Anti-cancer effects of renieramycin T on human non-small cell lung cancer cells

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ผลการออกฤทธิ์ต้านมะเร็งของสาร renieramycin T ต่อเซลล์มะเร็งปอดชนิดไม่ใช่เซลล์เล็กของมนุษย์

น.ส.กรกช เพ็ชรศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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การมุ่งเป้าไปที่โปรตีน Mcl-1 เป็นวิธีหนึ่งของการรักษามะเร็งปอด เนื่องจากการเพิ่มขึ้นของระดับโปรตีน Mcl-1 มีความสัมพันธ์กับความรุนแรง และการดื้อยาเคมีบำบัดหลายชนิด โดยในปัจจุบันนี้ มีการพัฒนายาที่มุ่งเป้าไปยังการลดระดับของ Mcl-1 อยู่ในขั้นตอนการทดลองทางคลินิกมากมาย ดังนั้นการศึกษานี้จึงได้นำเสนอฤทธิ์ของสารเรเนียรามัยซินที (RT) จากฟองน้ำสีน้ำเงินต่อ Mcl-1 ในเซลล์มะเร็งปอด หลังจากการศึกษาพบว่าสาร RT มีฤทธิ์ทำให้เซลล์เกิดอะพอพโทซิสผ่านทางการสลายของโปรตีน Mcl-1 ด้วยระบบยูบิควิติน-โปรติเอโซมในเซลล์มะเร็งปอดรวมถึงเซลล์มะเร็งปอดที่ดื้อยาจากผู้ป่วย นอกจากนั้นยังพบการเพิ่มขึ้นของระดับโปรตีน p53 และการลดลงของระดับโปรตีน Bcl-2 ซึ่งจะนำไปสู่การตายแบบอะพอพโทซิสเช่นกัน ต่อมาได้มีการศึกษาต่อถึงเหตุผลว่า RT มีกลไกหลักของการออกฤทธิ์มุ่งเป้าไปยังโปรตีน Mcl-1 ได้อย่างไร โดยการวิเคราะห์หาความสัมพันธ์ระหว่างโครงสร้างสารและการออกฤทธิ์ (SARs) เพื่อระบุหมู่ฟังก์ชันจำเพาะที่จำเป็นสำหรับการทำงานของสาร ดังนั้น RT จึงถูกนำไปสร้างเป็นสารคล้ายคลึงจำนวน 5 สารซึ่งมีหมู่ฟังก์ชันต่างกันเพื่อนำมาทดลองต่อ เป็นที่รู้กันอย่างดีว่าการทำงานของ Mcl-1 ขึ้นอยู่กับเสถียรภาพของสาร และเสถียรภาพของการก็ขึ้นอยู่กับการเติมฟอสเฟตด้วย ERK JNK และp38 ที่ตำแหน่งกรดอะมิโน Thr92 Thr163 และSer121 เพราะฉะนั้น การจับกับไซต์เชื่อมต่อของ MAPK บน Mcl-1 ด้วย RT จะสามารถลดเสถียรภาพของโปรตีนนี้ และทำให้เกิดการสลายโปรตีนในที่สุด จากการศึกษาพบว่า ไซยาไนด์และวงเบนซีนในโครงสร้างของ RT มีบทบาทสำคัญต่อการจับกันระหว่างสาร และกรดอะมิโน arginine ซึ่งมีประจุบวกในไซต์เชื่อมต่อของ MAPK บน Mcl-1 เพราะฉะนั้น ข้อมูลการศึกษาในครั้งนี้จึงเป็นประโยชน์ต่อการพัฒนายาใหม่จากกลุ่ม RT เพื่อรักษามะเร็งปอดในอนาคต

สาขาวิชา	เภสัชวิทยา	ลายมือชื่อนิสิต .....
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KEYWORD: lung cancer, anti-cancer, apoptosis, Mcl-1 degradation, patient-derived primary lung cancer cells, Structure–Activity Relationship, Molecular Docking Analysis, renieramycin T, Xestospongia sp.

Korrakod Petsri : Anti-cancer effects of renieramycin T on human non-small cell lung cancer cells. Advisor: Assoc. Prof. PITHI CHANVORACHOTE

Mcl-1 targeted therapy is promising strategy for lung cancer treatment since it is contributed to aggressive lung cancer and chemotherapeutic resistance. At present, many compounds targeting on Mcl-1 are currently under different clinical stages of development for cancer treatment. Herein, we report the Mcl-1 targeting activity of renieramycin T (RT), an alkaloid isolated from the blue sponge *Xestospongia* sp. RT was shown to target on Mcl-1 through ubiquitin-proteasomal degradation in lung cancer cells including chemotherapeutic resistant primary lung cancer cells. Additionally, RT also affects the activation of p53 and decrease of Bcl-2 which can lead to apoptosis of cancer cells. The reason why RT has a major activities on Mcl-1 is further proven in the structure–activity relationship (SAR) analysis. The SAR is depending on the specific chemical structure that reacts to the active site of drug action. For SAR study, five analogues of RT with different functional groups were tested for Mcl-1 targeting effects. It was known that Mcl-1 function is depended on its stability and its stability is regulated by phosphorylation at threonine 92, threonine 163, and serine 121 of the protein molecule by ERK, JNK, and p38. Therefore, binding to MAPK docking site with RT can prevent the Mcl-1 stabilization and finally induce its degradation. We found that cyanide and benzene ring in RT structure have an important role in Mcl-1 degradation since they have an ability to bind with arginine, positive charged amino acid in MAPK docking site of Mcl-1 sequence, with the high binding affinity. These data might have a benefit on further development of compounds as well as the design of novel drugs for treating Mcl-1-driven cancers.

Field of Study:	Pharmacology	Student's Signature .....
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CHAPTER I

Background and rational

Lung cancer is one of the leading causes of cancer-related death worldwide. At present, surgery, chemotherapy, radiation, targeted therapy, and immunotherapy are currently the most promising ways for the improvement of clinical outcome. Despite of advanced cancer therapies, the five-year survival rate after first diagnosis is still limited due to innumerable obstacles such as chemotherapeutic drug resistance, disease relapse and fatal adverse effects of chemotherapy. Thereby, searching for novel treatment strategy is needed [1].

Targeted therapy is a treatment strategy using drugs or substances that can interact with specific molecules in certain signaling pathways such as survival, apoptotic related, and angiogenesis pathways [2]. So far, evidences have suggested that the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family member named myeloid cell leukemia 1 (Mcl-1) is a potential molecular drug target for cancer therapy. Mcl-1 was demonstrated to involve in chemotherapeutic resistance [3-5]. In addition, recent evidences have suggested that ability to evasion apoptosis, survival during metastasis and especially resistance to therapy are likely to be dependent on expression and function of Mcl-1 protein [6-9]. Consequently, it is not unexpected that the amplification and overexpression of pro-survival Mcl-1 are found in many cancer types including lung cancer. Mcl-1 has been shown to be overexpressed and related to lung cancer progression [8, 10-14]. The clinical studies also reported that high expression level of Mcl-1 in NSCLC patients quite predicted worse survival rate [12, 15]. These implies that Mcl-1 activities are depended on its expression level. Gathering with the fact that Mcl-1 is potentially main contributors of oncogenesis and multidrug resistances, this proteins is highlighted as principle target of drug action in treatment of lung cancer. Treatment strategies for targeting Mcl-1 have been established including antisense-mediated inhibition, peptide inhibitors or BH3 mimetics, and small molecule inhibitors [8]. Recently, several Mcl-1 targeted agents are in clinical stages of anti-cancer drug development such as AT101, AZD-5591, and AMG-176. In particular, AT101 is proven as a Mcl-1 inhibitor in phase II clinical trials.

It has been reported that anti-apoptotic potency of Mcl-1 is depended on their stability [16] and degradation of this protein can be induced by anti-cancer agent [17]. The N-terminus of Mcl-1 called PEST region is found to relate with the process of stabilization and degradation of Mcl-1 through phosphorylation at this area. Phosphorylation of Mcl-1 with mitogen-activated protein kinase (MAPK) proteins such as extracellular signal-regulated kinases (ERK), c-Jun NH2-

terminal kinase (JNK), and p38 can stabilize Mcl-1, whereas glycogen synthase kinase-3 (GSK-3) induces its degradation [18]. Therefore, binding to MAPK docking motif (D-motif) with specific compounds might prevent the Mcl-1 stabilization and finally induce its degradation. The novel compounds with ability to eliminate Mcl-1 through this mechanism are of interest as a good candidate for targeted therapy.

At present, marine environment provides many potent bioactive compounds used for new drug developments with an aim to treat major diseases including cancer. From early reports, a large number of very potent agents from marine environment are proven to exert an ability to inhibit growth of human cancer cells and exhibit anti-cancer activities. For instance, five FDA approved drugs from marine organisms; cytarabine, ziconotide, trabectedin, eribulin mesylate, and brentuximab are accepted in usage for cancer treatment [19]. Thereby, marine environment functions as a reservoir of potential candidates for cancer treatment. One of marine derived products is renieramycins. Renieramycins are alkaloids in tetrahydroisoquinoline family [20] isolated from various marine organisms, including sponges in the genera *Reniera* [21, 22], *Xestospongia* [23-27], *Cribrochalina* [28, 29], and *Neopetrosia* [30]. During the period of time, several members of renieramycin family have been discovered such as renieramycin M (RM) and renieramycin T (RT) which extracted from Thai blue sponge, *Xestospongia* sp. Evidences suggested that RM could induce lung cancer cell apoptosis and anoikis via suppressing the anti-apoptotic proteins Bcl-2 and Mcl-1 [31, 32]. Interestingly, bishydroquinone renieramycin M (HQ-RM), a modificated form of RM which had greater anti-cancer activity than its parental RM was also found to have apoptotic induction effects through down-regulation of Bcl-2 and Mcl-1 [33]. For RT, a compound with a similar structure to RM, it showed a strong cytotoxicity effects against various cancer cells including colon (HCT116), prostate (DU145) [34], non-small cell lung (H292, H460 and QG56) [35], breast (T47D) and pancreatic (AsPC1) [36] cancers with a low IC_{50} in nanomolar scale. However, the mechanisms of anti-cancer activities including Mcl-1 targeted effect of RT in lung cancer have not been elucidated yet. Furthermore, some parts of RT structure are aromatic ring and cyano group which have an ability to bind with positive charge amino acids [37-39] that are component of MAPK binding site in Mcl-1 [40]. Thus, it is possible for RT to have an ability on Mcl-1 protein. RT was chosen to be studied in this research.

First of all, the primary lung cancer cells, recently derived from NSCLC patients in Chulalongkorn hospital were established with a drug sensitivity test to prove the chemotherapeutic resistance profiles. The known NSCLC cells and patient-derived primary lung cancer cells were then applied in the experiments of RT. In addition, after investigation of RT mechanism, a right-half of its structure was used as a prototype to synthesize some of simplified right-half model compounds;

TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55 which have different functional groups in each compounds for investigation of structure-activity relationships (SAR) and subsequent SAR-directed optimization to confirm a specific part of their structures that have an influence in loss of Mcl-1 mediated apoptosis. The SAR is depending on the specific chemical structure that reacts to the active site of drug action. In case of Mcl-1 elimination, the Mcl-1 protein was analyzed for its amino acid sequence that critical for the protein stability and found that the phosphorylation at threonine 92, threonine 163, and serine 121 of the protein molecule by MAPK was shown to be important for the stability of Mcl-1 [18]. Therefore, the design drug should be able to bind at MAPK binding pocket of Mcl-1 protein. We have proposed that the structure of RT at the right-half part are essential for its interaction with the Mcl-1 molecule. As RT has complex structure compositing several chemical moieties, SAR is necessity for identification of active moieties that critical for drug action and promisingly increase precision and potency.

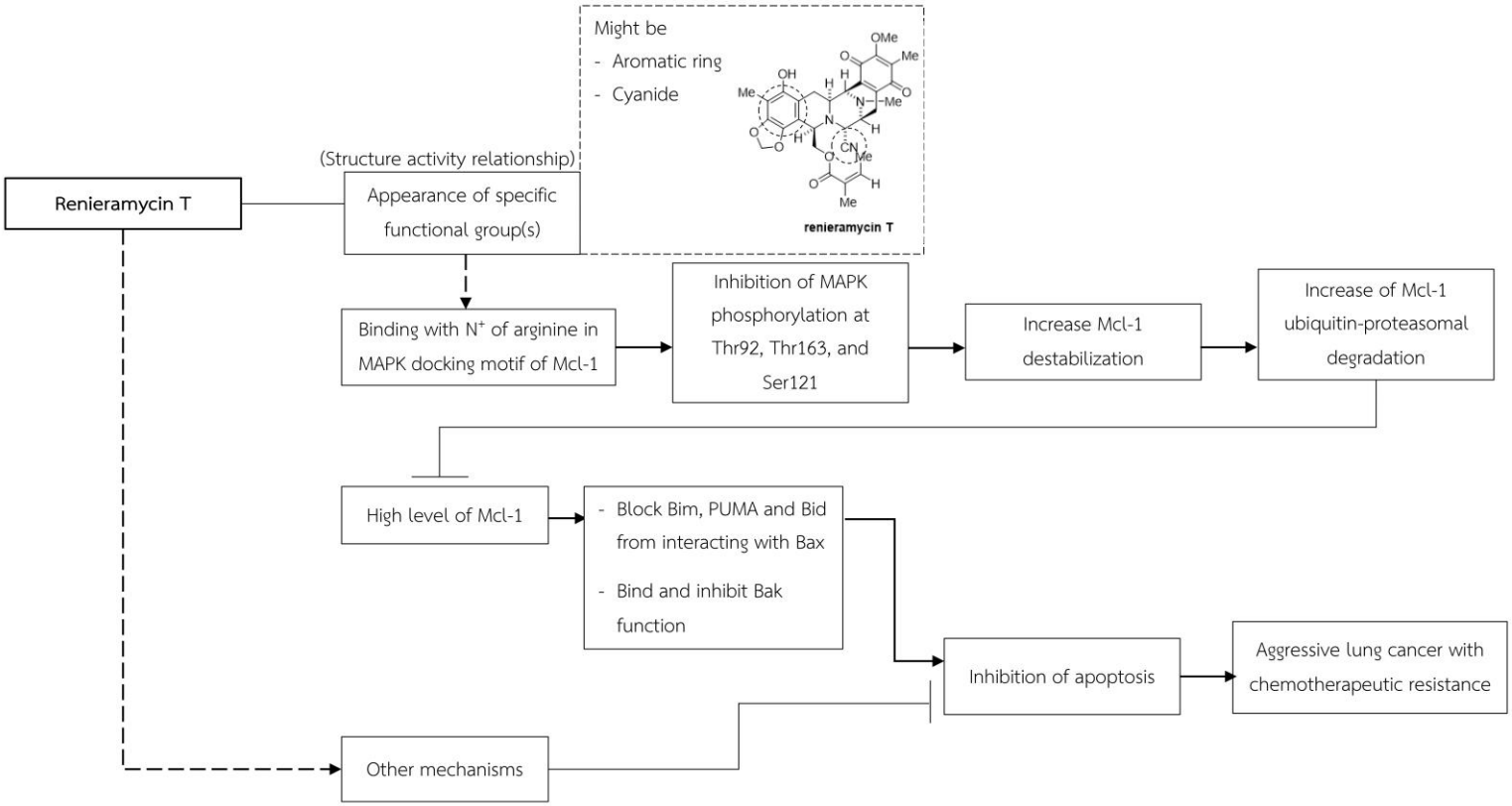
Objective

1. To evaluate the anti-cancer effects of RT on lung cancer cells (NSCLC and patient-derived primary lung cancer cells) and uncover its mechanisms focusing on Mcl-1 targeted effects.
2. To investigate structure-activity relationships (SARs) of RT for identification a specific part of structure that exerts Mcl-1 targeting activity through interaction with the protein molecule.

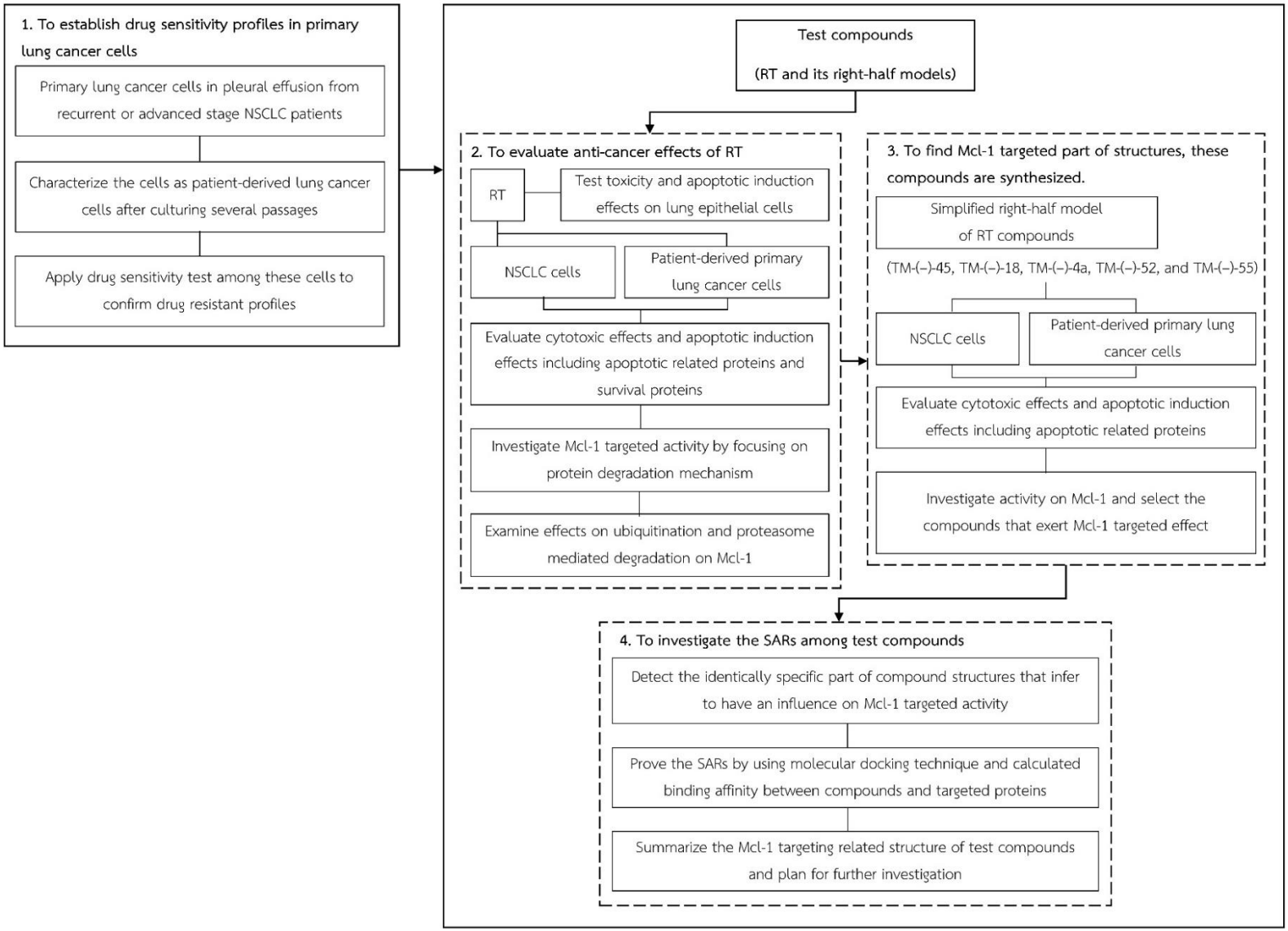
Hypothesis

Renieramycin T (RT) induces apoptosis in both human non-small cell lung cancer cells and drug resistant patient-derived primary lung cancer cells via diminishment of Mcl-1 protein by promoting protein degradation. For SARs, aromatic ring and cyanide in RT structure are important for Mcl-1 targeting activity by directly interaction to Mcl-1 molecule at MAPK docking motif which leads to inhibition of the phosphorylation at threonine 92, threonine 163, and serine 121 resulted in Mcl-1 destabilization.

Conceptual framework



Research design



Expected benefits and applications

This study provides the mechanism of RT to induce apoptosis in non-small cell lung cancer cells and resistant patient-derived primary lung cancer cells. From the SARs study on RT and its right-half model compounds, specific structural parts related to their mechanisms are identified. Thus, it can provide a benefit for further development of RT and its related compounds to gain more efficacy and Mcl-1 targeting activity.

CHAPTER II

Review of related literatures

Lung cancer

Estimates of the worldwide cancer records of incidence and mortality from all types of cancers revealed that lung cancer is the most cause of death with increasing incidence. Lung cancer arises from normal lung epithelial cells which grow abnormally out of control and finally form a mass called tumor. The tumor interferes lung functions, which normally provide an oxygen to the cells in whole body via blood vessels resulted in death of patients. In spite of advances in early diagnosis and several standard treatments, lung cancer still has a poor prognosis due to the resistance to cancer therapy and disease relapse [1]. Besides, the 5-year survival rate of lung cancer is about 17% which is critically lower than many other cancers [41].

Epidemiology

According to the American Cancer Society's publication, Cancer Facts & Figures 2020, lung cancer is the second most commonly diagnosed cancer in both male and female despite the incidence rate has been declining since 2000. From 2005 to 2014, lung cancer incidence rates decreased by 2.5% per year in male and by 1.2% per year in female. Similarly, death rates decreased by 3.8% per year in male and by 2.3% per year in female. However, an estimated death is still high. Predictably, 135,720 deaths and 228,820 new cases from lung cancer are estimated in 2020 [42]. The 1-year survival rate and the 5-year survival rate are 44% and 17% respectively depended on several factors such as subtypes and stages of cancer [41, 42]. In Thailand, lung has also been the third leading site of all cancers with 11.59% of incidence rates in 2012. The reported data revealed that an age-adjusted incidence rate in male is absolutely higher than in females; males 6.43% and females 3.96% [43].

Risk factors

[1, 41, 44]

1. Tobacco and smoking including secondhand smoke
2. Asbestos
3. Radon
4. **Industrial substances** such as arsenic, uranium, beryllium, vinyl chloride, nickel chromates, and coal products

5. **Radiation exposure**
6. **Air pollution**
7. **Tuberculosis**
8. Occupational exposures such as rubber manufacturing, paving, roofing, painting, and chimney sweeping
9. Genetics

Symptoms

[44]

Symptoms of localized lung cancer:

1. Persistent coughing
2. Pain in the chest, shoulder, or back unrelated to pain from coughing
3. Changes in color or volume of sputum especially blood in sputum
4. Shortness of breath
5. Changes in the voice or being hoarse
6. Harsh sounds with each breath (stridor)
7. Recurrent lung problems, such as bronchitis or pneumonia
8. Coughing up phlegm or mucus, especially with blood
9. Coughing up blood

Symptoms of metastasis lung cancer that can occur elsewhere in the body:

1. Loss of appetite or unexplained weight loss
2. Muscle wasting (cachexia)
3. Fatigue
4. Headaches, bone or joint pain
5. Bone fractures which are not related to accidental injury
6. Neurological symptoms, such as unsteady gait or memory loss
7. Neck or facial swelling
8. General weakness
9. Bleeding
10. Blood clots

Types and staging of lung cancer

[44, 45]

Lung cancer has been divided into two major types; **non-small cell lung cancer (NSCLC)** and **small cell lung cancer (SCLC).**

1. **Non-small cell lung cancer (NSCLC)** is the most common type which acquires estimated 85% of lung cancers. NSCLC is divided into 3 subtypes;
   - **Adenocarcinoma**, the most common form
   - **Squamous cell carcinoma**, 25% of NSCLC
   - **Large cell carcinoma**, 10% of NSCLC
2. **Small cell lung cancer (SCLC)** has only 15% incidence rate of all lung cancers. This type grows rapidly and more responsive to chemotherapy than NSCLC.

Staging of lung cancer

- Stage 0: Carcinoma in situ
- Stage I: Tumor size 3 cm or less in greatest dimension but no metastasis to nearby lymph nodes
- Stage II: Tumor size more than 3 cm but 7 cm or less with metastasis to ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes
- Stage III: Tumor of any size with metastasis to ipsilateral peribronchial, ipsilateral hilar, ipsilateral mediastinal, subcarinal, contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph nodes
- Stage IV: Tumor of any size with or without metastasis to nearby lymph nodes but has a distant metastasis to contralateral lobe or extrathoracic organ

Treatments of lung cancer

[1, 44]

Generally, treatments of lung cancer are depended on types, characteristics, and stages of cancer. The treatment strategies including;

1. **Surgery**

   In early stage of lung cancer (stage I and stage II), it can be treated with surgery to remove the lobe or section of the lung that contains the tumor.

2. **Chemotherapy**

Chemotherapy can be used in early and advanced-stage of lung cancer (sometimes in combination with radiation therapy and targeted therapy). The most commonly used chemotherapeutic drugs for lung cancer are cisplatin, carboplatin, docetaxel, gemcitabine, paclitaxel, vinorelbine, and pemetrexed.

3. **Radiation therapy**

This treatment can be used in combination with chemotherapy for prevention of recurrent cancer especially after surgery. In addition, radiation is also used for tumor destruction to make it easier to be removed by surgery.

4. **Targeted therapy**

Targeted therapy is designed to eliminate cancer cells by attaching to or blocking molecular targets that appear in the cells. Advanced-stage lung cancer patients with certain biomarkers can be received targeted therapy in combination with chemotherapy. The targeted drugs that are used include erlotinib, afatinib, gefitinib, bevacizumab, crizotinib, and ceritinib.

5. **Immunotherapy**

Immunotherapy is a new option for lung cancer treatment which uses an immune system against cancer. Immunotherapy is divided into 4 main categories; monoclonal antibodies, checkpoint inhibitors, therapeutic vaccines, and adoptive T-cell transfer.

Despite many kinds of lung cancer treatment options, the survival rate is still critically low due to several problems such as chemotherapeutic resistance and recurrent of cancer. Hence, the better treatment strategies are definitely required. In general, chemotherapeutic and targeted therapeutic drugs use programmed cell death called apoptosis as the main mechanism for cancer elimination so, process of apoptotic cell death is further mentioned.

Apoptotic cell death

Process of programmed cell death, or apoptosis is characterized by morphological changes including nuclei shrinkage, nuclear chromatin condensation, cytoplasmic shrinkage, dilated endoplasmic reticulum and membrane blebbing [46]. Apoptosis is considered as an important component of various processes since it contributes to elimination of unnecessary and unwanted cells to maintain the balance between cell survival and cell death, for example, processes of normal cell turnover, development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death. Therefore, loss of this balance due to inappropriate apoptosis is an essential factor in many diseases. An insufficient apoptosis can

cause cancer or autoimmune disease, whereas accelerated cell death is found in acute and chronic degenerative diseases, immunodeficiency, and infertility [47-49].

Mechanism of apoptosis

Apoptosis is regulated by activation of proteins in caspase family which are central to the mechanism of apoptosis because they are both initiators and executioners of apoptotic process. There are 3 pathways that caspases can be activated, the two are initiation pathways known as intrinsic and extrinsic pathway which eventually lead to activation of the execution pathway resulted in apoptosis of cells [50, 51]. **(Figure 1.)**

The intrinsic pathway (known as mitochondrial pathway) is triggered by internal stimuli such as genetic damage, infection with viruses, extremely high concentrations of cytosolic calcium and severe oxidative stress resulted in increase of mitochondrial membrane permeability that leads to the release of pro-apoptotic molecules into the cytoplasm [52]. This pathway is dominantly controlled by Bcl-2 family proteins. Bcl-2 family proteins are classified into 2 main groups based on their function called pro-apoptotic proteins and anti-apoptotic proteins. The anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome c, whereas the pro-apoptotic proteins promote its release through mitochondrial membrane disruption. When cytochrome c releases, the molecular complex named 'apoptosome' consisted of caspase-9, Apaf-1, and cytochrome c is formed with a purpose to activate the execution pathway through caspase-3 activation [52, 53]. In addition, other apoptotic proteins released from the mitochondria include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) [54]. Smac/DIABLO or Omi/HtrA2 promote apoptosis by blocking caspase inhibitor called inhibitor of apoptosis proteins (IAPs) resulted in caspase activation [54, 55].

Another initiation pathway, extrinsic pathway, involves with transmembrane receptor-mediated interactions. This pathway is initiated by binding of death ligands (e.g. TNF and FasL) and death receptors (e.g. TNFR and Fas (CD95)) [56]. After ligand binding, an intracellular death domain of death receptors recruits adapter proteins which are specific to their receptors such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) [57]. The complex which is consisted of ligand, receptor and adapter protein called death-inducing signaling complex (DISC) initiates caspase-8 activation. Caspase-8 acts as an initiator caspase to activate the execution pathway through caspase-3 activation [58].

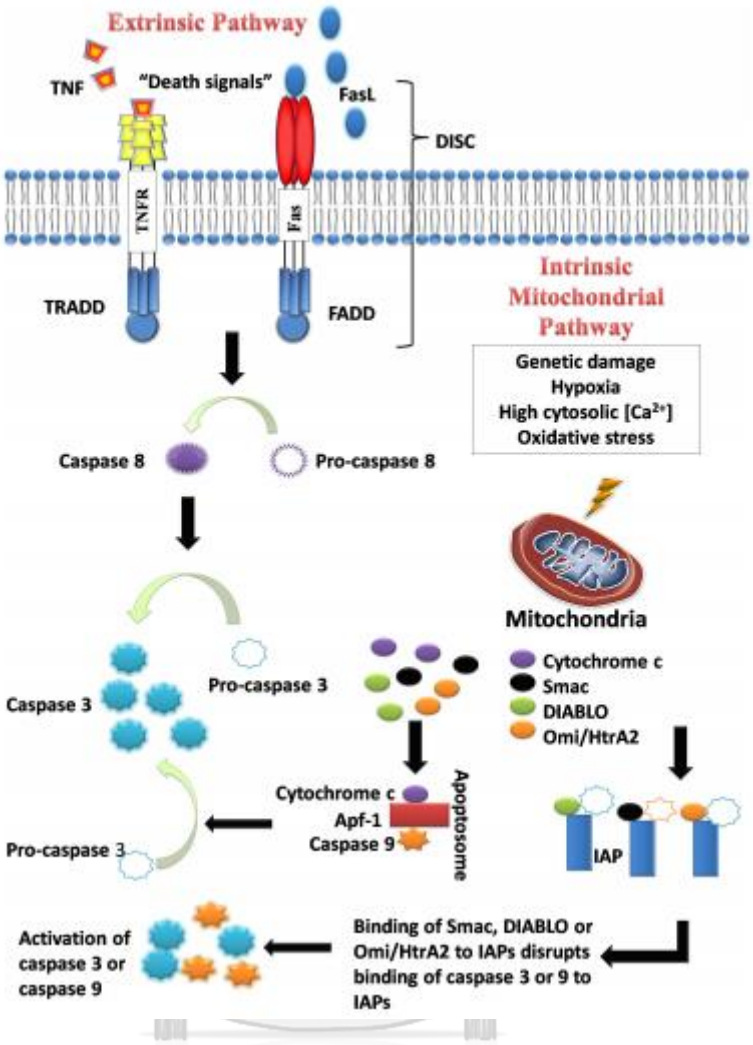


Figure 1. The intrinsic and extrinsic apoptotic pathways [51]

After executioner caspase activation, it leads to induce cleavages of nuclear apoptotic proteins, protein kinases, cytoskeletal proteins, DNA repair proteins, and inhibitory subunits of endonucleases family. Moreover, it also induces changes in the cytoskeleton, cell cycle and signaling pathways, which together contribute to the typical morphological changes in apoptosis [59].

Generally, apoptosis is started by various stresses from either extracellular or intracellular causes. Extracellular signals that can activate apoptosis include loss of growth factors, low oxygen levels (hypoxia), and radiation, while intracellular signals are DNA damage usually caused by chemical reagents, telomere malfunction, and virus infection [60]. Some chemotherapeutic drugs are used to induce intracellular stresses by directly damaging DNA or inducing oxidative stress through ROS generation and finally undergoing apoptosis [61].

