國 立 交 通 大 學

分子醫學與生物工程研究所

碩 士 論 文

海洋天然物做為抗癌藥物之研究

Characterization of marine natural products as anticancer agents

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摘要

做為治療疾病藥物之來源，天然物扮演了非常重要的角色，其獨特的結構與活性，對於新藥的發現與發展都十分具有幫助。由於知識與技術上的原因，在過去發現許多的海洋天然物並未就其分子作用機制做深入的研究與探討。在本研究中，我們將探討從海洋菌 *Zooshikella sp.*中所萃取出的紅色色素是否具有抗癌活性。藉由測定經過不同濃度的萃取物處理之後的癌症細胞株之細胞活性與增殖能力，我們證實了由 *Zooshikella sp.*中所萃取出的紅色萃取物，對於癌細胞的確具有細胞毒性。經過一系列的驗證與純化，我們最後得到兩個具有相似結構的化合物，分別為 WYT1-70-6 (prodigiosin) 與 WYT1-70-10 (2-methyl-3-hep-tyl prodigiosin)。兩者在抑制細胞增殖與誘發細胞凋亡的能力表現相當接近，此現象可由多二磷酸腺苷核糖聚合酶(PARP)被切割的情況證明。然而，根據結果發現即使結構相近，prodigiosin 可以抑制腫瘤壞死因子 α (TNFα) 在多發性骨髓瘤 (multiple myeloma) 所引發的 NF-κB 核轉移與活化；而 2-methyl-3-heptyl prodigiosin 則無此現象。最後，我們證實了 prodigiosin 可與 bortezomib 共同作用而得到更好的細胞毒殺性。總結來說，我們的實驗結果顯示：由於抑制了轉錄因子 NF-κB 的活性，由 *Zooshikella sp.*所純化出的天然物 prodigiosin 展現了抗癌活性，並與 bortezomib 在細胞毒殺能力上具有加成性。

Abstract

Natural products are important sources of bioactive agents for treating diseases. The unique structures and novel activities of marine natural products represent a promising field for drug discovery. For historical reasons, the molecular mechanisms of many bioactive marine natural products have not been well elucidated. In this study, we aimed to investigate the anticancer activity of natural products isolated from *Zooshikella sp.*, a marine bacterial species producing red pigments. By assessing the viability and proliferation of cancer cell lines treated with various concentrations of purified extracts/compounds, we have identified several bioactive extracts/molecules from *Zooshikella sp.* with cell toxicity. Moreover, two of the pure compounds, prodigiosin (WYT1-70-6) and its analog 2-methyl-3-heptyl prodigiosin (WYT1-70-10), induced apoptosis in cancer cell lines, as judged by the site-specific cleavage of PARP. Additionally, our preliminary data have shown that prodigiosin, but not 2-methyl-3-heptyl prodigiosin, inhibited TNFα induced NF-κB nuclear translocation and activation in the RPMI 8226 multiple myeloma cell line. Finally, we demonstrated that prodigiosin in combination with bortezomib had synergistic cell toxicity. In summary, results from this study suggested that marine natural product prodigiosin isolated from *Zooshikella sp.* exerts anticancer activity and additive interaction with bortezomib by targeting the transcription factor NF-κB signaling pathway.

誌謝

時光匆匆。轉眼間，兩年的碩班，已走到了盡頭。朝九晚五的生活，喔，不是。咳，是”午一晚一”的生活，嗯...還是”午二晚三”，隨便啦！總之，愜意的實驗室生活已經正式畫下了句點。兩年來的收穫不少，標準 SOP: 發現問題，面對問題，分析問題，解決問題。反覆的討論與實驗交叉印證，比起實驗結果，這些更是兩年來獲得足以用上一輩子的智慧。中間，最該感謝的當然是辛苦的梁美智老師了！從一開始帶著實驗新手又是做實驗，又是養老鼠的，各式各樣的疑難雜症都要請教她，最後在趕畢業的這段時間尤甚，不辭辛勞的為我修改論文，真可說是勞苦功高啊！再者也要感謝實驗室的大伙們：一起創室的世宏學長、福轅還有賤人(?!)，喔，是祖元才對！在實驗中給了我許多建議，也在實驗之余陪我度過了歡樂的時光；千芳、婷媛還有胖子，喔，是逸翔才對！平常實驗室的雜務真是辛苦你們了！(記得趕快脫手 XD)也感謝妳們在口試時幫忙準備了好吃的東西；還有親愛的專題生們，including 牟、SJ、朗賢 and 簡立，幫忙插了一盒又一盒的 tip，還有提供了我消遣的對象(對！SJ 就是你！)，為大家帶來了許多歡樂!!還要感謝新碩一的小朋友們，佩樺、大頭、奕君還有佳琪，祝你們以後實驗順利！

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就說聲再會吧！有緣再聚！

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
CHX	cycloheximide
COX	cyclooxygenase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid disodium salt
EGTA	ethylene glycol tetraacetic acid disodium salt
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
g	gram(s)
h	hour(s)
IC_{50}	inhibitory concentrations 50
IκBα	inhibitor of kappaB alpha
IKK	IkappaB kinase

IL	interleukin
IL-1	interleukin-1
IL-6	interleukin-6
LPS	lipopolysaccharide
M	molar
mA	milliamp (current)
MeOH	methanol
min	minute(s)
ml	milliliter(s)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
μg	microgram(s)
μl	microliter(s)
MM	multiple myeloma
mM	millimolar
μM	micromolar
MW	molecular weight
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-kappaB

NIK	NF-κB-inducing kinase
NLS	nuclear-localization signal
NP-40	nonidet-P 40
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
PDGF	platelet-derived growth factor
p-IκBα	Phosphorylation form of IκBα
PI-3K	phosphatidylinositol-3-OH kinase
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
s	second(s)
Ser	serine
TEMED	*N,N,N',N'*-tetramethylethelenediamine
TGF	transforming growth factor

TFIID	Transcription Factor II D
TNF-α	tumor necrosis factor-alpha
TNFR	tumor necrosis factor receptor
TRAF	tumor necrosis factor receptor associated factor
Tween-20	polyoxyethylene sorbitan monolaurate
UV	ultraviolet
VEGF	vascular endothelial growth factor

Introduction

1.1. Natural products

The natural products are small molecules produced by living organism can be applied to pharmacology. Throughout history, natural products have contributed abounding sources of compounds to application in the fields of medicine, pharmacy and biology. More than half of currently available drugs are natural compounds or are related to them (Newman and Cragg, 2007), which indicates that the discovery of a single natural product does not represent only one drug will be developed. By semisynthesis processes, we can modify the functional groups of lead compounds, the original natural products, to generate structural analogues with greater pharmacological activity and with fewer side effects (Gordaliza, 2007). There are many reports suggest that these synthetic analogs, in particular, could be important candidates for further studies involving structural modifications to improve the pharmacological profile of native organism metabolites (Schumacher et al., 2011). Additionally, it will get higher efficiency to cooperate with combinatorial chemistry, computational chemistry, bioinformatics and high-throughput screening protocols (Gordaliza, 2007).

1.1.1. The sources of natural products

Natural products can be extracted from eukaryotes to prokaryotes, including animals, plants, and microorganism covering land and ocean. For the natural products from animals, most of them are monoclonal antibodies. A famous example is Bevacizumab (Avestin), a vascular endothelial growth factor (VEGF) inhibitor (Los et al., 2007). It was approved by the U.S. Food and Drug Administration (FDA) for combination use with standard chemotherapy for metastatic colon cancer in 2004.

On the other hand, since the herbalism, the origin of contemporary medicine, the

plants have been studied for a very long time in this field. There are many diverse natural compounds have been discovered and isolated from plant sources (Mukherjee et al., 2001). For example, parthenolide which exists in a traditional herb feverfew (*Tanacetum parthenium*) is used for anti-inflammatory activity. The recent studies revealed that it is able to inhibit the NF-κB signaling pathway both *in vtro* and *in vivo* (Hehner et al., 1999; Sheehan et al., 2002). Additionally, the anticancer property of parthemolide has been noticed in many kinds of cancer in recent years (Cheng and Xie, 2011; Li et al., 2012). The scientists not only the identified the new compounds, but modified the functional groups to improve the biological activity. A typical example is the analog development of curcumin. Polyphenol curcumin, the synthetic analog of curcumin, displays a higher anticancer property compared to the parent compound. (Yadav et al., 2010).

While isolating the natural products from microorganism, compared to from plants, has more advantages. These advantages include: 1. Extracting the compounds is simpler since the cell wall of plants is hard to be lysed. 2. Culturing the microorganism is not only much less time cost, but also can obtain many times of amounts of resources for extracting. 3. The species of microorganism is much more than plants, and this fact represents that there are still many of unidentified natural compounds in microorganism. According to above opinions suggests that microorganism is a better resources for discovering new natural products which avail against the cancer. In decade, it becomes popular to test the bioactivities of natural compounds isolated from microorganism, especially the ocean microorganism, in cancer cell lines and find the mechanism how it kills the cancer cells. For example, a research group of University of California-San Diego published that they isolated an ester-substituted sesquiterpenoid called cryptosphaerolide from a marine-derived ascomycete related to the genus Cryptosphaeria (Oh et al., 2010). This marine extraction shows cytotoxicity

(IC_{50} of 4.5 μM) on the HCT-116 colon carcinoma cell line (Oh et al., 2010). A previous study revealed that this kind of compounds inhibited myeloid leukemia cell differentiation protein Mcl-1which plays an important role in life/death decisions of individual cells (Michels et al., 2005). Another example is manzamine A, which has been isolated from various marine sponges, displayed a cytotoxic effect against AsPC-1 pancreatic cancer cells, with an IC_{50} in a range of 4.2 μM, after 3 days of treatment (Guzman et al., 2011).

1.1.2. The value of application in clinical

The process mentioned above that biochemists cooperate with computational chemistry and bioinformatics to modify the leading compounds which were discovered by cancer biologists is very effective for drug development. There are many research data can support this fact. Besides, many of new commercial anticancer drugs have gone through this process. An example of these compounds is romidepsin which is a depsipeptide isolated from *Chromobacterium violaceum*. Romidepsin is a novel agent in a new class of anti-cancer drugs, known as histone deacetylase inhibitors (Glaser, 2007). It has received the Orphan Drug Designation from FDA for the treatment of non-Hodgkin T-cell lymphomas (Gordaliza, 2007). Another example is trabectedin (PharmaMar's Yondelis®) (D'Incalci and Galmarini, 2010). It is the first anticancer drug isolated from the marine source. Although it has not been approved by FDA now, it is still a potential anticancer drug.

1.2. NF-κB signal transduction pathway

NF-κB complex was discovered and defined as nuclear factor in 1986. It binds to the enhancer region of the κB chain of immunoglobulin in B cells (Anand et al., 2008). Therefore, it should have highly connection with immune response. Indeed, the stud-

ies of NF-κB have revealed that NF-κB plays a very important role in immune system (Bonizzi and Karin, 2004; Ghosh et al., 1998; Silverman and Maniatis, 2001). Furthermore, the inflammation which is mostly controlled by NF-κB has shown high positive connection with cancer. In addition, that NF-κB signal transduction pathway has abnormal expression level, most of cases were over expression or constitutive expression, was reported (Aggarwal and Gehlot, 2009).

The NF-κB signal transduction pathway can be distinguished into two types. One is the classical pathway, the other one is alternative pathway. Either classical pathway or alternative pathway can trigger the NF-κB signaling (Bonizzi and Karin, 2004). For the classical pathway, NF-κB complex exists in cytoplasm and inactivated by binding to IκB family proteins which result in that NF-κB complex cannot be translocated into nucleus. For that reason, NF-κB cannot function as the transcriptional faction through binding to its targeting site. When the cells receive the specific signal, such as proinflammatory cytokine tumor necrosis factor α (TNFα), the IκB protein will be phosphorylated by IκB kinase (IKK) complex and then be degraded through ubiquitination pathway. Consequently, the NF-κB complex will be activated by translocated into nucleus (see Fig. 1). Activated NF-κB can then be down-regulated through multiple mechanisms including the well characterized feedback pathway whereby newly synthesized IκBα protein binds to nuclear NF-κB and exports it out to the cytosol (Hayden and Ghosh, 2004).

As for the alternative pathway, the composition of NF-κB complex is different from the classical. Without binding to IκB proteins, the NF-κB complex maintains unstimulated state itself. While the activator, like CD40L, interacts with receptor and activates the pathway (Nakano et al., 1999), the IKKα homodimers will be phosphorylated by NF-κB inducing kinase (NIK) (Senftleben et al., 2001a) and phosphorylate one component of NF-κB complex subsequently. Once the phosphorylation of NF-κB

complex is happened, the phosphorylated component will be added poly-ubiquitin and processed. Finally, the active form of NF-κB complex will be able to enter the nucleus and turn the specific genes on (see Fig. 2).

1.2.1. Components and function of NF-κB complex

There are five members of the mammalian *NF-κB* family, including p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). They exist in cytoplasm as homo- or heterodimers bound to IκB family proteins at unstimulated state (Hayden and Ghosh, 2004). All of them have around 300- amino acids conserved sequence called Rel homology domain (RHD) which is responsible for dimerization, interaction with IκBs, and binding to DNA. Only RelA, RelB, and c-Rel contain the transactive domain (TAD) which is necessary for driving transcription. Therefore, the research shows that gene expression is repressed when the promoter or enhancer region binds to p50 or p52 homodimers (Zhong et al., 2002).

The p65 (Rel A) usually accompanies p50 to form the NF-κB complex in the classical pathway which is one of the most important pathway regulated immune system. However, the function of p65/p50 heterodimmers is shut down since the binding of IκBα. The crystallographic structures of this binding complex revealed that the IκB proteins mask the nuclear localization sequence (NLS) of p65 (Malek et al., 2001; Malek et al., 2003). Following degradation of IκBα, the released NF-κB is able to bind promoter and enhancer regions containing κB sites with the consensus sequence GGGRNNYYCC (N = any base, R = purine, and Y = pyrimidine). Moreover, the genetic studies of p65 through *p65*−/− mice revealed the crucial role that p65 protects cells or organs from apoptosis induced by TNFα signaling. This model exhibits lethality caused by liver degeneration at gestational day 15–16 (Beg et al., 1995).

RelB is the unique one in the mammalian *NF-κB* family since that it does not

homodimerize. Furthermore, it is unable to heterodimerize with c-Rel or p65. In fact, RelB forms heterodimers with p100, p52, and p50 (Dobrzanski et al., 1994; Ryseck et al., 1992) and does not interact with any other IκB proteins (Solan et al., 2002). The RelB/p100 is the major complex in alternative pathway. Without binding to IκB proteins, the RelB/p100 complex maintains stable state. When the pathway is stimulated, the IKKα is activated and phosphorylates p100, leading to their polyubiquitination and subsequent processing of p100 by the proteasome (Karin and Ben-Neriah, 2000), forming the active RelB/p52 heterodimers. Moreover, both NLS of RelB and p52 cannot be masked by IκBα, resulting in that RelB/p52 heterodimers exhibit constitutive nuclear localization (Dobrzanski et al., 1994; Lernbecher et al., 1994; Ryseck et al., 1992). In addition, RelB-deficient mice have decreased baseline NF-κB activity in the thymus and spleen, but increasing inflammatory infiltration in multiple organs and severe deficits in adaptive immunity (Weih et al., 1995).

1.2.2. The IκB family

There are seven members in IκB family, including IκBα, IκBβ, BCL-3, IκBε, IκBγ, and the precursor proteins p100 and p105 which are characterized by the existence of five to seven ankyrin repeats that assemble into elongated cylinders that bind the dimerization domain of NF-κB dimmers (Hatada et al., 1992). However, the role of the IκB family members, exclusive of IκBα, is less well understood.

The activity of p65/p50 is mostly, although not exclusively, regulated by IκBα through inhibiting the translocation of NF-κB complex. This fact was proofed by IκBα-deficient mice which displays increased but not constitutive DNA-binding ability and significantly increased termination time of NF-κB activation in response to TNF-α (Klement et al., 1996). Moreover, IκBα is known to participate in a feedback loop through that newly synthesized IκBα inhibits the activity of NF-κB. Besides,

IκBα contains nuclear export sequences (NES) resulting in the output of reformed IκBα and NF-κB complex from nucleus to cytosol. This NES characteristic coupled with the unmasked NLS of p50 contributes to constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm, although the steady-state localization is in the cytosol (Huang et al., 2000; Johnson et al., 1999).

Whereas, the researches of the others IκB proteins compared to IκBα have not clearly characterized their functions yet. The IκBβ was identified the ability that it can bind with p65/p50 or p65/c-Rel dimmers, which is similar to IκBα, through crystallographic structure (Malek et al., 2003). This similar complex in cytosol also has the similar regulating function too (Malek et al., 2001). However, hypophosphorylated IκBβ can bind DNA with p65 and cRel, and the DNA-bound NF-κB:IκBβ complexes are resistant to IκBα *in vitro*, suggesting hypophosphorylated, nuclear IκBβ may prolong the expression of certain genes (Tran et al., 1997; Weil et al., 1997). Overall, the result of *IκBβ−/−* mice shows that IκBβ serves to both inhibit and facilitate the inflammatory response (Rao et al., 2010) .

1.2.3. The IκB kinase (IKK) complex

The degradation of IκB is a highly regulated event that is initiated upon specific phosphorylation by activated IKK. The IKK contained function in cells can be purified as a 700–900 kDa complex, and has been shown to include two kinase subunits, IKKα (IKK1) and IKKβ (IKK2) which both are serine/threonine kinases, and one regulatory subunit, NEMO (NF-κB essential modifier) or IKKγ (Ghosh and Karin, 2002). In vitro studies indicate that the IKKα/IKKβ heterodimers have higher catalytic efficiency than either homodimer (Huynh et al., 2000)

In the classical NF-κB signaling pathway, IKKβ is both necessary and sufficient for phosphorylation of IκBα on Ser 32 and Ser 36, and IκBβ on Ser 19 and Ser 23. The

role of IKKα in the classical pathway is unclear, although some studies suggest it may regulate gene expression in the nucleus by modifying the phosphorylation status of histones.(Hayden and Ghosh, 2004)

The alternative pathway, however, only depends on the IKKα subunit, which functions by phosphorylating p100 and causing its inducible processing to p52 (Hayden and Ghosh, 2004). Additionally, this process of phosphorylation has high selectively, resulting in that processing of p100 releases a subset of transcriptionally active NF-κB dimers, consisting mainly of p52:RelB (Dejardin et al., 2002; Xiao et al., 2004)

The *IKKβ*-/- phenotype is similar to that observed for the p65 knockout, supporting the central role for IKKβ in regulation of NF-κB signaling via TNFα (Li et al., 1999a; Li et al., 1999b). In addition, compared to TNFR/p65 double knockout mice survive for many months, TNFR/IKKβ double knockouts shows more defect in innate immune responses to bacterial infection, only surviving a few days after birth. These data indicate that there is no partial compensation for IKKβ by IKKα or any other kinase (Senftleben et al., 2001b).

1.2.4. Activation of NF-κB signaling pathway

There are various stimuli that can induce NF-κB signaling pathway, including antigen from bacteria or virus, cytokines, oxidation stress, and receptor ligands etc. And these stimuli activate NF-κB correlated to their specific receptors, including tumor necrosis factor receptor (TNFR), toll/interleukin-1 receptor (TIR), B-cell receptor (BCR) and T-cell receptor (TCR). In these activators, the most important one is the cytokines which is quite simpler and affecting all kinds of cells. Therefore, the studies on mechanism of cytokines, such as TNFα, IL-6, is much more integrated and clarified.

1.2.4.1. Tumor necrosis factor receptor (TNFR)

The TNFR superfamily consists of at least 19 ligands and 29 receptors and exhibits a remarkable diversity in tissue distribution and physiology (Aggarwal, 2003). These family receptors lack intrinsic enzyme activity. Instead, signaling is started by recruitment of intracellular adapter molecules (Aggarwal, 2003; Wajant et al., 2003). The most famous ligand of this family is TNFα which interestingly stimulates both death and survival. As mentioned before, the *p65-/-* mice are fatal since TNFα induced liver degradation. However, the p65/TAFR double knockout mice are able to grow up. On the other hand, TNFα also up regulates anti-apoptotic proteins, such as Bcl-XL, through activating NF-κB pathway (Hayden and Ghosh, 2004) .

Besides, there is still another thing needed to be mentioned that TNFα signaling via TNFR1 results in the rapid activation of IKK and nearly complete degradation of IκBα around 10 min to 20 min. It is a very quick response compared to other stimuli (Hoffmann et al., 2003; Hoffmann et al., 2002).

1.2.4.2. Toll/interleukin-1 receptor (TIR)

The Toll/IL-1 receptor (TIR) indicates a group of receptors which have highly homologous domain of Toll-like receptors and IL-1 receptor. This intracellular domain mediates interaction with downstream signaling adapters that lead to activation of three key transcription factors, NF-κB, AP-1, and IRF3(Hayden and Ghosh, 2004). The encountering of LPS, bacterial or viral DNA, and flagellin can trigger the pathway through TLR. After activated, TIR domains recruit cytoplasmic adaptor proteins MyD88 which is crucial for normal NF-κB induction in response to IL-1 and IL-18 (Adachi et al., 1998; Kawai et al., 1999). Subsequently, MyD88 will recruit the others adaptor proteins, such as TIRAP, and eventually activates the IKK complex through

TRAF6 (Sun et al., 2004; Wu and Arron, 2003).

