中國醫藥大學　　藥學院
藥學系碩士班 碩士論文

探討 NSC746364 抑制 A549 人類肺癌細胞生長之分子機轉

Elucidating the Molecular Mechanisms of the Inhibitory Effects of NSC746364 on Cell Growth of A549 Human Lung Cancer Cells

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中　華　民　國　　100 年　　6 月

中國醫藥大學 藥學院藥學系

碩士學位考試

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本論文係 劉威鴻 同學於中國醫藥大學藥學院藥學系碩士班完成之碩士論文，經考試委員審查及口試合格，特此證明。

論文口試委員審定書

學位考試委員：

主　　任：

中 華 民 國　一百　年　六　月　二十　日

致謝

在經歷了大學三年與研究所兩年漫長的實驗室生活後，在寫此篇論文的時候，也意味著要離開這熟悉的環境，展開新的人生旅程。在這漫長的實驗室生涯，遇到挫折是難免的，但很感謝一路幫助我的人在我實驗的過程中提供我寶貴的經驗與知識，一起陪我度過重重的難關。

在此將這份研究論文敬獻給吳介信老師、黃旭山老師以及蔡士彰老師、的提攜，教導我在基礎知識及實驗技術方面的了解，並提供良好的環境、設備供我完成碩士研究。同時我也要感謝貴重儀器室戴如淳組長與李宜靜學姊的大力相助，提供儀器操作的資助幫助我實驗順利進行。

最後要感謝的是陪我度過這段日子的家人、實驗室學長姐、學弟妹以及隔壁實驗室的助理、學弟妹們，除了提供實驗室上的指導外，在實驗之餘也是一起哈拉的好對象。實驗室的媽媽桑文馨大人、實驗室老師的左右手以及有實驗之神稱號的俊旭學長、博學多聞認真向上的鳳樟學長、修電腦的專家以及愛玩愛瘋的義忠學長、樂於助人的佩玉學姊、菸酒公賣局局長佩娟學姊、認真做實驗的穎儀學姊、面貌酷酷的宜璇同學、即將踏入博生生活的顯峰同學、營養專家婉婷同學、大腦的專家筱婷學妹、說話慢條斯理以及我看過最認真的新進研究生宜倪學妹、問題寶寶韋婷學妹、以及其他學弟妹們，感謝你們陪伴我這兩年的研究生活。

目錄 (Contents)

中文摘要 (Abstract in Chinese)

端粒酶在約 80%左右的癌細胞中都有活化的跡象，而端粒酶的活化也與癌細胞能毫無限制的生長有密切的關係。因此，端粒酶被認為是一個很好被用來當做治療癌症的一個標的。人類的端粒 DNA 序列是由一段(TTAGGG)n 一直重複所構成，此外，端粒還會形成特殊的 DNA 的二級結構，稱為 G-quadruplex。研究顯示，能穩定 G-quadruplex 的化合物，可以抑制端粒酶的活性。然而，一系列含有不同取代基的 anthraquinone 化合物被證實能藉由標的到 G-quadruplex 進而達到抑制端粒酶的活性。NSC746364 是一個新穎的 2,7-diamidoanthraquinone 的衍生物，它已被證實對多種癌細胞中均有細胞毒殺的作用，但其分子機轉尚未明瞭。在本實驗中，我們發現 NSC746364 可以有效的抑制人類肺癌細胞株 A549 的生長並促使細胞周期停滯在 G2/M 期。我們更進一步發現，此抑制效果是藉由活化 ATM/ATR/Chk1/Chk2 等分子，進而影響了 G2/M 期的調控蛋白 Cylcin B1，最後促使細胞周期停滯在 G2/M 期。此外，caspase-3 的活化，也代表了 NSC746364 會促進細胞的凋亡。最後本實驗亦發現 NSC746364 所抑制細胞生長和細胞周期的現象都能被 ATM/ATR 的抑制劑 caffeine 所逆轉。因此本實驗結果推估，NSC746364 抑制 A549 細胞生長的機制是透過活化 ATM/ATR/Chk1/Chk2。

英文摘要 (Abstract in English)

Telomerase is activated in more than 80% of human cancers and it is responsible for the indefinite proliferation of cancer cells. Therefore, telomerase is considered to be a useful therapeutic target in human cancers. Human telomeric DNA is composed of G-rich (TTAGGG)n repeats and can form G-quadruplex DNA secondary structures. Compounds that can stabilize the G-quadruplex structures have been shown to inhibit the activity of telomerase. A series of substituted anthraquinone compounds have been shown inhibition of telomerase activity possibly through targeting the G-quadruplex structures. NSC746364 is a novel 2,7-diamidoanthraquinone derivatives, which has been found to have cytotoxic effects on various cancer cell lines. However, the detail molecular mechanisms are still unknown. In the present study, we showed that NSC746364 can effectively inhibit A549 cancer cell proliferation, causing cell cycle arrest at G2/M phase in a dose-dependent manner. The underlying mechanisms that NSC746364 induces cell cycle arrest are through activation of ATM/ATR/Chk1/Chk2 DNA damage sensing molecules, which in turn downregulate Cyclin B1 expression. Furthermore, activation of caspase-3 by NSC746364 treatment leading to cellular apoptosis is also one of the mechanisms to inhibit cell growth. Additionally, we found that caffeine abolished the inhibitory effects of NSC746364 on cell proliferation and cell cycle regulation. Taken together, our results suggest that NSC746364 targets the ATM/ATR/Chk1/Chk2 pathway, which leads to suppression of tumor growth.

表目錄 (Table of Contents)

圖目錄 (List of Figures)

第一章 前言(Introduction)

第一節 研究背景(Backgrounds)

Cultured somatic cells do not divide indefinitely [1] because of progressive telomere shortening each time when cell divides. In every eukaryotes, both ends of chromosomes end with G-rich (TTAGGG)n repeats in 5'-3'strand are known as "telomeres" [1-3]. In humans, (TTAGGG)n repeats are about 15-20 kb in length at birth and about 8~10 kb in adults (Figure 1). The 3' single-strand overhang of the end of each telomere is about 200 nucleotides in length and form a special telomere loop called "T-loop" [4], which protected telomere ends from exposure to DNA repair system (Figure 2).

Figure 1 The 3' overhang of telomeric DNA. {*The Biology of Cancer* (© Garland Science 2007)}

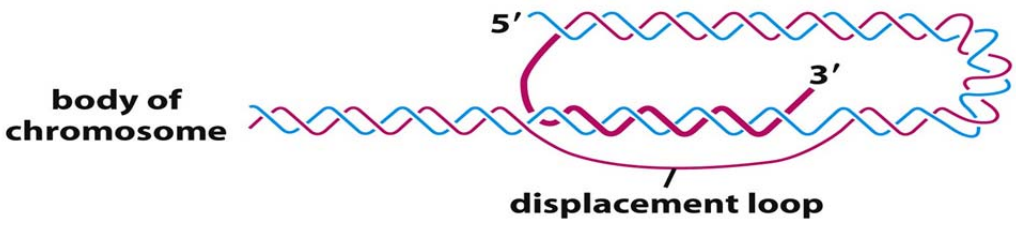


Figure 2 Structure of the T-loop. {*The Biology of Cancer* (© Garland Science 2007)}

Why are telomeres gradually shortened? In 1970s, Olovnikov and Watson reported the mechanism called "end-replication problem" (Figure 3), explained why telomeres were progressively shortened and also provided the

supported evidences to the phenomenon of replicative senescence in normal cells. They proposed that “lagging (discontinuous) strand” was considered to be the reason of telomere shortening, because DNA polymerase only synthesizes DNA in the 5’-3’ direction, and requires RNA primer as the starting point of DNA replication. When replication completes, RNA primers are removed. The 5’ end of the lagging strand locates at the end of extreme 3’ end of the complement strand leaves a gap there. Thus, “end-replication problem” occurs [5]. However, telomeric ends are single-stranded 3’ overhang G-rich sequences, the other mechanism called “leading strand problem”, which was proposed as inability of leading strand DNA synthesis to produce the 3’ overhang, and then “lagging strand problem” may occur in the next round of replication [6]. Considering both “lagging strand problem” and “leading strand problem”, these concepts demonstrated convincingly that normal cells have a limit number of cell divisions because of progressively shortening of telomeres.

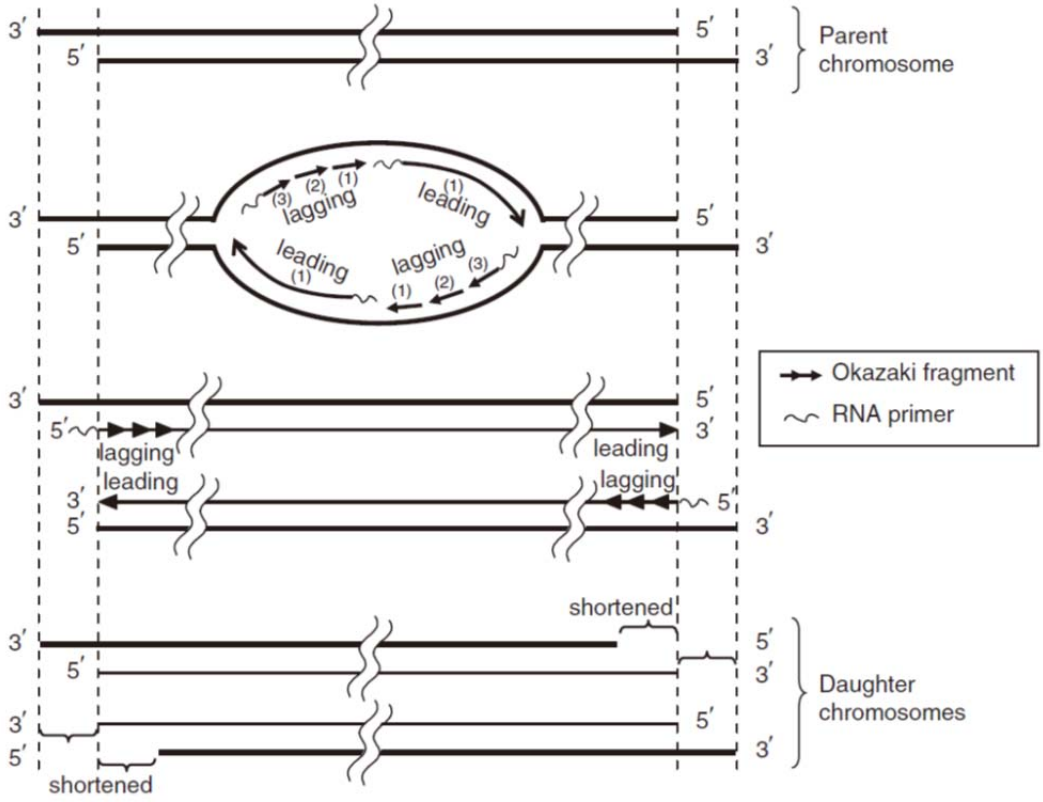


Figure 3 The "end replication problem". *Telomeres and Telomerase in Cancer* (© Human Press 2009)

However, some cells, such as male germline cells and embryonic stem cells [7], have greatly extended number of cell divisions times because of expression of telomerase, the enzyme that can elongate the length of telomere. Human telomerase is an RNA-dependent reverse transcriptase composed of catalytic component telomerase reverse-transcriptase (TERT) [8, 9], and RNA template hTR (also known as telomerase RNA component (TERC)) [10]. Telomerase uses its RNA component to elongate the 3' end of the G-rich telomeric strand. Telomerase than uses its reverse transcriptase activity to add six nucleotides (GGTTAG, directed by the complementary template sequence) sequentially to the telomere. Telomerase then dissociates from the DNA product and rebinds at the telomere and repeats the process (Figure 4). By this mechanism, cells that highly expressed telomerase, such as most cancer cells, have the capacity to proliferate indefinitely.