Targeted apoptosis for cancer therapy

In cancer, it is known that an evasion of apoptosis is considered as a prominent hallmark. Dysregulation of the apoptotic pathway mostly lead to sustained cell proliferation and enhanced tumor development. Therefore, treatment strategies that can restore the apoptotic signaling pathways may have the potential to eliminate cancer cells [62]. Many anti-cancer drugs including targeted therapeutic agents have the major mechanism based on targeting various aspects of apoptotic related process such as apoptosis-inducers, angiogenesis inhibitors, and signal-transduction inhibitors [2].

Some of upstream targeted therapies act on inhibition of molecules in survival pathways like phosphatidyl inositol 3-kinase (PI3K)/Akt pathway [63] since this pathway has main functions to control multiple cellular processes including cell motility, metabolism, growth, proliferation, and survival and this pathway is the most frequently found dysregulated in cancers. Importantly, the over-activation of essential molecules in the pathway unnecessarily promotes cancer cell survival by apoptosis inhibition [64, 65].

In PI3K/Akt pathway, growth factor receptor protein tyrosine kinases are activated, resulted in autophosphorylation on tyrosine residues. PI3K is recruited by directly binding to phosphotyrosine consensus residues of growth factor receptors. This leads to allosteric activation of the catalytic subunit. The second messenger phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) is produced. The lipid products of PI3K and PIP3 recruit a subset of signaling proteins with pleckstrin homology (PH) domains to the membrane, including Akt [66]. Once activated, Akt mediates the activation and inhibition of several proteins, resulting in cell survival, growth and proliferation. Akt phosphorylates and inhibits pro-apoptotic proteins Bim, Bax, and Bad. Furthermore, Akt also activates mTOR, which in turn activates anti-apoptotic protein Mcl-1 [65]. Taken together, inhibition of this survival pathway can lead to promotion of apoptosis in cancer cells via anti-apoptotic protein inhibition and pro-apoptotic protein activation. Several evidences supported this idea; inhibition of Akt, a crucial component of PI3K/Akt pathway could drive apoptotic process of cancer cells and could slow the progress of tumors *in vivo* [67-69].

Not only survival pathways, but one of the most outstanding targets for cancer therapy is also the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family of proteins (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B). Bcl-2 family proteins are among the most crucial protein group that dominate apoptosis process due to their functions which bind and inactivate BH3-domain of pro-apoptotic proteins. A number of studies have specified Bcl-2 family proteins as critical targets of anti-cancer drugs as well as gene therapy. Besides, anti-apoptotic members of Bcl-2 family especially Mcl-1 is

shown to dominantly negative regulate apoptosis control and responsible to chemotherapeutic resistance [8, 70]. Overexpression of anti-apoptotic proteins in Bcl-2 family can cause disturbance of the ratio between pro- and anti-apoptotic proteins and apoptotic cell death cannot be occurred. Thus, targeting the anti-apoptotic proteins in Bcl-2 family can enhance apoptosis and overcome drug resistance to chemotherapy [3-5].

Bcl-2 family proteins

It is known that chemotherapeutic resistance is the phenomenon implied the lack of capability to response and eliminate cancer cells of chemotherapeutic drugs due to host and tumoral-related factors resulted in failure to activate apoptotic cascade and promote cancer cell survival [71]. To overcome the treatment resistance, the mechanisms that cancer cells manage to evade chemotherapeutic induced cell death and become resistance are conscientiously studied. One of the most important mechanisms is to altered an expression of pro-survival proteins in apoptotic pathway especially Bcl-2 family proteins[8] .

Bcl-2 family proteins are the main regulators of apoptotic process. There are 25 members of the Bcl-2 family of proteins that have been found to localize in mitochondria, smooth endoplasmic reticulum, and perinuclear membranes [72]. Bcl-2 family proteins are classified into 3 main subfamilies based on their functions and structures; anti-apoptotic proteins, pro-apoptotic proteins, and BH-3 only proteins. The anti-apoptotic subfamily proteins which are Bcl-2, Bcl-XL, and Mcl-1, contain all 4 Bcl-2 homology domains (BH1, BH2, BH3, and BH4) and act on suppression of apoptotic mechanism by inserting the BH3 helix of a pro-apoptotic protein into the groove formed by the BH1, BH2 and BH3 helices of an anti-apoptotic protein [73]. For the pro-apoptotic subfamily proteins such as Bax and Bak, they contain Bcl-2 homology 1-3 domains and are termed “multidomain proteins”. Another pro-apoptotic subfamily proteins such as Bid, Bim, and Bad that contain only BH3 domain are termed “BH3-only” proteins [74] **(Figure 2.)**. Recent evidence has suggested that the survival of human cancers and the severity of malignancy are likely to depend on expression level and function of the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) [9, 75].

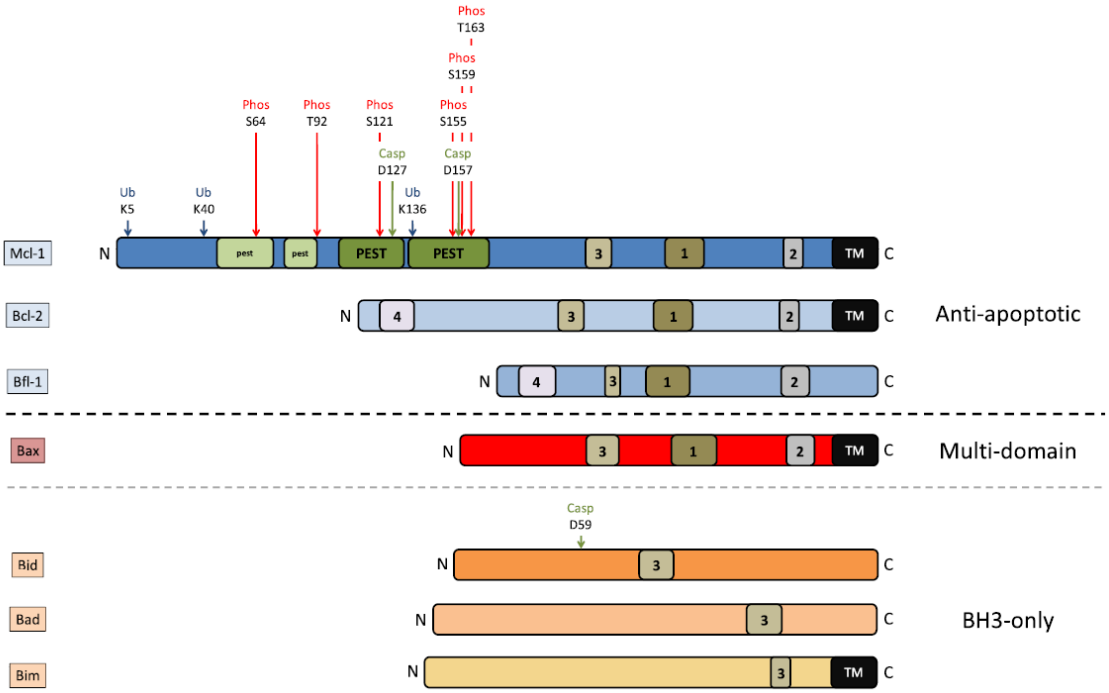


Figure 2. Bcl-2 family proteins. These include the transmembrane domain (TM), the Bcl-2 homology domains (numbered 1–4), PEST sequences (proline, glutamic acid, serine and threonine residues). Sites of post-translational modification include ubiquitination (Ub), caspase cleavage (Casp), and phosphorylation (Phos) [18].

Anti-apoptotic protein Myeloid cell leukemia 1 (Mcl-1)

Myeloid cell leukemia 1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 family proteins and has known to be larger than the other anti-apoptotic proteins due to its large N-terminus containing PEST sequences which is not required for BH3 binding but plays an important role in Mcl-1 regulation and degradation, whereas the other structural and functional homology are BH domains which are the same as other pro-survival proteins with a surface exposed hydrophobic groove for the binding of other BH3-domain containing proteins [18, 73, 74, 76, 77]. **(Figure 2.)**

There are 2 main roles of Mcl-1 in apoptotic process. One is to inhibit apoptosis by binding and sequestering the pro-apoptotic proteins Bak. Bak polymerization by Mcl-1 can be released in 2 conditions; interaction of Mcl-1 with BH3-only proteins and degradation of Mcl-1. BH3-only proteins such as Bim, Bid, Bik, PUMA, and NOXA can disrupt Mcl-1-Bak interaction to displace Bak from Mcl-1, leading to Bak oligomerization and cytochrome c release resulted in apoptosis [78-81]. Another role of Mcl-1 is related to its pro-survival function by heterodimerizing with activator BH3-only proteins including Bid, PUMA and Bim to block them from interacting with Bax. However, in apoptotic condition, NOXA displaces Mcl-1 from these proteins. Thus, Bim, PUMA and Bid can

interact with Bax to cause its insertion into outer mitochondrial membrane, oligomerization, and cytochrome c release [82]. The binding affinity between Mcl-1 and pro-apoptotic family proteins is highest among pro-survival Bcl-2 family proteins. That is the reason why cancer cells use Mcl-1 against extracellular stresses particularly chemotherapeutic drugs [76].

In various types of cancer, Mcl-1 is frequently found amplified or up-regulated due to an increase of its transcription through growth factor receptors especially epidermal growth receptor (EGFR) family members [83]. This up-regulation of Mcl-1 allows cancer cells to survive from death receptor and DNA damage induced apoptosis. Moreover, there are evidences that mRNA levels of Mcl-1 in various types of tumor tissues are higher than other anti-apoptotic proteins in Bcl-2 family when normalized to normal tissues [75]. Not unpredictably, the down-regulation of Mcl-1 expression increases cancer cell sensitivity to standard anticancer drugs, such as etoposide, doxorubicin, and ABT-737 [84]. Furthermore, the reduction of Mcl-1 expression sensitized human osteosarcoma cells to common chemotherapeutic agents in vitro and in xenograft tumors in vivo [85]. In particular, the augmented expression of Mcl-1 reflects poor prognosis and targeting on this protein might be a candidate for cancer therapy.

In lung cancer, Mcl-1 has been shown to be a promising targets of drug action. Not only its increased expression is critical for oncogenesis, cancer progression, and metastasis, but this protein also involves in conferring chemotherapeutic drug resistance [8, 10, 12, 15, 86]. Several evidences support this concept since Mcl-1 was found to relate with aggressive lung cancer as presented **Table 1.** Clinical studies showed high expression level of Mcl-1 which related to worse prediction in lung cancer patients [15, 87]. Previous research reported that NSCLC cells exhibited high levels of Mcl-1, and the inhibition of such a protein by siRNA could potently mediate NSCLC cell apoptosis. Additionally, evidence suggested that in NSCLC, Mcl-1 inhibition showed superior potentials for cancer therapeutic effect when compared with Bcl-xl inhibition [86]. Furthermore, Mcl-1 overexpression has been shown to augment tumor progression by inhibiting Myc-induced apoptosis in a mouse lung adenocarcinoma model [10]. Therefore, it can be concluded that to overcome an aggressive lung cancer with a metastatic and chemoresistant abilities, targeting on Mcl-1 is an interested strategy.

Protein	Aggressive behaviors	Descriptions	Ref.
Mcl-1	Metastasis	Mcl-1 was associated with TGF-β-induced epithelial–mesenchymal transition (EMT) in NSCLC.	92

		Mcl-1 was associated with the migration and invasion in lung adenocarcinoma.	90
		An interaction between Mcl-1 and voltage-dependent anion channel (VDAC) promoted lung cancer cell migration by a mechanism that involves Ca2+-dependent ROS production in NSCLC cells.	89
	Chemotherapeutic resistance	Down-regulation of Mcl-1 by siRNA or inhibition of Mcl-1 with Bcl-2 inhibitor could overcome the EMT-associated chemo-resistance in A549 cells.	92
		Mcl-1 mediated cisplatin resistance in lung cancer.	93
		Akt-mediated expression of Mcl-1 conferred acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells.	94
		Degradation of Mcl-1 by bufalin could reversed acquired resistance to osimertinib in EGFR-mutant lung cancer.	88
		Targeting Mcl-1 could induce apoptosis and sensitize NSCLC cells to apoptosis induced by cytotoxic agents.	91
		Mcl-1 mediated TWEAK/Fn14-induced therapeutic resistance in NSCLC.	95

Table 1. Mcl-1 related aggressive lung cancer. [88-95]

Possible ways for targeting Mcl-1 were established including antisense-mediated inhibition which reduces targeted mRNA expression levels, peptide inhibitors or so called BH3 mimetics that can bind to these proteins and impair their functions, and small molecule inhibitors which bind to and block BH3 binding groove. The small molecule inhibitors are more attractive treatment compared to other strategies because of their lower molecular weight and lower manufacturing costs [8]. At present, there are many small-molecule compounds targeting on Mcl-1 currently under different clinical stages of development for cancer treatment as seen in **Table 2.** Particularly, AT101 is also proven as a Mcl-1 inhibitor in phase II clinical trial [96]. Thereby, there is a possibility to develop Mcl-1 targeted agent.

Phase	Type	Small molecule inhibitor	Disease

Phase I	Mcl-1 protein inhibitor	AMG-176	Multiple myeloma
		AT-101	Prostate cancer, non-small cell lung cancer (combined with chemotherapeutic drugs)
		AZD-5991	Hematological cancer
		S-64315 (MIK-665)	Diffuse large B-cell lymphoma, multiple myeloma
		S-64315 (MIK-665)	Myelodysplastic syndrome, acute myeloid leukemia (AML)
Phase II	Mcl-1 protein inhibitor	AT-101	Prostate cancer, B cells' malignancies, advanced adrenocortical carcinoma, small cell lung cancer, non-small cell lung cancer (combined with chemotherapeutic drugs)

Table 2. Small molecules targeting Mcl-1 in clinical development [96, 97]

However, it has been shown that anti-apoptotic potency of Bcl-2 family proteins primarily relies on their stability [16]. Mcl-1 is considerably a very unstable protein compared to other Bcl-2 family proteins including Bcl-2, and the degradation of Mcl-1 can be induced by certain anti-cancer agents [17, 98-100]. Intracellular Mcl-1 level is tightly regulated by the ubiquitin-proteasomal degradation mechanism which is depended on specifically interaction between these proteins and ubiquitin ligase E3 contained the BH3 domain [101, 102]. The stabilization and degradation of Mcl-1 are found to be regulated by phosphorylation at PEST region, the area rich in putative phosphorylation sites [18]. Those phosphoresidues that have an influence in Mcl-1 stability have been confirmed as following; (**Figure 3**)

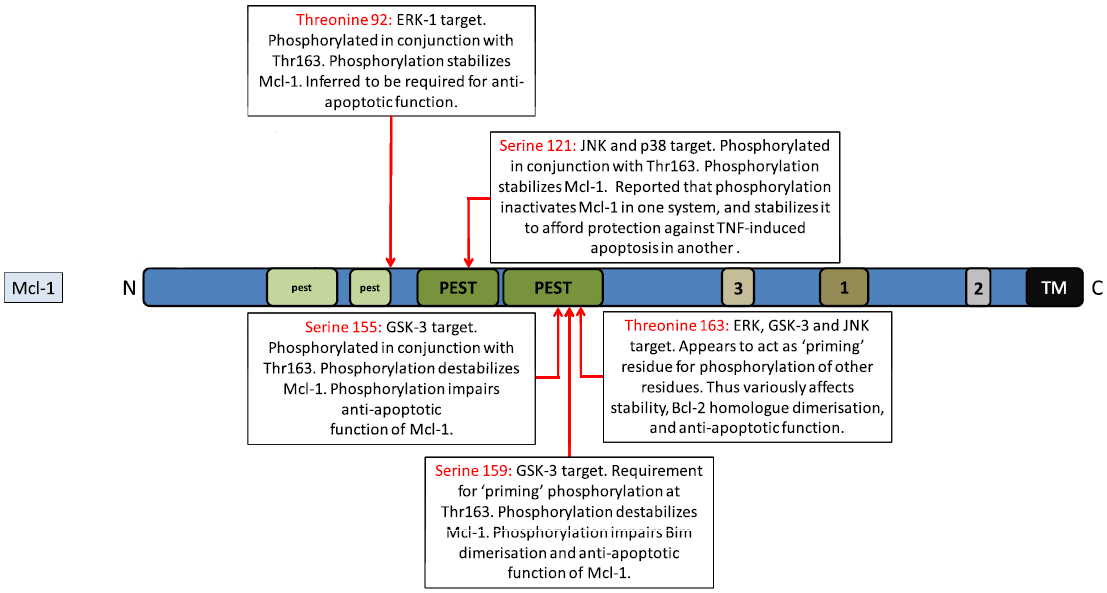


Figure 3. Phosphoresidues of Mcl-1 [18]

- Threonine 92

  This residue and threonine 163 are simultaneously phosphorylated by extracellular signal-regulated kinase (ERK) which can stabilizes Mcl-1. The phosphorylation was found to be required for Mcl-1 anti-apoptotic function [103].

- Serine 121

  This residue is c-Jun N-terminal kinase (JNK) and p38 target for phosphorylation. The phosphorylation of serine 121, in conjunction with threonine 163 was found to increase Mcl-1 stability and protect Mcl-1 from apoptosis induced by tumor necrosis factor-α (TNFα) [104].

- Serine 155

  Phosphorylation of this residue by glycogen synthase kinase (GSK) 3 in conjunction with threonine 163 was found to destabilize Mcl-1 and impair Mcl-1 anti-apoptotic function [105].

- Serine 159

  This residue is phosphorylated by GSK-3 in requirement of the threonine 163 phosphorylation by JNK. The phosphorylation can destabilize Mcl-1 and inhibit an interaction between Mcl-1 and Bim [105, 106]

- Threonine 163

  This residue is the most highly investigated phosphoresidues on Mcl-1 sequence as a MAPK phosphorylation sequence conserved in many species. It is targeted by ERK, JNK, and GSK-3. The co-phosphorylation of threonine 163 and threonine 92 by ERK

could stabilize Mcl-1 whereas the co-phosphorylation of threonine 163, serine 155, and serine 159 by GSK-3 destabilizes Mcl-1. Still, phosphorylation of this residue alone by JNK is in contradiction. [103-105, 107]

From this information, it can be concluded that MAPK proteins (ERK, JNK, and p38) phosphorylate and induce Mcl-1 stabilization. These proteins bind to Mcl-1 at specific docking motif called D-motif which is consisted of positively charged and hydrophobic residues. Therefore, binding to D-motif with specific compounds might prevent the Mcl-1 stabilization and finally induce its degradation. Taken together, the novel compounds with potent activity in eliminating Mcl-1 in lung cancer cells through this mechanism are of interest as a good candidate for targeted therapy.

In conclusion, one of the most effective strategies to treat lung cancer is to evaluate a new compound which has anti-cancer activities through apoptosis induction by essential mechanism such as targeting anti-apoptotic proteins especially Bcl-2 and/or Mcl-1 direct or indirect inhibition of survival pathways in without or less effect to normal cells.

Marine derived products

Seventy-five percentage of earth's surface is covered by water but research into the pharmacology of marine organisms is limited and most of it still remains unexplored. Marine environment represents countless and diverse resource of many potent bioactive compounds which are recently used for new drug developments to treat major diseases such as infection and cancer. Recently, antimicrobial, antitumor, and anti-inflammatory effects have been reported. The number of scientific publications about marine compounds displays an upward trend in the last twenty years, especially in the field of cancer. From many researches, the marine environment has produced a large number of strongly potent agents which are able to inhibit the growth of human cancer cells and exhibit anti-cancer activities [19]. It is found that substances from marine environment have structural and chemical features generally not found in terrestrial natural products. Their structures have more complexity and diversity. Thus, these marine-derived molecules are capable of interacting with numerous biomolecular targets to either inhibit or promote specific biological functions against various types of cancer cells. [35].

To date, five drugs isolated from marine organisms (cytarabine, ziconotide, trabectedin, eribulin mesylate, and brentuximab) have been approved by FDA and EMEA for use as pharmaceutical drugs in cancer treatment. Cytarabine and eribulin mesylate, drugs collected from marine sponge have been approved by FDA in 1969 and 2010 respectively. Cytarabine acts as DNA polymerase inhibitor, whereas eribulin mesylate is microtubule interfering agent. In 2004, ziconotide

isolated from cone snail has also been approved by FDA for the usage in relieving cancer pain by modulation of neuronal calcium channels. Trabectedin, an EMEA approved drug in 2007, comes from marine tunicate with the mechanism that can inhibit cancer cell growth and affect tumor microenvironment. Brentuximab, a drug from mollusk, which is an antibody-drug conjugation has been approved in 2011. In addition, there are lots of marine drugs still in clinical trials such as plitidepsin from tunicate and gemcitabine from marine sponge [19]. For this reason, it can be concluded that marine environment has abundant source of drugs that could be potential candidates for cancer treatment.

Renieramycins

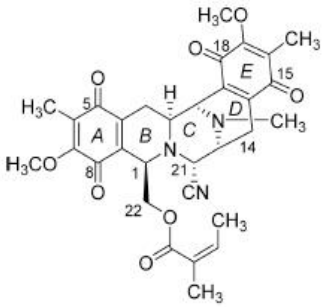
One of marine derived products is renieramycins. Renieramycins are alkaloids in tetrahydroisoquinoline family proteins [20] which are derived from marine natural products found in various marine organisms, including sponges in the genera *Reniera* [21, 22], *Xestospongia* [23-27], *Cribrochalina* [28, 29], and *Neopetrosia* [30]. However, they are unstable and decomposed after extraction and isolation. Therefore, a very unstable amino alcohol functionality at C-21 in their structure is converted into stable a-aminonitrile compounds by pretreatment with potassium cyanide [36]. The most prominent compound in this group is renieramycin M isolated from the potassium cyanide pretreated methanolic extract of the blue sponge *Xestospongia* sp., which has been collected in Thailand and the Philippines [26, 27]. Besides from renieramycin M, renieramycins A-Y are isolated from many species of sponges and become target molecules for synthetic studies and biological researches as the anticancer agents [108-118]. Many evidences showed that renieramycins had a strong cytotoxicity against various cancer cells, including breast (T47D), colon (HCT116 and DLD1), lung (QG56 and H460), pancreatic (AsPC1), and prostate (DU145) human carcinomas [23, 26, 36, 114]

In 2009, Charupant found that renieramycin M and their derivatives had anti-proliferative activities against breast (MDA-MB-435), and colon (HCT116) cancer cells with a very low IC50 [119]. In 2011, renieramycin M was found to induce non-small cell lung cancer cells (H (460apoptosis through p53-dependent pathway which down-regulated anti-apoptotic Mcl-1 and Bcl-2 proteins and it might inhibit progression and metastasis of lung cancer [31]. In 2016, bishydroquinone renieramycin M (HQ-RM), a modificated form of renieramycin M, had highly potent anti-cancer activity, greater than its parental RM. From this study, it had cytotoxicity and apoptosis induction in non-small cell lung cells by increase of pro-apoptotic protein Bax and decrease of anti-apoptotic protein Bcl-2 and Mcl-1 [33]. In the same year, renieramycin M was found to induce anoikis in NSCLC cells by suppressing survival proteins p-ERK and p-AKT and the anti-apoptotic proteins Bcl-

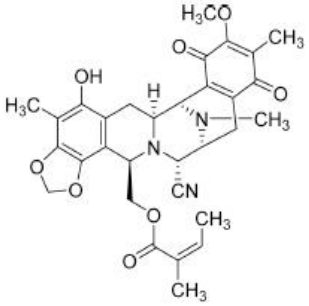
2 and Mcl-1 [32]. In 2017, several analogues modificated from RM were shown a cytotoxicity against non-small cell lung cancer cells (H460 and H292) [35]. Moreover, renieramycin M showed anti-metastatic effects by suppressing lung cancer stem cell-like phenotypes in non-small cell lung cancer cells (H460) [120]. Besides from RM, there is evidence that renieramycin G and its stereoisomers also had cytotoxicity among colon, cervical, non-small cell lung, and bladder cancer cells [121, 122]. In this research, one of renieramycin family, renieramycin T, were studied.

Renieramycin T (RT)

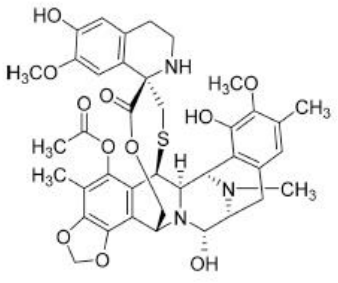
Renieramycin T (RT) is the renieramycin-related compound isolated from the blue sponge *Xestospongia* sp. that was pretreated with potassium cyanide in 2009 [36]. Normally, the A and E rings in the most renieramycin structures are in quinone form, but RT has a substituted aromatic A ring, which is the same as ecteinascidins, another tetrahydroisoquinoline compounds isolated from marine tunicates [131-123] **(Figure 4.).** Thus, RT is the renieramycin–ecteinascidin hybrids in the tetrahydroisoquinoline alkaloid family. It showed strong cytotoxicity in several cancer cells including colon (HCT116), prostate (DU145) [34], non-small cell lung (H292, H460 and QG56) [35], breast (T47D) and pancreatic (AsPC1) cancers [36] with a very low IC50 but less cytotoxicity than renieramycin M. Recently, the research in 2019 revealed that 5-*O*-acetyl-renieramycin T, a modification form of RT, induced lung cancer cell death via the induction of p53-dependent apoptosis and enhanced the death of cancer stem cells (CSCs) as represented by the CSC markers CD44 and CD133. Moreover, 5-*O*-acetyl-renieramycin T was also found to sensitize cisplatin-mediated apoptosis in the lung cancer cells [132]. However, the mechanisms of anticancer activities of RT in lung cancer cells had not been elucidated yet. Furthermore, some parts of RT structure are aromatic ring and cyanide which have an ability to bind with positive charge amino acids [37-39] that are component of MAPK docking motif in Mcl-1 sequence. Thus, it is possible for RT to have an ability on stabilization of Mcl-1 protein. This is the reason why RT was studied in this research.



renieramycin M



renieramycin T



ecteinascidin 743

Figure 4. Structures of renieramycin M, T and ecteinascidin 743

Before beginning the experiments of RT, primary lung cancer cells, recently derived from NSCLC patients in Chulalongkorn hospital were established with a drug sensitivity test to prove the chemotherapeutic resistant profiles. The protocol in collecting the patient-derived lung cancer cell was in accordance with the principles of World Medical Association Declaration of Helsinki and was received informed consent from all patients. To depict the reason why drug sensitivity test on primary lung cancer cells is needed, the literature review on primary cancer cells is provided as following.

Primary cancer cells and their importance for drug sensitivity test

From the previous issue mentioned the limited success on cancer therapy, multidrug resistant relapse of cancer is suggested to be the major cause of therapeutic failure, tumor recurrence, and disease progression. Although effective targeted therapies are widely used, an acquired resistance is eventually developed via various mechanisms within 1 to 2 years [133]. Cancer cells can develop a compensatory signaling pathway that activates the key downstream of proliferation and survival signals even inhibition the original signal [134]. Despite the new drug development and clinical trial research in every year in case to achieve a better objective clinical response, only few drugs have been approved by FDA for applying on patients and it is inadequate for overcoming the drug resistant [135]. Thus, an efficiency platform for pre-clinical study are developed with a benefit for further clinical use and beyond [136].

Preclinical studies that have an outstanding role on cancer biology throughout the screening of anti-cancer reagent for drug development is cancer cell line approach. Still, the clinical correlation is limited due to an accumulation of genetic aberrations and loss of heterogeneity occurred from an increasing of passage numbers [137, 138]. Additionally, using cancer cells under *in vitro* condition is not accurately represent a clinical status [139]. The genetic heterogeneity of patients causes a variation in response of therapeutic drug. Therefore, it is difficult to understand the relationship between genetic diversities of cancer and drug sensitivity in all patients from *in vitro* cancer cell experiments. For this reason, methods of generating and culturing primary cancer cells are concerned with an aim to establish a proper way for cancer management in clinic [140-142].

It has been reported that culturing primary cancer cells derived from patient tissue may conquer the limitation of cancer phenotype and drug sensitivity prediction [143]. The functional *in vitro* drug sensitivity testing concept has been inspected in both patient-derived xenografts and primary cell culture model [144]. Moreover, research in hematological malignancies revealed that

the results of a functional testing in a large number of primary cancer cells could be translated to clinical approaches for measurement of cancer response and correlating them to patient responses [145]. Another study was focused on overcoming drug resistance by using cancer cell derived from NSCLC patients after resistance occurred and performing a drug sensitivity test. This strategy was found to be successful for identifying suitable drugs that inhibit the growth of resistant cancer cells [146].

To be concluded, from the fact that primary cancer cells have more genetic heterogeneity which is found to relate with drug resistance [147] than cancer cell lines, using primary cancer cells on drug sensitivity or anti-cancer agent testing is an effective option for further clinical approaches. Besides, primary cancer cells in this study were received from advanced-stage or recurrent NSCLC patients who had been treated with chemotherapeutic drugs for a prolonged period, so an acquired-resistance or genetic aberration might be occurred. Therefore, it could be said that the drug or anti-cancer agent that exert a potent cytotoxicity on resistant primary cancer cells have a tendency to achieve an aim for effective cancer treatment in clinical application.

After testing the drug sensitivity and receiving the drug resistant profiles in primary lung cancer cells, effects of RT on cytotoxicity in several lung cancer cells through apoptosis induction and other mechanism including survival pathway (PI3K/Akt pathway) were then evaluated. Importantly, effects of RT on Mcl-1 protein inhibition were observed as we mentioned that Mcl-1 is the main anti-apoptotic protein in Bcl-2 family proteins and frequently found amplified or overexpressed. In particular, the augmented expression of Mcl-1 reflects poor prognosis of many malignancies including lung cancer. Targeting on this protein is shown to be one of the most interested mechanism for anti-cancer agents.

In the experiments, the known NSCLC cells (H460, H292, H23, and A549) and the drug resistant primary lung cancer cells (ELC12, ELC16, ELC17, and ELC20), recently derived from patients were applied. Furthermore, when the investigation of RT mechanism was completed, RT was used as a lead compound to synthesize some of RT analogs or simplified right-half model compounds; TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55 as seen in **Figure 5.** by Prof. Masashi Yokoya for investigation structure-activity relationships (SARs) and subsequent SAR-directed optimization. The SAR is depending on the specific chemical structure that reacts to the active site of drug action. In case of Mcl-1 elimination, the Mcl-1 protein was analyzed for its amino acid sequence that critical for the protein stability and found that the phosphorylation at threonine 92, threonine 163, and serine 121 of the protein molecule by MAPK was shown to be important for the stability of Mcl-1 [18]. Therefore, the design drug should be able to bind at MAPK binding pocket of Mcl-1 protein. We have proposed that the structure of RT at the right-half part are

essential for its interaction with the Mcl-1 molecule. As RT has a complex structure composed of several chemical moieties, understanding the structure–activity relationships (SARs) is a necessity for identification of the active moieties that are critical for drug action and that hold promise to increase drug precision and potency.

