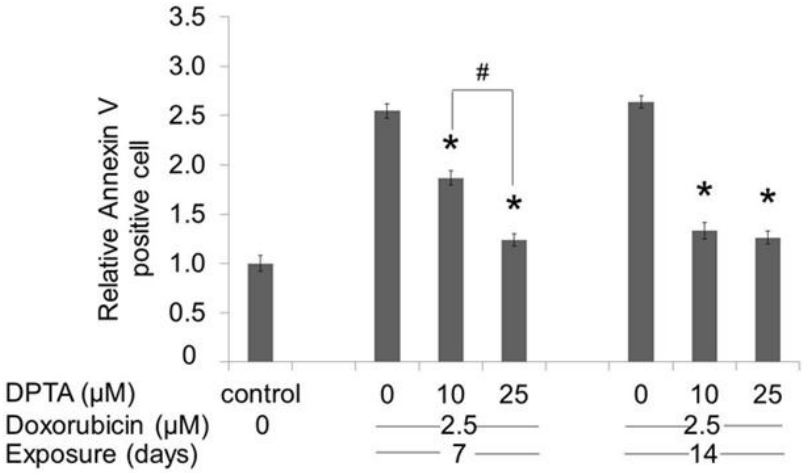
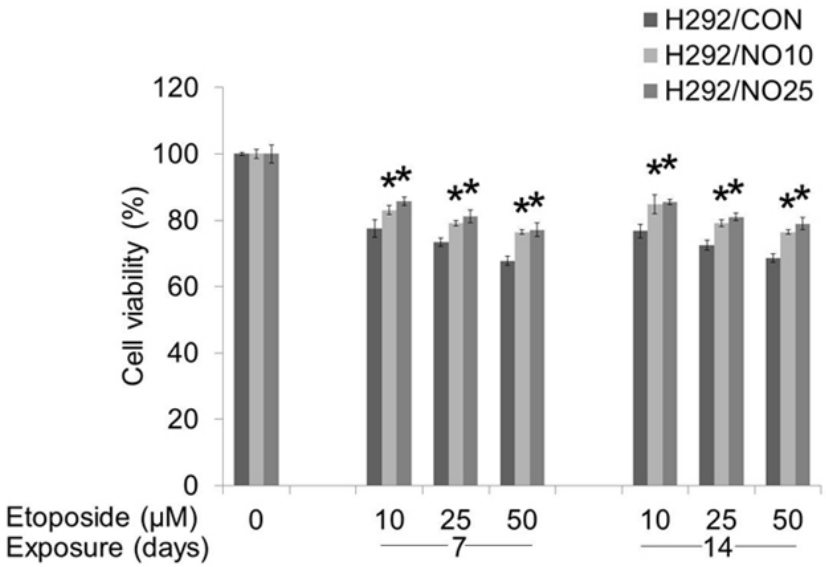


Figure 13 Long-term NO exposure mediates cell resistance to doxorubicin. H292 cells were incubated with DPTA NONOate (0-25 μM) for either 7 or 14 days, prior to treatment with various concentrations of doxorubicin (0-5 μM) for 24 h. (A) Cell survival was determined by MTT assay. (B) Apoptotic cell death was determined by Hoechst 33342 and PI co-staining assay. (C) Morphology of cell nuclei was visualized under a fluorescence microscope after Hoechst 33342/PI co-staining. (D) Apoptosis was evaluated by flow cytometry using Annexin V-FITC. Values are means ± SD (n = 3); $^{*}p < 0.05$ versus DPTA non-treated cells, $^{\#}p < 0.05$ versus 10 μM DPTA-treated cells.

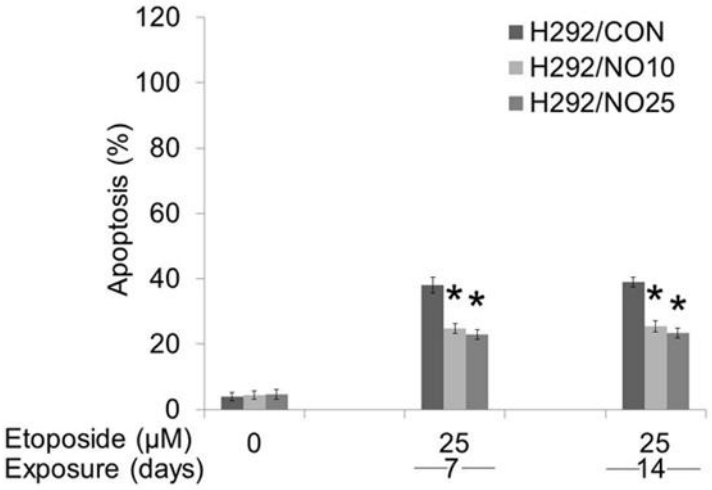
In accordance with above findings, the treatment with NO significantly reduced susceptibility of the cells to etoposide-mediated death. Figures 14A-D indicate that cells treated with NO for 7 and 14 days exhibited significant decrease in cellular response to etoposide-mediated death in comparison to their parental H292 cells.

Together, our observations have shown the negative regulatory effect of extended NO treatment on susceptibility of the cells to these chemotherapeutic agents.