1.2.4.3. T-cell receptor and B-cell receptor (TCR and BCR)

Signaling from T-cell receptors (TCRs) and B-cell receptors (BCRs) is critical for mounting adaptive immune responses. These signals indeed lead to NF-κB activation (Ruland and Mak, 2003). Subsequently, the activation of NF-κB resulted from TCR and BCR allows antigen-specific proliferation and maturation of lymphocytes into effector cells which are activated and involved in eliminating a pathogen.

The signal from TCR is passed through recruiting the adaptors which contain tyrosine kinase activity. However, the link between the receptor proximal tyrosine kinases and NF-κB is poorly defined. One group reported that that PKCθ is essential for activation of NF-κB by TCR through the knockout studies (Sun et al., 2000). And another group reported that the IKK complex is rapidly recruited to the immunological synapse through an unknownmechanism and can be colocalized to the TCR by confocal immunofluorescence analysis (Khoshnan et al., 2000). Summarizing these researches, a hypothesis was proposed that PKCθ is capable of directly interacting with the IKK complex and then activates NF-κB in primary T cells.

1.3. Inflammation and cancer

Cancer is now generally believed to be a preventable disease. Only 5% to 10% of all cancers are caused by inheritance of mutated genes and somatic mutations, whereas the remaining 90% to 95% has been linked to lifestyle factors and environment (Anand et al., 2008). Interestingly, in all these risk factors that are considered to cause cancers through inflammation, customs of diet have been recognized as major risk factors for the most common types of cancer (Aggarwal et al., 2009). Indeed, these factors of lifestyle and environment, including tobacco, stress, dietary agents, obesity,

alcohol, infectious agents, environmental pollutants ,and irradiation, mostly can induce inflammatory pathway which totally account for as much as 95% of all cancers (Aggarwal and Gehlot, 2009).

1.3.1. NF-κB dysregulation and caner

As reported by researches, NF-κB signaling pathway is regarded as playing a very important role in inflammation. Therefore, it is reasonable that inflammation promotes the progression of cancer through NF-κB pathway. Indeed, the researches revealed that NF-κB is not only over expressed but relied in many kinds of cancer cell lines or cancer stem cells (Mimeault et al., 2012; Noma et al., 2012; Rodriguez-Berriguete et al., 2012). Moreover, the study also revealed the characteristic that inflammatory cytokines secreted by inflammatory cells which express activated NF-κB contribute to tumor initiation and progression (Coussens and Werb, 2001). Not only the inflammatory cytokines but growth factors, such as epithelium growth factor (EGF) and vascular endothelial growth factor (VEGF), can induce NF-κB pathway although it does not activate the pathway directly. Actually, these growth factors affect NF-κB through the Akt/PI3K pathway and then result in the proliferation and antiapoptosis properties in cancer cells (Appendix 1.).

In addition, there is another significant discovery that NF-κB has been shown to be constitutively active in a majority of tumor cell lines, both solid and hematologic tumors (Sethi et al., 2008). Furthermore, the presence of constitutively active NF-κB has now been identified in tissue of most cancers including leukemia, lymphoma, and cancers of the prostate, breast, oral cavity, liver, pancreas, colon, and ovary (Aggarwal and Gehlot, 2009). This constitutive NF-κB signaling is utilized by various cancer types to block apoptosis (Basseres and Baldwin, 2006). Besides, constitutive NF-κB activation also has been linked to chemoresistance and radioresistance (Ahn et

al., 2007) . Many mechanism of constitutive activated NF-κB has been described, including overexpression of growth factor receptors, mutation of IκBα such that it cannot bind to NF-κB, constitutive activation of Ras protein, high proteolytic activity directed to IκBα, and autocrine secretion of inflammatory cytokines. However, these mechanisms are cases reports but not universe. In the other words, the mechanism of constitutive active NF-κB has not been demonstrated.

Although the mechanism has not been defined, the contributions of constitutive activated NF-κB to cancer cells are well clarified. These contributions include survival, antiapoptosis, proliferation, invasion and metastasis.

In normal condition, our body maintains homeostasis through the programmed cell death called apoptosis (Meier et al., 2000). This process usually induced when emergence of DNA damage which is repaired invalidly. However, transformed cells resulted from inflammatory microenvironment become antiapoptosis and turn out into cancer. The researches indicated that NF-κB regulates the expression of most antiapoptotic gene products (bcl-2, bcl-xl, c-FLIP, XIAP, IAP-1, IAP-2, and survivin) associated with the survival of the tumor (Aggarwal and Gehlot, 2009). All these gene products inhibit the caspase pathway directly. Therefore, cancer cells can keep survival and avoid death.

Besides, cancer cells show not only antiapoptosis but uncontrollable proliferation property. The studies revealed that gene products linked with proliferation of tumors such as c-myc and cyclin D1are tightly regulated through NF-κB (Hwang et al., 2010; Sun et al., 2012). Additionally most growth factors, such as EGF, linked with proliferation of tumors either activate NF-kB or are regulated by this transcription factor.

Also, the post-cancerogenesis, including angiogenesis, invasion and metastasis, is promoted through NF-κB which controls the expression of MMP, adhesion molecules, VEGF and TWIST (Aggarwal and Gehlot, 2009).

1.4. Multiple myeloma

Multiple myeloma (MM), also known as plasma cell myeloma or Kahler's disease, is a malignant plasma cell proliferation in the bone marrow (BM) associated with monoclonal protein in the serum and/or urine. With a low 5-year survival rate, it has a prevalence of 50,000 patients, occurring in approximately over 16,000 newly diagnosed cases of MM and over 10,000 deaths within the United States alone in 2005 (Jemal et al., 2007). While in Taiwan in 2006, there were 73,293 patients diagnosed with cancer and 360, around 0.5%, of cases are multiple myeloma and the average survival time is 28 months (statistics from Department of Heath, excusive Yuan, R.O.C). Due to the low 5-years survival rate, drug resistance, and high recurrence, the multiple myeloma is regarded as an incurable cancer.

1.4.1. NF-κB and multiple myeloma

60-70% of MM patients have bone involvement at the time of diagnosis, and 90% of MM patients will develop bone lesions during the course of the disease (Heider et al., 2005; Roodman, 2009). The studies revealed that BM microenvironment plays a crucial role in MM cell pathogenesis since that MM cells adhere to bone marrow stromal cells (BMSCs) and are able to secrete cytokines which induce several pathway, including NF-κB signaling pathway, promoting the proliferation of MM cells (Hideshima et al., 2007).

Furthermore, it has been suggested that activation of the NF-κB pathway is important for the survival of healthy plasma cells and for MM tumors (Moreaux et al., 2009; Tai et al., 2006). And there are many reports show that many of MM cell lines are sensitive to NF-κB inhibitors (Hideshima et al., 2006). Although the mechanism is unclear, the evidence of microarray and quantitative real time polymerase chain reaction (qPCR) suggested that MM cells, either from primary or cell lines, contain con-

stitutive NF-κB activity (Annunziata et al., 2007). To illuminate the mechanism of constitutive NF-κB signaling in MM, it is reported that both the mutation of NF-κB regulation genes in MM cells and adherence of MM-BMSC which enriches the cytokines and growth factors in BM microenvironment contribute to the constitutive NF-κB activation (Keats et al., 2007; Markovina et al., 2010).

1.4.2. The treatment of multiple myeloma

Conventional treatment of multiple myeloma is combined chemotherapy which is often given in cycles over a period of months, followed by a rest period. Commonly used chemodrugs are including melphalan, doxorubicin and dexamethasone. However, the drug resistance occurs frequently (Schmidmaier et al., 2007; Xiang et al., 2011). The radiation therapy is another common therapy for MM patients. Similar to other hematological malignancy, MM patients can receive the bone marrow transplant. Nevertheless, the high rates of tissue rejection associated with donor transplants have made autotransplants the standard for the field. Before accepting autotransplant, patient needs to be treated with high intensity of radiation therapy. After the autotransplant, many of patients still have a relapse since that some residual myeloma cells sometimes survive in the banked stem cells which are given cyclophosphamide and a white blood cell stimulating drug after collection. (Dolgin, 2011).

Although MM remains incurable, novel agents targeting MM cells in the BM microenvironment, such as lenalidomide and bortezomib, when used alone or in combination, can overcome conventional drug resistance and improve patient outcome (Messori et al., 2011; Richardson et al., 2012). Compared to thalidomide which is an old drug for multiple myeloma with serious side effect, bortezomib displays an outstanding therapeutic efficacy for multiple myeloma. Mitsutoshi Satoh et al. demonstrated that bortezomib showed a better effectiveness ratio than thalidomide for

drug-resistance multiple myeloma in a rapid effect in the clinical studies. In addition, the overall survival rate inclined toward the bortezomib -treated patients. The results indicated that bortezomib has therapeutic efficacy for patients with thalidomide resistance (Satoh et al., 2011).

Since the degradation of IκBα occurs in proteasome, scientists believed that bortezomib causes apoptosis in multiple myeloma through inhibiting NF-κB activity. However, it has not yet been shown that bortezomib induces the inhibition of NF-κB activity in primary tumor cells from multiple myeloma patients. In contrast, the studies demonstrated that both multiple myeloma cell lines and primary cells from patients resisted the bortezomib-induced NF-κB repression (Hideshima et al., 2009; Markovina et al., 2008). Moreover, the previous studies suggested an interesting result that bortezomib up regulate the activity of NF-κB (Hideshima et al., 2009). There are more studies supporting that constitutive NF-κB activity contributed by bone marrow stem cells or cell itself is a key reason of the resistance of bortezomib in multiple myeloma cell lines and stem-like cells of mantle cell lymphoma (Jung et al., 2012; Yang et al., 2008).

Specific aims

Studies have shown that the transcription factor NF-κB is a promising target for developing novel anticancer therapeutics. In the research collaboration with Dr. Chung-Kuang Lu at National Research Institute of Chinese Medicine, our specific aims are listed below.

Aim 1: to discover potent inhibitors of the NF-κB signaling pathway from extracts prepared from the marine bacterium *Zooshikella sp.*

Aim 2: to determine whether the purified NF-κB inhibitor(s) from *Zooshikella sp.* may repress cell growth and induce apoptosis in human cancer cell lines.

Aim 3: to assess whether the purified NF-κB inhibitor(s) from *Zooshikella sp.* in combination with clinical agents may exert synergistic cell toxicity in human multiple myeloma cells.

Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents

The natural extracts from *Zooshikella sp.* were kindly provided by Dr. Chung-Kuang Lu's lab at National Research Institute of Chinese Medicine. The natural extracts were dissolved in methanol to a stock concentration of 10 mg/ml or 10 mM, and stored at -80℃ before use. Doxorubicin (Sigma Chemical Co.) was dissolved in DMSO to 10 mM and stored at -80℃. Bortezomib (Luminescence Technology Corp.) was dissolved in DMSO to 20 mM and stored at -80℃.

Chemicals

2-Mercaptoethanol (2-ME)	Sigma Chemical Co.
Acrylamide-Bis solution (29:1), 30% (w/v)	SERVA
Ammonium persulfate (APS)	J.T. Baker
Bromophenol blue	Sigma Chemical Co.
Cycloheximide	Sigma Chemical Co.
Dimethyl sulfoxide (DMSO)	Sigma Chemical Co.
Dithiothreitol (DTT)	Sigma Chemical Co.
Ethylene glycol tetraacetic acid (EGTA)	Sigma Chemical Co.
Ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Co.
Glycerol	J.T. Baker.
Glycine	J.T. Baker
Hepes	Sigma Chemical Co.
Igepal ca-630; NonidetP-40 (NP-40)	Sigma Chemical Co.
Isopropanol	J.T. Baker

Leupeptin	Sigma Chemical Co.
Methanol	J.T. Baker
N,N,N',N'-tetramethyl- ethylenediamine (TEMED)	J.T. Baker
Octyl phenol ethoxylate; Triton X-100	J.T. Baker
Pepstatin	Sigma Chemical Co.
phenylmethanesulfonyl fluoride (PMSF)	Sigma Chemical Co.
Potassium chloride (KCl)	Sigma Chemical Co.
Recombinant human TNF-α	R&D Systems, Inc.
Sodium chloride (NaCl)	J.T. Baker
Sodium dodecyl sulfatea SDS	Sigma Chemical Co.
Sodium fluoride (NaF)	Sigma Chemical Co.
Sodium orthovanadate (Na_3VO_4)	Sigma Chemical Co.
Tris-base	J.T. Baker
Tris-HCl	J.T. Baker
Tween-20	J.T. Baker

Reagents

3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfoqhenyl)-2H- tetrazolium (MTS)	Promega
LightShift® Chemiluminescent EMSA Kit	Thermo
Protein assay kit	Bio-Rad
SuperSignal® West Dura Extended Duration Substrate	Thermo
SuperSignal® West Pico Chemiluminescent Substrate	Thermo
Thiazolyl blue tetrazolium bromide (MTT)	Sigma Chemical Co.

Primary antibodies

Target	Dilution	Host	Sources
IκBα	1:500	Rabbit	Santa Cruz Biotechnology #sc-371
p65	1:500	Rabbit	Santa Cruz Biotechnology #sc-372
PARP-1	1:500	Rabbit	Santa Cruz Biotechnology #sc-7150
Phospho - IκBα	1:500	Rabbit	Cell Signaling Technology # 2859S
TFIID	1:500	Rabbit	Santa Cruz Biotechnology #sc-204
α-Tubulin	1:500	Rabbit	Santa Cruz Biotechnology #sc-12462-R

Secondary antibody

Target	Dilution	Host	Sources
Goat anti-Rabbit IgG (H+L), HRP conjugate	1:10000	Goat	Thermo Scientific Pierce #31460

Cell culture medium

Dulbecco's Modified Eagle's Medium (DMEM)	Biowest
Fetal bovine serum (FBS)	Biowest
Penicillin streptomycin (P+S)	Biowest
Phosphate buffered saline (PBS)	Biowest
RPMI 1640	Biowest
Trypsin-EDTA	Biowest

2.1.2. Equipment and materials

Centrifuge	Eppendorf centrifuge 5702R (for 15 ml or 50 ml)
	BECKMAN model CS-15R Centrifuge (for 1.5 ml)
CO_2 incubator	NUAIRE CO_2 Incubator NU-5500
Cross-link	UV box of UNIVERSAL HOOD (Bio-Rad)
Electrophoresis system	Mini- PROTEAN® Tetra (Bio-Rad)
ELISA reader	Bio-Rad Model 550
Microscopy	OLYMPUS CKX41
Power supply	PowerPac™ Basic (Bio-Rad)
Transfer system	Mini Trans-Blot® Cell (Bio-Rad)
PVDF membrane	Immobilon® -P Transfer Membrane, Millipore
NC membrane	Immobilon-NY+, Millipore
X-ray film	Fuji

2.1.3. Buffers

1X SDS-running buffer	25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS
1X transfer buffer	20 mM Tris-HCl, 150 mM glycine
5X SDS loading dye	87.5 mM Tris-HCl, pH 6.8, 45% (v/v) glycerol, 5% (w/v) SDS, 0.25 % (w/v) bromophenol blue, 12.5% (v/v) 2-ME
5X TBE 0.5 M	Tris-base, 0.45 M boric acid, 10 mM EDTA, pH 8.0
AT buffer	20 mM Hepes, pH 7.9, 1% (w/v) Triton X-100, 20% (w/v) glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM DTT, 1 mM Na_3VO_4, 1 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin
Buffer E	10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0 supplemented with 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin
Buffer F	20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA supplemented with 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin
Resolving buffer	375 mM Tris-base, pH 8.8, 0.1% (w/v) SDS
Stacking buffer	125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS
Stripping buffer	62.5 mM Tris-HCl, pH 7.0, 2% SDS, 100 mM 2-ME
TBST buffer	20 mM Tris-HCl, 2.5 mM KCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.5

2.2. Cell culture

2.2.1. Cell lines

The cell lines listed below were used in this study:

NIH3T3 (mouse fibroblast); HeLa (human cervical adenocarcinoma); A293 (human embryonic kidney); RPMI 8226 (human multiple myeloma was purchased from Bioresource Collection and Research Center)

2.2.2. General culturing of cells

NIH3T3 and A293 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin streptomycin (P+S). HeLa was cultured in RPMI 1640, 10% FBS and 1X P+S were added as the same. RPMI 8226 was cultured in RPMI 1640 supplemented with 20 % FBS and 1X P+S. All cell lines were incubated at 37℃ in humidified 95% air and 5% CO_2.

For subculture of adherent cells, cells were washed by pre-warmed phosphate buffered saline (PBS) and incubated at 37℃ approximately 1 min with 1 ml of trypsin-EDTA. Trypsinization was stopped by adding 9 ml of culture medium after cells had released from 10 cm culture dish, and then the cell culture medium was transferred into 50 ml centrifuge tube and centrifuged at 1500 rpm (Eppendorf centrifuge 5702R) for 5 min at room temperature. After removing the supernatant, the cells were resuspended by 10 ml of culture medium and 1 or 2 ml of cell culture medium would be transferred into 10 cm dish containing 10 ml of fresh culture medium. For subculture of suspension cells, cells were directly centrifuged at 1500 rpm. Then, cells were operated as described before.

2.3. Cell viability assay

Thiazolyl blue tetrazolium bromide (MTT, Sigma Chemical Co.) and 3-(4,

5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfoqhenyl)-2H-tetrazolium (MTS, Promega) were used to indicate the cell viability. Cells were counted and seeded in 96-well plates (Corning, Inc.) at the amount of 5,000-25,000 cells each well (depends on cell line). After 24 h, cells were treated with different concentration of compounds for another 24 h. For the adherent cells, 20 μl of MTT was added to each well for 3 h. Then, the medium was removed carefully and 200 μl of DMSO was added to each well. Absorbance was measured at 540 nm by ELISA reader (Bio-Rad Model 550). For the suspension cells, on the other hands, 20 μl of MTS was added to each well for 4 h and absorbance was measured directly at 490 nm by ELISA reader. The inhibitory concentrations 50 (IC_{50}) were determined by survival curves drown by Excel.

2.4. Cell treatment

2.4.1. NF-κB inhibition treatment

Before compounds treatment, adherent cells should be subcultured to 60 mm dish (100 mm dish for nuclear extracts) over night in the cause of cells attachment, while the suspension cells should not. After that, cells were changed to starving environment. For adherent cells, cells were washed by PBS twice and cultured in proper medium described previously with 0.5% FBS and 1X P+S for 24 h. For suspension cells, cells were centrifuged and washed by PBS twice, and then cultured in proper medium with 0.5%FBS and 1X P+S for 24h as the same. Cells were treated with certain concentrations of compounds or solvent for 2 h at 37℃. After 2h, cells were stimulated with 5 ng/ml (except where noted) of recombinant human TNF-α (R&D Systems, Inc.) for 20 min. Cells were harvested after 20 min immediately.

2.4.2. Cell apoptosis treatment

Before compounds treatment, cells were prepared as previous described but using standard culture medium instead of 0.5% FBS contained medium. And then cells were treated with indicated concentrations of compounds, solvent, or 10 μg/ml of cyclo -heximide (Sigma Chemical Co.) for 24 h before harvest.

2.5. Preparation of whole cell lysates and nuclear extract

2.5.1. Preparation of whole cell lysates

All procedures were done on ice. Cells were washed by ice-cold PBS twice then harvested (adherent cells were scraped off by scraper and suspension cells were centrifuged down directly at 4℃, 1500 rpm for 5 min) with 1ml of PBS and transferred to 1.5 ml eppendorf tube. Cells were spun down at 4℃, 10000 rpm for 3 min and added 100 μl of AT buffer [20 mM Hepes, pH 7.9, 1% (w/v) Triton X-100, 20% (w/v) glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM DTT, 1 mM Na_3VO_4, 1 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin]. Cells in AT buffer were passed through 26_G×1/2" needle around 20 times, and 4 M NaCl was added to a final concentration of 150 mM. Finally, cells were centrifuged at 4℃, 13,000rpm for 35 min and the supernatant were collected and stored at -80℃.

2.5.2. Preparation of nuclear extracts

All procedures were done on ice. As the preparation of whole cell lysates, cells were harvested by 1 ml of ice-cold PBS and transferred to a 1.5 ml eppendorf tube (two 100 mm dishes of cells were collected to one tube). And then, cells were resuspended in 400 μl of buffer E (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0) supplemented with 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and incubated at 4℃ for 15 min. The following

step should be done twice. Cells were added 25 μl of 10% NP-40 and vortexed for 10 s. The mixtures were centrifuged at 10,000 rpm, 4℃ for 1 min, and then the pellets were washed by adding 300 μl of PBS and centrifuging down twice. The pellets were then resuspended in 80 μl of buffer F (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml leupeptin and 1 μg /ml pepstatin. The next step should be done twice. The mixtures were vortexed for 30 s and incubated at -80℃ for 20 min followed by thawing the mixtures at room temperature. The mixtures were then vortexed for 30 s and centrifuged at 13,000 rpm, 4℃ for 5 min. The supernatant (nuclear extracts) were collected and stored at -80℃.

