A Master's Thesis

NKT inhibits proliferation and sensitizes lung adenocarcinoma cells to adriamycin

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ABSTRACT

Despite multiple efforts in prevention and treatment, lung cancer remains one the world leading causes of death. Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases with relatively high rate of LKB1 and KRAS mutations. In this study, we utilize the lung adenocarcinoma A549 together with the adriamycin (ADR)-resistant A549/ADR cell line in the effort of looking for the novel effective strategy in combating against this type of cancer. By using MTT assay, colony formation assay and western blotting, we showed for the first time that NKT, a major constituent of grapefruit, activated AMPK in A549 and A549/ADR leading to the inhibition of cell growth by downregulating mTOR and P70S6K phosphorylation. Moreover, cell cycle analysis revealed another possible proliferation inhibiting effect of NKT by arresting cell cycle at G1 phase. In addition to AMPK activation and cell cycle arrest, NKT alone treatment also showed promising effect in KRAS-mutant A549 and A549/ADR by inhibiting the oncogenic AKT and ERK pathways. As the combined therapy has shown great potential in cancer treatment by targeting different pathways at the same time, we sought to examine the effect of NKT in combination with ADR on A549 and A549/ADR. The *in vitro* results show the synergistic effect of NKT and ADR by significantly inducing apoptosis in both cell lines. Our *in vivo* data also further confirmed this synergism without any possible systemic side effects. Altogether, these results demonstrate that NKT is a promising anticancer agent in KRAS mutant and LKB1 mutant NSCLC.

1. INTRODUCTION

In 2018, lung cancer remains the most frequent cancer and the leading cause of cancer-related death worldwide with approximately 2 million newly diagnosed cases (11.6% of total cases) and more than 1.7 million of deaths (18.4% of total cases) [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of total lung cancer cases and it is often intrinsically resistant to anticancer drugs [2]. In addition, NSCLC can also develop acquired resistance with continued administration of the drug [3]. The survival rate of patients with early-onset NSCLC may be relatively high after resection [4]. However, at the time of diagnosis, most of the patients already had progressive disease and the median survival rate was almost no more than 18 months after diagnosis [5]. Despite advances in early detection and standard therapies, treatment of NSCLC remains ineffective [6]. Mutation of genes including v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), liver kinase B1 (LKB1) and epidermal growth factor receptor (EGFR) have been associated with the development of NSCLC and clinical prognosis as well as treatment outcome of NSCLC.

In NSCLC, another mutated gene, the LKB1 gene encodes a serine/threonine kinase and it ranks as the third highest mutated gene after p53 and RAS with the percentage of 20-30% in human [7,8]. LKB1 plays a significant role in NSCLC metastasis and the co-occurrence of KRAS and LKB1 mutations has a tendency to have detrimental effects on overall survival [9]. Moreover, LKB1 is recently proven as a tumor suppressor that exerts its role in regulating cancer growth and metabolism through the activation of AMPK [10]. The LKB1-AMPK signaling has been playing a prominent role at the crossroad of diabetes and cancer. In type 2 diabetes patients, activation of AMPK can stimulate glucose uptake in skeletal muscles, improve insulin sensitivity and metabolic health thereby alleviating the severity of this disease [11]. On the other hand, targeting AMPK has

shown great potential in the treatment and prevention of cancer, especially NSCLC [12,13]. Moreover, tumors isolated from NSCLC patients show that AMPK activation correlated with a better prognosis and a significant increase in overall survival [14]. Metformin is the most commonly used AMPK activator in type 2 diabetes patients and the positive correlation between metformin uptake and cancer prevention has been proven in several studies [15]. Until now, although there are multiple ongoing clinical trials, no AMPK activators have been approved for cancer prevention and treatment. Therefore, it is vital for researchers to investigate and develop novel AMPK activators in NSCLC treatment.

It has been previously reported that KRAS mutation occurs in 25-30% of NSCLC and patients with mutated KRAS show a shorter median survival compared to other mutations [16]. Moreover, mutation of KRAS can lead to the overactivation of various downstream pathways such as RAF-MEK-ERK (RAF–MAPK/ERK kinase–extracellular signal-regulated kinase) and PI3K-AKT-mTOR (phosphoinositide 3-kinase–AKT–mechanistic target of rapamycin) [17]. These pathways have been proven to play crucial roles in cell growth, malignant transformation and drug resistance [18,19]. Despite being the most frequently mutated gene in cancer, RAS protein has been considered as “undruggable” since multiple targeted therapies attacking RAS have resulted in failures possibly due to the lack of full understanding of its signaling transduction, feedback loops, redundancy, tumor heterogeneity [20]. For examples, developing AKT inhibitors has counters a wide range of limitations including high rate of acquired resistance, severe hyperglycema and other metabolic abnormalities [21,22]. The use of Erk inhibitors are also limited due to cardiac and ophthalmologic side effect as well as rash, diarrhea, peripheral edema, fatigue, and dermatitis acneiform [23]. Therefore, novel targeted drug therapy that can suppress these oncogenic pathways has attracted much research interest from scientists.

Adriamycin (ADR) or doxorubicin, is one of the most commonly used against a wide range of tumors, including lung cancer. While ADR shows high activity against SCLC, it shows relatively limited efficacy with NSCLC, which accounts for 85% of all lung cancer patients [24]. Treatment of advanced NSCLC with anthracyclines, exemplified by doxorubicin, provides an overall response rate of only 30–50% [25]. Besides being inefficient, ADR also causes congestive heart failure, a major adverse effect, when used at high doses [26]. Despite numerous advantages in anticancer therapies, the intrinsic and acquired drug resistance remains the major obstacle for the use of ADR. Therefore, there is a need for novel therapeutic strategies that can minimize the dose as well as reduce the cytotoxicity of doxorubicin and enhance its therapeutic efficacy against NSCLC cells.

NKT, a major naturally occurring sesquiterpenoid, has been identified as a bioactive compound with a wide range of beneficial applications including a neuroprotective and hepatoprotective, anti-inflammatory, antiseptic, antioxidant and antiallergic agent [27-33]. More importantly, NKT can activate AMPK leading to the stimulation of energy metabolism and prevention of diet-induced obesity by either LKB1 or calcium/calmodulin dependent protein kinase kinase 2 (CAMKK2) in liver and muscle cells [34]. However, little is known about the role of NKT in cancer, particularly the role of NKT in NSCLC with the LBK1 mutation. Based on the previously proven role of NKT as an AMPK activator, we investigated the anticancer effect of NKT in KRAS mutant, LKB1 mutant lung adenocarcinoma cell line A549. Moreover, we also sought to demonstrate for the first time that NKT can sensitize ADR resistant A549/ADR cell to ADR in vitro and in vivo.

2. MATERIALS AND METHODS

2.1. Reagents

Kaighn's modification of Ham's F12 medium (F-12K), trypsin/EDTA, fetal bovine serum (FBS), 100x penicillin/streptomycin solution were purchased from Gibco (Grand Island, NY, USA). Nootkatone and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342 dye, dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Amresco Inc. (Solon, OH, USA). Annexin V-FITC Apoptosis Detection Kit-1 was purchased from BD Biosciences (Franklin Lakes, NJ, USA). c-PARP, caspase -3, cleaved-caspase-3, caspase-8, Bid, p-AMPK, AMPK, mTOR, p-mTOR, Akt, p-Akt, p-ERK, ERK, p-p38, p38, p-JNK, JNK, acetylated-alpha-tubulin, GAPDH antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). A BCA protein assay kit was purchased from Pierce (Rockford, IL, USA), and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA), BS ECL plus kit was purchased from Biosesang Co. (Gyeonggi-do, Korea).

2.2. Cell culture

The human lung adenocarcinoma A549 cell line was kindly provided by Dr. Min Young Kim in Jeju National University. Cells were cultured in F-12K medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics and were maintained in a humidified incubator at 37 ℃ in a 5% CO_2 atmosphere.

2.3. Establishment of adriamycin-resistant cell line

A549/ADR cells were obtained by using the dose-escalating method. Briefly, after examining the cell viability of A549 cells treated with different concentrations of ADR for 48 h, we chose 0.03 μM as our starting concentration for our procedure. A549 cells were maintained in medium containing ADR for two weeks with each concentration. ADR doses were gradually increased until it reached 0.5 μM, this process lasted for approximately three months. After that, we enriched three colonies that remained on the dish and performed MTT assay as well as western blotting for the MRP1 protein expression and chose the one with the highest cell viability and MRP1 expression as our ADR-resistant cell line.

2.4. Cell viability assay

The cell viability assay was performed as previously described. Briefly, exponential-phase cells were seeded to 96-well plates (5×10^3 cells/well). After 24 h, cells were incubated in with various concentration of nootkatone or metformin. To investigate the combination effect, nootkatone was co-treated with ADR. After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) agent was added into each well and incubated for 4 h at 37 ◦C. The MTT results were read immediately at 570 nm with a Sunrise microplate reader (Tecan, Salzburg, Austria). The percentage of viable cells was calculated based on the following formula: mean value of (control group–treated group)/control group × 100%. All results were assessed in triplicate at each concentration.

2.5. Cell proliferation assay

The cell proliferation assay was performed as previously described. Briefly, exponential-phase cells were seeded to 96-well plates (1×10^3 cells/mL). After 24 h, cells were incubated in with various concentration of nootkatone or metformin. To investigate the combination effect,

nootkatone was co-treated with ADR. After 0, 24, 72, 120 hrs, MTT agent was added into each well and incubated for 4 h at 37 ◦C. The MTT results were read immediately at 570 nm with a Sunrise microplate reader (Tecan, Salzburg, Austria).

