Effects of 17-AAG on the cell cycle and apoptosis of H446 cells and the associated mechanisms

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Abstract. As a heat shock protein 90 inhibitor, 17-allyl-amino-17-demethoxygeldanamycin (17-AAG) has been studied in numerous types of cancer, however the effects of 17-AAG on apoptosis and the cell cycle of H446 cells remain unclear. In the current study, the MTT method was used to evaluate the inhibitory effects of different durations and doses of 17-AAG treatment on the proliferation of H446 cells. The cells were stained with Annexin-fluorescein isothiocyanate/propidium iodide and measured by flow cytometry, and the gene and protein expression levels of signal transducer and activator of transcription 3 (STAT3), survivin, cyclin D1, cyt-C, caspase 9 and caspase 3 were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis. The results indicated that with treatment with 1.25-20 mg/l 17-AAG for 24 and 48 h, significant inhibition of H446 cell proliferation was observed in a time- and dose-dependent manner. With treatment of 3.125, 6.25 and 12.5 mg/l 17-AAG for 48 h, significant apoptosis and cell cycle arrest was observed. The results indicated that the gene and protein expression levels of STAT3, survivin and cyclin D1 were downregulated, and cyt-C, caspase 9 and caspase 3 were upregulated by 17-AAG in a dose-dependent manner when the cells were treated with 3.125 and 6.25 mg/l 17-AAG for 48 h. The results indicated that 17-AAG is able to inhibit the cell proliferation, induce apoptosis and G_2/M arrest and downregulate the gene and protein expression levels of STAT3, survivin and cyclin D1, and upregulate gene and protein expression of cyt-C, caspase 9, caspase 3.

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Key words: 17-AAG, H446 cells, apoptosis, cell arrest, STAT3, survivin, cyclin D1

Introduction

Lung cancer is one of the most prevalent types of malignant carcinoma worldwide (1), and is comprised of non-small cell lung cancer and small cell lung cancer. Non-small cell lung cancer is a common type making up 80% of lung cancer cases, and is predominantly treated by surgery. Small cell lung cancer, which makes up the remaining 20% of cases has a poorer prognosis following surgery, thus systematic chemotherapy is the main therapeutic strategy used, which carries the side effects of medicine toxicity. Previous studies have investigated novel molecular-targeted medicine for non-small cell lung cancer, however fewer studies have been conducting focussing on small cell lung cancer (2,3).

Members of the heat shock protein (HSP) family, in particular HSP90, are associated with numerous signal transducer pathways due to a high number of protein interactions, suggesting that HSP90 may be a promising novel molecular target (4). HSP90 has been previously reported to be overexpressed in various types of carcinoma including small cell lung cancer, and the increasing HSP90 expression levels have been associated with early stages of malignant carcinoma and their morphology, drawing attention to the use of HSP90 inhibitors.

Geldanamycin and its derivate 17-allyl-amino-17-demethoxygeldanamycin, (17-AAG) can compete with the ATP binding site on HSP90, thus inhibiting ATPase activity to result in the degradation of target proteins (5). The current study aimed to discover the effects of 17-AAG on proliferation, apoptosis and the cell cycle of H446 human small cell lung cancer cells, and to provide a theoretical basis of the mechanisms involved.

Materials and methods

Cell culture. The H446 human small cell lung cancer cell line was donated by Professor Huachuan Zheng from The First Affiliated Hospital of Liaoning Medical University (Jinzhou, China). The cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (both purchased from Thermo Fisher Scientific, Inc.,

Table I. Primer sequences and thermal cycling conditions of polymerase chain reaction analysis.