2.6. SDS-polyacrylamide gel electrophoresis

SDS- polyacrylamide gel was prepared and electrophoresed by Mini- PROTEAN® Tetra (Bio-Rad). The gel comprises a resolving gel and a stacking gel and. For the preparation of resolving gel, 1X resolving buffer [375 mM Tris-base, pH 8.8, 0.1% (w/v) SDS] containing 10% acrylamide which was diluted from 30% acrylamide (SERVA) was polymerized by adding appropriate of 10% ammonium persulfate (APS) and *N,N,N',N'*-tetramethyl- ethylenediamine (TEMED). The bubbles were expelled by the addition of 200 μl of isopropanol. The isopropanol was removed until the resolving gel was already polymerized, and stacking gel would be prepared by the mixture of 1X stacking buffer [125 mM Trix-HCl, pH 6.8, 0.1% (w/v) SDS], 5% acrylamide, and appropriate 10% APS and TEMED. The stacking gel mixture then was poured on the top of resolving gel and covered by comb.

To prepare samples, the 25 μg (depends on what kind of lysates and western blot target) of protein extract were diluted with 5X SDS loading dye [87.5 mM Tris-HCl, pH 6.8, 45% (v/v) glycerol, 5% (w/v)SDS, 0.25% (w/v) bromophenol blue, 12.5%

(v/v) 2-mercaptoethanol (2-ME)] to final concentration of 1X SDS loading dye and filled to total 20 μl by ddH_2O. The samples were heated at 98℃ for 10 min and cooled down at room temperature before loaded. Then, the gel was electrophoresed in 1X SDS-running buffer [25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS] at 80 volts until the loading dye passed through the stacking gel, and then the voltage was changed to 120 volts.

2.7. Western blot analysis

When gel electrophoresis was finish, protein should be transferred to PVDF membrane (Immobilon® -P Transfer Membrane, Millipore) in 1X transfer buffer (20 mM Tris-HCl, 150 mM glycine) containing 20% methanol at 250 mA for 1-2 h by using the transfer system (Mini Trans-Blot® Cell, Bio-Rad). It's important that PVDF membrane needed to activate by methanol before transferring. After transfering, the membrane was blocked in 5% (w/v) not-fat milk dissolved in TBST buffer [20 mM Tris-HCl, 2.5 mM KCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.5] for 1 h at room temperature or overnight at 4^{o}C. Then, membrane was dipped in primary antibody diluted with 5% not-fat milk in TBST at room temperature for 1 h; as for the anti phospho-protein antibody, membrane was suggested to incubate at 4℃ overnight. Followed by washing 3 times for 10 min by TBST, membrane was transferred to secondary antibody which was conjugated with Horseradish peroxidase (HRP) at room temperature for 1 h. After 3 times washing by TBST, the proteins were detected by SuperSignal® West Dura Extended Duration Substrate or SuperSignal® West Pico Chemiluminescent Substrate (Thermo).

For stripping the previous antibody, the membrane was incubated in stripping buffer (62.5 mM Tris-HCl, pH 7.0, 2% SDS, 100 mM 2-ME) at 50^{o}C for 5 min, and then was washed three times in TBST.

The antibodies used in this study were listed previous.

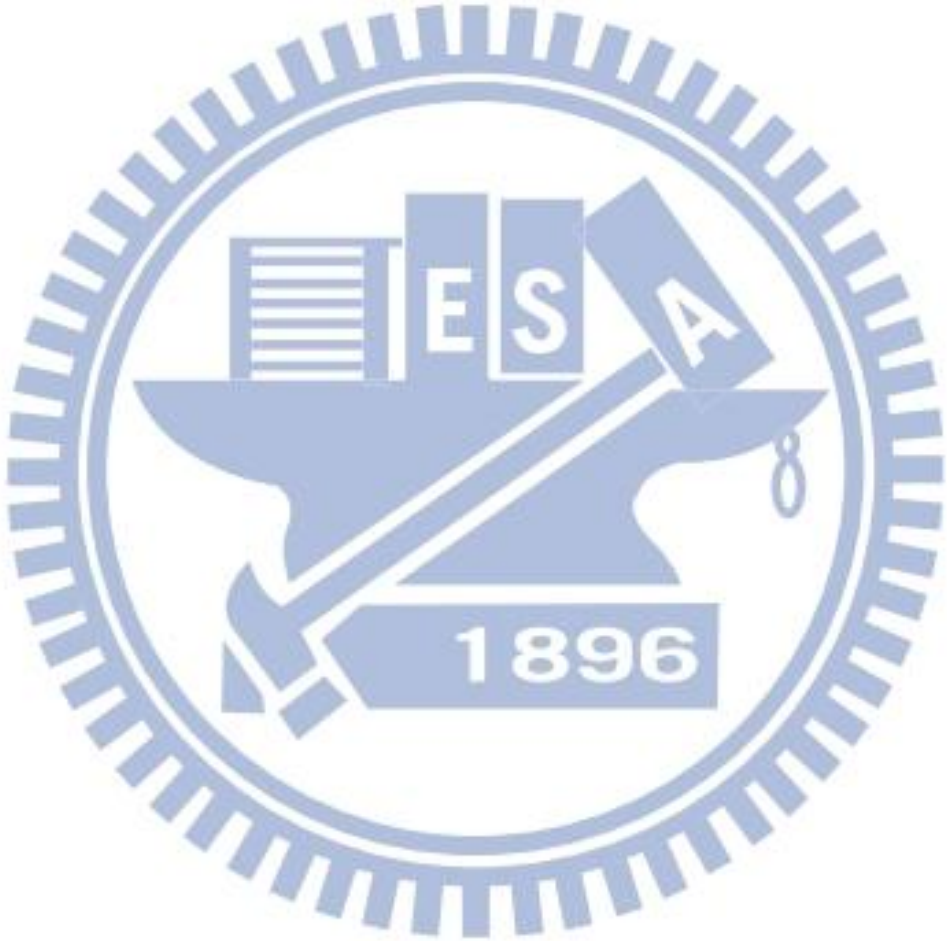
2.8. Electrophoretic mobility shift assay (EMSA)

The native gel for EMSA was prepared and electrophoresed by Mini- PROTEAN® Tetra (Bio-Rad). A native gel was made by that 1X TBE contained 6% of acrylamide and 2.5% of glycerol, followed by adding appropriate 10% APS and TEMED in order to polymerize the gel. The κB site probe (κB site: 5'-GGGAAATTCC-3' (Liang et al., 2006) labeled with biotin was ordered from Protech Technology Enterprise Co., LTD. And the binding buffer and biotin detection system were from LightShift® Chemiluminescent EMSA Kit (Thermo).

For the binding reaction, 15 μg of nuclear extract and 25 nM probe for final concentration were mixed in binding buffer in total volume of 20 μl, and the mixtures were incubate at room temperature for 20 min. Then, the sample mixtures were loaded with 5X loading dye contained in the kit and electrophoresed in 0.5X TBE at 80 volts until the loading dye migrated approximately 3/4 down the length of the gel. Before transfer, the NC membrane was soaked in 0.5X TBE for at least 10 min. The transfer was carried out by transfer system (Mini Trans-Blot® Cell, Bio-Rad) in ice-cold 0.5X TBE buffer at 380 mA for 1 h. Subsequently, the transferred DNA was cross-linked to membrane for 20 min with the membrane face down on a transilluminator equipped with 312 nm bulbs (UV box of UNIVERSAL HOOD, Bio-Rad)

Before the detection of biotin-labeled DNA, the blocking buffer and 4X wash buffer have to pre-warm until all particulate is dissolved. To block the membrane, membrane was soaked in blocking buffer for 15 min with gentle shaking, followed by transferring the membrane to anti-biotin antibody which is prepared with blocking buffer in 1:300 dilution and incubating for 15 min. After the incubation of antibody, the membrane was washed 4 times with 1X wash buffer which was diluted by ddH_2O

each time for 5 min. And then, membrane was removed to appropriate volume of Substrate Equilibration Buffer for 5 min with gentle shaking. Finally, the membrane was transferred to substrate mixture which is prepared before used for 3-5 min, and detected by exposure to film.



Results

3.1. Transcription factor NF-κB inhibitor screening from marine natural products

In a collaborative effort to discover potent NF-κB inhibitor, we screened more than 70 extracts (supplementary data 1) from a variety of marine resources provided by Dr. Chung-Kuang Lu's lab at National Research Institute of Chinese Medicine. Using a previously established NIH3T3 cell system (Liang et al., 2003; Liang et al., 2006), we were able to utilize TNFα as an activator of the NF-κB signaling for detecting inhibitors of this pathway.

Among all extracts tested, we found that WYT1-33-6, an extract from the marine bacterium *Zooshikella sp.*, was a potent NF-κB inhibitor. At a concentration of 20 μg/ml, WYT1-33-6 effectively protected IκBα from TNFα-induced degradation in the NIH3T3 cells (supplementary data 1). Furthermore, we treated cells with increasing concentrations (0-20 μg/ml) of WYT-33-6 and found that TNFα induced IκBα degradation was inhibited in a dose-dependent manner (Fig. 3A). This result suggested that WYT1-33-6 can against TNFα induced signal and inhibit the degradation of IκBα. However, this inhibition ability seems to reach the top at 10 μg/ml of WYT1-33-6 since the data showed that there was no apparent difference between IκBα ratio in cells treated with either 10 μg/ml or 20 μg/ml of WYT1-33-6 (Fig. 3B).

3.2. WYT1-33-6 inhibits NF-κB activation by blocking IκBα phosphorylation and NF-κB nuclear translocation in TNFα-induced NIH3T3 cells

Previous studies revealed that phosphorylation of IκBα N-terminal Ser residues is a prerequisite event for proteasome-mediated IκBα degradation (Hayden and Ghosh, 2004). Therefore, we were interested in determining whether WYT1-33-6 could block

the phosphorylation of IκBα. Results from Figure 4 showed that the amount of phosphorylated IκBα and total IκBα in different TNFα induction time from 0 to 20 min. The IκBα protein of the solvent control was phosphorylated within 5 min after TNFα induction (Fig. 4A, Fig. 4B), and then the phosphorylated IκBα protein was degraded by proteasome at 10 and 20 min after TNFα induction (Fig. 4A, Fig. 4C). In contrast, 20 μg/ml WYT1-33-6 treated cells presented low level of phosphorylated IκBα and constant amount of IκBα (Fig 4). However, the 20-min TNFα induction in the solvent control (Figure 4A) showed a rising IκBα expression which is contrary to the previous data (Fig. 3). Indeed, the IκBα upregulation supports the general understanding that IκBα is resynthesized once NF-κB signaling pathway was activated and can be explained by delay of harvesting. Overall, it was proved that WYT1-33-6 blocks the degradation of IκBα through repressing the phosphorylation of IκBα.

NF-κB nuclear translocation is a downstream event following IκBα degradation in the classical TNFα-induced NF-κB activation (Hayden and Ghosh, 2004). Because our data showed that TNFα-induced IκBα phosphorylation and degradation were blocked by WYT1-33-6 (Figures 3 and 4), we then tested whether WYT1-33-6 could inhibit the NF-κB subunit p65 nuclear translocation in the 3T3 cells. The result showed that the amount of the nuclear p65 protein in the 20 μg/ml WYT-33-6 treated cells was significantly less than that of the solvent control in the TNFα-activated NIH3T3 cells (Fig. 5). In summary, this result is consistent with our previous data and suggests that WYT-33-6 is a potent inhibitor of the NF-κB activation by blocking IκBα phosphorylation and degradation as well as NF-κB nuclear translocation.

3.3. WYT1-33-6 inhibits cell viability and induces apoptosis in the human cervical cancer HeLa cells

Our research data suggested that WYT-33-6, the extract from the marine bacterium

Zooshikella sp., is an effective inhibitor of the NF-κB activation in TNFα-induced mouse fibroblast 3T3 cells. We then examined whether WYT-33-6 could also inhibit NF-κB activation in human cancer cell lines. We first tested the frequently used human cervical cancer cell line HeLa and found that WYT-33-6 did not block TNFα-induced NF-κB activation in this particular cell line (data not shown). This result indicates that the NF--κB inhibition effect of WYT-33-6 is cell line-dependent.

We next studied whether WYT1-33-6 could exert cell toxicity in HeLa cells. Cells were treated with increasing concentrations of WYT1-33-6 for 24 h and the cellular morphological changes were examined using microscopy. Cycloheximide, the protein biosynthesis inhibitor in eukaryotic organisms, was used as a control of cell toxicity. After 24 h incubation, HeLa cells treated with 10 μg/ml of WYT1-33-6, compared with the solvent control, had a dramatic decrease in cell number and showed cell shrinkage in size (Fig. 6A). On the other hand, cycloheximide treated control also had cell shrinkage morphology. Interestingly, WYT1-33-6 treated cells showed a deep red color, the same color of the WYT1-33-6 (6A).

In addition to cellular morphological study, we assessed cell viability using MTT assay in WYT1-33-6 treated HeLa cells. After 24 h of drug incubation, the cell growth inhibition effect of WYT1-33-6 was determined by the MTT assay. The 50% of cell growth inhibition (IC_{50}) of WYT1-33-6 was approximately 15μg/ml in HeLa cells (Fig. 6B).

Because WYT1-33-6 inhibited cell growth as judged by MTT assay, we wanted to determine whether this agent could also induce apoptosis in HeLa cells. Cycloheximide was used as a positive apoptosis control. Poly (adenosine 5'-diphosphoateribose) polymerase (PARP) cleavage was used as an apoptosis marker. Same amount of HeLa cells were seeded and treated with different concentrations of WYT1-33-6 for 24 h and cell lysate was subjected to Western blots for PARP cleav-

age. The results revealed that WYT1-33-6 dose-dependently induced PARP cleavage.(Fig. 7). In summary, our studies showed that WYT1-33-6 is a potent agent in inhibiting cell growth and inducing apoptosis in HeLa cells.

3.4. Major bioactive components of WYT1-33-6, WYT170-6 (prodigiosin) and WYT1-70-10 (a prodigiosin analog), are cytotoxins in multiple myeloma cells

To take a further study in WYT1-33-6, our collaborator Dr. Chung-Kuang Lu and his lab members separated WYT1-33-6 into 11 fractions (WYT1-49-1 to WYT1-49-11). In order to examine which fraction contributed most to the bioactivity found in WYT1-33-6, we took the NF-κB inhibitor screening procedures previously mentioned in the TNFα induced 3T3 cells. The results suggested that two fractions, WYT1-49-6 and WYT1-49-8, could inhibit IκBα degradation (Fig. 8A). Also, results from cell growth inhibition effect assessed by MTT and MTS assay in several cancer cell lines supported the suggestion that these two were the most bioactive fractions in WYT1-33-6 (supplementary data 2). Dr. Lu and his students further purified WYT1-49-8 and obtained 2 compounds, prodigiosin (WYT1-70-6) and its analog 2-methyl-3-heptyl prodigiosin (WYT1-70-10) (Fig. 8B). Moreover, WYT1-49-6 was also separated into 6 fractions (WYT1-96-1 to WYT1-96-6). However, only one fraction, WYT1-96-4, had NFκB inhibitory effect in the TNFα induced 3T3 cells (data not shown). Our studies later confirmed that the main effective component of WYT1-96-4 was also prodigiosin (Appendix 2.). For this reason, the following studies were focused on two bioactive compounds WYT1-70-6 (prodigiosin) and WYT1-70-10 (a prodigiosin analog).

Not only the high connection between NF-κB signaling pathway and multiple myeloma but the incurable property and low 5 years survival rate, we selected the multiple myeloma cell line, RPMI 8226, as the target for our research. As precious proce-

dure described, we first examined whether WYT1-70-6 and WYT1-70-10 could inhibit TNFα induced IκBα degradation in 3T3 cells. Our results showed that WYT1-70-6 mediated NF-κB inhibition in TNFα-induced RPMI 8226 cells in a dose-depend manner (Fig. 9), whereas the WYT1-70-10 displayed no NF-κB inhibition (Fig. 10). Interestingly, these two compounds were both effective in inducing apoptosis and suppressing cell growth in the multiple myeloma RPMI 8226 cell line (Fig. 13). Both compounds began to induce PARP cleavage at the concentration of 2.5 μM (Fig. 11, Fig. 12). Moreover, the IC_{50} value for both compounds were approximately 0.5 μM as judged by MTS assay (Fig. 13). Although WYT1-70-10 could induce apoptosis and inhibiting cell viability in RPMI 8226 cells, we were unable to determine its molecular target yet. Therefore, we decided to focus our studies on the NF-κB inhibitor WYT1-70-6 (prodigiosin).

3.5. The DNA binding ability of NF-κB is inhibited by WYT1-70-6 through blocking NF-κB translocation in human multiple myeloma RPMI 8226 cells

Our previous study showed that WYT1-70-6 (prodigiosin) blocked the degradation of IκBα in TNFα induced human multiple myeloma RPMI 8226 cells (Fig. 9). We next examined whether NF-κB nuclear translocation could be blocked by WYT1-70-6 as judged by Western blot for the NF-κB subunit p65 using cell nuclear extracts. The result showed that the level of p65 protein in the nuclear extracts of WYT1-70-6 treated RPMI 8226 cells was significantly decreased compared with that of the solvent control (Fig. 14). The result suggested that WYT1-70-6 effectively blocks the nuclear translocation of the NF-κB subunit p65.

Although our studies demonstrated that p65 translocation was inhibited by WYT1-70-6 in RPMI 8226 cells, the function of NF-κB which binds to specific sequence of DNA (κB site) and turns on the downstream genes had not been examined.

Therefore, we used electrophoretic mobility shift assay (EMSA), a widely applied to determine the interaction between the NF-κB complex and DNA (κB site), to confirm whether this binding ability could be repressed due to a decrease in the level of NF-κB complex in the nucleus. As shown in Fig. 15, The NF-κB DNA binding activity of TNFα induced RPMI 8226 cells were inhibited by WYT1-70-6. In contrast, the TNFα induced solvent control displayed a strong NF-κB DNA binding activity. Taken together, these results suggested that WYT1-70-6 specifically inhibits the classical NF-κB signaling pathway through blocking the phosphorylation of IκBα in the human multiple myeloma RPMI 8226 cells.

3.6. Evaluating the cell killing effect of WYT1-70-6 in combination with clinical agents for treating multiple myeloma

Doxorubicin is a clinically used chemo drug for treating multiple myeloma. In addition, doxorubicin is also widely used for treating an array of cancer types, including bladder, breast, and the lung. We thus assessed the cell killing effect of combination therapy using WYT1-70-6 and doxorubicin in the multiple myeloma RPMI 8226 cells. The combination of these two agents displayed a more potent cytotoxicity than either of them used alone (Fig. 16A). However, these two agents did not exhibit significant additive interaction according to the isobologram analysis (Steel and Peckham, 1979).

In contrast, the proteasome inhibitor bortezomib, is a FDA-approved drug for treating multiple myeloma. The recent research revealed that bortezomib actually activates the NF-κB signaling pathway (Hideshima et al., 2009). The studies indicated the fact that bortezomib-induced cytotoxicity is not associated with NF-κB inhibition in multiple myeloma. Therefore, we assumed a combination therapy using bortezomib and WYT1-70-6 would have a synergistic cell killing effect. The result showed that this combination treatment displayed an additive interaction as judged by MTS assay

(Fig. 16B). To examine this additive interaction between bortezomib and WYT1-70-6, we harvested cell lysates treated for 8 h with bortezomib alone or in combination in the RPMI 8226 cells. As shown in Figure 17, cells treated with bortezomib (lane 2) exhibited lower protein level of IκBα than that of the solvent control (lane 1). Moreover, results from the Western blots of the nuclear p65 were consistent with this observation (Fig. 17B). Our data suggested that bortezomib induced the degradation of IκBα and consequently the nuclear translocation of p65 in RPMI 8226 cells. On the other hand, bortezomib in combination with WYT1-70-6 inhibited NF-κB activation (Fig 17A, lane 3) in the same cell line. Taken together, this immunoblotting result supports the additive interaction between bortezomib and WYT1-70-6 in the MTS assay and suggests a more effective therapeutic option by combination therapy using bortezomib and WYT1-70-6 in multiple myeloma.

Discussion

4.1. Marine microorganism *Zooshikella sp.*, a new source of prodiginine family

In this study, we demonstrated a natural product, prodigiosin, extracted from the marine microorganisim *Zooshikella sp.* and exhibiting NF-κB inhibitor and anticancer property through a series of experiments. This new species of the genus *Zooshikella* is gram-negative marine bacterium which was identified as a new source of prodiginine family, including two structures which were unknown before (Lee, Kim et al. 2011).

4.2. Prodigiosin has gotten attention again as an anticancer agent in recent years

Although the discovery of prodigiosin was very early, it was regarded as antibiotics at that time (Lichstein and Van De Sand 1946). During these two decades, scientists have perceived that prodigiosin may have other applications. The new characteristics was identified that prodigiosin represses the growth of lymphocytes, especially T-cells, which displays a potential to be an immunosuppressant (Nakamura, Nagai et al. 1986; Magae, Yamashita et al. 1993; Han, Kim et al. 1998). Furthermore, the studies from 2000s revealed that prodigiosin can induce apoptosis in cancer cell lines (Montaner and Perez-Tomas 2001; Montaner and Perez-Tomas 2002; Llagostera, Soto-Cerrato et al. 2003). However, most of these studies only indicated that prodigiosin results in apoptosis through caspase-dependent pathway. In recent researches, more characteristics of prodigiosin have been clarified, including antiproliferation, inducing DNA damage and inhibiting the function of topoisomerase I and II (Montaner, Castillo-Avila et al. 2005; Hsieh, Shieh et al. 2012). Thus, those recent studies suggest that prodigiosin induces apoptosis through modulating several pathways as well as damaging DNA directly. And results from this thesis study also support the previous researches about the characteristics of prodigiosin.