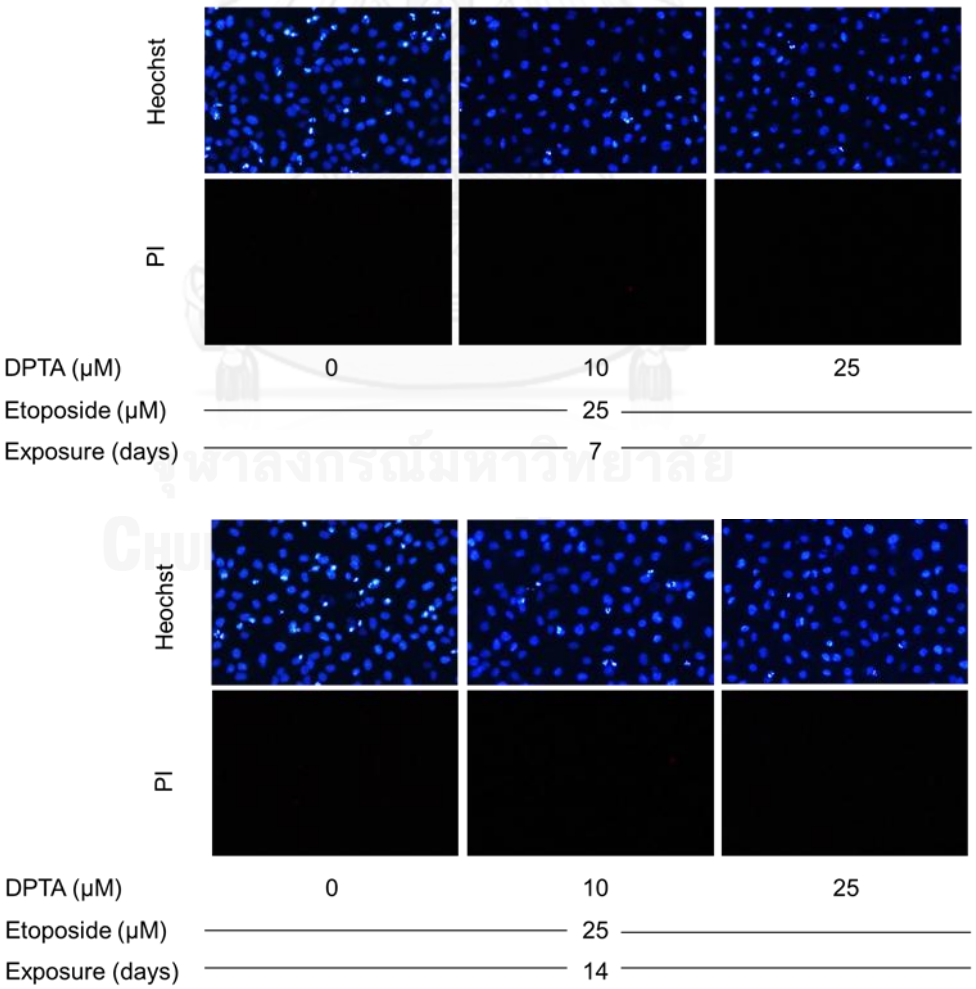
A



B


120
100
80
60
40
20
0
Apoptosis (%)
H292/CON
H292/NO10
H292/NO25
* *
* *
Etoposide (μM)
Exposure (days)
0
25
7
25
14

C


Heochst
PI
DPTA (μM)
0
10
25
Etoposide (μM)
25
Exposure (days)
7
Heochst
PI
DPTA (μM)
0
10
25
Etoposide (μM)
25
Exposure (days)
14

D

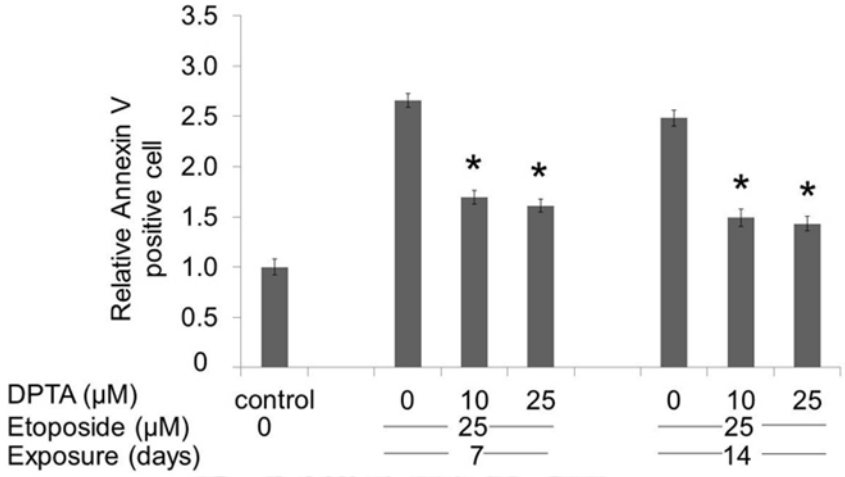


Figure 14 Long-term NO exposure mediates cell resistance to etoposide. H292 cells were incubated with DPTA NONOate (0-25 μM) for either 7 or 14 days, prior to treated with various concentrations of etoposide (0-50 μM) for 24 h. (A) Cell survival was determined by MTT assay. (B) Apoptotic cell death was determined by Hoechst 33342 and PI co-staining assay. (C) Morphology of cell nuclei was visualized under a fluorescence microscope after Hoechst 33342/PI co-staining. (D) Apoptosis was evaluated by flow cytometry using Annexin V-FITC. Values are means ± SD (n = 3); $^{*}p < 0.05$ versus DPTA non-treated cells, $^{\#}p < 0.05$ versus 10 μM DPTA-treated cells.

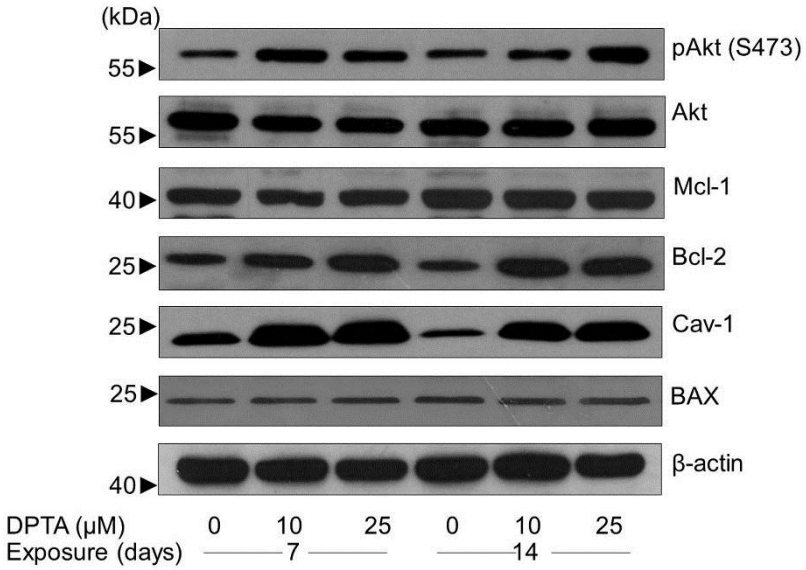
4. Nitric oxide mediates chemotherapeutic resistance in lung cancer cells by increasing cellular survival pathways.