Gene	Forward primer sequence	Annealing temperature	Cycles	Product size
STAT3	F: CCTCCTCAGCATCTTATCCG R:CAGCCTGGGCATCCTTG	59˚C	30	499 bp
Caspase 3	F: TGGACCTGTTGACCTGA R: CACAAAGCGACTGGATG	57˚C	30	269 bp
Caspase 9	F: TGGACCTGTTGACCTGA R: CACAAAGCGACTGGATG	57˚C	30	885 bp
Cyt-C	F: GTCCGGTTGCGCTTTCCTT R: CGCAGTTTCCTCAAATTCTTTCTTC	60˚C	40	156 bp
Cyclin D1	F: TGTCCTACTACCGCCTCACACGCTTCCTCTCCG R: TCCTCTTCCTCCTCCTCGGCGGCCTTG	63˚C	35	160 bp
Survivin	F: TAACAGCCTGTTATCAGC R: TGCATCGTCATCGATCAG	60˚C	30	266 bp
β-actin	F: CAATGACCCCTTCATTGACC R: TGGAAGATGGTGATGGGATT	60˚C	30	135 bp

STAT3, signal transducer and activator of transcription 3.

Waltham, MA, USA) and 100 U/ml penicillin at 37˚C in a 5% CO_2 incubator. Cells were monitored daily and the medium was replaced every 2-3 days as appropriate. Cells at the logarithmic phase were selected to plant into the 96-well plate as a control group, and when all the cells had adhered to the wells, 5 different concentrations of 17-AAG (Shenger Yimei Co., Shenzhen, China) were added to the experiment groups. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was added into wells after 24 or 48 h, and 4 h later all the liquid was discarded. Dimethyl sulfoxide was then added into wells at 150 μl/well, the plate was vibrated for 15 min and the optical density was measured at 490 nm. The data was then analyzed to calculate the growth inhibition rates at different doses of 17-AAG.

Flow cytometry

Cell cycle assay. Cells at the logarithmic phase were selected for planting in the petri dishes with a control group and experimental groups with different concentrations of 17-AAG. Cells were collected after 48 h and incubated in 70% ethanol for 12 h, and then resuspended in 500 μl binding buffer (Thermo Fisher Scientific, Inc.) subsequent to centrifugation at 2,000 x g for 5 min at 4˚C. The RNA enzyme was added into the buffer and then incubated in 37˚C for 30 min. Subsequent to incubation, 5 μl propidium iodide (PI) was mixed into each sample and incubated at 20˚C for 30 min without bright light. A flow cytometer from BD Biosciences (Franklin Lakes, NJ, USA) was used to detect cell cycle distribution.

Apoptosis assay. Cells at the logarithmic phase were selected for planting in the petri dishes with a control group and experimental groups with different concentrations of 17-AAG. Cells were collected after 48 h and resuspended in 500 μl binding buffer (Thermo Fisher Scientific, Inc.) subsequent to centrifugation at 2,000 x g for 5 min at 4˚C. Annexin V-fluorescein isothiocyanate (5 μl) and PI (10 μl) were added into each sample and incubated at 20˚C for 5 min without bright light. The flow cytometer from BD Biosciences was used to detect apoptosis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) test. Cells at the logarithmic phase were selected for planting in the petri dishes with a control group and experimental groups with different concentrations of 17-AAG. All the cells were collected after 48 h into eppendorf tubes according to their groups, then 1 ml TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was added into each tube and incubated on ice for 10 min. A total of 200 μl chloroform was then added and shocked for 15 sec, and the cells were then centrifuged for 15 min at 12,000 x g and 4˚C. Subsequent to centrifugation, the upper layer was collected and a volume of isopropyl alcohol equal to that of upper layer was added into each tube. The tubes were placed on ice for 10 min, then were centrifuged for 15 min at 12,000 x g. Subsequently, all the liquid was discarded and 70% ethanol diluted by diethylpyrocarbonate-treated water was added to obtain RNA. The concentration of each sample was measured using a UV spectrophotometer. A Takara RNA PCR kit (AMV) version 3.0 (Takara Bio, Inc., Otsu, Japan) was used according to the manufacturer's protocol, and primers for STAT3, cyclin D1, cyt-C, caspase 9, caspase 3 and survivin were used for amplification subsequent to the reverse transcription. The primer sequences and thermal cycling conditions for PCR are presented in Table I. β-actin was used as a reference gene. Quantity One (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyze the data.

