# VDAC upregulation and αTAT1-mediated α-tubulin acetylation contribute to tanespimycin-induced apoptosis in Calu-1 cells

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**Abstract.** Voltage-dependent anion channel 1 (VDAC1) functions as a porin in the mitochondrial outer membrane (MOM) and plays important roles in mitochondria-mediated cell apoptosis. VDAC1 interacts with a variety of proteins, such as Bcl-2 family proteins, hexose kinase (HK), adenine nucleotide translocase (ANT) and α-tubulin. However, the association between VDAC1 and α-tubulin, particularly between VDAC1 and acetylated α-tubulin (Ac-α-tubulin), in apoptosis remains unclear. The present study revealed that the heat shock protein 90 inhibitor, tanespimycin, induced VDAC1 upregulation and α-tubulin acetylation during Calu-1 cell apoptosis in human lung cancer. Hsp90 mediated the expression level of VDAC1, and the acetylation of α-tubulin was enhanced in an α-tubulin acetyltransferase 1 (αTAT1)-dependent manner following an increase in VDAC1 expression. Docetaxel, as an inhibitor of microtubules, augmented the expression of Ac-α-tubulin, VDAC1 and Bax induced by tanespimycin and increased the degree of caspase activation. Immunoprecipitation (IP) experiments revealed that Ac-α-tubulin, α-tubulin and VDAC1 were co-precipitated in the IP complex, in which α-tubulin expression was decreased and VDAC1 proteins were oligomerized, and that the p-AKT/glycogen synthase kinase 3β (GSK3β) signalling pathway mediated the opening of VDAC1. Therefore, it can be asserted that the acetylation of α-tubulin and VDAC1 upregulation or oligomerization induced by tanespimycin may lead to mitochondrial permeability and consequently induce the apoptosis of lung cancer cells. These findings provide evidence for the use of a combination of drugs that target VDAC1 and tubulin to induce tumour cell apoptosis.

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*Abbreviations:* Ac-α-tubulin, acetylated α-tubulin; ANT, adenine nucleotide translocase; GSK3*β*, glycogen synthase kinase *3β;* HDAC6, histone deacetylase 6; HK, hexose kinase; Hsp27, heat shock protein 27; Hsp90, heat shock protein 90; IP, immunoprecipitation; LC3B-II, microtubule-associated protein 1 light chain 3B-II; MOM, mitochondrial outer membrane; PARP, poly ADP-ribose polymerase; PKA, protein kinase A; PTP, permeability transition pore; SRB, sulforhodamine B; VDAC1, voltage-dependent anion channel 1; αTAT1, α-tubulin acetyltransferase 1

*Key words:* VDAC1, α-tubulin acetylation, Hsp90, αTAT1, tanespimycin, apoptosis

## Introduction

Voltage-dependent anion channel (VDAC)1, as a mitochondrial porin, controls the entry and exit of metabolites and energy between the mitochondria and the cytosol. Therefore, it is a target convergence for both cell death and mitochondria-mediated apoptosis (1). In 2008, the 3D structure of VDAC1 was determined by NMR spectroscopy and X-ray crystallography (2-4). VDAC1 comprises a barrel formed by 19 β-strands and an N-terminal α-helix folded into the barrel interior. The N-terminus is composed of hydrophilic amino acid residues and has a higher positive charge density than negative charge density (4). The N-terminus stabilizes the barrel, preventing it from adopting a partially dilapidated, low-conductance closed state. In addition, the N-terminus reportedly contributes to VDAC1 oligomerization and cellular apoptosis through interactions with hexokinase and adenosine triphosphate (ATP) (5). In the mitochondrial outer membrane (MOM), VDAC1, adenine nucleotide translocase (ANT) and cyclophilin D, constitute the permeability transition pore (PTP), which is involved in the release of pro-apoptotic factors from the intra-mitochondrial space (6).

VDAC1 channel permeability, which is an important feature of the PTP, is mediated by ions, small molecules, and protein kinases (7,8). Studies have demonstrated that VDAC1 interacts with hexose kinase (5,9-11), the pro-apoptotic proteins Bax and Bak, and the anti-apoptotic proteins, Bcl-2 and Bcl-xL (12,13). Previous mechanistic studies have revealed that PKA-dependent VDAC1 phosphorylation decreases VDAC1 conductance, glycogen synthase kinase 3β (GSK3β)-mediated VDAC1 phosphorylation appears to promote channel opening and chemotherapy-induced cytotoxicity, and the AMPK/mTOR signalling axis also controls

mitochondrial metabolism (14,15). These studies highlight the importance of the VDAC1 protein for cancer cell survival and mitochondrial apoptosis.