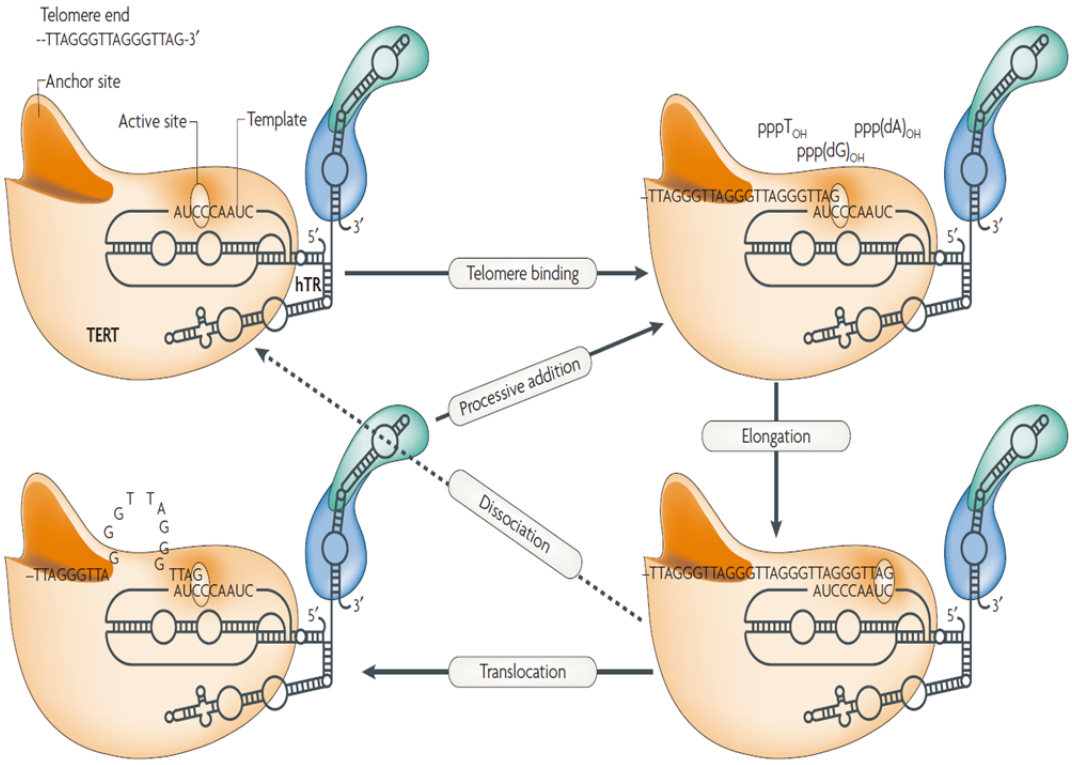


Figure 4 Telomerae is an RNA-dependent reverse transcriptase in which the templating RNA (telomerase RNA component (hTR)) is integral to the enzyme. (*Nature Rev. Cancer* 2008;**8**:167–179.)

Carcinoma and sarcoma are malignant tumors that characterized by uncontrolled cell proliferation and invaded to surrounding tissues or even worse, distant organs. Telomerase, a key enzyme regulating continuous cell division, usually expressed in a low level or repressed in somatic cells except for lymphocytes and self-renewal progenitor cells but is activated in approximately 80% of human cancers and sarcoma tissues [11] (Table 1). These findings suggested that telomerase activity is one of the most crucial and universal tumor markers of human cancers [12-14]. As the wide expression of telomerase in various cancers, it is useful as cancer diagnostic marker for early detection and as a prognostic indicator for predicting the outcome for patients. Because of the specificity and university of telomerase activity and expression in tumors, they are not only ideal cancer diagnostic

and prognostic markers, but suitable targets for cancer therapy. Ideal cancer therapeutic targets are those that are specifically expressed in tumors and are critical for maintaining malignancy [15]. Targeting telomere/telomerase machinery offers a novel and potentially broad-spectrum anticancer therapeutic strategy since telomerase is constitutively overexpressed in the vast majority of human cancers and plays a critical role in unlimited proliferation of cancer cells [15-20].

Table 1 A survey for telomerase activity in human tumors.

Organs/tumors	Positive ratio for telomerase activity (positive/total number)			References
	Normal/adjacent tissue	Nonmalignant lesion	Malignant tumor	
Brain				
Glioblastoma	**0% (0/9)**		**75% (45/60)**	[21]
Astrocytoma			**10% (2/20)**	
Oligodendroglioma			**100% (19/19)**	
Meningioma			**50% (26/52)**	
Head and neck				
Oral	**7.7% (3/39)**	**54.3% (25/46)**	**86.2% (112/130)**	
Thyroid	**9% (9/100)**	**29.2% (26/89)**	**55.3% (73/132)**	[22-26]
Parathyroid	**2.6% (1/38)**	**4.2% (2/47)**	**80% (12/15)**	[27-29]
Breast	**4% (4/100)**	**14.8% (11/74)**	**87.3% (502/575)**	[30-33]
Lung	**4.4% (3/68)**	**6.6% (10/152)**		
Non small cell carcinoma			**72.0% (956/1328)**	[34-37]
Small cell carcinoma			**92.3% (48/52)**	
Digestive organs				
Esophagus	**72.7% (216/297)**		**94.4% (270/286)**	[38-41]
Barrett's esophagus	**68.8% (22/32)**	**44.7% (21/47)**		[42]
Stomach	**15.7% (64/408)**	**95.5% (85/89)**	**85.2% (595/698)**	[43-55]
Colon	**22.8% (69/302)**	**27.6% (37/134)**	**87.3% (331/379)**	[56-61]
Liver	**12.3% (19/154)**	**51.9% (40/77)**		

Hepatocellular carcinoma			**87.0% (537/617)**	[62-73]
Hepatoblastoma [a]		**31.0% (53/171)**	**66.7% (26/39)**	[74]
Biliary duct	**9.8% (4/41)**	**0% (0/25)**	**65.2% (30/46)**	[75, 76]
Pancreas	**5.3% (5/95)**	**3.6% (2/55)**		[77-82]
Duct cell carcinoma			**84.0% (142/169)**	
Endocrine organs				
Pancreas				
Endocrine carcinoma			**15% (6/40)**	[83, 84]
Adrenal gland	**0% (0/62)**			
Adrenocortical		**15.6% (17/109)**	**67.6% (25/37)**	
Phenochromocytoma		**6.5% (2/31)**	**62.5% (5/8)**	[85-89]
Genitourinary organs				
Kidney	**2.1% (8/386)**			
Renal cell carcinoma		**7.1% (1/14)**	**76.9% (409/532)**	[90-99]
Nephroblastoma [a]			**79.3% (69/87)**	[100]
Bladder	**9.8% (9/92)**	**36.2% (21/58)**	**84.4% (552/654)**	[101-112]
Prostate	**7.4% (5/68)**	**20.5% (34/166)**	**87.3% (254/291)**	[113-120]
Testis	**100% (7/7)**	**100% (1/46)**	**100% (82/82)**	[121, 122]
Ovary	**38.0% (11/29)**	**45.5% (15/33)**	**68.5% (100/146)**	[123]

Uterus/endmetrial	**57.0% (77/135)**	**76% (19/25)**	**96.0% (143/149)**	[123-128]
Uterus/cervical	**6.2% (12/193)**	**60.8% (110/181)**	**89.5% (350/391)**	[129-137]
Skin	**24% (6/25)**	**28.6% (12/42)**		
Squamous cell carcinoma			**60% (18/30)**	[138]
Basal cell carcinoma			**92.2% (83/90)**	
Melanoma			**71.8% (28/39)**	
Sarcoma	**0% (0/12)**	**6.7% (6/89)**	**52.9% (312/590)**	[139-149]
Malignant histiocytoma			**(1/12)**	
Liposarcoma			**(1/5)**	
Rhabdomyosarcoma			**(0/1)**	
Osteosarcoma		**(0/14)**	**(20/47)**	
Leiomyosarcoma			**(2/6)**	
Childhood tumor				
Retinoblastoma			**46.5% (20/43)**	[150]
Neuroblastoma	**0% (0/13)**	**0% (0/6)**	**61.0% (178/292)**	[151-154]
Germ cell tumor		**25% (1/4)**	**100% (27/27)**	[121]

The table is adapted from Telomeres and Telomerase in Cancer (Springer).

[a] Childhood malignancy

The various approaches to eliminating telomerase-positive cancer cells (Figure 5) have their own strengths and weakness (Table 2). One of the approaches called telomere-disrupting agents, which targets special DNA secondary structures named as G-quadruplex (Figure 6) has been widely developed recently. Because human telomeric DNA consists of tandem repeats of the sequence (TTAGGG)n, it has a strong propensity to form the DNA G-quadruplex secondary structure, which is known to inhibit the activity of telomerase [155]. Thus, developing compounds that can stabilize G-quadruplex DNA secondary structure has been considered as potentially therapeutic intervention to telomerase-positive cancer cells. Wide arrays of ligands that target the telomeric G-quadruplex have been developed and identified [19] (Figure 7). Disrupting telomere DNA maintenance can be sensed as DAN damage, which in turn rapidly activated signal transducers which responded for DNA damage. These serine-threonine kinases, such as ATM (ataxia telangiectasia mutated protein or *Sc* and *Sp* Tel1), ATR (ATM and Rad3-related protein or *Sc* Mec1 and *Sp* Rad3) [156, 157] and DNA-PK, senses DNA damage and later on, stalls replication forks, causes cell cycle arrest and rapidly activates the apoptotic pathways [158-161] (Figure 8). Interestingly, G-quadruplex-targeting ligands have been shown to disrupt telomere capping and maintenance and induce rapid apoptosis [19, 162]. Studies show that anthraquinones are potent human telomerase inhibitors and some members of the amidoanthraquinone class of DNA intercalators could target to quadruplexs effectively and, thus could show antitumor properties [163-169]. Huang et.al, synthesize a series of symmetrical 2,7-diamidoanthraquinone derivates [170]. Among them, three lead molecules (NSC746364, NSC746365, and NSC746366) elicit potent antitumor activity

against a full panel of different types of human tumor cell lines, including non-small cell lung cancer, colon cancer, breast cancer, ovarian cancer, leukemia, renal cancer, melanoma, prostate cancer, and central nervous system (CNS) cancer [171].