Western blotting. Cells at the logarithmic phase were selected for planting in the petri dishes with a control group and experimental groups with different concentrations of 17-AAG. Cells were collected after 48 h, and radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) was added into cells and kept on ice for 20 min. The cells were then centrifuged at 12,000 x g for 20 min at 4˚C. The upper layer was collected to measure protein concentrations. Total cell lysates

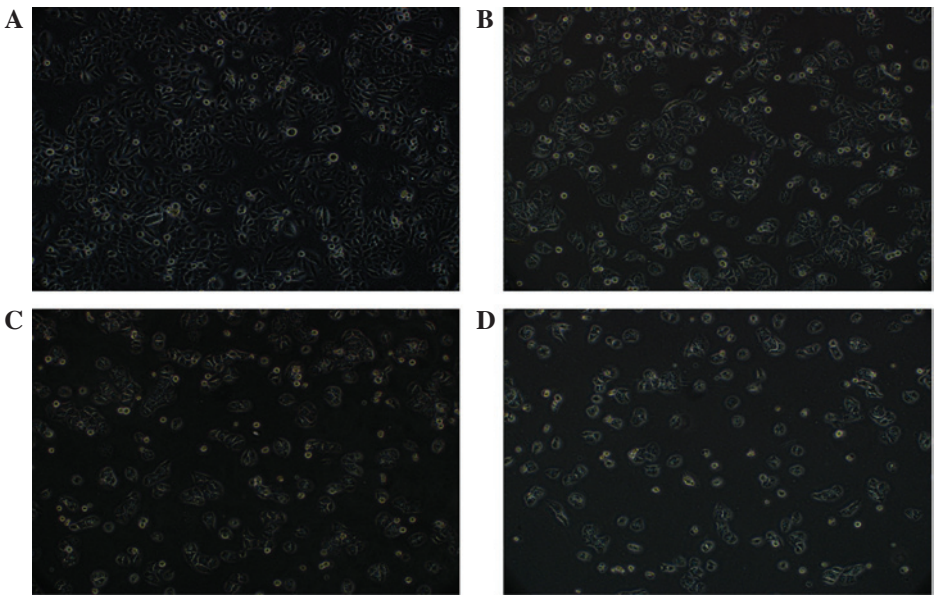

Figure 1. H446 cells subsequent to treatment with 17-AAG for 48 h. (A) Control; (B) 3.125 mg/l; (C) 6.25 mg/l; (D) 12.5 mg/l. 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

were resolved on 12% sodium dodecyl sulfate-polyacrylimide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes. The blots were then blocked with 5% nonfat milk powder, and incubated with primary antibodies against STAT3 (monoclonal mouse; 1:5,000; cat. no. ab119352; Abcam, Cambridge, UK), cyclin D1 (monoclonal rabbit; 1:10,000; cat. no. ab134175; Abcam), cyt-C (monoclonal mouse; 1:5,000; cat. no. ab13575; Abcam), caspase 9 (monoclonal rabbit; 1:5,000; cat. no. ab32539; Abcam), caspase 3 (monoclonal rabbit; 1:500; cat. no. ab32042; Abcam), survivin (polyclonal rabbit; 1:5,000; cat. no. ab24479; Abcam) and β-actin (monoclonal mouse; 1:5,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 12 h at 4˚C. The membranes were then washed with Tris-buffered saline with Tween 20, and incubated with horseradish peroxidase-conjugated goat anti-mouse (1:200; cat. no. 116475; Jackson ImmunoResearch, West Grove, PA, USA) and horseradish peroxidase-conjugated goat anti-rabbit (1:200; cat. no. 109525; Jackson ImmunoResearch) secondary antibodies for 1 h at 20˚C. Protein expression levels were normalized to β-actin.