Having shown that NO exposure induces cell resistance to cisplatin, doxorubicin, and etoposide, the present study further identifies the possible underlying mechanisms. Focusing on the fact that patients with lung cancer suffer from low responses after receiving single or combination regimen for treatment, the activation and up-regulation of survival pathways were proposed as an important cause. While activated Akt has garnered the most attention and was observed to be up-regulated in a number of resistant cancer cells (Brognard *et al.*, 2001), increasing evidence has indicated the prominent role of anti-apoptotic proteins namely, Bcl-2 and Mcl-1, on chemoresistance (Sartorius *et al.*, 2002; Song *et al.*, 2005).

We thus characterized the survival-related protein profiles of 7- and 14-day NO-treated H292 cells and compared them to those of H292 cells without NO. Figure 15 shows that the total Akt level in NO-treated and control H292 cells were comparable and not affected by either dose or length of time of treatment. The significant increase of phosphorylated Akt at Ser473 (activated form) was observed in the NO-treated cells in comparison to that of the control cells. This NO-mediated Akt activation was found to be in a concentration-dependent manner, and further increase was observed when the duration of NO exposure was prolonged. Likewise, the expression of anti-apoptotic Bcl-2 protein was found to be strongly up-regulated in NO-treated cells. NO exposure at the concentrations of 10 and 25 μM induced Bcl-2 up-regulation in dose- and time-dependent manners, with an approximately 3-fold induction observed when the cells were treated with 25 μM of NO donor for 14 days. The expression level of Cav-1 was shown to be increased in response to NO

exposure, whereas NO treatment caused no significant effect on the levels of anti-apoptotic Mcl-1 and pro-apoptotic Bax.

A



B

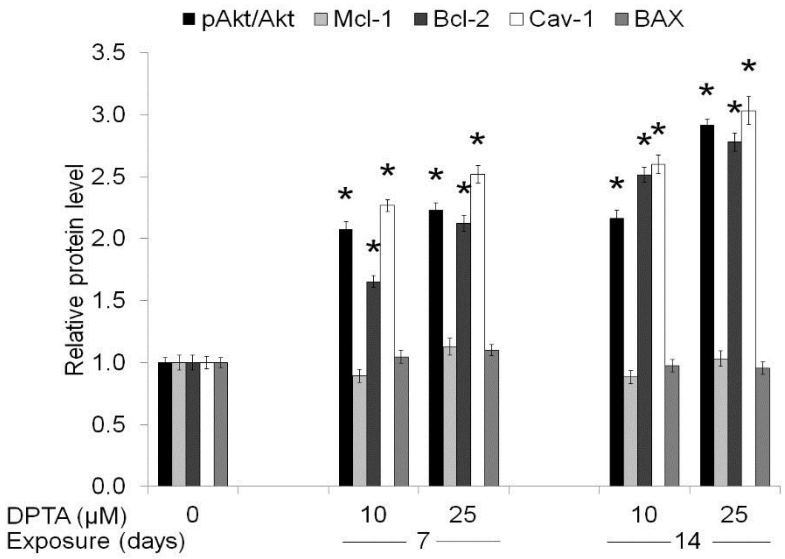


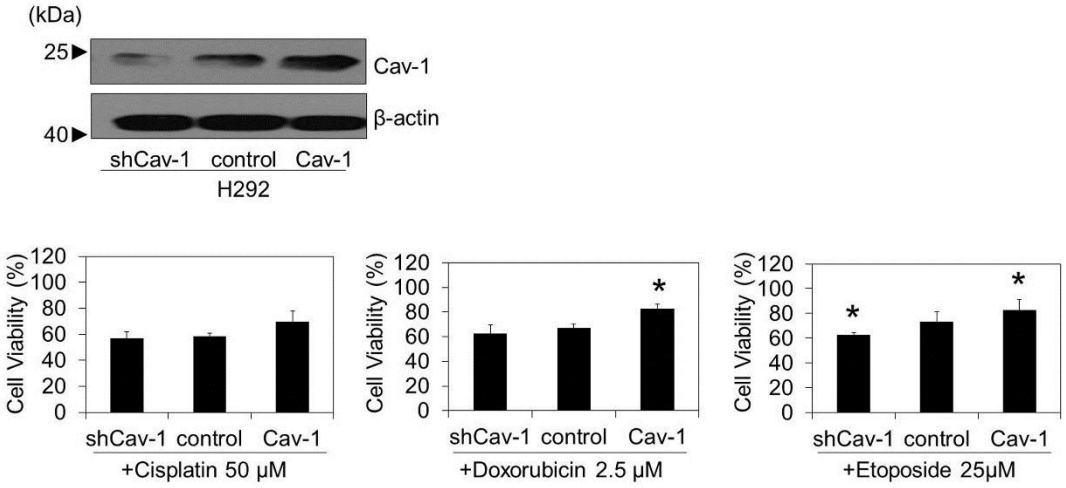
Figure 15 Nitric oxide influences cellular survival proteins. (A) After H292 cells were incubated with DPTA NONOate (0-25 μM) for 7-14 days, cells were collected and analyzed for the expression of phosphorylated Akt (Ser 473), total Akt, Bcl-2,

Mcl-1, Bax and Cav-1 proteins by western blotting. Blots were reprobed with a ß-actin antibody to confirm equal loading of samples. (B) Immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized by blot of the controls. Values are means ± SD (n = 3); $^{*}p < 0.05$ versus non-treated control cells.