2.6. Cell cycle analysis

To determine cell cycle distribution analysis, 5×10^4 cells/mL cells were plated and treated with NKT, ADR and their combinations for 24 or 48 h. After treatment, the cells were collected, fixed in 70% ethanol, washed in PBS (2 mM EDTA), resuspended in 1 mL PBS containing 1 mg/mL RNase and 50 mg/mL propidium iodide, incubated in the dark for 30 min at 37°C, and analyzed by FACScalibur flow cytometry (Becton Dickinson, USA). Data from 10,000 cells were collected for each data file.

2.7. Colony formation assay

A549 or A549/ADR cells were seeded in 6-well plates at 1,000 cells/well and exposed to NKT for 10 days. After treatment, cells were washed with PBS 1X, fixed with 4% paraformaldehyde and stained with crystal violet for 30 min.

2.8. Combined drug analysis

Drug interaction was determined using the combination index (CI)-isobologram equation that allows quantitative determination of drug interactions, where CI < 1 implied synergism, CI=1 additive, and CI >1 implied antagonism. Compusyn© version 1.0 software (ComboSyn, Inc. Paramus, NJ, USA) was used to generate the dose-response curves, dose-effect analysis, and CI-effect plot.

2.9. Western blot

Western blotting was performed as followed. Briefly, A549/ADR cells were plated in 60 mm dishes (15×10^4 cells/plate). 24 h after being treated, cells were harvested and lysed in lysis buffer (20 nM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4). Protein concentrations were measured and normalized using a BCA Protein Assay kit. Lysates were then separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. using glycine transfer buffer. Membranes were then blocked for non-specific bindings by 5% skim milk solution. After that, membranes were incubated with primary antibodies overnight at 4 ◦C, followed by an additional 40 min incubation with secondary antibodies. The resultant membranes were analyzed using a BS ECL Plus kit (Biosesang Inc., Seongnam, Korea)

2.10. Xenograft

Mice were maintained and used for experiments according to a protocol approved by the Institutional Animal Care and Use Committee of Jeju National University (Jeju, Korea). The tumorigenicity of A549/ADR cells was assayed by subcutaneous inoculation of 1×10^6 cells resuspended in a mixture of 100 μL Matrigel (Sigma-Aldrich, MO, USA) in PBS into the flanks of 6-week-old athymic BALB/c male nude mice (n = 3/group). After the successful generation of tumor models, NKT (40 mg/kg) and/or ADR (10 mg/kg) were treated and mice were kept for weekly measurements for up to 7 weeks. The animals were then sacrificed, and the tumors were removed from all animals and weighed.

2.11. Statistical Analysis

Group comparisons were performed using Student's t-test and one-way analysis of variance with Statistical Package for the Social Sciences software (SPSS v. 20.0, IBM Corp., Armonk, NY, USA). $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. NKT inhibited A549 and A549/ADR cell proliferation.

Previously, we have shown the unique characteristics of A549/ADR compared to the parental A549 cell line. Briefly, after 48 h treatment of ADR, A549/ADR showed significant ADR resistance as evidenced by the lower cell viability, sub-G1 cell cycle population, expression of proapoptotic proteins and higher expression of antiapoptotic proteins.

Since NKT is already known to activate AMPK in liver and muscle of mice, preventing diet-induced obesity, we sought to examine the ability of NKT as an anticancer agent in LKB1-deficient A549 cells. The viability of A549 and A549/ADR cells treated with NKT was examined by MTT assay, which is based on the transformation of yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. After 48 h, NKT inhibited the cell viability of A549 with the IC50 ~ 200 μM (Fig. 1A) while it showed minimal inhibition in A549/ADR cells (Fig. 2A). However, NKT inhibited the growth of A549 / ADR and cells as well as A549 cells, although the cytotoxic effect on A549 / ADR was minimal. (Fig. 1B, Fig. 2B). We then confirmed the growth inhibitory activity by the colony formation assay. Cells were seeded at low concentration (1000 cells/ well), treated with various concentrations of NTK and incubated for 10 days. Colonies were then stained with crystal violet for the quantification of colony formation inhibition. The result showed that NKT significantly inhibited the colony formation of both A549 and A549/ADR cells (Fig. 1C, Fig. 2C). To further confirm our findings, we treated A549 and A549/ADR cells with a well-known and clinically used AMPK activator metformin with the doses ranging from 0.625 to 10mM. In comparison with NKT, metformin showed similar results in cell viability, cell growth and colony formation assays (Fig. 3 and Fig. 4).

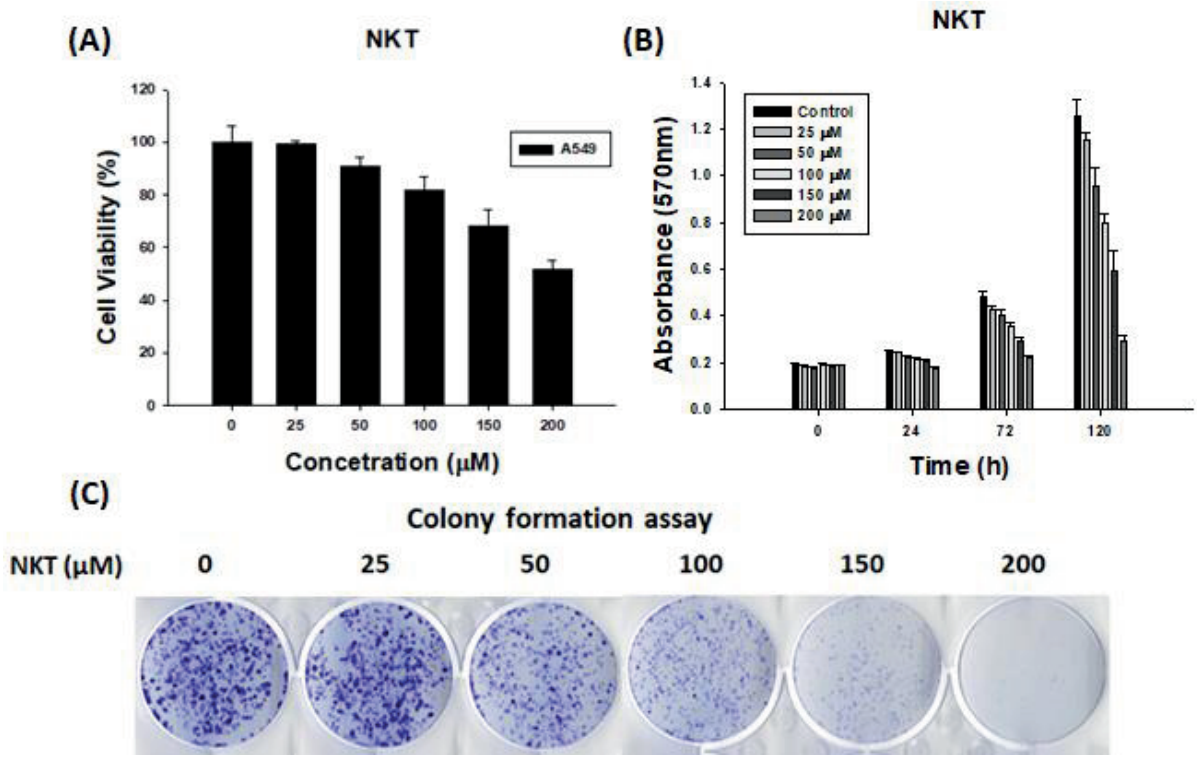


Figure 1. Antiproliferative effect of NKT on A549 cells. (A) A549 cells were treated with various concentrations of NKT for 48 h, then the cell viability determined by MTT assay. (B) A549 cells proliferation were determined after treatment with NKT for 0, 24, 72, 120h by MTT assay. (C) Colony formation assay of A549 cells after NKT treatment for 10 days. Values represent means ± SD (n = 3) (* $p < 0.05$).