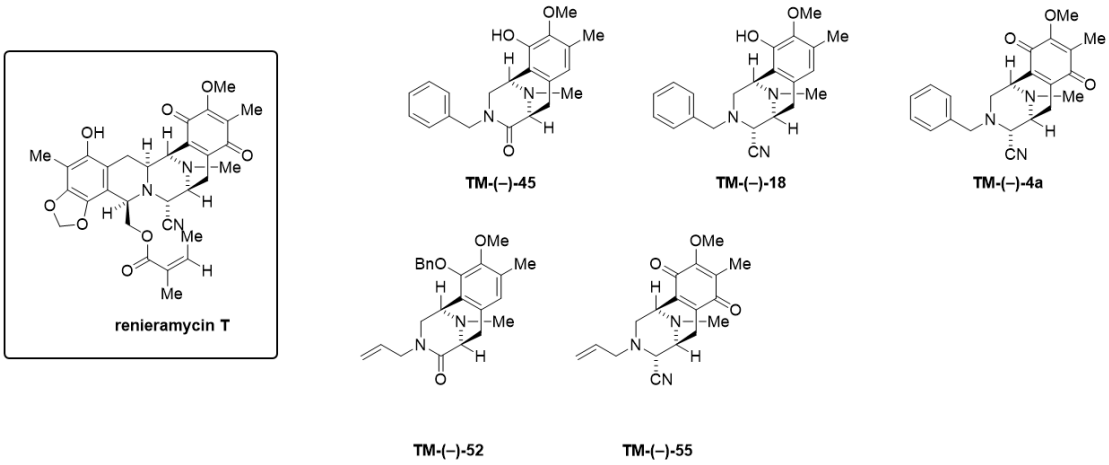


Figure 5. Structures of RT and simplified right-half model compounds; TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55

Structure-activity relationship (SAR)

It is known that the structure-activity relationship or SAR is the relationship between the chemical structure of reagent molecule and its bioactivity. The biological effects are depended on its molecular structure [148]. Over a decade, SAR has an important role in several aspects of drug discovery since it is used for primary screening the chemical groups which is responsible for bringing a target biological effect in an organism [149]. Studying the SAR concept is started from identifying an existence of SAR in collected molecules and their associated activities and trying to elucidate the detail of SAR [150]. This information also describes the critical parts of molecules to be exploited for drug action as well as can help to increase the potency of the initially detected activity and reduce toxicity [149]. Moreover, SAR is used to examine the alteration in pharmacological properties by performing minor changes in the molecule. This allows modification and development of the bioactive compound series involved optimizing physiochemical and biological properties by changing its chemical structure [149]. In conclusion, the SARs of a bioactive compounds is very useful information that could facilitate the development of drugs.

Several evidences according to SAR concept are reported in bioactive compounds including the group of renieramycins. For example, it was found that different functional group

among structures of renieramycins caused various cytotoxicity responses in colon cancer (HCT116) and lung cancer (QG56) cells [23]. For renieramycin G stereoisomers, it was revealed that L-shaped topological configuration of the pentacyclic skeleton was important for their cytotoxicity effects in colon cancer (HCT-8), cervical cancer (HeLa), lung cancer (A549), and gastric cancer (BGC-803) cells [121]. In renieramycin M study, replacement of its quinone by 5-*O*-acetylhydroquinone could eliminate an accidental necrosis induction effect from reactive oxygen species (ROS) mechanism, whereas an apoptosis induction effect was preserved [151]. Moreover, the report of hydroquinone 5-*O*-monoester renieramycin M analogs was shown an improved cytotoxicity on NSCLC (H460 and H292) cells [35]. Even in RT, the cytotoxicity effects of compounds occurred during RT synthesis processes were vary depended on their functional groups [34]. Recently, a protocol for synthesis of renieramycin T right-half models was developed with an aim to study SAR. Still, the mechanism related to structures and effects on lung cancer cells were unexplored [152]. Thus, Examination the SARs on RT in lung cancer cells were mentioned in this study.

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CHAPTER III

Feasibility Technique of Low-Passage In Vitro Drug Sensitivity Testing of Malignant Pleural Effusion from Advanced-Stage Non-Small Cell Lung Cancer for Prediction of Clinical Outcome

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Feasibility Technique of Low-Passage *In Vitro* Drug Sensitivity Testing of Malignant Pleural Effusion from Advanced-Stage Non-Small Cell Lung Cancer for Prediction of Clinical Outcome

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Key Words: In vitro drug sensitivity testing, precision medicine, malignant pleural effusion, advanced stage non-small cell lung cancer.

Abstract

Background/Aim: Individualized proper chemotherapy using in vitro drug sensitivity testing has been proposed a novel therapeutic modality and shown to have better efficacy than empiric chemotherapy .However, issues around establishing a patient-derived cell culture or xenograft, the timing of the testing obtained, and the validity of testing represent major limitations to translating the use of such a technique to clinical practice .Materials and Methods: In this study,

we assessed the feasibility of an in vitro drug sensitivity technique for testing malignant pleural effusion from advanced stage non-small cell lung cancer .Results: Our technique was able to produce a turnaround time for in vitro drug sensitivity testing of less than 1 week, with a success rate of more than 90 %of cases .Correlated with the individual clinical outcome, using the area under the dose response curve)AUC (could define the level of in vitro drug sensitivity as: responsive)AUC >0.25(, intermediate response) 0.1≤ AUC ≤0.25(, or resistance)AUC <0.1 .(Conclusion: The data obtained from this method of drug testing was correlated with the clinical outcome. The present drug sensitivity evaluation may benefit the development of individual precision chemotherapy.

The response rate from standard platinum-doublet chemotherapy for advanced non-small cell lung cancer)NSCLC (ranges from 25-30 %with a 1-year survival rate of up to 30 %(1) .With the advancement of molecular biomarkers and targeted therapy, the survival of advanced lung cancer patients with sensitizing mutation who receive matched targeted therapy can increase up to 30-36 months (2) .It is potentially noteworthy that defining optimized therapeutic agents for individual patients could be beneficial in terms of treatment efficiency, economical concerns, and for avoidance of unnecessary toxicity .The *in vitro* drug sensitivity concept has been explored with both patient-derived xenografts)PDXs (and as a primary cell culture model .It was claimed that the PDX model is expensive and has a low engraftment rate, and may not give results back in a timely manner to instruct therapeutic decisions .Further, it might not represent the real clinical response due to the lack of interaction between human stromal cells and the immune system (3) . Patient-derived organoids)PDOs (is a model providing another option for drug sensitivity testing but work is still in progress in this area .Among the several *in vitro* drug sensitivity assays that have been developed, the methyl thiazol-diphenyl-tetrazolium bromide) MTT (assay, based on the measurement of mitochondrial enzyme activity, is the most widely used technique due to its simplicity, sensitivity, and reliability (4) .This technique could define the viability of cancer cells after culture with anticancer agents .However, the concentration of anticancer agents and how to define sensitivity or resistance in the clinic have yet to be concluded .Furthermore, the super-therapeutic concentration of anticancer agents that have been used to define the threshold sensitivity might not represent a good clinical response .

Flourishing patient-derived cell culture and passage variation are two other issues of concern .In particular, patient-derived cell cultures can be difficult to sustain, which is a major limitation with *in vitro* drug sensitivity testing .Furthermore, advancements in multiple passages may lead to selection of undesired cell subpopulations and a change in the pattern of genomic characteristic and even cancer phenotypes (5) .Herein, we hypothesize that testing very early

passage or patient-derived primary cells directly from a patient without propagation might be an efficient method to define drug sensitivity .Consequently, the present study was conducted to define the proper *in vitro* drug sensitivity testing of malignant pleural effusion from advanced stage non-small cell lung cancer, and the results were assessed for clinical correlation and potential clinical application.

Materials and Methods

Study population.

A prospective cohort of 11 recurrence or advanced stage non-small cell lung patients with malignant pleural effusion who had been diagnosed at the King Chulalongkorn Memorial Hospital and treated with either standard platinum-doublet chemotherapy or second-line chemotherapy, specifically with docetaxel or pemetrexed, were enrolled in the study .*In vitro* drug sensitivity was tested for 5 agents, namely gemcitabine, pemetrexed, docetaxel, vinorelbine, and erlotinib, which were obtained from Sigma-Aldrich .We chose these agents to correlate with the patients 'history of prior or potential further subsequent chemotherapy .The treatment decision, objective response rate, and progression-free survival according to treatment were determined by the physicians as standard practice through the patients 'medical records .The response rate was defined as the maximum response of the treatment, referring to the RECIST v1.1 criterion (6). In brief, complete response)CR (referred to the disappearance of the target and non-target lesion, partial response)PR (referred to a decrease in the target lesion of more than 30%, progressive disease)PD (referred to an increase in the target lesions of at least 20 %or the development of a new lesion, and stable disease)SD (referred to a lesion that was not PD or PR .Progression-free survival of a particular treatment was defined as the duration since the start of such treatment to progression of the disease .The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand)IRB 365/62 .(Written informed consent was obtained from all the participants.

Protocol for cancer cell preparation from malignant pleural effusion.

The study aimed to determine *in vitro* drug sensitivity in isolated patient-derived primary lung cancer cells directly without cultivation .Primary cancer cells were obtained from pleural effusion) 500-1000 ml (by thoracentesis and collected aseptically in heparinized .Samples were centrifuged at 300 g for 10 min, at 4°C, and cell pellet was resuspended in 10 %FCS-RPMI)Invitrogen.(Viability was determined by the Trypan Blue exclusion dye .After a harvesting and 2 rounds of washing, the cells were subjected to drug sensitivity testing .

In vitro drug sensitivity testing)DST(.

For the cytotoxicity assay, approximately 0.8-1.5 x 10^4 living cells determined by Trypan Blue exclusion assay were seeded in 1 well of 96-well plates .The cultures were then incubated at 37°C in humidified atmosphere containing 5 %CO_2 .After cells adhering, cells were immediately treated with various concentrations of drugs for 24 h in at least triplicate fashion .The chemotherapeutic concentrations were calculated based on recommended therapeutic dose and plasma concentration of each drug (7) following by justified using human conversion factor and human blood volume (8) .The cancer cells were treated with various concentrations of the drug form 0.5-4-fold .The calculated concentrations of each drug are presented in Table I .Cells were incubated with 500 μg/ml of MTT at 37°C for 4 h and the formazan crystals were solubilized in 100 μl DMSO and measured at wavelength 570 nm with microplate reader) Anthros, Durham, NC, USA .(Relative cell viability was calculated by dividing the absorbance of the treated cells by that of the control cells .Untreated cells were visualized randomly using light microscope) Olympus IX5, Tokyo, Japan.(

Definition of in vitro drug sensitivity testing results.

We calculated AUC (Area under the dose response curve); represent response area (1 - vitality), which could capture the efficacy and potency of drug to represent particular drug sensitivity better than either IC50 (Inhibitory concentration of compound that reaches 50% reduction in cell viability) or EC50 (Inhibitory concentration of compound that reach 50% maximum reduction in cell viability) (9) . High AUC implying high sensitivity to the drug. In this study, we defined drug sensitivity by correlated with clinical outcome; resistance referred for AUC < 0.10, Intermediate response referred for AUC range (0.01-0.25 (and responsive referred for AUC > 0.25 . The AUC calculation was done using PharmacoGx R package (10) .

Table I. *Concentration of chemotherapeutic agents in every fold)0.5–4 (that had been conducted in the in vitro testing for chemotherapeutic sensitivity .One-fold chemical concentration refers to the average plasma concentration of the recommended dose for lung cancer treatment.*

Chemotherapeutic agent	Concentration in each fold)μg/ml(			
	0.5	1	2	4
Gemcitabine	2.70	5.40	10.80	21.60
Pemetrexed	1.35	2.70	5.40	10.80

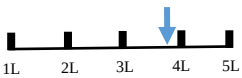
Docetaxel	0.27	0.54	1.08	2.16
Vinorelbine	0.08	0.16	0.32	0.64
Erlotinib	0.07	0.14	0.27	0.54

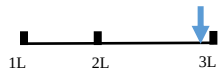
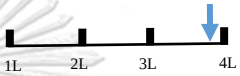
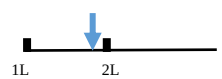
Results

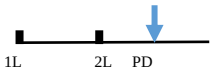
Individual in vitro drug sensitivity testing and clinical correlation.

An *in vitro* drug sensitivity assay for five agents was successfully performed with 11 pleural effusions)>90(%, with data obtained within 1 week from the time of collection .However, in only 3 cases did the results from the *in vitro* drug sensitivity returned to the provided physician influence their decision making .The actual dose response curve is shown in Figure 1 .Table II presents information on the patients according to the treatment and the condition of the 11 successfully performed *in vitro* drug sensitivity patients, and further, brief outlines of each case are given below .

Table II. *Clinical characteristic of advanced/recurrent non-small cell lung cancer patients retrieved for malignant pleural effusion collection)MPE (and conduct low-passage cell culture in vitro drug sensitivity testing .Time point of specimen collection)blue arrow (is shown related to the provided treatment .Outcome of treatment is represented by the maximal)Max (responses, comprising PR)partial response(, SD)stable disease(, PD)progression of disease (by the provided physician and progression-free survival)PFS.(*

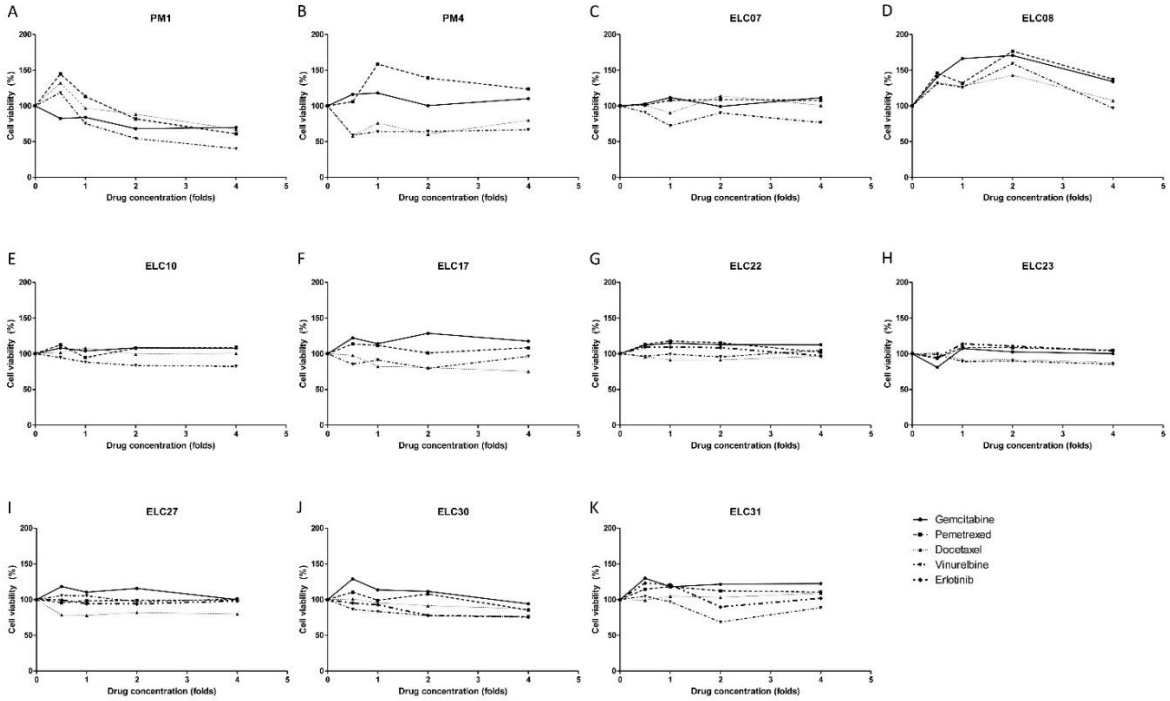
Study ID	Compound	AUC	Prediction	MPE time point collection	Information (treatment; Max response; PFS)
PM1	Gemcitabine Pemetrexed Docetaxel Vinorelbine	0.24 0.12 0.09 0.33	Intermediate Intermediate Resistance Responsive		1L Erlotinib; PR;125wk 2L Carboplatin/Pemetrexed; PR; 33wk 3L Docetaxel; PD; 8wk 4L Gemcitabine; SD; 12 wk
PM4	Gemcitabine Pemetrexed Docetaxel Vinorelbine	0 0 0.31 0.37	Resistance Resistance Responsive Responsive		1L Carboplatin/gemcitabine; PR; 37wk 2L Docetaxel; SD; 51wk

					3L Erlotinib; PR; 89wk 4L Pemetrexed; PD; 2wk 5L Osimertinib; PR; 22 wk& ongoing
ELC07	Gemcitabine Pemetrexed Docetaxel Vinorelbine	0 0 0 0.25	Resistance Resistance Resistance Responsive		1L Carboplatin/Pemetrexed; PD; 10wk 2L Docetaxel; PR; 25wk 3L Vinorelbine; intolerate
ELC08	Gemcitabine Pemetrexed Docetaxel Vinorelbine	0 0 0 0.00 8	Resistance Resistance Resistance Resistance		1L Carboplatin/Pemetrexed; SD; 27wk 2L Docetaxel; PD; 10wk 3L Erlotinib; SD; 17wk 4L Atezolizumab; NA
ELC10	Gemcitabine Pemetrexed Docetaxel Vinorelbine	0 0 0 0.16	Resistance Resistance Resistance Intermediate		1L Gefitinib; SD; 20wk 2L Carboplatin/Gemcitabine; PR; 11wk 3L Pemetrexed; intolerate
ELC23	Gemcitabine Pemetrexed Docetaxel Vinorelbine Erlotinib	0.01 0 0.09 0.08 0	Resistance Resistance Resistance Resistance Resistance		1L Carboplatin/Pemetrexed; PR; 23 wk
ELC17	Gemcitabine Pemetrexed Docetaxel Vinorelbine	0 0 0.17 0.12	Resistance Resistance Intermediate Intermediate		1L Carboplatin/Paclitaxel/Avastin; PD; 11 wk
ELC22	Gemcitabine Pemetrexed Docetaxel Vinorelbine Erlotinib	0 0 0.05 0 0	Resistance Resistance Resistance Resistance Resistance		1L Carboplatin/Paclitaxel; PD; 6 wk 2L Atezolizumab; ongoing

ELC27	Gemcitabine Pemetrexed Docetaxel Vinorelbine Erlotinib	0 0 0.21 0 0.05	Resistance Resistance Intermediate Resistance Resistance		1L Gefitinib; SD, 32 wk 2L Carboplatin/paclitaxel; PD; 11 wk
ELC30	Gemcitabine Pemetrexed Docetaxel Vinorelbine Erlotinib	0.01 0.01 0.03 0.18 0.21	Resistance Resistance Resistance Intermediate Intermediate		1L Carboplatin/gemcitabine; SD; 44 wk 2L Docetaxel; PR; 35 wk 3L Carboplatin/paclitaxel; 2 cycle & ongoing
ELC31	Gemcitabine Pemetrexed Docetaxel Vinorelbine Erlotinib	0 0 0 0.15 0	Resistance Resistance Resistance Intermediate Resistance		1L Carboplatin/paclitaxel; SD; 64 wk 2L Docetaxel; PD; 15 wk

PFS: Progression-free survival defined as time from start treatment to progression of that compound; Max: maximal response of treatment (CR, PR, SD, PD) was assessed by provided physician after 2-3 cycle of chemotherapy by using RECIST v.1.1.

Figure 1. *Cytotoxic effect of chemotherapy (A-K) in cancer cells treated with various concentrations of drugs (0-4 folds) for 24 h, or the solvent, that was used as a control, and then cell viability was determined by the MTT assay, relative to the viability of untreated cells set as 100%.*



Case PM1 :

A 64-year-old male adenocarcinoma patient who had *EGFR* exon 19 deletion .He had quite long-term disease control from 2L)second-line(platinum-double chemotherapy)carboplatin/paclitaxel .(However, at the time of disease progression after 3L)third-line (docetaxel, pleural fluid had been collected and indicated an *in vitro* sensitivity pattern of resistance to docetaxel)AUC=0.09 .(Subsequent treatment in this patient involved gemcitabine .*In vitro* drug sensitivity showed intermediate activity on gemcitabine)AUC=0.24 .(He had a stable disease from this agent and continued for 5 cycles before the disease became uncontrolled .

Case PM4 :

A 69-year-old female who had very long disease control after completing 6 cycles) 3-week intervals per cycle (of 2L docetaxel for up to 51 weeks .Her clinical response was correlated with an *in vitro* modest response to docetaxel)AUC=0.31 .(Molecular testing at the time of disease progression on 3L erlotinib revealed *EGFR* L858R T790M, but she could not afford osimertinib at

that time .Consequently, 4L pemetrexed was provided without a clinical response consistent with *in vitro* sensitivity, which indicated a resistance to pemetrexed) AUC=0 .(

Case ELC07 :

A 65-year-old female for whom pleural effusion had been collected after progression on docetaxel and revealed a modest response only to vinorelbine)AUC=0.25 .(Even though vinorelbine is not the standard chemotherapy regimen after docetaxel failure, the physician decided to use this agent according to the *in vitro* sensitivity testing results .However, due to poor performance status, this patient could not tolerate chemotherapy .Only a 2-week interval of vinorelbine 25 mg/m^2 was provided .She then had a urinary tract infection, which interrupted treatment, before progression of the disease .

Case ELC08 :

An 82-year-old male for whom pleural effusion had been collected after 3L erlotinib .*In vitro* drug sensitivity testing revealed pan-resistance to all potential chemotherapy .The patient had received subsequent immunotherapy per standard practice .The patient's symptoms worsened after 1 cycle of treatment and he expired 2 weeks after treatment.

Case ELC10 :

A 71-year-old female who had progression after the 4th cycle of 2L carboplatin and gemcitabine .Pleural effusion had been collected after the 1st cycle of 3L pemetrexed .She had febrile neutropenia and her worsening condition prohibited subsequent chemotherapy .Medical pleurodesis was provided to control her symptoms .However, *in vitro* drug sensitivity revealed resistance to pemetrexed .

Case ELC23 :

An 83-year-old male who had received first-line chemotherapy; carboplatin/pemetrexed for 4 cycles, and maintenance with pemetrexed for 2 cycles, before stopping due to progression of the disease .Radiotherapy was planned to control the symptoms of non-massive hemoptysis, but interrupted due to acute myocardial infarction, but he expired in that admission. Even though this patient did not receive subsequent chemotherapy, *in vitro* drug sensitivity revealed resistance to pemetrexed, consistent with the clinical progression upon treatment .

Case ELC17 :

A 73-year-old female who had disease progression after 4 cycles of first-line carboplatin/paclitaxel and bevacizumab .Due to poor performance, this patient could not receive

subsequent chemotherapy .Intermittent thoracocentesis and the release of pleural effusion was provided .The patient's *in vitro* drug sensitivity profile revealed an intermediate response to docetaxel and vinorelbine .Furthermore, it also showed a resistance pattern to pemetrexed and gemcitabine .

Case ELC22 :

A 63-year-old male adenocarcinoma patient with wild-type *EGFR* testing had disease progression from 3 cycles of first-line carboplatin/paclitaxel. Pleural effusion was collected at the time of disease progression. This patient had a poor performance status, which limited further chemotherapy .Atezolizumab, an anti-PD-L1 antibody, was considered and treatment is currently ongoing .According to molecular testing of wild-type *EGFR*, the *in vitro* drug sensitivity profile revealed a resistance to erlotinib .

Case ELC27:

A 75-year-old female who had long disease control from 1L gefitinib but no response from 2L carboplatin/paclitaxel .The pleural effusion had been collected afterward .*In vitro* drug sensitivity revealed only an intermediate response to docetaxel chemotherapy, but she refused to receive further chemotherapy .

Case ELC30 :

A 41-year-old female who had quite a long duration of disease control after 4 cycles of carboplatin/gemcitabine and 6 cycles of docetaxel .Despite molecular testing revealing *EGFR* exon 19 mutation, she could not afford targeted therapy nor the non-national drug list pemetrexed .The physician considered retreatment with platinum-doublet chemotherapy, with carboplatin/paclitaxel .According to her sensitivity *EGFR* mutation pattern, *in vitro* sensitivity testing revealed an intermediate response to erlotinib) AUC=0.21.(

Case ELC31 :

A 67-year-old female who had disease control after 6 cycles of first-line chemotherapy, with carboplatin and paclitaxel .According to oligometastasis, this patient had consolidative radiation to prolong overall disease controlled .Subsequent chemotherapy, with docetaxel, was provided with a complication of grade IV neutropenia after the 1st cycle of treatment .According to treatment interruption, medical pleurodesis was provided with pleural fluid collection . Subsequent imaging revealed progression of the disease consistent with the *in vitro* sensitivity testing) AUC for docetaxel=0.(

Discussion

The efficacy of individualized chemotherapy selected by *in vitro* drug sensitivity testing has been shown to offer better efficacy than empiric therapy (11, 12) .Several techniques for *in vitro* drug sensitivity testing have been used .However, the significant progression of *in vitro* drug sensitivity testing is still limited and it cannot yet be implemented in routine clinical usage .The feasibility to maintain patient-derived *in vitro* cell cultures, the timing of obtaining the test results, and the validity of interpreting the results from such *in vitro* drug sensitivity testing are major issues in the field .In this study, we demonstrated a feasible technique involving a low-passage cell culture and defined an optimal *in vitro* drug sensitivity dosage)range 1–4 fold (related to the standard therapeutic dosage for that disease .The results of the *in vitro* drug sensitivity were obtained within 1 week and could give a yield of up to 90 .%Incorporated with the clinical response, we could define a cut-off *in vitro* drug sensitivity level as a responsive, intermediate, or resistance pattern. The results from the *in vitro* sensitivity pattern influenced the clinical judgment of the provided physician in terms of the compound that should be used for individual patients .The main purpose of incorporating *in vitro* sensitivity testing in recurrent disease is not to risk patients receiving an ineffective treatment, especially frail elderly patients, and it could probably affect the retreatment strategy by showing the response from a prior treatment compound. Furthermore, the use of the MTT technique to define a growth inhibition of 50) %IC50 (has been widely used to represent drug sensitivity in large-scale pharmacogenomics resources (13) .To obtain such a value, a super-therapeutic concentration of anticancer agents is needed particular for resistance clones, but is not needed in our protocol .We propose using the area under the dose response curve)AUC (to represent the entire cell response curve as a measure of sensitivity to provide adequate information for decision-making about whether a patient has sensitivity or resistance to a particular chemotherapy.

Early-passage patient-derived cell culture is defined by various definitions .An inconsistent genomic landscape from patient-derived cell culture and the patient-derived xenograft model have been reported in cases with more than 10 passages (14) .These inconsistent findings have also been reported in various patient-derived cancer cell cultures between early) lower than p5(and later passage)greater than p10 (cases (15). Nevertheless, an earlier passage)less than p10(patient-derived cell culture retained particular copy number variations, gene mutation, and miRNA expression compared to primary breast cancers (16) .A low-passage cell culture could diminish the *in vitro* influences that might impact the phenotype of the cancer cells to represent the real clinical situation .Moreover, a short culture assay will minimize the variable effects of cell proliferation and cell death over the assay period (6) .We proposed herein the feasibility of an in vitro drug sensitivity

technique that could systematically support investigations of the biology of various cancer types. This assay may have potential clinical applications for predictive chemotherapy selection. Further exploration with treatment-naïve cancer patients and model prediction might be warranted.

Conflicts of Interest

The Authors have no conflicts of interest.

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Authors 'Contributions

OP: Methodology, investigation, data curation, writing, including review and editing; ST: investigation; KP: investigation; NL: participant recruitment, methodology; PT: Investigation; CV: methodology, investigation, conceptualization, funding acquisition, writing the original draft, writing-review & editing; PC: methodology, investigation, funding acquisition, writing-review & editing.

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CHAPTER IV

RENIERAMYCIN T INDUCES LUNG CANCER CELL APOPTOSIS BY TARGETING MCL-1 DEGRADATION:
A NEW INSIGHT IN THE MECHANISM OF ACTION

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Article

Renieramycin T Induces Lung Cancer Cell Apoptosis by Targeting Mcl-1 Degradation: A New Insight in the Mechanism of Action

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Abstract: Among malignancies, lung cancer is the major cause of cancer death. Despite the advance in lung cancer therapy, the five-year survival rate is extremely restricted due to therapeutic failure and disease relapse. Targeted therapies selectively inhibiting certain molecules in cancer cells have been accepted as promising ways to control cancer. In lung cancer, evidence has suggested that the myeloid cell leukemia 1 (Mcl-1) protein, an anti-apoptotic member of the Bcl-2 family, is a target for drug action. Herein, we report the Mcl-1 targeting activity of renieramycin T (RT), a marine-derived tetrahydroisoquinoline alkaloid that was isolated from the Thai blue sponge *Xestospongia* sp. RT was shown to be dominantly toxic to lung cancer cells compared to the normal cells in the lung. The cytotoxicity of this compound toward lung cancer cells was mainly exerted through apoptosis induction. For the mechanism of action, we found that RT mediated activation of p53 protein and caspase-9 and -3 activations. While others Bcl-2 family proteins (Bcl-2, Bak, and Bax) were minimally changed in response to RT, Mcl-1 protein was dramatically diminished. We further performed the cycloheximide experiment and found that the half-life of Mcl-1 was significantly shortened by RT treatment. When MG132, a potent selective

proteasome inhibitor, was utilized, it could restore the Mcl-1 level. Furthermore, immunoprecipitation analysis revealed that RT significantly increased the formation of Mcl-1-ubiquitin complex compared to the non-treated control. In conclusion, we report the potential apoptosis induction of RT with a mechanism of action involving the targeting of Mcl-1 for ubiquitin-proteasomal degradation. As Mcl-1 is critical for cancer cell survival and chemotherapeutic failure, this novel information regarding the Mcl-1-targeted compound would be beneficial for the development of efficient anti-cancer strategies or targeted therapies.

Keywords: Renieramycin T; *Xestospongia* sp.; Lung cancer; Anti-cancer; Marine sponge; Mcl-1 degradation

1. Introduction

Lung cancer causes nearly 30% of all cancer deaths globally. Despite the advance in lung cancer therapy, most patients hardly survive longer than five years after the first time diagnosis due to the high drug resistance and metastasis [1]. In recent years, targeted therapies aiming to selectively inhibit certain receptors or proteins influencing growth and survival of cancer cells have been recognized as highly promising treatments to control cancer [2].

B-cell lymphoma 2 (Bcl-2) family proteins are among the most important protein groups that dominate the apoptosis of cells. A number of studies have specified Bcl-2 family proteins as the crucial targets of anti-cancer drugs as well as gene therapy [3,4]. Besides, anti-apoptotic members of the Bcl-2 family (i.e., Bcl-2 and Mcl-1) are demonstrated to be involved in chemotherapeutic resistance [5–7]. Recent evidence has suggested that the survival of human cancers is likely to be dependent on expression levels and function of the myeloid cell leukemia 1 (Mcl-1) protein [8,9]. Mcl-1 is a member of the Bcl-2 family proteins with a prominent activity in apoptosis inhibition. The pro-survival function of Mcl-1 is due to the binding activity of the protein to pro-apoptotic members of the Bcl-2 family proteins, thus suppressing the activation of the apoptosis cascade [10–13]. In several cancers, Mcl-1 was frequently found amplified or overexpressed and, in particular, the augmented expression of Mcl-1 reflected the poor prognosis of many malignancies including lung cancer [14–16]. Since Mcl-1 is potentially the main contributor to multidrug resistance, this protein is highlighted as a principal target of drug action in the treatment of lung cancer.

In lung cancer, Mcl-1 has been shown to be a promising target of drug action [14,16]. Not only is its increased expression critical for oncogenesis and cancer progression, but Mcl-1 is also involved in conferring chemotherapeutic drug resistance in this cancer [17–19]. Mcl-1 is a relatively unstable protein, and the degradation of Mcl-1 can be induced by certain anti-cancer drugs [20–23]. Intracellular Mcl-1 level is tightly regulated by the ubiquitin-proteasomal degradation mechanisms. Therefore, compounds with potent activity in eliminating Mcl-1 in cancer cells are of interest as good candidates for Mcl-1-targeted therapy.