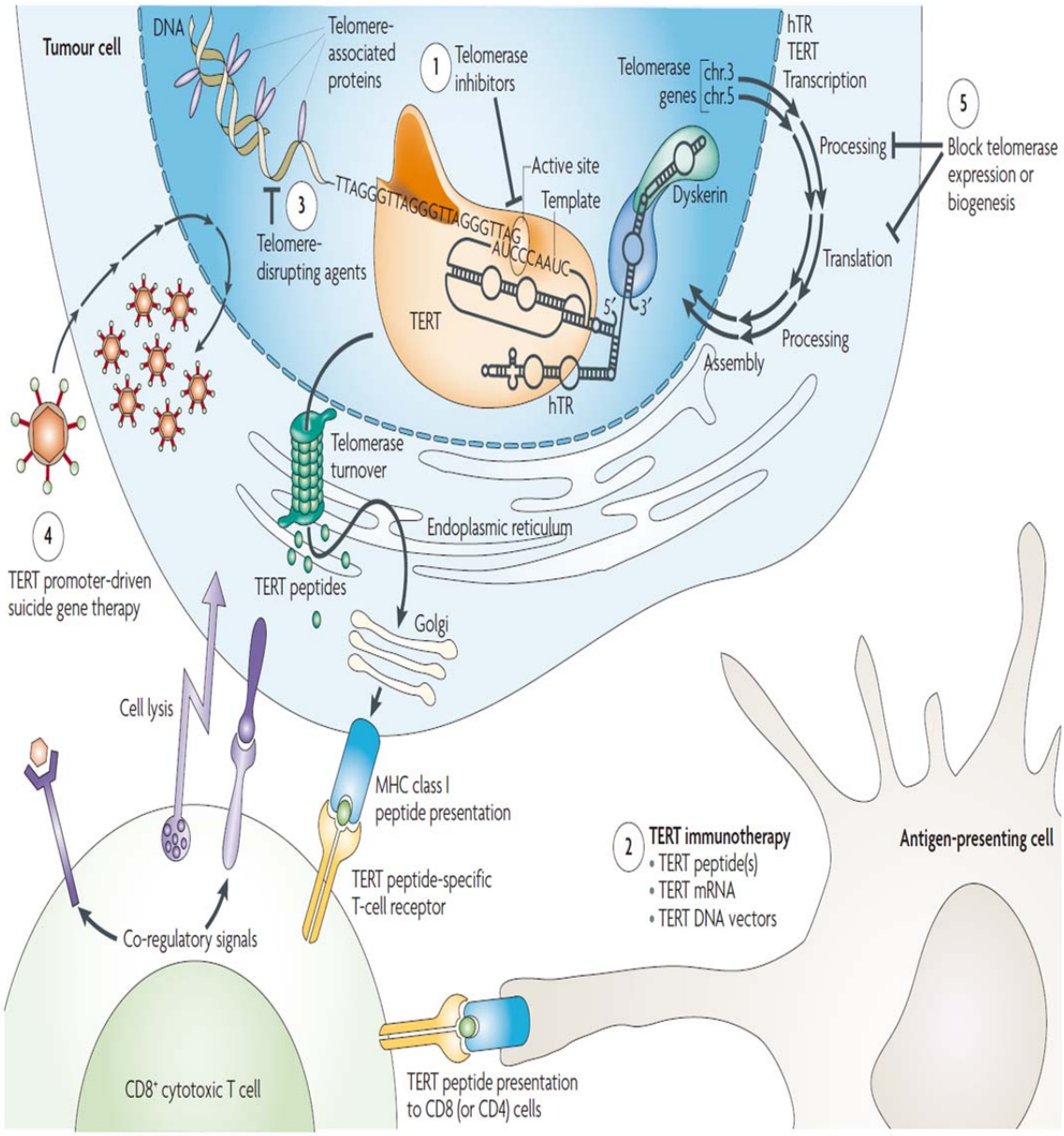


Figure 5 Five-telomerase-based approaches to kill tumor cells are illustrated in order of their anticipated pharmaceutical potential. (*Nature Rev. Cancer* 2008;**8**:167–179.)

Table 2 Advantages and disadvantages of different telomerase-based anticancer approaches.

Approach	Possible advantages	Possible disadvantages
Telomerase inhibition		
Active site and allosteric small molecules and oligonucleotides explored. A short lipidated oligonucleotide active site inhibitor is in clinical trials (TABLE 2)	Standard pharmacological approach, amenable to small molecule and oligonucleotide drug development. Oligonucleotide products probably not subject to common drug-resistance mechanisms. Safety margin based on known differences in telomerase activity and telomere length in tumour versus normal tissue	Time between drug administration and clinical response can be long. Sustained inhibition is probably required. Long treatment duration might cause toxicity in some proliferative tissues in some patients
Active immunotherapy		
Products based on TERT peptides, mRNA, plasmid or viral DNA; *ex vivo* or *in vivo* priming of autologous or allogeneic APCs. Multiple products in clinical trials (TABLE 2)	Life-long effects with periodic boosting possible. Might be extremely effective in minimal residual disease with clearing of metastatic circulating tumour cells. Ultimately might extend to prophylactic setting for high-risk individuals. Autologous cell, full-length gene or mRNA-based strategies likely to be most potent, but also most challenging from a manufacturing and quality-control perspective. 'Off-the-shelf' vaccines (either genetic (DNA or mRNA) or peptide) are most practical	Immune system may be compromised in many patients (weak TERT response). Significant manufacturing and regulatory challenges with autologous cell gene-based products
Telomere-disrupting agents		
G-quadraplex-stabilizing molecules in development. Telomere protein–protein interaction targets and mutant hTR gene therapy-based approaches in discovery research	Rapid induction of cell death possible. Amenable to small-molecule drug discovery in some cases	Toxicity to normal cells and tissue might be unacceptable for agents targeting endogenous telomere (disrupting telomere structure probably lethal in normal cells). Gene therapy with a mutant hTR, creating altered telomere sequence, is specific for telomerase-positive tumour cells, but effective delivery throughout body is challenging
Suicide gene therapy		
Gene therapy using telomerase promoter-driven expression of a toxic gene or gene that triggers a toxic downstream event (for example, oncolytic viral replication or toxic prodrug conversion)	Rapid killing of telomerase-positive cells based on the increased expression of either *hTR* or *TERT* promoters in tumour versus normal cells. Can be engineered to have bystander effects	Effective delivery of gene therapy to cancer cells throughout the body is challenging. Immunological response to vector system might limit dosing
Agents that block telomerase expression or biogenesis		
Antisense, ribozyme or small interfering RNA agents targeting TERT mRNA or hTR; gene therapy overexpressing mutant TERT; HSP90 inhibitors targeting assembly, etc.	Orthogonal approach to direct telomerase inhibition	Agents may be leakier, or less efficient than direct inhibitors. Broad delivery and stability issues with oligonucleotide and gene therapy-based approaches. See other possible disadvantages for telomerase inhibition above

APC, antigen-presenting cells; HSP90, heat shock protein 90; TERT, human telomerase reverse transcriptase; hTR, human telomerase RNA component.

(*Nature Rev. Cancer* 2008;**8**:167–179.)

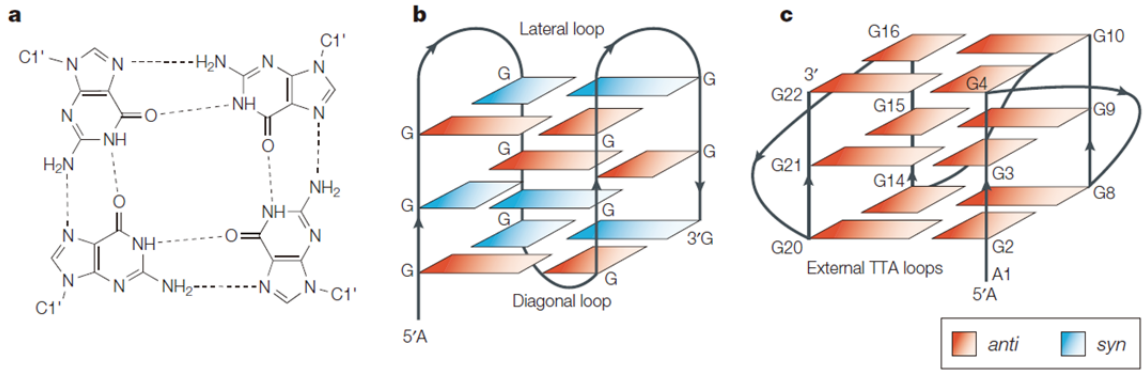


Figure 6 The folding of telomeric DNA: guanine quadruplex. (*Nat Rev Drug Discov* 2002;**1**:383–393.)

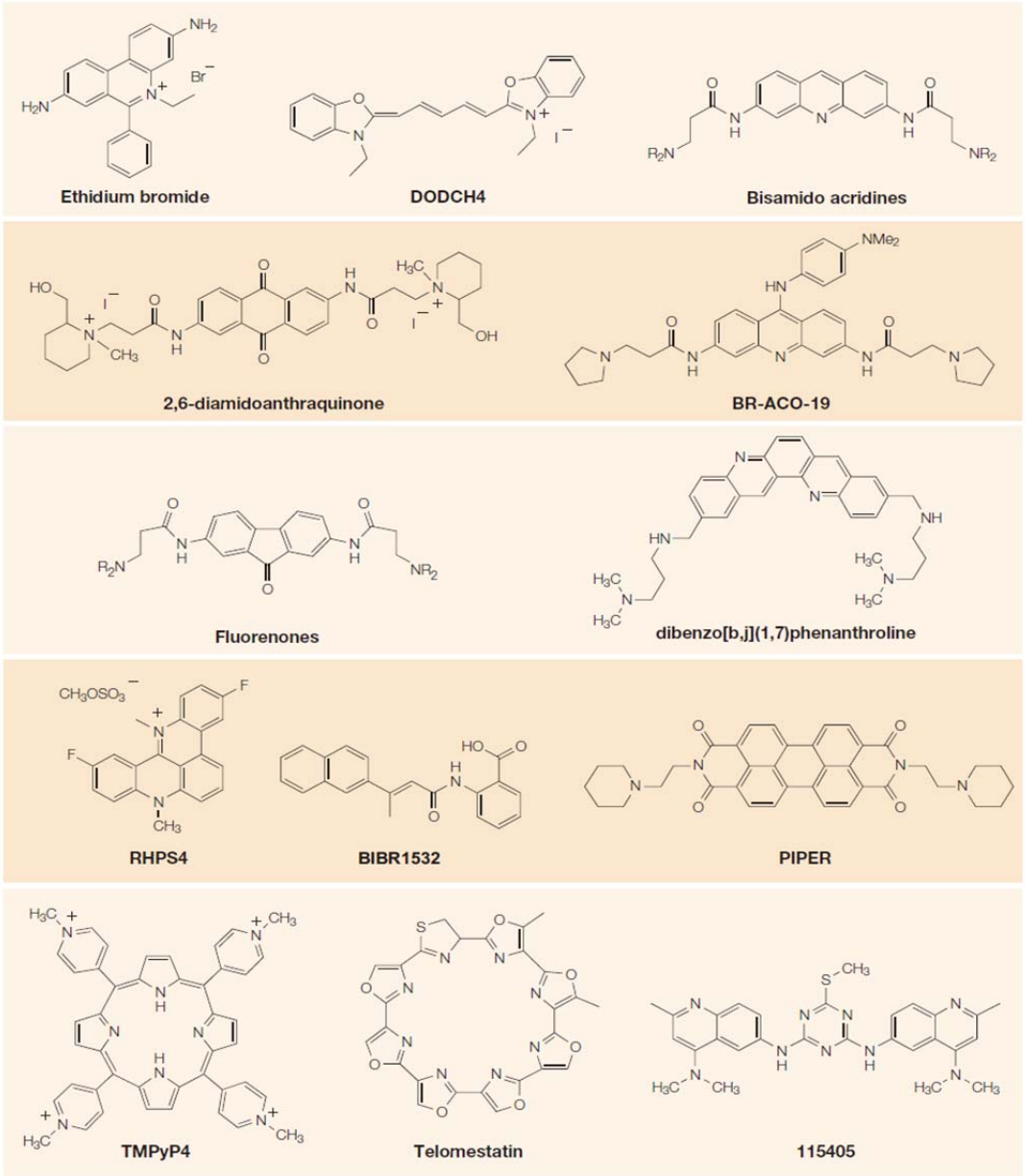


Figure 7 Structures of some small-molecule compounds that target telomeric G-quadruplexes. (*Nat Rev Drug Discov* 2002;**1**:383–393.)