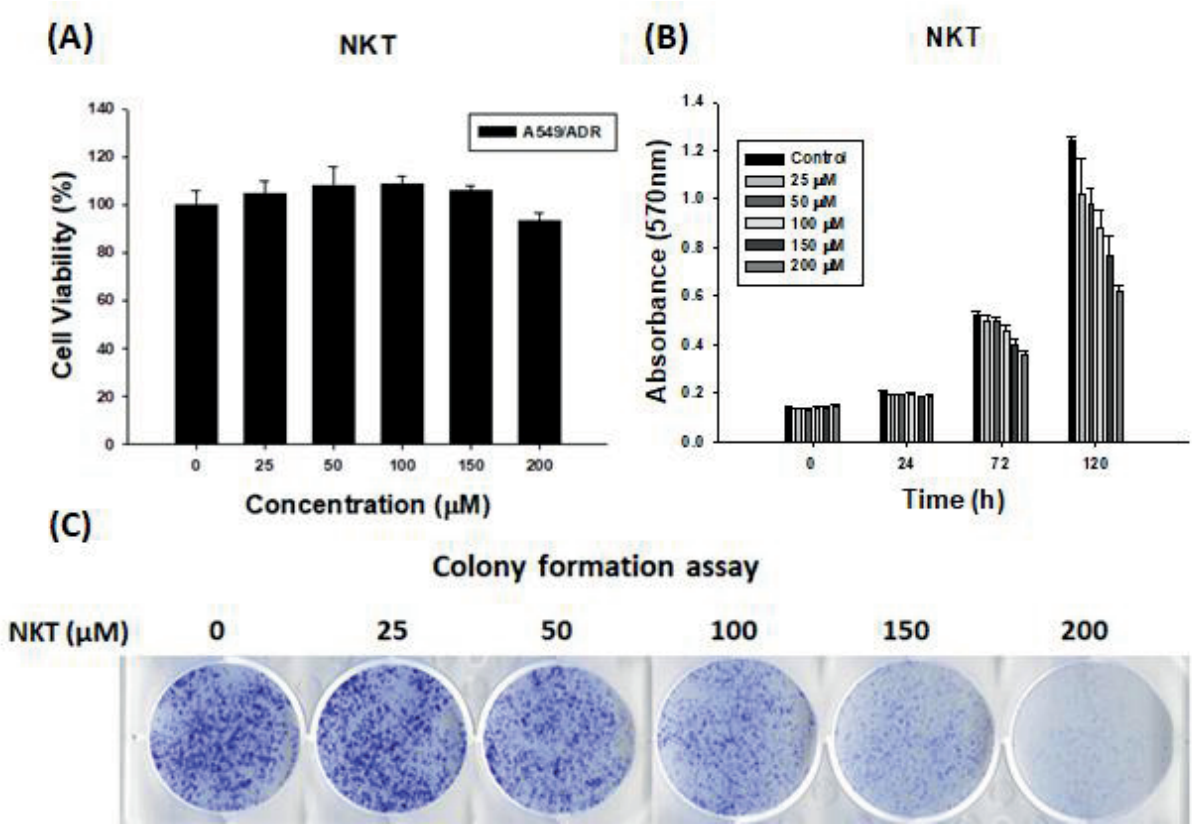


Figure 2. Antiproliferative effect of NKT on A549/ADR cells. (A) A549 cells were treated with various concentrations of metformin for 48 h, then the cell viability determined by MTT assay. (B) A549 cells proliferation were determined after treatment with metformin for 0, 24, 72, 120h by MTT assay. (C) Colony formation assay of A549 cells after metformin treatment for 10 days. Values represent means ± SD (n = 3) (* $p < 0.05$).

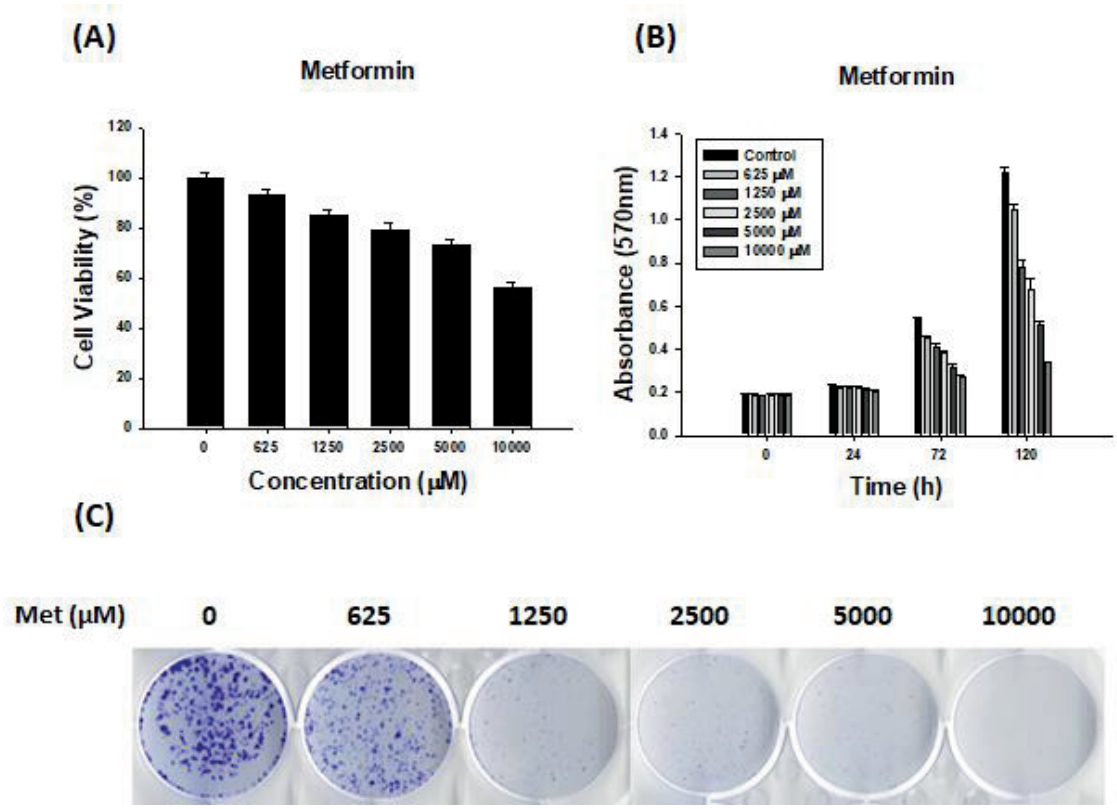


Figure 3. Antiproliferative effect of metformin on A549 cells. (A) A549/ADR cells were treated with various concentrations of NKT for 48 h, then the cell viability determined by MTT assay. (B) A549/ADR cells proliferation were determined after treatment with NKT for 0, 24, 72, 120 h by MTT assay. (C) Colony formation assay of A549/ADR cells after NKT treatment for 10 days. Values represent means ± SD (n = 3) (* $p < 0.05$).

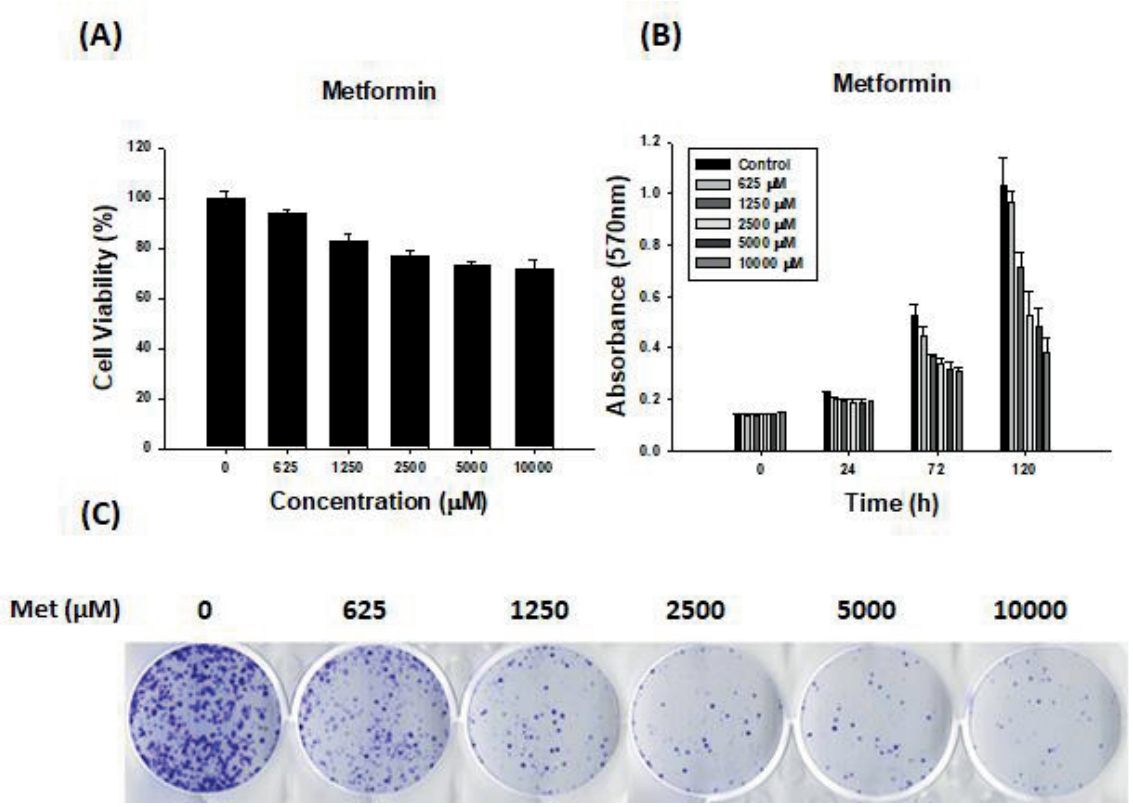


Figure 4. Antiproliferative effect of metformin on A549/ADR cells. (A) A549/ADR cells were treated with various concentrations of metformin for 48 h, then the cell viability determined by MTT assay. (B) A549/ADR cells proliferation were determined after treatment with metformin for 0, 24, 72, 120 h by MTT assay. (C) Colony formation assay of A549/ADR cells after metformin treatment for 10 days. Values represent means ± SD (n = 3) (* $p < 0.05$).

3.2. NKT activated AMPK pathway in A549 and A549/ADR cells.

We then sought to determine the possible mechanism by which NKT inhibits the proliferation of A549 and A549/ADR cells. Given the fact that A549 cell line is LKB1 deficient in its ability to activate AMPK through the well-known LKB1-AMPK axis, we have nevertheless tested whether NKT can activate the AMPK pathway. A549 and A549/ADR cells were treated with NKT 0, 50, 100, 200 μM for 24 h and then cell lysates were harvested for western blotting. As indicated in Fig. 5, NKT increased the level of p-AMPK and the downregulation of mTOR and P70S6K phosphorylation, which is a hallmark of AMPK activation [35]. This phenomenon was also confirmed in A549 and A549/ADR treated with metformin 0, 2.5, 5, 10 mM. As studied by several groups earlier, metformin as well as NKT can activate AMPK via both LKB1-dependent and -independent manner [34,36]. These data suggest that NKT can effectively stimulate AMPK activation via LKB1-independent manner in A549 and A549/ADR cells, which could partially occur via CAMKK2 axis.