To test whether the increases in survival-related protein are involved in resistance to apoptosis induced by cisplatin, doxorubicin, and etoposide, we stably transfected the cells with overexpressing or shRNA plasmids of Cav-1, Akt, and Bcl-2 and the cell viability after chemotherapeutic drug treatments was determined. Figure 16 shows that cells transfected with Cav-1-, Akt-, and Bcl-2-overexpressing plasmids exhibited up-regulation of their corresponding proteins, while the shRNA-transfected counterparts showed significantly decreased levels. To determine the effect of each protein on the susceptibility of the cells to drug-mediated death, stably transfected cells were left untreated or treated with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide, and viability at 24 h was measured. MTT-based cell viability assay indicated that Cav-1-overexpressing cells exhibited significant resistant to doxorubicin and etoposide but with no effect on cisplatin. In addition, shCav-1 only significantly sensitized the cells to etoposide-mediated death. For Akt-transfected cells, the increase of activated Akt significantly attenuated the death response of the cells to all three drugs. The shAKT-transfected cells exhibited increased susceptibility to doxorubicin- and etoposide-mediated toxicity. Accordingly, cells overexpressing Bcl-2 exhibited resistance to all three drugs and the shRNA knock-down of the protein significantly increased the response of the cells to cisplatin, doxorubicin, and etoposide. Taken together, these results suggest that the increase of Cav-1 level in response to NO rendered cells resistant to doxorubicin and

etoposide but not cisplatin. However, the increase of activated Akt and Bcl-2 in response to long-term NO could render cells resistant to all three drugs.

A



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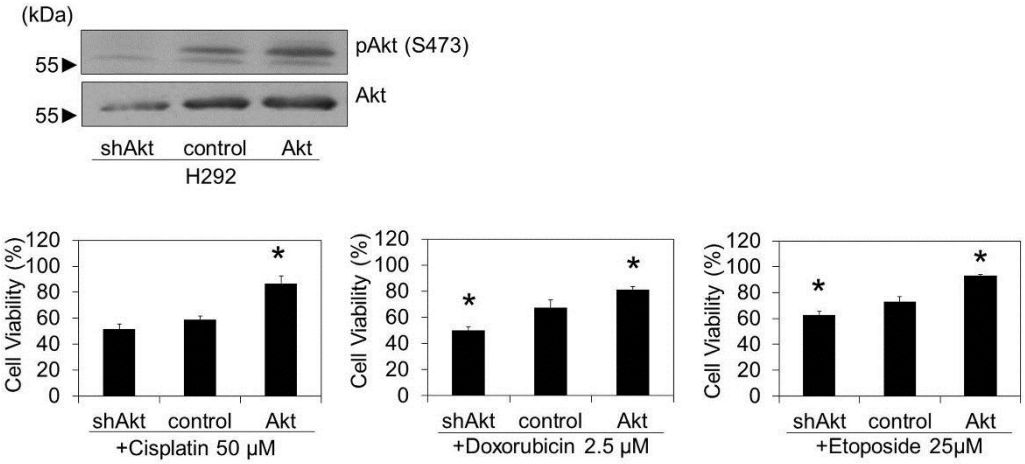

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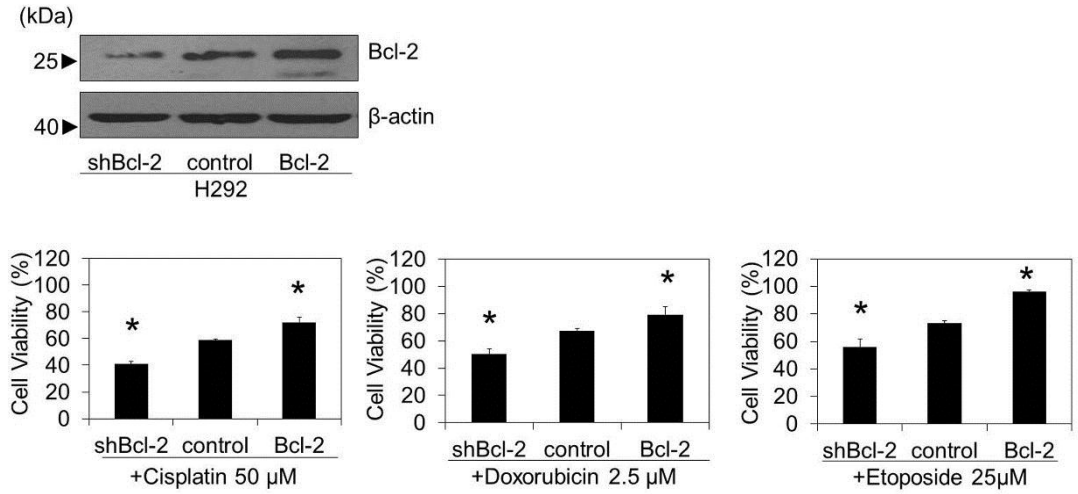
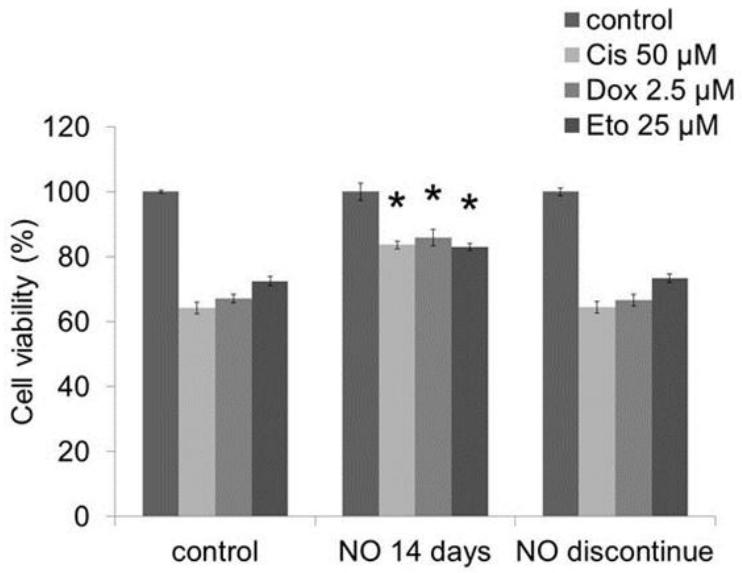


Figure 16 Effect of Akt, Cav-1, and Bcl-2 proteins on susceptibility of H292 cells to cisplatin, doxorubicin, and etoposide-mediated death. H292 cells were stably transfected with overexpressing, control, or shRNA plasmids of Cav-1 (A), Akt (B), and Bcl-2 (C) and the levels of proteins were analyzed by western blot analysis. Stable transfected cells were treated with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide for 24 h and cell viability was measured by MTT assay. Values are means of samples ± SD (n = 3); $^{*}p < 0.05$ versus H292 control cells.