The mitochondria prefer to be localized within the tubulin-microtubule network and to move along microtubules, which are cylindrical polymers composed of α and β tubulin. Microtubules are the primary components of the cytoskeleton and mediate crucial cellular functions. Tubulin proteins exhibit a high affinity for VDAC1 by binding to the mitochondria. A previous study confirmed that tubulins are inherent components of the mitochondrial membrane and may be involved in modulating mitochondrial permeability and subsequently controlling respiration (16). The same previous study also demonstrated that dimeric tubulin at nanomolar levels induces reversible closure of VDAC in a voltage-sensitive manner. The tubulin protein has an extended C-terminal tail with a negative charge. These anionic C-terminal tails are essential for the interaction of tubulin with the cationic N-terminus of VDAC in the barrel at the mitochondria-cytosol interface. In the tubulin-VDAC interaction model, the anionic C-terminus of tubulin penetrates into the VDAC cationic channel lumen and specifically interacts with VDAC, thus blocking channel conductance (17). This indicates that the level of dimeric tubulin or free α/β tubulin may regulate the permeability of VDAC in the MOM and then influence mitochondria-mediated cellular apoptosis. Paclitaxel and other microtubule-targeting anti-tumour drugs reportedly modify the interactions of microtubules and/or tubulin with VDAC and induce overexpression of VDAC1 (18). The high levels of VDAC1 may shift VDAC1 from a monomeric to an oligomeric assembly, which promotes CytC release from the mitochondria, leading to apoptosis (18). However, the critical factor is whether these drugs change the levels of free tubulins that interact with VDAC in the MOM and subsequently affect VDAC permeability. This question is of special interest and is worth exploring.

Geldanamycin and its analogue, 17-AAG, inhibitors of heat shock protein (Hsp)90, have been shown to interact with the mitochondria, particularly with VDAC, through hydrophobic interactions independent of Hsp90, increasing the intracellular Ca(2+) concentrations and decreasing the plasma membrane cationic current (19). In previous studies, it was found that geldanamycin and its analogue, 17-AAG, both induced lung cancer cell apoptosis effectively by inhibiting Hsp90 (20). Therefore, the present study aimed to investigate whether tanespimycin (17-AAG) associates with VDAC by interacting with it in the MOM during apoptosis induction. Herein, the experiments revealed that tanespimycin induced α-tubulin acetylation and increased the expression of VDAC and Bax. In particular, docetaxel, a microtubule stabilizer that promotes the hyperpolarization of microtubules, enhanced the levels of acetylated α-tubulin, VDAC and Bax when combined with tanespimycin. As a result, cellular apoptosis was induced. This implies that the two drugs have a similar mechanism of apoptosis induction. However, at present, the molecular mechanisms underlying the association between α-tubulin acetylation and VDAC in apoptosis upon treatment with tanespimycin remain unclear. Therefore, the present study aimed to explore the mechanisms between α-tubulin acetylation and VDAC upregulation in apoptosis in lung cancer cells.

## Materials and methods

*Reagents*. Tanespimycin was provided by LC Laboratories. Docetaxel was purchased from American Radiolabeled Chemicals, Inc. Rapamycin was purchased from Cell Signaling Technology, Inc. Each of these compounds was dissolved in dimethyl sulfoxide (DMSO) at a given concentration, and aliquots were stored at -20˚C. The stock solutions were diluted to the final appropriate concentration immediately prior to use.

*Antibodies*. Mouse monoclonal anti-caspase-3 antibody (cat. no. 31A1067) and anti-caspase-8 antibody (cat. no. 9746) were purchased from Imegenex and Cell Signaling Technology, Inc., respectively. Rabbit anti-VDAC1 antibody (cat. no. 12454), mouse anti-caspase-9 antibody (cat. no. 9508) and anti-poly (ADP-ribose) polymerase (PARP) antibody (cat. no. 9542) were obtained from Cell Signaling Technology, Inc. A mouse anti-Hsp90α antibody (cat. no. ab128483) and rabbit anti-Hsp90β antibody (cat. no. ab2927) were purchased from Abcam. A goat anti-α-tubulin acetyltransferase 1 (αTAT1) antibody (cat. no. sc-101911) and mouse polyclonal anti-β-actin antibody (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. Mouse polyclonal anti-acetylated α-tubulin (Ac-α-tubulin) antibody (cat. no. 2152) and α-tubulin antibody (cat. no. 3873) were obtained from Cell Signaling Technology, Inc. Rabbit anti-p-Akt antibody (cat. no. 4060), anti-Akt antibody (cat. no. 9272) and anti-GSK3β antibody (cat. no. 5676) were purchased from Cell Signaling Technology, Inc. Protein A-Agarose (cat. no. 05015979001) and Protein G-Agarose (cat. no. 05015952001) were obtained from Roche Diagnostics.