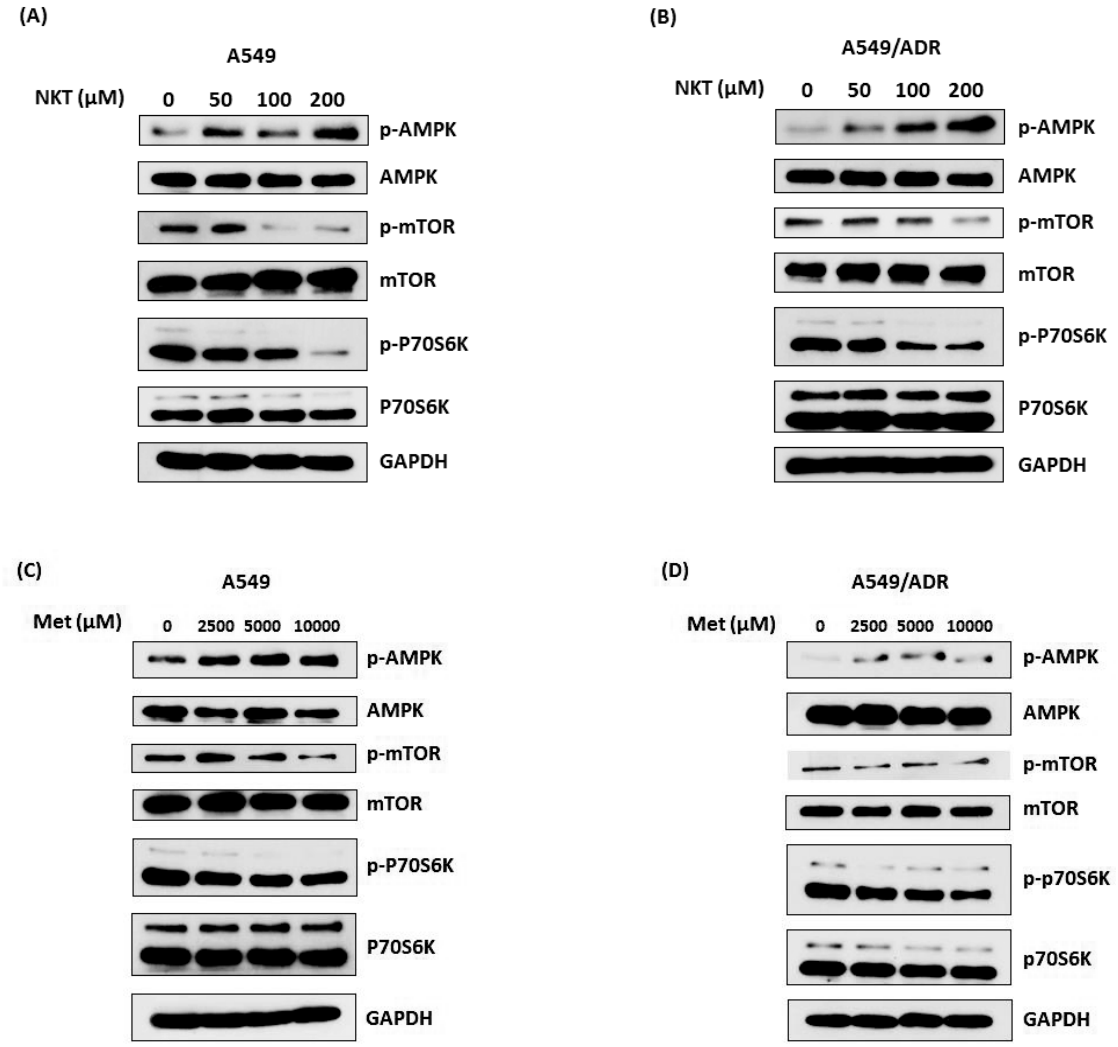


Figure 5. NKT and metformin activated AMPK in A549 and A549/ADR cells. A549 and A549/ADR cells were exposed to NKT 50, 100, 200 μM (A, B), or metformin 2.5, 5, 10 mM (C, D) for 24 h before western blotting for the detection p-AMPK, AMPK, p-mTOR, mTOR, p-P70S6K and P70S6K. GAPDH was used as loading control.

3.3. NKT induced G1 cell cycle arrest in A549 and A549/ADR cells.

After indicating the AMPK stimulating activity, we sought to further investigate the anticancer effect of NKT in A549 and A549/ADR cell lines. Cells were treated with NKT for 24 h before being harvested, fixed and stained with PI for cell cycle analysis using flow cytometry method. As illustrated in Fig. 6A and Fig. 7A, 100, 150 and 200 μM NKT caused an increase in G1 population in A549 and A549/ADR cells from 58.88% to 65.15±1.45%, 69.47±3.87, 71.5±6.31% and from 60.52±2.68% to 68.49±2.87, 76.68±1.55, 78.19±1.67, respectively. We then examined the possible mechanism for this cell cycle arrest. Among the tested cell cycle-related proteins, we identified the downregulation of cyclin D1 and retinoblastoma (Rb) phosphorylation proteins after NKT treatment (Fig. 6B and Fig. 7B). Indeed, cyclin D1 expression level and phosphorylation of Rb and its downstream E2F transcriptional factors have been proven to be involved in the G1-S phase transition [37,38]. Therefore, the decrease in cyclin D1 and p-Rb expression is correlated with the cell cycle arrest at G1 phase.

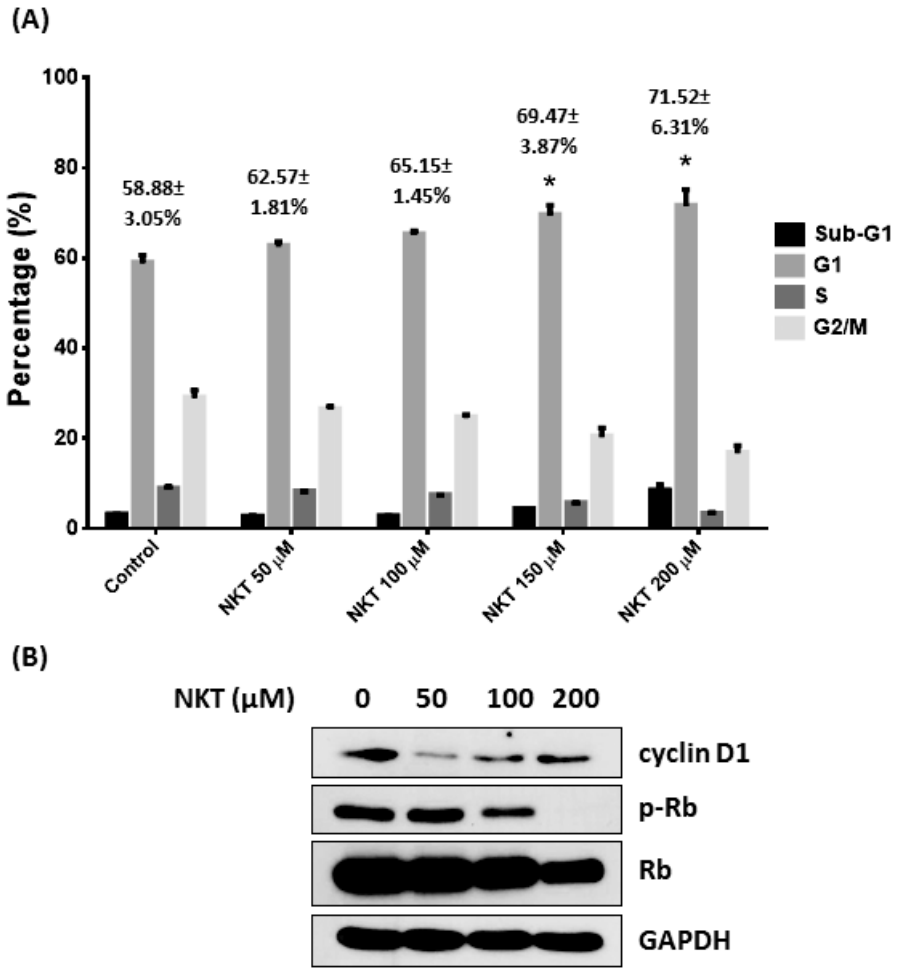


Figure 6. NKT induced G1 cell cycle arrest in A549 cells. (A) A549 cells were treated with various concentrations of NKT for 24 h. Cells were then harvested and analyzed by PI staining and flow cytometry analysis. (B) Protein levels of cyclin D1, p-Rb, Rb were examined by western blotting of A549 cells treated with NKT 0-200 μM for 24 h, GAPDH was used as loading control. Values represent means ± SD (n = 3) (* $p < 0.05$).