5. Effect of NO-mediated chemo-resistance is reversible.

Having shown that up-regulation of survival-related proteins was responsible for the chemotherapeutic resistance of NO-treated cells, we next examined whether these effects could be reversible in the absence of NO donor. The cells after 14 day of NO exposure were cultured in the absence of NO for 5 days. The cells were then tested for their chemotherapeutic susceptibility and the expression levels of Cav-1, Akt, pAkt, and Bcl-2 were determined using western blotting. Figure 17A and B show that the resistant characteristic observed in the 14-day treated cells revert to the base line level when the cells were grown under NO-free condition. In addition, the expressions of Cav-1, pAkt, and Bcl-2 were found to be comparable to those of control cells (Figure 17C and D).

A



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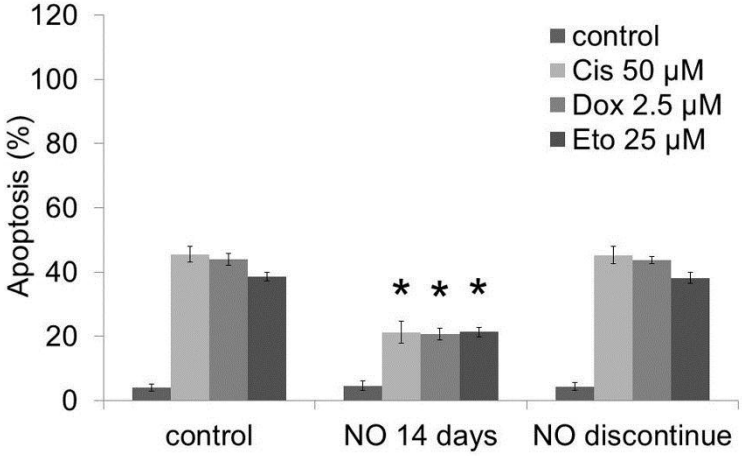

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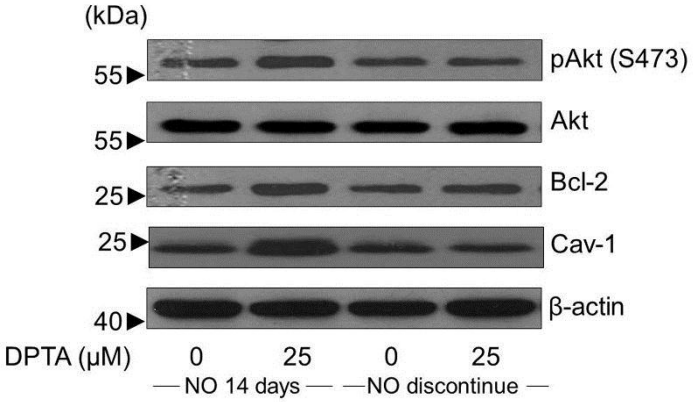

D

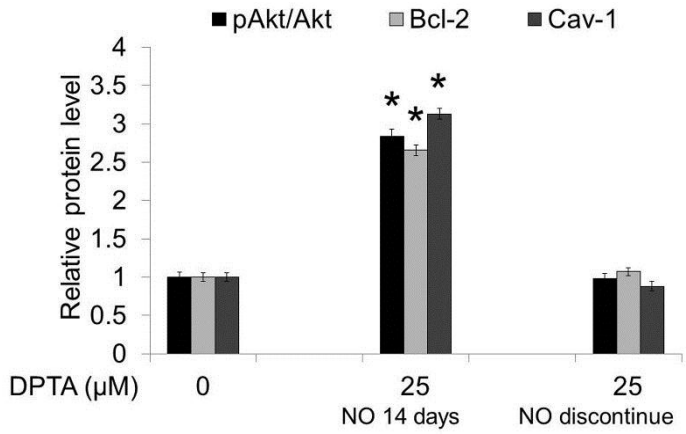

Figure 17 Effect of NO discontinuation on chemotherapeutic resistance. The NO-treated H292 cells (14 days) were cultured in the absence of NO donor for 5 days. The cells were then treated with cisplatin (50 μM), doxorubicin (2.5 μM), or etoposide (25 μM) for 24 h. (A) Cell survival was determined by MTT assay. Data represent means ± SD (n = 3); $^{*}p < 0.05$ versus drug-treated control cells. (B) Apoptotic cells were evaluated by Hoechst 33342 staining. Data represent means ± SD (n = 3); $^{*}p < 0.05$ versus drug-treated control cells. (C) Cells were collected and analyzed for Cav-1, Akt, pAkt, and Bcl-2 expressions by western blotting. Blots were reprobed with a β-actin antibody to confirm equal loading of samples. (D) Immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the results for the controls. Data represent mean ± SD (n = 3); $^{*}p < 0.05$ versus H292 control cells.

CHAPTER V
DISCUSSION AND CONCLUSION

Mechanisms involve in cancer-related drug resistance have been proposed and demonstrated by several works. Among them, the attenuation of apoptosis has garnered the most recognition (Makin and Hickman, 2000). Several pathways are suggested to be involved in such chemoresistance, including an up-regulation of anti-apoptotic members of the Bcl-2 family (Sartorius *et al.*, 2002), an increase of antioxidant and related detoxifying mechanisms (Homma *et al.*, 2009), and the enhancement of survival signals of the cells (Brognard *et al.*, 2001). Since such resistance occurred even during the use of combination drugs acting on different cellular targets, survival augmentation seems to be a possible mechanism for cancer cells resistance to various death stimuli.

Accumulated clinical observations have supported the above concepts by pointing out a significant increase of cell survival in a variety of human cancer types (Brognard *et al.*, 2001; Hutchinson *et al.*, 2001; Grille *et al.*, 2003; Tang *et al.*, 2006) and such mechanisms are linked to chemotherapeutic resistances (Brognard *et al.*, 2001; Hutchinson *et al.*, 2001). An increase of survival signals in cancer cells was shown to be mediated through Akt activation as a core regulator. While the increased activity of Akt caused by gene mutations was found in many types of cancer, including breast and lung cancer (Samuels and Waldman, 2010), the mechanism of up-regulation of Akt activity by acquired means is largely unknown.

