N, N-BIS (5-ETHYL-2-HYDROXYBENZYL) METHYLAMINE INDUCES APOPTOSIS OF NON-SMALL CELL LUNG CANCER CELLS VIA C-MYC PROTEIN DEGRADATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy in Pharmacology and Toxicology
Department of Pharmacology and Physiology
FACULTY OF PHARMACEUTICAL SCIENCES
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เอ็น, เอ็น-บิส (5-เอทิล-2-ไฮดรอกซีเบนซิล) เมทิลลามีนเหนี่ยวนำให้เซลล์มะเร็งปอดชนิดไม่ใช่เซลล์เล็กตายแบบอะพอพโทซิสผ่านการทำลายโปรตีนซี-มิก

น.ส.นิชารัตน์ ศรีรัตนศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยาและพิษวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
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Thesis Title	*N, N*-BIS (5-ETHYL-2-HYDROXYBENZYL) METHYLAMINE INDUCES APOPTOSIS OF NON-SMALL CELL LUNG CANCER CELLS VIA C-MYC PROTEIN DEGRADATION
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มะเร็งปอดเป็นโรคที่พบได้มาก และเป็นสาเหตุการตายอันดับต้นๆ ซี-มิก (c-Myc) เป็นโปรตีนที่มีส่วนเกี่ยวข้องกับการก่อมะเร็ง โดยมีรายงานพบว่ามะเร็งปอดส่วนมากจะมีการเพิ่มระดับของโปรตีนชนิดนี้ส่งผลให้ความรุนแรงของมะเร็งเพิ่มมากขึ้น และส่งผลต่อการรักษาที่ล้มเหลว ในการศึกษานี้จึงมีวัตถุประสงค์ที่จะแสดงผลของสารสังเคราะห์ เอ็น, เอ็น-บิส (5-เอทิล-2-ไฮดรอกซีเบนซิล) เมทิลลามีน หรือ อีเอ็มดีซึ่งเป็นสารในกลุ่มเบนโซซาซีนไดเมอร์ ว่ามีเป้าหมายที่ซี-มิก สามารถเหนี่ยวนำให้เกิดการทำลายซี-มิกในมะเร็งปอดได้ อีเอ็มดีแสดงความเป็นพิษต่อเซลล์มะเร็งปอดผ่านทางการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิส ผลการศึกษาต่อโปรตีนเป้าหมายพบว่า อีเอ็มดีสามารถลดระดับโปรตีนซี-มิกได้อย่างมีประสิทธิภาพ อีกทั้งเหนี่ยวนำให้เกิดการทำงานอย่างเป็นขั้นตอนของเอนไซม์แคสเปส การศึกษาด้วยไซโคลเฮกซิไมด์ (cycloheximide; CHX) บ่งบอกว่าอีเอ็มดีสามารถลดค่าครึ่งชีวิตของโปรตีนซี-มิกได้ เมื่อทำการศึกษาต่อด้วยเอ็มจี 132 (MG132) ซึ่งเป็นสารที่ยับยั้งโปรตีเอโซมพบว่าโปรตีนซี-มิกไม่สลายไปเมื่อให้อีเอ็มดี แสดงให้เห็นว่าการสลายไปของโปรตีนซี-มิกเกี่ยวข้องกับกระบวนการทำลายโปรตีนผ่านทางยูบิควิติน นอกจากนั้นการวิเคราะห์ด้วยอิมมูโนพรีซิพพิเทชั่น (immunoprecipitation analysis) แสดงให้เห็นว่าเมื่อให้สารอีเอ็มดีเกิดการสร้างซี-มิก-ยูบิควิติน คอมเพล็กซ์เพิ่มมากขึ้นเมื่อเทียบกับกลุ่มควบคุม นอกจากนี้อีเอ็มดียังสามารถลดซี-มิกในเซลล์ไลน์ของมะเร็งปอดที่พัฒนามาจากผู้ป่วยชาวไทยได้ ไม่เพียงแต่ความสามารถในการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสของดีเอ็มดี ผู้วิจัยยังได้ทำการศึกษาผลของสารอีเอ็มดีต่อการยับยั้งการแพร่กระจายของเซลล์มะเร็ง การแพร่กระจายของเซลล์มะเร็งเป็นกระบวนการที่เกิดเมื่อเซลล์มะเร็งหลุดออกจากพื้นผิวที่ต้นกำเนิด แล้วไปก่อให้เกิดมะเร็งยังบริเวณอื่นผ่านทางหลอดเลือดหรือน้ำเหลือง การเคลื่อนที่ของเซลล์เป็นกระบวนการหนึ่งที่เกิดขึ้นในระหว่างการแพร่กระจายของเซลล์ซึ่งถูกควบคุมด้วยหลากหลายกลไก สัญญาณจากอินทิกรินมีส่วนในการควบคุมการดำรงอยู่ของเซลล์ และกระตุ้นให้เกิดการเคลื่อนที่ของเซลล์ อีเอ็มดีในขนาดความเข้มข้นที่ไม่เป็นพิษต่อเซลล์สามารถยับยั้งการสร้างฟิโลโพเดีย และยับยั้งการเคลื่อนที่ของเซลล์ได้ ทั้งยังมีผลต่อการดื้อของเซลล์ในกระบวนการอะนอยคิส โดยยับยั้งการเจริญ และการดำรงอยู่ของเซลล์มะเร็งที่หลุดออกจากพื้นผิวยึดเกาะ นอกจากนี้อีเอ็มดียังมีความสามารถในการลดระดับ อินทีกรินเบต้า 3 (integrin b3) ในขณะที่ไม่พบผลต่อ อินทิกรินเบต้า 1 (integrin b1) และแอลฟา 5 (integrin a5) สำหรับโปรตีนที่เกี่ยวข้องกับสัญญาณจากอินทิกรินได้แก่ focal adhesion kinase (FAK) และ active protein kinase B (Akt) อีเอ็มดีสามารถลดระดับโปรตีนดังกล่าวได้อย่างมีนัยสำคัญทางสถิติ จากผลการศึกษาที่ได้กล่าวมานี้เป็นหลักฐานสนับสนุนให้อีเอ็มดีสามารถเป็นสารใหม่ที่มีประสิทธิภาพในการพัฒนาเพื่อรักษามะเร็งปอดต่อไป

สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อนิสิต .....
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KEYWORD: Non-small cell lung cancer, Apoptosis, c-Myc, N N-bis (5-ethyl-2-hydroxybenzyl) methylamine, cancer cell metastasis, integrin

Nicharat Sriratanasak : *N, N*-BIS (5-ETHYL-2-HYDROXYBENZYL) METHYLAMINE INDUCES APOPTOSIS OF NON-SMALL CELL LUNG CANCER CELLS VIA C-MYC PROTEIN DEGRADATION. Advisor: Assoc. Prof. PITHI CHANVORACHOTE, Ph.D. Co-advisor: Sudjit Luanpitpong, Ph.D.

Lung cancer is a common cancer disease that contributes as a major cause of cancer related death. The oncoprotein, c-Myc plays a major role in oncogenic malignancies. It has been shown that the up—regulated c-Myc relates with aggressiveness of cancers and treatment failure. In this study, we purposed *N, N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD), a benzoxazine dimer to be a novel c-Myc targeting compound and displayed evidence presenting the potential effect of EMD on c-Myc degradation in human lung cancer cells. EMD exhibited cytotoxicity to lung cancer cells through apoptosis induction. EMD dramatically eliminated c-Myc in the cells and initiated caspase-dependent apoptosis cascade. Cycloheximide chase assay showed that half-life of c-Myc protein was shortened by EMD. MG132, a potent selective proteasome inhibitor, could restore the c-Myc level conveying the involvement of ubiquitin-mediated proteasomal degradation in the process. In addition, immunoprecipitation analysis demonstrated that EMD significantly increased c-Myc-ubiquitin complex formation. We further verified that EMD strongly decreased c-Myc protein levels in primary lung cancer cells. In addition, we also purposed that EMD has an anti-metastatic effect. Metastasis is the process that cancer cells detach from the original site to form a new tumor at distant site through blood or lymphatic vessels. Cell migration is a part of the metastasis cascade which regulated by several signals. Integrin signaling involves in cell survival and activation of cell migration. EMD at non-toxic concentrations reduced filopodia formation and cell migration. In addition, EMD significantly inhibited growth and survival of detached lung cancer cells. Moreover, EMD strongly decreased integrin b3 while other evaluated integrins, b1 and a5 were not altered. Other downstream of integrin signaling such as active focal adhesion kinase (FAK) and active protein kinase B (Akt) were significantly decreased after EMD treatment. Altogether, we identified EMD as a novel potential compound for lung cancer treatment.

Field of Study:	Pharmacology and Toxicology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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Nicharat Sriratanasak

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CHAPTER 1

INTRODUCTION

1.1 Background and rationale

One of the most critical public health problems is lung cancer. It is contributed as an extremely cause of cancer death in male and female which can be divided into 2 subtypes, Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC). More than half of lung cancer cases are considered as the NSCLCs. Survival rates of this cancer type are quite low, approximately 19% five-year survival, and unfortunately, almost lung cancer patients have been diagnosed with metastasis [(1)].

Apoptosis is a one of programmed cell death mechanism eliminating unwanted or damaged cells. This mechanism can be triggered by both intracellular and extracellular signals leading to intrinsic and extrinsic death pathway, respectively. To control the cancer, apoptosis mechanism in cancer cell will be induced for elimination of unwanted cells [(2)]. c-Myc, proto-oncogene, has a crucial role in tumorigenesis and tumor maintenance. It is clear that c-Myc plays a role in cancer cell resistance to chemotherapy as well as immune cell destruction [(3)]. It was found that c-Myc is frequently found to overexpress in cancer patients. In addition, only 20% of patients had been identified the gene amplification of Myc, implying that the major mechanism of such an increase of c-Myc level is through increased c-Myc protein stability. The underlying mechanisms of increased c-Myc stability in cancers have been shown in many studies indicating that c-Myc overexpression may be related to gene alterations. Such alterations include point mutation on c-Myc that

directly affects c-Myc stability by interfering the normal degradation pathways and amplification of c-Myc DNA [4]. In addition, the mutation of E3 ligase enzyme, the enzyme responsible for c-Myc degradation, resulted in the defect of c-Myc degradation pathway [5, 6]. As knockdown of c-Myc can induce tumor regression [7,8], c-Myc is a potential molecular drug target for cancer therapy. Nowadays, there are several strategies to control the c-Myc level and function. One of the strategies which controls the c-Myc protein levels is ubiquitin-degradation by proteasome system [9].

Compounds which can inhibit cell proliferation or induce apoptosis cell death has been studied as potential therapeutic cancer treatment [10]. *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) is a new benzoxazine dimer derivative synthesized by mannich reaction and ring-opening dimerization from three starting materials, 4-ethylphenol, formaldehyde and methylamine [10, 11, 12, 13] . It has been reported that benzoxazine dimer derivative can cause apoptosis cell death in many cancers [14]. Therefore, the present work aims at investigating the potential effect of EMD in inducing apoptosis cell death by targeting c-Myc proto-oncogene.

1.2 Objectives of the study

1.2.1 To evaluate the apoptotic inducing property of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine in non-small cell lung cancer

1.2.2 To evaluated the underlying mechanism of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine in lung cancer cells

1.2.3 To investigate the inhibitory effect of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine on migration activity of lung cancer cells

1.2.4 To evaluated the molecular mechanism involves in migration process of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine in lung cancer cells

1.3 Hypothesis of the study

1.3.1 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine can induce apoptosis in non-small cell lung cancer cells with concentration dependent manner.

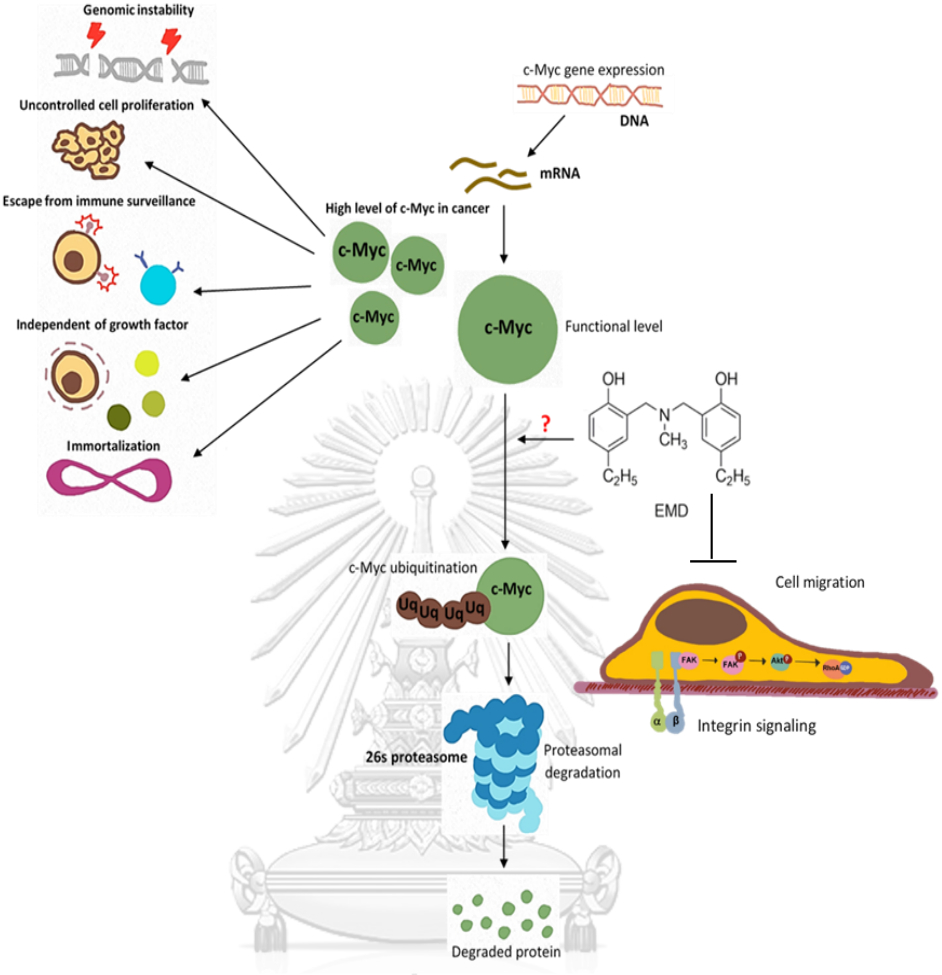
1.3.2 c-Myc may be a target of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine by protein ubiquitination.