Immunofluorescence test. Cells at the logarithmic phase were selected for planting into the 6-cell plates on a glass slide with a control group and experimental groups with different concentrations of 17-AAG. Cells were collected after 48 h, and phosphate-buffered saline (PBS) was added to wash the cells. Paraformaldehyde (4%) was added into the wells and incubated at room temperature for 15 min, then PBS was added to wash the cells 3 times. Subsequently, 1% Triton X-100 was added and the cells were incubated at 20˚C for 20 min. PBS was added to wash the cells 3 times, then bovine serum albumin (Invitrogen; Thermo Fisher Scientific, Inc.) was added into the wells, and they were incubated for 30 min at room temperature. The primary antibodies (as described above; STAT3 antibody, 1:1,000) were then added into the wells for incubation overnight at 4˚C. The next day, following washing with PBS, goat anti-mouse DyLight 488 (1:200; cat. no. ab96879; Abcam) secondary antibody was added into the wells and incubated for 2 h at room temperature, and PBS was added to wash the cells 3 times. Following the washing, 4',6-diamidino-2-phenylindole was added into the wells and they were incubated for 5 min without bright light, then cells were observed under fluorescence microscope and images were captured.

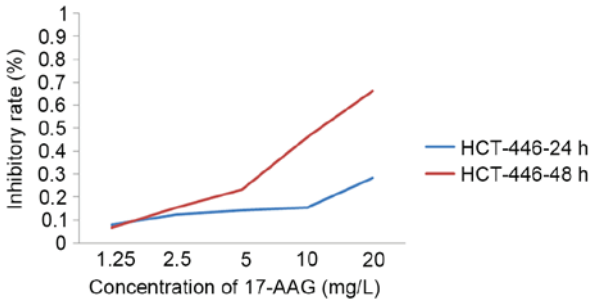

Figure 2. Inhibitory effects of different concentration of 17-AAG. 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

Statistical analysis. Statistical analysis was performed with SPSS software version 19.0 (IBM SPSS, Amronk, NY, USA). The data were presented as the mean ± standard deviation. The data comparisons were made using analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H446 cells proliferation is inhibited by 17-AAG. The MTT assay results indicated that 1.25~20 mg/l 17-AAG had a clear inhibitory effect on H446 proliferation, with a significant concentration-dependent correlation. The cell number of the drug groups was significantly less than in the control group, and abnormal morphology was observed (Fig. 1). The proliferation inhibition rate of 17-AAG on cells at 48 h (IC_{50}, 12.61 mg/l) was higher than that at 24 h (IC_{50}, 27.54 mg/l) (Table II, Fig. 2).

Table II. Inhibitory effects of 17-AAG on the proliferation of H446 small cell lung cancer cells (mean ± standard deviation, n=6).

Concentration of 17-AAG (mg/l)	24 h		48 h	
	Mean	Inhibitory rate (%)	Mean	Inhibitory rate (%)
Control	0.316±0.01	0	0.378±0.04	0
1.25	0.309±0.013	6.72[a]	0.357±0.01	7.86[a]
2.5	0.304±0.006	12.34[a]	0.330±0.019	15.38[a]
5	0.299±0.004	14.28[a]	0.304±0.005	23.41[a]
10	0.260±0.009	15.33[a]	0.233±0.004	46.02[a]
20	0.226±0.007	28.31[a]	0.169±0.008	66.19[a]
IC_{50}	27.54		12.61	

[a]P<0.01 vs. control group. IC_{50}, the mean value of 6 repetitions. 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

Table III. Effects of 17-AAG on cell cycle and apoptosis of H446 cells [($\bar{x}$ ± s), % n=3].

Group	G_0/G_1 phase	S phase	G_2/M phase	Apoptotic rate (%)
Control	55.95±0.83	42.20±0.77	1.85±0.52	16.08±0.79
3.125 mg/l	35.47±0.24[a]	43.71±0.33	20.82±0.35	24.07±1.13[a]
6.25 mg/l	40.26±0.31[a]	31.64±0.82	28.10±0.13	29.77±0.89[a]
12.5 mg/l	43.38±1.02[a]	31.83±1.03	24.79±0.26	46.2±0.23[a]

[a]P<0.05 vs. control group. 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

17-AAG causes G_2 arrest in H446 cells. PI staining detection results suggest that different concentrations of 17-AAG can arrest the cell cycle of H446 cells at G_2, after treatment for 48 h. However, this effect was not observed to be concentration-dependent (Table III, Fig. 3).