4.3. Prodigiosin inhibits NF-κB signaling pathway in cancer cells

Previous studies on prodigiosin associated with NF-κB signaling pathway were focused on primary culture of immunocytes for demonstrating its immunosuppressive property (Mortellaro, Songia et al. 1999; Huh, Yim et al. 2007). However, there were no reports that prodigiosin inhibits the activation of NF-κB in cancer cells. The dose of prodigiosin in those immunosuppression studies was usually low to nanomolar scale. Compared to those studies, the dose used in this study was relatively high for demonstrating the NF-κB inhibitory effect (Fig. 9, Fig. 15). This different dose required for NF-κB inhibitoin could be resulted from the differences in cell type since the inhibitory concentrations 50 (IC_{50}) of MTT and MTS are consisted with other published results. Moreover, our results demonstrated that prodigiosin blocks the phosphorylation of IκBα, resulting in the inactivation of NF-κB signaling pathway (Fig. 4, Fig. 9, and Fig. 15). In addition, the EMSA data suggested that prodigiosin specifically inhibited the classical NF-κB signaling pathway. All these characteristics suggested that prodigiosin might be an IKKβ inhibitor.

4.4. Prodigiosin in combination with bortezomib exert synergistic cell toxicity in human multiple myeloma cells

Current chemotherapy seldom uses one drug alone. In most cases, doctor usually gives two or three chemodrugs for combination since most of cancers display drug resistance after a period of time. In this study, we demonstrated that prodigiosin is an effective agent as a chemotherapy candidate in multiple myeloma. In fact, our results suggested that prodigiosin induces growth inhibition and apoptosis in several other cancer types, including small cell lung cancer (SCLC) and none-small cell lung cancer (NSCLC) (data not shown). Nevertheless, the TNFα induction system did not

work well in both of SCLC and NSCLC cell lines. Thus, we were unable to determine whether prodigiosin is an NF-κB inhibitor in the lung cancer cell line studies. The 26S proteasome inhibitor bortezomib, has exhibited good curative effect on multiple myeloma. However, there were cases reported that some patients were resistance to bortezomib due to activation of the NF-κB pathway (Markovina, Callander et al. 2010). Additionally, the research suggested that bortezomib-induced apoptosis is not related to the repression of NF-κB activity. On the contrary, the studies revealed that bortezomib would activate NF-κB through classical pathway (Hideshima, Ikeda et al. 2009). The same group reported that an IKKβ inhibitor in combination with bortezomib overcame bortezomib resistance (Hideshima, Ikeda et al. 2009). In this thesis study, we demonstrated that prodigiosin suppressed the NF-κB activity induced by bortezomib in a human multiple myeloma cell line. This result supports our hypothesis that prodigiosin might be an IKKβ inhibitor.

For chemotherapy, doxorubicin is a common drug used in many types of cancers, including bladder, breast, lung, and multiple myeloma. It interacts with DNA by intercalation and inhibition of molecular biosynthesis; thus, it usually accompanies cardiotoxicity in higher dosage. Therefore, a combination with other chemodrugs is a good solution to reduce the dosage of doxorubicin. Our data showed that prodigiosin in combination with doxorubicin were not effective in treating multiple myeloma cells. On the other hand, doxorubicin in combination with histone deacetylase inhibitor displayed a synergistic effect (Sanchez, Shen et al. 2011).

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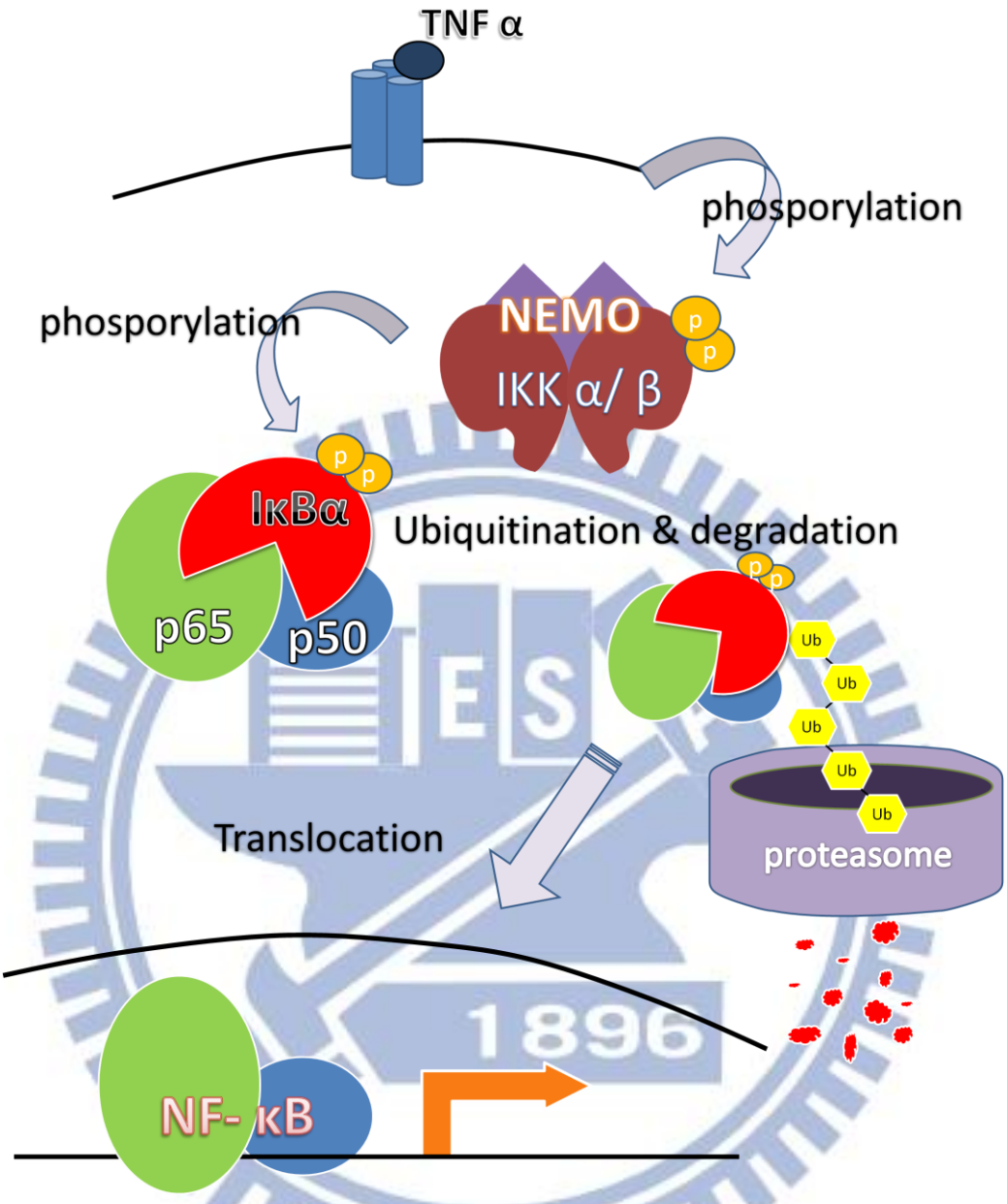


Figure 1. The classical NF-κB signaling pathway

Starting from receiving signal, the phosphorylation is occurred on IKK complex which is the key modulator of whole pathway. Followed by phosphorylating IκBα, IκBα is degraded by proteasome resulting in translocation of NF-κB complex which turn on multiple genes associating with cancer progression (Hayden and Ghosh, 2004).

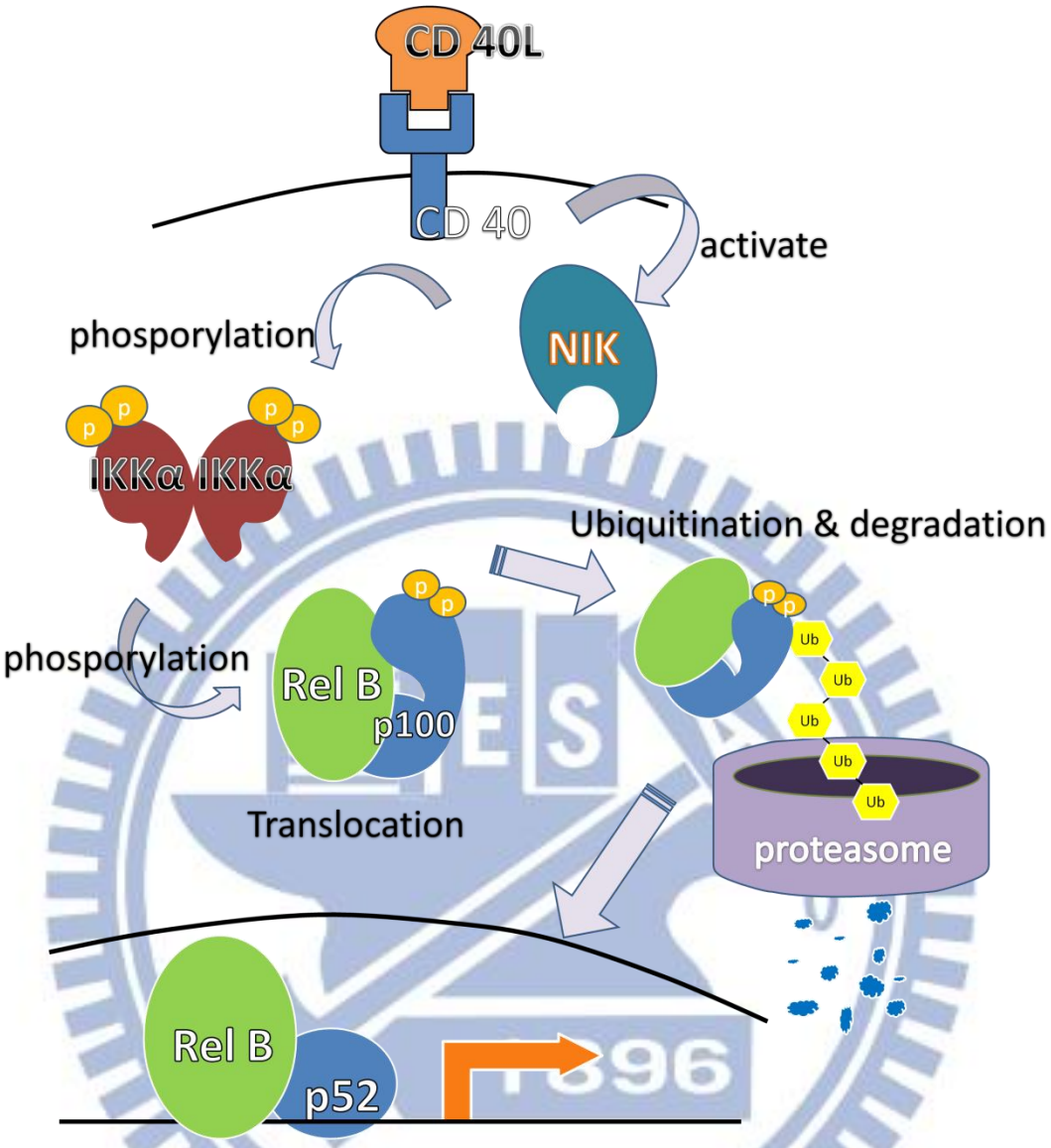


Figure 2. The alternative NF-κB signaling pathway

The alternative pathway has NIK which does not exist in classical pathway. The activation of NIK results in the phosphorylation of IKKα homodimer which is followed by phosphorylating p100. The phosphorylated p100 will be processed by proteasome and then form the active NF-κB complex which is able to translocate into nucleus (Hayden and Ghosh, 2004).

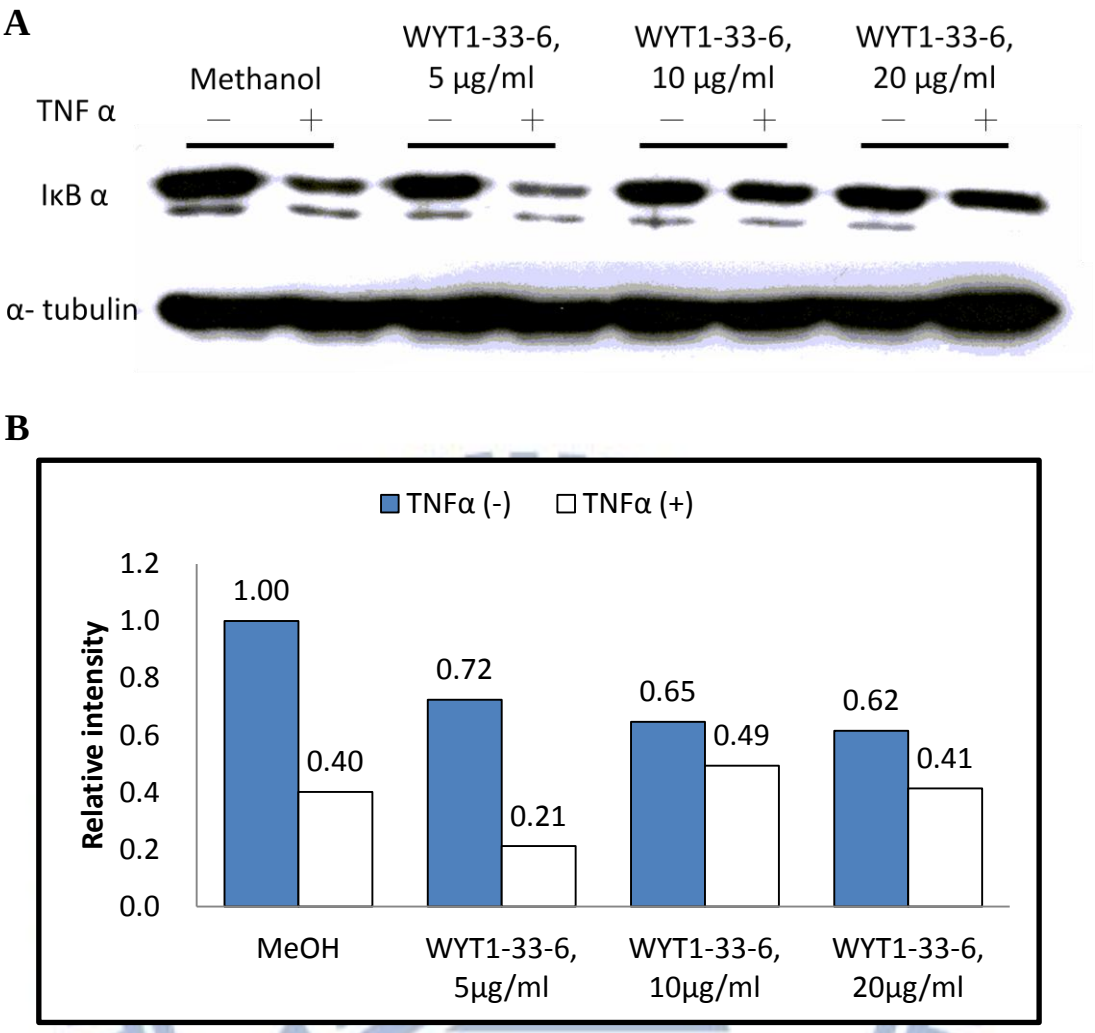


Figure 3. WYT-33-6 dose-dependently inhibits the degradation of IκBα in NIH3T3 cells. (A) NIH3T3 cells were treated with increasing concentrations of WYT1-33-6 for 2 h. Cells were then treated with TNFα (+) for 20 min or not treated as a control (-). Equal amounts of whole cell lysates were analyzed by Western blotting for IκBα or α-tubulin (loading control). (B) The blots in (A) were scanned and quantified using the NIH ImageJ software.

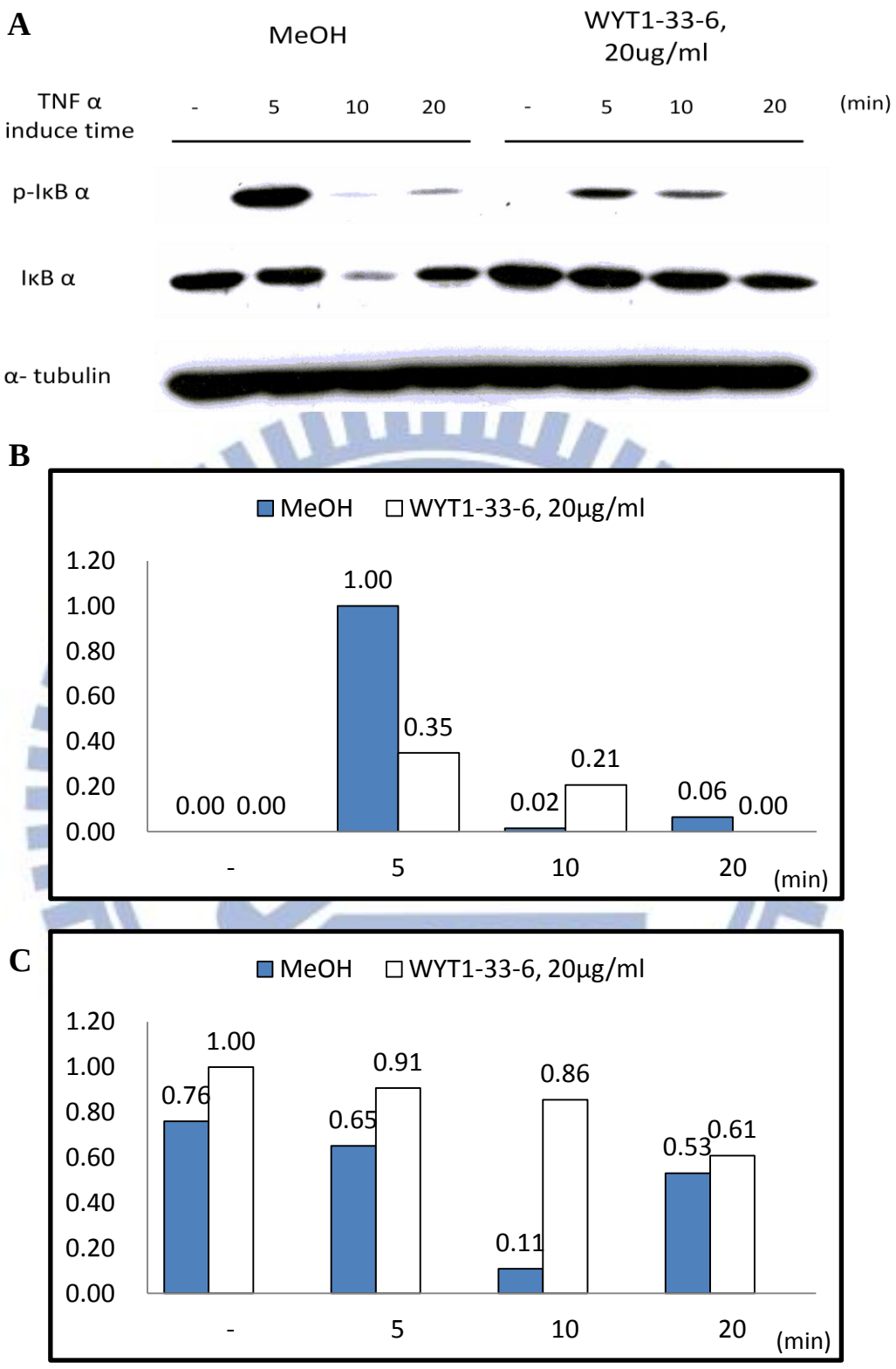


Figure 4. WYT1-33-6 time-dependently blocks the phosphorylation of IκBα in TNFα induced NIH3T cells.

Figure 4. WYT1-33-6 time-dependently blocks the phosphorylation of IκBα in TNFα induced NIH3T cells. (A) NIH3T3 cells were treated with 20 μg/ml of WYT1-33-6 or the solvent methanol (MeOH) for 2 h. Cells were then treated with TNFα for indicated times (0-20 min). Equal amounts of protein were analyzed by Western blotting for p- IκBα, IκBα and α-tubulin. The blots in (A) were scanned and quantified using the NIH ImageJ software and then the figures were plotted into (B) p- IκBα and (C) IκBα.