1.3.3 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine has inhibitory effect on migration activity in lung cancer cells via integrin signaling pathway.

1.4 Benefits of the study

This study will provide the preliminary data of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine inducing apoptosis mechanism in non-small cell lung cancer by targeting c-Myc that has a consequential role in maintenance tumor. Degradation of c-Myc will be a future effective targeted treatment. Moreover, *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine shows potential effect in inhibiting non-small cell lung cancer cell migration in early metastasis process. Therefore, *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine has a potential to develop into an anti-cancer compound.

1.5 Conceptual framework



CHAPTER 2

LITERATURE REVIEW

2.1 Lung Cancer

Lung cancer is a disease of mutated cells within the lung tissue turn dysregulated growth, proliferation, and maintenance of their normal function. This causes the development of tumors that compromise a person's ability to breath and reduce quality of life. The uncontrolled cells will invade neighboring tissues, disseminate, and form new metastasized tumors throughout the body. Cancer statistics in 2019, indicated that lung cancer incidence is slightly reducing in male but remaining as ever in female due to the changing of smoking behavior (1), (15).

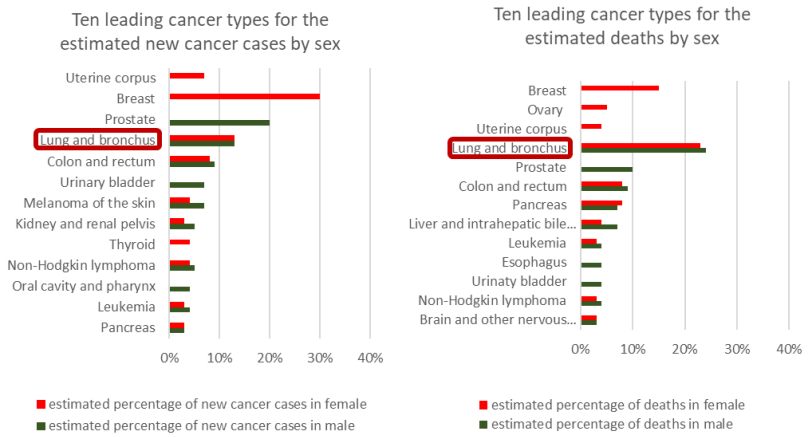


Figure 1 *Ten leading cancer types for the estimated new cancer cases and deaths by sex in 2019.*

Survival rate of this cancer type is approximately 19% five-year relative survival rate. In 2016, men were killed estimate 40% more than women but this pattern is considered to invert by 2045. One important factor determining lung

cancer survival rate is the stage of diagnosis. More than 50% of the cases were found to be diagnosed at a late stage [1]. Interestingly, the 5-year survival of lung cancer localizing inside the lung is as high as 54%, however, after metastasis the survival rate is only less than 4%. Unfortunately, majority of lung cancer cases were with metastasis at the time of first diagnosis [1].

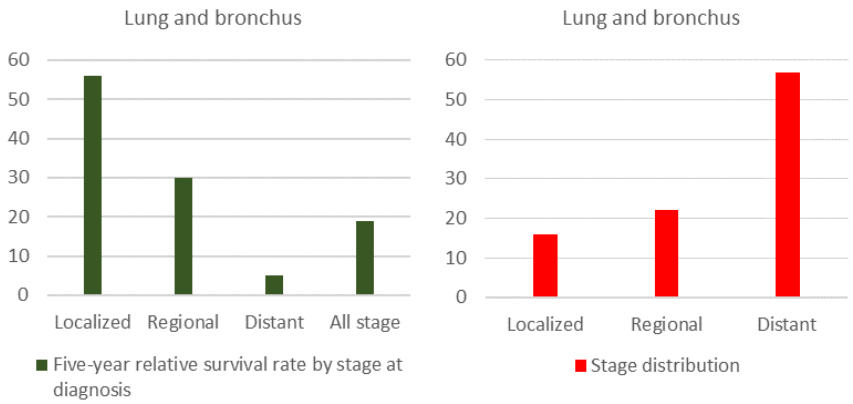


Figure 2 *Five-year survival rates (left) and stage distribution (right) for lung and bronchus in the United states.*

Types of lung cancer

Generally, lung cancer is classified into 2 types by histology, Non-Small Cell Lung cancer (NSCLC) and Small Cell Lung Cancer (SCLC). SCLCs are quite rare compared to NSCLCs and account for approximately 15% of all lung cancers, whereas NSCLCs account for about 85% of all lung cancers. SCLCs tend to grow faster than NSCLCs and often metastasize to other organs or lymph nodes, thus they are more responsive to chemotherapy. NSCLCs are still being divided into 3 sub-categories, adenocarcinoma, squamous cell carcinoma (SqCC), and large cell carcinoma. The most common type of NSCLC is adenocarcinoma which presents in the outer parts of lung. It has been shown that this subtype occurs with higher frequency in females

than in males and develops more frequently in younger age than other subtypes. However, adenocarcinoma can be detected earlier before it starts to metastasize. Squamous-cell carcinoma (epidermoid) accounts for approximately 25-30% of all lung cancers. It initiates from squamous cells in the central parts of the lungs, near bronchi. This subtype is firmly related to cigarette smoking. Large cell (undifferentiated) carcinoma comprises about 10-15% of all lung cancer cases. It can present in any part of the lung but often starts in the central of the lung. It has a tendency to proliferate and metastasize quicker than other subtypes [16], [17].

Risk factors of lung cancer disease

There are several leading risk factors for lung cancer including genetic factors, behavior factors and environmental factors.

1. Genetic factors corelate with lung cancer incident. It was found that people in the families which have a history of cancer will get a chance to receive an inherited susceptibility to malignancies such as uncommon mutations in tumor suppresser genes or autosomal recessive disorders. The risk will increase 2 times and 1.5 times in smoking families and non-smoking families, respectively. Moreover, tumors also obtain genetic mutations and amplifications which lead to genomic instability[18].
2. Behavioral factors include tobacco and smoking are the most important risk factors. Up to 90% of smokers and 1.14 to 5.20 of passive smoking who live with a smoker will develop lung cancer. There are about 60 chemical substances in the tobacco such as polycyclic aromatic hydrocarbons (PAHs) which defined as carcinogens. Except PAHs, nicotine in cigarettes and cannabis

sativa are also associated with lung cancer. Even thought, there is no premise of nicotine itself induces tumorigenesis, but it is implicated with progression of existing tumors by upregulating the nicotinic receptors that increase the tobacco addition. In addition, heterocyclic amines (HCAs) which are generated by cooking diet have been linked with cancer incidence [18], [19].

3. Environmental factors can induce thoracic malignancies. The small particulate matter with diameter less than 2.5 μm ($PM_{2.5}$) will increase the incident rate of lung cancer up to 30% when receive more than 10 μg/cubic meter[20]. Radon and asbestos can also increase the cancer incidences up to 5 times when compare with people who have not expose to them. In conjunction with tobacco smoking, synergistic effect on risk of lung cancer will accelerate [18, 19].

Treatments

Several treatments are used for non-small cell lung cancer. Surgery alone is the first choice for elimination of the tumor masses in early lung cancer stage patients (stage 0-2). For lung cancer patients in stage 3, they are treated by other treatments such as chemotherapy or radiotherapy because only surgery may not remove the whole of the cancer masses. In stage 4 patients, the cancer spread to other organs therefore the treatment options are depended on the site or the number of cancers and patient's factors [21], [22], [23].

2.2 Apoptosis Programmed Cell Death

Apoptosis is one of the essential programmed cell deaths regulating cell development, maintenance of cell homeostasis, and cell viability. This process

occurs when the cells are damaged or infected resulting in cell morphological changes. In cancer cells, irregularity of programmed cell deaths can be discovered. The cells which undergo the apoptosis programmed cell death will shrink and condense following by cytoskeleton collapse and breaking up nuclear chromatin into fragments. The cell membrane will swell outward and turn into membrane-enclosed apoptotic bodies that carry fragments including nucleus, mitochondria and other organelles. After that phagocytosis mechanism is induced for engulfing the apoptotic bodies [24]. This programmed cell death is divided into two principal pathways, intrinsic (mitochondrial) and extrinsic (death receptor) pathway, activated by several signaling cascades [25], [26].

Extrinsic pathway is activated through death domain on transmembrane receptor called tumor necrosis factor (TNF) receptor. The death signaling is transmitted from outside of the cell membrane to intracellular signaling pathway. The best characterized ligands-receptors are FasL/FasR and TNF-α/TNFR1. Binding between death signaling ligands and receptors trigger the cascade proteases which drive the apoptosis mechanism. Pro-caspase 8, initiator caspase will be amplified resulting in the activation of down-stream effector caspases like caspase 3 [25], [26].

In contrary, intrinsic pathway is induced by mitochondria or intracellular signals. The intracellular signals directly transmit the activation cascade to their targets within the cells in either positive and negative manner. The signaling stimulation causes the changing of inner mitochondrial membrane resulting in the alteration of mitochondrial permeability transition (MPT) pore. After disruption of mitochondrial membrane, pro-apoptotic signals from the intermembrane space including cytochrome c, the serine protease HtrA2/Omi, as well as Smac/DIABLO are released.

The molecular signals will activate caspase-cascade pathway. Pro-caspase 9 is an initiator caspase which activates the function of effector caspase-3. The permeability of such mitochondrial membrane is regulated by another protein family called Bcl-2 family which can be either anti- or pro-apoptotic activity. Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG are some of anti-apoptotic proteins while Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk are some of pro-apoptotic proteins. After the cells undergo the apoptosis mechanism, DNA will be digested into small fragments and turned to apoptotic body which will be engulfed and destroyed by other cell nucleases like white blood cells[25], [26], [27].

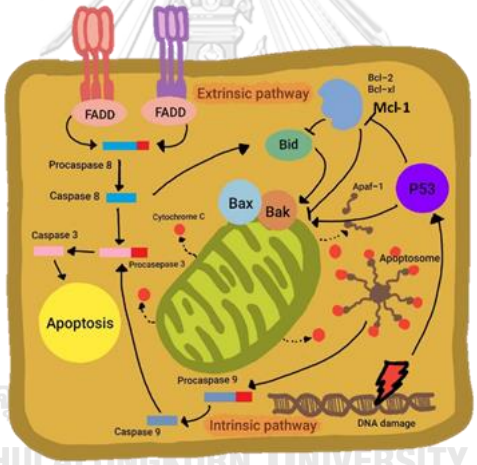


Figure 3 *Apoptosis mechanism*

Apoptosis mechanism can be divided into two main pathways, extrinsic and intrinsic pathway. The enzyme cascade is induced and trigger the death pathways[25], [26], [27], [28].

The apoptosis mechanism is regulated by enzyme cascade called caspases which are synthesized in inactivated forms called pro-caspases. Pro-caspases compose with 3 domains; N-terminal pro-domain, large and small subunits. Aspartate

residues are inserted between these three domains. The caspases can be categorized into 2 groups, initiator (caspase-8, 9, 10) and effector (caspase-3, 6, 7) caspase. Initiator caspases retain in the cell as monomer. Apar-1 is a scaffolding cofactor of intrinsic pathway which secreted from mitochondria. It will oligomerize with cytochrome c to form apoptosomes. This complex can turn inactive initiator caspases to cleaved form (active form) leading to caspase enzyme cascade [25], [26], [27].

2.3 c-Myc

The activation of oncoproteins together with the inactivation of tumor suppressor proteins are the generally accepted concept of initiation of human cancers. The Myc proto-oncogenes are transcription factors, which are commonly overexpressed in cancer patients and have been intensively investigated as they may be potential targets of cancer treatment [29], [10]. Myc associates with the tumorigenesis and tumor maintenance in many human cancers [7], [30], [31]. There is study indicated that mutation of c-Myc is not potent as KRAS or EGFR mutations in oncogenesis of lung cancer [32]. Nevertheless, shutdown of the c-Myc function was found to induce growth arrest and apoptosis even in the models of tumor driven by other oncogenes such as Ras mutation [33]. Basically, the Myc family proteins comprise L-myc, N-myc and c-Myc [34]. c-Myc is expressed in many kinds of adult tissues while N-myc is found mostly in early developmental stages of neuronal tissues. L-myc is prominently expressed in embryonic brain, kidney and lung tissue [34] but the function of L-myc is not well studied. c-Myc exhibits many important roles in regulation of normal and cancerous cell signaling. c-Myc is involved in cell growth, division, differentiation, genome stability, cell survival and death, and angiogenesis [35], [36], [37], [38], [39]. c-Myc functions as a down-stream signal of several growth receptors

such as epidermal growth factor receptor (EGFR), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β) receptor, interleukin-6 (IL-6) receptor, Notch receptor, and Frizzled receptor [40]. Therefore, c-Myc usually responds to growth factors-receptor interaction and contributes to cellular response through its transcriptional activity [41].