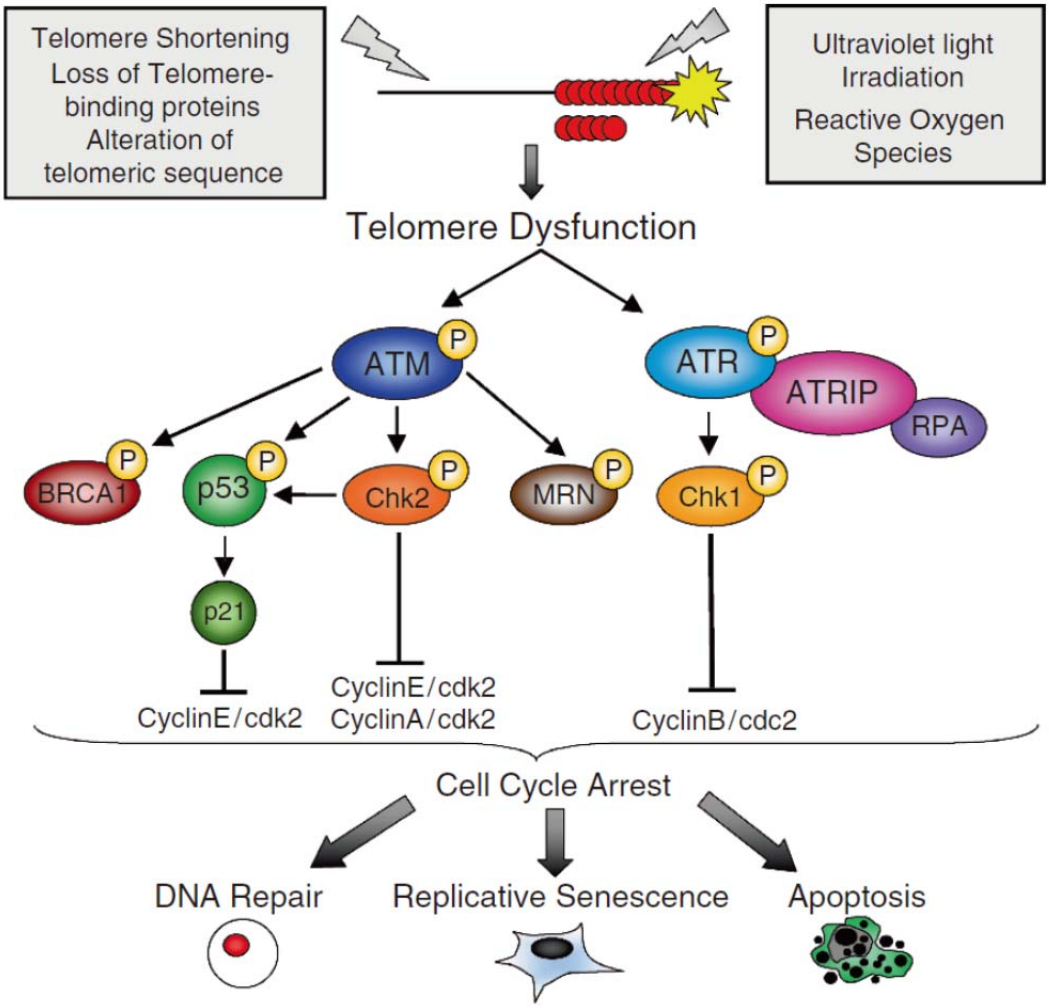


Figure 8 Telomere dysfunction activates the DNA damage response. *Telomeres and Telomerase in Cancer* (© Human Press 2009)

第二節 研究目的

Telomeres are distinct structures located at the ends of linear chromosomes, which play a crucial role in protecting chromosomes ends from DNA degradation, DNA repair mechanisms and fusion [172, 173]. Telomeres shortening or uncapped telomeres activated DNA damage response resulting in cellular senescence and apoptosis [173]. To counter these effects, an enzyme called telomerase which is the well known enzyme that elongate telomeric DNA [174, 175]. Evidence indicated that telomerase activation has been detected in more than 80 percent of overall cancer tissues [11]. In tumor tissue, telomerase activity was detected in 94% of neuroblastoma [176], 93% of colorectal cancer [177], 85% of gastric cancer [178], and 85% of hepatocellular carcinoma specimens [179]. In lung cancer, telomerase activity was detected in approximately 80% of non-small cell lung cancer (NSCLC) specimens [180]. No other tumor-associated gene is widely expressed in cancers [181]. Thus, targeting telomerase in comparison with most other cancer targets are more universality, criticality and specificity for cancer cells.

As human telomerase has been considered as a novel and potentially highly specific target for antitumor drug design [182-184], various approaches have been developed, including inhibition of telomerase enzyme activity and telomere-disrupting agents. GRN163L, the oligonucleotide template antagonist of telomerase RNA component (hTR) sequence [185], was taken currently into clinical trials for chronic lymphocytic leukemia. In addition to inhibiting telomerase activity in cancer cells, the development of G-quadruplex stabilizers has emerged as a highly promising approach [15, 19, 20, 186]. Human telomeric DNA consists of tandem repeats of the sequence

d(TTAGGG) and can form G-quadruplex DNA secondary structures, which is known to inhibit the activity of telomerase [155]. As a consequence, compounds that can stabilize the telomeric DNA G-quadruplex have been considered as potentially valuable antitumor drugs. Several compounds that target G-quadruplex and inhibit telomerase activity have been identified, including porphyrins [187-189], anthraquinones [190], and N,N'-bis[2-1(-piperidino)-ethyl] -3,4,9,10-perylene-tetracarboxylic diimide (PIPER) [191].

Anthraquinone-containing extracts from different plants such as senna, cascara, aloe, frangula, and rhubarb have been found to have a wide variety of pharmacological activities, including anti-inflammatory, antimicrobial, and antitumor activities [192, 193]. This widely occur compounds in the plant kingdom and its' synthetic derivates currently occupy a prominent position in anticancer drugs development. For example, Mitoxantrone and ametantrone are synthetic 1,4-bis[(aminoalkyl)amino]anthraquinones that have been developed to treat many malignancies [194, 195]. Although the molecular mechanism of the antitumor activity of anthraquinone is complicate, studies have indicated that its intercalative interaction with DNA may play a major role [196-198]. From the study on the structure-activity relationships of anthraquinonees, the first anthraquinone derivate that has been shown to interact with quadruplex structures and inhibit telomerase was a symmetric 2,6-disubstituted aminoalkylamido anthraquinone molecule [190]. To date, various newly synthesized G-quadruplex targeting anthraquinone-base derivates have been evaluated [163, 164, 168, 170, 199-203]. NSC746364 was one of three lead molecules (NSC746364, NSC746364, and NSC746366) emerged from a series of synthetic symmetrical 2,7-diamidoanthraquione

derivates [170], which elicits potent antitumor activity against a full panel of different types of human tumor cell lines, including non-small cell lung cancer, colon cancer, breast cancer, ovarian cancer, leukemia, renal cancer, melanoma, prostate cancer, and central nervous system (CNS) cancer [171]. Although NSC746364 has shown to inhibit telomerase and exerts cytotoxic effect on a variety of cancer cells, the underlying molecular mechanisms still unclear.

第二章 研究方法(Methods)

第一節 研究設計(Experimental Design)

The goal of this study was to investigate the molecular mechanisms of NSC746364 on A549 human lung adenocarcinoma cell line. For this, we used a wide variety of *in vitro* assays. These included assay to detect cell proliferation, flow cytometry to analyze cell cycle distribution, and Western blot to explore cell signaling pathways. Several signaling molecules we are interesting in, includes DNA damage sensing and response proteins, cell cycle regulatory proteins, and some key executors involved in apoptosis. Below is an experimental design concept map (Figure 9).

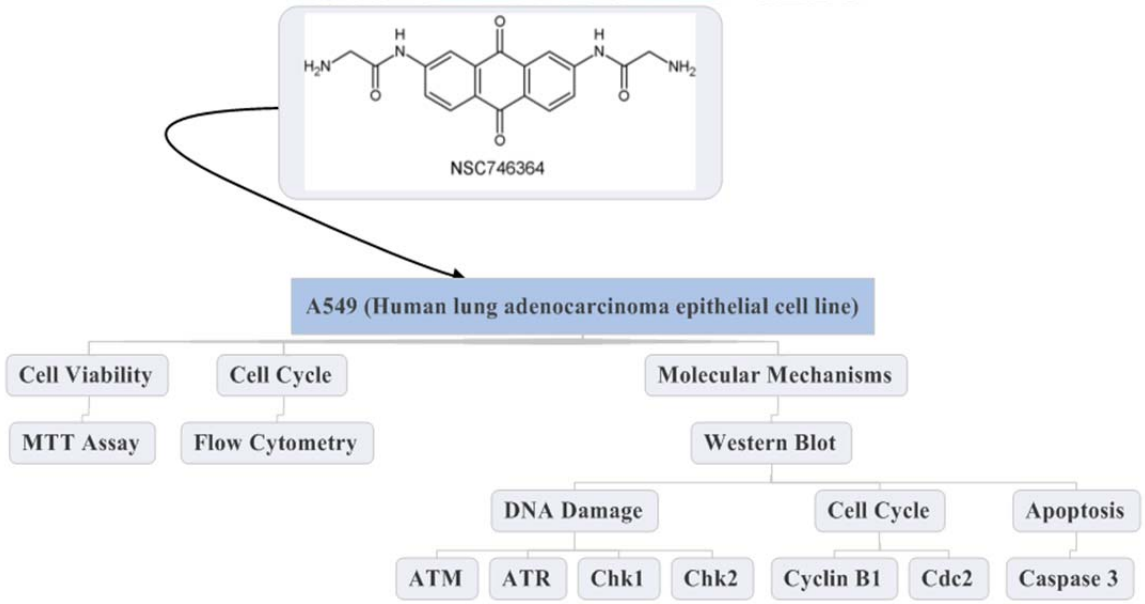
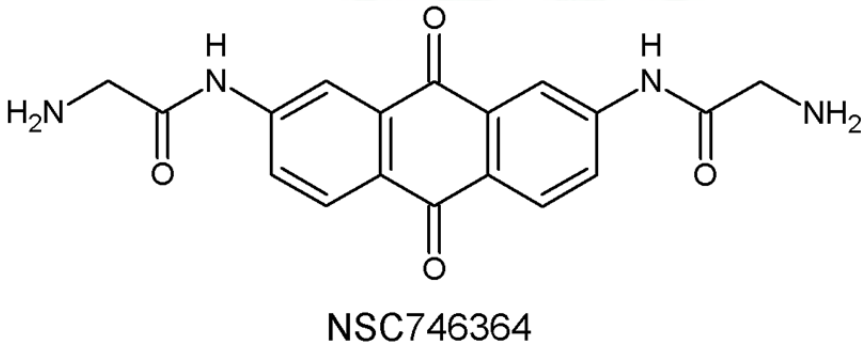


Figure 9 An experimental design concept map.