17-AAG promotes H446 cell apoptosis. The cells of the drug groups were observed to exhibit a higher apoptotic rate than those in the control group. The apoptotic rates of all the drug groups were significantly higher than those of the control group. With increases in drug concentration, the apoptotic rate increased, thus this effect was concentration-dependent (Table III, Fig. 4).

17-AAG effects the mRNA and protein expression levels of STAT3, cyclin D1, survivin, cyt-C, caspase 9 and caspase 3. RT-qPCR and western blot analysis results indicated that the expression of STAT3, cyclin D1 and survivin in the drug groups were significantly reduced compared with the control group, while expression levels of cyt-C, caspase 9 and caspase 3 were significantly increased compared with the control group. The increases in cyt-C, caspase 9 and caspase 3 observed were concentration-dependent. Comparisons were performed between drug groups and control groups (Tables IV and V, Figs. 5 and 6).

17-AAG inhibits STAT3 expression. Using fluorescence microscopy, it was identified that the green staining of STAT3 was markedly reduced in the drug groups compared with the control group in a concentration-dependent manner (Fig. 7).

Discussion

As a molecular chaperone, HSP90 is involved in various signal and transducer pathways to regulate cell proliferation, cell cycle and apoptosis. It has been identified that 17-AAG inhibits proliferation of gastric cancer (6), bladder cancer (7) and hepatocellular carcinoma (8) and may induce cell cycle arrest and apoptosis of which the mechanisms remain unclear (9). The results of the current study demonstrated that 17-AAG is able to inhibit the proliferation of H446 cells and can also induce cell cycle arrest and apoptosis of H446 cells.

It has been previously reported that cell cycle arrest serves an important role in the development of human carcinoma, and that HSP90 inhibitors are able to influence the cell cycle, indicating that HSP90 inhibitors may induce arrest of cancer cells at G_1 (10). Chaklader *et al* (11) identified that 17-AAG may significantly downregulate expression of cyclin D1, induce cell cycle arrest at G_1 period and weaken its proliferation ability (11). As an oncogene, cyclin D1 can combine with cyclin-dependent kinase 4 to initiate transition of cells from G_1 to S phase (12). However, the results of cell cycle assay in the current study indicated that 17-AAG could induce H446 cell cycle arrest at G_2 period, which was not correlated with the downregulation of cyclin D1. It is hypothesized that 17-AAG does influence the expression of cyclin D1, and then influence the percentage of cells in the G_1 phase, however this alteration wasn't a vital factor for H446 cell cycle arrest. The exact mechanisms of the effects of 17-AAG on each stage of cell cycle, through which pathways these is mediated and how 17-AAG affects cyclin D1 expression remain unclear, thus require further investigation.

Table IV. Effects of 17-AAG on mRNA expression of H446 cells ($\bar{x} \pm s$, n=6).

17-AAG (mg/l)	STAT3/β-actin	Cyclin D1/β-actin	Cyt-C/β-actin	Caspase 9/β-actin	Caspase 3/β-actin	Survivin/β-actin
Control	0.52±0.02	0.65±0.05	0.61±0.02	0.05±0.00	0.45±0.01	0.85±0.03
3.125	0.27±0.01[a]	0.46±0.04[a]	0.56±0.03[a]	0.07±0.01	0.54±0.02	0.62±0.02[a]
6.25	0.21±0.01[a]	0.39±0.01[a,b]	0.79±0.01[a,b]	0.37±0.01[a,b]	0.63±0.01[a,b]	0.53±0.01[a,b]

[a]P<0.05 vs. control group; [b]P<0.05 vs. 3.125 mg/l. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; STAT3, signal transducer and activator of transcription 3.