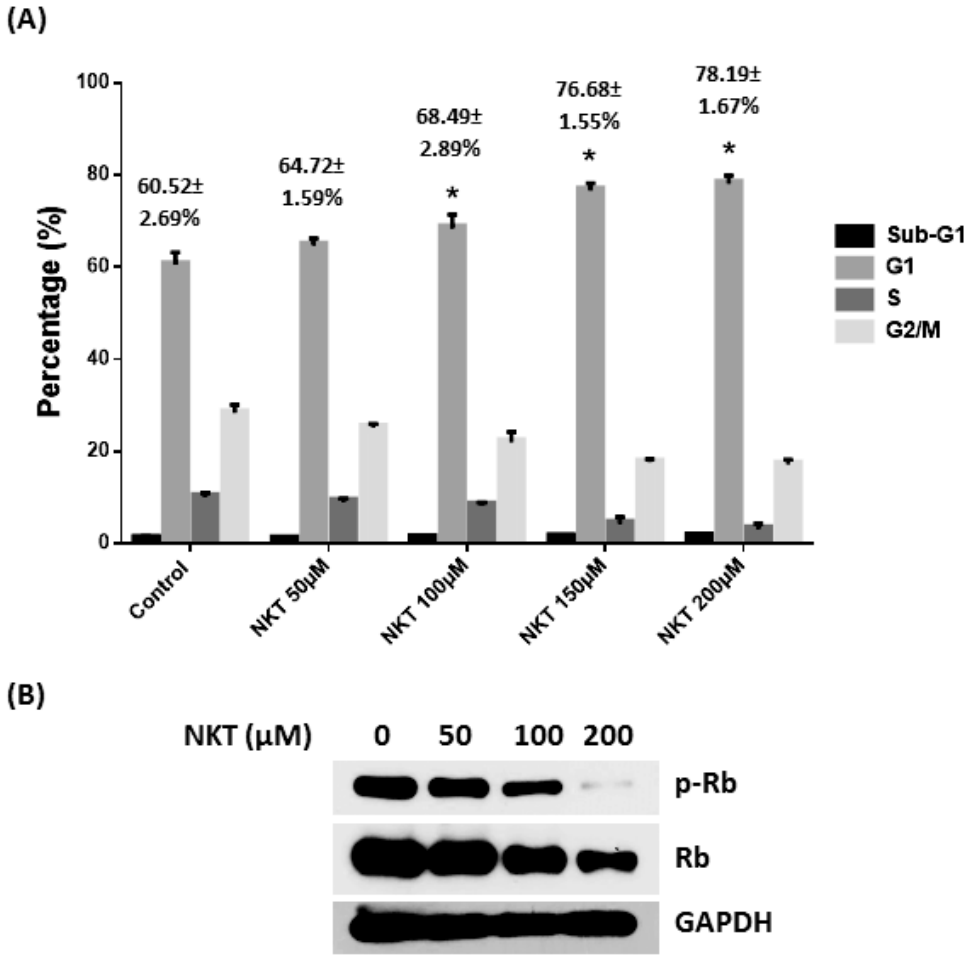


Figure 7. NKT induces A549/ADR cell cycle arrest at G1 phase. (A) A549/ADR cells were treated with various concentrations of NKT for 24 h. Cells were then harvested and analyzed by PI staining and flow cytometry analysis. (B) Protein levels of cyclin D1, p-Rb, Rb were examined by western blotting of A549/ADR cells treated with NKT 0-200 μM for 24 h, GAPDH was used as loading control. Values represent means ± SD (n = 3) (* $p < 0.05$).

3.4. NKT suppresses growth and survival signaling pathways in KRAS mutated A549 and A549/ADR cell lines.

KRAS mutation has been known to lead to the overactivation of multiple cellular pathways such as PI3K/AKT and Raf/MEK/ERK. These pathways are known for their functions in promoting cell growth, cell survival and drug resistance [17]. Here, in the context of this study, we sought to investigate the RAS mediated signaling transduction in KRAS mutated A549 and A549/ADR cells. The phosphorylation status of AKT and ERK was examined by western blotting. As shown in Fig. 8A and 8B, treatment with NKT decreased the expression of p-AKT and p-ERK in both A549 and A549/ADR cells. On the other hand, while metformin inhibited AKT phosphorylation, it failed to decrease p-ERK protein expression in both cell lines. Our result indicated that NKT inhibited KRAS mutated cell line growth and survival via regulating KRAS downstream signaling pathways.

To further identify the potential of NKT as an anticancer agent, we sought to investigate the effect of the combination between NKT and ADR, a well-known chemotherapeutic agent that is widely used for various types of cancer. Although ADR is effective for SCLC treatment, it showed low efficacy against NSCLC as well as high risk of lethal adverse effects. Therefore, we tested if NKT could synergize with ADR to elevate the effect and lower the ADR concentration need for the treatment.

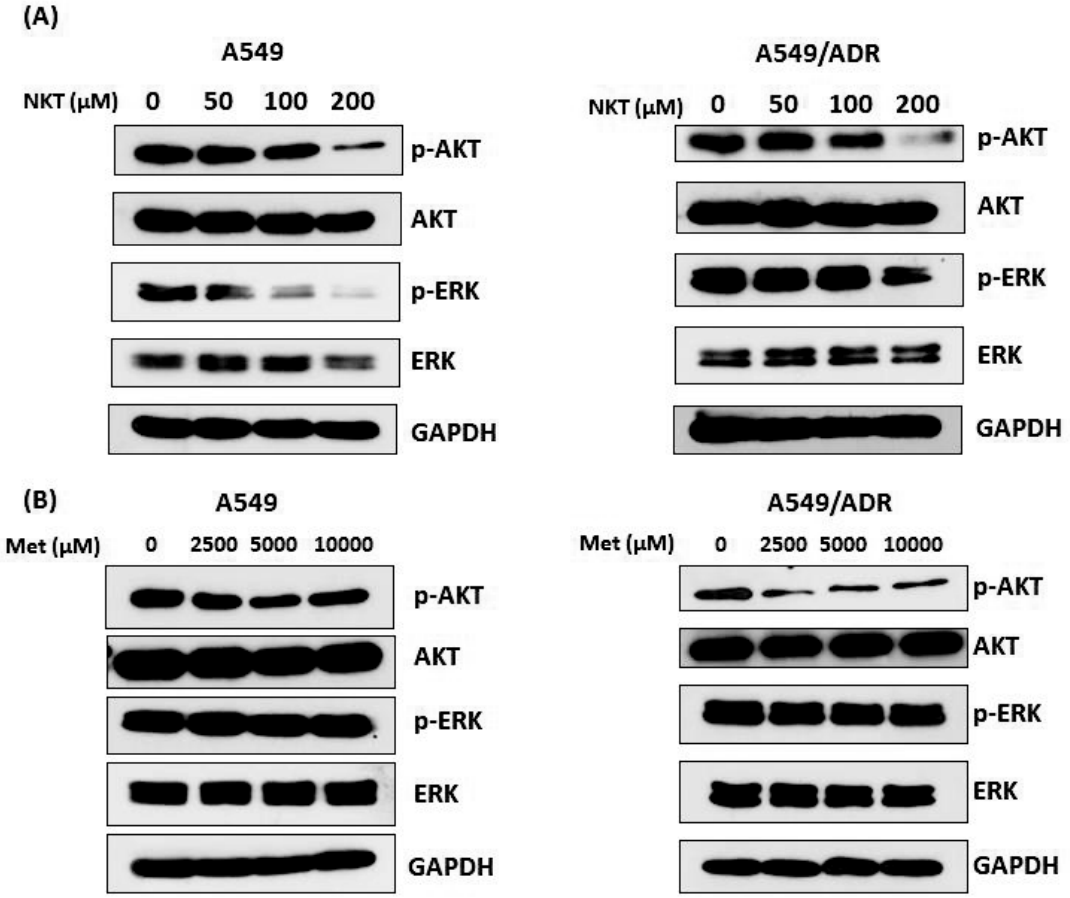


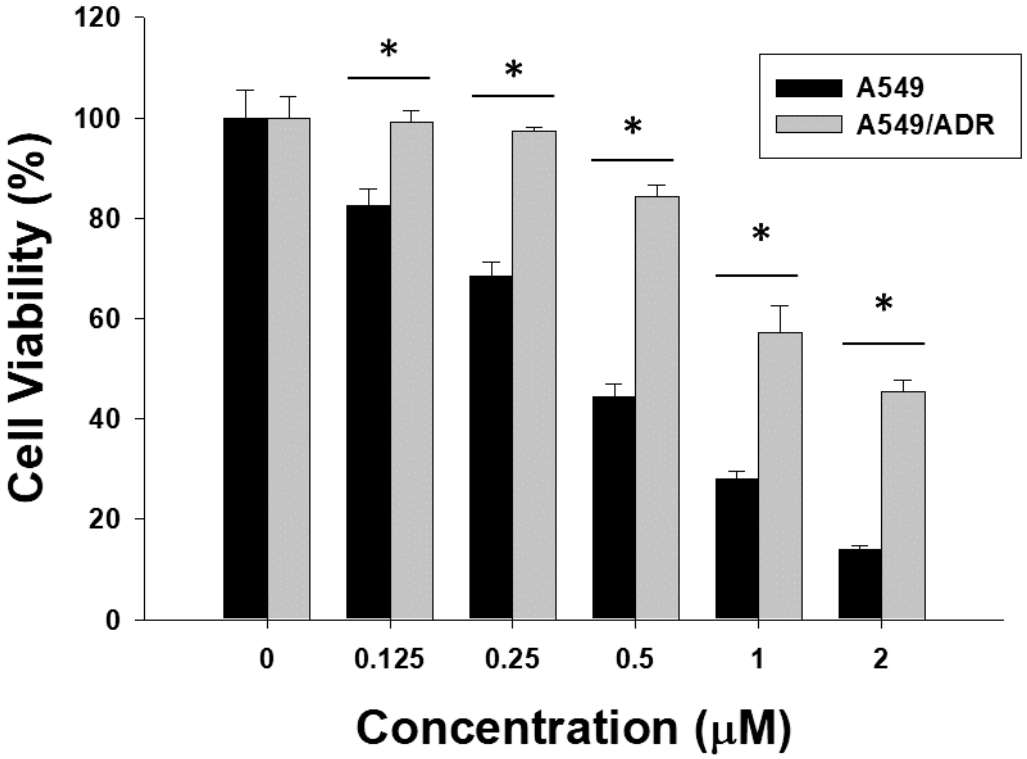
Figure 8. NKT inhibits pathways involved in KRAS-mutant cancer progression. A549 (A) and A549/ADR (B) cells were exposed to NKT 50, 100, 200 μM for 24 h before western blotting for the detection p-AKT, AKT, p-ERK, ERK. GAPDH was used as loading control.