The marine environment represents a countless and diverse resource for many potent bioactive compounds, which have recently been used for new drug developments to treat major diseases such as infection and cancer. Recently, antimicrobial, antitumor, and anti-inflammatory effects have been reported. The number of scientific publications on marine compounds has followed an upward trend in the last twenty years, especially in the field of cancer [24]. From many studies, the marine environment has produced a large number of very potent agents, which are

able to inhibit the growth of human cancer cells and exhibit anticancer activities [25]. It has been found that substances from marine organisms have structural and chemical features generally not found in terrestrial natural products; their structures have more complexity and diversity [26,27]. Thus, these marine-derived molecules are capable of interacting with numerous biomolecular targets to either inhibit or promote specific biological functions against various types of cancer cell lines. One of the marine-derived natural products is renieramycins. Renieramycins are alkaloids in the tetrahydroisoquinoline family [28], which is derived from various marine organisms, including sponges in the genera *Reniera* [29,30], *Xestospongia* [31–35], *Cribrochalina* [36,37], and *Neopetrosia* [38]. However, they are unstable and decomposed after extraction and isolation. Therefore, a very unstable amino alcohol functionality at C-21 in their structure is converted into stable aminonitrile compounds by pretreatment with potassium cyanide [30,39]. In this work**,** we have focused on renieramycin T (RT), which is the renieramycin-related compound isolated from the Thai blue sponge *Xestospongia* sp. Normally, the A and E rings in almost all renieramycin structures are in the quinone form, but RT has a substituted aromatic A-ring having a dioxymethylene moiety [39], which is the same as ecteinascidins, another tetrahydroisoquinoline compound isolated from marine tunicates [28,40]. Thus, RT is the renieramycin–ecteinascidin hybrid in the tetrahydroisoquinoline alkaloid family (Figure 1).

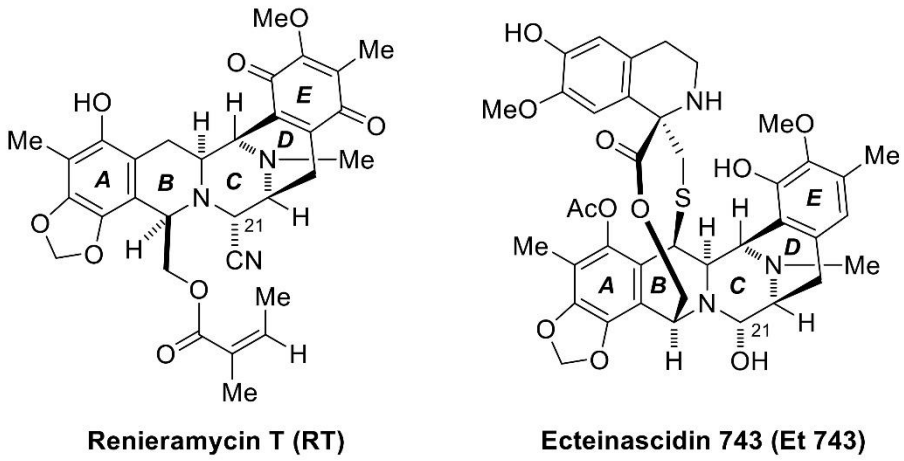

Figure 1. The structures of renieramycin T (RT) and ecteinascidin 743 (Et 743)

2. Results

2.1. The Cytotoxicity and Apoptosis-Inducing Effect of Renieramycin T

To elucidate the anti-cancer potential of RT, we first determined the cytotoxic profile of RT in several non-small cell lung cancer (NSCLC) cells including H460, H292, H23, and A549 cell lines and human bronchial epithelial cells (BEAS-2B) were used for comparison. Cell viability was evaluated by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with various concentrations of RT (0–25 μM) for 24 h. The results showed that RT significantly reduced cell viability in both NSCLC and human bronchial epithelial cell lines in a concentration-dependent manner compared with untreated controls (Figures 2A, B). The cytotoxic effects of RT were found to be significant at the concentration of 0.05 μM in all NSCLC cells, while the cytotoxicity of the compound on BEAS-2B was found at 1 μM. IC_{50} values of RT were determined in various lung cancer cells, and the results are presented in Figure 2B, which indicate that the IC_{50} of RT in NSCLC cells was generally lower than that of normal epithelial cells. Overall, these results suggest the promising effect of RT as an alternative approach in anti-cancer therapy.

The mode of cell death was further evaluated by monitoring the morphology of the cell nucleus as well as cell membrane integrity. Hoechst 33342 staining was used to evaluate the nucleus morphology of apoptotic cells, while the propidium iodide (PI) stained the cell's necrotic features. H460 and BEAS-2B were treated with RT at various concentrations (0–25 μM) for 24 h, and the cells were co-stained with Hoechst 33342 and PI. Condensed or fragmented nuclei exhibiting blue fluorescence of Hoechst 33342 represented fragmented chromatin in apoptotic cells while a red fluorescence of PI indicated necrotic cells. The results revealed that RT caused an increase in apoptosis in a dose-dependent manner, whereas necrotic cells exhibited minimal in response to all treatments (Figures 2C–E). Figure 2F shows the morphology of the cells in H460 and BEAS-2B cells treated with 10 μM RT. We observed that RT could kill lung cancer cells and resulted in the rounded apoptosis cells; however, the normal BEAS-2B showed a relatively higher amount of living intact cells (Figure 2G).

To confirm, flow cytometric analysis of apoptosis and necrosis using annexin V-FITC/PI staining was utilized. H460 was similarly treated with RT (0–25 μM) for 24 h. The results showed that RT dramatically induced apoptosis in a concentration-dependent manner (Figures 2H–I). As shown in Figure 2H, percentages of early apoptotic cells in response to 1, 5, 10, and 25 μM of RT were 19.08%, 49.90%, 58.41%, and 57.60%, respectively.

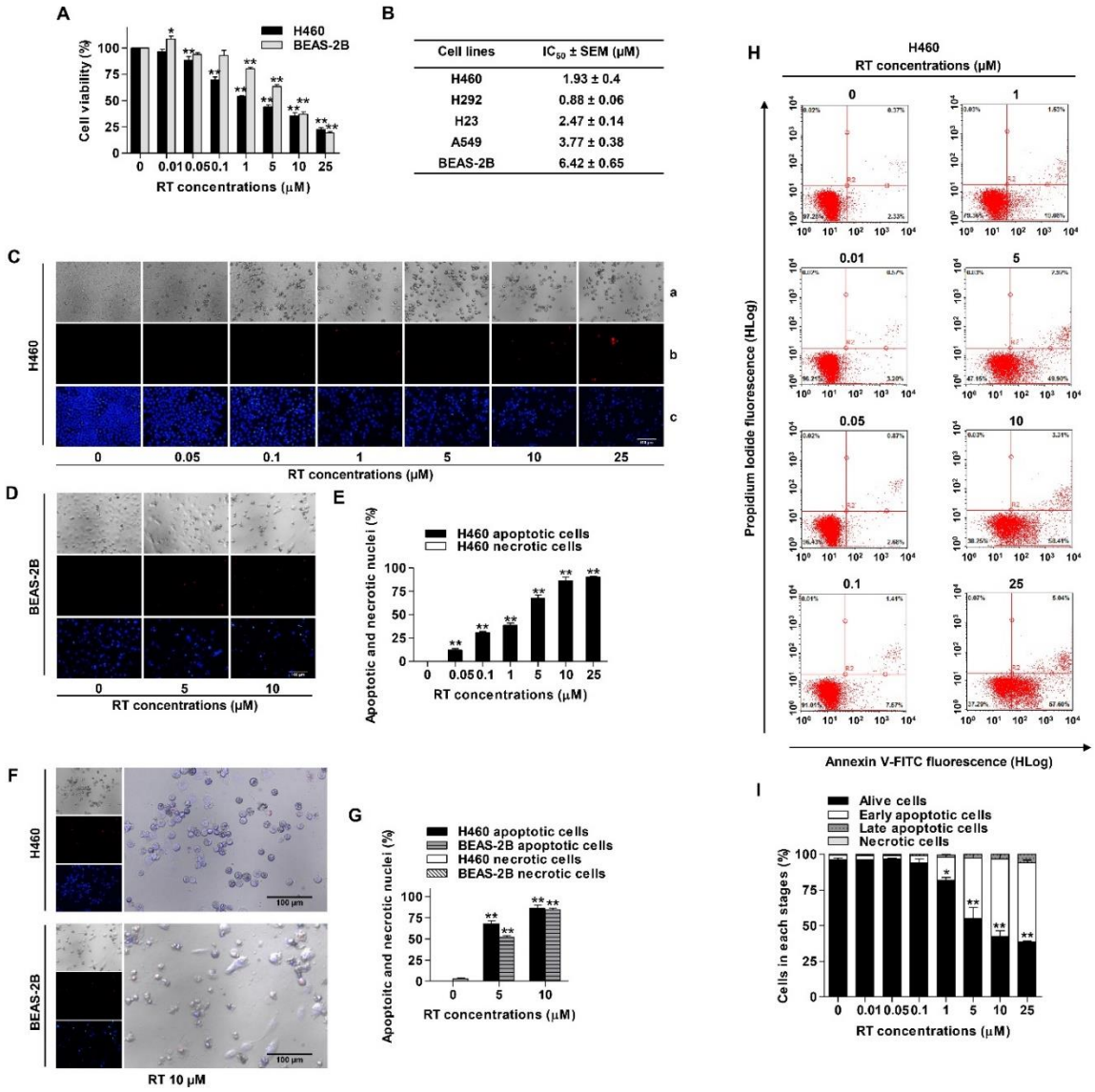

Figure 2. Renieramycin T (RT) reduced cell viability and induced apoptosis in NSCLC and human normal lung epithelial (BEAS-2B) cell lines. (**A**) NSCLC and BEAS-2B cell lines were treated with various concentrations of RT (0–25 μM) for 24 h. Percentages of cell viability were determined using the MTT assay. (**B**) The half maximal inhibitory concentrations (IC_{50}) in NSCLC and BEAS-2B cell lines were calculated by comparison with the untreated control. (**C**–**G**) H460 and BEAS-2B cell lines were treated with RT (0–25 μM) for 24 h. Hoechst 33342 and propidium iodide (PI) were added. Then, Images were detected by using an inverted fluorescence microscope (a-c). A condensed blue fluorescence of Hoechst 33342 reflected fragmented chromatin in apoptotic cells (c) while a red fluorescence of PI reflected late apoptotic or necrotic cells (b) comparing with no staining condition (a). Percentages of nuclear fragmented and PI positive cells were calculated. (**H**) H460 was treated with RT (0–25 μM) for 24 h. Apoptotic and necrotic cells were determined using annexin V-FITC/PI staining with flow cytometry. (I) Percentages of cells at each stage were calculated. Data represented the mean ± SEM (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the untreated control).

2.2. Renieramycin T Promotes Apoptosis by Targeting Mcl-1 to Proteasomal Degradation.

In order to further investigate the mechanism of action of RT, specific apoptotic markers including caspase-3, caspase-9, Poly (ADP-ribose) polymerase (PARP), and their cleaved forms, as well as the up-stream apoptosis regulatory proteins were investigated by Western blot analysis. Lung cancer cells were treated with RT of 1–10 μM concentrations or left untreated for 24 h. Western blotting revealed that in response to RT treatment, the activated caspase-3 and caspase-9 significantly increased compared with the untreated control. Likewise, the cleaved form of PARP was up-regulated simultaneously (Figures 3A, B).

For apoptosis induction, the key pro-survival protein Akt, its active form, phosphorylated Akt (pAkt), p53, and members of the Bcl-2 family proteins were monitored in RT-treated H460 cells. We found that treatment of the cells with RT did not alter the level of Akt and pAkt proteins. The underlying mechanism of apoptosis induction was further evaluated by investigating major regulators of p53-dependent apoptosis such as p53, anti-apoptotic proteins (Mcl-1 and Bcl-2), and pro-apoptotic proteins (Bak and Bax). The H460 cell line was treated in the same condition as the previous experiments. The results from Western blot analysis revealed that RT dramatically up-regulated p53. Interestingly, while the downstream targets of p53, including anti-apoptotic Bcl-2 and pro-apoptotic Bax and Bak, were found to be only slightly altered in response to RT treatment, the anti-apoptotic Mcl-1 was found to be dramatically depleted (Figures 3C, D). As Mcl-1 is known to be critical for cancer cell survival [8,9], this result implies that the principal mechanism of action of RT may involve the targeting of the Mcl-1 function.

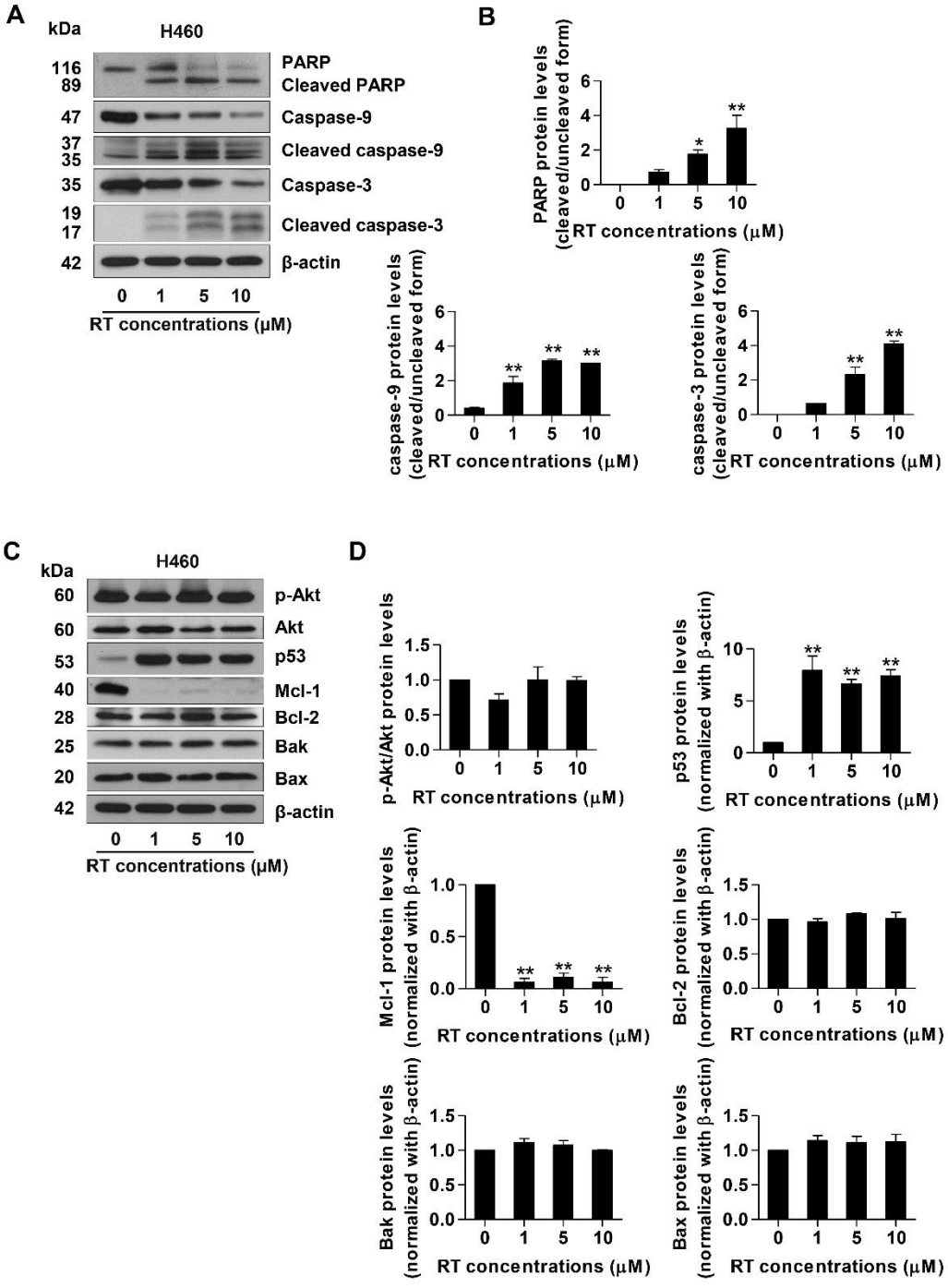

Figure 3. Renieramycin T (RT) induced activation of PARP, caspase-3, and caspase-9 in the H460 cell line. Moreover, RT also significantly up-regulated p53 and down-regulated Mcl-1 in the H460 cell line. H460 cells were treated with RT (0–25 μM) for 24 h. (**A**) and (**C**) Apoptotis-related proteins were measured with Western blot analysis. The blots were reprobed with β-actin to confirm equal loading of each of the protein samples. (**B**) and (**D**) The relative protein

levels were calculated by densitometry. Data represented the mean ± SEM (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the untreated control).

2.3 Renieramycin T Decreases Mcl-1 Through the Induction of Mcl-1 Proteasomal Degradation.

Mcl-1 was not only shown to protect cells against apoptotic stimuli, but also this protein has been suggested as an important therapeutic target in human cancers [41]. As targeting Mcl-1 for cell degradation has been demonstrated as a promising action of novel anti-cancer drugs [15, 42, 43], we further evaluated the effect of RT on Mcl-1 stability in lung cancer cells. To compare the Mcl-1 stability between RT-treated and control cells, the cycloheximide (CHX) chasing assay was used followed by Western blot analysis. CHX, an inhibitor of protein biosynthesis, was widely used for determining the half-life of the protein of interest [44]. Therefore, H460 cells were treated with RT (0–5 μM) in the presence or absence of 50 μg/ml CHX and the level of Mcl-1 over time was determined.

Figures 4A and B show that in the condition where protein production was blocked, RT decreased the stability of the Mcl-1 protein. The difference was first detected at 4 h after RT treatment (Figures 4A, B). We also determined the Mcl-1 protein half-life and found that the half-life of Mcl-1 protein in the RT-treated groups was about 1.4 h, whereas in the untreated control the value was 3.4 h (Figure 4C). To prove that this decrease in Mcl-1 stability was through proteasomal degradation of the protein, we utilized MG132, a potent selective proteasome inhibitor. Treatment of the lung cancer cells with RT for 4 h dramatically decreased Mcl-1 level, while the addition of MG132 (0–20 μM) could restore the Mcl-1 level (Figures 4E, F). Taken together, it could be concluded that RT induced Mcl-1 proteasomal degradation. Importantly, it was shown that the degradation of Mcl-1 protein required ubiquitination [45]. The tagging of ubiquitin and poly-ubiquitin molecules on the target proteins conferred the recognition of the proteasome providing a specific degradation machinery of certain functional proteins. We also provided the evidence of Mcl-1 ubiquitination using immunoprecipitation and analysis of the Mcl-1-ubiquitin complex (Ub-Mcl-1) in the lung cancer cells treated with RT and non-treated control cells. After treating the cells with 5 μM RT for 4 h, the Mcl-1 protein was selectively isolated from cell lysates by the Mcl-1 antibody. Then, unwanted proteins were washed out and only Mcl-1 complex was analyzed for ubiquitin through Western blot analysis. Figures 4G and H show that treatment of the cells with RT dramatically enhanced the formation of the Mcl-1-ubiquitin complex compared to the baseline complex in non-treated controls. Taken together, the mechanism of action of RT is through the induction of Mcl-1 degradation via ubiquitin-proteasomal degradation.

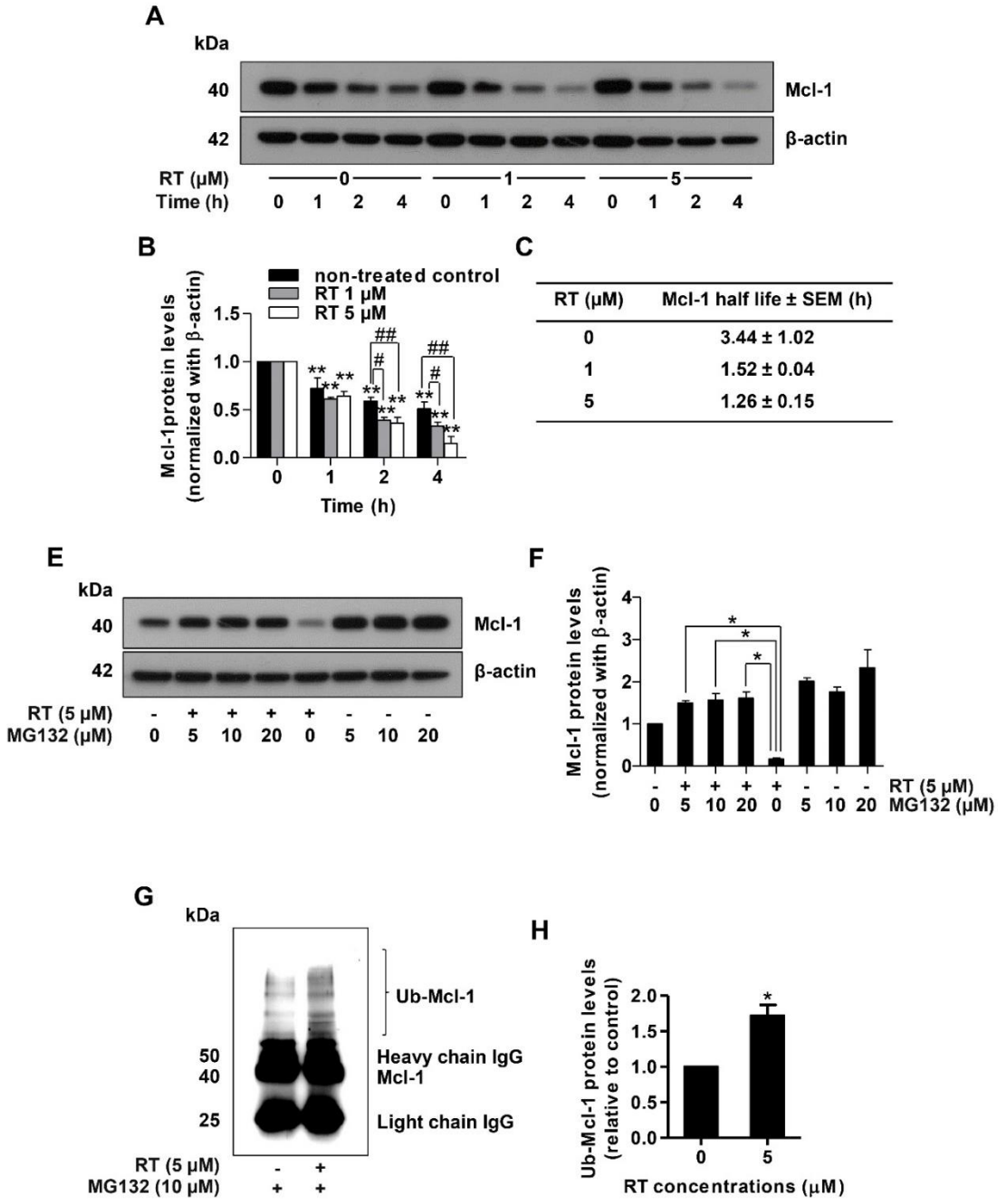

Figure 4. Renieramycin T (RT) induced ubiquitin-mediated Mcl-1 proteasomal degradation. (**A**) Cycloheximide (CHX) chasing assay was performed to measure Mcl-1 half-lives. H460 cells were treated with RT (0–5 μM) with or without 50 μg/ml CHX as indicated by the time in h. Western blot analysis was performed for determined Mcl-1 levels. The blots were reprobed with β-actin to confirm equal loading of each of the protein samples. (**B**) The relative protein levels were calculated by densitometry (** $p < 0.01$, compared with the untreated control at 0 h, # $0.01 \leq p < 0.05$, ## $p < 0.01$, compared with the untreated control at the same time). (**C**) and (**D**) Mcl-1 half-lives were calculated. (**E**) H460 cell line was treated with RT (0–5 μM) with or without MG132 (0–20 μM) for 4 h. Mcl-1 expression levels

were measured using Western blot analysis. The blots were reprobed with β-actin to confirm equal loading of each of the protein samples. (**F**) The reversal of RT-mediated down-regulation of Mcl-1 levels by MG132 was calculated by densitometry compared to the non-MG132 treated group (* $0.01 \leq p < 0.05$, compared with the non-MG132 treated group). (**G**) H460 was treated with RT (5 μM) and MG132 (10 μM) for 4 h. Then, protein lysates were collected subsequent to Mcl-1 immunoprecipitation, and the ubiquitinated protein levels were measured by Western blotting. (**H**) Ub-Mcl-1 levels were quantified using densitometry (* $p < 0.01$, compared with the untreated control) All data represented the mean ± SEM (n = 3).

3. Discussion

Apoptosis is a critical component in cancer pathogenesis. The origin of cancer, as well as its progression, depends on the dysregulation or disruption of proliferation and apoptosis. The tumor suppressor p53 has a principal role in the control of DNA repair, cell cycle arrest, and apoptosis [46,47]. In response to DNA-damage, p53 is activated and most probably exerts its transcriptional regulatory functions to control the level of Bcl-2 family proteins, causing the increased cellular level of pro-apoptotic members of the Bcl-2 family and in a concomitant decrease of the anti-apoptotic proteins [48]. Then, the death-survival threshold is altered by competitive dimerization between anti- and pro-apoptotic proteins. The pro-apoptotic dimers could create the release of mitochondrial contents to the cytoplasm, and such contents activate the function of caspases leading to apoptosis [49]. We found that the treatment of the lung cancer cells with RT resulted in the significant induction of p53 that may, at least in part, play a role in RT-mediated apoptosis (Figures 3C, D). Interestingly, our protein analysis shows a striking effect on the cellular protein level of Mcl-1 (Figures 3C, D). Mcl-1 is an anti-apoptotic protein that has garnered increased attention in lung cancer cell biology as it was shown to be highly expressed in lung cancer [14,16]. Besides, Mcl-1 was shown to be important for the survival of lung cancer cells [50] and the multivariate analysis indicated MCl-1 expression to be a significant prognostic factor of lung cancer [14,51].

RT is a member of tetrahydroisoquinoline marine alkaloids in the renieramycins series from the *Xestospongia* sp. found in Thailand and the Philippines [39]. Evidence has shown that renieramycins have strong cytotoxicity against various cancer cell lines, including breast (T47D), colon (HCT116) and DLD1), lung (QG56 and H460), pancreatic (AsPC1), and prostate (DU145) human carcinomas [31,34,39,52]. Interestingly, RT, the renieramycin–ecteinascidin hybrids in the tetrahydroisoquinoline alkaloid family, showed strong cytotoxicity in several cancer cell lines including colon (HCT116), prostate (DU145), non-small cell lung (H292, H460 and QG56), breast (T47D), and pancreatic (AsPC1) cancers [39,53–55]. Moreover, our previous study suggested that 5-*O*-acetyl-renieramycin T, a modified compound of RT, exerted a potential to suppress cancer stem

cell (CSCs) growth, which is represented by a decrease in the CSCs markers CD44 and CD133 due to depletion of the protein kinase B (AKT) signal resulting in apoptosis induction of CSCs [56]. However, cell death mechanisms in CSCs and cancer cells might be different since CSCs have special characteristics only found in the stem cell population such as metabolic activities, signaling pathways, and cell cycle regulation [57–59]. Although anti-CSC activities of 5-*O*-acetyl-renieramycin T have been reported, the mechanism of action of RT in lung cancer cells has not been elucidated.

In an agreement with evidence favoring the use of Mcl-1 targeted therapy [15,41], we have shown herein that RT could induce Mcl-1 degradation in lung cancer cells. RT, an active compound from the blue sponge *Xestospongia* sp., was demonstrated here to possess a potent induction effect of Mcl-1-targeted degradation. The results revealed that while other Bcl-2 family proteins were not affected by the treatment with RT, the level of Mcl-1 was dramatically diminished (Figures 3C, D). In fact, the degradation process of Mcl-1 was shown to involve the ubiquitin-proteasomal pathway [45]. In addition, Mcl-1 degradation was shown to be important for targeted therapeutics [22]. In this study, we found that under RT treatment, the Mcl-1 half-life was dramatically shortened. The cycloheximide-based assay showed that the half-life of Mcl-1 in response to 1 μM RT was 1.52 h in comparison to 3.44 h in the non-treated control cells (Figures 4A, C). When the selective proteasome inhibitor (MG132) was applied, the Mcl-1 level in the RT-treated cells was significantly restored, indicating that the proteasomal degradation plays a key part in RT function (Figures 4E, F). Further, we monitored the levels of the Mcl-1-Ubiquitin complex and found that the formation of the complex dramatically increased in the RT-treated cancer cells (Figure 4G, H). Together, these results strongly support the conclusion that RT mediated cell death by targeting the degradation of Mcl-1.

4. Materials and Methods

4.1. Reagents and Antibodies

Dulbecco's Modified Eagle's Medium (DMEM) medium, Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, phosphate-buffered saline (PBS), and trypsin-EDTA were obtained from Gibco (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33342, propidium iodide (PI), cycloheximide, MG132, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). The primary antibodies, PARP (#9532), caspase-9 (#9502), caspase-3 (#9662), p53 (#9282), Mcl-1 (#94296), Bcl-2 (#4223), Bak (#6947), Bax (#5023), Akt (#9272), phosphorylated Akt (#4060), and β-actin (#4970), were obtained from Cell Signaling Technology (Danvers, MA, USA). The primary antibody ubiquitin was obtained from Sigma-

Aldrich, Co. (St. Louis, MO, USA). The respective secondary antibodies, anti-rabbit IgG (#7074), and anti-mouse (#7076), were obtained from Cell Signaling Technology (Danvers, MA, USA).

4.2. Isolation of Renieramycin T (RT)

Renieramycin T (RT) was isolated from its natural source, the Thai blue sponge *Xestospongia* sp. that was collected from Sichang Island, Chonburi Province, Thailand, in October 2016 with the assistance of the Aquatic Resources Research Institute, Chulalongkorn University. The characteristics of the fresh sponge, the light bluish-gray color, bulbous surface lobes, the numerous and moderate size of oscules, and the easily crumbled texture were identified [34]. The collected blue sponge was homogenized and suspended in the phosphate buffer solution (pH 7). After that, the suspension was pretreated with potassium cyanide (KCN) until the KCN concentration reached 10 mM. The suspension was stirred continuously for 6 h. Then, methanol was added and the mixture was macerated for 48 h. The methanol extract was filtered and concentrated under reduced pressure to acquire an aqueous methanolic solution. The processes of maceration with methanol and concentration were repeated three times. After that, the concentrated aqueous methanolic solutions were combined and extracted with ethyl acetate. The ethyl acetate fractions were combined and concentrated. Then, the crude extract was obtained as a dark brown gum. Purification of RT was performed by using silica gel chromatography with the gradient solvent mixture of hexane, ethyl acetate, and methanol. RT was obtained with 0.001% weight by dry weight of the blue sponge [34]. The resulting amorphous yellow powder showed the spectroscopic data of the RT that was matched with the previous report [39]. Renieramycin T was obtained as an orange amorphous solid.$[\alpha]_D^{25}$ +232.1 (*c* 0.0028, $CHCl_3$); CD $\Delta\varepsilon$ (c: 17 mM, methanol, 20 °C) −1.2 (354), −1.2 (329), −1.1 (313), +0.9 (291), +3.5 (272), +1.2 (248), +0.1 (233); IR (KBr) 3449, 2953, 2924, 2853, 1717, 1653, 1616, 1460, 1375, 1307, 1234, 1151, 1093 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.00 (1H, qq, *J* = 7.2, 1.5 Hz, 26-H), 5.92 (1H, d, *J* = 1.2 Hz, OCH_2O), 5.85 (1H, d, *J* = 1.2 Hz, OCH_2O), 4.42 (1H, dd, *J* = 11.4, 3.8 Hz, 22-Hb), 4.29 (1H, s, 5-OH), 4.16 (1H, t, *J* = 3.8 Hz, 1-H), 4.10 (1H, d, *J* = 2.4 Hz, 21-H), 4.00 (1H, overlapped, 22-Ha), 3.99 (1H, overlapped, 11-H), 3.99 (3H, s, 17-OCH_3), 3.36 (1H, dd, *J* = 7.2, 2.4 Hz, 13-H), 3.24 (1H, ddd, *J* = 12.0, 2.7, 2.4 Hz, 3-H), 2.87 (1H, br d, *J* = 15.0 Hz, 4-Hα), 2.75 (1H, dd, *J* = 20.7, 7.2 Hz, 14-Hα), 2.29 (1H, d, *J* = 20.7 Hz, 14-Hβ), 2.29 (3H, s, NCH_3), 2.11 (3H, s, 6-CH_3), 1.94 (3H, s, 16-CH_3), 1.85 (3H, dq, *J* = 7.2, 1.5 Hz, 27-H_3), 1.69 (3H, br d, *J* = 1.5 Hz, 28-H_3), 1.67 (1H, dd, *J* = 15.0, 12.0 Hz, 4-Hβ); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 186.1 (C-15), 182.8 (C-18), 167.1 (C-24), 155.4 (C-17), 144.9 (C-7), 144.7 (C-5), 141.7 (C-20), 139.8 (C-26), 136.9 (C-8), 135.8 (C-19), 129.0 (C-16), 126.8 (C-25), 117.5 (CN-21), 113.1 (C-10), 112.2 (C-9), 106.1 (C-6), 101.1 (OCH_2O), 64.6 (C-22), 61.0 (17-OCH_3), 59.7 (C-21), 56.4 (C-1), 56.3 (C-3), 55.0 (C-11), 54.8 (C-13), 41.5 (NCH_3), 26.8 (C-4), 21.2

(C-14), 20.5 (C-28), 15.8 (C-27), 8.8 (6-CH_3), 8.7 (16-CH_3); FAB+ HRMS m/z 576.2344 ([M + H], calcd for $C_{31}H_{34}N_3O_8$, 576.2347).