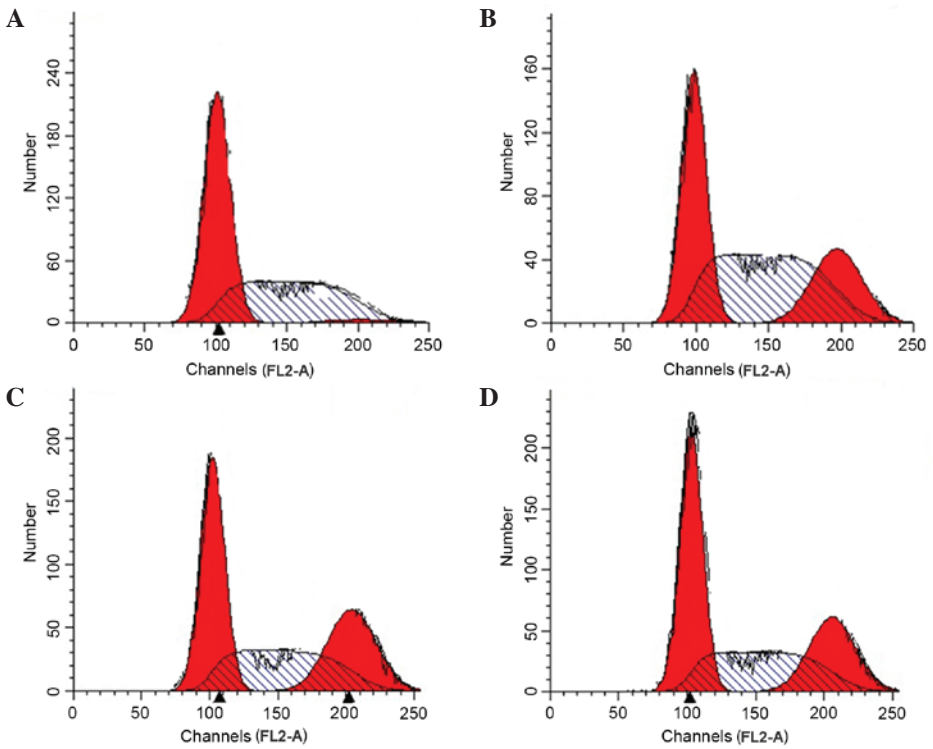

Figure 3. Effects of 17-AAG on the cell cycle of H446 cells. (A) Control group; (B) 3.125 mg/l; (C) 6.25 mg/l; (D) 12.5 mg/l. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; FL2-A, pulse area.

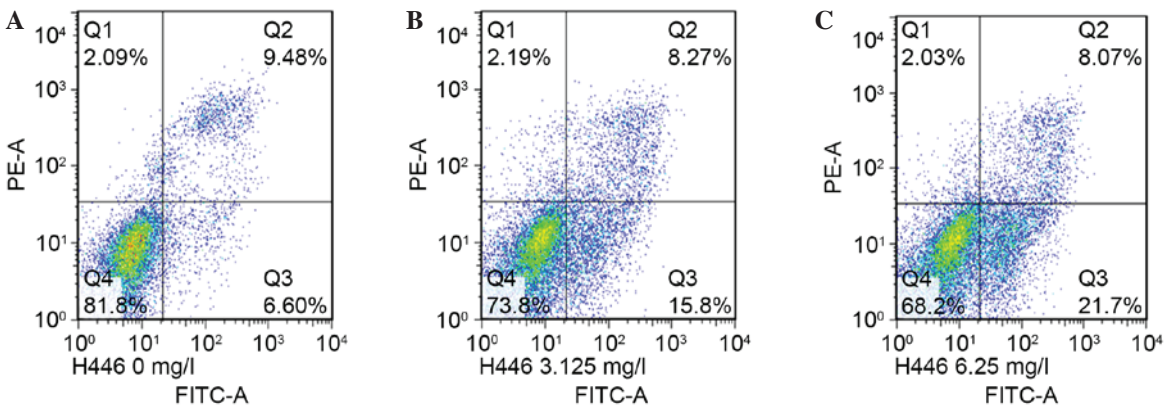

Figure 4. Effects of 17-AAG on apoptosis of H446 cells. (A) Control group; (B) 3.125 mg/l; (C) 6.25 mg/l. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

Apoptosis, whereby cells initiate programmed death as a result of stimulation by a specific stimulus, serves a vital role in various physical and pathological processes (13). The most important process involved in the mitochondrial apoptosis pathway is the release of the membrane interspace protein cyt-C; following its release cyt-C forms an apoptotic body

Table V. Effects of 17-AAG on protein expression of H446 cells ($\bar{x} \pm s$, n=6).