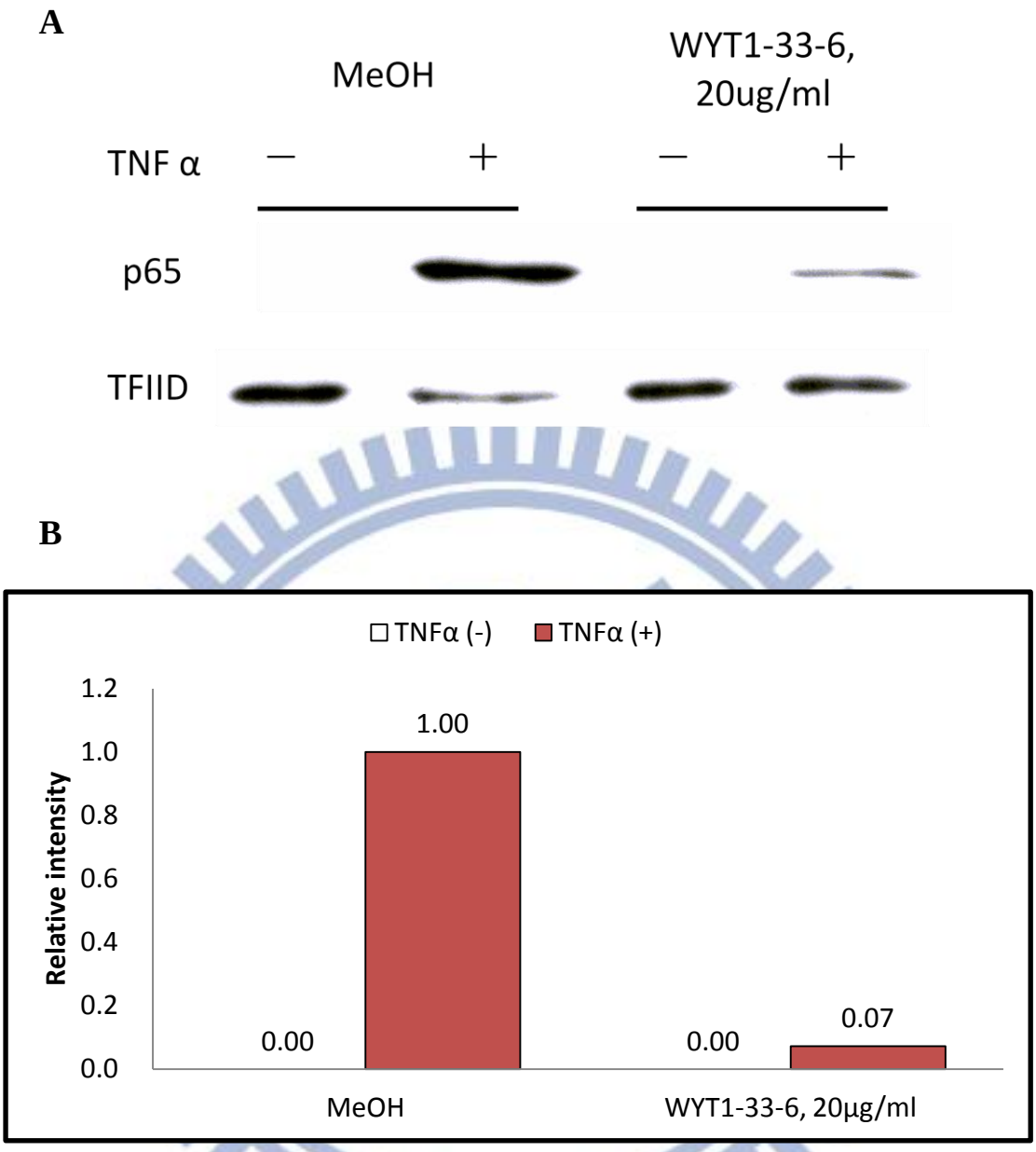


Figure 5. WYT1-33-6 inhibits NF-κB subunit p65 nuclear translocation in TNFα induced NIH3T3 cells. (A) NIH3T3 cells were treated with 20 μg/ml or the solvent methanol (MeOH) of WYT1-33-6 for 2 h. Cells then treated with TNFα (+) for 20 min or not treated as control. Equal amounts of nuclear extracts were analyzed by Western blotting for p65 or TFIID (loading control). (B) The blots in (A) were scanned and quantified using the NIH ImageJ software.

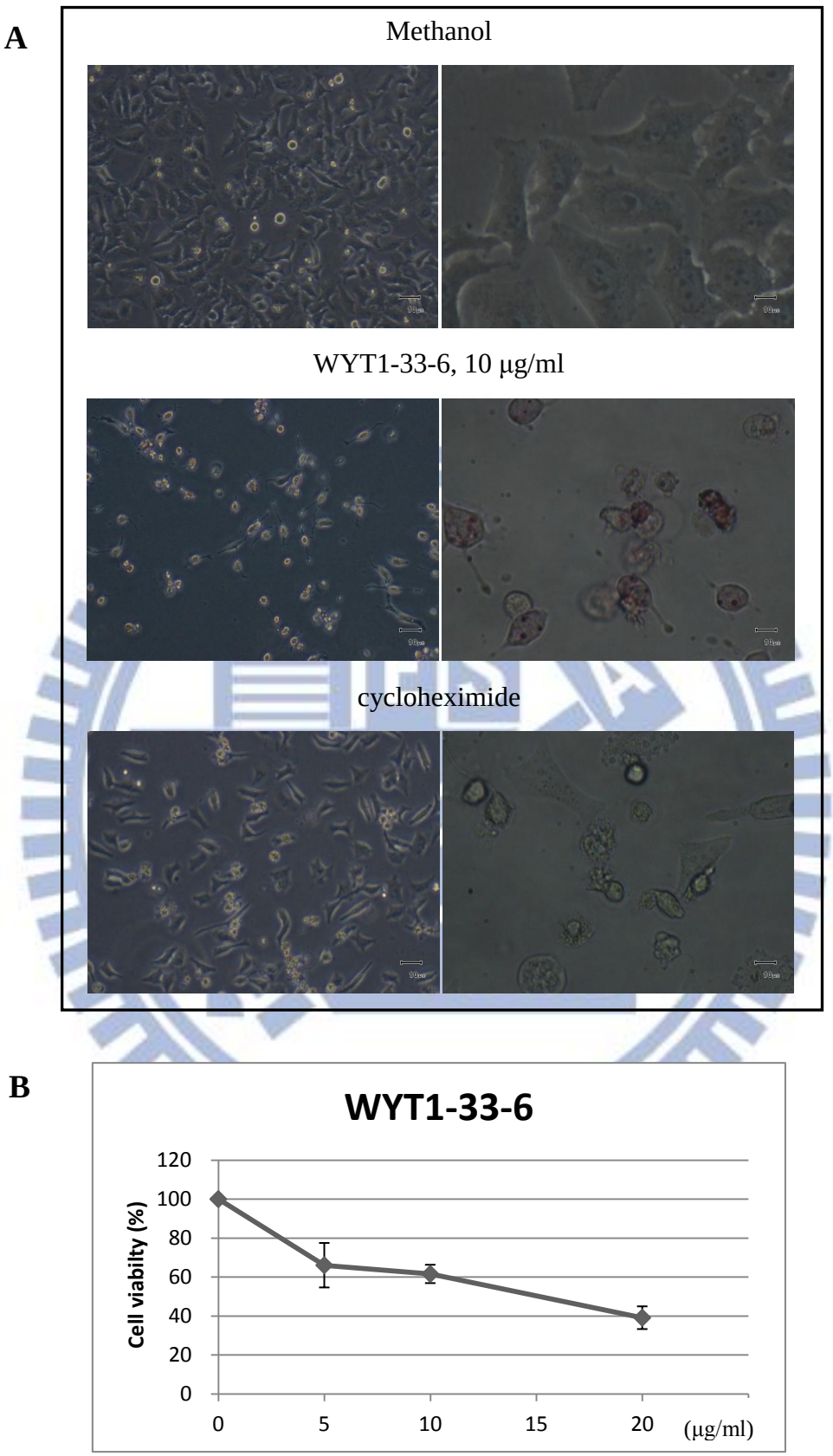


Figure 6. WYT1-33-6 causes cellular morphological changes and reduces cell viability in the human cervical cancer HeLa cells.

Figure 6. WYT1-33-6 causes cellular morphological changes and reduces cell viability in the human cervical cancer HeLa cells.

(A) HeLa cells were incubated with either the solvent methanol, WYT1-33-6 (10μg/ml), or cycloheximide (10 μg/ml) for 24 h and the cellular morphology changes were observed by different magnifications of microscopy. (B) The cell viability of HeLa cells treated with increasing concentrations of WYT1-33-6 for 24h was determined by MTT assay as describe in the Materials and Methods. Data are expressed as mean ± SD (n=3).

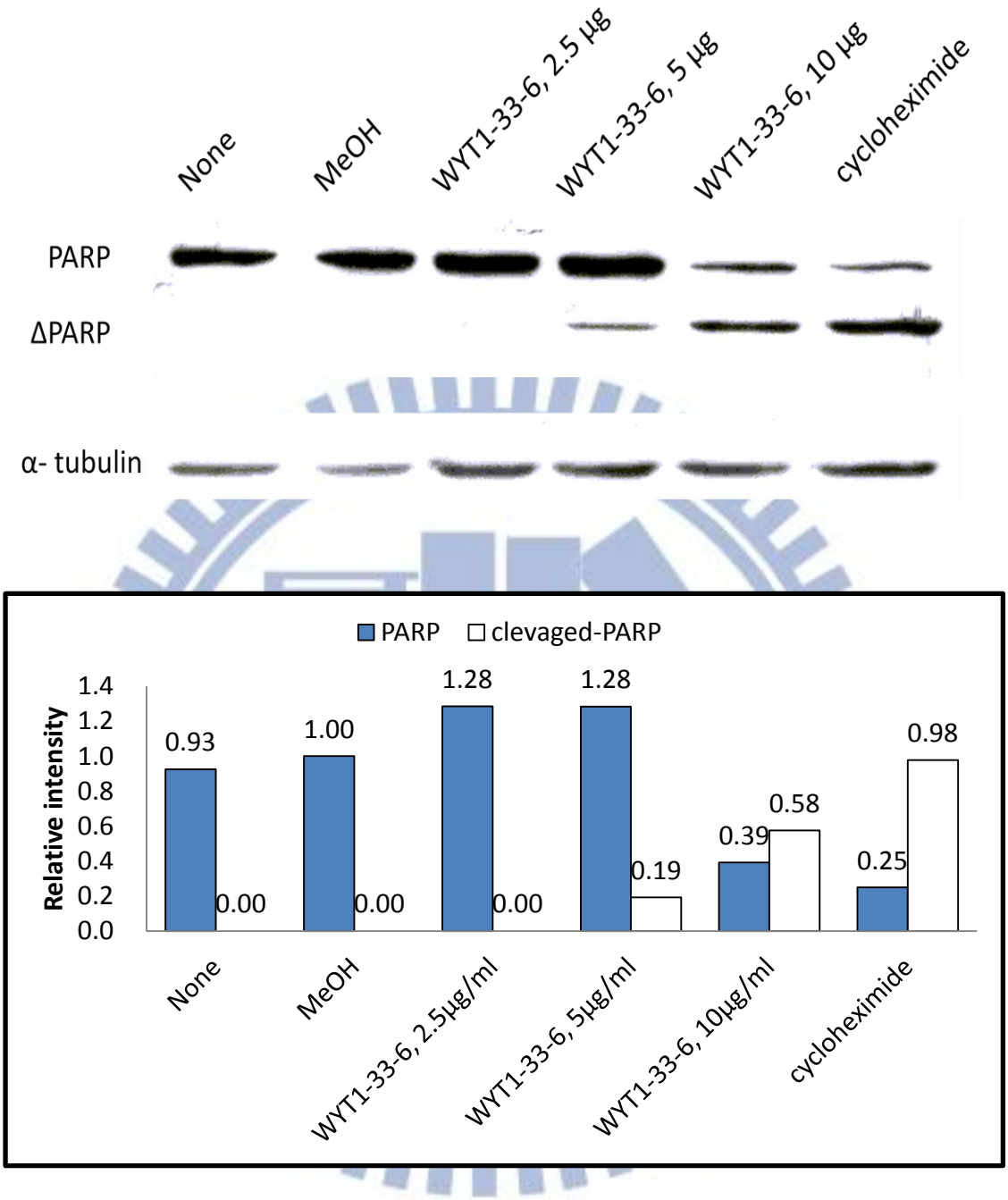
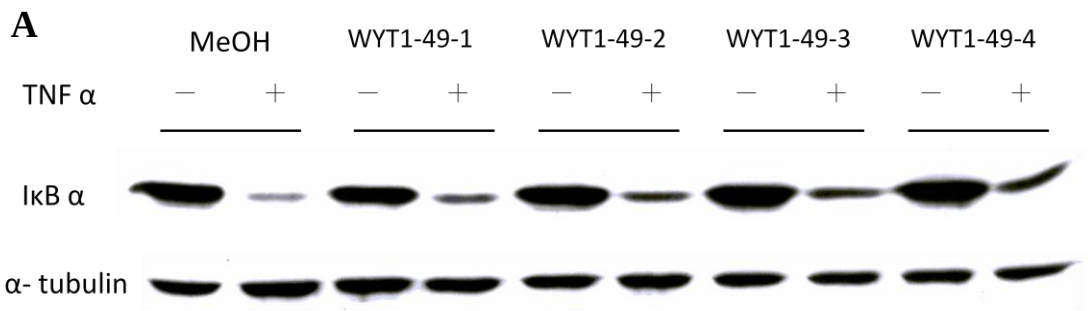
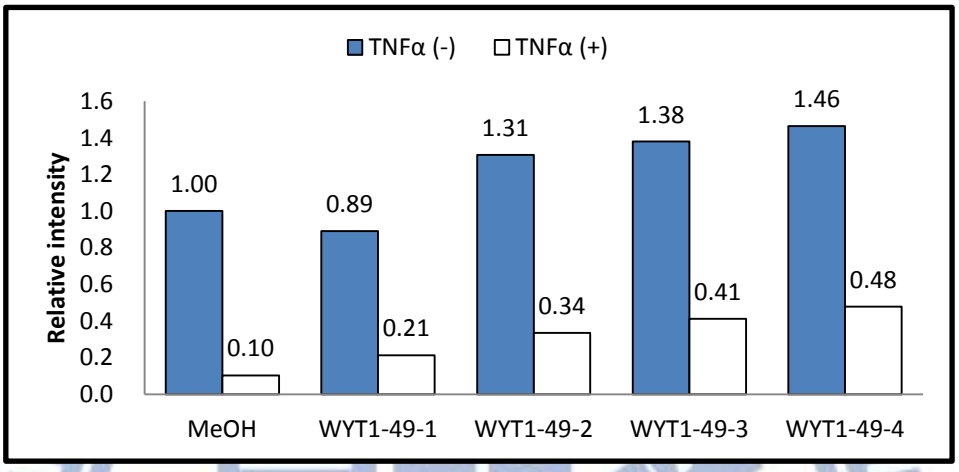
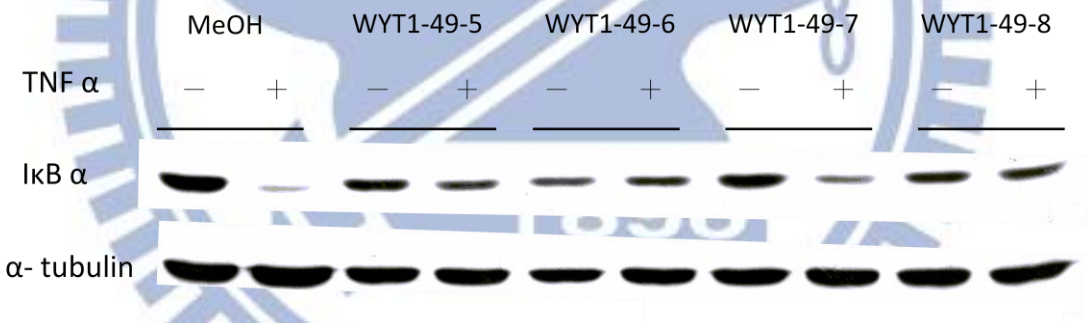


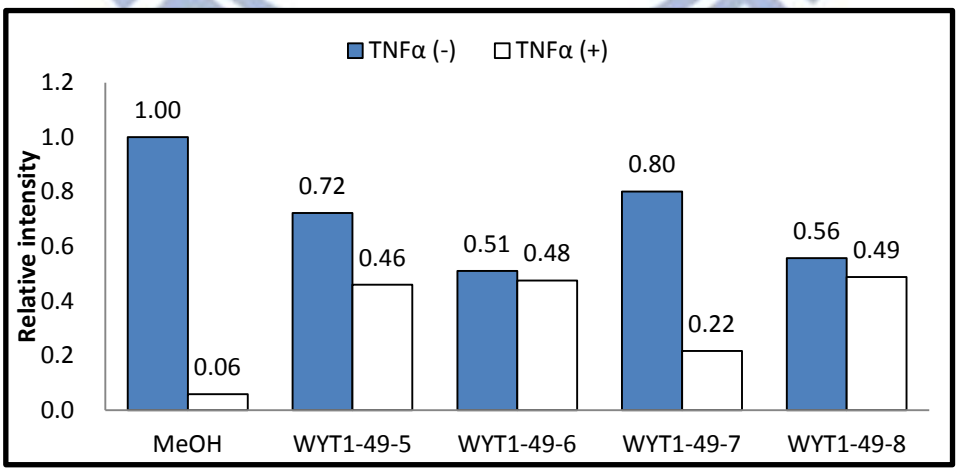
Figure 7. WYT1-33-6 dose-dependently induces apoptosis which in human cervical cancer HeLa cells. (A) HeLa cells were incubated with no addition (None), the solvent methanol (MeOH), or increasing concentrations of WYT1-33-6 and 10 μg/ml of cycloheximide for 24 h. The whole cell lysates were analyzed by Western blotting for PARP or α-tubulin (loading control). (B) The blots in (A) were scanned and quantified using the NIH ImageJ software.

A


MeOH
WYT1-49-1
WYT1-49-2
WYT1-49-3
WYT1-49-4
TNF α
IκB α
α- tubulin


TNFα (-)
TNFα (+)
Relative intensity
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.00
0.10
0.89
0.21
1.31
0.34
1.38
0.41
1.46
0.48
MeOH
WYT1-49-1
WYT1-49-2
WYT1-49-3
WYT1-49-4


MeOH
WYT1-49-5
WYT1-49-6
WYT1-49-7
WYT1-49-8
TNF α
IκB α
α- tubulin


TNFα (-)
TNFα (+)
Relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.00
0.06
0.72
0.46
0.51
0.48
0.80
0.22
0.56
0.49
MeOH
WYT1-49-5
WYT1-49-6
WYT1-49-7
WYT1-49-8

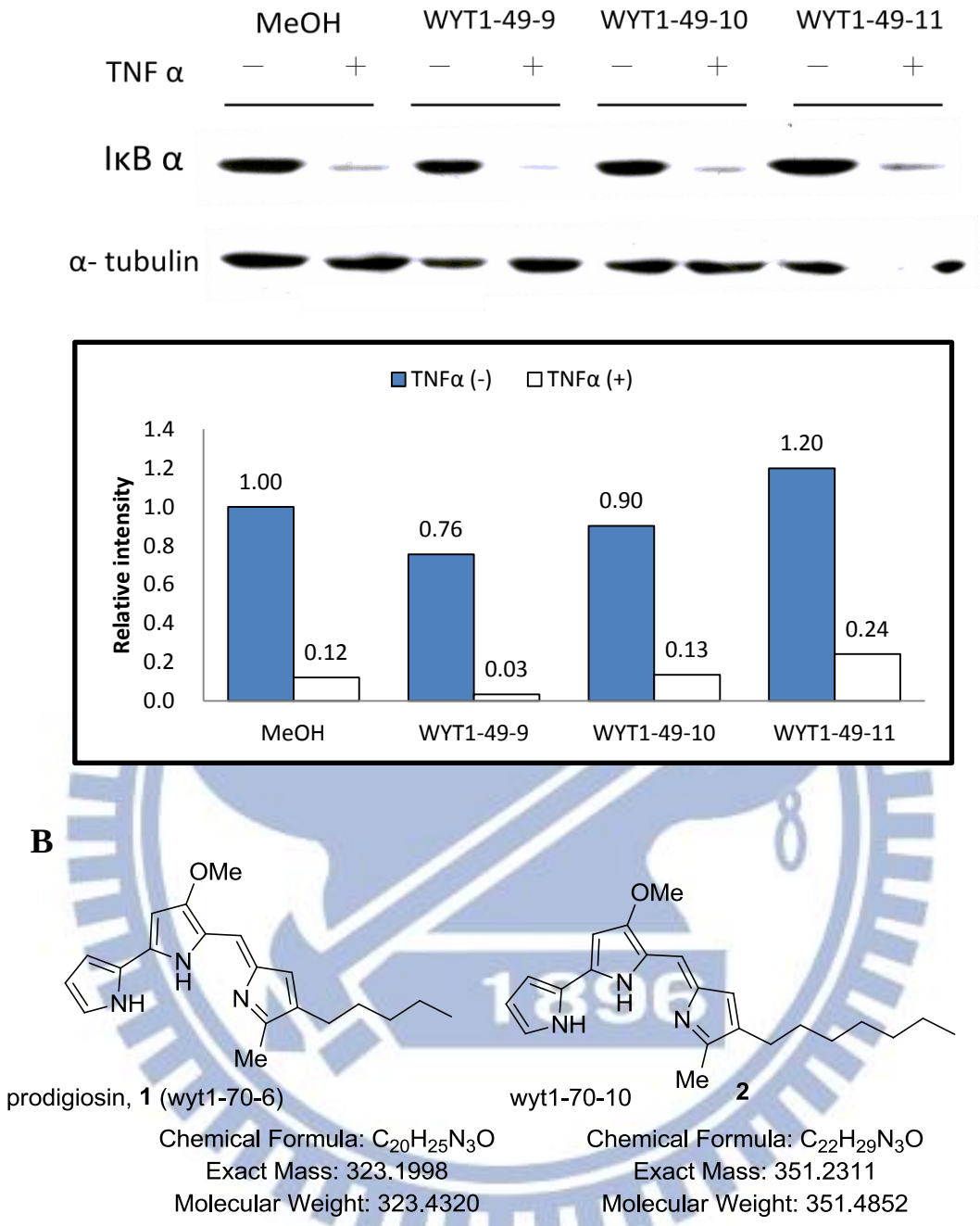


Figure 8. Identification of fractions of WYT1-33-6 with NF-κB inhibition effects in TNFα induced NIH3T3 cells. (A) NIH3T3 cells were treated with different fractions of WYT1-33-6 (WYT1-49-1–WYT1-49-11) in final concentration of 20 μg/ml for 2 h prior to TNFα induction. Whole cell lysates were harvested and then analyzed by Western blotting. The blots were scanned and quantified using the NIH ImageJ software. (B) The structures of WYT1-70-6 (prodigiosin) and its analog WYT1-70-10 (2-methyl-3-heptyl prodigiosin).