Regarding the mechanism of action, c-Myc forms a dimer with Myc - associated factor X (MAX) before binding to E-boxes (CACGTG) in DNA within enhancers and promoters of genes. Its target genes encode for proteins that control cell growth as well as cell proliferation including CDK4, CDC25A, p15, p21, PTMA and E2F1 [7]. They are also linked to DNA damage and apoptosis responses as c-Myc has been shown to regulate the expression of Bax, Bcl-2 and Mcl-1 [42]. In normal cells, overexpression of Myc proteins will lead to apoptosis, proliferation arrest, or senescence [43]. In addition, the Myc protein has been shown to act as a central contributor to the survival and division of cancer cells [44]. This Myc is recognized as an important therapeutic target because this protein was found to be dysregulated in about 50% of human cancers [45]. It is likely that the dysregulation of Myc oncogene contributes to cancer progression and aggressiveness [30], [34], [44], [46]. In addition, evidence indicating that Myc is associates with poor prognosis in cancers [47]. Evidence indicates that Myc functions in several steps of carcinogenesis and disease progression including enhancement of cell proliferation, inhibition of apoptosis, and deregulation of differentiation [30], [34], [44], [46]. It has been widely accepted that c-Myc inhibition is interesting for cancer therapy [10], [35], [47], [48]. This is supported by many studies indicating that the inactivation or inhibition of c-Myc can induce tumor

regression by restoration of the normal cell checkpoint mechanisms or induction of proliferation arrest, cellular senescence, and apoptotic mechanisms [44], [49].

c-Myc protein levels are increased in about 70% in cancer patients but only 15-20% of the patients have a gene amplification. Recently, a meta-analysis has shown that the rate of c-Myc amplification in cancers varies from 1-50% with an average of about 15.7% [50]. It is possible that the augmented level of c-Myc may be caused by the amplification of the c-Myc gene, however, the rate of overexpression of the c-Myc protein in cancers is much higher (approximately 2 folds) than the incidence of c-Myc amplification.

In malignant cancers, an increase in c-Myc protein has been linked to malignant phenotypes. Recent studies reveal that amplification of the c-Myc gene is observed in many cancer cell lines including human colon cancer cells and SCLC cells. c-Myc oncoprotein was found to be overexpressed in about 50-100% of breast cancers, and about 50–75% of NSCLC cases [51], [52], [53] but only 30% shown DNA amplification of c-Myc in NSCLC[54]. Most of the c-Myc overexpression cases relate to c-Myc protein stability. Hotspots mutation on c-Myc gene is alanine substitution at T58 resulting in increasing protein stability. Other mutated points such as P245A can decrease turnover rate of c-Myc protein [46]. In NSCLC, overexpression of Myc was found to be associated with mutated *PHACTR3*, *E2F4* and FBW7 genes [54], [55].

The ubiquitin-proteasomal degradation of c-Myc protein has been shown to be a critical process that affects c-Myc function. Myc protein is rapidly degraded via proteasome after synthesis that makes its half-life approximately 20-30 minutes[9]. Ser62 could be phosphorylated by several kinases including mitogen-activated protein kinase (MAPK), cyclin-dependent kinase 1 (CDK1), and c-JUN N-terminal kinase

(JNK). Interestingly, Ras signaling that found to be frequently activated in lung cancer has been suggested to enhance MYC phosphorylation at Ser62 [56]. Ras also induces the activation of PI3K/Akt that phosphorylates GSK3β and subsequently results in c-Myc stabilization. After phosphorylation at Ser62, GSK3β could phosphorylate Thr58 of the protein. These 2 sites of phosphorylation of c-Myc target the protein for degradation by the ubiquitin-proteasomal degradation. As c-Myc is naturally maintained at low levels, the degradation of the protein is the determining factor that regulates its function[9], [57], [58].

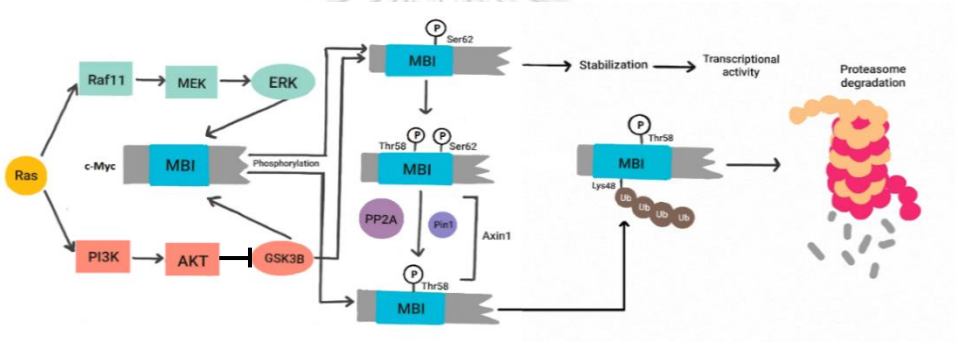


Figure 4 *Mechanism of c-Myc degradation*

Myc undergoes 2 phosphorylation site, serine 62 and Threonine 58. ERK can promote phosphorylation at Ser62 leading to stabilization of c-Myc while GSK3β can induce phosphorylation at Thr58 which be recognized by Fwb7, E3 ligase. Then the phosphorylated c-Myc molecules are degraded by 26s proteasomes[9], [57], [58], [59].

A study has demonstrated that suppression of c-Myc promotes Fas-mediated apoptosis [60]. While another study has shown that degradation of c-Myc can affect topoisomerase's function in DNA repair, leading to various types of cancer cells to apoptosis [45]. Moreover, decrease in c-Myc activity was demonstrated to sensitize cancer cells response to vinca alkaloids treatment [61].

2.4 c-Myc and drug resistance

Drug resistance is the main problem in cancer management and it has been correlated with overexpression of the c-Myc protein as well as amplification of c-Myc oncogene [62, 63, 64]. c-Myc cellular levels is not only related to cancer aggressive phenotypes, but it is also associated with chemotherapy resistance in melanoma, ovarian cancer, hepatocellular carcinoma, and lung cancer [35, 65, 66]. The mechanism of c-Myc in contributing drug resistance may involve increased the expression of several genes promoting cell survival, genomic instability, and blockage of apoptosis [35, 67].

Drug resistance has been recognized as a long important concern regarding lung cancer therapy. The rate of chemotherapeutic resistance in lung cancer is relatively high [68]. Although the targeted therapy, including epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), has been shown to improve the clinical outcome and recommended as a first choice for lung cancer with EGFR mutations [69] the platinum-based drugs as well as other chemotherapeutic agents remain the standard drug of choice for other patients with advanced disease [70].

Nowadays, anti-proliferating and apoptosis inducing agents have been studied as cancer therapeutic strategies. c-Myc protein involves in proliferation and apoptosis mechanism therefore the compound which targeting the c-Myc protein will be a promising effective anti-cancer compound. Decreasing the levels of c-Myc proteins not only induces tumor regression or apoptosis activity but also enhances sensitivity of other anti-cancer treatments.

2.5 Possible strategies for targeting c-Myc

c-Myc is found to be activated in many cancers and enhances cancer cell proliferation, while preventing apoptosis cell death. The aim of targeting c-Myc as a therapeutic target is focusing on cell proliferation inhibition and/or apoptosis induction. There are several ways of c-Myc targeting for cancer therapy including inhibition of c-Myc expression at DNA and mRNA level, blockage of c-Myc expression by small molecules, depredation of c-Myc/Max dimerization, inhibition of c-Myc interaction, disruption of c-Myc/Max complex binding to DNA, blockage of c-Myc targeted gene expression, c-Myc targeted gene dysfunction and induction of c-Myc protein degradation [6].

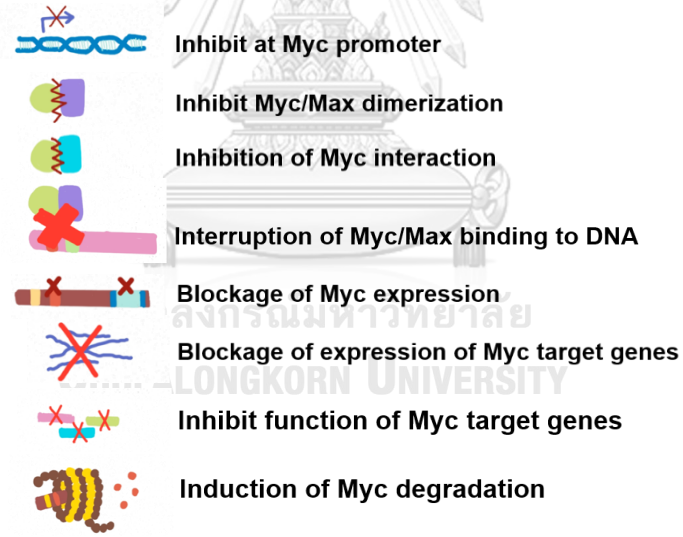
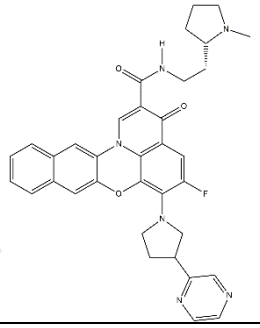
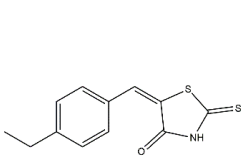
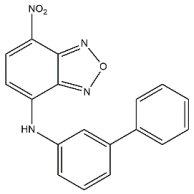
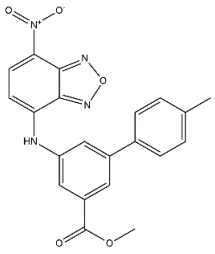
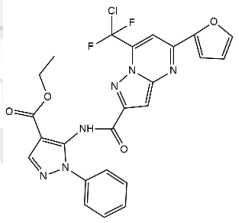
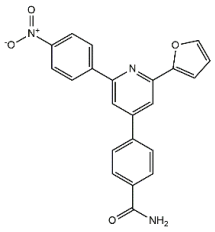
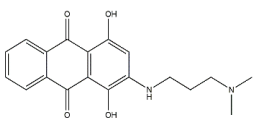
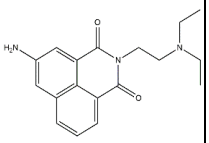
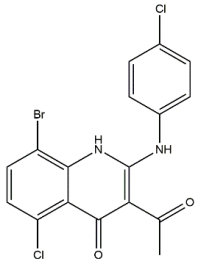
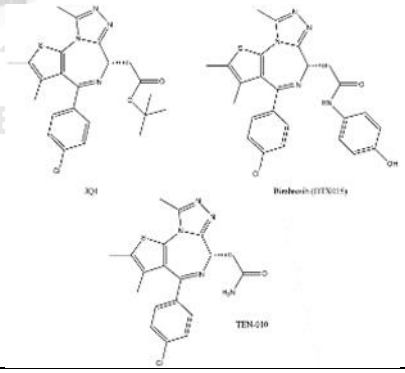


Figure 5 The potential strategies of targeting c-Myc

The example of molecules that target Myc in cancer is provided in Table 1.

Strategy	Mechanism	Examples
Direct inhibition of Myc expression	G-quadruplex stabilizers	Quarfloxin (CX-3543) disrupts the interaction between nucleolin protein and G-quadruplex DNA structure resulting in inhibition of ribosome synthesis and leading tumor cell apoptosis [71]. 
	Antisense oligonucleotides	AVI-4126 is a phosphorodiamidate morpholino oligomer which prevents ribosomal assembly leading to inhibit c-Myc mRNA translation [72]. Sequence of AVI-4126 antisense [73]: 5′-ACGTTGAGGGGCATCGTCGC-3′
	siRNA and microRNA	c-Myc siRNA is delivered in gold particles modified with branched polyethyleneimine [74]. c-Myc siRNA targeted sequence [75]: 5’-AAC GUU AGC UUC ACC AAC AUU-3’
Direct inhibitors of Myc that act by	Small molecule protein-protein interaction inhibitors	Compound 10058-F4 and 10074-G5 bind to a distinct region and inhibit dimerization of Myc/Max [76]. Unfortunately, both compounds are immediately metabolized and exhibited

Strategy	Mechanism	Examples
interfering with protein-protein interaction or DNA binding site		poor distribution[77], [78].  10058-F4  10074-G5 3jc48-3 is developed from 10074-G5 and increased potency and stability in cell-based assays [79].  Mycro3 and KJ-Pyr-9 are new inhibitors which exhibit potency in *in vivo* [80], [81].  Mycro3  KJ-Pyr-9
	Compounds that inhibit Myc binding to DNA	MYRA-A and NSC308848 target the DNA-binding domain of Myc/Max and inhibit interaction with DNA [82], [83].  MYRA-A  NSC308848

Strategy	Mechanism	Examples
		KSI-3716 blocks Myc/Max binding to DNA in bladder tumor growth [84].  KSI-3716
	Mini-protein or protein domain that disrupt Myc function	Omomyc comprises a group of structurally-related molecules based on domains from Myc resulting in disruption of Myc/Max dimerization, Myc transcriptional function regression and inactivation of E-box DNA binding site [85], [86], [87], [88].
Indirect inhibition of Myc	BET bromodomain and extra-terminal domain inhibitors	JQ1, OTX015 and TEN-010 substitute the bromodomain chromatin leading to defection in Myc transcription [89], [90]. 
	Block Myc mRNA translation	mTOR and mTORC1/2 kinase inhibitors have been approved for clinical use as mTOR mediates translation of mRNAs including Myc. Perhaps, using mTOR kinase inhibitors were not sufficient in down-regulation of Myc [91], [92], combination of eIF4A, silvestrol may

Strategy	Mechanism	Examples
		improve the effectiveness [93].
	Target regulator of Myc protein stability	oridonin [94] and MLN8237 [95] advocated the activity of Fbw7, E3 ligase mediated ubiquitin-proteasomal degradation of c-Myc. SET and CIP2A inhibitors block the activity of endogenous SET and CIP2A resulted in elevation of PP2A activity which increase c-Myc destabilization [96], [97].
	Target immune checkpoints that are altered in Myc-driven tumors	PD-L1 or CDC47 inhibitors show a potency to decrease Myc driven tumor response due to c-Myc induce PD-L1 and CD47 expression [98].