第二節 研究材料 (Materials)

一、 新穎端粒酶抑制劑, NSC746364 (A Novel Telomerase inhibitor, NSC746364)

NS746364 was given by professor Hsu-Shan Huang. The synthesis and chemical characterization of NSC746364 have been described previously [170, 204]. NSC746364 was dissolved in DMSO to make a 100 mM stock solution. All stock solutions were aliquoted and stored as ≦ －20℃ and protect from light. The structure of NSC746364 represents below (Figure 10).

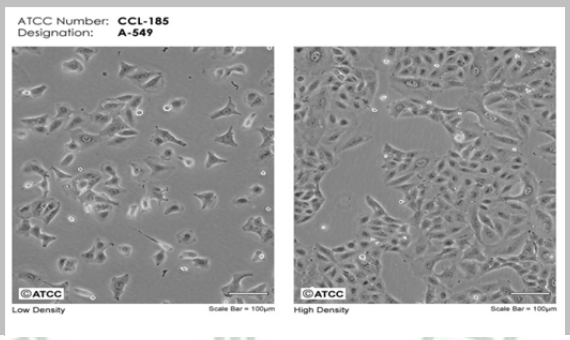


NSC746364

Figure 10 Chemical structure of bioactive chromophoric anthraquinone-base antitumor agent, NSC746364.

二、 實驗細胞株 (Experimental Cell Line)

Table 3 A549 (Human lung adenocarcinoma epithelial cell line)

ATCC® Number:	CCL-185™
Designations:	A549
Depositors:	M Lieber
Biosafety Level:	1
Growth Properties:	Adherent
Organsim:	*Homo sapeines* (Human)
Morphology:	Epithelial 
Source:	**Organ:** lung **Disease:** carcinoma
Cellular Products:	keratin
Propagation:	**Complete growth medium:** The base medium for theis cell line is DMEM. To make complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:**37.0℃
Subculturing:	**Interval:** Maintain cultures at a cell concentration between 6 $\times 10^3$ and 6×10^4 cell/cm^2 **Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:8 is

	recommended **Medium Renewal:** 2 to 3 times per week
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5％ (v/v) of DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	About 22 hours

The table is adapted and modified from ATCC™.

三、 藥品試劑 (Reagents)

1. 藥品試劑 (Reagents)

（A） 40% 29:1 Acrylamide/Bis (BIO BASIC.INC, Canada)

（B） APS (USB, USA)

（C） Bradford (BIO-RAD, USA)

（D） Bromophenol Blue (AMRESCO®, USA)

（E） Caffeine (SIGMA, USA)

（F） DMEM （GIBCO,USA）

（G） DMSO（SIGMA, USA）

（H） DTT (BIO-RAD, USA)

（I） Ethanol (ECHO Chemical, Taiwan)

（J） FBS（GIBCO,USA）

（K） Glycerol (GERBU, Germany)

（L） Glycine (GERBU, Germany)

（M） Methanol (ECHO Chemical, Taiwan)

（N） MTT (BIO BASIC INC., Canada)

（O） Nonfat dry milk (Anchor, New Zealanders)

（P） Potassium phosphate Monobasic,Crystal (KH_2PO_4) (J.T. Baker, USA)

（Q） Potassium chloride (KCl) (Scharlau, Spain)

（R） Sodium chloride (BIO BASIC INC., USA)

（S） Sodium phosphate, dibasic anhydrous (Na_2HPO_4) (J.T. Baker, MALAYSIA)

（T） Sodium dihydrogen phosphate, monohydrate ($NaH_2PO_4 \cdot H_2O$) (MERCK, Germany)

（U） Sodium bicarbonate ($NaHCO_3$) (BIO BASIC INC.,

USA)

（V） Sodium azide (NaN_3) (USB, USA)

（W） TEMED (AMRESCO, USA)

（X） Tis-HCL(GERBU, USA)

（Y） Tris-C (GERBU, USA)

（Z） Triton X-100（SIGMA, USA）

（AA） Trypan blue（GIBCO,USA）

（BB） Trypsin（GIBCO,USA）

（CC） Tween-20 (GERBU, USA)

2. 抗體 (Antibodies)

（A）一級抗體 (Primary Antibodies)

i. Anti- phsopho-ATM (Ser 1981) (ab36810; Abcam, USA)

ii. Anti- phospho-ATR (Ser 428) (sc-109912; SENTA CRUZ, USA)

iii. Anti-phospho-Chk1 (Ser 345) (#9114; Cell Signaling, USA)

iv. Anti-phospho-Chk2 (Thr 68) (#2661; Cell Signaling, USA)

v. Anti-Chk1 (#2345; Cell Signaling, USA)

vi. Anti-Chk2 (#2662; Cell Signaling, USA)

vii. Anti-Cyclin B1(#4138 ;Cell Signaling, USA)

viii. Anti-phospho-cdc2 (Thr 161) (#9114 ;Cell Signaling, USA)

ix. Anti-Cdc2 (#9112;Cell Signaling, USA)

x. Anti-Caspase 3 (AB1899; MILLIPORE, USA)

xi. Anti-PARP (ab4830; Abcam, USA)

xii. Anti-β-actin (sc-47778; SENTA CRUZ, USA)

（B）二級抗體 (Secondary Antibodies)

i. Goat-anti-mouse IgG HRP (Sc-2005; SENTA CRUZ, USA)

ii. Goat-anti-rabbit IgG HRP (NEF812001EA; PerkinElmer, USA)

3. 試劑組 (Kits)

（A）Western Lightning® Plus-ECL Enhanced Chemiluminescence Substrate (NEL 104001EA; PerkinElmer, USA)

（B）Bio-Rad Protein Assay Kit (500-0002; Bio-Rad, USA)

（C）Pro-PREP™ Protein Extration Solution (17081.1; iNtRON BIOTECHNOLOGY, Korea)

四、 設備與器材（Equipments）

（A）BD FACSCanto (Argon-Ion Laser 488 nm, He-Ne Lasser 633 nm) (Becton Dickinson, USA)

（B）Centrifuge (Hettich, Germany)

（C）Centrifuge 5804R (eppendorf, Germany)

（D）CO2 Incubator (NU4500, Nuaire, USA)

（E） Dry Bath (Model 110001, Boekel)

（F）Elctronic Precision Scales/ Balances (ACCULAB sartorius

group, Germany)

（G）Freezer (Frigidaire®, USA)

（H）General-Purpose Analysis Software Multi Gauge -Ver3.X- (FUJIFILM, Japan)

（I）Hemocytometer (Boeco, Germany)

（J）Heraeus Megafuge 16R Centrifuge (Thermo Fisher Scientific Inc., USA)

（K）Hotplate Stirrer (Laboratory & Medical Supplies, Japan)

（L）Laminar Flow Hood (TSAO HSIN ENTERPRISE CO.,LTD., Taiwan)

（M） LAS-4000 luminescence/fluorescence imaging system (FUJIFILM, Japan)

（N）Microscope (Nikon, TE-2000-U, Japan)

（O）Mini Format Vertical Electrophoresis (Mini-PROTEAN® Tetra Cell, Bio-RAD, USA)

（P）ModFit LT Analysis Software (Verity Software House, USA)

（Q）MS Orbital Shaker (Major Science, Taiwan)

（R）Orbital Shaker (GENPURE, Taiwan)

（S）pH Meter (C831, Consort, UK)

（T）PowerPac™ Basic Power Supply (Bio-RAD, USA)

（U）Spectrafuge 24D Microcentrifuge (Labnet International, Inc., USA)

（V）Tank Transfer Systems (Mini Trans-Blot® Cell, Bio-RAD, USA)

（W） Ultrapure water System (Millipore, USA)

（X）Vortex Genie-2 (Scientific Industries, Inc., USA)

（Y）Water Bath (YIHDERN, Taiwan)

（Z）ELISA Reader (BioTeck, British)

（AA） ELISA Reader (ANTHOS-2030, Salzbrug, Austria)

（BB） Cell Culture dishes (Greiner Bio-One, USA)

（CC） Centrifuge tube (AXYGEN, USA)

五、 試劑配製 (Reagent Preparation)

Table 4 1.5M Tris-HCl, pH 8.8

	Final concentration	Amount
Tris-base	**1.5 M**	**181.71 g**
HCl		**adjust to pH 8.8**
d.d H_2O	**added to total volume 1000 ml**	

Table 5 0.5M Tris-HCl, pH6.8

	Final concentration	Amount
Tris-base	**0.5 M**	**60.57 mg**
HCl		**adjust to pH 6.8**
d.d H_2O	**added to total volume 1000 ml**	

Table 6 PI Staining Solution

	Final concentration	Amount
Propidium iodide (PI)	**0.4 mg/dl**	**5 ml**
Triton X-100	**1 %**	**5 ml**
RNase A	**0.1 mg/ml**	**1.25 ml**
1×PBS		**13.75 ml**
Total volume		**25 ml**

Table 7 10% SDS

	Final concentration	Amount
SDS	**10 %**	**10 g**
d.d H_2O	**added to total volume**	**100 ml**

Table 8 10% APS

	Final concentration	Amount
APS	**10 %**	**100 mg**
d.d H_2O	**added to total volume**	**1 ml**

Table 9 1×PBS Buffer (pH 7.4)

	Final concentration	Amount
KCl	**2.68 mM**	**0.2 g**
NaCl	**136.89 mM**	**8 g**
Na_2HPO_4	**10.14 mM**	**1.44 g**
KH_2PO_4	**1.76 mM**	**0.24 g**
HCl		**adjust to pH 7.4**
d.d H_2O	**added to total volume**	**1000 ml**

Table 10 0.05% MTT Reagent

	Final concentration	Amount
MTT	**0.05 %**	**5 mg**
d.d H_2O	**added to total volume 10 ml**	

Table 11 SDS-PAGE Preparation

	Separating gel			Stacking gel
One Gel	**8 %**	**10 %**	**12 %**	**5 %**
d.d H2O	**5.25 ml**	**4.75 ml**	**4.25 ml**	**3.04 ml**
1.5 M Tris-HCl (pH 8.8)	**2.5 ml**	**2.5 ml**	**2.5 ml**	**-**
0.5 M Tris-HCl (pH 6.8)	**-**	**-**	**-**	**1.25 ml**
10 % SDS	**0.1 ml**	**0.1 ml**	**0.1 ml**	**0.05 ml**
40 % Acrylamide/Bis（29：1）	**2 ml**	**2.5 ml**	**3 ml**	**0.61 ml**
10 % APS	**0.05 ml**	**0.05 ml**	**0.05 ml**	**0.05 ml**
TEMED	**0.01 ml**	**0.01 ml**	**0.01 ml**	**0.006 ml**