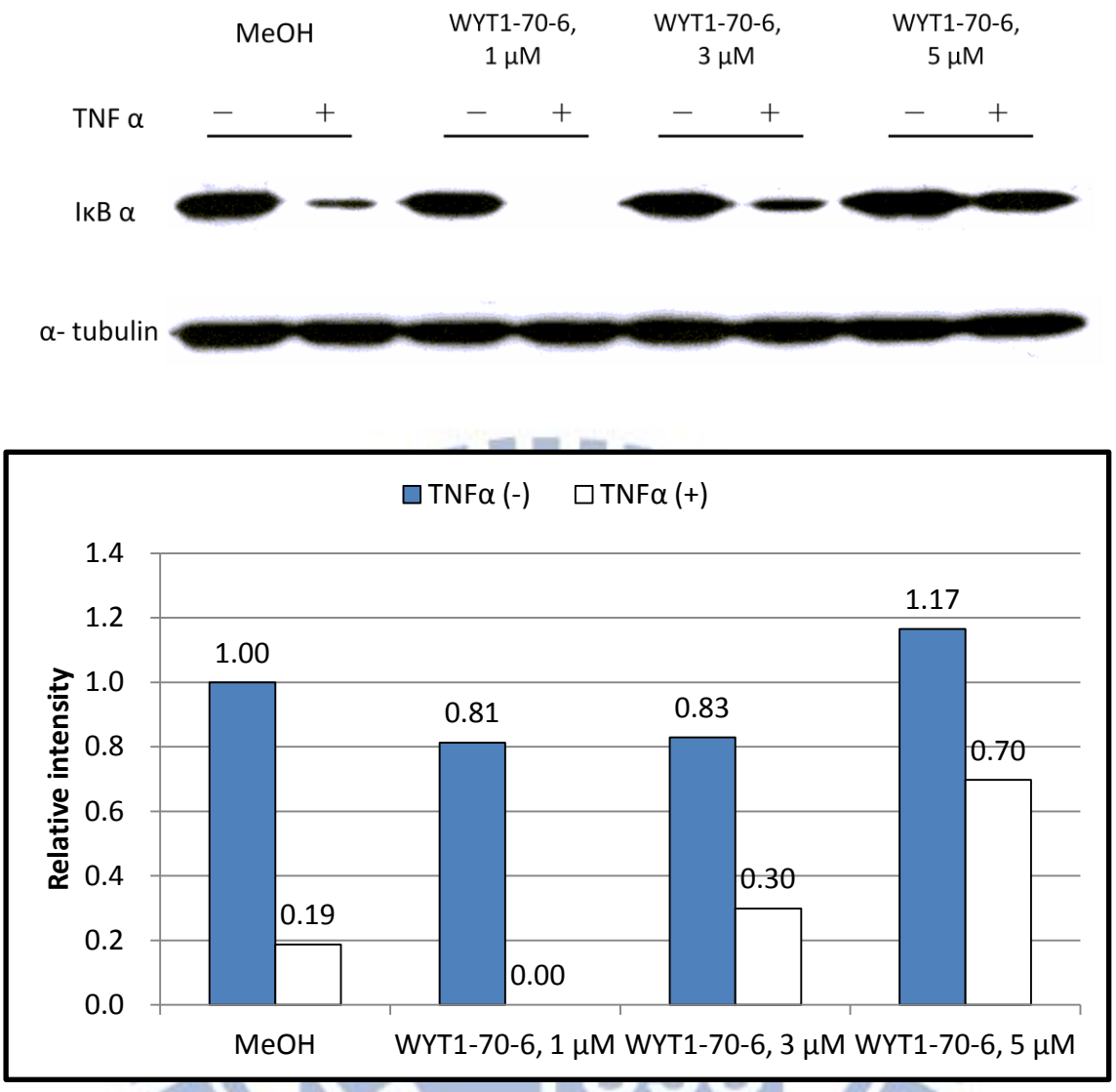


Figure 9. WYT1-70-6 dose-dependently inhibits the degradation of IκBα in TNFα induced human multiple myeloma RPMI 8226 multiple cells. RPMI 8226 cells were treated with increasing concentrations of WYT1-70-6 or the solvent methanol (MeOH) for 2 h. Cells were then treated with TNFα (+) for 20 min or (-) not treated as control. Equal amounts of whole cell lysates protein were analyzed by Western blotting for IκBα or α-tubulin (loading control). The blots were scanned and quantified using the NIH ImageJ software.

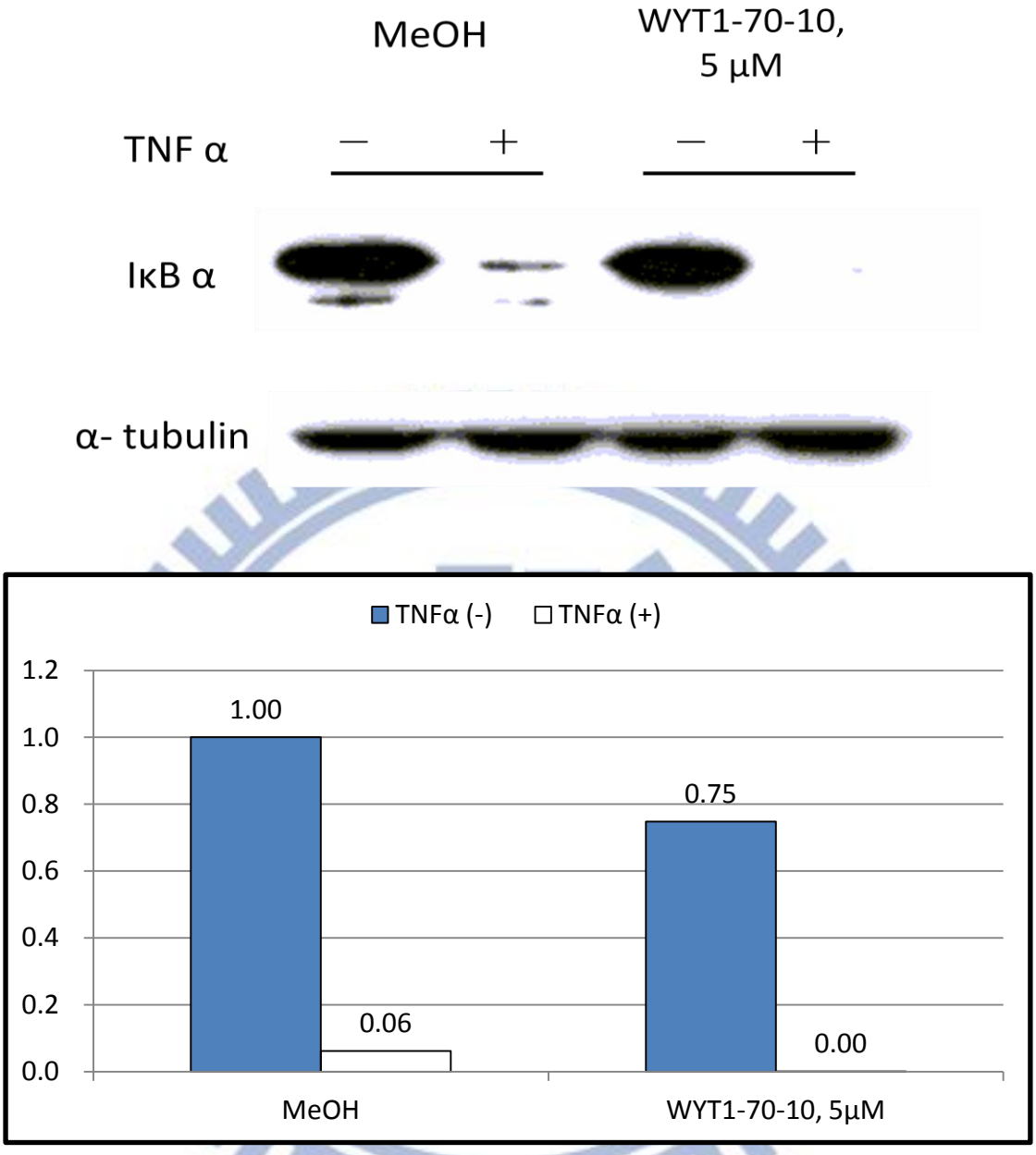


Figure 10. WYT1-70-10, the prodigiosin analog dose not block IκBα degradation in TNFα induced human multiple myeloma RPMI 8226 cells. RPMI 8226 cells were treated with 5 μM WYT1-70-10 or the solvent methanol (MeOH) for 2 h. Cells were then treated with TNFα (+) for 20 min or (-) not treated as a control. Equal amounts of whole cell lysates protein were analyzed by Western blotting for IκBα or α-tubulin (loading control). The blots were scanned and quantified using the NIH ImageJ software.

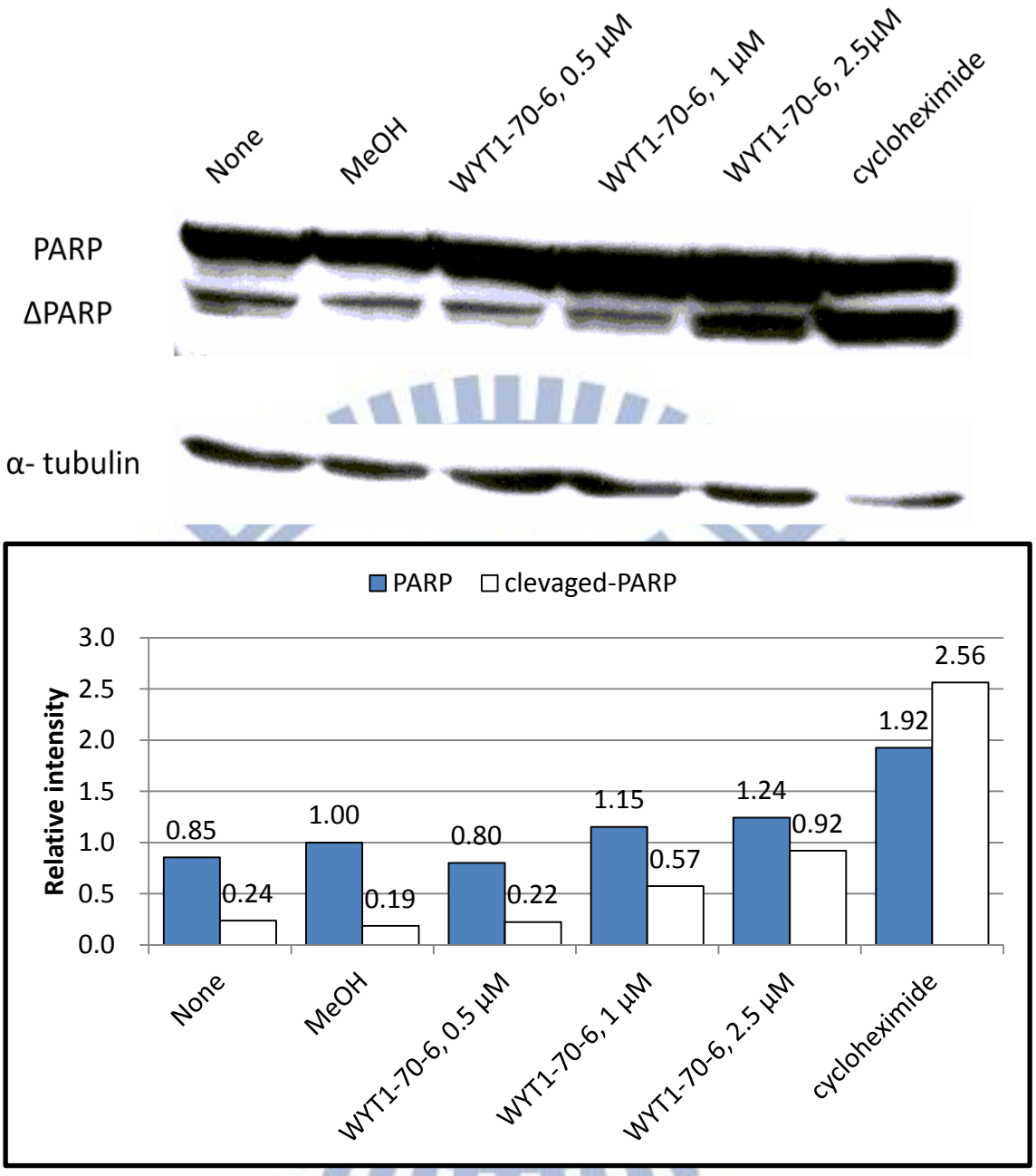


Figure 11. WYT1-70-6 induces apoptosis related PARP –cleavage in human multiple myeloma RPMI 8226 cells. RPMI 8226 cells were incubated with no addition (None), the solvent methanol (MeOH), increasing concentrations of WYT1-70-6 or cycloheximide (10 μg/ml) for 24 h. The whole cell lysates were analyzed by Western blotting for PARP or α-tubulin (loading control). The blots were scanned and quantified using the NIH ImageJ software.

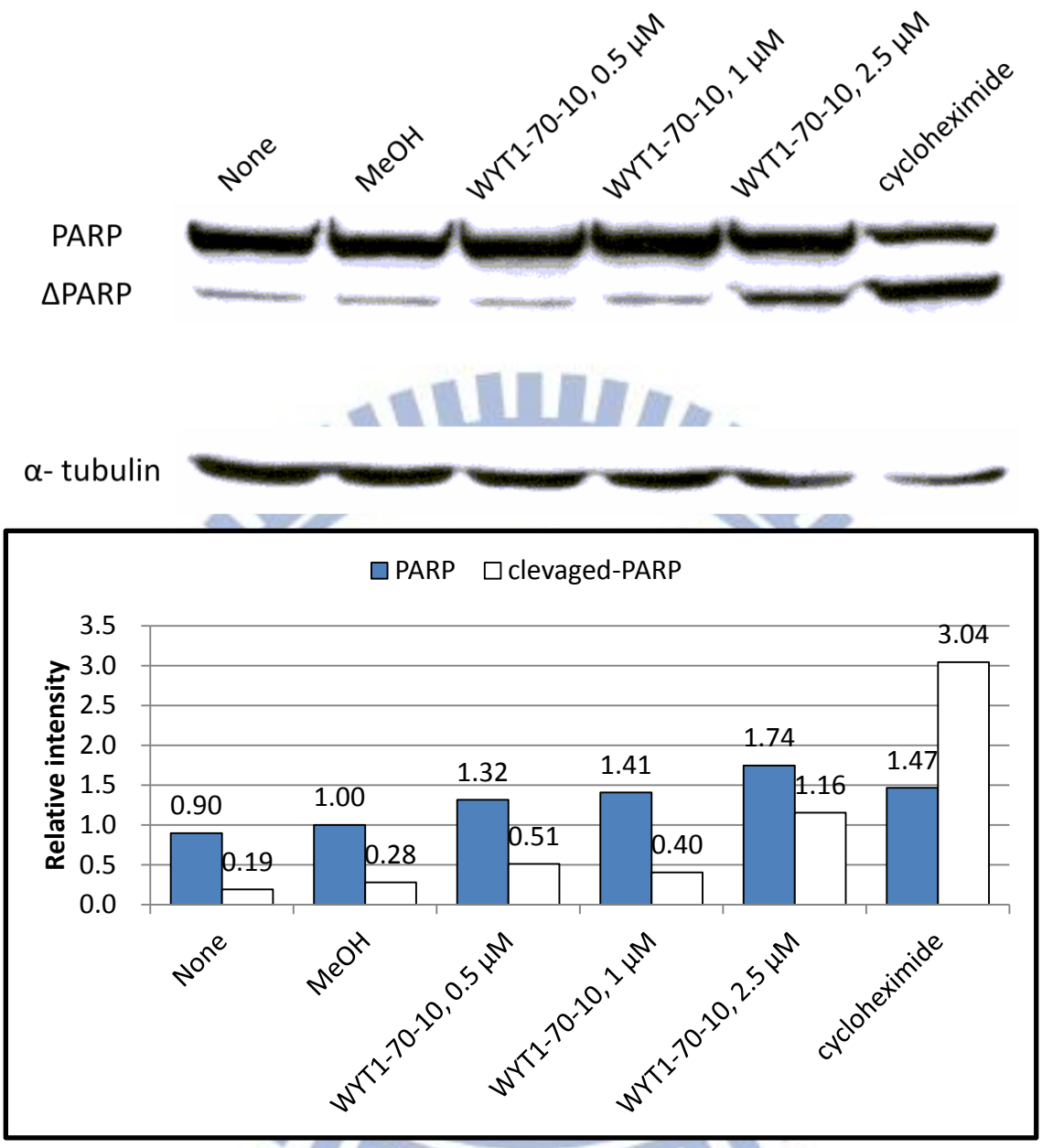
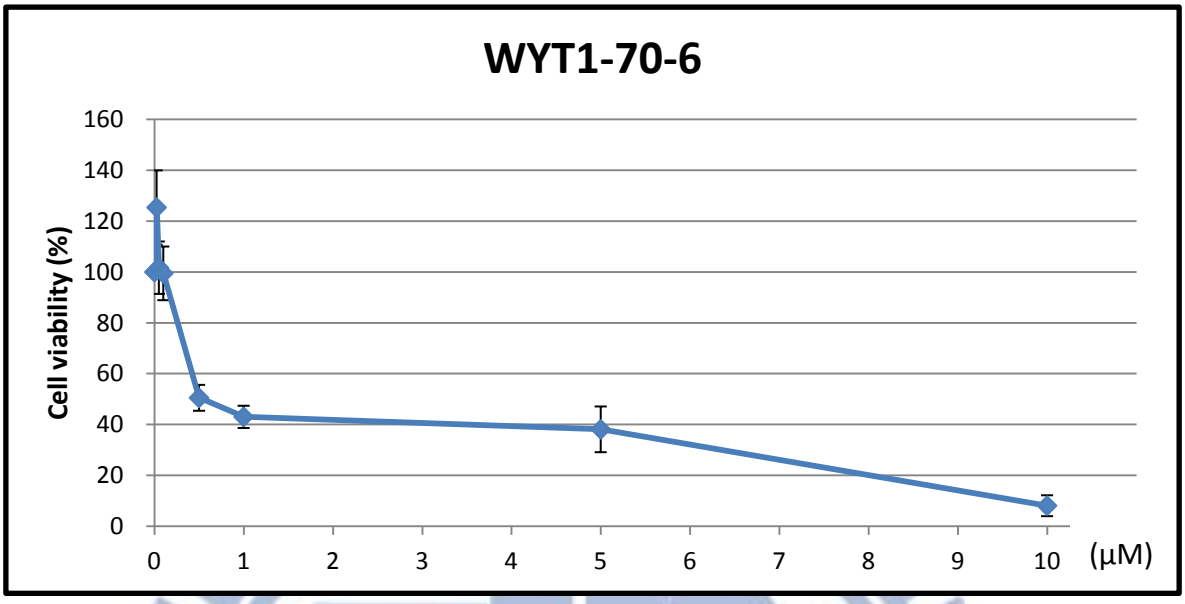


Figure 12. WYT1-70-10 induces apoptosis related PARP –cleavage in human multiple myeloma RPMI 8226 cells. RPMI 8226 cells were incubated with no addition (None), the solvent methanol (MeOH), increasing concentrations of WYT1-70-10 or cycloheximide (10 μg/ml) for 24 h. The whole cell lysates were analyzed by Western blotting for PARP or α-tubulin (loading control). The blots were scanned and quantified using the NIH ImageJ software.

A



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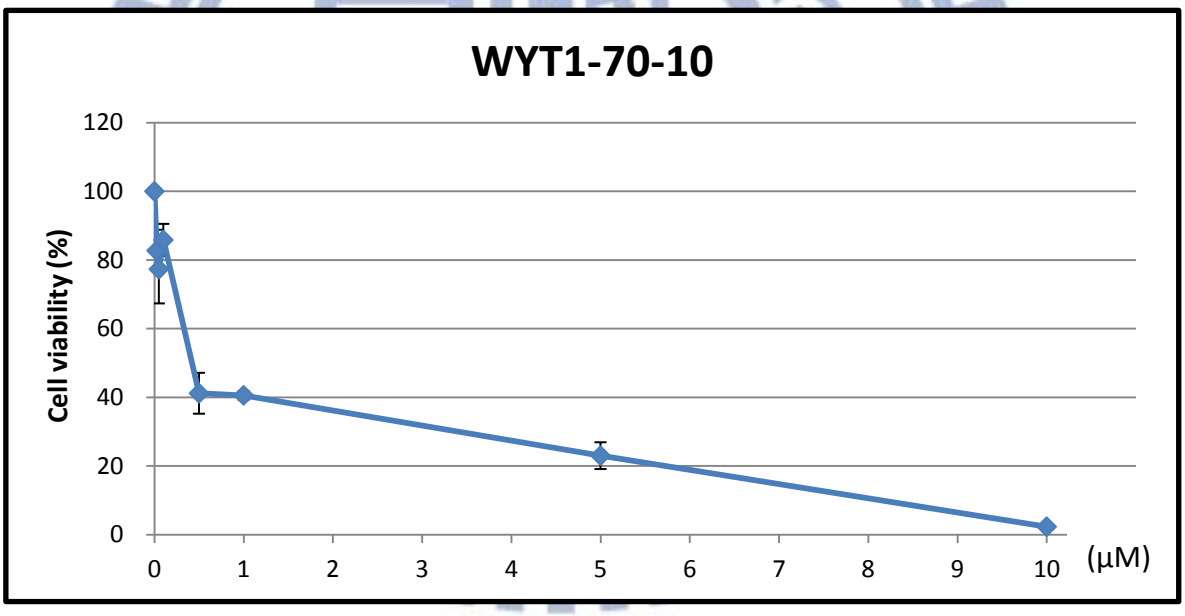


Figure 13. WYT1-70-6 and its analog WYT1-70-10 exhibit a similar cell growth inhibition effect in human multiple myeloma RPMI 82206 cells. RPMI 8226 cells were treated with (A) WYT1-70-6 or (B) WYT1-70-10 for 24 h and cell proliferation was determined by MTS assay as described in the Materials and Methods. Data are expressed as mean ± SD (n=3).