17-AAG (mg/l)	STAT3/β-actin	Cyclin D1/β-actin	Cyt-C/β-actin	Caspase 9/β-actin	Caspase 3/β-actin	Survivin/β-actin
Control	0.64±0.01	0.87±0.01	0.17±0.01	0.47±0.01	0.16±0.02	0.54±0.03
3.125	0.34±0.02[a]	0.68±0.02[a]	0.38±0.02[a]	0.88±0.02[a]	0.38±0.01[a]	0.34±0.02[a]
6.25	0.12±0.01[a,b]	0.29±0.01[a,b]	0.59±0.01[a,b]	1.39±0.01[a,b]	0.59±0.01[a,b]	0.12±0.01[a,b]

[a]P<0.05 vs. control; [b]P<0.05 vs. 3.125 mg/l. 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

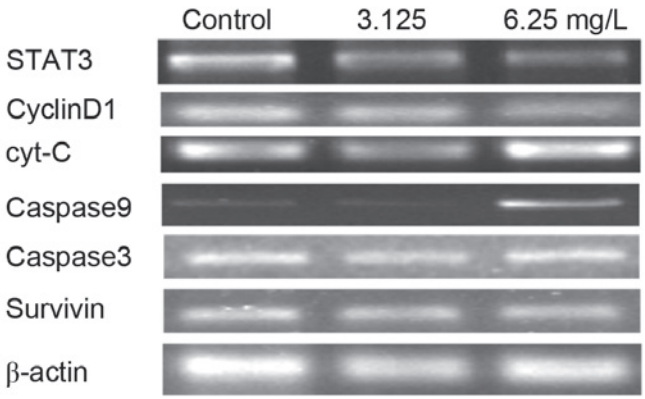

Figure 5. Effects of 17-AAG on mRNA expression. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; STAT3, signal transducer and activator of transcription 3.

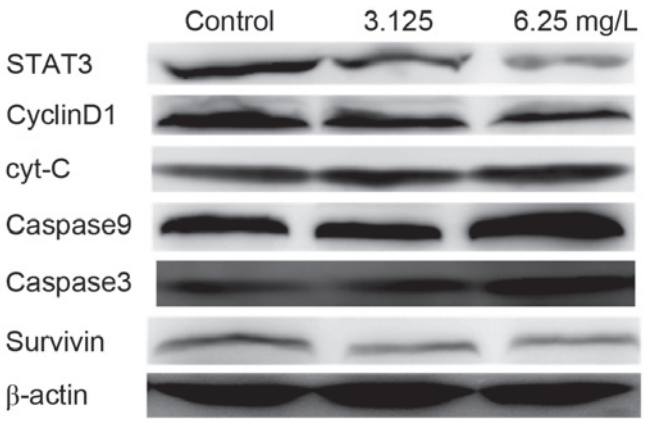

Figure 6. Effects of 17-AAG on protein expression. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; STAT3, signal transducer and activator of transcription 3.

with apoptotic protease activating factor 1, thus activating caspase 9, caspase 3 and caspase 7 to initiate apoptosis. Survivin is a member of the inhibition of apoptosis protein family and is associated with cell mitosis and apoptosis (14). It has been reported that survivin can inhibit the expression of caspase through the mitochondria apoptosis pathway to inhibit apoptosis. The results of the current study indicated that in the drug groups the expression of survivin was reduced, and protein and mRNA expression of cyt-C, caspase 9 and caspase 3 were increased. All the results indicated that 17-AAG functioned to aid in the initiation of apoptosis and survivin participated in the process.