4.3. Preparation of the RT Stock Solution

RT was prepared as a 50 mM stock solution by dissolving it in dimethyl sulfoxide (DMSO) solution and then stored at −20 °C. It was freshly diluted with medium to the desired concentrations before using. The final concentration of DMSO was less than 0.5% solution, which showed no signs of cytotoxicity.

4.4. Cell Lines and Culture

The human bronchial epithelial cell line (BEAS-2B) and human non-small cell lung cancer (NSCLC) cell lines (H460, H292, H23, and A549) were obtained from the American Type Culture Collection (Manassas, VA, USA). H460, H292, and H23 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. BEAS-2B and A549 were cultured in DMEM medium. The medium was supplemented with 10% FBS, 2 mM L-glutamine, and 100 units/ml of each of penicillin and streptomycin at 37 °C with 5% CO_2 in a humidified incubator.

4.5. Cell Viability Assay

NSCLC and BEAS-2B cells were seeded into 96-well plates (1×10^4 cells/well) for overnight. Cells were treated with various concentrations of RT (0, 0.01, 0.05, 0.1, 1, 5, 10, and 25 μM) for 24 h. Then, 100 μL of MTT solution (400 μg/ml) was added in each well and incubated for 3 hours at 37 °C. Supernatants were replaced with 100 μL of DMSO to dissolve the formazan product of MTT. The optical density was measured at 570 nm by a microplate reader (Anthros, Durham, NC, USA). The percentage of cell viability and IC_{50} were calculated according to the manufacturer's protocol (7sea Biotech). Cell viability = (ODexperiment − ODblank) / (ODcontrol − ODblank) × 100%. Some concentrations of RT were chosen for using in the next experiments.

4.6. Nuclear Staining Assay

Hoechst 33342 and PI double staining were used for screening and detecting cell morphology. H460 and BEAS-2B cells were seeded and treated with various concentrations of RT (0, 0.05, 0.1, 1, 5, 10, and 25 μM) for 24 h. Then, cells were stained with 10 μg/mL Hoechst 33342 for 15 min at 37 °C and then stained with 5 μg/mL PI. Fluorescence of nuclear-stained cells was detected randomly using the fluorescent microscope (Olympus IX5, Tokyo, Japan). Nuclear condensation and DNA fragmentation were analyzed as the percentage of apoptotic cells.

4.7. Annexin V-FITC/PI Staining Apoptotic Assay

H460 cells were seeded and treated with various concentrations of RT (0, 0.01, 0.05, 0.1, 1, 5, 10, and 25 μM) for 24 h. After that, cells were collected via centrifugation, washed twice with cold PBS, pH 7.4, and suspended in binding buffer. Then, 5 μl of annexin V-FITC and 1 μl of PI were added to stain the cells for 15 min at room temperature as recommended in the manufacturer's protocol (ImmunoTools, Friesoythe, Germany). Live, apoptotic, and necrotic cells were analyzed using Guava easyCyte flow cytometer (EMD Millipore, Hayward, CA, USA).

4.8. Western Blot Analysis

H460 cells were seeded and treated with various concentrations of RT (0, 1, 5, and 10 μM) for 24 h. The treated cells were collected and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) for 1 h on ice. Protein concentrations were determined using the bicinchoninic acid (BCA) assay. All protein samples (70 μg) were run on SDS-polyacrylamide gel electrophoresis and further transferred to 0.45 μm nitrocellulose and 0.2 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Then, membranes were blocked in Tris-buffer saline containing 0.1% Tween 20 and 5% non-fat dry milk for 2 h at room temperature and incubated with the specific primary antibodies at 4 °C for overnight. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Immunoreactive proteins were detected with the enhanced chemiluminescent detection system (Supersignal West Pico, Pierce, Rockford, IL, USA) and subsequently exposed to X-ray film. Protein bands were analyzed using the ImageJ software (version 1.52, National Institutes of Health, Bethesda, MD, USA).

4.9. Cycloheximide (CHX) Chasing Assay

H460 cells were seeded and treated with various concentrations of RT (0, 1, 5, and 10 μM) with or without 50 μg/ml CHX for 0, 1, 2, and 4 h. The treated cells were collected and lysed with RIPA lysis buffer containing the protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Western blot analysis was performed for detecting Mcl-1 protein levels. Protein bands were analyzed using the ImageJ software (version 1.52, National Institutes of Health, Bethesda, MD, USA), and the Mcl-1 protein half-life was calculated.

4.10. Immunoprecipitation Assay

H460 cells were seeded and treated with 0 and 5 μM of RT for 4 h. The treated cells were collected and lysed with RIPA lysis buffer containing the protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Then, Immunoprecipitation was performed by using Dynabeads™

Protein G Immunoprecipitation Kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Magnetic beads were prepared and resuspended with the primary antibody of Mcl-1 in a binding buffer for 10 min. A suspension of the magnetic bead-Ab complex was mixed with lysed protein and incubated at 4 °C overnight to allow Mcl-1 antigen to bind with magnetic bead-Ab complex. After that, the magnetic bead-Ab-Ag complex was washed three times using 200 µL washing Buffer, separated on the magnet between each wash, and the supernatant was removed. Elution Buffer was added for releasing the Ab-Ag complex from magnetic beads. The supernatant contained the Ab-Ag complex was then used to perform Western blot analysis for detecting the ubiquitinated Mcl-1 protein.

4.11. Statistical analysis

The data from three independent experiments (n = 3) was presented as the mean ± standard error of the mean (SEM). Statistical differences between multiple groups were analyzed using an analysis of variance (ANOVA). The p-value of less than 0.05 was considered as statistically significant.

5. Conclusions

In conclusion, this study provides supporting evidence for RT to be developed for the anti-cancer therapy in a lung cancer cell model. The RT was shown to be predominantly toxic to lung cancer cells rather than normal epithelial cells in the lung. The mechanism of action of RT is quite specific. The compound mediates Mcl-1 depletion by enhancing the ubiquitin-proteasomal degradation of the protein. As Mcl-1 was demonstrated to control for survival and progression of cancer, these data might be beneficial for highlighting RT as a novel lead compound with supportive information to be further developed for targeted anti-cancer approaches (Figure 5).

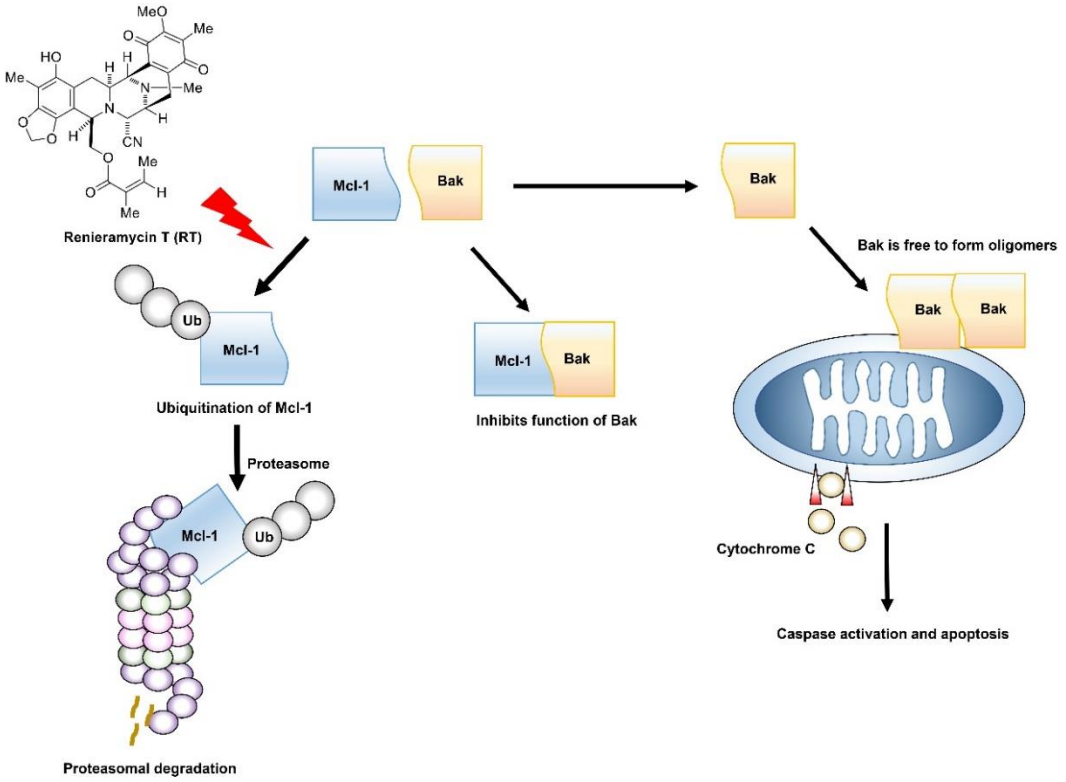

Figure 5. Renieramycin T (RT) enhances apoptosis induction through Mcl-1 proteasomal degradation. Normally, Mcl-1 forms complex with Bak to inhibit its apoptotic function, but when Mcl-1 is degraded through the ubiquitin-mediated proteasomal degradation by treatment of RT, Bak is relieved. Activated Bak forms oligomerization that can permeabilize the outer membrane of mitochondria and release cytochrome c to initiate the apoptosis mechanism.

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CHAPTER V

STRUCTURE–ACTIVITY RELATIONSHIPS AND MOLECULAR DOCKING ANALYSIS OF MCL-1 TARGETING RENIERAMYCIN T ANALOGUES IN PATIENT-DERIVED LUNG CANCER CELLS

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Article

Structure–Activity Relationships and Molecular Docking Analysis of Mcl-1 Targeting Renieramycin T Analogues in Patient-derived Lung Cancer Cells

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Abstract: Myeloid cell leukemia 1 (Mcl-1) and B-cell lymphoma 2 (Bcl-2) proteins are promising targets for cancer therapy. Here, we investigated the structure–activity relationships (SARs) and performed molecular docking analysis of renieramycin T (RT) and its analogues and identified the critical functional groups of Mcl-1 targeting. RT have a potent anti-cancer activity against several lung cancer cells and drug-resistant primary cancer cells. RT mediated apoptosis through Mcl-1 suppression and it also reduced the level of Bcl-2 in primary cells. For SAR study, five analogues of RT were synthesized and tested for their anti-cancer and Mcl-1- and Bcl-2-targeting effects. Only two of them (TM-(–)-18 and TM-(–)-4a) exerted anti-cancer activities with the loss of Mcl-1 and partly reduced Bcl-2, while the other analogues had no such effects. Specific cyanide and benzene ring parts of RT's structure were identified to be critical for its Mcl-1-targeting activity. Computational molecular docking indicated that RT, TM-(–)-18, and TM-(–)-4a bound to Mcl-1 with high affinity, whereas TM-(–)-45, a compound with a benzene ring but no cyanide for comparison, showed the lowest binding affinity. As Mcl-1 helps cancer cells evading apoptosis, these data encourage further development of RT compounds as well as the design of novel drugs for treating Mcl-1-driven cancers.

Keywords: renieramycin T; Structure–Activity Relationship; patient-derived primary lung cancer cells; lung cancer; apoptosis; Mcl-1; Molecular Docking Analysis

1. Introduction

Estimates of the worldwide cancer records of incidence and mortality from all types of cancers have revealed that lung cancer is the most common cause of cancer death, and is showing an increasing incidence. Furthermore, the 5-year survival rate from lung cancer is critically low due to its resistance to cancer therapy and due to disease relapse [1,2]. The improved clinical response as a result of targeted therapies has suggested that the use of more precise drugs focused on the molecular targets underlying the aggressiveness of the cancer and its drug resistance would be highly beneficial.

It has been widely shown that one of the important hallmarks of cancer is the ability of the tumor cells to evade apoptosis [3]. In general, programmed apoptosis is a well-controlled cell death mechanism for the removal of unwanted or harmful cells. However, what frequently occurs in cancer is the upregulation of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family of proteins [4], which consequently facilitates oncogenesis through cell death resistance [5]. The Bcl-2 family of proteins belong to the class of BH-domain-containing proteins [6], which can be classified into anti-apoptotic members (Bcl-2, Mcl-1, Bcl-w, Bcl-xl, and Bfl-1/A1) and pro-apoptotic members (Bax, Bak, Bad, Bid, Bak, Bim, Puma, Noxa, Hrk, and Bmf). The anti-apoptotic proteins interact and are antagonized specifically to certain BH3-only proteins and pro-apoptotic proteins [7]. The pro-apoptotic proteins Bax and Bak are the key members that trigger permeabilization of the mitochondrial membrane and the release of pro-apoptogenic proteins. Recent evidence has suggested that the ability to evade apoptosis, survival during metastasis, and resistance to therapy are likely to be dependent on the expression and function of the pro-survival Bcl-2 proteins [8]. Consequently, it is not unexpected that the amplification and overexpression of pro-survival Bcl-2 proteins, such as Bcl-2 and Mcl-1, are found in many cancer types (e.g., non-small-cell lung cancer, breast cancer, ovarian cancer, prostate cancer, and pancreatic cancer) [9–11]. Bcl-2 family members, like Bcl-2 and Mcl-1, have been shown to dominate negative regulation for apoptosis control and to be responsible for chemotherapeutic resistance [12]. An analysis of cancer specimens revealed that up to 50% of lung cancer specimens exhibited elevated levels of Mcl-1 expression, which is associated with a poor prognosis for lung cancer patients [13,14]. Not unpredictably, the downregulation of Mcl-1 increases cancer cell sensitivity to standard anticancer drugs, such as etoposide, doxorubicin, and ABT-737 [14]. Moreover, the reduction of Mcl-1 sensitized osteosarcoma cells to chemotherapeutic agents [15]. In lung cancer, evidence from a microarray analysis indicated that Mcl-1 is highly expressed in the cancer tissue of non-small cell lung cancer (NSCLC) patients [16,17]. In addition, it was shown that NSCLC cell lines exhibited high levels of Mcl-1, and the inhibition of such a protein by siRNA could potently mediate NSCLC cell

apoptosis [18]. Additionally, evidence has suggested that, in NSCLC, Mcl-1 inhibition showed superior potential for having a cancer therapeutic effect when compared with Bcl-xl inhibition [18]. Taken together, new drugs with modes of action involving eliminating Mcl-1 in lung cancer cells are of interest as candidates for Mcl-1-targeted therapy.

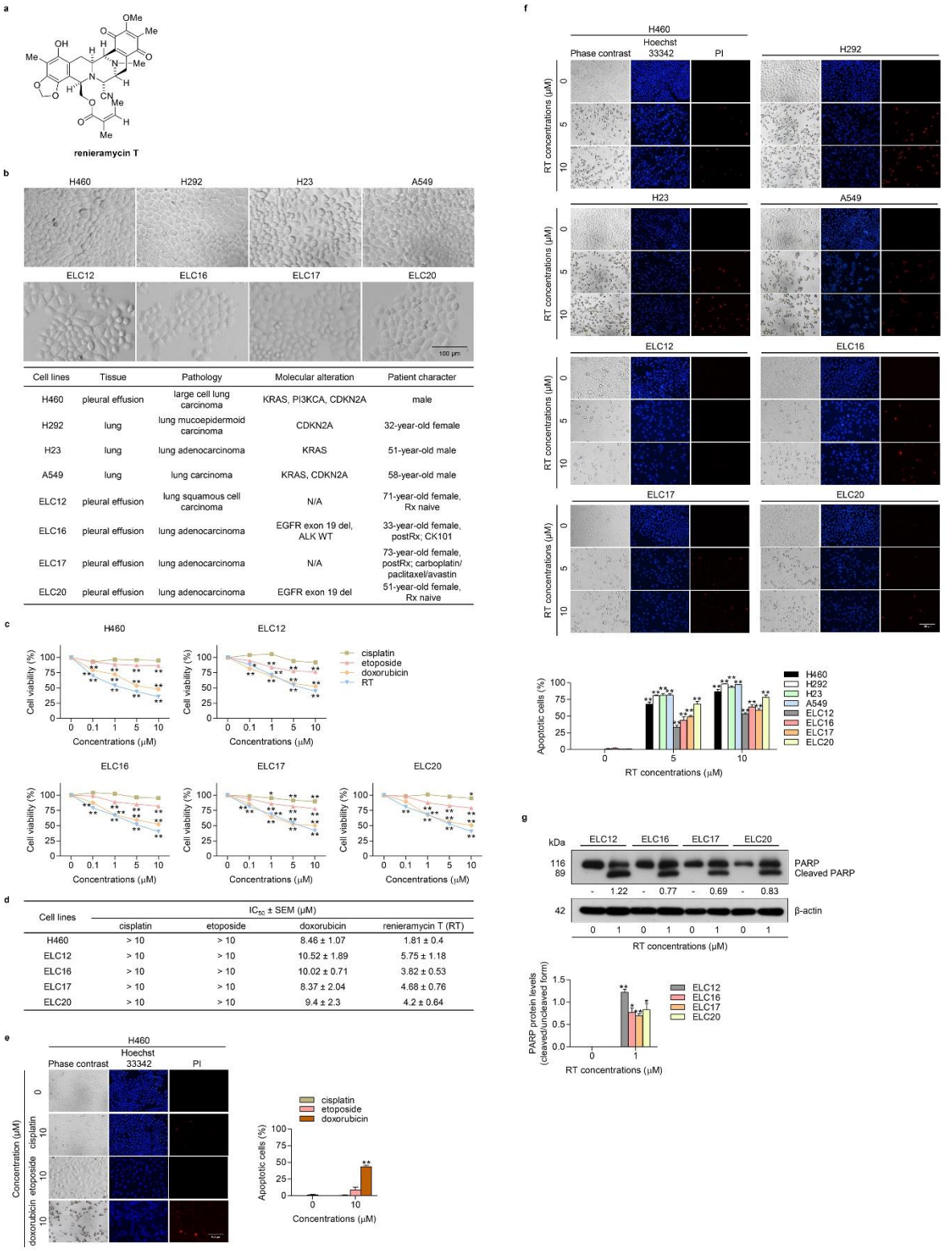
Marine organisms function as a reservoir of potent marine-derived agents capable of inhibiting the growth of cancer cells, as has been demonstrated in in vitro and in vivo studies [19,20]. For instance, there are four marine agents approved for use in the treatment of cancers: cytarabine, trabectedin, eribulin mesylate, and the conjugated antibody brentuximab vedotin [21]. Moreover, it has been found that substances from marine organisms, such as renieramycins, have the potential to prevent tumor formation and induce cell death via the apoptosis pathway [22]. A previous study reported that renieramycin T (RT), a renieramycin-related compound isolated from the blue sponge *Xestospongia* sp., was dominantly toxic to lung cancer cells and mainly exerted this effect through apoptosis induction via the targeting of Mcl-1 for ubiquitin-proteasomal degradation [23]. As RT has a complex structure composed of several chemical moieties, understanding the structure–activity relationships (SARs) is a necessity for identification of the active moieties that are critical for drug action and that hold promise to increase drug precision and potency. Using RT as a lead compound, we aimed to establish such structure–activity relationships (SARs) and the subsequent SAR-directed optimization for treatment. The newly synthesized simplified parts of RT were developed and the active parts as well as the required moieties of the compound for the Mcl-1-targeted effect were evaluated in the present study utilizing protein analysis in combination with molecular docking simulation.

2. Results

2.1. Cytotoxicity and Apoptosis-inducing Effect of RT on Patient-derived Primary Lung Cancer Cells

Chemotherapeutic drug resistance is accepted to be a major cause of therapeutic failure, tumor recurrence, and disease progression in lung cancer [24]. Mcl-1, an anti-apoptotic member of the Bcl-2 family, was demonstrated to be mainly involved in chemotherapeutic resistance as this protein is frequently found to be highly expressed in lung cancer [25] and the diminishment of Mcl-1 can lead to cancer cell death [26,27]. To characterize the potency of the anti-cancer activity of RT (Figure 1a), we determined the cytotoxic profile of RT in chemotherapeutic resistant primary lung cancer cells (ELC12, ELC16, ELC17, and ELC20) and lung cancer cell lines (H460). The basic cell morphology of the NSCLC and patient-derived primary cancer cell lines and the molecular characteristics are shown in Figure 1b. The results indicated that RT exerted a superior cytotoxic

potency when compared with the commonly used chemotherapeutic drugs, including cisplatin, etoposide, and doxorubicin, at the equivalent concentrations (Figure 1c). Figure 1c shows that nearly all of the lung cancer cells were resistant to cisplatin at 0–10 μM, as the cell viability was found to be above 90% after treatment, while doxorubicin and RT showed comparable potent cytotoxic effects and both compounds could reduce cancer cell viability by approximately 70% at the 10 μM concentration. The half maximal inhibitory concentrations (IC_{50}) values of RT and the commercial drugs were calculated and the results indicated that the IC_{50} of RT was generally lower than that of the chemotherapeutic drugs. Importantly, RT showed greater potency compared to that of doxorubicin in all the cells (Figure 1d). The apoptotic cell death and necrosis were further evaluated by Hoechst33342 and propidium iodide (PI) staining, respectively. We tested the apoptosis induction effect of cisplatin, etoposide, and doxorubicin in H460 cells and found consistent results with the cytotoxicity results, showing that doxorubicin caused the highest apoptosis, as indicated by the fragmented or condensed nuclei (Figure 1e). Then, the apoptosis induction effect of RT was evaluated in all lung cancer cells (H460, H292, H23, A549, ELC12, ELC16, ELC, 17, and ELC 20). The result revealed that RT caused an increase in apoptosis in a concentration-dependent manner, whereas it exhibited a minimal necrotic cell death effect, as shown in Figure 1e and 1f. We confirmed the apoptotic cell death by determination of cleaved PARP protein using Western blot analysis. The result showed an increase of cleaved PARP in response to RT treatment compared to control (Figure 1g).



Cell lines	Tissue	Pathology	Molecular alteration	Patient character
H460	pleural effusion	large cell lung carcinoma	KRAS, PI3KCA, CDKN2A	male
H292	lung	lung mucoepidermoid carcinoma	CDKN2A	32-year-old female
H23	lung	lung adenocarcinoma	KRAS	51-year-old male
A549	lung	lung carcinoma	KRAS, CDKN2A	58-year-old male
ELC12	pleural effusion	lung squamous cell carcinoma	N/A	71-year-old female, Rx naive
ELC16	pleural effusion	lung adenocarcinoma	EGFR exon 19 del, ALK WT	33-year-old female, postRx; CK101
ELC17	pleural effusion	lung adenocarcinoma	N/A	73-year-old female, postRx; carboplatin/ paclitaxel/avastin
ELC20	pleural effusion	lung adenocarcinoma	EGFR exon 19 del	51-year-old female, Rx naive

Cell lines	IC_{50} ± SEM (μM)			
	cisplatin	etoposide	doxorubicin	renieramycin T (RT)
H460	> 10	> 10	8.46 ± 1.07	1.81 ± 0.4
ELC12	> 10	> 10	10.52 ± 1.89	5.75 ± 1.18
ELC16	> 10	> 10	10.02 ± 0.71	3.82 ± 0.53
ELC17	> 10	> 10	8.37 ± 2.04	4.68 ± 0.76
ELC20	> 10	> 10	9.4 ± 2.3	4.2 ± 0.64

Figure 1. Effects of renieramycin T (RT) on cell viability and apoptotic cell death in non-small cell lung cancer (NSCLC) cell lines (H460, H292, H23, and A549) and patient-derived primary cancer cell lines (ELC12, ELC16, ELC17, and ELC20). (**a**) The structure of RT. (**b**)

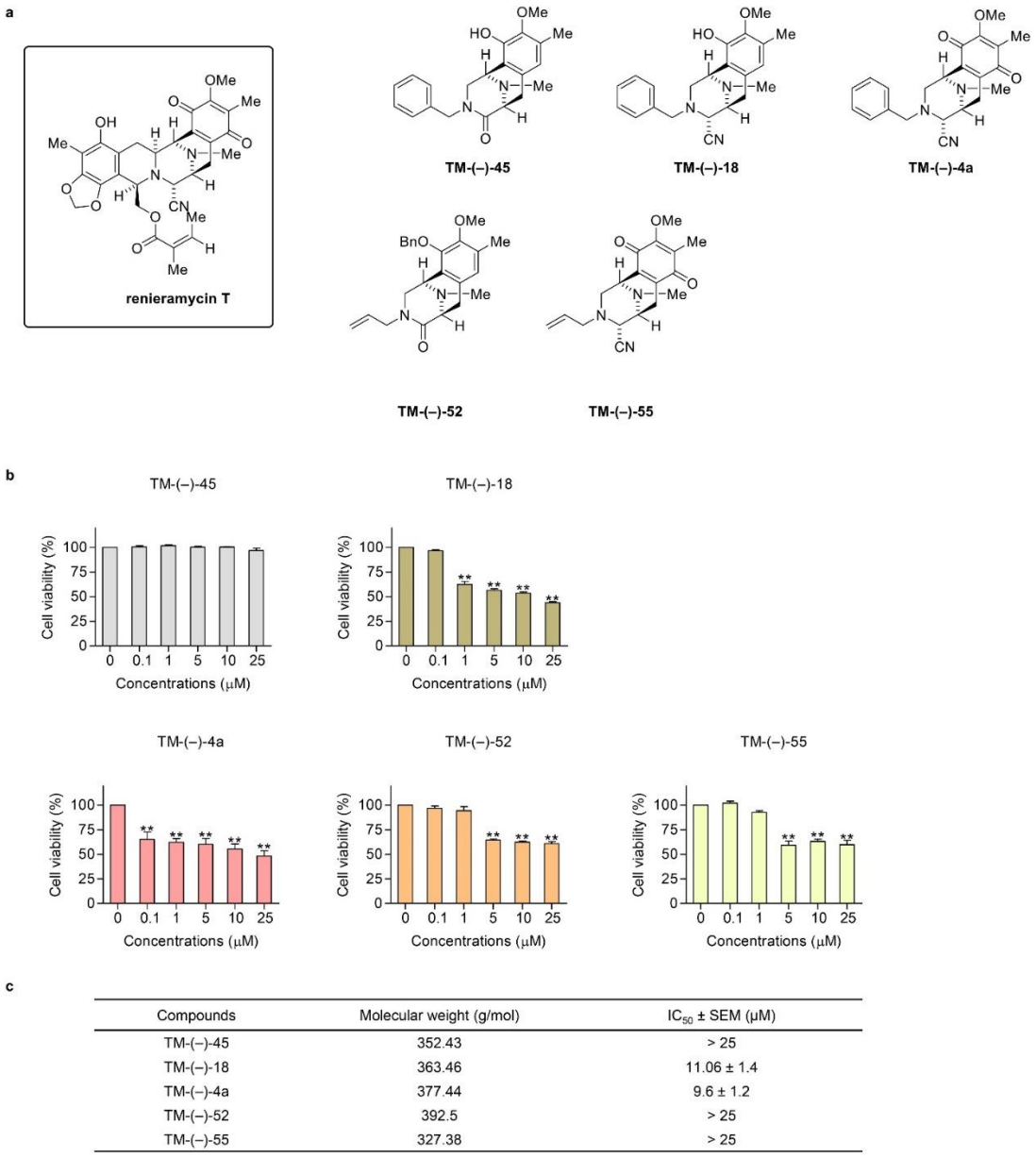
The morphology of NSCLC and patient-derived primary cancer cell lines and their molecular characteristics. (**c**) H460, ELC12, ELC16, ELC17, and ELC20 cells were seeded and treated with 0–25 μM of RT or chemotherapeutic drugs (cisplatin, etoposide, and doxorubicin) for 24 h. Then, the MTT assay was performed to determine the percentages of cell viability. (**d**) The IC_{50} in all cells was calculated in comparison to the untreated control. (**e–f**) Cells were seeded and treated with 0–10 μM of RT or chemotherapeutic drugs (cisplatin, etoposide, and doxorubicin) for 24 h before adding Hoechst 33342 and PI to stain the cell nucleuses. Images were detected by using a fluorescence microscope and the percentages of nuclear-fragmented and propidium iodide (PI)-positive cells were calculated. (**g**) ELC12, ELC16, ELC17, and ELC20 cells were treated with 0–1 μM of RT for 24 h. Western blot analysis was performed to detect the PARP and cleaved PARP protein levels. The blots were reprobed with β-actin to confirm an equal loading of each of the protein samples and densitometry was used to calculate the protein expression levels. Densitometric values of protein levels were presented as the fold changes relative to uncleaved form of the protein. Data represent the mean ± SEM (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the untreated control). Gene symbols: KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog), PI3KCA (Phosphatidylinositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), CDKN2A (Cyclin Dependent Kinase Inhibitor 2A), EGFR (Epidermal Growth Factor Receptor), ALK (Anaplastic Lymphoma Receptor Tyrosine Kinase). Abbreviations: N/A (not available), del (deletion), WT (wild type), Rx (treatment).

2.2. Cytotoxic and Mcl-1-targeting Activities of the Simplified Right-half model of RT Compounds

The SAR of a bioactive compound is very useful information that could facilitate the development of drugs, as this information describes the critical parts to be exploited for drug action as well as helps to increase the potency of the initially detected activity. As RT has been shown to have promising anti-cancer activity against lung cancer through its Mcl-1-targeted activity, as demonstrated in our previous study [23], we designated RT as a lead compound and generated simplified right-half models in order to elucidate the SAR. The structures of RT and the simplified right-half model of RT compounds, namely TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55, are shown in Figure 2a. The synthesized compounds with various modifications were then tested for cytotoxicity in H460 cells. The results obtained from the MTT assay showed that TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55 significantly decreased the cell viability of the lung cells, whereas TM-(–)-45 had no effect (Figure 2b). Moreover, the IC_{50} values of the simplified right-half model of RT compounds were determined, and the results suggested that TM-(–)-18 and TM-(–)-4a retained the cytotoxicity of RT (Figure 2c).

In order to verify the Mcl-1-targeted activity of the simplified right-half compounds of RT, we first confirmed the Mcl-1-targeted action of RT in primary lung cancer cells. We determined the Mcl-1 level in H460, ELC12, and ELC16 by immunofluorescence assay. The results showed that RT reduced the fluorescence intensity, reflecting the Mcl-1 protein, in H460, ELC12, and ELC16 cells (Figure 3a). Western blot analysis was used to confirm the effect of RT on Mcl-1 and its possible effect on the anti-apoptotic Bcl-2 proteins. The Mcl-1, Bcl-2, and proapoptotic Bax in the Bcl-2 family proteins were determined and the results showed that RT significantly decreased the level of Mcl-1 and Bcl-2 in primary lung cancer cells. In contrast, RT had no effect on the Bax protein (Figure 3b). It was noteworthy that while we showed in this study that RT reduced both Mcl-1 and Bcl-2 in primary lung cancer cells, RT was previously shown to have a minimal effect on Bcl-2 in H460 cells [28].