Table 12 Running Buffer

	Final concentration	Amount
Tris-base	**24.76 mM**	**30 g**
Glycine	**191.82 mM**	**144 g**
SDS	**0.1 %**	**10 g**
d.d H_2O	**added to total volume 10 L**	

Table 13 6× Protein Dye

	Final concentration	Amount
Tris-base	**0.62 M**	**1.135 g**
SDS	**10 %**	**1.5 g**
Glycerol	**50 %**	**7.5 ml**
DTT*	**0.5 M**	**1.155 g**
Bromophenol blue	**0.07 %**	**10 mg**
HCl		**adjust to pH 6.8**
d.d H_2O		**added to total volume 15 ml**

Table 14 Transfer Buffer

	Final concentration	Amount
Tris-base	**19.98 mM**	**24.2 g**
Glycine	**191.82 mM**	**144 g**
MeOH	**15 %**	**1.5 L**
d.d H_2O		**added to total volume 10 L**

Table 15 1×TBST Buffer

	Final concentration	Amount
Tris-base	**10.98 mM**	**1.33 g**
NaCl	**154 mM**	**9 g**
Tween-20	**0.05 %**	**0.5 ml**
HCl		**adjust to pH 7.4**
d.d H_2O		**added to total volume 1000 ml**

第三節 實驗方法 (Experimental Methods)

一、 細胞培養 (Cell Culture)

1. 人類肺癌細胞株 A549 之細胞培養 (Culture of Human Lung Cancer Cell Line, A549)

A549 human cell lung carcinoma cell line was form the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagles's medium (DMEM), supplemented with 10% Fetal bovine serum (FBS) and 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfates. The culture medium was replaced every two days and cells were passaged every week. Cells that became at least 80% confluent were starved for 24 h in DMEM followed by a treatment with indicated concentration of NSC746364 in DMEM containing 10% FBS for indicated time.

2. 細胞計數 (Cell Counting)

The most widely used type of cell counting chamber is called a hemocytometer. To prepare the device the surface of counting chamber is carefully cleaned with 70% ethanol and wiped out the surface with lens paper. The cover glass is also cleaned and is placed over the counting surface prior to introducing the cell suspension. First, 100 μl of cell suspension was mixed with 10 μl of Trypan Blue dye (GIBICO) in 1.7 ml eppendorf, then placed 10 μl of the mixture in both wells of the hemocytometer. viable cells were count in both sides of the hemocytometer using the hand counter while looking through the microscope.

二、 細胞存活檢測 (MTT Cell Viability Assay)

MTT assay was performed to measure the cytotoxicity of NSC746364 on A549 lung cancer cells. Cells were seeded in 24-well plates with 2×10^4 cells/well in DMEM supplemented with 10% FBS. After 24 hr, cells were washed with phosphate-buffered saline (PBS) and then exposed to either DMSO alone or different concentrations（5, 10 and 20 μM） of NSC746364. After 24 hr and 48 hr, the number of viable cells was determined. Briefly, MTT (0.5 mg/ml in DMEM containing 10% FBS) was added to each well (400 μl per well), and the plate was incubated at 37℃ for 4 hr. Cells were then spun at 300g for 5min, and the medium was carefully aspirated. A 400 μl aliquot of DMSO was added, and the absorbance at 595 nm was measured for each well on ELISA reader (Anthos, 2001, Anthos Labtech. Austria).

三、 流式細胞儀—細胞週期分析 (Flow Cytometry—Cell Cycle Analysis)

The DNA content of the treated cells was assessed using flow cytometry following propidium iodide (PI) staining. Cells were seeded in 6-cm petri-dishes with 2×10^5 cells/dish in DMEM supplemented with 10% FBS. After 24 hr, cells were washed with phosphate-buffered saline (PBS) and starved for 24 hr in DMEM followed by exposing to either DMSO alone or serial dilutions（5, 10 and 20 μM） of NSC746364 for 24 hr or 48 hr. After 24 hr or 48 hr, cells were harvested with trypsin-EDTA, washed twice with 10 ml ice-cold PBS, fixed in 70% ethanol, and kept at -20℃ prior to FACS analysis. For DNA content analysis, cells were centrifuged and resuspended

in 0.4 ml of DNA staining solution [0.4 mg/dl PI, 1% Triton X-100, 0.1 mg/ml RNase A (DNase-free) in PBS]. The cell suspension was stored at 4℃ and protected from light for a minimum of 30 min and analyzed within 2 hr. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentages of hypodiploid (apoptotic, sub-G1) events and percentages of cells in G0/G1, S-, and G2-M phases were determined using the DNA analysis software ModFitLT, version 2.0 (Verity Software, Topsham, ME, USA).

四、 西方墨點法 (Western Blot)

A549 cells cultured in petri dishes were incubated with 5, 10 and 20 μM of NSC746364 in DMEM containing 10% FBS for 24hr. For the time course experiments, cells were treated with 20 μM of NSC746364 in DMEM containing 10% FBS for 15, 30, and 60 min. Cells were then lysed in protein extraction buffer (iNtRON Biotechnology Inc), followed by incubation at 95°C for 5 min. Samples were separated using SDS-PAGE, transferred to PVDF membranes, blocked with 5% nonfat dry milk in TBST (Tris-base saline containing 0.05% Tween-20) for 1 h, and then probed with the desired antibodies [anti-Cyclin B1 (Cell Signaling #4138), anti-Cdc2 (Cell Signaling #9112), anti-phsoho-Cdc2-Thr-161(Cell Signaling #9114), anti-p-ATM-Ser-1981 (ab36810), anti-ATM, anti-p-ATR-Ser-428 (sc-109912), anti-ATR, anti-p-Chk1-Ser345 (Cell Signaling #2341), anti-Chk1 (Cell Signaling #2345), anti-p-Chk2-Ser345 (Cell Signaling #2661), anti-Chk2 (Cell Signaling #2662) and anti-β-actin (sc-47778)] overnight at 4°C. The blots were then incubated with horseradish peroxidase-linked

secondary antibody for 1 h followed by development with the ECL reagent and chemiluminescence signals were detected by LAS-4000 luminescence/fluorescence imaging system (FUJIFILM). The intensities were quantified by densitometric analysis software Multi Guage version 2.0 (FUJIFILM).

五、 DAPI 染色 (DAPI Staining)

A549 cells were seeded in 6-wells plate and incubated with 0, 5, 10, and 20 μ M of NSC746364 in DMEM containing 10% FBS for 24 h. After 24 h incubation time, cells were washed three times with PBS, fixed with 3% Formaldehyde for 15–20 min, following 0.1% Triton X-100 for 15 min, and stained with DAPI (1μg/mL) for 1 min. Wash cells two times with PBS. Cells were observed by using fluorescence microscope, and then all pictures were taken at 40X.

六、 統計分析 (Statistics Analysis)

Data are expressed as mean ± S.E.M Statistical analysis was conducted using ANOVA test. A p-value $\leqq 0.05$ was considered significant.

第三章 實驗結果 (Results)

一、 *NSC746364 suppresses cell proliferation of A549 human lung cancer cell line*

The effect of NSC746364 on cell proliferation was assessed using the MTT proliferation assay. To test the effect of NSC746364 on the proliferation of A549 cells, the cells were treated with different concentrations of NSC746364 (0 μM, 5 μM, 10 μM, 20 μM). After 24 h (A) and 48 h (B) incubation, the cell viability was determined by using the MTT assay. As shown in Figure 11 A and B, treatment with NSC746364 significantly inhibited the viability and proliferation of cells, and these effects occurred in a dose-dependent manner.

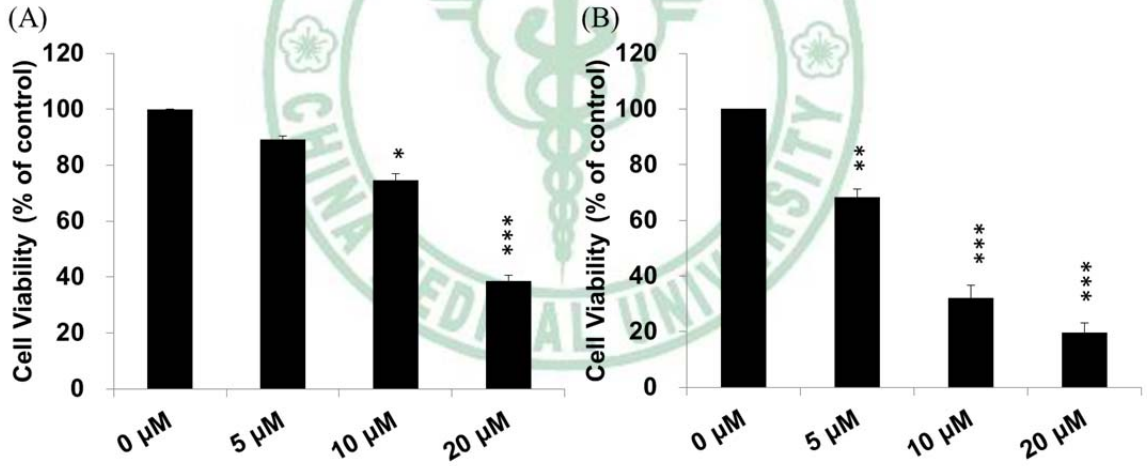
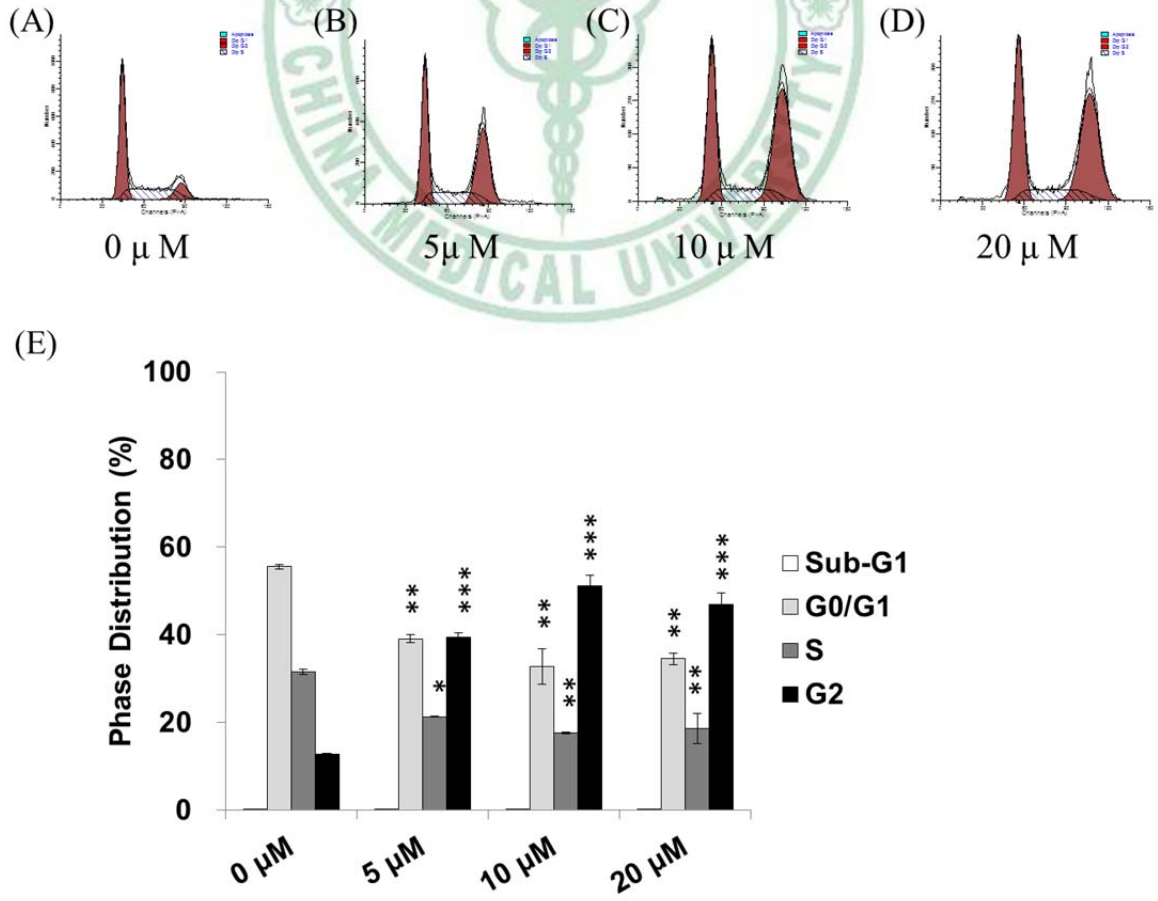