Table 1 Example of compounds that candidate as c-Myc targeted substances

2.6 Migration of cancer cells

Metastasis strongly associates with high mortality of cancer patients and treatment failure [99], [100], [101]. During metastasis, cancer cells detach from the original site, and then move through blood or lymphatic vessels to distant tissues to form secondary and tertiary tumors [102]. In addition, the migration of cancer cells is an critical factor facilitating cancer metastasis [103], [104]. This process involves the adhesion and interaction of the cell with its extracellular matrix (ECM), which induces changes in the cytoskeleton and cell adhesion [105]. The transmembrane proteins of the integrin family link ECM to intracellular cytoskeletal proteins. The specific dimerization of α and β subunits can promote migration of cancer cells. A study has demonstrated that certain integrins, such as integrin β1, β3, α5 and αv, promote cancer cell migration [106]. Moreover, integrin β3 has been shown to interact with integrin αv and

enhances angiogenesis and to mediate microtubule-dependent cell migration [107]. Activating signals from the integrin family need secondary messengers to transduce the signal such as low molecular weight GTPases, tyrosine kinases, lipid mediators, serine/threonine kinases and intracellular calcium fluxes. The activation of focal adhesion kinase (FAK), one of the tyrosine kinases [108], induces a signaling cascade in a PI3K-dependent manner and supports cell migration [109]. In the process of migration, protrusion of cell membrane is initiated to form a filopodium before a new focal adhesion formation on the attachment surface by the function of Rho family proteins [110]. Therefore, de-regulation of this protein family can promote a more aggressive cell migration [111]. Inhibition of the proteins that control the cell migration process will be a target of cancer therapy.

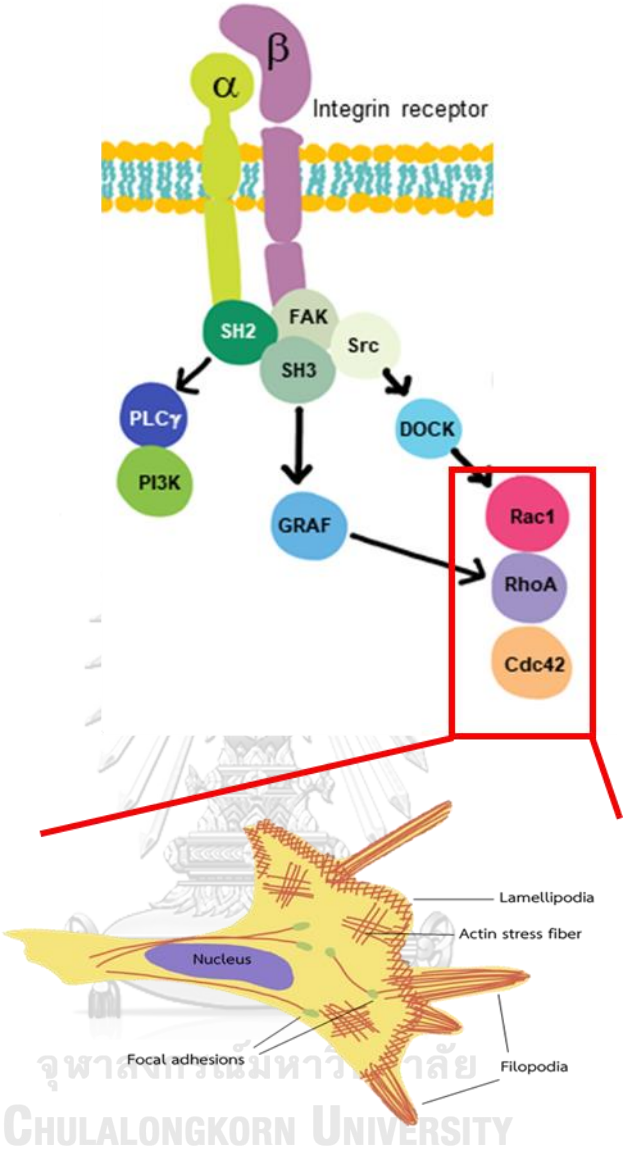


Figure 6 *Integrin signaling*

Integrin signaling involves in cell migration. The specific dimerization of α and β subunits transduces signal through secondary messenger, FAK to down-stream signaling. The integrin signaling results in protrusion of cell membrane and filopodia formation.

2.7 Anoikis resistance and metastatic colonization

Anoikis is the term of apoptosis induction when the solid cancer cells detach from the adhesion with cell and ECM [102]. Escaping from anoikis, a programmed cell death induced by cell detachment from the basement is shown to be critical for the survival of the cancer cells in the circulations [112], [113]. Anoikis resistance is mostly generated in aggressive cancer cells by activating or increasing survival pathways such as integrin-mediated survival, anchorage-independent cells transformation [114], [115] or overexpression of Bcl-2 protein family which leading to apoptosis resistance [116].

2.8 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD)

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) is benzoxazine dimer which synthesized by two steps, Mannich reaction and ring-opening dimerization. by using formaldehyde, 4-ethylphenol and methylamine in the ratio of 1:2:1 as precursors. After the two processing steps, the colorless single crystals were obtained. The characteristic of EMD was elucidated by using Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) [11], [12], [13], [117], [118]. The compound was received from department of Materials Engineering, Faculty of Engineering, Karsatsart University.

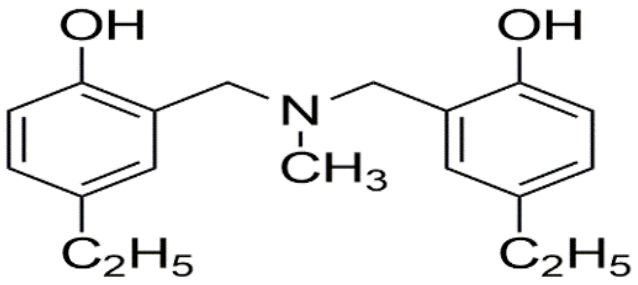


Figure 7 *Structure of N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD)*

From previous study, benzoxazine derivatives can induce apoptosis cell death of cancer cells such as breast cancer, cervical cancer and osteosarcoma in a dose-dependent manner[10].

CHAPTER 3

METHODOLOGY

3.1 Material and Instruments

3.1.1 Cell Lines and Culture

Non small cells lung cancer cell lines: H23 (ATCC®CRL 5800™), H292 (ATCC ®CRL 1848™) cells, ELC12, ELC16, ELC17 and ELC20 lung cancer cells were cultured in 10% FBS RPMI with 1% penicillin and streptomycin. The cells were stored at 37°C in a humidified incubator of 5% carbon dioxide. About 85% confluence of cells were applied for dosing experiments in this study.

3.1.2 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine preparation

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine were prepared as a 2,000 μM, 4,000 μM, 6,000 μM, 8,000 μM, 10,000 μM, 12,000 μM, 15,000 μM and 40,000 μM stock solution in dimethyl sulfoxide (DMSO). The solution were stored at -20°C and will be freshly diluted 200 times with 10% FBS RPMI media to treated concentrations before using. The final concentration of DMSO were 0.5% solution.

3.1.3 Chemical and Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, Fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, PBS and trypsin-EDTA were obtained from Gibco (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cycloheximide (CHX), MG132, dimethyl sulfoxide (DMSO), Hoechst 33342 and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). The primary

antibodies, c-Myc (#5605), Poly (ADP-ribose) polymerase or PARP (#9532), caspase-3 (#9662), caspase-9 (#9502), Bcl-2 (#4223), Akt (#9272), phosphorylated Akt or p-Akt (#4060), integrin β1 (#4706), integrin β3 (#4702), integrin α5 (#4705), FAK (#3285), phosphorylated FAK or p-FAK (#3283), RhoA (#2117), Mcl-1 (#94296) and β-actin (#4970) were acquired from Cell Signaling Techbology (Danvers, MA, USA). The primary antibody ubiquitin (ab7780) was obtained from Abcam (Cambridge, UK). The secondary antibody, anti-rabbit IgG (#7074) were obtained from Cell Signaling Technology (Danvers, MA, USA). The tested compound, EMD was synthesized by the procedure shown in following section.

3.1.4 Equipment

- CO_2 incubator (Thermo forma)
- Oven (United instrument Co., Ltd., Thailand)
- Water bath (Memmert, Chatcharee Holding Co., Ltd., Thailand)
- Fume hood FH120 (BossTech)
- Nikon Eclipse Ts2 microscope
- Microplate reader Perkin Elmer VICTOR3 (Anthros, Durham, USA)
- Guava flow cytometer (Merck Millipore)
- SDS-PAGE (Bio-rad)
- Chemiluminescent ImageQuant LAS4000
- 60- and 100-mm dish culture (Corning Inc., USA)
- 6, 24 and 96 well plate (Corning Inc., USA)
- 15 and 50 conical tube (PSL, Korea)

- 1.5 ml microcentrifuge tube (Corning Inc., USA)
- 0.2-2 µl, 2-20 µl, 10-200 µl and 200-1000 µl micropipettes (Corning Inc., USA)
- 2 µl, 20 µl, 200 µl and 1000 µl micropipette tips (Corning Inc., USA)
- Magnetic bar (Dynabeads™)

3.2 Methods

3.2.1 Apoptosis Inducing Activity

3.2.1.1 Cytotoxicity Assay

For cytotoxicity assay, 1.5 x 10^4 cells/well of H292 and H23 lung cancer cells were seeded on 96-well plates and incubated overnight. After that cells were treated with various concentrations of *N*,*N*-bis(5-ethyl-2-hydroxybenzyl)methylamine for 24 hours at 37°C and analyze by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer's protocol. In calculating the cell viability, measured absorbance of treated cells were divided by the value of non-treated cells and report in percentage.

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of controlled cells}} \times 100$$

3.2.1.2 Nuclear Staining Assay

This method was applied to define apoptotic and necrotic cell death by using nuclear staining with Hoechst 33342. H292 and H23 were seeded on 96-well plates at the density of 1.5 x 10^4 cells/well and provided to incubate overnight. The cell were treated with various concentrations of *N*, *N*-bis (5-

ethyl-2-hydroxybenzyl) methylamine and incubated for 24 hours at 37°C. Afterwards, the cells were incubated with 10 µg/ml of Hoechst 33342 for 30 minutes at 37°C. Then, they were visualized and imaged under a fluorescence microscope. Results were reported in percentage of apoptotic cells.

$$\text{Percentage of apoptotic cells} = \frac{\text{Number of apoptotic cells}}{\text{Number of live cells}} \times 100$$

3.2.1.3 Annexin V-FITC/PI Flow Cytometry

This method was introduced to examine apoptotic cell death by using flow cytometry with Annexin V-FITC/PI staining. H292 and H23 were seeded on 24-well plates at concentration of 1 x 10^5 cells/well and incubated overnight. The cells were treated with various concentrations of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine and incubated for 24 hours at 37°C. At the end of incubation time, they were detached from the well surface by using trypsin-EDTA (0.25%). The cells were incubated with 5 µL of Annexin V-FITC and 1 µL of PI for 15 minutes at room temperature in dark. After that, the cells were analyzed by guavaCyte™ flow cytometry systems (guavasoft™ Software version 3.3).

3.2.1.4 Western Blot Analysis

After *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine treatment, the apoptosis cells were collected by centrifuging media for 5 minutes with 1500 rpm and aspirated supernatants. The cells were incubated with RIPA lysis buffer containing NaCl 150 mM, Tris-HCl pH 7.6 25 mM, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS for 30 minutes at 4°C. The lysates were

collected and their protein content were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equivalent amount of proteins from each sample were separated by SDS-PAGE and transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The separating blots were blocked with 5% skim milk in TBST (Tris-buffer saline with 0.1% tween containing NaCl 125 mM, Tris-HCl pH 7.5 25 mM and 0.1% tween 20) for 2 hours and incubated with primary antibody against caspase-3, caspase-9, Bcl-2, PARP, Bax, c-Myc, integrin β1, integrin β3, integrin α5, FAK, p-FAK, Mcl-1 and beta-actin overnight at 4°C. Secondary antibody were incubated for 2 hours at room temperature after washed by TBST three times. Finally, the protein bands were detected using chemiluminescence substrate and exposed by Chemiluminescent ImageQuant LAS4000. Protein band were analyzed using Image J software (version 1.52, National Institutes of Health, Bethesda, MD, USA).