Next, we examined whether the simplified right-half model of RT could target the levels of Mcl-1, Bcl-2, and Bax. Consequently, H460 cells were treated with all the compounds of the RT right-half models and it was found that only TM-(–)-18 and TM-(–)-4a significantly decreased the level of Mcl-1 in H460 cells (Figure 3c). Together with the results of the cytotoxicity tests in Figure 2b and c that indicate that only TM-(–)-18 and TM-(–)-4a exerted significant cytotoxicity, it is very likely that the cytotoxic mode of action of TM-(–)-18 and TM-(–)-4a depended on their Mcl-1-targeting activity.



c

Compounds	Molecular weight (g/mol)	IC_{50} ± SEM (μM)
TM-(–)-45	352.43	> 25
TM-(–)-18	363.46	11.06 ± 1.4
TM-(–)-4a	377.44	9.6 ± 1.2
TM-(–)-52	392.5	> 25
TM-(–)-55	327.38	> 25

Figure 2. Cytotoxic effects of the simplified right-half model of RT compounds on the NSCLC cell line (H460). (**a**) The structures of RT and the simplified right-half model of RT compounds: TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55. (**b**) The H460 cell line was treated with 0–25 μM of the compounds for 24 h. Then, the MTT assay was used to determine the percentages of cell viability. (**c**) IC_{50} values in each cell line were calculated in comparison to the untreated control. Data represent the mean ± SEM (n = 3) (* $0.01 \le p < 0.05$, ** $p < 0.01$, compared with the untreated control).

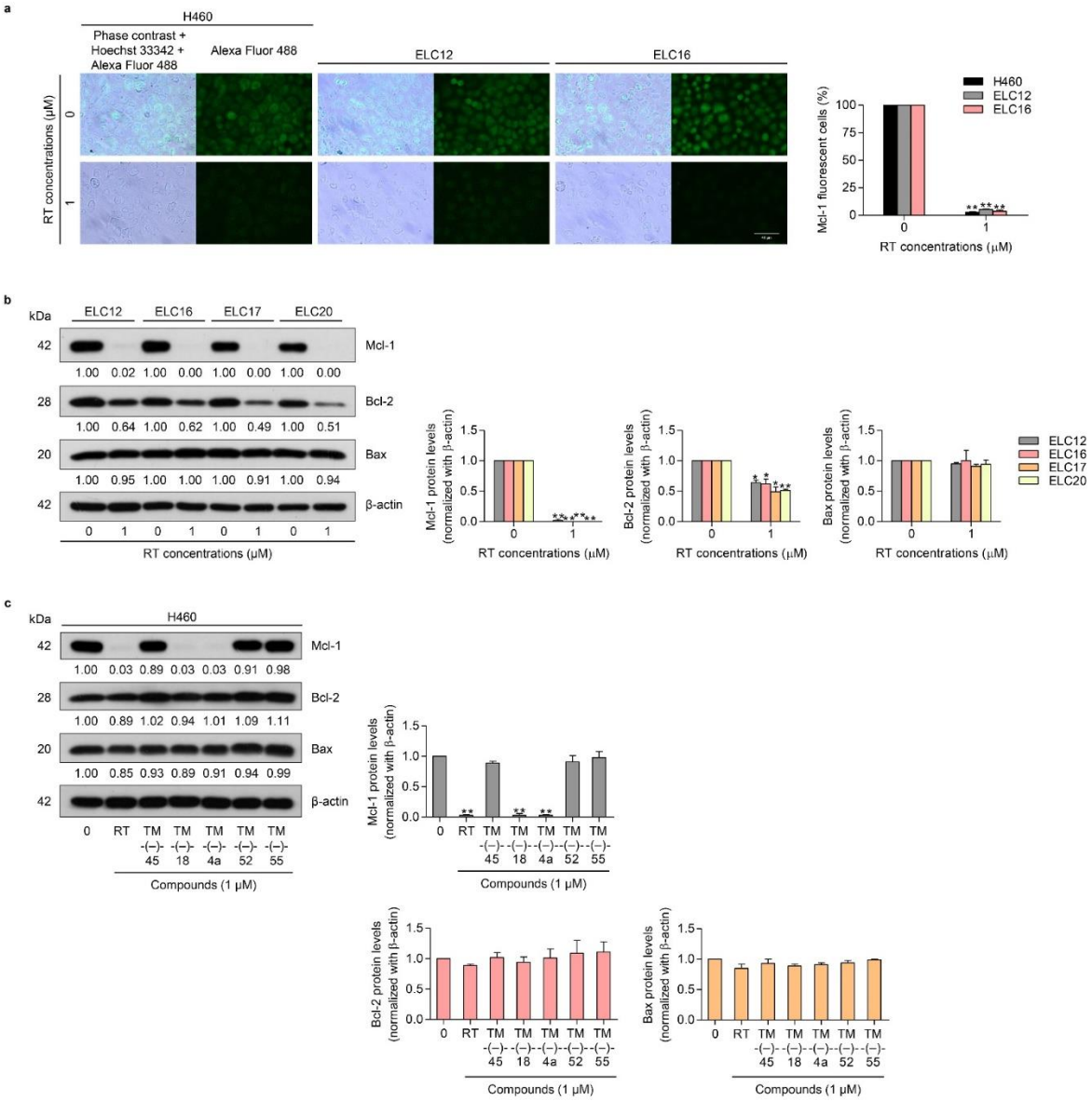


Figure 3. Effects of RT on the expression of the apoptotic-related proteins: Mcl-1, Bcl-2, and Bax in the NSCLC cell line (H460) and patient-derived primary cancer cell lines (ELC12, ELC16, ELC17, and ELC20). (**a**) H460, ELC12, and ELC16 cell lines were seeded and treated with 0–1 μM of RT for 24 h. Then, immunofluorescence analysis was performed using an antibody against Mcl-1. The Alexa Fluor 488 conjugated secondary antibody and Hoechst 33342 were added and the images were visualized under a fluorescence microscope. (**b**) ELC12, ELC16, ELC17, and ELC20 cell lines were seeded and treated with 0–1 μM of RT for 24 h. Western blot analysis was performed to detect the Mcl-1, Bcl-2, and Bax protein levels. The blots were reprobed with β-actin to confirm

an equal loading of each of the protein samples and densitometry was used to calculate the relative protein levels. Densitometric values of protein expression levels were presented as the fold changes relative to β-actin. (**c**) H460 cells were seeded and treated with 0–1 μM of RT, TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55 for 24 h. After that, Western blot analysis was performed to detect the Mcl-1, Bcl-2, and Bax protein levels. Data represent the mean ± SEM (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the untreated control).

2.3. TM-(–)-18 and TM-(–)-4a compounds Induced Apoptotic Cell Death in Patient-derived Primary Cancer Cell Lines Through the Suppression of Mcl-1 and Bcl-2

Having shown the potential Mcl-1 suppression in H460 cells, we next confirmed the Mcl-1- and Bcl-2-targeting activity of TM-(–)-18 and TM-(–)-4a in primary lung cancer cells. ELC12, ELC16, ELC17, and ELC20 cells were treated with 0–25 μM of TM-(–)-18 and TM-(–)-4a for 24 h, and cell viability, apoptosis induction, and the Mcl-1, Bcl-2, and Bax levels were determined. The results showed that TM-(–)-18 and TM-(–)-4a decreased cell viability in all the cell lines (Figure 4a). Next, the morphology of the apoptotic cell death was evaluated by Hoechst33342 and PI double staining. Patient-derived primary cancer cells were treated with TM-(–)-18 and TM-(–)-4a at various concentrations for 24 h, and then the cells were co-stained with Hoechst 33342 and PI. The results revealed that TM-(–)-18 and TM-(–)-4a increased apoptotic cells in a concentration-dependent manner, as shown in Figure 4b and S3a. Immunofluorescence detecting of the Mcl-1 protein in the H460, ELC12, and ELC16 cell lines in response to TM-(–)-18 and TM-(–)-4a treatment showed that TM-(–)-18 and TM-(–)-4a significantly reduced the Mcl-1 fluorescence intensity in all the cells (Figure 4c). Consistently, the protein analysis of Mcl-1, Bcl-2, and Bax indicated that treatment of the cells with TM-(–)-18 and TM-(–)-4a caused a dramatic reduction in the Mcl-1 protein in all the primary lung cancer cells (Figure 4d). The Bcl-2 protein was found to significantly decrease in a concentration-dependent manner, while the treatment had no effect on the Bax protein (Figure 4d). Taken together, this result implied that the mechanism of action of RT and the simplified right-half model of RT compounds (TM-(–)-18 and TM-(–)-4a) involved the targeting of the Mcl-1 protein and, at least in part, Bcl-2 suppression.

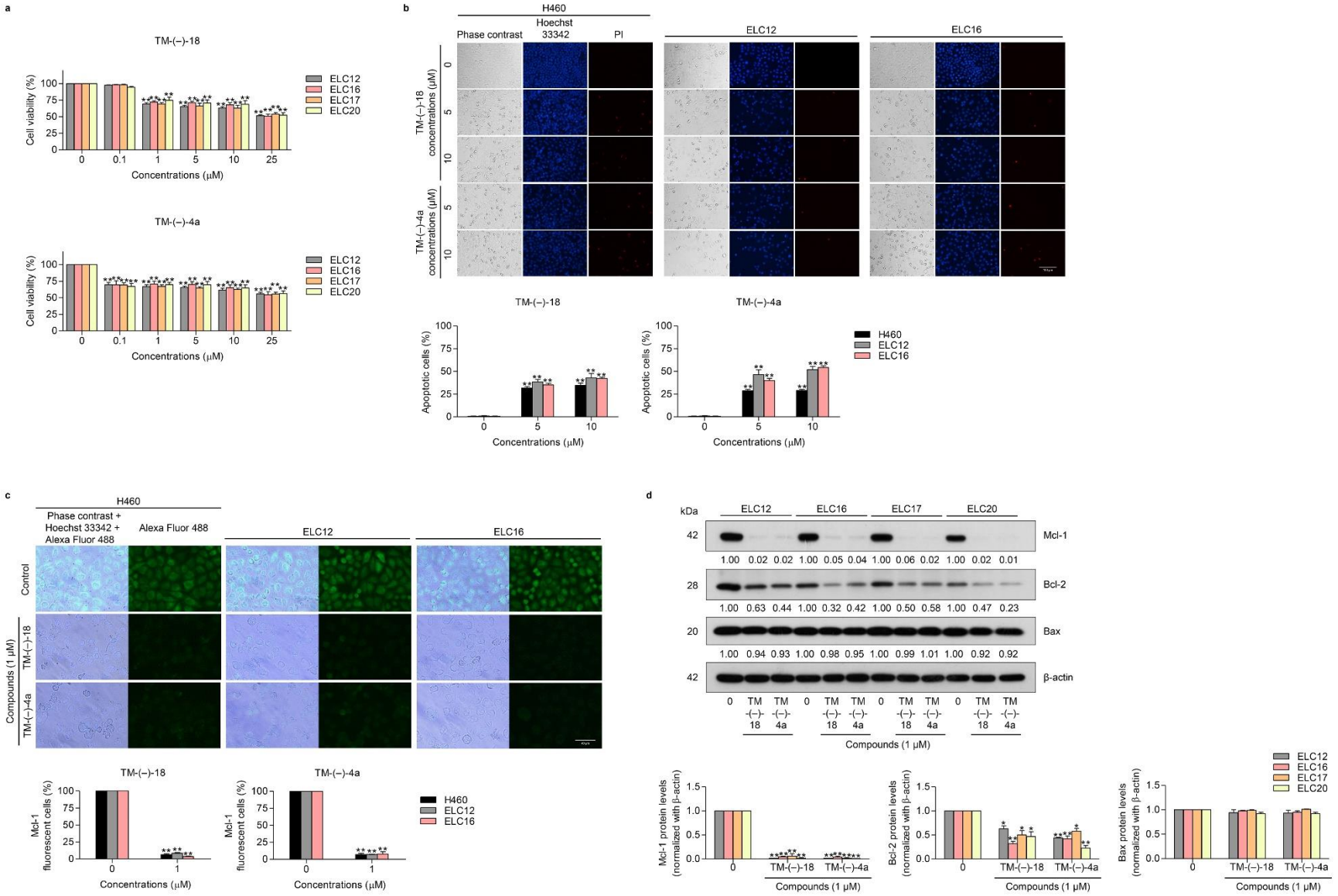

a
TM-(−)-18
Cell viability (%)
Concentrations (μM)
ELC12
ELC16
ELC17
ELC20
TM-(−)-4a
b
H460
Phase contrast
Hoechst 33342
PI
ELC12
ELC16
TM-(−)-18 concentrations (μM)
TM-(−)-4a concentrations (μM)
Apoptotic cells (%)
H460
c
Phase contrast + Hoechst 33342 + Alexa Fluor 488
Alexa Fluor 488
Control
Compounds (1 μM)
Mcl-1 fluorescent cells (%)
d
kDa
Mcl-1
Bcl-2
Bax
β-actin
Mcl-1 protein levels (normalized with β-actin)
Bcl-2 protein levels (normalized with β-actin)
Bax protein levels (normalized with β-actin)

Figure 4. Effects of the simplified right-half model of RT compounds: TM-(–)-18 and TM-(–)-4a on cell viability, apoptotic induction, and apoptotic-related protein expressions in patient-derived primary cancer cell lines (ELC12, ELC16, ELC17, and ELC20). (**a**) ELC12, ELC16, ELC17, and ELC20 cells were seeded and treated with 0–25 μM of TM-(–)-18 and TM-(–)-4a for 24 h. Then, the MTT assay was performed to determine the percentages of cell viability. (**b**) H460, ELC12, and ELC16 cells were seeded and treated with 0–10 μM of TM-(–)-18 and TM-(–)-4a for 24 h. Hoechst 33342 and PI were added and then the images were visualized using a fluorescence microscope. (**c**) H460, ELC12, and ELC16 cells were seeded and treated with 0–1 μM of TM-(–)-18 and TM-(–)-4a for 24 h before performing immunofluorescence using the Mcl-1 primary antibody. Alexa Fluor 488 conjugated secondary antibody and Hoechst 33342 were added and the images were visualized under a fluorescence microscope. (**d**) ELC12, ELC16, ELC17, and ELC20 cell lines were seeded and treated with 0–1 μM of TM-(–)-18 and TM-(–)-4a for 24 h. Western blot analysis was performed to detect the Mcl-1, Bcl-2, and Bax protein levels. The blots were reprobed with β-actin to confirm an equal loading of each of the protein samples and densitometry was used to calculate the relative protein levels. Densitometric values of protein expression levels were presented as the fold changes relative to β-actin. Data represent the mean ± SEM (n = 3) (* $0.01 \leq p < 0.05$, ** $p < 0.01$, compared with the untreated control).

2.4. Molecular Docking Simulation Revealed the RT, TM-(–)-18, and TM-(–)-4a Interactions with the Mcl-1 Protein

Regarding SAR, we compared the chemical structures of the active (TM-(–)-18 and TM-(–)-4a) and non-active (TM-(–)-45, TM-(–)-52, and TM-(–)-55) compounds of the RT simplified right-half model and found that those in the circled part in Figure 5a could be the important groups for Mcl-1 suppression, as this part has a similar structure among RT, TM-(–)-18, and TM-(–)-4a, but not in the inactive compounds. To confirm the interaction between RT, TM-(–)-18, and TM-(–)-4a and the suspected moieties on the Mcl-1-targeting action, molecular docking simulation was applied. Moreover, the relative binding energies of the three simplified right-half model of RT compounds (TM-(–)-18, TM-(–)-4a, and TM-(–)-45) in comparison to the prototype compound (RT) were estimated and insights into how these ligands bind to the binding site of Mcl-1 were gained. It should be noted that the relationship between the binding energy derived from the docking calculations and the binding affinity was associated with the concept of, 'The higher the binding affinity of the compound, the lower the docking energy' [29]. The docking results showed that the binding affinity of TM-(–)-18 and TM-(–)-4a to Mcl-1 was in a similar range to that of RT (binding

energies of −6.9, −7.5, and −7.1 kcal/mol for TM-(−)-18, TM-(−)-4a, and RT, respectively) (Figure 6a–c and 6e). This prediction reflected that these three compounds could bind well to the binding site of Mcl-1, as previously supported by the experimental data. Nonetheless, the binding energy of TM-(−)-45 (−6.0 kcal/mol) was somewhat higher than those of the other compounds (Figure 6d), indicating that this compound exhibited the lowest binding affinity toward Mcl-1, leading to its incapability to induce Mcl-1 depletion. This was possibly due to the distinct orientation of TM-(−)-45 within the binding site of Mcl-1, in which both its benzene ring (circled part in Figure 5a) moieties pointed outward to the protein binding pocket, thus creating weaker interactions with the critical residues (Arg137, Pro138, Ala139, Val140, Leu141, and Pro142).

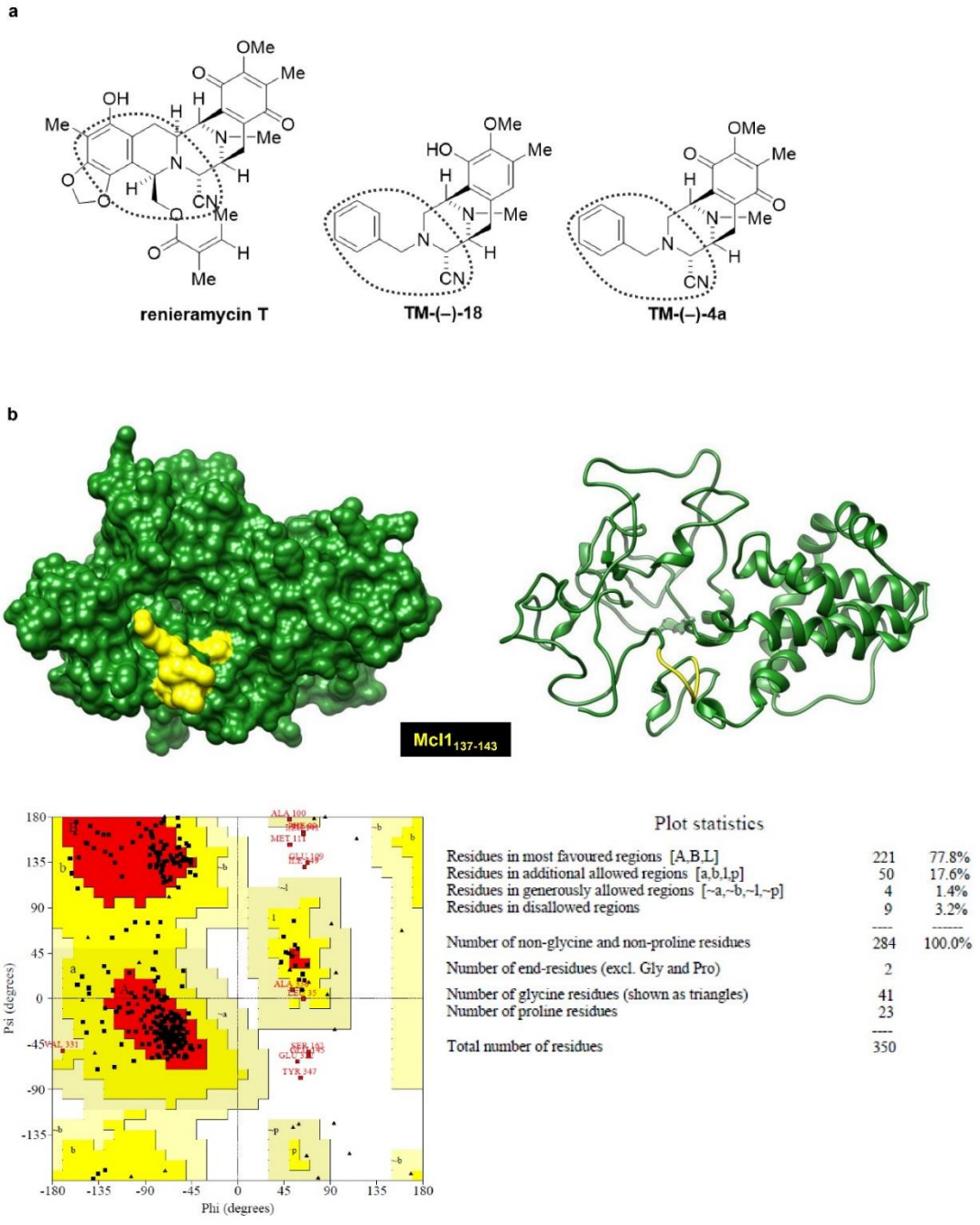


Figure 5. (**a**) Structures of RT, TM-(–)-18, and TM-(–)-4a. Circled part represents a similar structure between these three compounds. (**b**) Stereochemical quality of the homology model of Mcl-1 created by the I-TASSER server. Ramachandran plot of Mcl-1 generated by PROCHECK. Areas colored by red, yellow, beige, and white indicate the most favored, additionally allowed, generously allowed, and disallowed regions, respectively.

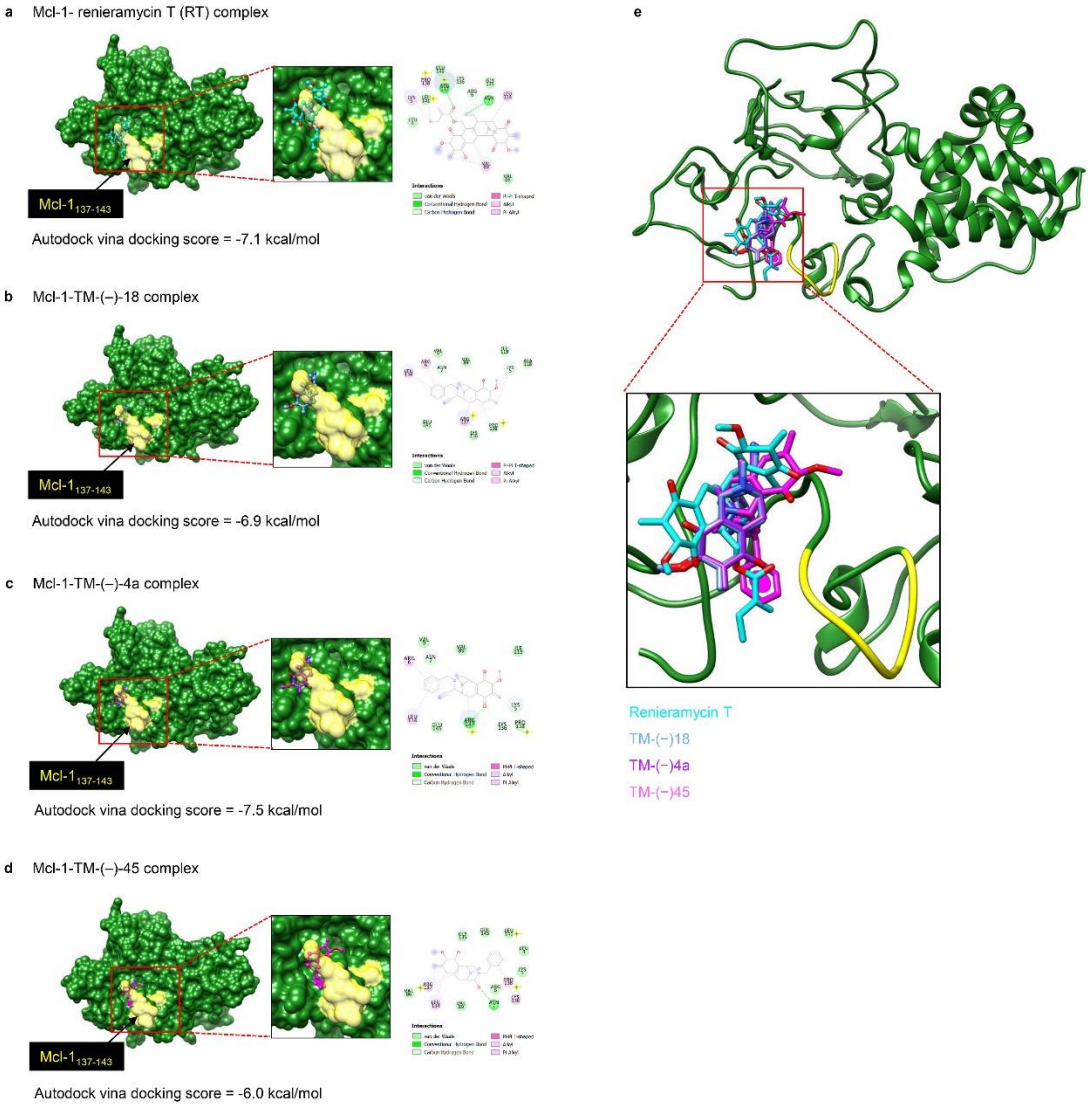


Figure 6. Binding mode and docking energy of: (**a**) renieramycin T, (**b**) TM-(–)-18, (**c**) TM-(–)-4a, and (**d**) TM-(–)-45 bound to the binding site of Mcl-1 (residues 137–143) taken from the AutoDock Vina molecular docking study. (**e**) Superimposition structure of each compound at the binding site of Mcl-1.

3. Discussion

In cancer biology, it is well known that the evasion of apoptosis is a prominent hallmark of cancer [3]. Apoptosis is an important component of various processes as it contributes to the elimination of unwanted cells to maintain the equivalence between cell survival and cell death. Therefore, the dysregulation of apoptosis results in sustained cell proliferation and enhanced tumor

development [30], and consequently, drugs or treatment strategies that can restore the apoptotic signaling pathways may benefit the management of cancer.

The essential regulators of apoptosis are the Bcl-2 family of proteins [31]. Regarding evading apoptosis, the roles of anti-apoptotic members, like Mcl-1 and Bcl-2, have been shown in many *in vitro* and *in vivo* studies [5,15,32–36]. The protein levels of Bcl-2 and Mcl-1 in various types of tumor tissues were found to be frequently augmented at a higher ratio than the other anti-apoptotic proteins in the Bcl-2 family [26]. In particular, increased expressions of Bcl-2 and Mcl-1 reflect a poor prognosis for many malignancies, including lung cancer [37–39]. Not only is their increased expression critical for oncogenesis and cancer progression, but these proteins are also involved in conferring chemotherapeutic drug resistance [35,40–43]. Research was performed using Bcl-2 as a target for overcoming chemoresistance through BCL2 gene silencing to improve the clinical outcome in small-cell lung cancer [44]. Furthermore, in a mouse lung adenocarcinoma model, Mcl-1 overexpression was shown to help tumor progression by inhibiting Myc-induced apoptosis [40]. Taken together, compounds with potent activity for eliminating Bcl-2 or Mcl-1 in cancer cells are of great interest as good candidates for targeted therapy. In agreement with the use of Mcl-1 as a target for cancer therapy [11], our previous study highly supported this concept, where in our experiments, the treatment of RT in an NSCLC cell line (H460) resulted in apoptotic cell death through an Mcl-1 proteasomal degraded mechanism [23]. In the same way, not only in a known lung cancer cell line, but we also proved that the absence of Mcl-1 after RT treatment in primary lung cancer cell lines derived from patients (Figure 3a and 3b) could trigger the apoptotic pathway, which led to the death of cancer cells (Figure 1f and 1g).

Renieramycin T (RT) (Figure 1a) is a tetrahydroisoquinoline alkaloid compound that is part of the renieramycin family and was first isolated from the blue sponge *Xestospongia* sp. by pretreatment with potassium cyanide in a study in 2009 [45]. Recently, its anti-cancer activities have been reported against several types of cancer cells, such as colon (HCT116), prostate (DU145) [46], non-small cell lung (H292, H460, and QG56) [47], breast (T47D), and pancreatic (AsPC1) cancer cells [45]. Moreover, a modified form of RT, 5-O-acetyl-renieramycin T, was shown to induce the death of lung cancer stem cells and sensitize cisplatin-mediated apoptosis in lung cancer cells [48]. Our previous study revealed that the effects of RT on the apoptotic mechanism depended on the disappearance of Mcl-1 through the increase in Mcl-1 protein degradation [23]. In this study, we further confirmed that RT also had anti-cancer activities as well as Mcl-1-targeted activity in patient-derived primary lung cancer cells, with a lower IC_{50} compared to other first-line chemotherapeutic drugs (Figures 1b–g, 3a, and 3b). It was newly discovered that RT could decrease the level of the Bcl-2 protein in primary lung cancer cells, while we did not observe this effect before in the lung

cancer cell line H460 [23]. The explanation for this may due to the specific mutations of H460 cells, including KRAS and PI3K. The mutation of these two oncogenes may cause a high expression of Bcl-2 in H460 cells [28]. Gathering all the collected information, it is not surprising that RT could be an outstanding applicant for further clinical investigation in anti-cancer drug development.

In spite of the marvelous anti-cancer effects of RT, it is hard to deny that RT is difficult to fabricate in massive large-scale synthesis due to its large and complicated structure. Besides, its structure–activity relationships are still unrecognized. Therefore, simplified right-half model of RT compounds (TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55) were established using RT as a prototype (Figure 2a) to help define which part of RT is necessary for achieving the Mcl-1-targeted effect. After screening by treatment against the H460 cell line, we found that only TM-(–)-18 and TM-(–)-4a exerted cytotoxic activities, with IC_{50} values less than 25 μM (Figure 2b and 2c). Moreover, when we performed Western blot analysis using a primary antibody against Mcl-1, the results revealed that treatment with TM-(–)-18 and TM-(–)-4a could diminish Mcl-1 protein levels in the H460 cell line compared to the untreated control and the other simplified right-half model of RT compounds (Figure 3c). Having compared the structures between the compounds causing Mcl-1 depletion, namely RT, TM-(–)-18, and TM-(–)-4a, and the compounds not causing Mcl-1 depletion, namely TM-(–)-45, TM-(–)-52, and TM-(–)-55, we identified two similar parts among the Mcl-1-depleted compounds that did not simultaneously appear in the others. Hence, we hypothesized that both cyanide and the benzene ring in the circled parts in Figure 5a were essential in the Mcl-1-depletion mechanism. Not only the H460 cell line, but we also examined the effects of TM-(–)-18 and TM-(–)-4a with patient-derived primary lung cancer cells. TM-(–)-18 and TM-(–)-4a demonstrated cytotoxic effects and apoptotic induction effects in both NSCLC (H460) and patient-derived primary lung cancer cells (Figures 4a, 4b and S3a). Furthermore, the results from the TM-(–)-18 and TM-(–)-4a treatments showed a decrease in Mcl-1 and Bcl-2 protein levels in primary lung cancer cells (Figure 4c and 4d), but as we previously discussed in the section about the RT-treatment conditions, Mcl-1 depletion was the main mechanism by which these compounds induced apoptosis.