3.5 NKT and ADR exert a synergistic cytotoxic effect on A549 and A549/ADR cells

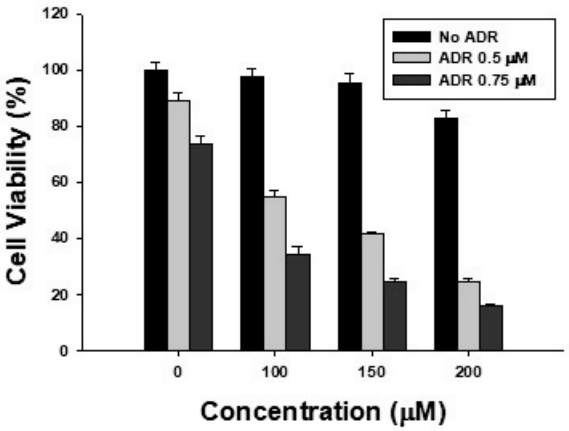
We first confirmed the ADR resistance of A549/ADR cell line by treating both cell lines with ADR 0-2 μM (Fig. 9A). After that, to examine the potential of NKT as a chemosensitizer against ADR in lung adenocarcinoma cell line, MTT viability assays were performed using parental A549 and resistant A549/ADR cell line. Exposure to various concentrations of NKT and ADR dose-dependently decreased cell compared with the negative control group (Fig. 9B, 9C).

To determine whether NKT and ADR had synergistic effect on A549 and A549/ADR cells, we calculated the Combination Index (CI) values for the combined drug treatment based on the Chou-Tatalay method [39]. Utilizing the growth inhibitory data of either drug alone or their combinations, we calculated the CI values by Compusyn© software. CI values of NKT and ADR combined treatment on both A549 and A549/ADR cells ranged from 0.476 to 0.634 and from 0.685 to 0.789, respectively (Fig. 9B, 9C). The combined treatment is considered additive when CI = 1, whereas CI > 1 or CI < 1 indicates antagonist or synergistic effect, respectively. Based on this given fact, the CI values showed that the combinations between NKT and ADR are synergism at all tested concentrations.

(A)

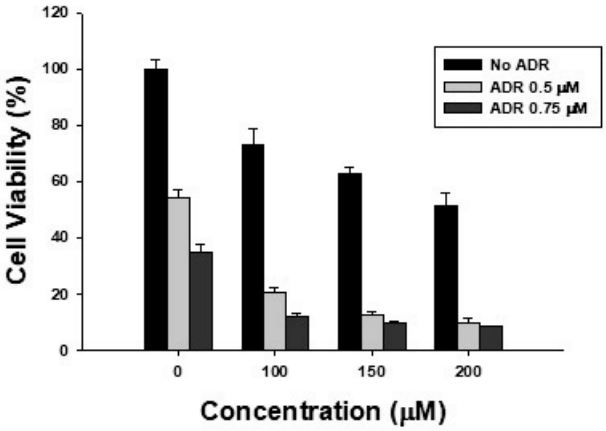


(B)



Concentration (μM)		Effect	CI
ADR	NKT		
0.5	100	0.450326	0.789
0.5	150	0.581705	0.78
0.5	200	0.757058	0.685
0.75	100	0.65798	0.757
0.75	150	0.754615	0.737
0.75	200	0.841477	0.689

(C)



Concentration (μM)		Effect	CI
ADR	NKT		
0.5	100	0.79549	0.634
0.5	150	0.87222	0.518
0.5	200	0.90354	0.476
0.75	100	0.87702	0.622
0.75	150	0.90262	0.585
0.75	200	0.91565	0.575

Figure 9. NKT enhanced sensitivity of A549 and reversed the resistant characteristic of A549/ADR to ADR. (A) Cell viability of A549 and A549/ADR cells after 48 h treatment with ADR 0-2 μM was quantified by MTT assay. Effects of ADR and NKT combined treatment was examined on A549 (B) and A549/ADR (C) by MTT assay. Cells were treated with NKT 100, 150, 200 μM alone or in combination with ADR 0.5 and 0.75 μM. Values represent means ± SD (n = 3) (* $p < 0.05$).

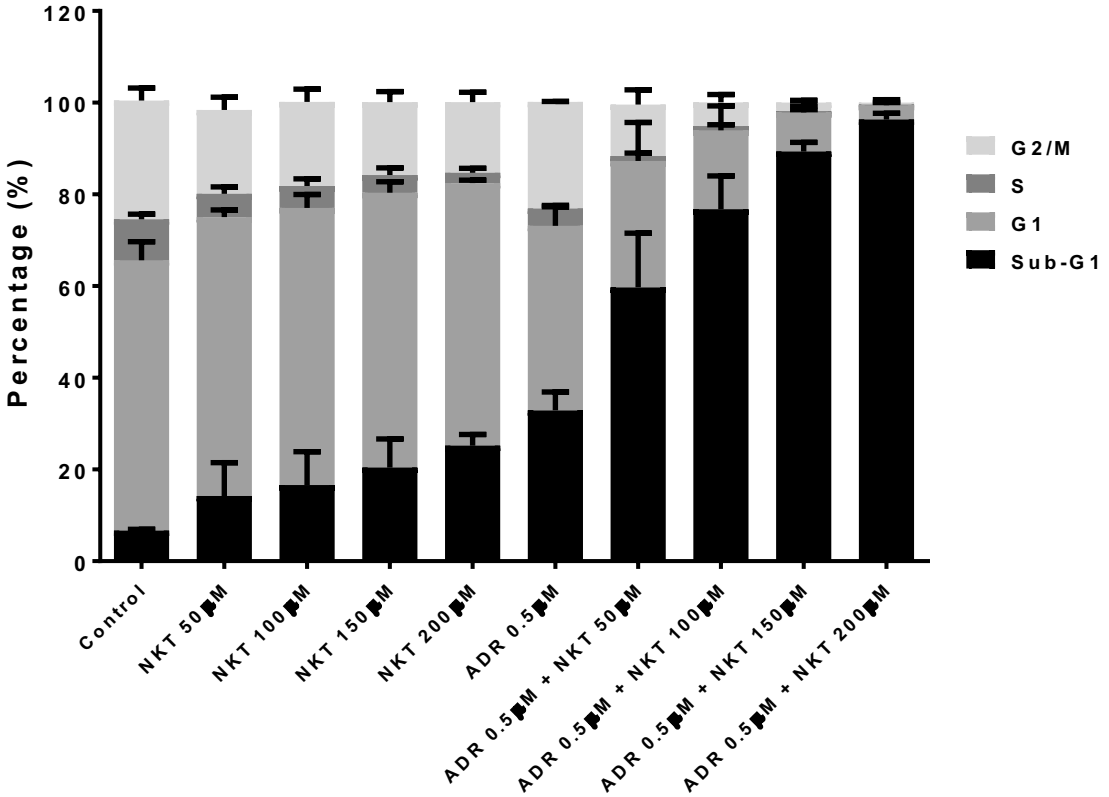
3.6. The combination of NKT and ADR induced apoptosis in A549 and A549/ADR cells.

We next explored the mechanism of how NKT enhanced the effect of ADR on A549 and A549/ADR cell lines. As hypothesized in the previous part, we sought to use the concentration of NKT and ADR that caused the most minimal toxicity in the monotherapy in. As previously shown in Fig. 9C, ADR 0.5 μM and NKT 0-200 μM showed the least cytotoxicity to A549/ADR when treated alone, therefore we chose these concentrations for further assessment on the synergistic mechanism.

Cell cycle analysis was performed to reveal to cell cycle distribution in Sub-G1, G1, S and G2/M phases of A549 and A549/ADR cells treated with either drugs alone or in combination for 48 h. Sub-G1 is the fraction of cells with internucleosomal DNA fragmentation, which is one of the hallmarks of apoptosis [40]. The result showed significant dose-dependent increase in sub-G1 population of cells treated with different combined treatments (Fig. 10A, 10B). The combination between NKT 200 μM and ADR 0.5 μM showed the highest sub-G1 population with 94.69±2.31% and 67.38±0.59% in A549 and A549/ADR cells, respectively.