3.2.2 Effect of *N, N*-bis (5-ethyl-2-hydroxybenzyl) methylamine

3.2.2.1 Protein Stability by Cycloheximide Chasing Assay

H292 and H23 lung cancer cells were seeded on 6-well plate at the density of 5 x 10^5 cells/well and incubated overnight. Then, the cells were treated with cycloheximide (CHX) with or without 100 μM of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine for 0, 15, 30, 45 and 60 minutes. The treated cells were collected and lysed with RIPA lysis buffer. Western blot analysis were performed for elucidating c-Myc protein levels. Protein band were analyzed using Image J software (version 1.52, National Institutes of Health, Bethesda, MD, USA) and protein half-life will be calculated.

3.2.2.2 Ubiquitin-Dependent Protein Degradation

5 x 10^5 cells/well of H292 and H23 were seeded on 6-well plates and left for attraction overnight. Then, treating with 100 μM of *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine after pre-treat with various concentration of MG 132. Non-treated cells were used as a control. All of the cells were collected and lysed with RIPA buffer. Western blot analysis was performed for elucidating c-Myc protein levels. Protein band were analyzed using Image J software (version 1.52, National Institutes of Health, Bethesda, MD, USA).

3.2.2.3 Immunoprecipitation

H292 and H23 lung cancer cell lines were pre-treated with MG132 10 μM for 30 minutes followed by *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine 100 μM for 1 hour. The treated cells were collected and lysed with RIPA buffer. The magnetic beads from Dynabeads™ Protein G Immunoprecipitation Kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA) were irrigated with washing buffer and incubated with primary antibody of c-Myc in binding buffer for 10 minutes. Protein lysate were mixed with bead-Ab complex at 4°C overnight. Then, the bead-Ab-Ag complex were washed three time with 200 μL washing buffer. Supernatant were removed and elution buffer were added for detaching the Ab-Ag complex from the beads. After that, western blot analysis was performed for detecting the ubiquitinated c-Myc protein.

3.2.3 Anoikis resistance inhibition

3.2.3.1 Anchorage-independent growth assessment

For studying anchorage-independent cell growth, soft agar colony formation assay was used. H292 cells were seeded and incubated on 24-well plates at a concentration of 2.5×10^4 cells/well for 24 h at 37°C. The cells were pre-treated with various non-toxic concentrations of EMD for 24 h at 37°C. Soft agar was prepared by using a 1:1 mixture of 10% FBS-RPMI culture media and 1% agarose gel. After 24 h, the 8×10^3 cells/ml were mixed with 10% FBS-RPMI culture media and 0.33% agarose gel and the cell mixture was added into the well and allowed to solidify for 3 h. The 10% FBS-RPMI culture media was then added on the top layer. Colony formation was measured after two weeks using a phase contrast microscope. Relative colony number and diameter were calculated by dividing the values of the treated cells to those of the control cells.

$$\text{Relative colony number} = \frac{\text{Number of colony from treated cells}}{\text{Number of colony from controlled cells}}$$

$$\text{Relative diameter of colony} = \frac{\text{Diameter of colony from treated cells}}{\text{Diameter of colony from controlled cells}}$$

3.2.4 Migration inhibition

3.2.4.1 Wound healing cell migration assay

H292 cells were seeded on a 24-well plate at a density of 1.2×10^5 cells/well to create the monolayer within 24 h. Wound space was scratched by a 200 µl-tip. Medium was removed and the cells were washed with PBS before photographed under a phase contrast microscope (Nikon ECLIPSE Ts2). The cells were treated with various concentrations of EMD and the images

were captured at various time points. The wound area at each time point was measured by using Image J software (version 1.52, National Institutes of Health, Bethesda, MD, USA). The results are reported as a relative wound area.

3.2.4.2 Filopodia formation assessment

H292 cells were seeded on 96-well plate at a density of $5x10^3$ cells/well. After EMD treatment for 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at 37°C. Then, the cells were permeabilized with 0.1% Triton-x100 for 5 min and non-specific binding was blocked with 0.2% BSA for 30 min. Phalloidin-rhodamine was diluted to 1:100 in PBS before staining for 30 min. Afterwards, the cells were mounted with 50% glycerol in PBS. They were visualized and imaged under a fluorescence microscope (Nikon ECLIPSE Ts2). The results are reported as relative numbers of filopodia per cell.

3.2.5 Statistical Analysis

The resulted will be presented in mean ± SD of at least 3 independent replicated samples. Multiple comparisons for statistically significant differences between multiple groups (ANOVA) will be calculated by using SPSS software program version 16 (SPSS Inc., Chicago, IL, USA), followed by individual comparisons with Schefft's post-hoc test. For two-group comparisons, t-test will be calculated by SPSS software program. Statistical significance will be considered at $p < 0.05$. GraphPad prism 5 will be used for creating graphs in all experiments.

CHAPTER 4

RESULTS

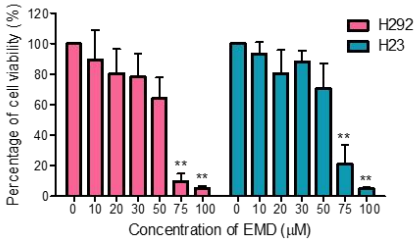
4.1 Apoptosis inducing activity

4.1.1 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) induces apoptosis in human lung cancer cells

To investigate the potential anticancer activity of EMD in human lung carcinoma, its cytotoxicity was first evaluated. NSCLC H292 and H23 cells were exposed with various doses of EMD (0–100 μM) for 24 hours. The cell viability was then determined by MTT assay. All of the data was calculated based on results of 3 replicated samples. The results indicated that EMD significantly decreased viability of the H23 and H292 cells in a concentration-dependent manner when compared with non-treatment controls (Figure 8A). The IC_{50} values of EMD in both cell lines are presented in Figure 8B. The values were calculated from single values based on equations fitted to the pooled data. Further, a nuclear staining assay was utilized to investigate the mode of cell death induced by EMD. Morphological changes by means of condensed and/or fragmented nuclei were observed after the treatment for 24 hours, indicating that both cell lines encountered apoptotic cell death significantly after treatment with 75 μM of EMD when compared with the non-treatment control (Figures 8C–D).

Flow cytometry analysis using annexin V-FITC/PI was applied to validate the apoptotic cell death induced by EMD. Similar to the nuclear staining assay, EMD significantly induced apoptotic cell death, starting at a concentration of 75 μM in H23 cells, but at 100 μM in H292 cells (Figures 8E–F).

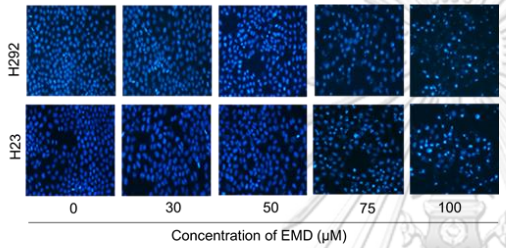
A



B

Cell line	IC50 (μM)
H292	56.60
H23	62.13

C



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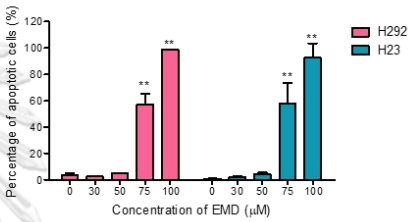
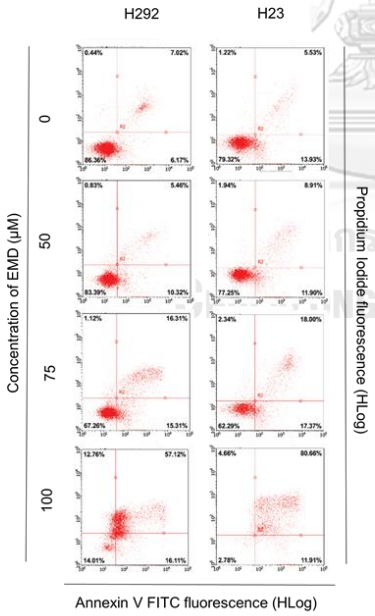


E



F

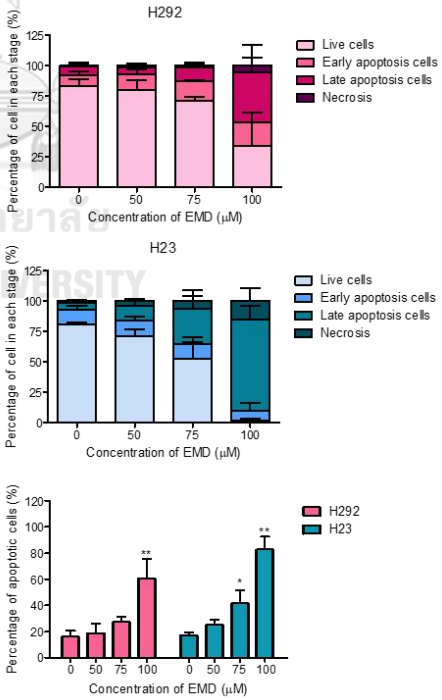


Figure 8 *Apoptosis induction activity of N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine*

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) can inhibit cell proliferation and induce apoptosis, programed cell death of H292 and H23 non-small cell lung cancer. (A) MTT assay was used to evaluate cell viability after treatment with various concentrations of EMD (0-100 μM) for 24 hours. (B) The half maximal inhibitory concentrations (IC_{50}) of EMD against H292 and H23 were calculated from MTT assay by comparison with untreated control. (C-D) H292 and H23 were treated with EMD (0-100 μM) for 24 hours and then stained with Hoechst 33342. The images were detected by using an inverted fluorescence microscope. A blue fluorescence of Hoechst 33342 represented the fragmented and condensed chromatin in the apoptotic cells. (E-F) H292 and H23 were treated with EMD (0-100 μM) for 24 hours and percentages of death cells in each stage were evaluated by using annexin V-FITC/PI staining with flow cytometry. Percentages of cell in each stage were presented as the mean ± SD and percentage of apoptotic cells between each concentration of EMF compared with non-treatment control were statistically calculated. (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$)

4.1.2 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) triggers apoptosis cascade through mechanisms that involve c-Myc downregulation

Caspase activation and the cleavage of caspase substrate PARP are known to initiate an apoptosis cascade, making them well-known apoptotic markers. NSCLC H292 and H23 cells were treated with EMD (0–100 μM) for 24 hours, after which the total cells were collected and PARP, caspase-3, caspase-9, and their cleaved forms were evaluated by western blot analysis. Same as previous experiments, all of the data was calculated based on results of 3 replicated samples. Figures 9A and B show that EMD significantly cleaved and activated caspase-3 and caspase-9, while it caused

PARP cleavage and inactivation when compared to the non-treated control (Figures 9A–B).

The drug sensitivity of tumor cells in response to certain treatments, including conventional chemotherapy, is regulated by the balance and interaction of pro-survival and apoptotic signals. To elucidate the underlying mechanisms of apoptosis induction by EMD, we monitored the expression levels of the key pro-survival proteins, i.e., Akt and phosphorylated Akt (p-Akt), and anti-apoptotic Bcl-2 protein in EMD-treated H292 and H23 cells. We found that the ratio of p-Akt/Akt and Bcl-2 levels were not notably changed after EMD treatment at the concentrations that induced apoptosis, leading us to the discovery of other targets that are important in NSCLC drug response and that may be associated with EMD.

c-Myc is an essential protein for tumor cell maintenance and is a central oncogenic switch. In lung cancer, the inactivation of c-Myc can induce apoptotic cell death and lead to cancer regression and induce apoptotic cell death [7], [43]. We tested the effect of EMD treatment on c-Myc and found a dramatic downregulation of c-Myc in response to EMD at 24 hours, even at the lowest concentration of 50 μM. At higher concentrations of EMD, i.e., 100 μM, the level of c-Myc had almost disappeared in all the tested cells (Figures 9C–D). These results indicate that c-Myc is a preferable molecular target of EMD and that the possible mechanism of action of EMD may be related to c-Myc degradation.