It has been shown that the anti-apoptotic potency of the Bcl-2 family of proteins primarily relies on their stability [49]. Mcl-1 is considered a very unstable protein compared to the other Bcl-2 family proteins and the degradation of Mcl-1 can be induced by anti-cancer agents [50–53]. The stabilization and degradation of Mcl-1 were reported to be regulated by a phosphorylation mechanism at the PEST region, which is the N-terminus of Mcl-1 enriched in proline (P), glutamate (E), serine (S), and threonine (T) residues and rich in putative phosphorylation sites [54]. Several pieces of evidence have suggested that the phosphorylation of Mcl-1 induced by extracellular

signal-regulated kinases (ERK), c-Jun NH2-terminal kinase (JNK), and p38 at threonine 92, threonine 163, and serine 121 can stabilize the Mcl-1 protein level [55–57]; whereas, the phosphorylation by glycogen synthase kinase-3 (GSK-3) at serine 155 and serine 159 destabilizes the Mcl-1 protein level and inhibits the interaction between Mcl-1 and the pro-apoptotic protein Bim [58–61]. From this information, we made the hypothesis that, supposing our compounds attached Mcl-1 at ERK, JNK, or p38 binding sites, they might prevent the phosphorylation mechanism of those molecules and lead to destabilization of the Mcl-1 protein level. To prove our hypothesis, we applied computational molecular docking using the mitogen-activated protein kinase (MAPK) docking motif, also known as the D-motif, found in the Mcl-1 protein sequence 137–143 as the target for the compounds and Mcl-1 interactions to characterize the behavior of these small molecule compounds at the binding site of the Mcl-1 protein [62,63]. Likewise, the circled part of the compounds in Figure 5a, which it is supposed are necessary for their mechanisms, was deployed as a docking site for examination of the binding properties and SARs. After investigation (Figure 6a–e), the results revealed that the binding affinity of RT, TM-(–)-18, and TM-(–)-4a was high and in a similar range, which indicated their ability to bind properly with Mcl-1 and induce destabilization of the protein; whereas, the binding affinity of TM-(–)-45, which has a benzene ring but no cyanide, was lower than the others, thus reflecting its incapability to induce Mcl-1 destabilization, as previously supported by the experimental data.

The appearance of both cyanide and a benzene ring (Figure 5a) in RT, TM-(–)-18, and TM-(–)-4a was found to be necessary for the induction of Mcl-1 destabilization. This study energetically supported the SAR concept, which is key to many aspects of new drug discovery [64] and provides beneficial information for further anti-cancer drug modification and drug development as SARs can then be used to predict the activities of new molecules from their molecular structure because there is a relationship between molecular structures and their biological activity. This allows the modification of the effect or the potency of a bioactive compound by changing its chemical structure. Therefore, the SAR concept is essential in drug discovery to guide the acquisition or synthesis of desirable new compounds, as well as to further characterize existing molecules.

Our results suggested that RT, TM-(–)-18, and TM-(–)-4a exerted an apoptotic induction effect via the destabilization of the Mcl-1 protein (Figure 7). The interaction of the protein with the compounds promoted the ubiquitination and proteasomal degradation of Mcl-1 protein. As Mcl-1 has an important function to inhibit apoptosis by interacting and sequestering the pro-apoptotic Bak protein, the diminishing of Mcl-2 cause by RT and its analogues could facilitate the Bak dimerization. The decrease in cellular Mcl-1 protein or the disruption of Mcl-1-Bak interaction can

lead to Bak oligomerization, mitochondrial membrane destabilization, cytochrome c release, and apoptosis [25,30].

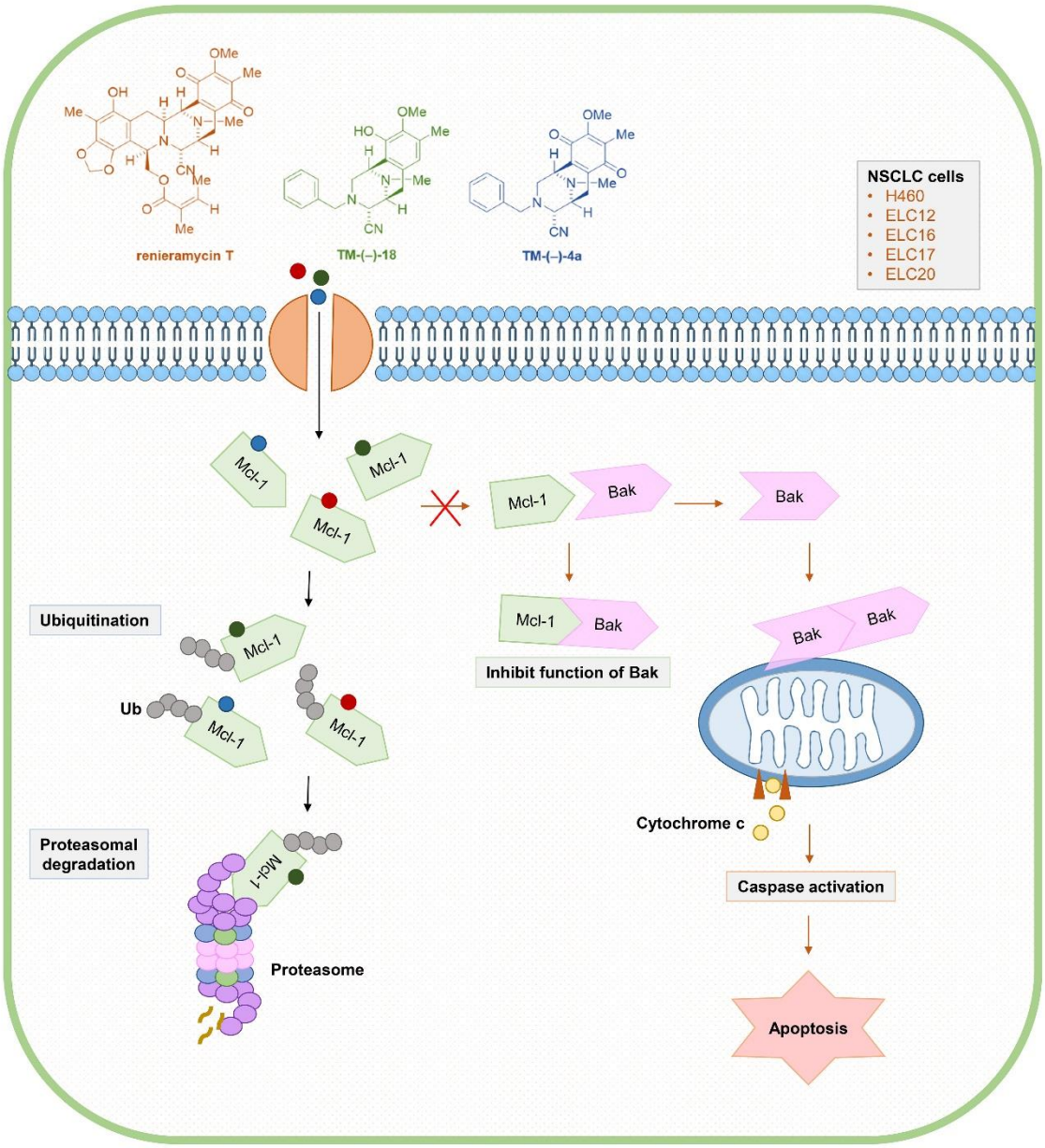


Figure 7. Renieramycin T (RT) and the simplified right-half model of RT compounds: TM-(–)-18 and TM-(–)-4a could enhance the absence of the Mcl-1 protein levels through Mcl-1 proteasomal degradation, resulting in the apoptosis of NSCLC cells. Mcl-1 normally functions as an anti-apoptotic protein by forming a complex with Bak. However, when Mcl-1 disappears, Bak is relieved to form an oligomerization that can permeabilize the

outer membrane of mitochondria. Cytochrome c is released to initiate the apoptosis mechanism through caspase activation.

4. Materials and Methods

4.1. Non-small Cell Lung Cancer Cell Lines and Cultures

Non-small cell lung cancer cells used in the experiments including NCI-H460 [H460] (ATCC® HTB-177™, RRID: CVCL_0459), NCI-H292 [H292] (ATCC® CRL-1848™, RRID: CVCL_0455), NCI-H23 [H23] (ATCC® CRL-5800™, RRID: CVCL_1547), and A549 (ATCC® CCL-185™, RRID: CVCL_0023) were obtained from the American Type Culture Collection (Manassas, VA, USA). H460, H292, and H23 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium, and A549 cells was grown in Dulbecco's Modified Eagle Medium (DMEM). All cell culture mediums were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/mL of each of penicillin and streptomycin. Cells were placed in a humidified atmosphere of 5% carbon dioxide (CO_2) at 37 °C.

4.2. Patient-derived Primary Lung Cancer Cell Line Preparation from Malignant Pleural Effusion

The patient-derived malignant cancer cells were isolated from pleural effusions of recurrent or advanced stage non-small cell lung cancer patients who had been diagnosed at the King Chulalongkorn Memorial Hospital. The protocol of conduction was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB 365/62) and was obtained informed consents from all participants. This study was carried out in accordance with the principles of World Medical Association Declaration of Helsinki. Primary cancer cells were collected from pleural effusion (500–1000 mL) through thoracentesis. The collected samples were centrifuged at 300 g for 10 min, at 4 ˚C and the cells were resuspended in RPMI medium with 10% FBS, 2 mM L-glutamine, and 100 units/ml of each of penicillin and streptomycin. After culturing for 10–15 passages, they were characterized as the patient-derived primary cancer cell lines (ELC12, ELC16, ELC17, and ELC20). The characteristics as well as their status of mutation were presented in the Figure 1b.

4.3. Renieramycin T (RT) Prepararation

From the previous study [23], renieramycin T (RT) was isolated from the Thai blue sponge *Xestospongia* sp. collected from Sichang Island, Chonburi Province, Thailand. After isolated process, RT was obtained in an orange amorphous solid form with the purity more than 99% and spectroscopic data matching to Daikuhara report [45]. The nuclear magnetic resonance (NMR) values were demonstrated in the previous report [23]. Before using in the experiments, RT was

dissolved to 50 mM stock solution by dimethyl sulfoxide (DMSO), stored at -20 °C, and freshly diluted to desired concentrations with medium in each experiment. DMSO final concentration was less than 0.5% referring to non-toxicity for cancer cells.

4.4. Simplified Right-half model of RT Compounds Synthesis

Right-half model of RT compounds in this study namely TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55 were synthesized as described in Figure 8. TM-(–)-18 and TM-(–)-4a were synthesized according to our previous work [65], whereas TM-(–)-45, TM-(–)-52, and TM-(–)-55 were newly synthesized. All compounds were confirmed to have 99% enantiomeric excess HPLC analysis to prove that there is no racemization at the chiral center in the synthetic route.

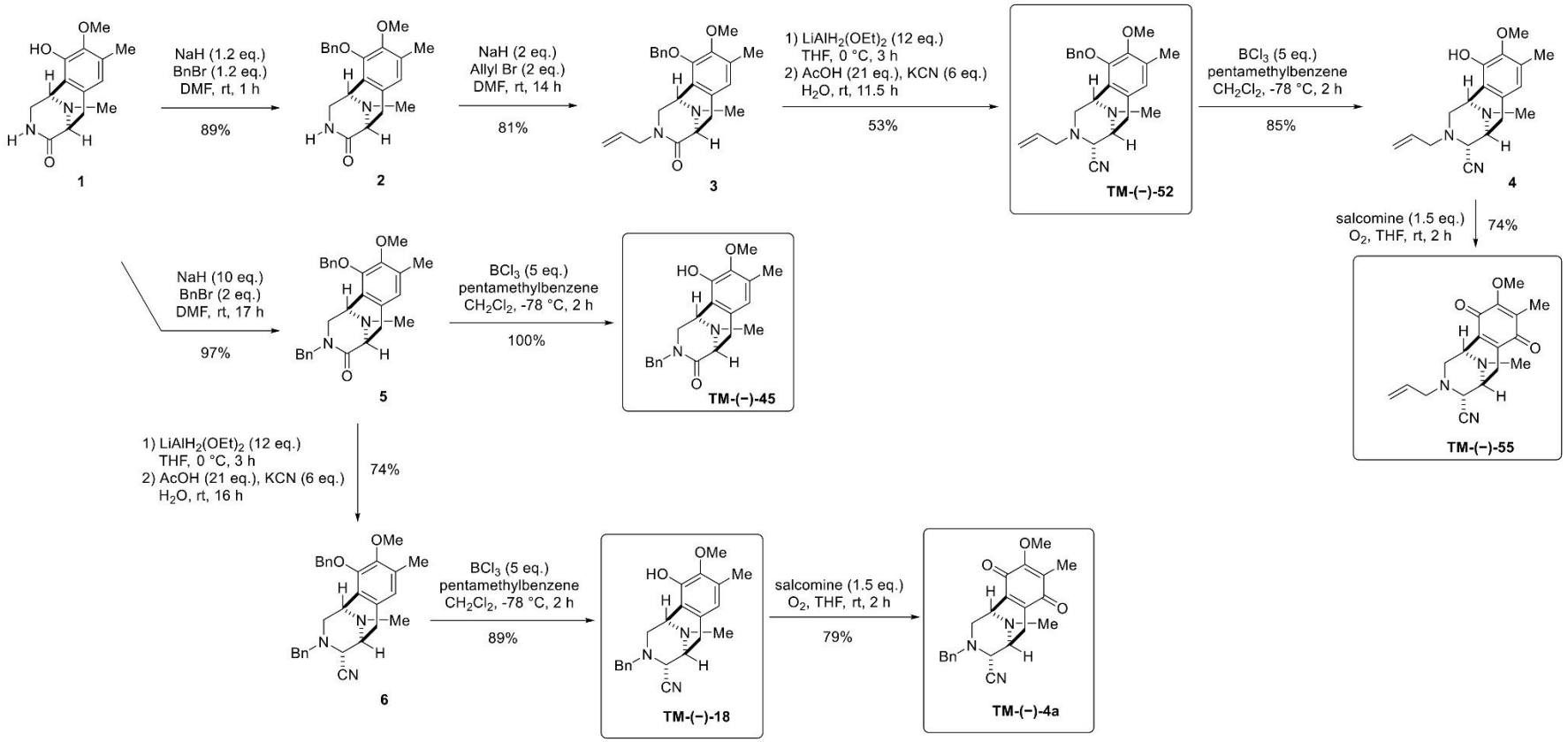

1
NaH (1.2 eq.)
BnBr (1.2 eq.)
DMF, rt, 1 h
89%
2
NaH (2 eq.)
Allyl Br (2 eq.)
DMF, rt, 14 h
81%
3
1) LiAlH2(OEt)2 (12 eq.)
THF, 0 °C, 3 h
2) AcOH (21 eq.), KCN (6 eq.)
H2O, rt, 11.5 h
53%
TM-(−)-52
BCl3 (5 eq.)
pentamethylbenzene
CH2Cl2, -78 °C, 2 h
85%
4
salcomine (1.5 eq.)
O2, THF, rt, 2 h
74%
TM-(−)-55
NaH (10 eq.)
BnBr (2 eq.)
DMF, rt, 17 h
97%
5
BCl3 (5 eq.)
pentamethylbenzene
CH2Cl2, -78 °C, 2 h
100%
TM-(−)-45
1) LiAlH2(OEt)2 (12 eq.)
THF, 0 °C, 3 h
2) AcOH (21 eq.), KCN (6 eq.)
H2O, rt, 16 h
74%
6
BCl3 (5 eq.)
pentamethylbenzene
CH2Cl2, -78 °C, 2 h
89%
TM-(−)-18
salcomine (1.5 eq.)
O2, THF, rt, 2 h
79%
TM-(−)-4a

Figure 8. Simplified right-half model of RT compounds: TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55 synthetic pathways. Compound 1 could be easily obtained from L-Tyr [65]. The phenol of compound 1 was selectively protected with 1.2 eq. of NaH and BnBr in DMF to give compound 2. The alkylation of the lactam nitrogen of compound 2 with allyl bromide gave compound 3 in an 81% yield. The lactam carbonyl of compound 2 was partially reduced with $LiAlH_2(OEt)_2$ in THF to generate the aminal, [66] which was then treated with KCN and H_2O to give the **α**-aminonitrile TM-(–)-52 as a single diastereomer. Chemoselective debenzylation was achieved with BCl_3 in the presence of pentamethylbenzene to give the phenol. [67] Finally, oxidation of this obtained phenol using salcomine with O_2 afforded TM-(–)-55 in a 74% yield; whereas compound 2 was bisbenzylated with 2 eq. of benzylbromide in the presence of 10 eq. of NaH to give compound 5 in a 97% yield. The debenzylation of 19 by using BCl_3 gave the phenol TM-(–)-45. The reductive cyanation of compound 5 generated the aminonitrile compound 6, which was chemoselectively debenzylated with BCl_3 to afford TM-(–)-18 in an 89% yield. Finally, the phenol compound 5 was oxidized with salcomine in an oxygen atmosphere to give TM-(–)-4a.

4.4.1. Synthesis of (1R, 5S)-10-(Benzyloxy)-9-methoxy-8, 11-dimethyl-2, 3, 5, 6-tetrahydro-1, 5-epiminobenzo[d]azocin-4(1H)-one (compound 2), (1R, 5S)-3-Benzyl-10-(benzyloxy)-9-methoxy-8, 11-dimethyl-2, 3, 5, 6-tetrahydro-1, 5-epiminobenzo[d]azocin-4(1H)-one (compound 5), (1R, 4R, 5S)-3-Benzyl-10-(benzyloxy)-9-methoxy-8, 11-dimethyl-1, 2, 3, 4, 5, 6-hexahydro-1, 5-epiminobenzo[d]azocine-4-carbonitrile (compound 6), Synthesis of (1R, 4R, 5S)-3-Benzyl-10-hydroxy-9-methoxy-8, 11-dimethyl-1, 2, 3, 4, 5, 6-hexahydro-1, 5-epiminobenzo[d]azocine-4-carbonitrile (TM-(–)-18), and (1R, 4R, 5S)-3-Benzyl-9-methoxy-8, 11-dimethyl-7, 10-dioxo-1, 2, 3, 4, 5, 6, 7, 10-octahydro-1, 5-epiminobenzo[d]azocine-4-carbonitrile (TM-(–)-4a)

Asymmetric synthesis methods of compound 2, compound 5, compound 6, (TM-(–)-18), and (TM-(–)-4a) were previously described in the Scheme 4 of Matsubara's study 'Asymmetric Synthesis and Cytotoxicity Evaluation of Right-Half Models of Antitumor Renieramycin Marine Natural Products' [65].

4.4.2. Synthesis of (1R, 5S)-3-Allyl-10-(benzyloxy)-9-methoxy-8, 11-dimethyl-2, 3, 5, 6-tetrahydro-1, 5-epiminobenzo[d]azocin-4(1H)-one (Compound 3)

To a solution of lactam compound 2 (10.2 mg, 28.0 μmol) in 1.0 mL dimethylformamide (DMF) was slowly added with sodium hydride, NaH, (60% oil dispersion, 2.6 mg, 57.0 μmol, 2.0 eq.) over 10 min at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, after which allyl bromide,

AllylBr, (7.0 µL, 57.0 µmol, 2.0 eq.) was added slowly. The mixture was stirred at 25 ℃ for 14 h. The mixture was diluted with 10 mL water (H_2O) and extracted with diethyl ether, Et_2O, (3 × 10 mL). The combined extracts were washed with 10 mL brine, dried over sodium sulfate (Na_2SO_4) and concentrated in vacuo to give a residue. The residue was purified by silicon dioxide (SiO_2) flash column chromatography ($CHCl_3$–MeOH = 49:1) to afford compound 3 (9.0 mg, 81%) as a colorless oil. $[\alpha]_D^{26}$ −72.8 (*c* 0.5, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.45–7.32 (5H, m, 10-*O*-Bn-H), 6.70 (1H, s, 7-H), 5.58–5.49 (1H, m, 2'-H), 5.14 (1H, d, *J* = 11.4 Hz, 10-OC<u>H</u>$_2$Ph), 5.08 (1H, d, *J* = 11.4 Hz, 10-OC<u>H</u>$_2$Ph), 4.92 (1H, dd, *J* = 8.9, 1.4 Hz, 3'-H), 4.62 (1H, dd, *J* = 15.6, 1.4 Hz, 3'-H), 3.99–3.90 (1H, s, 1-H, overlapped), 3.99–3.90 (1H, m, 1'-H), 3.81 (3H, s, 9-OCH_3), 3.80–3.72 (1H, m, 1'-H), 3.80–3.72 (1H, m, 2-H, overlapped), 3.60 (1H, brd, *J* = 6.4 Hz, 5-H), 3.12 (1H, dd, *J* = 17.1, 6.4 Hz, 6-H), 3.04 (1H, d, *J* = 11.7 Hz, 2-H), 2.78 (1H, d, *J* = 17.1 Hz, 6-H), 2.30 (3H, s, ^{11}*N*-CH_3), 2.26 (3H, s, 8-CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 169.9 (s, C-4), 149.4 (s, C-9), 148.4 (s, C-10), 137.5 (s, Bn), 132.0 (d, C-2'), 131.9 (s, C-8), 131.4 (d, Bn), 128.6 (d × 2, Bn), 128.5 (s, C-6a), 128.2 (d × 2, Bn), 126.3 (s, C-10a), 125.9 (d, C-7), 116.2 (t, C-3'), 74.4 (t, 2'-O<u>C</u>H_2Ph), 60.1 (q, 9-OCH_3), 59.3 (d, C-5), 51.5 (d, C-1), 51.0 (t, C-2), 48.0 (t, C-1'), 39.7 (q, ^{11}*N*-CH_3), 27.2 (t, C-6), 15.7 (q, 8-CH_3); IR (KBr) 3007, 2940, 2340, 1636, 1493, 1337, 1061, 700 cm^{-1}; EIMS m/z (%) 392 (M^+, 28), 301 (12), 295 (23), 294 (100), 204 (36), 203 (49); HREIMS m/z 392.2099 (M^+, calcd for $C_{24}H_{28}N_2O_3$, 392.2100).

4.4.3. Synthesis of (1R, 4R, 5S)-3-Allyl-10-(benzyloxy)-9-methoxy-8, 11-dimethyl-1, 2, 3, 4, 5, 6-hexahydro-1, 5-epiminobenzo[d]azocine-4-carbonitrile (TM-(–)-52)

To a solution of lactam compound 3 (128 mg, 326 µmol) in 16 mL tetrahydrofuran (THF) at 0 ℃ was added lithium diethoxyaluminum hydride, $LiAlH_2(OEt)_2$, (1.0 mol/L in CH_2Cl_2, 3.90 mL, 3.90 mmol, 12 eq.) over 10 min. The reaction mixture was stirred for 3 h at 0 ℃. The mixture was quenched with acetic acid, AcOH, (390 µL, 6.78 mmol, 20.8 eq.), followed by the addition of potassium cyanide, KCN, (129 mg, 1.96 mmol, 6.0 eq.) in H_2O (2.0 mL), and stirring was continued for 11.5 h at 25 ℃. The mixture was neutralized with 5% sodium bicarbonate ($NaHCO_3$) solution and diluted with saturated Rochell's salt aq., and the mixture was stirred for 1.5 h. The mixture was extracted with 3 × 100 mL of chloroform ($CHCl_3$). The combined extracts were washed with brine (100 mL), dried over Na_2SO_4, and concentrated in vacuo to give a residue. The residue was purified by SiO_2 flash column chromatography (n-Hex.–EtOAc = 2:1) to afford TM-(–)-52 (70.4 mg, 53%) as a colorless oil. $[\alpha]_D^{24}$ −26.9 (*c* 0.2, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41–7.30 (5H, m, 10-*O*-Bn-H), 6.64 (1H, s, 7-H), 5.58–5.48 (1H, m, 2'-H), 5.11–5.02 (2H, m, 3'-H), 5.07 (2H, s, 10-OC<u>H</u>$_2$Ph), 3.92 (1H, brs, 1-H), 3.85 (3H, s, 9-OCH_3), 3.78 (1H, brs, 4-H), 3.25 (1H, brd, *J* = 7.6 Hz, 5-H), 3.05–2.92 (3H, m, 1'-H, 6-H, overlapped), 2.77 (1H, dd, *J* = 11.1, 3.1 Hz, 2-H), 2.57 (1H, d, *J* = 11.1 Hz, 2-H), 2.39

(1H, d, *J* = 17.9 Hz, 6-H), 2.27 (3H, s, 8-CH_3), 2.12 (3H, s, ^{11}*N*-CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 148.9 (s, C-9), 148.3 (s, C-10), 137.5 (s, Bn), 133.5 (d, C-2'), 130.1 (s, C-8), 130.1 (s, C-6a), 128.6 (d × 2, Bn), 128.5 (d × 2, Bn), 128.2 (d, Bn), 126.6 (s, C-10a), 124.4 (d, C-7), 118.8 (t, C-3'), 116.4 (s, 4-CN), 74.5 (t, 10-O$\underline{C}H_2$Ph), 60.0 (q, 9-OCH_3), 58.8 (d, C-4), 57.9 (t, C-1'), 55.2 (d, C-5), 54.2 (t, C-2), 52.6 (d, C-1), 41.2 (q, ^{11}*N*-CH_3), 25.1 (t, C-6), 15.8 (q, 8-CH_3); IR ($CHCl_3$) 3013, 2938, 2824, 2226, 1416, 1323, 1157, 1063, 1028 cm^{-1}; EI-MS *m/z* (%) 403 (M^+, 1), 295 (28), 294 (100), 243 (12), 204 (24), 203 (21); HREIMS *m/z* 403.2263 (M^+, calcd for $C_{25}H_{29}N_3O_2$, 403.2260).

4.4.4. Synthesis of (1R, 4R, 5S)-3-Allyl-10-hydroxy-9-methoxy-8, 11-dimethyl-1, 2, 3, 4, 5, 6-hexahydro-1, 5-epiminobenzo[d]azocine-4-carbonitrile (Compound 4)

To a solution of TM-(–)-52 (60.0 mg, 149 μmol) and pentamethylbenzene (224 mg, 1.49 mmol, 10.0 eq.) in dichloromethane, CH_2Cl_2, (10.0 mL) was added boron trichloride, BCl_3, (1.0 mol/L in CH_2Cl_2, 750 μL, 744 μmol, 5.0 eq.) at −78 °C and the reaction mixture was stirred for 2 h. The mixture was diluted with 10.0 mL CH_2Cl_2 and quenched at 0 °C with saturated $NaHCO_3$ solution. The reaction mixture was extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue. The residue was purified by SiO_2 flash column chromatography (n-Hex.–EtOAc = 2:1) to afford compound 4 (39.5 mg, 85%) as a colorless amorphous. $[\alpha]_D^{26}$ −85.8 (*c* 0.6, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.42 (1H, s, 7-H), 5.62 (1H, brs, 10-OH), 5.59–5.49 (1H, m, 2'-H), 5.15–5.07 (2H, m, 3'-H), 4.05 (1H, brs, 1-H), 3.81 (1H, brs, 4-H), 3.78 (3H, s, 9-OCH_3), 3.31 (1H, brd, *J* = 7.8 Hz, 5-H), 3.09–2.97 (3H, m, 1'-H, 6-H, overlapped), 2.85 (1H, dd, *J* = 11.2, 2.9 Hz, 2-H), 2.72 (1H, d, *J* = 11.2 Hz, 2-H), 2.40 (1H, d, *J* = 17.6 Hz, 6-H), 2.34 (3H, s, ^{11}*N*-CH_3), 2.26 (3H, s, 8-CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 145.4 (s, C-10), 142.7 (s, C-9), 133.6 (d, C-2'), 130.6 (s, C-6a), 128.1 (s, C-8), 120.5 (d, C-7), 119.4 (s, C-10a), 118.8 (t, C-3'), 116.5 (s, 4-CN), 60.7 (q, 9-OCH_3), 58.7 (d, C-4), 57.9 (t, C-1'), 55.3 (d, C-5), 53.5 (t, C-2), 52.3 (d, C-1), 41.5 (q, ^{11}*N*-CH_3), 25.0 (t, C-6), 15.8 (q, 8-CH_3); IR ($CHCl_3$) 3536, 3013, 2940, 2824, 2226, 1458, 1418, 1227, 1157, 1059 cm^{-1}; EIMS *m/z* (%) 313 (M^+, 1), 205 (22), 204 (100); HREIMS *m/z* 313.1789 (M^+, calcd for $C_{18}H_{23}N_3O_2$, 313.1790).

4.4.5. Synthesis of (1R, 4R, 5S)-3-Allyl-9-methoxy-8, 11-dimethyl-7, 10-dioxo-1, 2, 3, 4, 5, 6, 7, 10-octahydro-1, 5-epiminobenzo[d]azocine-4-carbonitrile (TM-(–)-55)

To a solution of phenol compound 4 (24.0 mg, 76.6 μmol) in 1 mL THF was added salcomine (37.7 mg, 115 μmol, 1.5 eq.) at 25 °C, and the mixture was stirred for 2 h under oxygen (O_2) atmosphere. The mixture was filtered through a cellulose pad and washed with ethyl acetate (EtOAc). The filtrate was concentrated in vacuo to give a residue. The residue was purified by SiO_2 flash column chromatography (n-Hex.–EtOAc = 2:1) to afford compound TM-(–)-55 (18.6 mg, 74%)

as a yellow oil. $[\alpha]_D^{26}$ −41.8 (*c* 0.6, $CHCl_3$); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.63–5.52 (1H, m, 2'-H), 5.25 (1H, dd, *J* = 17.2, 1.4 Hz, 3'-H), 5.17 (1H, dd, *J* = 10.3, 1.4 Hz, 3'-H), 4.02 (3H, s, 9-OCH_3), 3.84 (1H, brs, 1-H), 3.74 (1H, brs, 4-H), 3.33 (1H, brd, *J* = 7.6 Hz, 5-H), 3.09 (1H, ddt, *J* = 13.5, 5.3, 1.4 Hz, 1'-H), 3.02 (1H, dd, *J* = 13.5, 7.6 Hz, 1'-H), 2.84 (1H, dd, *J* = 11.6, 3.2 Hz, 2-H), 2.72 (1H, dd, *J* = 20.8, 7.6 Hz, 6-H), 2.55 (1H, d, *J* = 11.6 Hz, 2-H), 2.32 (3H, s, ^{11}*N*-CH_3), 2.17 (1H, d, *J* = 20.8 Hz, 6-H), 1.97 (3H, s, 8-CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 186.9 (s, C-7), 182.3 (s, C-10), 155.2 (s, C-9), 140.9 (s, C-6a), 137.2 (s, C-10a), 133.0 (d, C-2'), 128.7 (s, C-8), 119.8 (t, C-3'), 115.8 (s, 4-CN), 60.9 (q, 9-OCH_3), 57.9 (d, C-4), 57.8 (t, C-1'), 54.4 (d, C-5), 51.8 (t, C-2), 51.2 (d, C-1), 41.4 (q, ^{11}*N*-CH_3), 20.8 (t, C-6), 8.7 (q, 8-CH_3); IR ($CHCl_3$) 2936, 2820, 2228, 1659, 1630, 1308, 1236, 1165 cm^{-1}; EIMS *m/z* (%) 327 (M^+, 8), 220 (16), 219 (95), 218 (100), 204 (35), 201 (12), 190 (11), 176 (11); HREIMS *m/z* 327.1582 (M^+, calcd for $C_{18}H_{21}N_3O_3$, 327.1583).