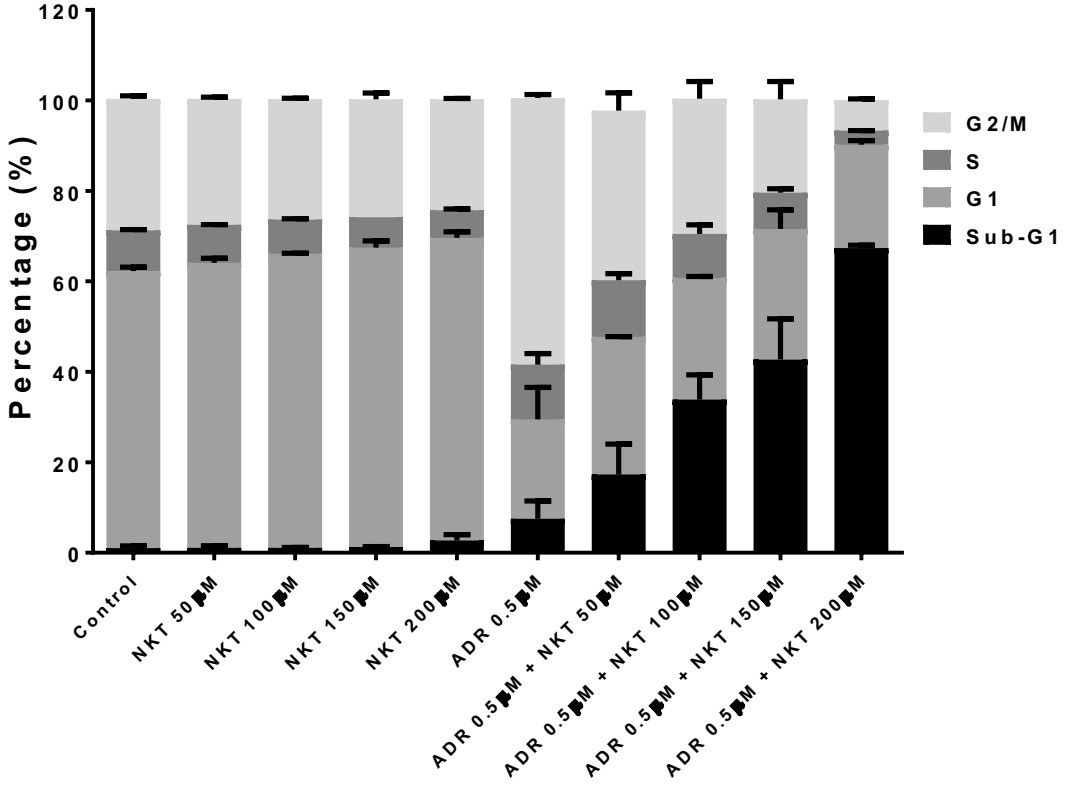
We further investigated the apoptosis induction by performing western blotting to detect the change in protein expression levels of cells treated with NKT 150 μM and ADR 0.5 μM**.** Another hallmark of apoptosis is the increased cleavage of PARP, caspase proteins including caspase-8 and caspase-3 [41]. As shown in Fig. 11, the combined treatment of ADR and NKT significantly increased the cleavage of PARP and caspase-3 compared to the single treatment alone. Therefore, we reached the conclusion that NKT enhances ADR cell proliferation inhibition by inducing apoptosis in A549 and A549/ADR cells.

(A)



	Sub-G1 population (%)									
ADR 0.5 μM	-	-	-	-	-	+	+	+	+	+
NKT (μM)	-	50	100	150	200	-	50	100	150	200
	6.64± 0.52	14.23± 12.58	16.61± 12.62	20.43± 10.79	25.19 ±4.22	32.92 ±6.91	59.74 ±20.44*	76.78 ±12.58*	89.34 ±3.49*	96.32 ±2.31*

(B)



	Sub-G1 population (%)									
ADR 0.5 μM	-	-	-	-	-	+	+	+	+	+
NKT (μM)	-	50	100	150	200	-	50	100	150	200
	1.05± 0.47	1.09± 0.49	1.09± 0.06	1.26± 0.12	2.73 ±1.30	7.53 ±3.90	17.28 ±6.73	33.85 ±5.46*	42.72 ±8.98*	67.38 ±0.59*

Figure 10. Combination treatment of NKT and ADR synergistically increased the sub-G1 population in A549 and A549/ADR cells. A549 (A) and A549/ADR cells (B) were treated with NKT 0, 50, 100, 150, 200 μM either alone or in combination with ADR 0.5 μM for 48 h. Cells were then stained with PI and analyzed by flow cytometry for cell cycle distribution. The table below each graph is the percentage of each cell cycle proportion with different drug treatments. Values represent means ± SD (n = 3) (* $p < 0.05$).

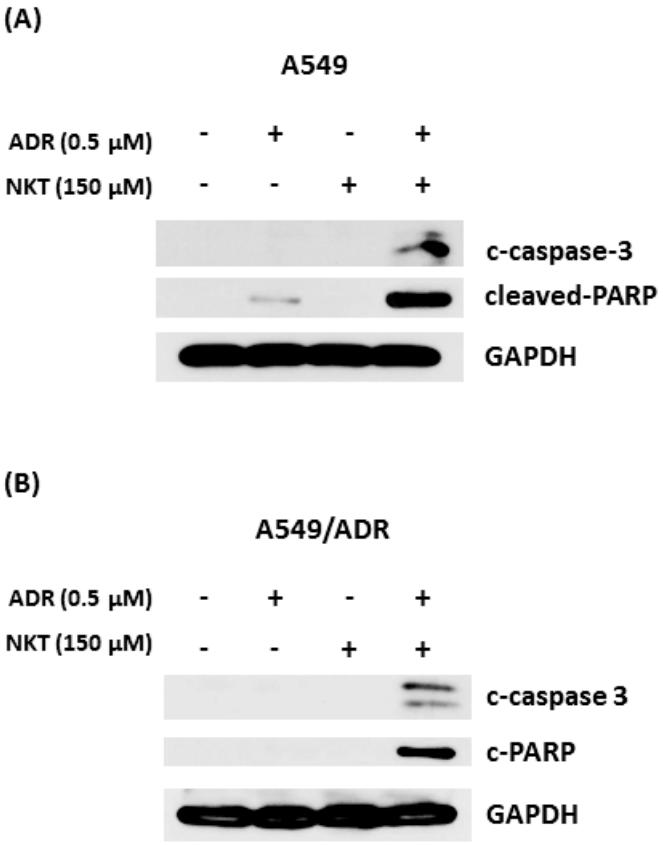


Figure 11. Combination treatment of NKT and ADR synergistically increased the apoptotic markers in A549 and A549/ADR cells. A549 (A) and A549/ADR cells (B) were treated with NKT 0, 50, 100, 150, 200 μM either alone or in combination with ADR 0.5 μM for 48 h. Cell lysates were then harvested for western blotting to detect c-caspase-3 and c-PARP expression level.

3.7. NKT and ADR synergistically inhibit the growth of ADR-resistant A549/ADR tumors *in vivo*

To validate the synergism of NKT and ADR combined treatment inn A549/ADR cells, we examined the antitumoral effect of the compounds alone or in combination in a mouse xenograft model inoculated with A549/ADR cells lines. When tumor reached a volume of 100mm^3, animals were randomly assigned into 4 groups, 3 mice per group. Mice were treated with ADR 10mg/kg, NKT 40mg/kg and their combination. As shown in Fig. 12, treatment of mice with the combination of NKT and ADR significantly reduced tumor progression compared to the vehicle-treated animals. NKT alone or in combination with ADR did not exert systemic side effects as shown by the weight of the animals measured during each treatment period (Fig. 12B). As can be seen in Fig. 12C, the tumor volumes were significantly reduced in the combination treated group compared with either group alone or the controlled samples. Although ADR and especially NKT alone treatment decreased the tumor growth rate compared to the control group, the results were not statiscally significant. However, at the completion of the study, we observed that the tumor weight was significantly reduced in mice treated with either NKT or with ADR as single agents (Fig. 12E).

Altogether, these results suggest that the treatment of NKT alone or the combination of ADR and NKT significantly inhibits the progression of ADR-resistant A549/ADR tumors in vivo. Therefore, it might represent an efficient therapeutic approach to overcome the failure in NSCLC treatment.