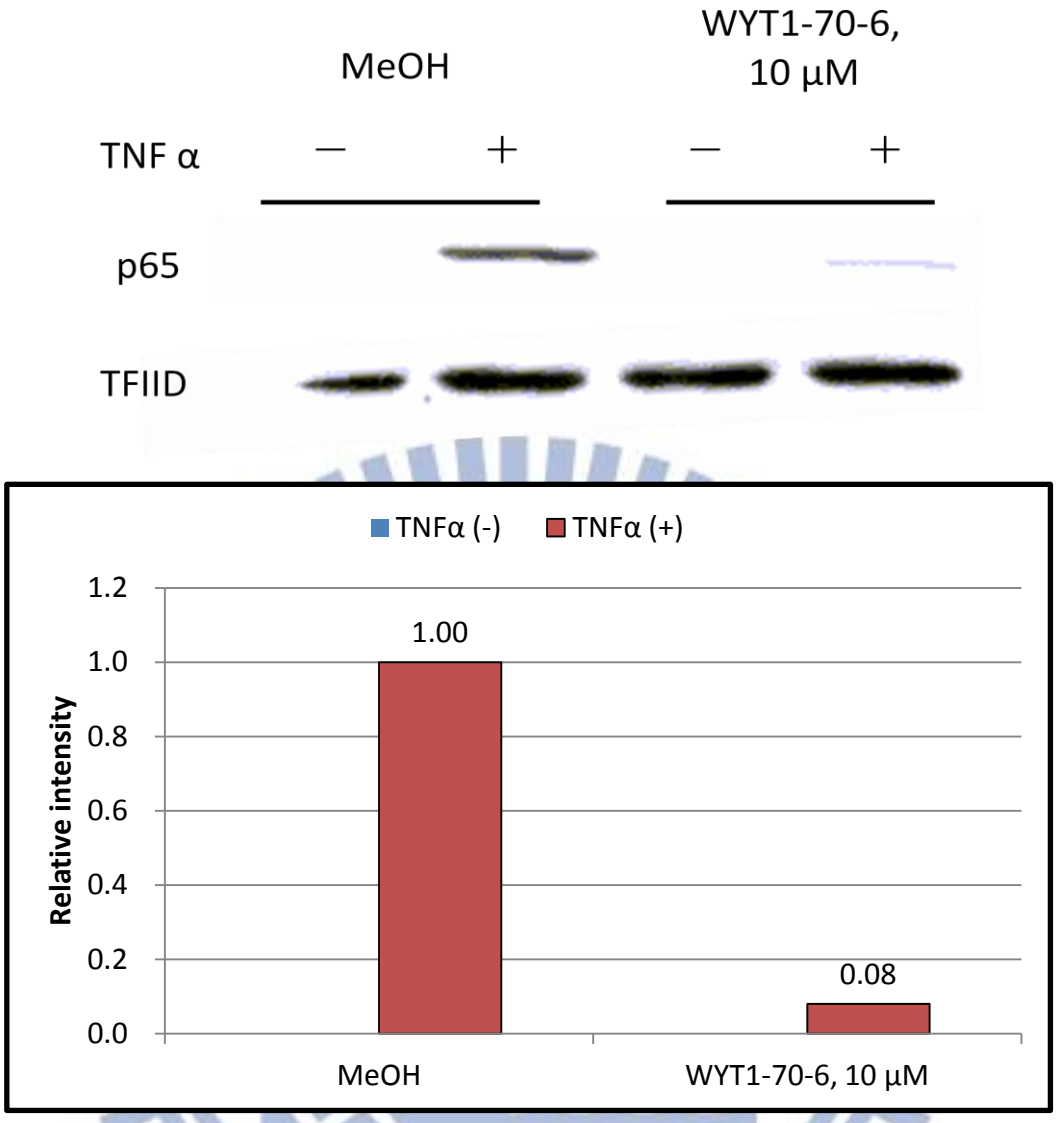


Figure 14. WYT1-70-6 inhibits NF-κB subunit p65 nuclear translocation in TNFα induced RPMI 8226 cells. RPMI 8226 cells were treated with 10 μM WYT1-70-6 or the solvent methanol (MeOH) for 8 h. Cells then treated with TNFα (+) for 20 min or not treated as control. Equal amounts of nuclear extracts were analyzed by Western blotting for p65 or TFIID (loading control). The blots were scanned and quantified using the NIH ImageJ software.

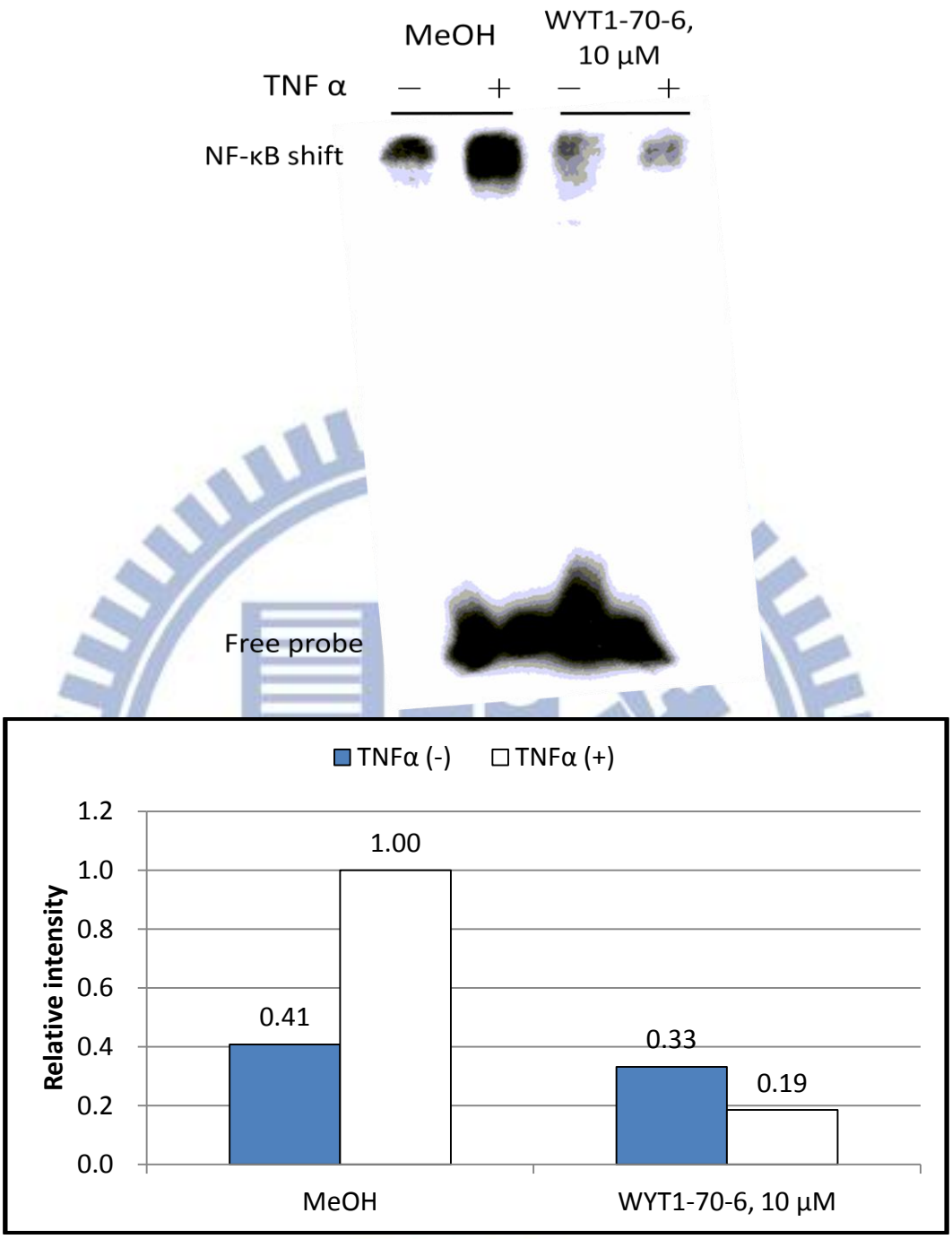


Figure 15. WYT1-70-6 represses the activity of NF-κB complex in TNFα induced RPMI 8226 cells. RPMI 8226 cells were treated with the solvent methanol (MeOH) or 10 μM WYT1-70-6 for 8 h. Cells then treated with TNFα (+) for 20 min or not treated as a control. Equal amounts of nuclear extracts were analyzed by EMSA using a biotin-labeled κB site probe as described in the Materials and Methods. The blots were scanned and quantified using the NIH ImageJ software.

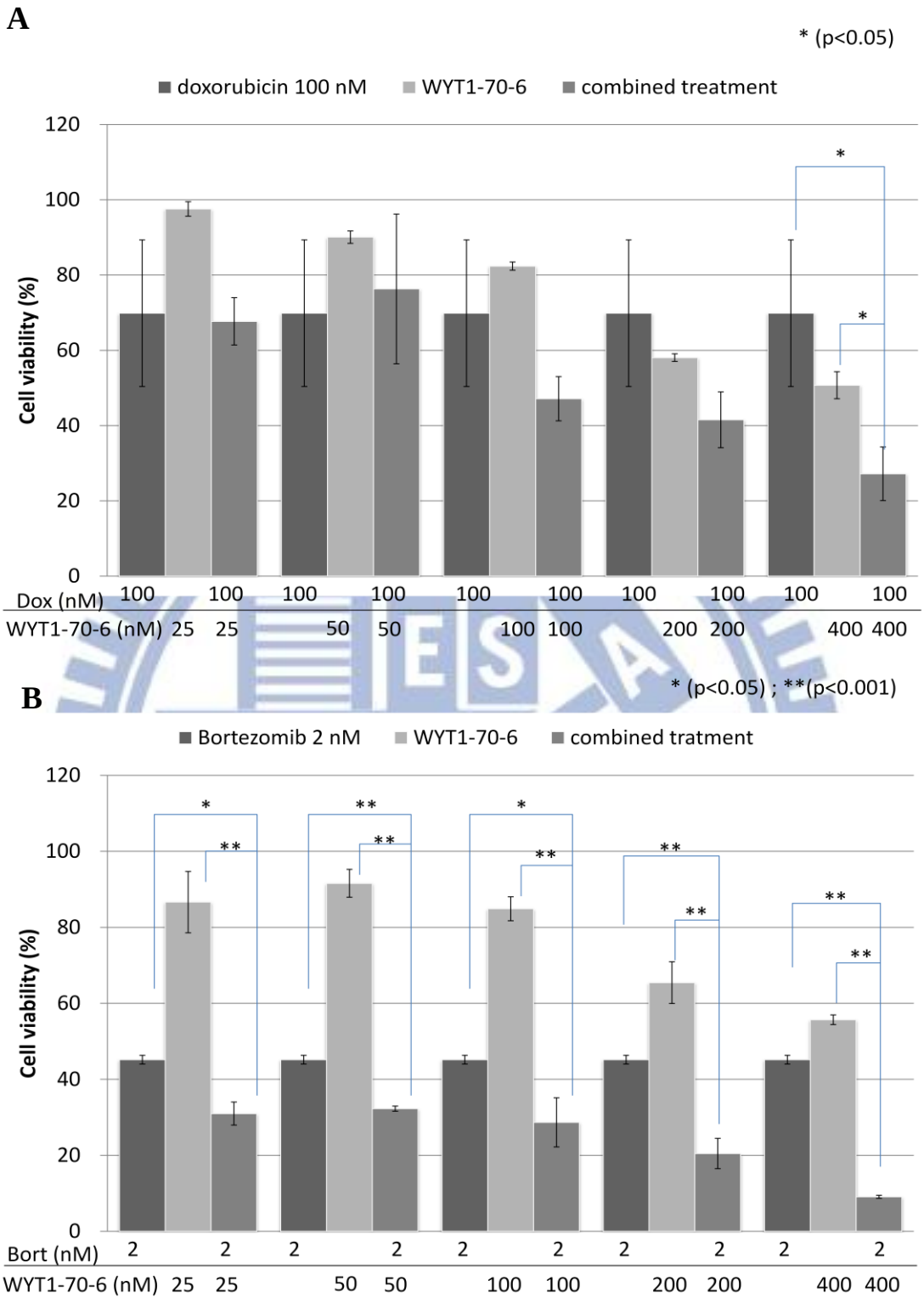
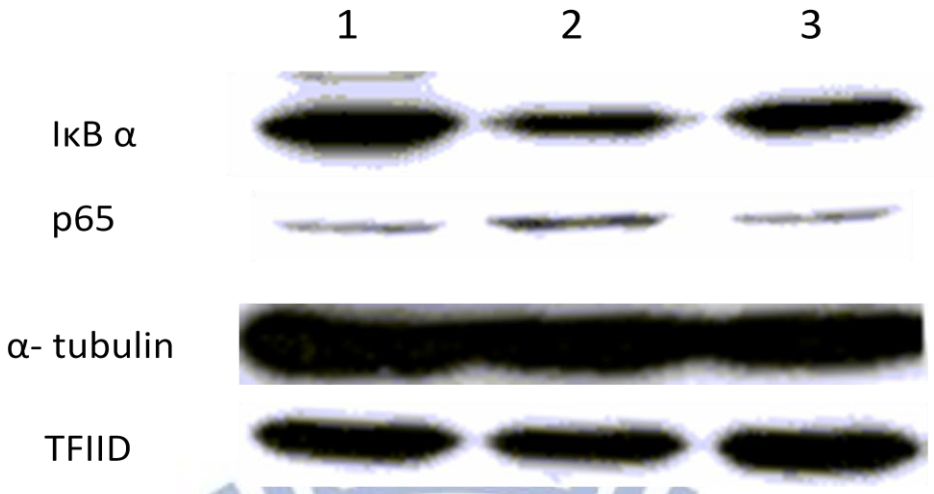


Figure 16. Synergistic anti-myeloma activity of WYT1-70-6 in combination with clinically used drugs. RPMI 8226 cells were incubated with increasing concentrations of WYT1-70-6 in combination with (A) 100 nM doxorubicin or (B) 2 nM bortezomib for 24 h. Cell viability was assessed by MTS assay as described in the Materials and Methods. Data are expressed as mean ± SD (n=3).

A



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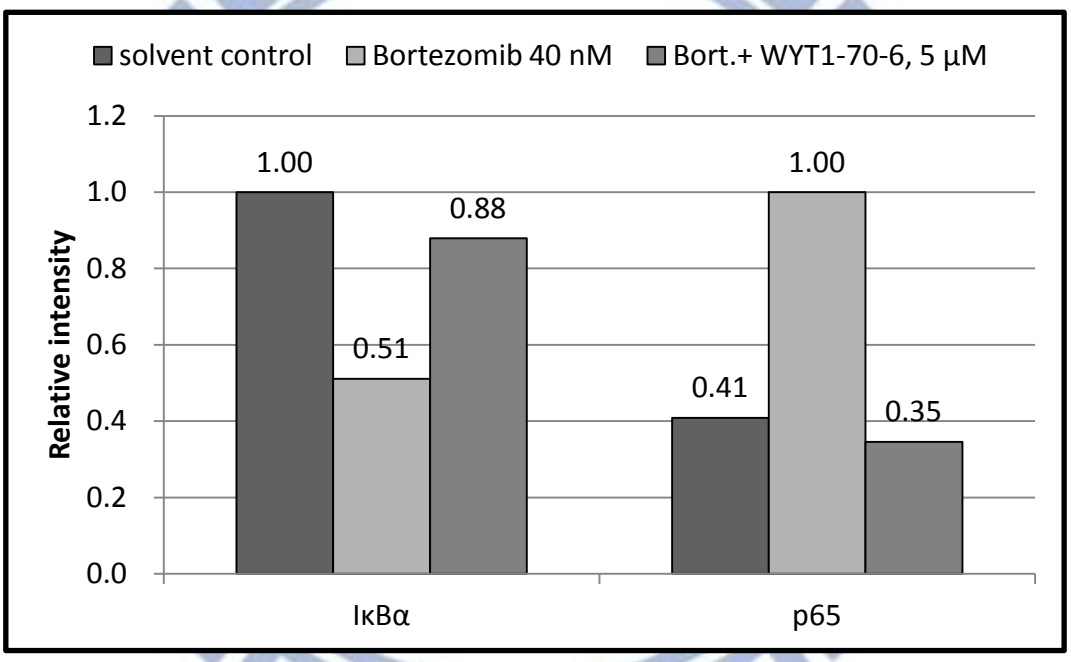
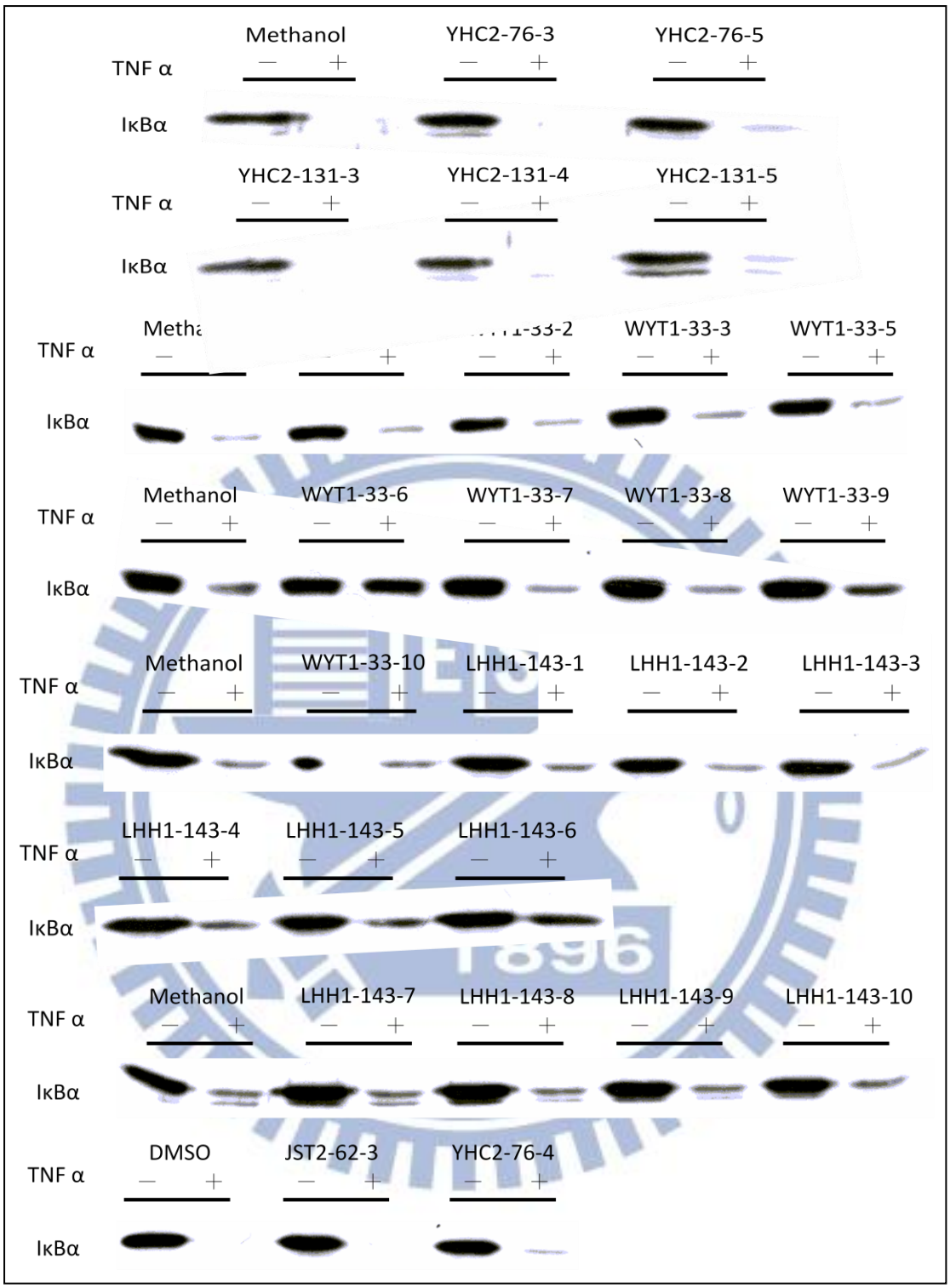
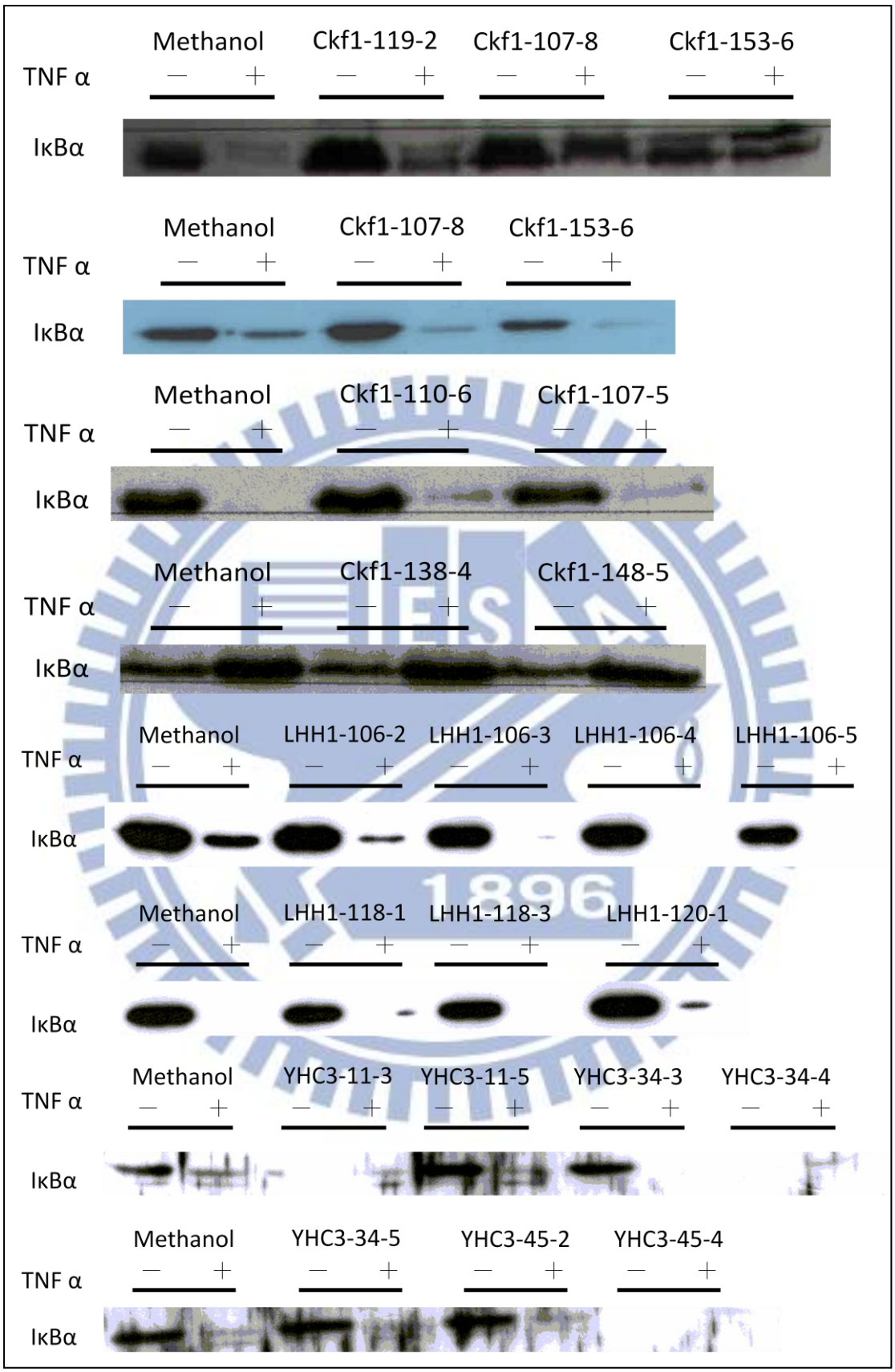