The STAT family regulates cell growth, differentiation and development of numerous physical and pathological processes. Among all the members, STAT3 has been identified as important in the regulation of transformation, modification subsequent to translation, cell location and cell function. STAT3 can transfer into the nucleus and combine with a promoter sequence to regulate transformation and then regulate cell proliferation, differentiation and the metabolism. Considering the fact that STAT3 is important in inflammation and in carcinoma, it is regarded as a crucial regulatory factor (15). It has been reported that cyclin D1 is a downstream gene of STAT3, and expression of cyclinD1 will be reduced when STAT3 is inhibited (16). In the current study, it was identified that expression of STAT3 was reduced in the drug groups, measured by RT-qPCR, western blotting and immunofluorescence test. Additionally, protein and mRNA expression of cyclin D1 was reduced in the drug groups compared with that of the control groups, thus it was concluded that 17-AAG may downregulate cyclin D1 via the STAT3 pathway and induce H446 arrest at G_2 phase to inhibit cell proliferation. A previous study identified that

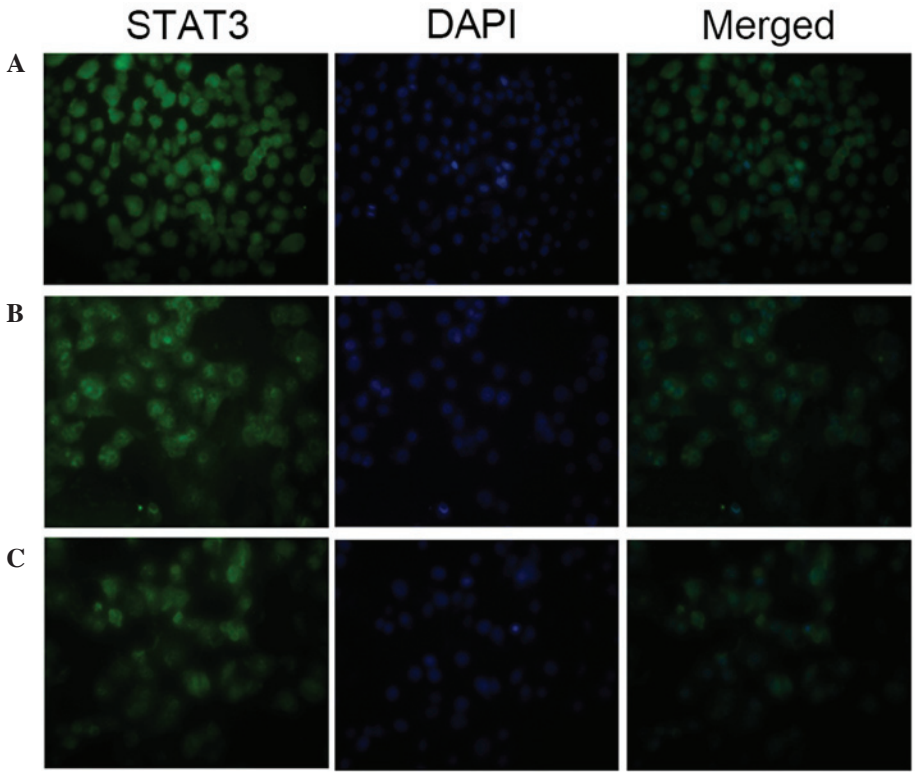

Figure 7. Effects of 17-AAG on STAT3 expression of H446 cells. (A) Control group; (B) 3.125 mg/l; (C) 6.25 mg/l. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; STAT3, signal transducer and activator of transcription 3; DAPI, 4',6-diamidino-2-phenylindole.

STAT3 can transfer into mitochondria to regulate mitochondrial apoptosis (17). It has been suggested that caspase 3 may be a target of STAT3 and that STAT3 serves a crucial role in the mitochondrial apoptosis pathway. It has been reported that when STAT3 is inhibited, expression of survivin is markedly reduced, which indicated that survivin may also be a target of STAT3. Ma *et al* (18) reported that blocking of the STAT3 pathway in ovarian cancer resulted in a reduction of survivin expression and an increase of caspase 3 expression, indicating that the STAT3 pathway may inhibit survivin expression to initiate the mitochondrial apoptosis pathway and apoptosis. Downregulation of STAT3 in the present study suggested that 17-AAG may regulate expression of survivin through inhibiting STAT3 to regulate the mitochondrial apoptosis pathway and induce apoptosis of H446 cells. Further studies exploring the role of STAT3 in the effects of 17-AAG on H446 cells are required.

Acknowledgements

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