4.4.6. Synthesis of (1R, 5S)-3-Benzyl-10-hydroxy-9-methoxy-8, 11-dimethyl-2, 3, 5, 6-tetrahydro-1, 5-epiminobenzo[d]azocin-4(1H)-one (TM-(–)-45)

To a solution of compound 5 (30.8 mg, 67.8 μmol) and pentamethylbenzene (101 mg, 678 μmol, 10.0 eq.) in CH_2Cl_2 (10.0 mL) was added BCl_3 (1.0 mol/L in CH_2Cl_2, 340 μL, 339 μmol, 5.0 eq.) and the mixture was stirred for 2 h at −78 °C. The mixture was diluted with 5.0 mL CH_2Cl_2 and quenched at 0 °C with saturated $NaHCO_3$ solution. The reaction mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$–MeOH = 9:1) to afford TM-(–)-45 (14.3 mg, 89%) as a colorless amorphous. $[\alpha]_D^{27}$ −290.7 (*c* 0.8, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.15–7.06 (3H, m, ^{3}*N*-Bn-H), 6.82–6.80 (2H, m, ^{3}*N*-Bn-H) 6.53 (1H, s, 7-H), 4.69 (1H, d, *J* = 15.0 Hz, ^{3}*N*-C<u>H</u>$_2$Ph), 4.35 (1H, d, *J* = 15.0 Hz, ^{3}*N*-C<u>H</u>$_2$Ph), 4.14 (1H, brd, *J* = 4.6 Hz, 1-H), 3.78 (1H, dd, *J* = 11.9, 4.6 Hz, 2-H), 3.70 (1H, d, *J* = 6.4 Hz, 5-H), 3.68 (3H, s, 9-OCH_3), 3.19 (1H, dd, *J* = 17.0, 6.4 Hz, 6-H), 3.10 (1H, d, *J* = 11.9 Hz, 2-H), 2.87 (1H, d, *J* = 17.0 Hz, 6-H), 2.49 (3H, s, ^{11}*N*-CH_3), 2.28 (3H, s, 8-CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.4 (s, C-4), 145.5 (s, C-10), 143.4 (s, C-9), 136.4 (s, C-1'), 129.2 (s, C-8), 129.0 (s, C-6a), 128.3 (d × 2, C-3', C-5'), 127.2 (d × 2, C-2', C-6'), 126.9 (d, C-4'), 121.8 (d, C-7), 119.2 (s, C-10a), 60.6 (q, 9-O<u>C</u>H_3), 59.5 (d, C-5), 51.1 (d, C-1), 49.6 (t, C-2), 49.0 (t, ^{3}*N*-<u>C</u>H_2Ph), 40.0 (q, ^{11}*N*-CH_3), 27.9 (t, C-6), 15.7 (q, 8-CH_3); IR ($CHCl_3$) 3528, 3005, 2934, 2359, 1636, 1495, 1233, 1057, 696 cm^{-1}; EIMS *m/z* (%) 352 (M^+, 14), 205 (19), 204 (100), 189 (12); HREIMS *m/z* 352.1788 (M^+, calcd for $C_{21}H_{24}N_2O_3$, 352.1787).

4.5. Simplified Right-half Model of RT Compounds Preparation

The compounds in solid form were dissolve in DMSO to 50 mM stock solution and stored at -20 ℃. They were freshly diluted to concentrations used in the experiments with an awareness that final concentration of DMSO should be less than 0.5%.

4.6. Reagents and Antibodies

Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM) medium, penicillin/streptomycin, fetal bovine serum (FBS), phosphate-buffered saline (PBS), L-glutamine, and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), propidium iodide (PI), Hoechst 33342, cisplatin, etoposide, doxorubicin, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). The primary antibodies used in the experiments were β-Actin (13E5) rabbit mAb (Cat#4970, RRID: AB_2223172), PARP (46D11) rabbit mAb (Cat# 9532, RRID: AB_2160739), Mcl-1 (D2W9E) rabbit mAb (Cat#94296, RRID: AB_2722740), Bcl-2 (D55G8) rabbit mAb (Cat# 4223, RRID: AB_1903909), and Bax (D2E11) rabbit mAb (Cat#5023, RRID: AB_10557411) which were obtained from Cell Signaling Technology (Danvers, MA, USA). The respective secondary antibodies, anti-rabbit IgG, HRP-linked antibody (Cat#7074, RRID: AB_2099233) was also purchased from Cell Signaling Technology (Danvers, MA, USA). Moreover, goat anti-rabbit IgG H&L (Alexa Fluor 488, Cat#ab150077, RRID: AB_2630356) was obtained from Abcam (Cambridge, MA, USA).

4.7. Cell Viability Assay

NSCLC cell line (H460) and patient-derived primary lung cancer cell lines (ELC12, ELC16, ELC17, and ELC20) were seeded in 96-well plates at the density of $1x10^5$ cells/well. After incubating overnight, they were treated with various concentrations of chemotherapeutic drugs (cisplatin, etoposide, and doxorubicin), RT, and right-half model of RT compounds (TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55) for 24 h. Then, 100 μL per well of MTT solution was added to achieve a final concentration of 400 μg/mL and incubated for an additional 3 h at 37 ℃. Yellow supernatants of MTT were removed and replaced with 100 μl of DMSO to dissolve formazan crystals. The quantity of formazan product proportional to the number of viable cells was measured by recording change in absorbance at 570 nm by a microplate reader (Anthros, Durham, NC, USA). The percentage of cell viability and IC_{50} were determined as described in the manufacturer's protocol (7sea Biotech). Cell viability = (ODexperiment – ODblank) / (ODcontrol – ODblank) × 100%.

4.8. Nuclear Staining Assay

Hoechst 33342 and PI double staining were applied to define apoptotic and necrotic cell death through nuclear co-staining. NSCLC cell lines (H460, H292, H23, and A549) and patient-derived primary lung cancer cell lines (ELC12, ELC16, ELC17, and ELC20) were seeded into 96-well plates at the density of $1x10^5$ cells/well overnight and treated with 5 and 10 μM of chemotherapeutic agents (cisplatin, etoposide, and doxorubicin), RT, and right-half model of RT compounds (TM-(–)-18 and TM-(–)-4a) for 24 h. Afterwards, the cells were stained with 10 μg/mL Hoechst 33342 for 15 min at 37 °C and then stained with 5 μg/mL PI before immediately detecting fluorescence of nuclear-stained cells by fluorescent microscope (Nikon ECLIPSE Ts2, Tokyo, Japan). The number of nuclear condensed and DNA fragmented cells was reported as the percentage of apoptotic cells.

4.9. Immunofluorescence for Mcl-1

Immunofluorescence was introduced to evaluate whether or not cells in particular samples express Mcl-1 through antibody specification. NSCLC cell line (H460) and patient-derived primary lung cancer cell lines (ELC12 and ELC16) were seed overnight in 96-well plates at the density of $1x10^5$ cells/well. Then, they were treated with 1 μM of RT, TM-(–)-18, and TM-(–)-4a and incubated for 24 h. After that, cells were fixed with 4% of paraformaldehyde for 30 min, permeabilized by 0.5% of Triton X-100 in PBS for 5 min, followed by blocking with 10% of FBS in 0.1% of Triton X-100 for further 1 h at room temperature (RT). Primary antibody of Mcl-1 at proportional 1:100 in 10% of FBS was applied to the cells before incubation overnight at 4 °C. After incubation time, Alexa Fluor 488 conjugated with goat anti-rabbit IgG secondary antibody was added and incubated in dark for 1 h at RT. Cell nucleuses were stained with Hoechst 33342 and then visualized under fluorescent microscope (Nikon ECLIPSE Ts2, Tokyo, Japan).

4.10. Western Blot Analysis

Western blot analysis was used to determine the amount of specific proteins in the cells. NSCLC cell line (H460) and patient-derived primary lung cancer cell lines (ELC12, ELC16, ELC17, and ELC20) were seeded overnight at the density of $4x10^5$ cells/well and treated with RT, TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55) for 24 h. Cells were then collected by centrifuging media with 1500 rpm for 5 minute and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) for 30 min at 4 °C. The lysates were collected and their protein contents were determined by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equivalent amount of proteins from each sample (70 μg) was separated using SDS-polyacrylamide gel electrophoresis and further

transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). 5% skim milk in TBST (Tris-buffer saline with 0.1% tween containing 25 mM Tris-HCl pH 7.5, 125 mM NaCl and 0.1% tween 20) was applied to block the separating blots for 2 h at RT. Membranes were incubated with primary antibodies specific for PARP, Mcl-1, Bcl-2, Bax, and β-Actin at 4 °C overnight, washed with TBST and then were incubated with secondary antibody for 2 h at RT. Finally, the immunoreactive proteins were detected with the enhanced chemiluminescent detection system (Supersignal West Pico, Pierce, Rockford, IL, USA) and subsequently exposed to X-ray film. The intensity of protein bands was analyzed by the ImageJ software (version 1.52, National Institutes of Health, Bethesda, MD, USA). Densitometric values of protein expression levels were calculated as the fold changes relative to β-actin.

4.11. Computational Mcl-1 modelling and Molecular Docking

Molecular docking was applied to detect an interaction between RT or simplified right-half model of RT compounds and Mcl-1 protein. The target sequence of Mcl-1 (350 amino acids) was retrieved from UniProt, accession code Q07820 [68]. Since the three-dimensional (3D) structure of Mcl-1 has not been available yet, homology modelling, a promising tool to predict 3D structure of protein, was performed using the I-TASSER server [69,70]. The quality of the constructed homology model was then estimated by the Ramachandran plot using PROCHECK server [71]. The information in Figure 6b showed that the majority of amino acids were mainly found in most favored and additional allowed regions with values of 77.8% and 17.6%, respectively, which is suggestive of a reliable quality of this model. Before docking calculations, the modeled structure of Mcl-1 was relaxed by short molecular dynamics simulation for 20 ns using the AMBER16 software package according to standard procedures [72–75].

To prepare for the docking study, the chemical structures of all studied ligands were built using the Gaussian09 program [76]. Afterward, quantum chemistry calculation with the B3LYP/6-31G* level of theory was used for geometry optimization of all compounds. The docking calculations were carried out with AutoDock Vina [77]. Each ligand was docked to the expected binding site of Mcl-1 (residues 137–143). A grid box size was set to of 20 × 20 × 20 Å, whereas the grid was centered at the position of the residues 137–143, at x, y, z coordinates of 54.0, 31.0, 51.5. The UCSF Chimera package [78] was used for the graphical presentation of the data.

4.12. Statistical Analysis

The data from at least three independent replicated experiments (n = 3) was presented as the mean ± standard error of the mean (SEM). Statistical differences between multiple groups

were analyzed using an analysis of variance (ANOVA) which calculated SPSS software program version 16 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $p < 0.05$.

5. Conclusions

This study provides supporting evidence that RT and its simplified right-half model compounds TM-(–)-18 and TM-(–)-4a exert an anti-cancer action through Mcl-1 suppression and in part by the decrease in Bcl-2. Furthermore, by synthesizing structurally modified compounds as analogues of RT, and by performing in vitro protein analysis and molecular docking experiments, we were able to clarify the SAR information of RT compounds, which indicated that the cyanide and benzene ring compositions of RT play key functions in targeting the Mcl-1 protein. RT, TM-(–)-18, and TM-(–)-4a were the active compounds that were demonstrated to have potent anti-cancer activity in lung cancer cells, and information on the SARs of these compounds could encourage the development of related compounds having these groups for Mcl-1 suppression.

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1.

CHAPTER VI

Discussion

Evasion of apoptosis is a critically well-known in hallmark of cancer [1] since apoptosis is contributed to the elimination of unwanted cells to maintain the equivalence between cell survival and cell death. It is not surprising that dysregulation or disruption in apoptosis pathway results in sustained cell proliferation and developed cancer [2]. Therefore, effective reagents or treatment strategies that can restore the signaling pathway of apoptosis may have benefits for cancer management.

The tumor suppressor p53 has a major role in the controlling processes of DNA repair, cell cycle arrest, and apoptosis [3, 4]. When DNA-damage is occurred, p53 is activated and most probably exerts its transcriptional regulatory functions to control the level of Bcl-2 family proteins, causing increase of cellular level of pro-apoptotic members and decrease of the anti-apoptotic members in the Bcl-2 family [5]. Afterwards, the alteration of death-survival threshold is occurred by the dimerization between anti- and pro-apoptotic proteins. The pro-apoptotic dimers can cause the release of mitochondrial contents to the cytoplasm especially cytochrome c, resulted in activation of the caspase function leading to apoptosis [6]. In this research, the significant induction of p53 was found after treatment the non-small cell lung cancer cells with RT that may, at least in part, play a role in RT-mediated apoptosis. However, Western blot analysis showed a striking effect on the cellular protein level of Mcl-1.

Regarding evasion of apoptosis, the pivotal roles of anti-apoptotic proteins, especially Mcl-1, have been demonstrated in several *in vitro* and *in vivo* studies [6-12]. The level Mcl-1 protein in many types of cancers were found to be augmented at a higher ratio than the other anti-apoptotic proteins in the Bcl-2 family [13]. In particular, increased expressions of Mcl-1 reflect a poor prognosis for many malignancies, including lung cancer [14, 15]. Not only their increased expression is critical for oncogenesis and cancer progression, but these proteins are also involved in conferring chemotherapeutic drug resistance [11, 16-19]. It has been known that chemotherapeutic resistance is the failure to eliminate cancer cells of chemotherapeutic drugs due to host and tumoral-related factors resulted in incapability to activate apoptotic cascade and promote cancer cell survival [20]. It was shown that inhibition of Mcl-1 by siRNA could potently mediate NSCLC cell apoptosis [21]. Furthermore, in a mouse lung adenocarcinoma model, Mcl-1 overexpression was shown to help tumor progression by inhibiting Myc-induced apoptosis [16]. Taken together, compounds with potent activity for eliminating Mcl-1 in cancer cells are of great interest as good candidates for

targeted therapy. In agreement with the use of Mcl-1 as a target for cancer therapy [22], this study also supported this concept, where in experiments, the treatment of RT in an NSCLC cells (H460) resulted in apoptotic cell death through a Mcl-1 proteasomal degraded mechanism. In the same way, not only in a known lung cancer cell line, but it was also proved that the absence of Mcl-1 after RT treatment in resistant primary lung cancer cells derived from patients could trigger the apoptotic pathway, which led to the death of cancer cells.

Renieramycin T (RT) is a tetrahydroisoquinoline alkaloid compound that is part of the renieramycin family and was first isolated from the blue sponge *Xestospongia* sp. by pretreatment with potassium cyanide in a study in 2009 [23]. Recently, its anti-cancer activities have been reported against several types of cancer cells, such as colon (HCT116), prostate (DU145) [24], non-small cell lung (H292, H460, and QG56) [25], breast (T47D), and pancreatic (AsPC1) cancer cells [23]. Moreover, a modified form of RT, 5-O-acetyl-renieramycin T, was shown to induce the death of lung cancer stem cells and sensitize cisplatin-mediated apoptosis in lung cancer cells which is represented by a decrease in the CSCs markers CD44 and CD133 due to depletion of the protein kinase B (AKT) signal resulting in apoptosis induction of CSCs [26]. However, cell death mechanisms in CSCs and cancer cells might be different since CSCs have special characteristics only found in the stem cell population such as metabolic activities, signaling pathways, and cell cycle regulation [27-29]. Although anti-CSC activities of 5-O-acetyl-renieramycin T have been reported, the mechanism of action of RT in lung cancer cells has not been elucidated yet.

In an agreement with an evidence favoring the use of Mcl-1 targeted therapy [30, 31], this study was shown herein that RT could induce Mcl-1 degradation in lung cancer cells. RT, an active compound from the blue sponge *Xestospongia* sp., was demonstrated to possess a potent induction effect of Mcl-1-targeted degradation. The results revealed that whereas other Bcl-2 family proteins were not affected by RT treatment, the protein level of Mcl-1 was mostly disappeared. Moreover, the degradation process of Mcl-1 was found to involve with the ubiquitin-proteasomal pathway [32]. From the fact that Mcl-1 degradation was demonstrated to be important for targeted therapy [33], in this study, it is suggested that under RT treatment, the Mcl-1 half-life was dramatically shortened. The cycloheximide chasing assay showed the decrease of Mcl-1 protein half-life in response to 1 μM of RT was 1.52 h in comparison to 3.44 h in the non-treated control cells. In addition, when the selective proteasome inhibitor (MG132) was applied, the Mcl-1 protein level in the RT-treated cells was significantly restored, indicating that the proteasomal degradation plays a key part in RT function. Furthermore, the levels of the Mcl-1-Ubiquitin complex were monitored and it was found that the formation of the complex definitely increased in the RT-treated cancer cells. Taken together, these results strongly support the conclusion that RT

mediated cell death by targeting the degradation of Mcl-1. Further, in this study, it was confirmed that RT also had anti-cancer activities as well as Mcl-1-targeted activity against the chemotherapeutic resistant patient-derived primary lung cancer cells, with a lower IC50 compared to other first-line chemotherapeutic drugs. It was newly discovered that RT could decrease the level of the Bcl-2 protein in primary lung cancer cells, which was not observed before in the lung cancer cell H460. The explanation for this may due to the specific mutations of H460 cells, including KRAS and PI3K. The mutation of these two oncogenes may cause a high expression of Bcl-2 in H460 cells [34]. Gathering all the collected information, it is not surprising that RT could be an outstanding applicant for further clinical investigation in anti-cancer drug development.

In spite of the marvelous anti-cancer effects of RT, it is hard to deny that RT is difficult to fabricate in massive large-scale synthesis due to its large and complicated structure. Besides, its structure–activity relationships are still unrecognized. Therefore, simplified right-half model of RT compounds (TM-(–)-45, TM-(–)-18, TM-(–)-4a, TM-(–)-52, and TM-(–)-55) were established using RT as a prototype to help define which part of RT is necessary for achieving the Mcl-1-targeted effect. After screening by treatment against the H460 cells, it was found that only TM-(–)-18 and TM-(–)-4a exerted cytotoxic activities, with IC50 values less than 25 μM. Moreover, when Western blot analysis was performed using a primary antibody against Mcl-1, the results revealed that treatment with TM-(–)-18 and TM-(–)-4a could diminish Mcl-1 protein levels in the H460 cells compared to the untreated control and the other simplified right-half model of RT compounds. Having compared the structures between the compounds causing Mcl-1 depletion, namely RT, TM-(–)-18, and TM-(–)-4a, and the compounds not causing Mcl-1 depletion, namely TM-(–)-45, TM-(–)-52, and TM-(–)-55, two similar parts among the Mcl-1-depleted compounds that did not simultaneously appear in the others were identified. Hence, it was hypothesized that both cyanide and the benzene ring appeared in the Mcl-1-depleted compounds were essential in the Mcl-1-depletion mechanism **(Figure 2a)**. Not only the H460 cells, but the effects of TM-(–)-18 and TM-(–)-4a with patient-derived primary lung cancer cells were also examined. TM-(–)-18 and TM-(–)-4a demonstrated cytotoxic effects and apoptotic induction effects in both NSCLC (H460) and patient-derived primary lung cancer cells. Furthermore, the results from the TM-(–)-18 and TM-(–)-4a treatments showed a decrease in Mcl-1 and Bcl-2 protein levels in primary lung cancer cells, but as it was previously discussed in the section about the RT-treatment conditions, Mcl-1 depletion was aimed to be the main mechanism by which these compounds induced apoptosis.

It has been shown that the anti-apoptotic potency of the Bcl-2 family of proteins primarily relies on their stability [35]. Mcl-1 is considered a very unstable protein compared to the other Bcl-2 family proteins and the degradation of Mcl-1 can be induced by anti-cancer agents [33, 36-38].

The stabilization and degradation of Mcl-1 were reported to be regulated by a phosphorylation mechanism at the PEST region, which is the N-terminus of Mcl-1 enriched in proline (P), glutamate (E), serine (S), and threonine (T) residues and rich in putative phosphorylation sites [39]. Several pieces of evidence have suggested that the phosphorylation of Mcl-1 induced by extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinase (JNK), and p38 at threonine 92, threonine 163, and serine 121 can stabilize the Mcl-1 protein level [40-42]; whereas, the phosphorylation by glycogen synthase kinase-3 (GSK-3) at serine 155 and serine 159 destabilizes the Mcl-1 protein level and inhibits the interaction between Mcl-1 and the pro-apoptotic protein Bim [43-46]. From this information, the hypothesis was made with the idea that, supposing compounds in this study attached Mcl-1 at ERK, JNK, and p38 binding site, they might prevent the phosphorylation mechanism of those molecules and lead to destabilization of the Mcl-1 protein level. Moreover, evidences also showed that the mitogen-activated protein kinase (MAPK) docking motif, known as the D-motif is consisted of positive charged and hydrophobic amino acids [47] which might interact with aromatic ring and cyanide in the compounds[50-48] . To prove the hypothesis, computational molecular docking was applied using the MAPK docking motif found in the Mcl-1 protein sequence 137–143 as the target for the compounds and Mcl-1 interactions to characterize the behavior of these small molecule compounds at the binding site of the Mcl-1 protein [51, 52]. Likewise, the mentioned parts of the compounds **(Figure 1a)**, which it is supposed are necessary for their mechanisms, was deployed as a docking site for examination of the binding properties and SARs. After investigation, the results revealed that the binding affinity of RT, TM-(–)-18, and TM-(–)-4a was high and in a similar range, which indicated their ability to bind properly with Mcl-1 and induce destabilization of the protein; whereas, the binding affinity of TM-(–)-45, which has a benzene ring but no cyanide, was lower than the others, thus reflecting its incapability to induce Mcl-1 destabilization, as previously supported by the experimental data. **(Figures 1b-e)**

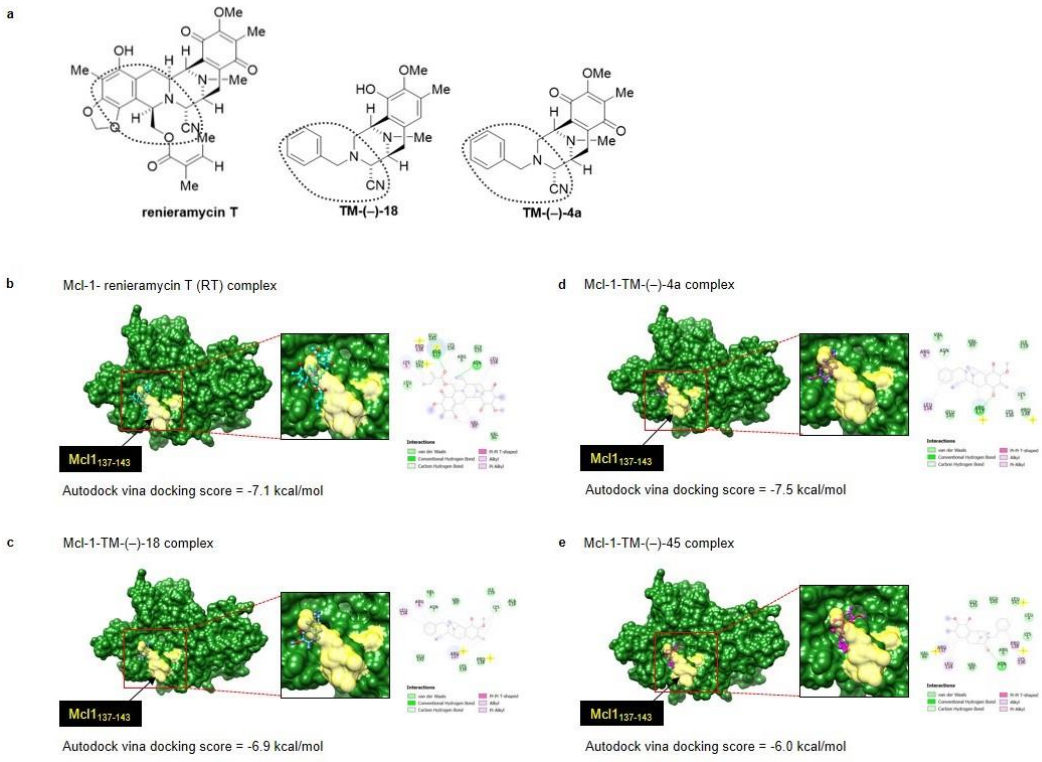

Figure 1. (a) Structures of RT, TM-(–)-18, and TM-(–)-4a. Circled part represents a similar structure between these three compounds. Binding mode and docking energy of: (b) renieramycin T, (c) TM-(–)-18, (d) TM-(–)-4a, and (e) TM-(–)-45 bound to the binding site of Mcl-1 (residues 137–143) taken from the AutoDock Vina molecular docking study

The appearance of both cyanide and a benzene ring in RT, TM-(–)-18, and TM-(–)-4a was found to be a necessary for the induction of Mcl-1 destabilization **(Figure 1a)**. This study energetically supported the SAR concept, which is key to many aspects of new drug discovery [53] and provides beneficial information for further anti-cancer drug modification and drug development as SARs can then be used to predict the activities of new molecules from their molecular structure because there is a relationship between molecular structures and their biological activity. This allows the modification of the effect or the potency of a bioactive compound by changing its chemical structure. Therefore, the SAR concept is essential in drug discovery to guide the acquisition or synthesis of desirable new compounds, as well as to further characterize existing molecules.

Results in this research suggested that RT, TM-(–)-18, and TM-(–)-4a exerted an apoptotic induction effect via the destabilization of the Mcl-1 protein. The interaction of the protein with the compounds promoted the ubiquitination and proteasomal degradation of Mcl-1 protein. As Mcl-1 has an important function to inhibit apoptosis by interacting and sequestering the pro-apoptotic

Bak protein, the diminishing of Mcl-2 cause by RT and its analogues could facilitate the Bak dimerization. The decrease in cellular Mcl-1 protein or the disruption of Mcl-1-Bak interaction can lead to Bak oligomerization, mitochondrial membrane destabilization, cytochrome c release, and apoptosis [2, 54].

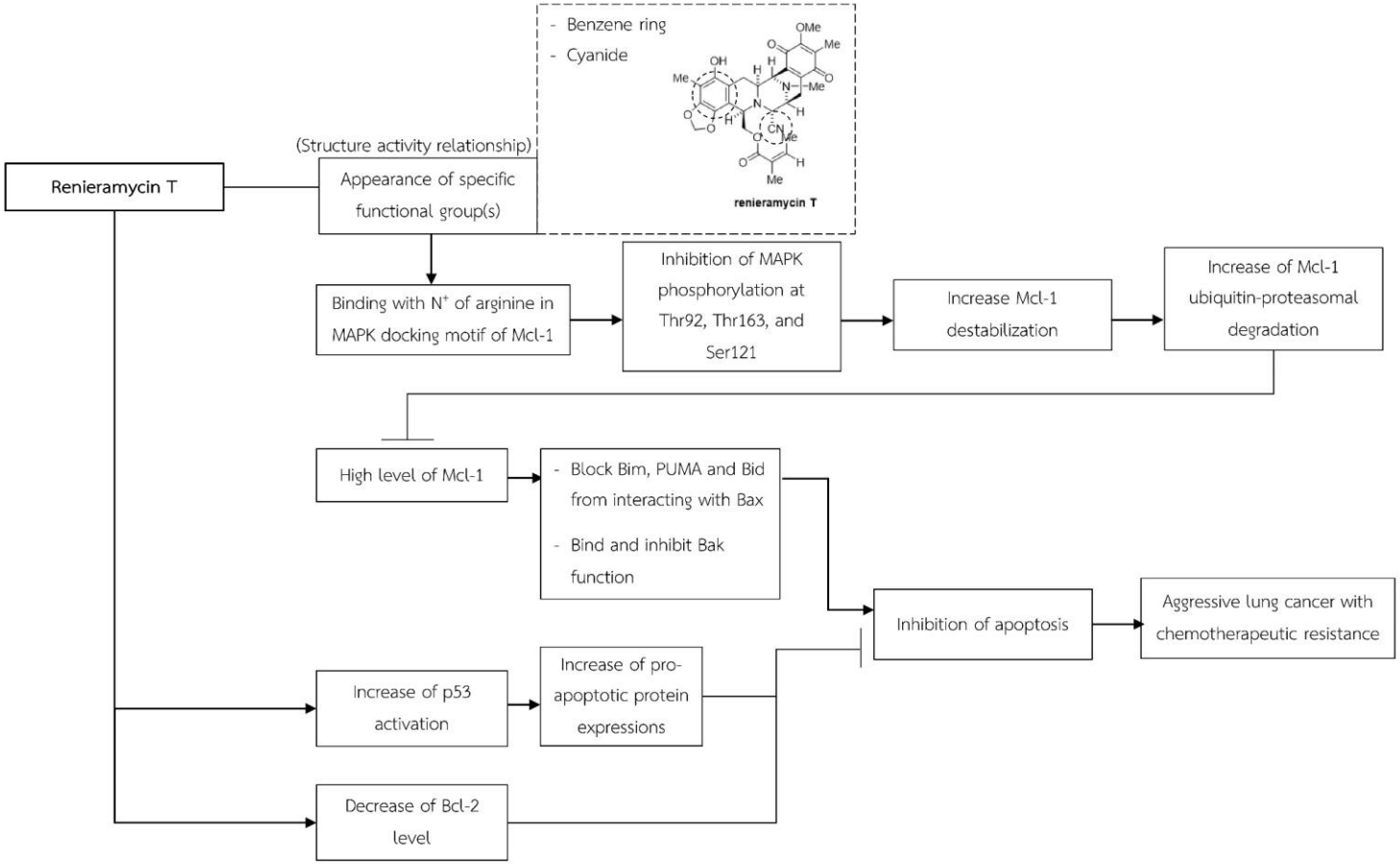

Figure 2. Mechanisms of RT. RT could overcome an aggressive lung cancer with chemotherapeutic resistance by induction of apoptosis through Mcl-1 targeted effect, p53 activation, and decrease of Bcl-2. RT has specific functional groups, benzene ring and cyanide, that could bind to MAPK binding site of Mcl-1 and induce its destabilization. This can lead to an increase of Mcl-1 degradation through ubiquitin proteasome system.

Conclusion for novel finding in this research

(Figure 2)

This project have unraveled the novel information as following;

1. RT has anti-cancer effects on lung cancer cells not only established lung cancer cells but also resistant patient-derived primary lung cancer cells. It is implied that this compound has an ability to overcome the chemotherapeutic resistant cells. Moreover, these effects are definitely caused by apoptosis induction process.
2. The apoptotic mechanisms of RT in lung cancer cells could be the results of an alteration in essential proteins in apoptotic pathway e.g. increase of p53, decrease of Bcl-2 and Mcl-1. However, the main mechanism is Mcl-1 targeted effect since the protein level of Mcl-1 was mostly diminished. After several investigations, it can be concluded that this compound exerts Mcl-1 targeted effect through ubiquitin proteasomal mediated Mcl-1 degradation.
3. The Mcl-1 degradation induced by RT was occurred from the binding between benzene ring and cyanide in RT structure and positively charged protein, arginine, of MAPK binding site in Mcl-1 sequence. This interaction could lead to Mcl-1 destabilization and degradation.

All of these findings provide the beneficial information for anti-cancer drug development in terms of the mechanism of RT, a novel anti-cancer compound, which is to induce Mcl-1 degradation and the modification of RT chemical structure for searching an essential part of RT to achieve its bioactivity which is necessity for further anti-cancer drug modification and large-scale synthesis.

Suggestion for further investigation

As Mcl-1 stability is regulated by MAPK proteins (ERK, JNK, and p38), it is interesting to investigate whether RT could interfere the binding between Mcl-1 and MAPK proteins or not. Thus, immunoprecipitation may be used to separate Mcl-1. Then, MAPK-Mcl-1 complex will be detected using specific antibodies against each MAPK proteins via Western blot analysis. If RT could interfere the binding between Mcl-1 and MAPK proteins, the MAPK protein expression levels will decrease compared to untreated control.

Although it is known that MAPK proteins control the Mcl-1 stability, it is unclear which of MAPK proteins have the most dominant activity on Mcl-1 due to the different mutations in upstream signaling pathway among each cell types. For example, EGFR and KRAS mutated cells

may cause ERK overexpression more than other MAPK proteins, so ERK may has an activity on Mcl-1 the most. To investigate the most dominant MAPK proteins on Mcl-1 in each cell types, MAPK inhibitors (ERK, JNK, and p38 inhibitors) may be used. If the Mcl-1 depleted effect occurs after treatment with one of MAPK inhibitors, it can be concluded that Mcl-1 stability is mostly depended on that MAPK protein.

Additionally, the resistant patient-derived primary lung cancer cells that were used have only EGFR mutation. To predict the clinical correlation for using RT as a Mcl-1 inhibitor, more types of genetic mutated primary lung cancer cells such as KRAS mutated cells may have benefits. Besides, most of the experiments were mainly performed *in vitro* system which could not represent all of cancer features even though the primary cells derived from patients were utilized. Thereby, for further drug development process, *in vivo* study and clinical trial study are still required. Moreover, from the structural findings discovered in this study, the further modification of RT structure to create more of Mcl-1 targeted effective compounds are of interested and could be processed in the future.

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