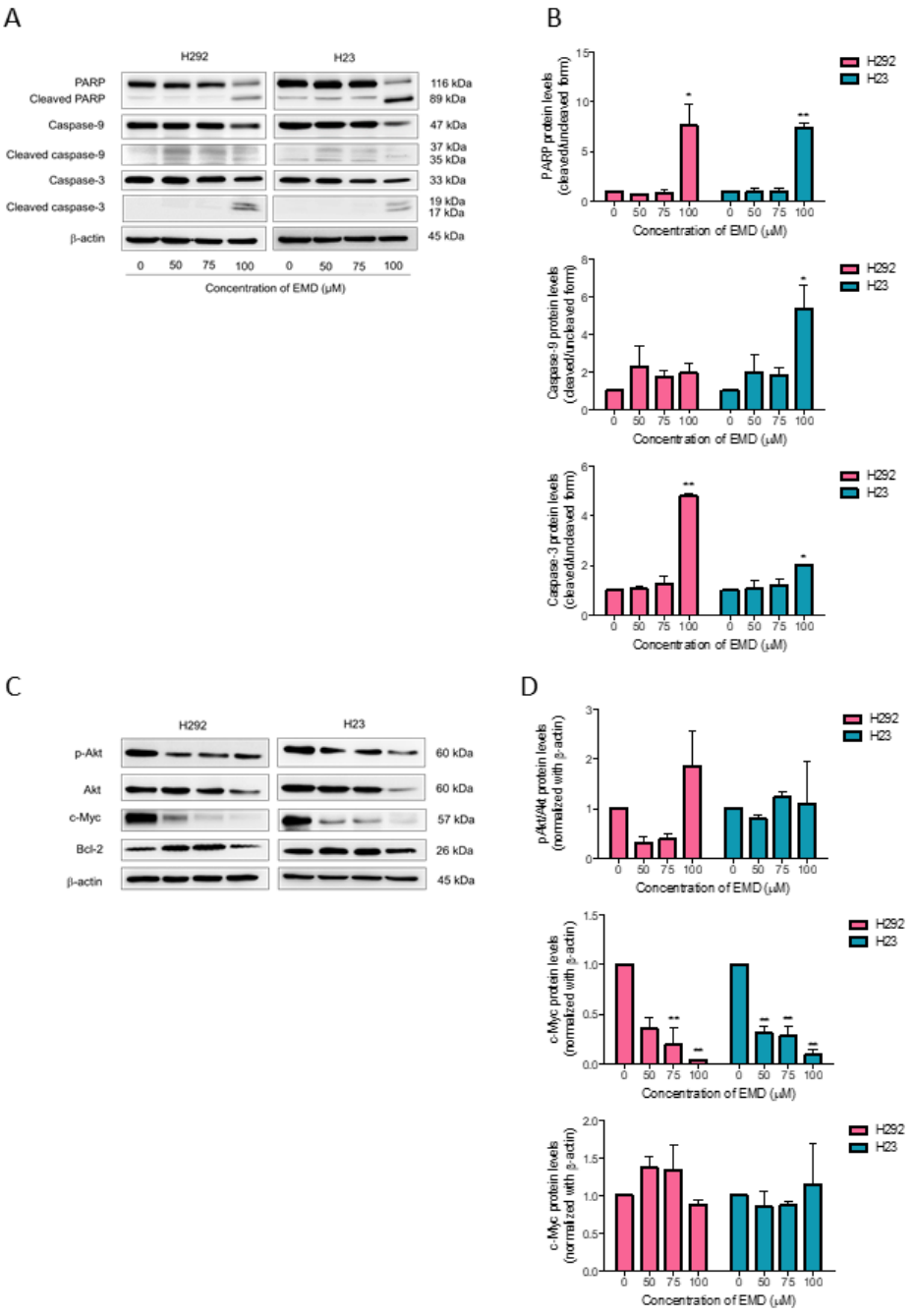


Figure 9 *Effect of N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine against apoptotic protein markers and c-Myc targeted protein*

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) triggered the apoptotic marker proteins and decreased c-Myc in both H292 and H23 cell lines after treated the cell lines with EMD (0-100 μM) for 24 hours. (A) and (C) Western blot analysis was performed to measure the apoptotic-related proteins. β-actin protein was evaluated to confirm equal loading of samples. (B) and (D) Densitometry of each protein levels were calculated and presented the results in relative protein levels. Data represented the mean ± SD (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the non-treatment control).

4.2 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) decreases c-Myc protein level through initiation of c-Myc proteasome degradation

One of the strategies for targeting c-Myc for cancer treatment is the promotion of c-Myc degradation [10]. Having demonstrated the dramatic c-Myc downregulation by EMD, we further identified its mechanism of c-Myc regulation. A protein abundance reflects the balance of the rates of protein synthesis and protein degradation. To substantiate whether EMD affected c-Myc degradation, we used the protein biosynthesis inhibitor CHX to prevent translational elongation [119] and performed a CHX chase assay to estimate the half-life of c-Myc in NSCLC H292 and H23 cells. Here, the cells were treated with 50 μg/ml of CHX with or without 100 μM EMD and western blot analysis was performed at various time (0–60 minutes) to determine the c-Myc level (Figures 10A–B). We observed that c-Myc started to degrade significantly at 15 minutes in the non-treated cells and that EMD treatment in both H292 and H23 cells induced c-Myc degradation. The half-lives of c-Myc in the non-treated control and EMD-treated cells were calculated and the results are shown in Figure 10C. The values were calculated from single values based on

equations fitted to the pooled data. The results demonstrated that c-Myc degraded much faster and its stability was reduced in EMD-treated cells when compared to the non-treated cells.

Ubiquitin–proteasome degradation has been shown to influence protein turnover. Thus, MG132, a potent proteasome inhibitor, was applied to verify that c-Myc destability by EMD occurred in this degradation mechanism. After EMD treatment in H292 and H23 cells, the c-Myc protein level decreased within 1 hour (Figures 10D–E). Remarkably, the addition of MG132 (0–20 μM) to EMD treatment restored the c-Myc protein level, indicating that c-Myc degradation occurred through the ubiquitin–proteasome system. We also checked the premise of ubiquitin-mediated c-Myc degradation using co-immunoprecipitation and evaluated the level of the c-Myc-ubiquitin complex (poly Ub-c-Myc) in H292 and H23 cells after treatment with 100 μM of EMD and in non-treated control cells for 1 hour. Figures 10F–G show that the polyubiquitination of c-Myc was noticeably elevated after EMD treatment when compared with the non-treated control, thus confirming that EMD mediated c-Myc stability through ubiquitin–proteasome degradation.

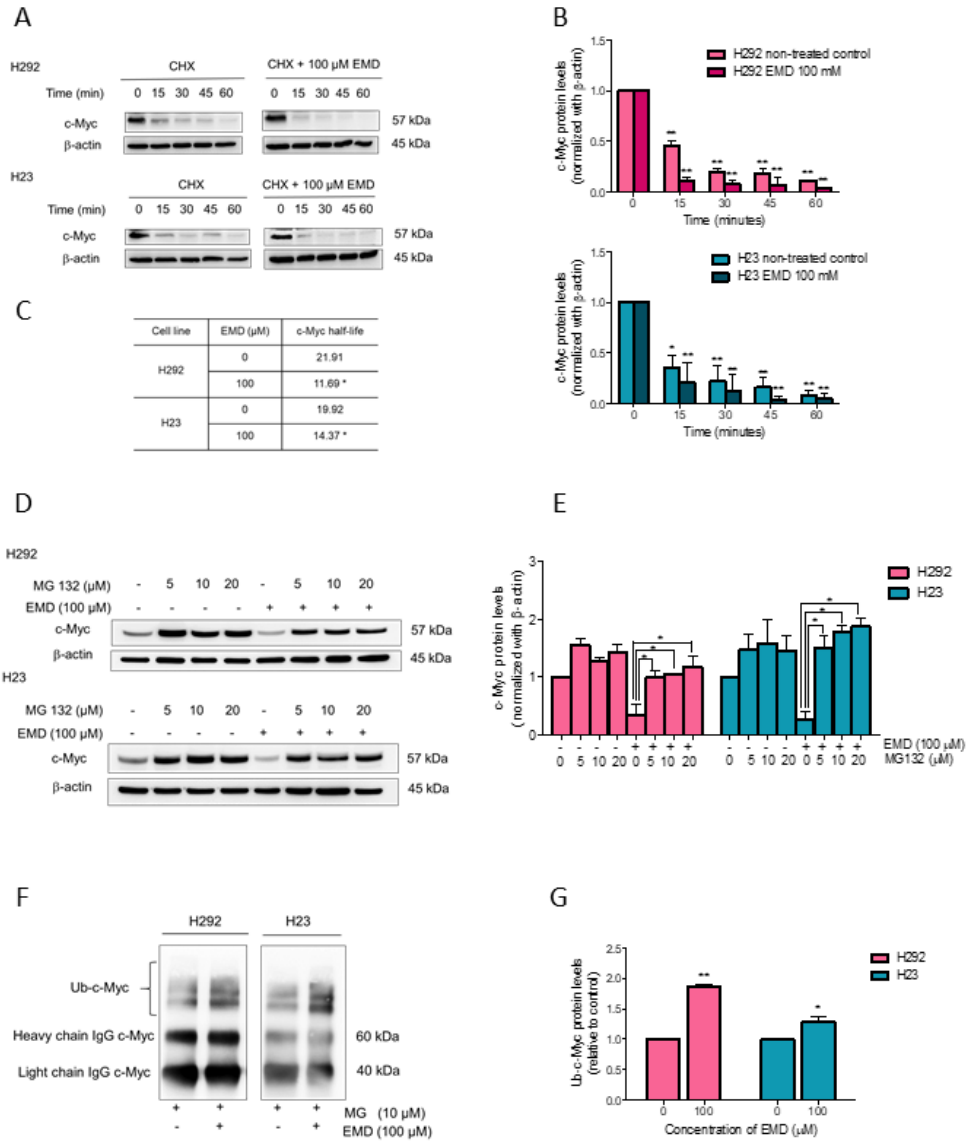


Figure **10** *N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine* induced c-Myc degradation through ubiquitin proteasomal pathway

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) induced ubiquitin-mediated c-Myc proteasomal degradation. (A) The half-life of c-Myc was determined by Cycloheximide (CHX) chasing assay. H292 and H23 cell lines were treated with 50 μg/ml of CHX with or without 100 μM EMD as indicated by the time in minute.

Western blot analysis was used to evaluate the c-Myc protein levels. (B) The relative c-Myc protein levels were calculated and compared with non-treatment control at 0 minute. (C) Half-lives of c-Myc protein of H292 and H23 were calculated. (D-E) H292 and H23 cell lines were pre-treated with MG 132 (0-20 μM), potent proteasome inhibitor for 1 hour and treated with EMD (0-100 μM) for 1 hour. c-Myc protein levels were determined using western blot analysis. (F-G) H292 and H23 were pre-treated with MG132 10 μM for 30 minutes and treated with EMD 100 μM for 1 hour. The protein lysates were collected and incubated with mixture of beads and c-Myc primary antibody to pull out the protein of interest. Then, the ubiquitinated protein levels were measured by western blot analysis. The relative to control protein levels were reported. Data represented the mean ± SD (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$ when compared with non-treatment control)

4.3 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) shows a potency against primary lung cancer cells

This section outlines how EMD not only had a universal effect on lung cancer cell lines but also affected other cancerous cells. Hence, four primary cell lines, namely ELC12, ELC16, ELC17, and ELC20, derived from adenocarcinoma lung cancer patients were used for evaluating the anticancer property of EMD. ELC12, ELC16, and ELC 17 cells from patients who had not received any chemotherapy, while ELC20 cells were collected from a patient who had received chemotherapy [120].

MTT assay and nuclear staining assay were used for evaluating the cell viability and apoptotic cell death of the four primary lung cancer cell lines, respectively. We used the chemotherapeutic drug etoposide for comparison. After treatment with various concentrations of EMD (0–100 μM) or etoposide at the same

concentrations, the results showed that EMD had superior apoptotic-inducing activity than etoposide at the concentration of 75 μM in the first three cell lines and at 100 μM in ELC20. EMD significantly decreased cell viability and mediated apoptotic cell death in all four primary cancer cells (Figures 11A-D). The half-maximal inhibitory concentrations (IC_{50}) of EMD on the four primary cell lines were calculated. The values were calculated from single values based on equations fitted to the pooled data. As etoposide is one of standard drugs for lung cancer treatment [121], [122], [123] and evidences indicated that etoposide is effective in treatment of lung cancer when used alone or in combination with other chemotherapeutic drugs [124], [125], the observed resistance of primary lung cancer cells in this experiment was unexpected and the resistance may cause by specific properties of certain cells. Moreover, western blot analysis was done to determined the c-Myc protein levels after 100 μM of EMD treatment in all four primary cell lines. The results showed that the c-Myc protein levels in all four primary cell lines were significantly decreased compared with those of the non-treatment control (Figures 11E-F).