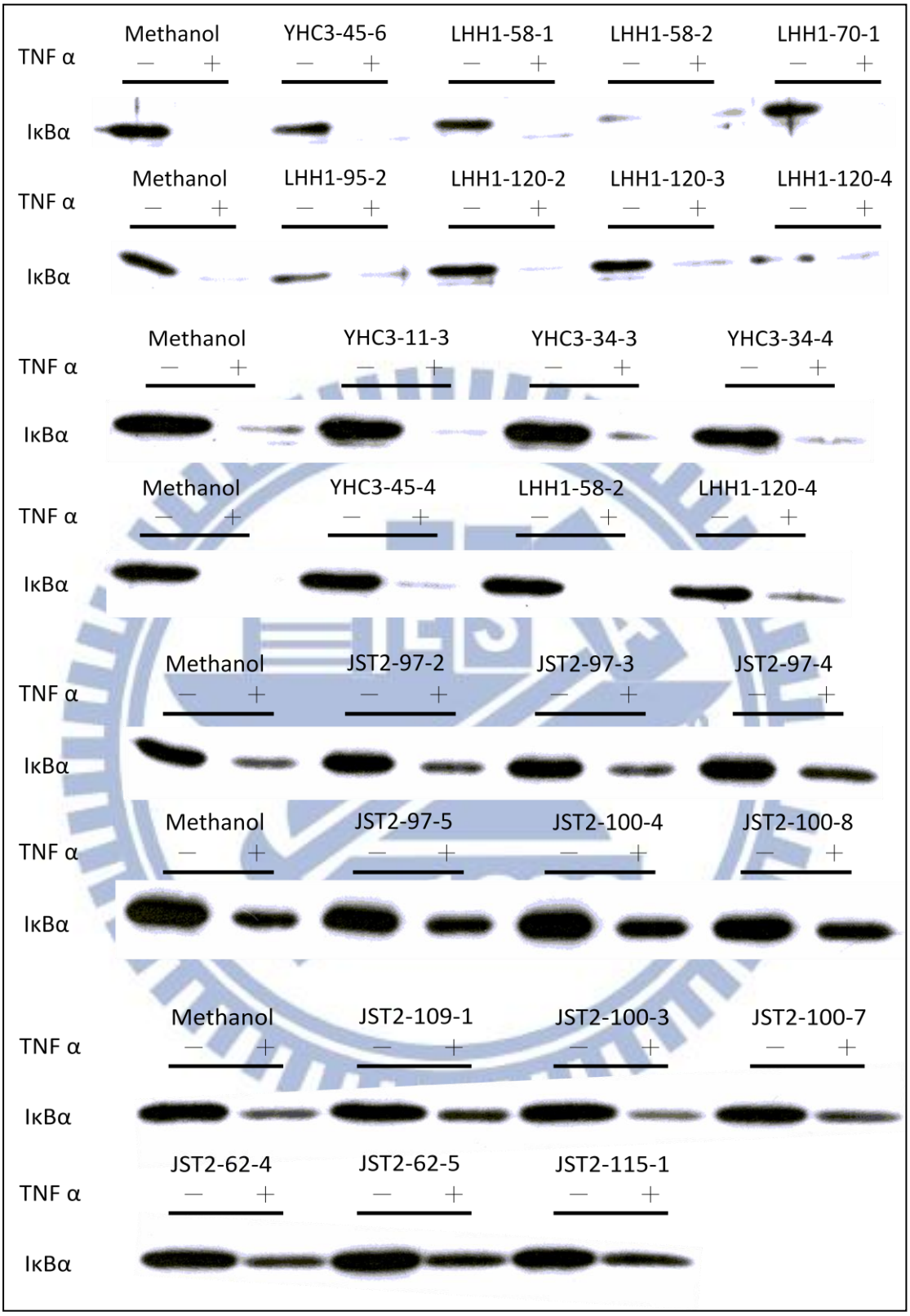
Figure 17. The NF-κB activation induced by bortezomib is suppressed by WYT1-70-6. (A) RPMI 8226 cells were treated with 40 nM bortezomib alone (lane 1), combined with 5 μM WYT-70-6 (lane 3) or the solvent methanol and DMSO as a control (lane 1) for 8 h without TNFα induction. The whole cell lysates were harvested and then analyzed by Western blotting for IκBα or α-tubulin; the nuclear extracts were harvested and then analyzed by Western blotting for p65 or TFIID (loading control). (B) The blots in (A) were scanned and quantified using the NIH ImageJ software.



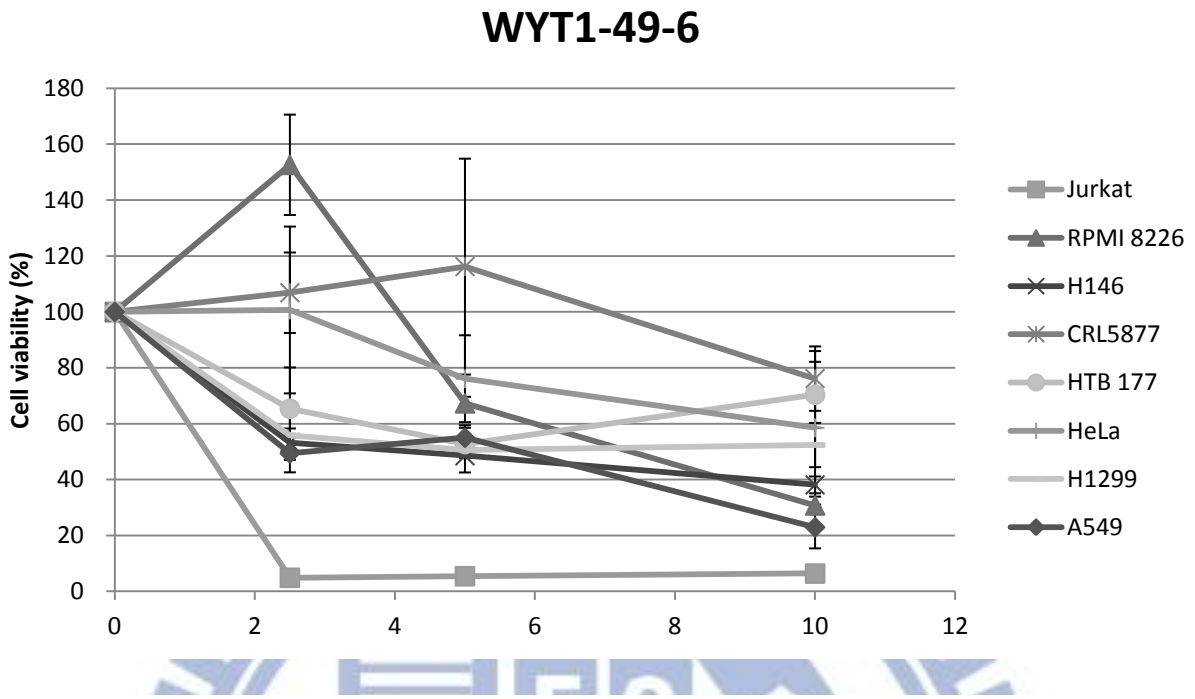
Supplementary data 1. Initial stage of NF-κB inhibitor screening using the extracts provided by Dr. Chung-Kuang Lu's lab. The NF-κB inhibition effect of extracts (20 μg/ml) was determined in the NIH3T3 cell line in the presence (+) or absence (-) of TNFα. Experimental procedures were described in the Materials and Methods.

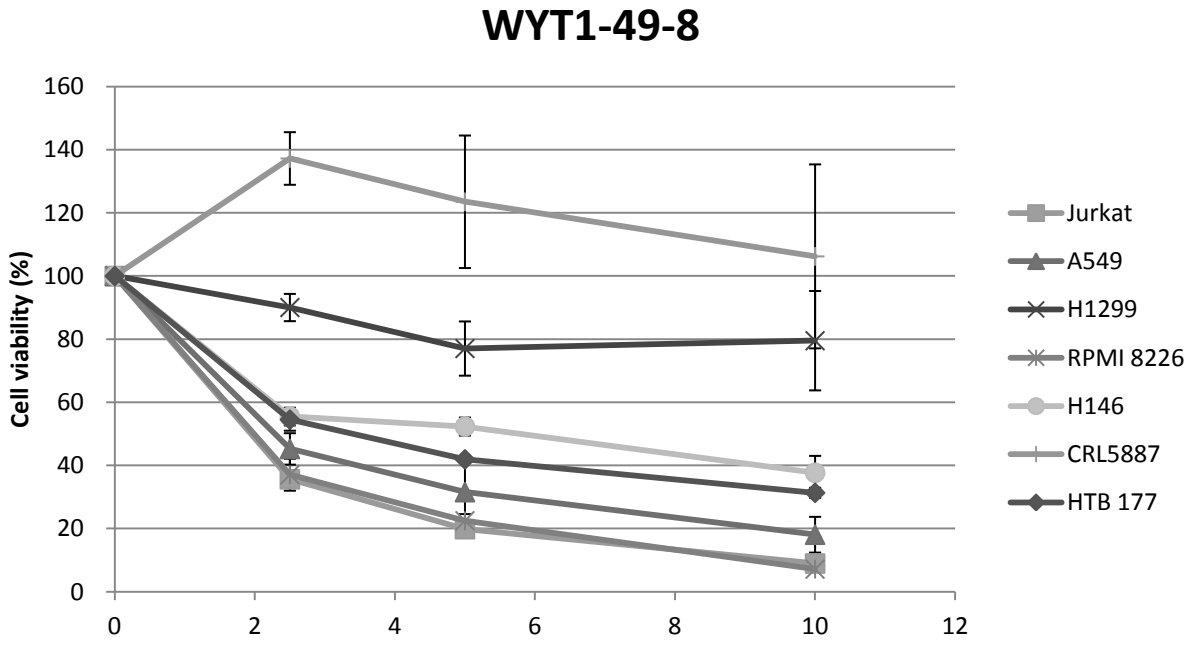


Supplementary data 1. Continued

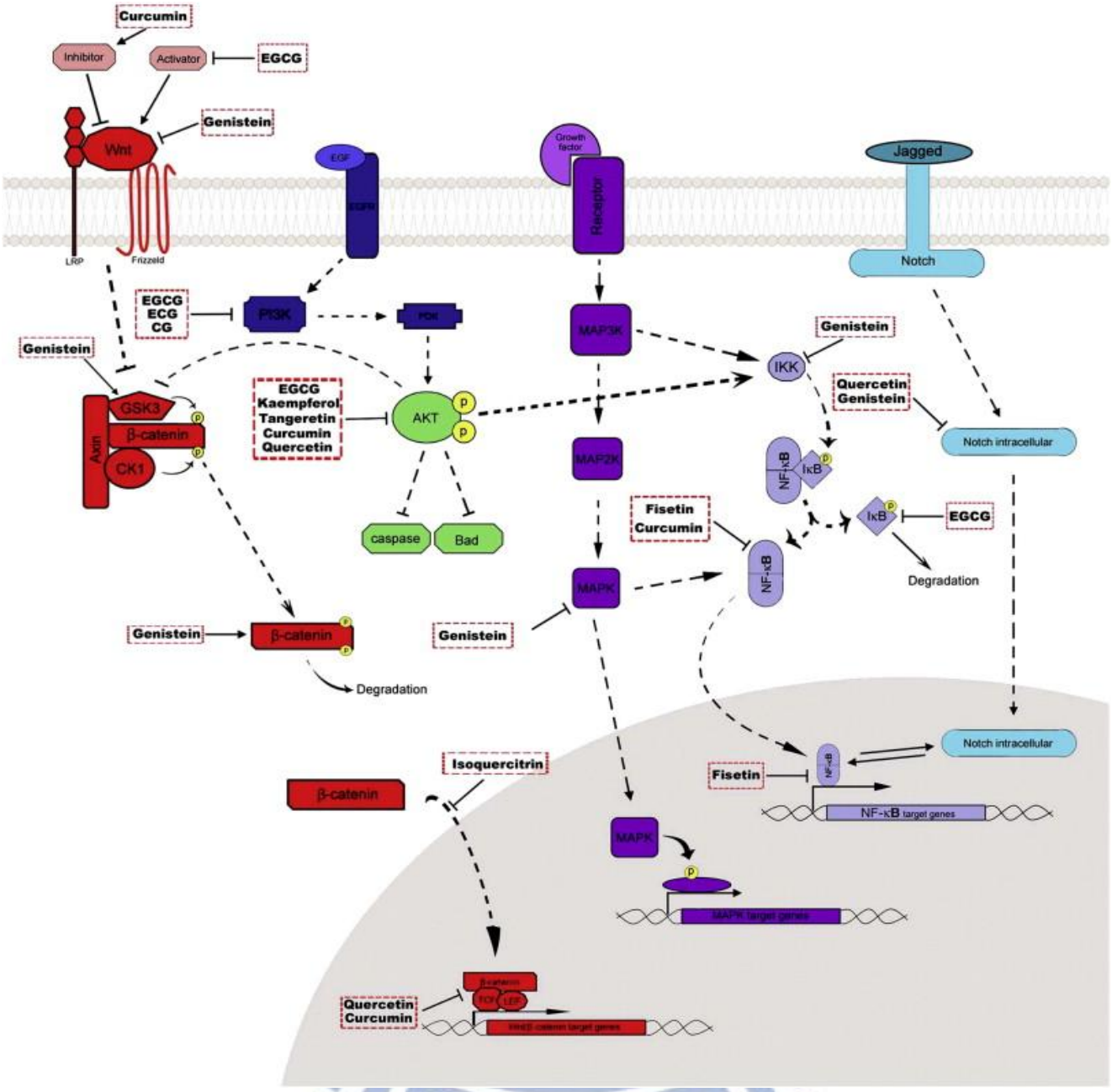


Supplementary data 1. Continued

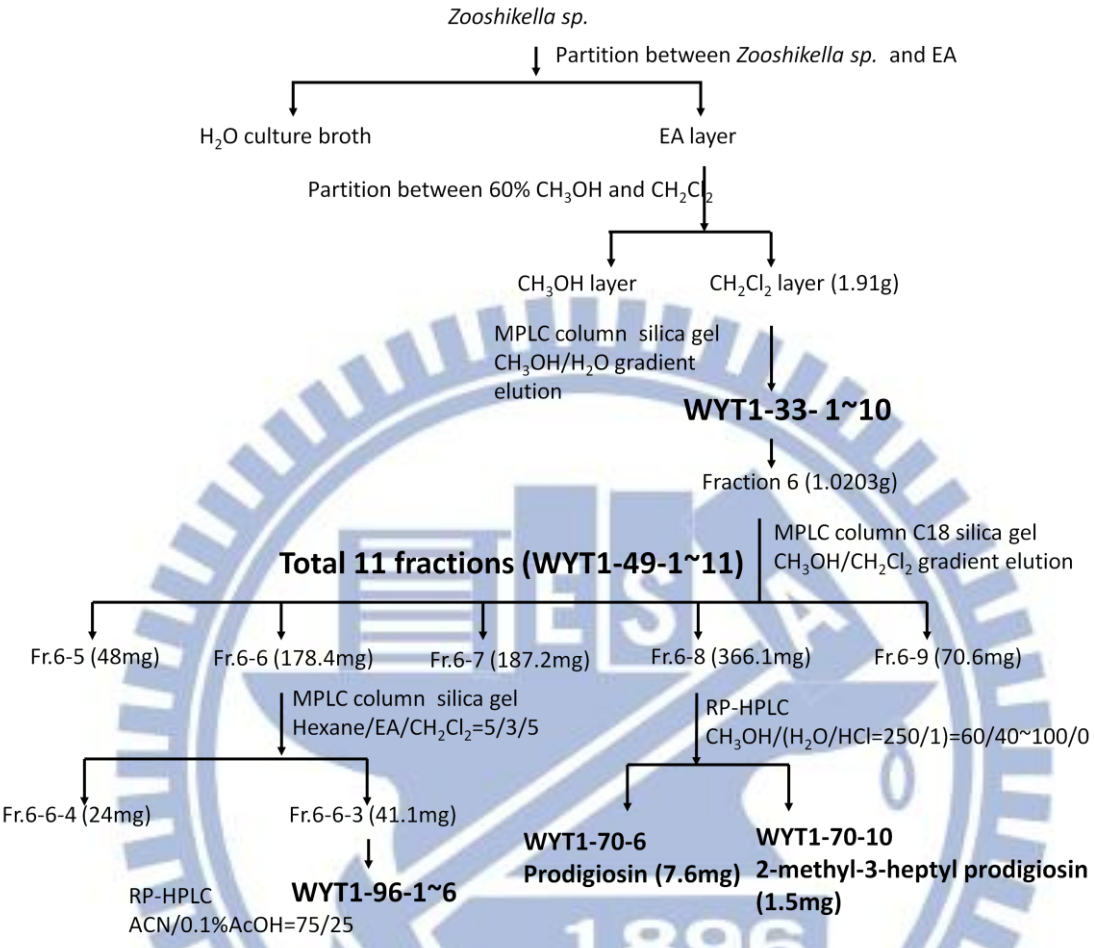




Supplementary data 2. WYT1-49-6 and WYT1-49-8 reduce cell viability in multiple cancer cell lines. Cells were treated with increasing concentrations of extracts for 24 h and cell viability was determined by MTT or MTS assay as described in the Materials and Methods. Data are expressed as mean ± SD (n=3).



Appendix 1. The epithelium growth factor (EGF) affects NF-κB signaling through AKT/PI3K pathway (Amado et al., 2011).



Appendix 2. The process of natural product purification from the marine bacterium *Zooshikella sp.* was provided by Yun-Ting Wu from Dr. Chung-Kuang Lu's lab.