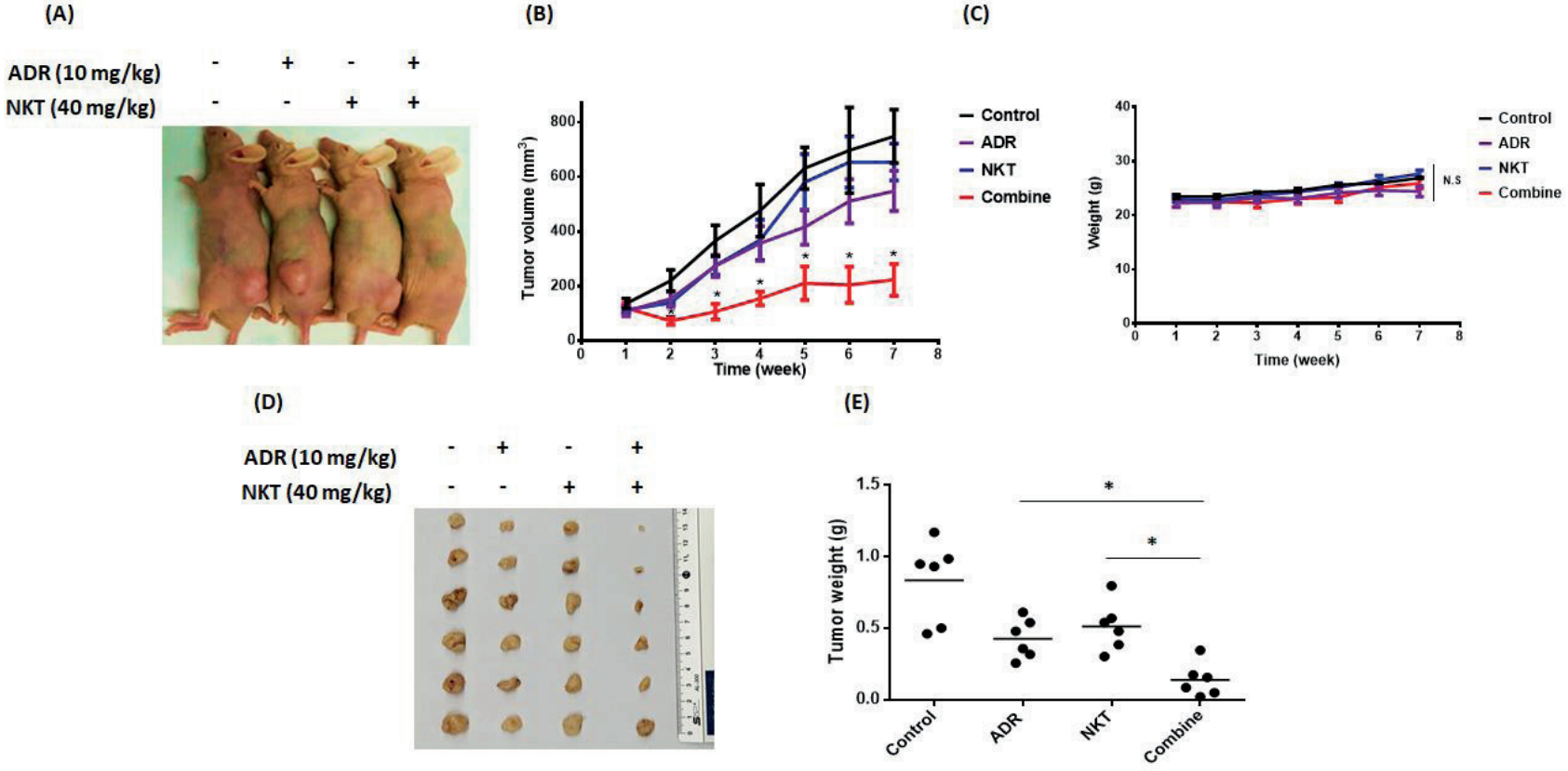


Figure 12. NKT synergized with ADR to inhibit tumor growth in xenograft tumor model. (A) Representative images of mouse from each group of treatment. (B) The tumor volume in each group was assessed by calipers and calculated as the length × width × width × 0.5. (C) There were no significant changes in the body weights of the mice during the ADR and NKT treatment periods. (D) The tumor burden obtained from each group. (E) Each bar represents the mean ± SEM of the tumor weight of four groups. (D) *p < 0.05.

DISCUSSION

For decades, lung cancer has been known as one of the most common cancer and the leading cause of cancer-related deaths. The prognosis of lung cancer is poor and the 5-year survival rate for late stage diagnosis is 5-16%. NSCLC is the most common type of lung cancer with the proportion of 85% in all lung cancer cases. In NSCLC patients harboring KRAS mutant, the constitutional activation of KRAS downstream targets such as AKT and ERK represents the uncontrolled cell proliferation, apoptosis resistance and other tumorigenesis-related cascades [42,43]. Until now, targeted therapies remain limited due to its inefficiency as well as side effects [21,22]. Moreover, anthracyclines, exemplified by ADR, are commonly used chemotherapeutic agents for a wide range of cancers but it remains ineffective against NSCLC owing to its toxicity and acquired drug resistance. Therefore, it is urgent to develop new strategy to effectively inhibit cell viability in vitro as well as tumor growth in vivo. We also sought to limit the side effects caused by ADR through lowering the effective dosage of this drug.

In this study, we showed for the first time the anticancer effect of NKT by using KRAS mutant lung adenocarcinoma A549 and its ADR-resistant counterpart A549/ADR cell. Based on the previously proven role of NKT as an AMPK activator (by either LKB1 or CAMKK2 in liver and muscle cells [34]), we confirmed that NKT can inhibit the proliferation of A549 and A549/ADR in vitro (Fig. 1 and Fig. 2), which is also similar to the effect of metformin, a clinically used AMPK activator, in these models. As previously reported, A549 is a LKB1 mutant cell line, which loses its ability to activate AMPK via LKB1 [44]. Besides being activated by LKB1, AMPK can be stimulated by other upstream kinase including CAMKK2 and transforming growth factor beta-activated kinase 1 (TAK1) [45,46]. In our study, by using the LKB1 mutant cell line, together with the previous finding that NKT activates liver and muscle cells through LKB1 and CAMKK2 [34],

we showed that NKT can activate AMPK in A549 and A549/ADR cells via CAMKK2 (Fig. 5A, 5B). In addition, AMPK activation has been shown to exert multiple anticancer properties including inhibiting cancer progression via cyclooxygenase (COX-2), limiting fatty acid synthesis and accelerating fatty acid oxidation through acetyl CoA carboxylase1/2 (ACC1/2), inhibiting cell growth and protein synthesis via mTOR [47]. As a primary downstream target of mTOR, P70S6K plays an important role in the regulating cell growth, survival and proliferation [48]. In the context of this research, as a consequence of AMPK activation, NKT treatment resulted in the downregulation of mTOR and P70S6K phosphorylation (Fig. 5A and 5B). The similar phenomena were observed in A549 and A549/ADR cells treated with metformin (Fig. 5C and Fig. 5D). Therefore, we proposed that NKT activates AMPK through CAMKK2 in these models. In fact, cancer cells with LKB1 tumor suppressor gene mutation fail to control the normal cell growth by AMPK activation [49], therefore activating AMPK to slow down the tumor progression plays a vital role in cancer prevention and treatment.

Since the failure of regulating cell cycle progression was found almost all human cancers including in NSCLC, therapies targeting this malfunction of cancer have been developed [50]. Rb is known to regulate G1/S transition [51]. In quiescent cells, Rb protein is activated and sequesters members of the E2F family of transcription factors leading to cell cycle suppression. On the other hand, phosphorylation of Rb causes the disruption of Rb-E2F transcription complex leading to the release of active E2F and continuing cell cycle progression [52]. In this study, we showed that NKT induced G1 cell cycle arrest by downregulating Rb phosphorylation in A549 and A549/ADR cells (Fig. 6 and Fig. 7).

KRAS is the most frequently mutated oncogene in cancer and KRAS mutation is commonly associated with poor prognosis and resistance to therapy. KRAS is considered undruggable,

therefore targeting its downstream pathways such as RAF/MEK/ERK and PI3K/AKT pathway is a more potential approach [53]. In our study, we demonstrated the inhibition of ERK and AKT phosphorylation in A549 and A549/ADR cells treated with NKT (Fig. 8A, 8B). In addition, while metformin showed inhibitory effect on AKT pathway, it failed to inhibit ERK activation, which is an important prosurvival downstream signal of KRAS overactivation (Fig. 8C, 8D).

The principle of combining two more drugs that target different pathways at the same time to improve the clinical outcome, prevent the acquired drug resistance and prolong patient survival is broadly utilized in cancer treatment [54]. Compounds with plant origin have been extensively studied and developed as anticancer compounds and some of them were indeed approved for cancer treatment in clinics [55]. Recently, there have been several studies studying the synergistic effect between ADR and phytochemicals on NSCLC cell lines via different mechanisms. Punia et al. reported that acacetin, a naturally occurring flavone, showed synergistic effect with ADR on A549 and H1299 NSCLC cell lines by increasing cell apoptosis, modulating cell cycle progression and increasing ADR intracellular uptake through downregulating MDR1 [56]. Chrysin, a flavone found in *Passiflora caerulea* and honeycomb, exerted synergistic effect with ADR in lung cancer cell lines by regulating glutathione level [57]. Moreover, alantolactone, a sesquiterpene lactone component of Inula helenium, enhanced chemosensitivity of ADR in Doxorubicin-resistant A549 by inhibiting STAT3 activation and MDR1 expression thereby elevating ADR accumulation [58]. In the context of this study, after identifying the potential of NKT as an anticancer alone, we went further to determine the combined treatment of NKT with ADR, a commonly used chemotherapeutic agent that is currently ineffective against NSCLC due to the intrinsic as well as acquired resistance. Our data showed that NKT significantly sensitized A549 and A549/ADR cells to ADR by reducing the cell viability as well as inducing apoptosis (Fig. 9, Fig. 10 and Fig. 11).

Furthermore, we also showed synergistic effect and the safety of this combination in *in vivo* mouse xenograft model using A549/ADR cells (Fig. 12). In the future studies, it is important to figure out the mechanism by which the combined treatment exerts the synergism and to study the effect of NKT on various cancer cell types to explore the potential of NKT in cancer treatment.

CONCLUSION

Our results demonstrated that NKT exhibited anticancer effect by inhibiting A549 and A549/ADR cell proliferation through activating AMPK pathway. Moreover, treatment of cells with NKT resulted in G1 cell cycle arrest as well as KRAS-related pathways inhibition. In addition, NKT synergized with ADR to increase cell death and decrease the dosage used for each compound. We also confirmed this synergistic effect in vivo model with minimal systemic toxicity. Altogether, NKT can serve as a promising agent in cancer treatment.

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