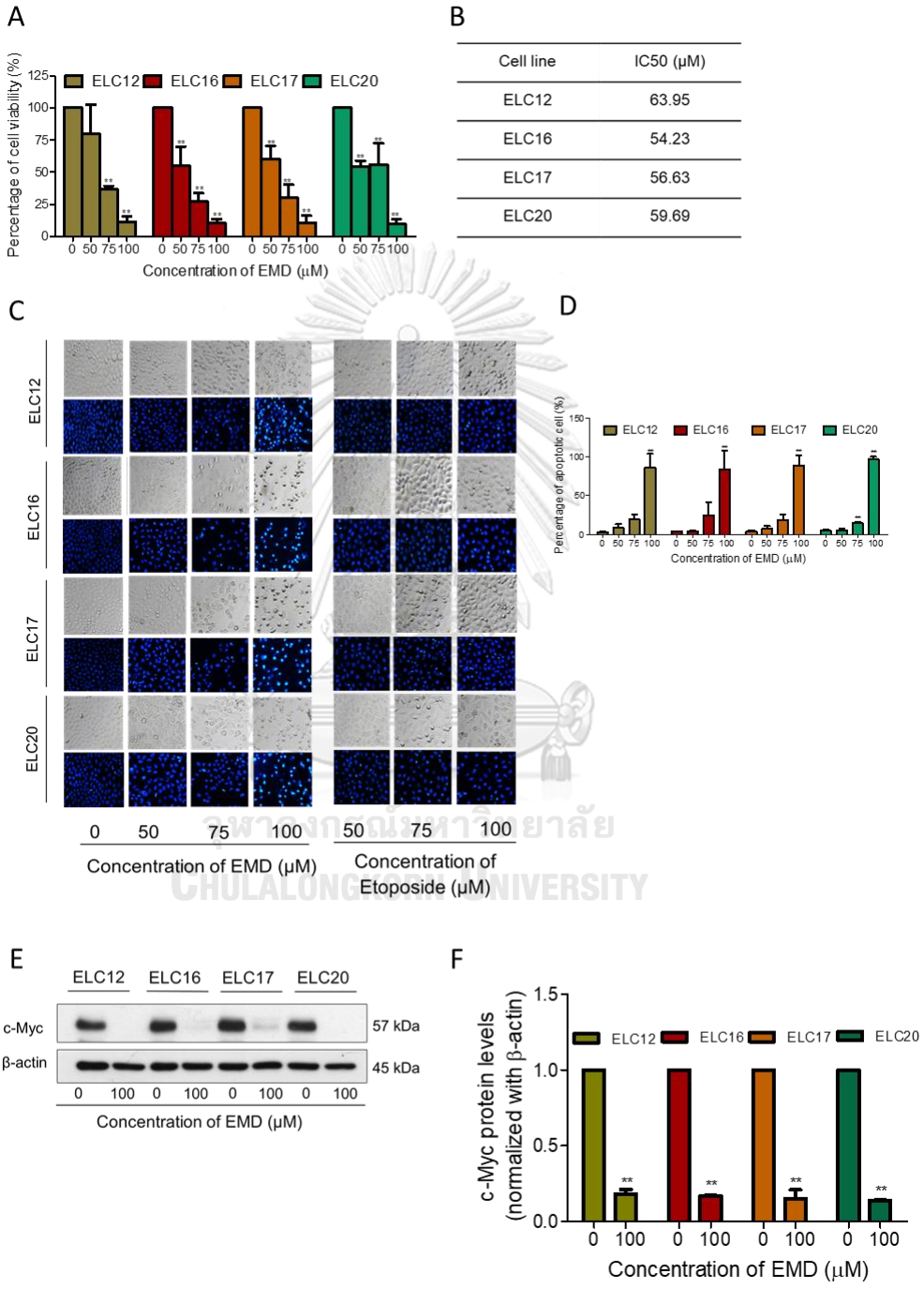


Figure **11** Effect of *N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine in primary lung cancer cell lines*

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) demonstrated the potential to inhibit cell proliferation and promote apoptosis cell death in all four primary cell lines. (A-B) Cell viabilities of four primary cell lines were significantly decreased and the half maximal inhibitory concentrations (IC_{50}) of EMD against four cell lines were calculated. (C-D) Four primary cell lines were treated with EMD (0-100 μM) and etoposide (0-100 μM) for 24 hours before stained with Hoechst 33342 and captured the images under an inverted fluorescence microscope. (E-F) Western blot were performed to analyzed the c-Myc protein levels. Densitometry of c-Myc protein levels were calculated and presented the results as relative protein levels. Data represented the mean ± SD (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$).

4.4 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) inhibits anoikis independence cell death and demonstrates migration inhibition

4.4.1 *N*, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) inhibits anchorage-independent growth, migration and filopodia formation

To select the non-toxic concentrations, we further examined apoptosis. The cells were similarly treated with EMD and nuclear morphology was monitored by Hoechst 33342 staining. Apoptotic cells representing fragmented or condensed nuclei were determined. The results indicated that EMD at the concentrations of 0-40 μM did not induce apoptotic cell death (Figure 12A-B).

Next, we examined whether EMD, at non-toxic concentrations (indicated by cell viability and no apoptosis induction), could attenuate the survival of cells and inhibit the anchorage-independent growth of cancer cells. As detachment-induced apoptosis or cell death termed anoikis is an important obstacle of cancer metastasis,

and lung cancer cells are frequently found to be anoikis resistant, the compound that inhibits anchorage-independent growth may have anti-metastatic activity. The cells were treated with non-toxic concentrations of EMD (0-40 μM) for 24 h before subjected to the anchorage-independent growth assay. Colony formation of the cells was tested following incubation on agarose gel for 14 days. The number and size of colonies were significantly reduced in response to EMD treatment (Figure 12C-D).

Having shown that EMD potentially inhibits anchorage-independent cell growth, we further evaluated the effect of the compound on the cancer cell migration by the wound healing assay. The monolayer of H292 cells was scratched to make wound area and then treated with non-toxic concentrations of EMD (0-40 μM) for 24 and 48 h. The relative wound area at 24 and 48 h was measured. Cell migration after treatment with EMD was found to be significantly inhibited at the concentrations of 20 and 40 μM when compared with the non-treated control (Figure 12E-F).

Furthermore, the formation of filopodia, the mandatory step of migration, which involves cell movement, was observed by the phalloidin-rhodamine staining assay. The cells were visualized after EMD treatment at various concentrations (0-40 μM). The filopodia were stained with phalloidin-rhodamine and their numbers per cell were calculated. EMD treatment significantly decreased the formation of filopodia at the concentration of 40 μM (Figure 12G-H).

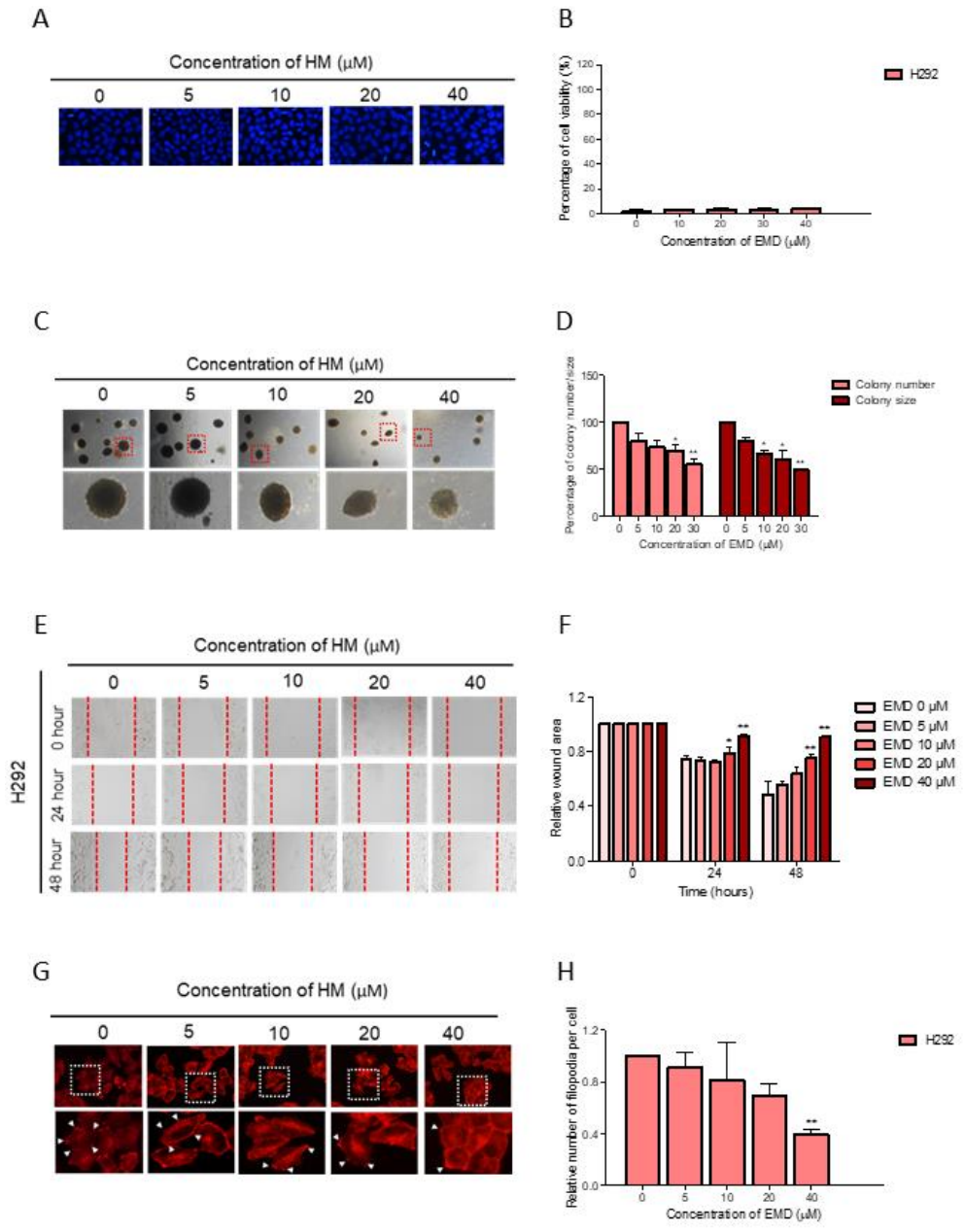


Figure 12 *Effect of N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine on H292 cell anchorage-independent growth, migration and filopodia formation.*

(A-B) Hoechst 33342 staining analysis of the cells. (C-D) anchorage-independent growth of EMD-treated cells. The results were represented in relative number of colonies and relative colony size. EMD inhibits cell migration. (E-F) Wound healing assay was performed to evaluate cell migration after treatment with various concentrations of EMD (0-40 μM) for 24 and 48 h. Relative wound area at 24 and 48 h was calculated compared with that at 0 h. (G-H) Filopodia formation was examined by phalloidin rhodamine staining and the numbers of filopodia per cell were calculated compared with the non-treated control. The data is presented as mean ± SD. (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the non-treatment control)

4.4.2 *N, N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) down-regulates integrin signaling and the proteins involved in cell migration

Integrin generates survival and motility signals. In order to clarify the underlying mechanism of the inhibitory effect of EMD on migration and anchorage-independent growth, essential integrins including $\alpha 5$, $\beta 1$ and $\beta 3$ were evaluated by western blot analysis. H292 lung cancer cells were treated with various concentrations of EMD (0-40 μM) for 24 h. Treatment of cells with EMD dramatically decreased integrin $\beta 3$ levels in a dose-dependent fashion, while had no effect on the levels of integrins $\alpha 5$ and $\beta 1$ (Figure 13A-B). The downstream integrin-mediated signaling was examined by assaying for the levels of focal adhesion kinase (FAK), phosphorylated FAK (p-FAK), Akt and phosphorylated Akt (p-Akt), RhoA. Mcl-1 and Bcl-2 in EMD-treated cells. The levels of p-FAK/FAK and p-Akt/Akt were significantly decreased in EMD-treated cells compared with the non-treated control. Moreover, RhoA, which plays a role in filopodia formation was also evaluated. The protein levels of RhoA were notably decreased in response to 40 μM of EMD. However, the

levels of the pro-survival proteins Mcl-1 and Bcl-2 were not significantly changed after EMD treatment (Figure 13C-D).

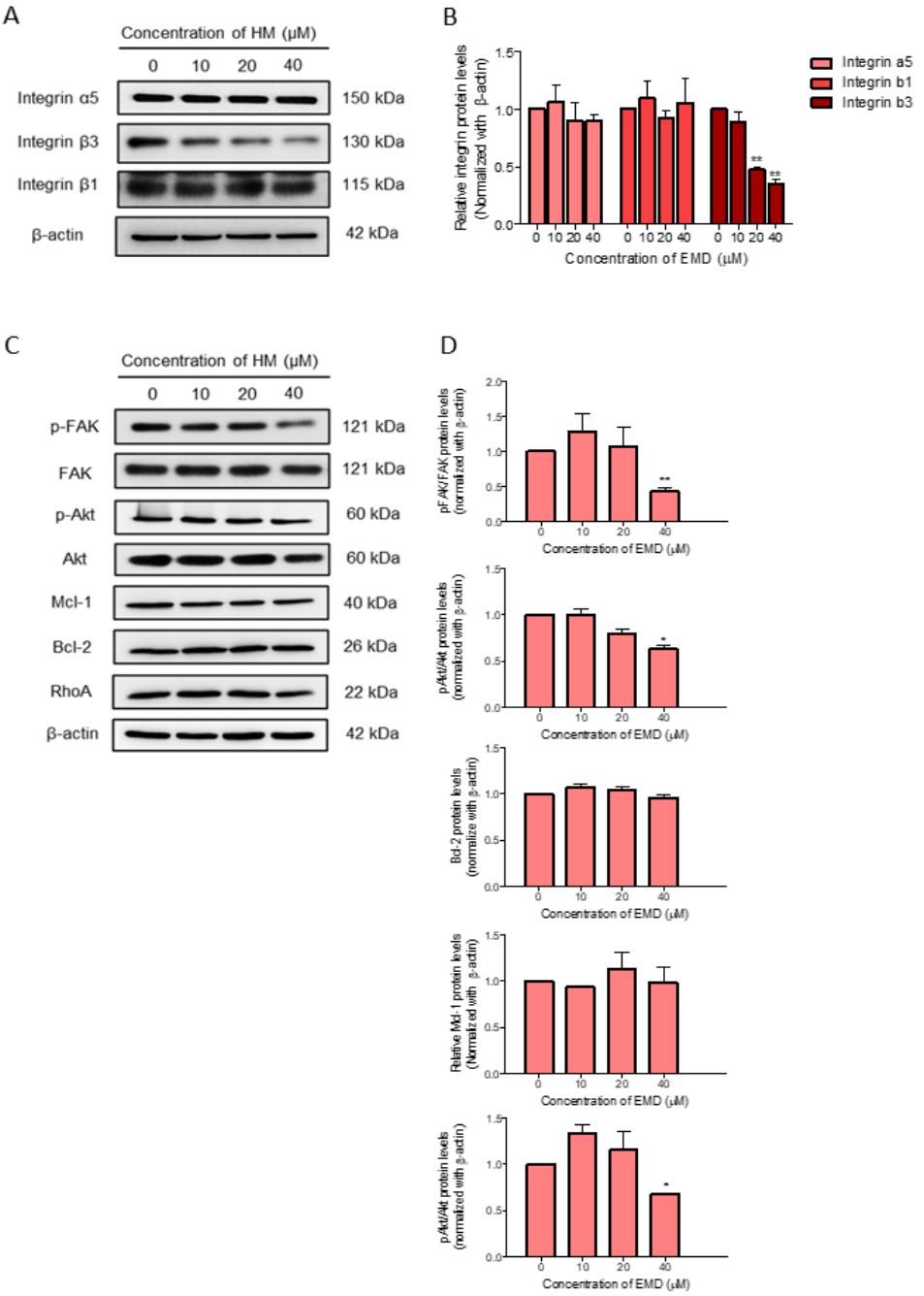


Figure **13** Effect of *N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine against migratory protein markers*