Figure 11 Cell survival inhibition of A549 human lung cancer cell lines by NSC746364 assessed using the MTT proliferation assay. NSC746364 significantly suppressed proliferation of A549 cells in concentration dependent manner. Cells were treated with NSC746364 at concentrations 5, 10, and 20 μM for 24 h (A) and 48 h (B). Untreated groups (0 μM) contained DMSO less than 0.01%. All results were obtained from at least 3 times independent experiments. Statistical significance was determined using

ANOVA test, (* p<0.05).

二、 *NSC746364 modulates cell cycle progression*

The consequences of telomere dysfunction caused by telomere-disrupting agents, such as G-quadruplex inhibitors, resulting in activation of DNA damage response signaling and leading to activation of cell cycle checkpoints [161, 205-208]. The cell cycle distribution of A549 cells was examined by flow Cytometry on cells treated with various concentrations (0 μM, 5 μM, 10 μM, 20 μM) of NSC746364 for 24 h Figure 12 (A to E) and 48 h (F to J). In the present study, flow cytometric analysis showed that NSC746364 modulated cell cycle progression through inducing cells to accumulate at G2/M-phase with concurrent decrease of cells at G0/G1 and S phase.



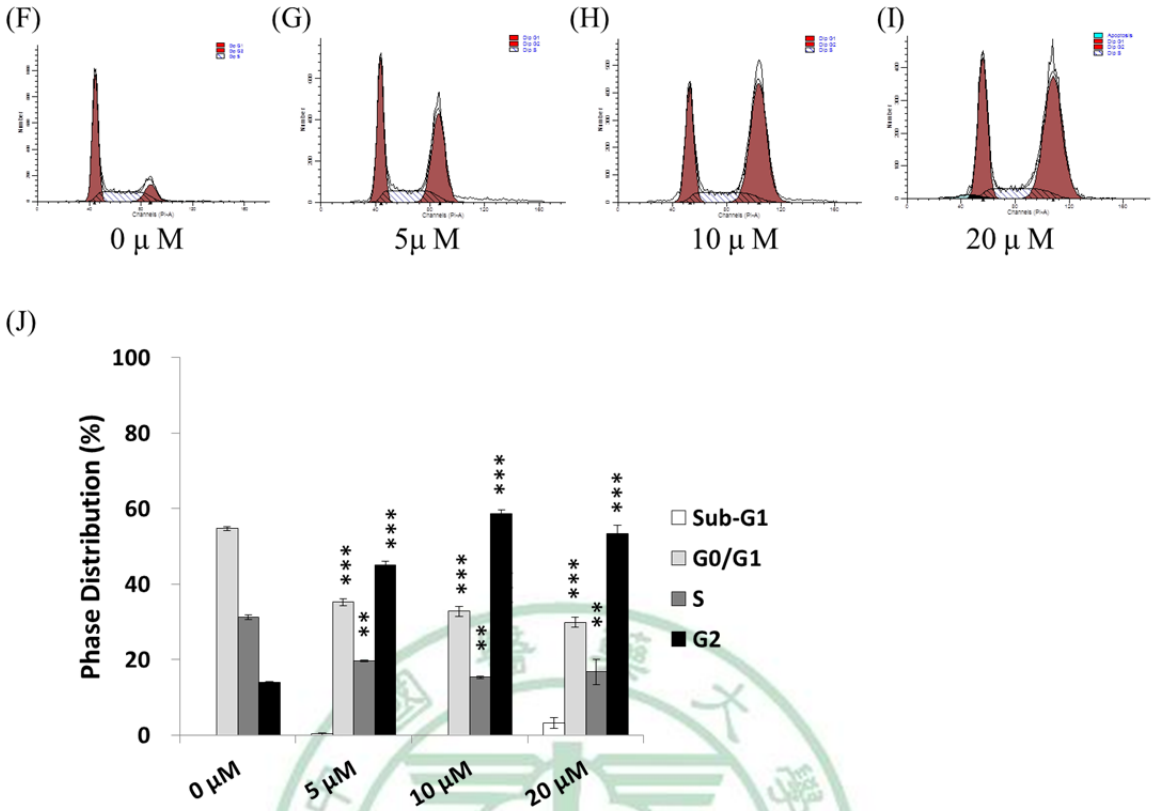


Figure 12 Flow cytometric analyses elucidated the effect of NSC746364 on the cell cycle phase distribution in A549 cells. Averages from three independent experiments of 0.01% DMSO-treated cells (0 μM, as a control) and NSC736364-treated cells at doses 5, 10 and 20 μM for 24 h (A to E) and 48 h (F to J) were calculated. Statistical significance was analyzed using ANOVA test, (* p<0.05). NSC746364 induced G2/M cell cycle arrest in a dose-dependent manner.

三、 *NSC746364 modulates cell cycle progression through activation of DNA damage sensing pathways*

To further elucidate the mechanism by which NSC746364 induced G2/M arrest through investigating G2/M cell cycle regulatory proteins. Two dominant regulatory proteins, Cyclin B1 and the mitotic marker protein kinase (Cdk1/Cdc2) were determined by western blot analysis. We found that NSC746364 down-regulated Cyclin B1 levels in a dose-dependent manner. However, the protein levels of Cdc2 were not affected by NSC746364. We also found that NSC746364 up-regulate both Chk1 and Chk2 phosphorylation at Ser-345 and Thr-68, respectively. However, NSC746364 markedly increases Chk1 phosphorylation at Ser-345 but not Chk2 phosphorylation at Thr-68. Furthermore, the significantly increased Chk1 phosphorylation at Ser-345 could be detected very early after 15 min of NSC746364 treatment.

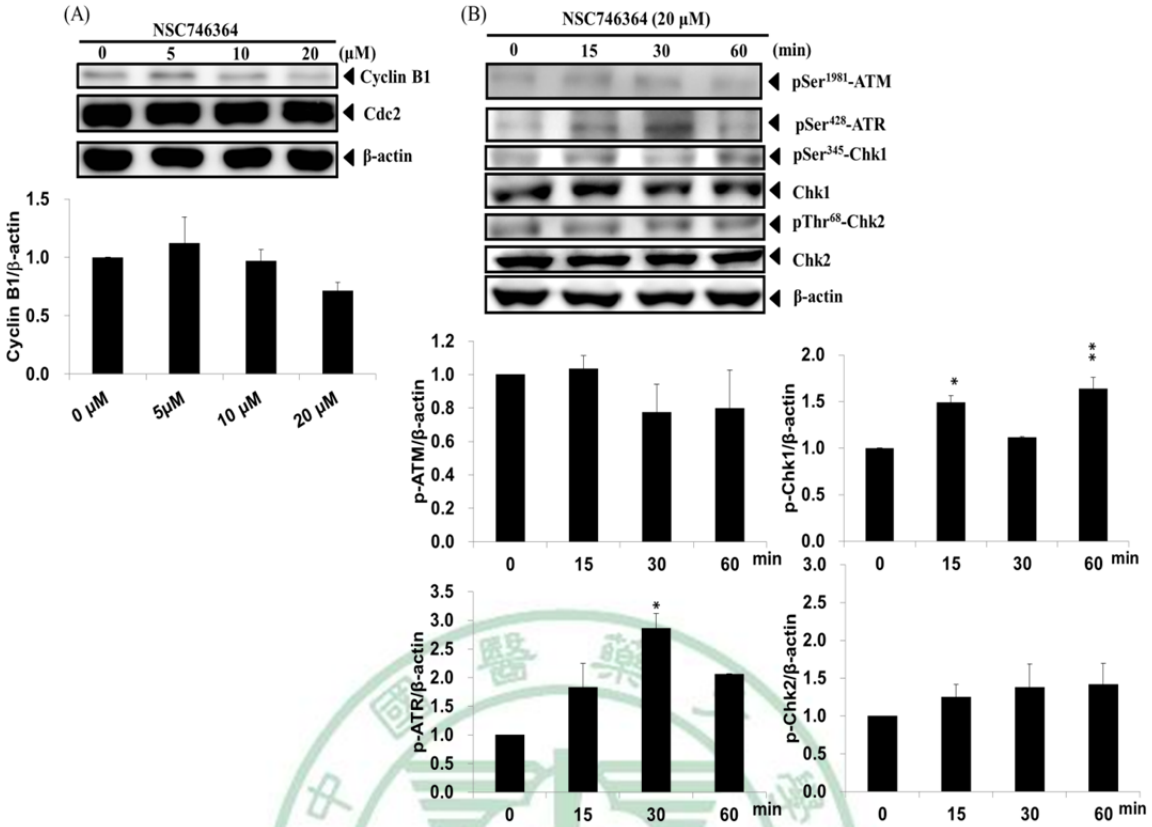


Figure 13 Effects of NSC746364 on protein expression level of Cyclin B1, p-Chk1, and p-Chk2 in A549 cells. (A) Cells were treated with 5, 10 and 20 μM for 24 h. Cell lysates were analyzed by Western blotting with anti-Cyclin B1, anti-Cdc2, and anti-β-actin antibodies. The bar graphs are results of densitometry analyses of the ratio of Cyclin B1 to β-actin. (B) A549 cells were incubated with NSC746364 for 0, 15, 30, and 60 minutes. The protein levels of p-Chk1, p-Chk2, Chk1, Chk2, and β-actin were determined by Western blot. The bar graphs are results of densitometry analyses of the ratio of p-Chk1 to β-actin and p-Chk2 to β-actin. Each value represents the mean± S.D. *P<0.05, as compared to the control (0 μM and 0 min), n=3.