We and others hypothesized that endogenous substances found surrounding the tumor, including reactive oxygen species (ROS), NO, and pro-inflammatory cytokines, can force the cells to change their behaviors (Storz, 2005) or phenotypes

(Feig, Reid, and Loeb, 1994). Our observations in the present study indicated that NO-mediated drug resistance can be reversed when the cells are cultured under NO-free condition, with cell sensitivity being restored to baseline level at five days of NO discontinuation (Figure 17). These results suggested that the cellular responses involving pAkt, Bcl-2, and Cav-1 up-regulations are due to the adaptive machinery of the cells. Results from this study also support the concept that NO present in the cancer microenvironment plays a possible role in regulation of chemotherapeutic resistance.

In terms of DNA-interacting agents, such as cisplatin and doxorubicin, cell cycle arrest at the G_2/M phase was shown to be a critical event necessary for apoptosis induction (Sorenson, Barry, and Eastman, 1990). Studies indicated that the induction of the Akt pathway inhibits apoptosis by reducing the levels of p21 and p27, inhibitors of cyclin-dependent kinases, and prevents the down-regulation of cyclins A, B, and E (Liang and Slingerland, 2003). In addition, the attenuated apoptosis of cancer cells by an increase of the cellular level of anti-apoptotic proteins has been extensively reported (Yang *et al.*, 1997). Along with the mentioned evidences, the present study shows that NO triggered the resistance to chemotherapeutic drugs by an increase in Akt function. We also found that NO exposure significantly increases the level of anti-apoptotic Bcl-2 protein, while having only minimal effect on Mcl-1 protein (Figure 15). Previously, we found that the short-term NO exposure (12 h) rendered H460 human lung cancer cells resistant to cisplatin-mediated death (Chanvorachote *et al.*, 2006). In that study, NO *S*-nitrosylated Bcl-2 and prevented the protein form proteasomal degradation. Even though it is possible that such

S-nitrosylation may have up-regulated Bcl-2 protein in the present study, *S*-nitrosylated proteins in fact have a short half-life (Arnelle and Stamler, 1995).

With gene manipulation methods, we generated stable transfectants of H292 cells exhibiting different levels of Cav-1, Bcl-2, and Akt protein expressions, in order to verify the functions of each protein on drug resistances. The results indicated that Bcl-2, as well as pAkt, up-regulation in the H292 cells conferred cisplatin, doxorubicin, and etoposide resistance. However, the up-regulation of Cav-1 in these cells inhibited only doxorubicin- and etoposide-mediated death. This finding consists with the previous study indicating that Cav-1 has less effect on cisplatin-mediated death and in certain study Cav-1 was found to sensitized cells to cisplatin-induced apoptosis (Pongjit and Chanvorachote, 2011).

In summary, the present study has provided evidence indicating that NO present in the tumor microenvironment has a significant impact on cell susceptibility to chemotherapeutic agents by increasing expression of survival regulatory proteins. The findings in the present work could be important for the development of novel cancer therapies and encourages further studies in related areas.

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APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1 The percentage of H292 cell viability after treatment with various concentrations of DPTA NONOate for 24, 48 and 72 h was determined by MTT assay. Values are means ± S.D. of three-independent experiments.

DPTA NONOate (μM)	Cell viability (%)		
	24 h	48 h	72 h
control	100 ± 0.13	100 ± 0.89	100 ± 1.97
5	98.83 ± 0.63	97.19 ± 1.75	96.24 ± 0.56
10	97.28 ± 0.96	95.79 ± 1.60	95.11 ± 0.58
15	96.53 ± 1.43	94.45 ± 1.83	94.41 ± 2.75
20	95.97 ± 2.13	94.23 ± 1.78	93.97 ± 2.84
25	95.77 ± 1.26	93.94 ± 2.52	93.73 ± 1.70
50	85.11 ± 0.40	86.26 ± 1.57	84.46 ± 2.00
100	79.48 ± 1.71	79.68 ± 1.93	77.89 ± 1.57
150	75.96 ± 1.10	72.85 ± 1.28	69.87 ± 1.93
200	72.90 ± 1.84	68.69 ± 0.66	65.78 ± 0.71

Table 2 The percentage of cell apoptosis and necrosis after treatment with non-toxic concentrations of DPTA NONOate were determined by Hoechst 33342 and PI co-staining assay and visualized and scored under fluorescent microscope. Values are means ± S.D. of three-independent experiments.

DPTA NONOate (μM)	Cell apoptosis (%)	Cell necrosis (%)
control	3.63 ± 0.74	1.03 ± 0.17
10	4.10 ± 0.50	1.12 ± 0.35
25	4.43 ± 0.49	1.19 ± 0.40

Table 3 The percentage of H292 cell viability after treatment with various concentrations of cisplatin was determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Cisplatin (μM)	Cell viability (%)
control	100 ± 2.50
1	100.07 ± 3.31
5	98.72 ± 4.31
10	97.95 ± 4.98
25	82.60 ± 2.80
50	67.50 ± 4.75
100	55.66 ± 2.55
200	38.12 ± 3.37

Table 4 The percentage of H292 cell viability after treatment with various concentrations of doxorubicin was determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Doxorubicin (μM)	Cell viability (%)
control	100 ± 2.46
0.1	82.77 ± 2.45
0.25	77.66 ± 2.91
0.5	77.94 ± 2.67
1	73.97 ± 3.17
2.5	64.58 ± 3.12
5	57.76 ± 2.79
10	54.15 ± 5.19

Table 5 The percentage of H292 cell viability after treatment with various concentrations of etoposide was determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Etoposide (μM)	Cell viability (%)
control	100 ± 3.45
1	90.64 ± 4.71
2.5	87.33 ± 4.81
5	84.97 ± 4.64
10	77.79 ± 3.55
25	72.86 ± 2.90
50	66.78 ± 2.68
100	63.94 ± 2.29