N, *N*-bis (5-ethyl-2-hydroxybenzyl) methylamine (EMD) down-regulated the essential protein involved in cell migration in H292 lung cancer cell after EMD treatment with various concentration (0-40 μM) for 24 hours. (A) and (C) western blot analysis was used to evaluated protein levels of integrin protein family and other downstream protein of integrin signaling pathway. β-actin protein was examined to confirm equal loading of each protein samples. (B) and (D) Densitometry of each protein levels were calculated and exhibited the results in relative protein levels. The data was represented in mean ± SD. (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with non-treatment control)

CHAPTER 5
DISCUSSION AND CONCLUSION

5.1 Discussion

As is well known, cancer cells have a fallibility in terms of the apoptotic pathway. These defects not only promote cancer progression, but can also render therapeutic failure [126]. One way to perform cancer treatment is through activation of the death mechanism within cancer cells, where targeting the apoptosis pathway is the most successful. The apoptosis mechanism can be evaluated by tracking the pro-apoptotic or anti-apoptotic markers and caspase enzyme cascade [25], [27]. c-Myc is an oncoprotein that has been shown to be important for progression of lung cancer [127] , including therapeutic resistance and a poor prognosis [64], [35], [128]. Much research data have indicated that most cancers develop mechanisms to elevate c-Myc activities to promote cell survival, proliferation, and invasiveness [7]. A series of reports exhibited that c-Myc inhibition may lead to tumor regression. In this study, we found that treatment with EMD caused a significant induction of the caspase cascade but had minor effects on other apoptotic markers (Figure 9). In contrast, our protein of interest, i.e., c-Myc, was strongly affected by EMD treatment (Figure 9C-D).

EMD is a benzoxazine dimer synthesized from 4-ethylphenol, formaldehyde, and methylamine in two steps, comprising a Mannich reaction and ring-opening dimerization [11], [12]. As this is a newly synthesized compound, data on this compound are limited, although a previous study found that benzoxazine derivatives could induce apoptosis in various cancerous cell types, such as breast cancer, cervical cancer, and osteosarcoma [14]. Emerging evidence favors the potential use of c-Myc-targeted therapy for cancer treatment [34], [40], [29]. A previous study revealed that the

increased stability of c-Myc plays a role in the pathogenesis of certain cancers [129]. As the level of the c-Myc protein is shown to be regulated through ubiquitin–proteasome protein degradation [130], targeting c-Myc by facilitating the protein degradation may offer a strategy for drug action in c-Myc-driven cancers. So far, several small molecules have been shown to be able to effectively promote c-Myc degradation, such as oridonin [94] and MLN8237 [95] which advocated the activity of Fbw7 on ubiquitin-proteasomal degradation of c-Myc resulted in suppression of cancer cell growth and apoptosis induction [94].

Another approach focused on the stabilization of c-Myc. It is known that the c-Myc protein is stabilized by phosphorylation at serine 62 of the c-Myc protein [56]. While the phosphatase 2A (PP2A) dephosphorylates such a serine phosphorylation and destabilizes c-Myc. Cellular inhibitors of PP2A, SET oncoprotein and the cancerous inhibitor of PP2A (CIP2A), have been found to be overexpressed in certain cancers, and the function of these, i.e., SET and CIP2A, resulted in c-Myc stabilization [96], [97]. In addition, a study indicated that the augmented expression of CIP2A is linked with the aggressiveness and poor prognosis of lung cancer [131]. The SET antagonist OP449 was shown to suppress the tumorigenic potential of cancer cells, induce apoptosis cell death, and enhance the effects of tyrosine kinase inhibitors [132], [133]. Overall, these data suggest that directly targeting c-Myc for degradation or regulating the molecular control of its stability has potential therapeutic value for cancer treatment [134].

Consistent with the above, our experiments exhibited that EMD could induce c-Myc degradation in lung cancer cells. EMD was demonstrated to possess a potent effect on c-Myc-targeted degradation. Our results indicated that while the other

protein markers that we evaluated in this study were not affected by EMD treatment, the level of the c-Myc protein was strongly depleted (Figure 9C-D).

In this study, we found that after EMD treatment, the half-life of the c-Myc protein was shortened. The cycloheximide chasing assay revealed that c-Myc half-life in response to 100 μM was approximately 12 and 15 minutes compared to 23 and 20 minutes in non-treated H292 and H23 cells, respectively (figure 10A-C). After a proteasome inhibitor MG132 was added, the c-Myc protein in EMD-treated cells was significantly restored, demonstrating that proteasomal degradation had a critical role in c-Myc-targeting activity of EMD. Moreover, etoposide was used for comparison with EMD and found that when used at the same concentrations EMD showed greater cytotoxic effect (Figure 11). Because etoposide resistance can be frequently found in an aggressive lung cancer [135], [136], [137], EMD may benefit the treatment of etoposide resistant cancer.

Finally, the effect of EMD against the c-Myc protein in normal cells was evaluated. The results in HaCAT and HK2 cells differed from each other and showed that the effect of EMD may be cell-type specific. c-Myc is a part of several essential mechanisms in normal cells and is strictly regulated by ubiquitin-proteasomal degradation and other interacting proteins [41]. However, there are alterations of c-Myc regulation were reported in cancerous cells. Indeed, mutation and inactivation of some MYC E3 ligases were reported. Fbw7 functioning as c-Myc ligase was found to be inactivated in cancers [5], [6] and the deletion of its gene *FBW7* was found in approximately 30% of human cancers [138], [139], [140]. In addition, Usp28, an antagonist of c-Myc ligase Fbw7α, was found to be increased in cancers. Also, TRUSS, c-Myc E3 ligase, was reported to be decreased in cancer cells [141]. The different responses to

EDM-mediated c-Myc down-regulation found in this study is likely caused by the alteration of c-Myc control mechanisms or different up-stream signals that specific to cell type and condition. Results of EMD in targeting c-Myc in cancerous and normal cells may at least provide additional information of this compound and encourage the investigations of the c-Myc regulatory mechanism that specific for cancer cells.

Even though the present study used EMD at high concentration (100 μM) for apoptosis induction in cancerous cells. However, the effective dose is much lower than the dose of etoposide (Figure 11C). In further study, we may attempt to evaluate the synergistic effect between EMD and standard treatments that may be given only low dose of EMD. As known that lung cancer cells demonstrated the resistance properties against many standard drugs and EMD showed apoptosis inducing effect in etoposide resistance cells. EMD could enhance the effect of standard drugs and may be used in novel combination therapy.

The process of metastasis involves cell detachment form original tumor, migration and invasion into circulation, and formation of new tumor at distant site [142]. Researches have focused in finding a treatment, which can target these crucial steps of cancer cell dissemination. Cell migration involves movement by changing the morphology of the cells, detachment from one adhesion surface and adhesion to a new one [143]. Migration requires integrin complexes to respond to extracellular signals, and transactivate cellular pathways, causing the membrane protrusion and formation of filopodia [144], [145], [146]. Focal adhesion kinase (FAK) is shown to function as a secondary messenger in the integrin signaling pathway that activates other downstream targets such as AKT and Rho family of proteins [147]. Thus, targeting key integrins could potentially be a strategy for inhibit cell migration.

During metastasis, most of the solid tumor cells will undergo apoptosis after detachment [114]. The detachment-induced apoptosis termed anoikis has been accepted as an important obstacle of successful metastasis in many cancers, so metastatic cancer cells must acquire mechanisms to escape from anoikis [112], [113]. Here, we demonstrated that EMD inhibited cancer cell migration and anchorage-independent cell growth via suppression of integrin β3. In agreement with our finding, previous studies showed that the decrease of integrin and integrin-related pathway caused cell migration arrest [148], [149]. We observed that EMD-mediated suppression of integrins was specific to integrin β3, as integrin β1 and α5 were not affected. In addition, the integrin-associated downstream proteins, p-FAK, p-Akt and RhoA were found to be depleted (Figure 13) EMD inhibited also anchorage-independent colony formation and reduced colony size (Figure 12).

5.2 Conclusion

In conclusion, this study provides contributing evidence for EMD to be considered a candidate anticancer therapy for several cancer types. EMD was shown to have a tremendous apoptotic induction capability with various cell types. For mechanism, the compound induces c-Myc depletion by promoting ubiquitin–proteasomal degradation of the c-Myc protein. As c-Myc was exhibited to be an essential factor for cancer cell proliferation and survival, these data might be advantageous for emphasizing EMD as a candidate compound in anticancer research

Moreover, we evaluated the effect of EMD against migration and anchorage-independent growth of lung cancer cells. Migration activity of the cells was inhibited by targeting integrin β3 leading to the downregulation of active FAK (p-FAK), active Akt (pAkt) and RhoA. Furthermore, EMD reduced the ability of the cells to survive

and grow in the detached condition when used at non-toxic concentrations. These data indicate EMD as a candidate for anticancer approaches (Figure 14).

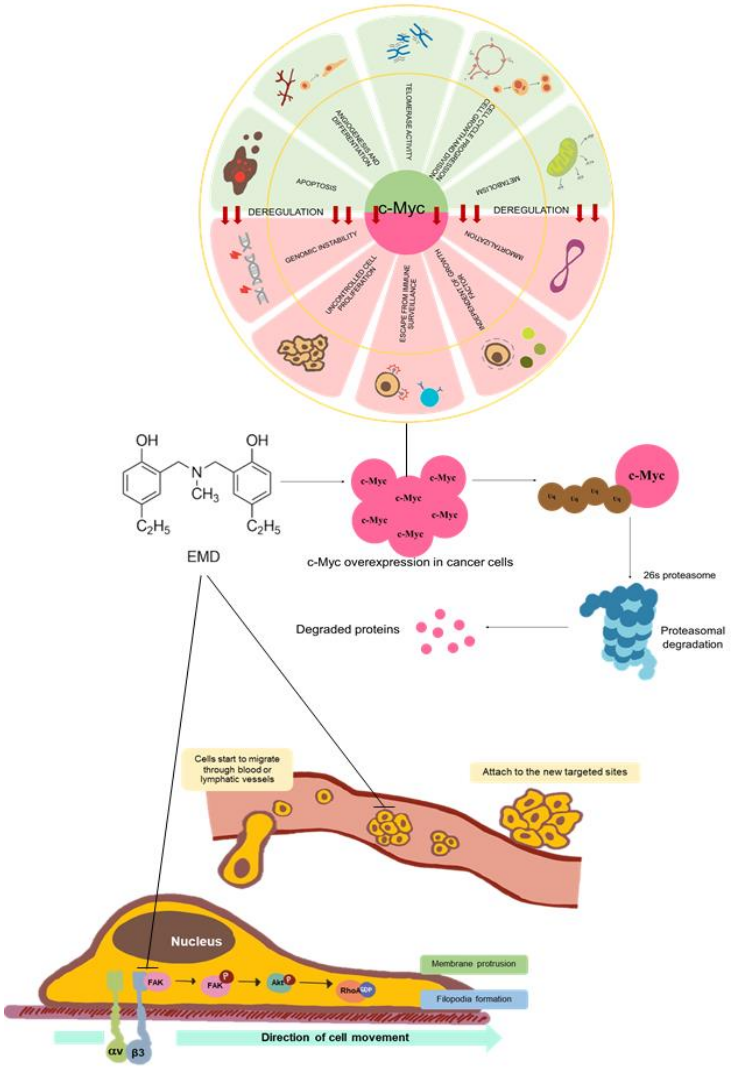


Figure 14 *Conclusion of N, N-bis (5-ethyl-2-hydroxybenzyl) methylamine effect in non-small cell lung cancer cells*

Deregulation of c-Myc, oncoprotein takes part in several survival mechanism of cancer cells which supports the disease progression and treatment failure such as genomic instability and dysregulated cell proliferation. EMD enhances apoptosis cell death of cancer cell and specifically trigger the ubiquitin-proteasome degradation of c-Myc. In addition, cell migration is one of the important steps of cancer metastasis.

Inhibition in this process shows a potential in cancer treatment. EMD exhibits the ability to inhibit integrin β3 which mainly involves in cell migration process leading to decrease in downstream signaling. Lacking stimulated signals, filopodia formation is inhibited, leading to the suppression of cell migration. Not only inhibition of cell movement, but EMD also exhibited a possible activity in preventing anoikis-resistance cell growth.

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