四、 *NSC746364 induces A549 cells apoptosis*

As shown in our MTT results, tumor growth was strongly suppressed by NSC746364 treatment, suggesting the cytotoxic effects of NSC746364 on lung cancer cells. To test this hypothesis, we examined the potential apoptotic signaling pathways in A549 cancer cell lines. Our results show that NSC746364 induced tumor cell apoptosis by activating Caspase-3 (Figure 14A). The increasing intensity of DAPI staining demonstrates the dose dependent apoptotic effects of NSC746364 on A549 cells (Figure 14B). Furthermore, Annexin V-FITC/propidium iodide (PI) staining was another alternative way to evaulate apoptosis. As shown in Figure 14C, NSC746364 significantly increased PI-positive and Annexin-V-positive cells in a dosel–dependent-manner.

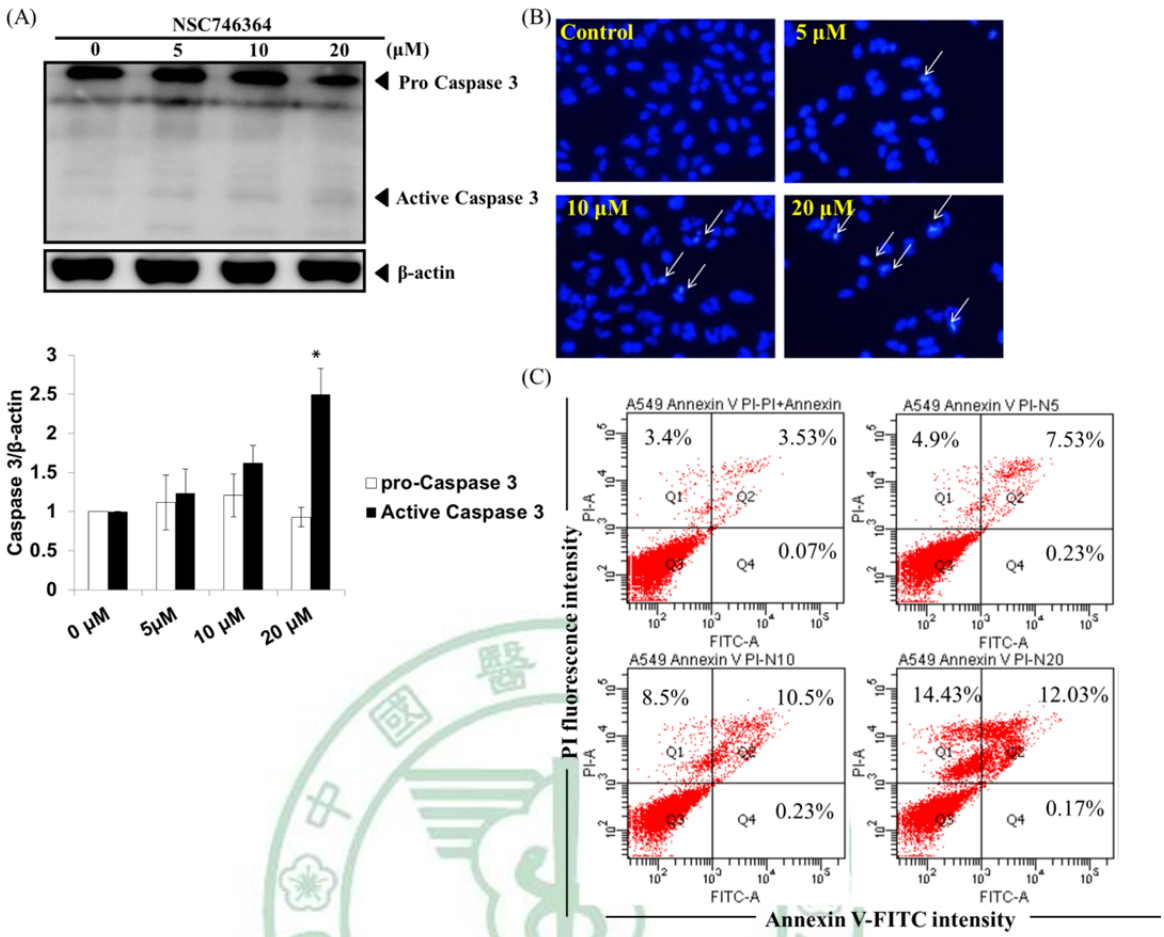


Figure 14 NSC746364 induces cell apoptosis. (A) A549 cells were incubated with NSC746364 at concentration 5, 10 and 20 μM for 24 h. Cell lysates were analyzed by Western blotting with anti-Caspase-3 and anti-β-actin antibodies. The bar graphs are results of densitometry analyses of the ratio of either pro-Caspase-3 to β-actin or active Caspase-3 to β-actin. (B) The cells were cultured in 10% FBS with or without NSC746364 for 24 h. After 24 h, the cells were fixed and incubated with DAPI for 1 min, and observed using a fluorescent microscope. *White arrows* indicate the apoptotic nucleus. All graphs were taken at 40X. (C) Apoptosis of A549 cells treated with different concentrations of NSC746364 (0, 5, 10 and 20 μM) for 24h was assessed using Annexxin V/PI staining and flow cytometry. Cells in the lower right quadrant (Q4) indicate early apoptotic cells. Cells in the upper right quadrant

(Q2) indicate late apoptotic cells.

五、 *ATM/ATR-Chk1/Chk2 DNA damage sensing signaling pathways are responsible for NSC746364's action in A549 human lung cancer cell lines*

We have previously demonstrated that cells treated with NSC746364 showed activated Chk1 and Chk2 protein phosphorylation levels. To further confirm the mechanism that underlies the antitumor effects of NSC746364 via ATM/ATR DNA damage sensing pathways, we examined the effect of caffeine, an inhibitor of ATM and ATR kinases [209], on tumor cells. Cells were either incubated with caffeine (10 mM) for 15 min or treated for 15 min before NSC746364 (20μM) treatment. As the data indicated (Figure 15), cells treated with NSC746364 significantly accumulated in G2/M phase. In contrast, pre-treated cells with caffeine markedly attenuated the effect of NSC746364 on cell cycle regulation. Furthermore, caffeine also significantly increased the cell viability as compared to NSC746364 alone group.

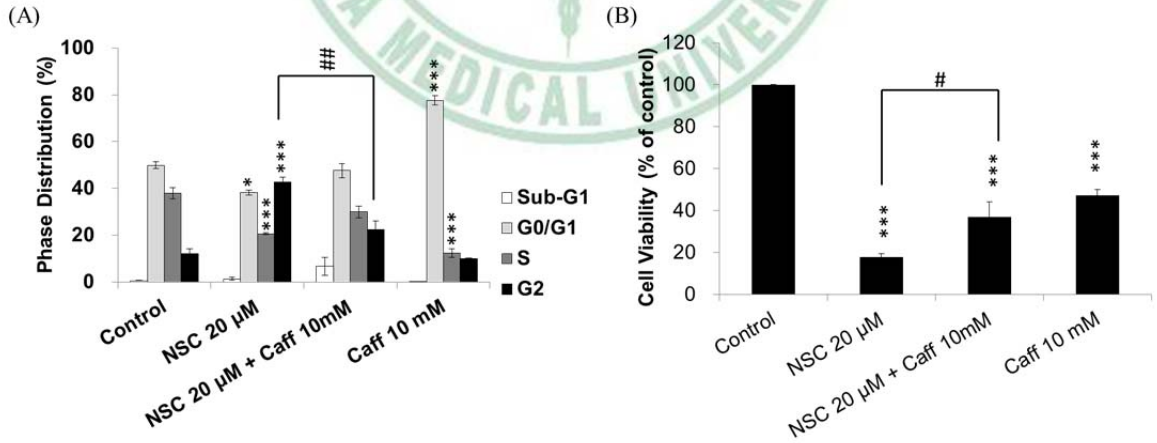


Figure 15 ATM and ATR blockage attenuated the effect of NSC746364 on cell cycle regulation and cell viability. Cells were pre-treated with caffeine (10 mM) for 15 min, following incubated with NSC746364 (20μM) for 24 h. Cell cycle phase distribution (A) and cell viability (B) were determined by flow

cytometry and MTT assay, respectively. Each value represents the mean±S.D. *P<0.05, as compared to the control (0 μM). $^{\#}P$<0.05, as compared to the NSC746364 treated group, n=3.

第四章 討論 (Discussion)

In the present study, we have shown that NSC746364 can inhibit the growth of A549 human lung cancer cells. One of the major mechanisms by which NSC746364 mediates its effects against lung cancers seems to be through activation of DNA damage signaling pathways, which in turn stalls cell cycle at G2/M phase and subsequently leading to cellular apoptosis.

NSC746364 is one of the novel 2,7-diamidoanthraquinone derivates. It has been shown to inhibit cell growth and telomerase activity of multiple cancer cell lines [171]. Various studies provided evidences that telomerase inhibition would induce growth arrest or apoptosis of cancers [20]. Our results demonstrate that NSC746364 can effectively inhibit cell growth (Figure 11), arrest cell cycle at G2/M phase (Figure 12) and induce cell apoptosis (Figure 14). Although caspase-3 activation and nucleus condense indicate cells are undergoing apoptotic process, we didn't detect such alteration in our experiments. The sub-G1 peaks on DNA histograms usually represent the apoptotic cells with degraded DNA [210, 211]. However, we did not detect any sub-G1 peaks when we measured the DNA contents of cells (Figure 12). All these results indicate that the anticancer effects of NSC746364 might mainly through inhibiting cell growth, with minor portion of cells undergoing apoptosis.

The telomere-specific protein complex, named as shelterin, is essential for genome stability and cell survival. These telomere binding proteins have been identified as TRF2, POT1, RAP1, TIN2, and TPP1. All these proteins form the shelterin complex, to prevent telomeres from triggering a DNA damage response [212]. In addition, telomerase is also one of the mechanisms

to maintain telomere integrity. Disrupting telomere DNA maintenance by telomerase or telomere targeting agents can be sensed as DAN damage, which in turn rapidly activated signal transducers which responded for DNA damage. As shown in Figure 13 B, NSC746364 activates ATM/ATR signaling pathways by phosphorylating ATM at Ser-1981 and ATR at Ser-428. However, it seems that NSC746364 dominantly increases ATR/Chk1 phosphorylation but not ATM/Chk2 phosphorylation. One of the possible mechanisms to regulate ATR activation is through repression of the shelterin protein, POT1. Recent studies showed that distinct shelterin components independently repress ATM and ATR signaling: Activation of ATM at telomeres is repressed by TRF2, while POT1 is required to prevent ATR activation [213-216]. Thus, NSC746364 might downregulate POT1, leading to ATR activation. In Figure 15, caffeine, an ATM and ATR kinase inhibitor [209], effectively abrogate the effects of NSC746364 on cell cycle regulation and cell viability. These results indicate that inhibition of cell growth by NSC746364 is through activation of ATM and ATR. However, more specific inhibitors like siRNA should be used in further experiments to evaluate the ATM/ATR signaling. In addition, the role of POT1 in regulating ATR activation in our experimental models should also be tested.

Taken together, our study demonstrates that tumor growth could be suppressed by NSC746364. Its pharmacological mechanism may be through targeting ATM/ATR/Chk1/Chk2 signaling pathways. Our results unmask the molecular mechanisms of NSC746364 in inhibiting A549 human lung cancer cells growth in vitro to shed light into the conjunctive roles of NSC746364 with some other pharmacological anticancer agents. Further studies using

animal models or clinical evaluations need to be conducted to confirm the proposed theory in this aspect.

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