Table 6 The percentage of cell viability of 7- and 14-day DPTA-treated H292 cells after treatment with various concentrations of cisplatin for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Cell types / Cisplatin (μM)	Cell viability (%)		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	100 ± 0.50	100 ± 1.34	100 ± 2.69
25	81.83 ± 0.33	92.31 ± 1.19	94.60 ± 0.65
50	64.48 ± 1.82	75.95 ± 0.61	80.90 ± 2.24
100	56.78 ± 1.99	64.36 ± 1.31	71.17 ± 0.99

Cell types / Cisplatin (μM)	Cell viability (%)		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	100 ± 2.69	100 ± 0.83	100 ± 0.35
25	80.99 ± 1.84	89.43 ± 1.22	95.32 ± 0.64
50	63.92 ± 5.16	72.16 ± 2.36	81.73 ± 1.20
100	55.73 ± 0.93	62.07 ± 1.28	75.64 ± 2.64

Table 7 The percentage of cell apoptosis of 7- and 14-day DPTA-treated H292 cells after treatment with 50 μM cisplatin were determined by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope. Values are means ± S.D. of three-independent experiments.

Cell types / Cisplatin (μM)	Cell apoptosis (%)		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	3.96 ± 1.16	4.32 ± 1.24	4.56 ± 1.47
50	45.24 ± 2.31	30.62 ± 1.47	24.43 ± 2.89

Cell types / Cisplatin (μM)	Cell apoptosis (%)		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	3.96 ± 1.16	4.32 ± 1.24	4.56 ± 1.47
50	45.76 ± 2.39	34.85 ± 2.69	23.23 ± 3.45

Table 8 The relative Annexin V positive cell in 7- and 14-day DPTA-treated H292 cells after treatment with 50 μM cisplatin were evaluated by flow cytometry using Annexin V-FITC. Values are means ± S.D. of three-independent experiments.

Cell types / Cisplatin (μM)	Relative Annexin V positive cell		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.05
50	2.59 ± 0.06	1.51 ± 0.05	1.24 ± 0.07

Cell types / Cisplatin (μM)	Relative Annexin V positive cell		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	1 ± 0.08	1 ± 0.07	1 ± 0.05
50	2.79 ± 0.10	1.99 ± 0.10	1.30 ± 0.06

Table 9 The percentage of cell viability of 7- and 14-day DPTA-treated H292 cells after treatment with various concentrations of doxorubicin for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Cell types / Doxorubicin (μM)	Cell viability (%)		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	100 ± 0.50	100 ± 1.34	100 ± 2.69
1	72.41 ± 1.65	76.53 ± 1.46	84.74 ± 2.01
2.5	66.63 ± 2.68	72.21 ± 2.18	81.13 ± 2.19
5	60.61 ± 0.93	70.84 ± 1.71	80.06 ± 1.12

Cell types / Doxorubicin (μM)	Cell viability (%)		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	100 ± 2.69	100 ± 0.83	100 ± 0.35
1	72.33 ± 0.63	83.31 ± 1.27	85.63 ± 2.22
2.5	67.51 ± 1.38	81.18 ± 0.35	83.88 ± 2.44
5	61.12 ± 2.00	79.37 ± 2.98	81.81 ± 1.14

Table 10 The percentage of cell apoptosis of 7- and 14-day DPTA-treated H292 cells after treatment with 2.5 μM doxorubicin were determined by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope. Values are means ± S.D. of three-independent experiments.

Cell types / Doxorubicin (μM)	Cell apoptosis (%)		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	3.96 ± 1.16	4.32 ± 1.24	4.56 ± 1.47
2.5	43.75 ± 1.16	30.37 ± 1.01	23.33 ± 2.00

Cell types / Doxorubicin (μM)	Cell apoptosis (%)		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	3.96 ± 1.16	4.32 ± 1.24	4.56 ± 1.47
2.5	44.20 ± 1.80	23.97 ± 2.40	22.63 ± 1.82

Table 11 The relative Annexin V positive cell in 7- and 14-day DPTA-treated H292 cells after treatment with 2.5 μM doxorubicin were evaluated by flow cytometry using Annexin V-FITC. Values are means ± S.D. of three-independent experiments.

Cell types / Doxorubicin (μM)	Relative Annexin V positive cell		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.05
2.5	2.55 ± 0.07	1.87 ± 0.08	1.24 ± 0.06

Cell types / Doxorubicin (μM)	Relative Annexin V positive cell		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	1 ± 0.08	1 ± 0.07	1 ± 0.05
2.5	2.64 ± 0.06	1.33 ± 0.08	1.26 ± 0.07

Table 12 The percentage of cell viability of 7- and 14-day DPTA-treated H292 cells after treatment with various concentrations of etoposide for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Cell types / Etoposide (μM)	Cell viability (%)		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	100 ± 0.50	100 ± 1.34	100 ± 2.69
10	77.52 ± 2.56	83.05 ± 1.37	85.64 ± 1.22
25	73.37 ± 1.29	79.04 ± 0.81	81.13 ± 1.98
50	67.77 ± 1.32	76.40 ± 0.66	77.15 ± 2.10

Cell types / Etoposide (μM)	Cell viability (%)		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	100 ± 2.69	100 ± 0.83	100 ± 0.35
10	76.74 ± 1.99	84.85 ± 2.85	85.46 ± 0.87
25	72.48 ± 1.47	79.02 ± 1.01	81.04 ± 1.05
50	68.54 ± 1.31	76.44 ± 0.64	78.95 ± 1.82

Table 13 The percentage of cell apoptosis of 7- and 14-day DPTA-treated H292 cells after treatment with 25 μM etoposide were determined by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope. Values are means ± S.D. of three-independent experiments.

Cell types / Etoposide (μM)	Cell apoptosis (%)		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	3.96 ± 1.16	4.32 ± 1.24	4.56 ± 1.47
25	38.13 ± 2.47	24.90 ± 1.49	22.93 ± 1.46

Cell types / Etoposide (μM)	Cell apoptosis (%)		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	3.96 ± 1.16	4.32 ± 1.24	4.56 ± 1.47
25	39.02 ± 1.40	25.54 ± 1.68	23.37 ± 1.50

Table 14 The relative Annexin V positive cell in 7- and 14-day DPTA-treated H292 cells after treatment with 25 μM etoposide were evaluated by flow cytometry using Annexin V-FITC. Values are means ± S.D. of three-independent experiments.

Cell types / Etoposide (μM)	Relative Annexin V positive cell		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.05
25	2.66 ± 0.07	1.69 ± 0.07	1.61 ± 0.07

Cell types / Etoposide (μM)	Relative Annexin V positive cell		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
control	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.05
25	2.48 ± 0.08	1.49 ± 0.08	1.43 ± 0.07

Table 15 The relative of phosphorylated Akt (Ser 473), total Akt, Mcl-1, Bcl-2, Cav-1 and Bax proteins level of 7- and 14-day DPTA-treated cells were determined by western blot analysis. Values are means ± S.D. of three-independent experiments.

Cell types / Proteins	Relative protein level		
	Day 7 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
pAkt/Akt	1.00 ± 0.04	2.08 ± 0.06	2.23 ± 0.05
Mcl-1	1.00 ± 0.06	0.89 ± 0.05	1.13 ± 0.07
Bcl-2	1.00 ± 0.06	1.65 ± 0.05	2.12 ± 0.07
Cav-1	1.00 ± 0.05	2.27 ± 0.05	2.52 ± 0.07
Bax	1.00 ± 0.04	1.05 ± 0.05	1.10 ± 0.05

Cell types / Proteins	Relative protein level		
	Day 14 of DPTA exposure		
	H292/CON	H292/NO10	H292/NO25
pAkt/Akt	1.00 ± 0.04	2.17 ± 0.06	2.92 ± 0.05
Mcl-1	1.00 ± 0.06	0.88 ± 0.05	1.03 ± 0.06
Bcl-2	1.00 ± 0.06	2.51 ± 0.06	2.78 ±0.07
Cav-1	1.00 ± 0.05	2.60 ± 0.08	3.03 ± 0.11
Bax	1.00 ± 0.04	0.97 ± 0.05	0.95 ± 0.05

Table 16 The percentage of cell viability of H292/control, H292/shCav-1 and H292/Cav-1 cells after treatment with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Transfected cells / Treatments	Cell viability (%)		
	H292/shCav-1	H292/control	H292/Cav-1
Cisplatin 50 μM	56.70 ± 5.13	58.77 ± 1.96	69.82 ± 7.88
Doxorubicin 2.5 μM	62.55 ± 6.75	67.30 ± 3.23	82.52 ± 4.15
Etoposide 25 μM	62.55 ± 2.11	73.06 ± 7.89	82.52 ± 8.65

Table 17 The percentage of cell viability of H292/control, H292/shAkt and H292/Akt cells after treatment with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Transfected cells / Treatments	Cell viability (%)		
	H292/shAkt	H292/control	H292/Akt
Cisplatin 50 μM	51.62 ± 4.15	58.77 ± 2.93	86.75 ± 5.87
Doxorubicin 2.5 μM	49.90 ± 3.10	67.30 ± 5.96	81.30 ± 2.15
Etoposide 25 μM	62.80 ± 2.85	73.06 ± 4.12	93.20 ± 1.28

Table 18 The percentage of cell viability of H292/control, H292/shBcl-2 and H292/Bcl-2 cells after treatment with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Transfected cells / Treatments	Cell viability (%)		
	H292/shBcl-2	H292/control	H292/Bcl-2
Cisplatin 50 μM	40.98 ± 2.14	58.77 ± 1.21	72.01 ± 3.92
Doxorubicin 2.5 μM	50.12 ± 4.15	67.30 ± 1.93	79.15 ± 5.89
Etoposide 25 μM	55.78 ± 5.92	73.06 ± 2.05	96.23 ± 1.32

Table 19 The percentage of cell viability of 14-day DPTA-treated cells and 5-day DPTA-discontinued cells after treatment with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide for 24 h were determined by MTT assay. Values are means ± S.D. of three-independent experiments.

Cell types / Treatments	Cell viability (%)		
	control	14-day DPTA-treated cells	5-day DPTA-discontinued cells
control	100 ± 0.50	100 ± 2.69	100 ± 1.12
Cisplatin 50 μM	64.20 ± 1.82	83.73 ± 1.20	64.48 ± 1.91
Doxorubicin 2.5 μM	67.07 ± 1.38	85.88 ± 2.44	66.63 ± 1.71
Etoposide 25 μM	72.48 ± 1.47	83.04 ± 1.06	73.37 ± 1.29

Table 20 The percentage of cell apoptosis of 14-day DPTA-treated cells and 5-day DPTA-discontinued cells after treatment with 50 μM cisplatin, 2.5 μM doxorubicin, or 25 μM etoposide for 24 h were determined by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope**.** Values are means ± S.D. of three-independent experiments.

Cell types / Treatments	Cells apoptosis (%)		
	control	14-day DPTA-treated cells	5-day DPTA-discontinued cells
control	3.96 ± 1.16	4.56 ± 1.47	4.32 ± 1.24
Cisplatin 50 μM	45.48 ± 2.39	21.23 ± 3.45	45.20 ± 2.69
Doxorubicin 2.5 μM	43.97 ± 1.80	20.63 ± 1.82	43.75 ± 1.16
Etoposide 25 μM	38.58 ± 1.40	21.37 ± 1.50	38.13 ± 1.68

Table 21 The relative of phosphorylated Akt (Ser 473), total Akt, Bcl-2, and Cav-1 proteins level of 14-day DPTA-treated cells and 5-day DPTA-discontinued cells were determined by western blot analysis. Values are means ± S.D. of three-independent experiments.

Cell types / Proteins	Relative protein level		
	control	14-day DPTA-treated cells	5-day DPTA-discontinued cells
pAkt/Akt	1.00 ± 0.06	2.84 ± 0.09	0.98 ± 0.06
Bcl-2	1.00 ± 0.06	2.66 ± 0.07	1.07 ± 0.05
Cav-1	1.00 ± 0.06	3.13 ± 0.07	0.88 ± 0.06

VITA

Mrs. Piyaparisorn Wongvaranon was born on February 5, 1980 in Phatthalung. She received her B.Pharm (2nd class honor) from the Faculty of Pharmacy, Mahidol University in 2003.