*Cell line and cell culture*. The human non-small cell lung cancer cell line, Calu-1, was provided by the American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 5% fetal bovine serum in a humidified atmosphere of 5% $CO_2$ and 95% air at 37˚C.

*Cell survival assay*. First, Calu-1 cells were seeded in 96-well plates at an appropriate density of $8x10^3$ cells per 100 $\mu$l of culture medium per well. On the second day, Calu-1 cells were treated with tanespimycin (0, 0.5 and 1.0 $\mu$M) or combination with docetaxel (0.5 nM) and the total volume per well was 200 $\mu$l. Calu-1 cells were treated for 48 h, and cell survival was estimated by a sulforhodamine B (SRB) assay as previously described (20). First, the culture supernatants were discarded, and 100 $\mu$l of 10% TCA (trichloroacetic acid,) were then added per well. After washing with water thrice, 50 $\mu$l SRB were then added per well by agitating for 5 min. After recycling the SRB solution and washing with 1% acetic acid 5 times, 100 $\mu$l tris-base (10 mM, pH 10.5) were added per well and followed by agitation for 5 min. The absorbance value was measured by a microplate reader at 540 nm. The relative fold-change of cell survival was determined by comparing with the control. At least 4 independent experiments were performed.

*Western blot analysis*. Whole-cell protein lysate preparation and western blot analysis were performed as previously described in the literature (21), in which various apoptotic proteins were induced and examined by western blot analysis.

Cells were lysed in RIPA buffer and total lysate proteins were assessed by Biorad Bradford assay (Bio-Rad Laboratoreis, Inc.). SDS-PAGE (10%) and immunoblotting were performed with 40 $\mu$g of proteins from each sample and proteins were transferred to a polyvinylidene difluoride membrane (PVDF). The dilution of the primary antibodies (Cell Signaling Technology) used for probing was usually 1:1,000. The secondary antibodies were usually diluted at 1:5,000. In the blocking protocol, 5% skim milk was used at room temperature for 1 h. The primary antibody was incubated at 4˚C overnight and the secondary antibody was incubated at room temperature for 1 h. Images were developed using ECL reagent (cat. no. 32109; Thermo Fisher Scientific, Inc.) with an Amersham Imager 600 (GE Healthcare). All immunoblots were repeated thrice, and the band densities were quantified using ImageJ 1.53a software. β-actin was used as a loading control.

*Silencing of VDAC1, Hsp90α/β and αTAT1 with siRNA oligos*. siRNAs were synthesized by GenePharma. The Hsp90α/β siRNA and VDAC1 siRNA target sequences were synthesized as previously described (22,23): siRNA transfection (1.5 $\mu$l) was conducted as previously described (24) and the sequences were as follows. HiPerFect Transfection Reagent was used and at 6 h following transfection (siRNA, 25 pmol), the cells were cultured in new medium for 18-24 h and then treated with the given drugs for 48 h. The sequences were as follows: Control siRNA oligos, 5'-UUCUCCGAACGUGUCACG UTT-3'; VDAC1 siRNA oligos, 5'-GCTTGGTCTAGGACT GGAA-3'; Hsp90α siRNA oligos, 5'-GTTTGAGAACCTCTG CAAA-3'; Hsp90β siRNA oligos, 5'-CGACAAGAAUGAUAA GGCA-3'; and αTAT1 siRNA oligos, 5'-GGGAAACUCACC AGAACGA-3'.

*Immunoprecipitation (IP)*. Calu-1 cells were lysed with RIPA lysis buffer, and the supernatant was treated with Protein A-Agarose and Protein G-Agarose (1:1) and with 10 $\mu$g of VDAC1 antibody. The mixture was rotated at 4˚C overnight, and the pelleted beads were washed according to the manufacturer's instructions. The precipitated proteins were dissolved in 2X SDS sample buffer and boiled for 5 min. The pulled down proteins were then examined by western blot analysis.

*Microscopy*. First, Calu-1 cells were seeded in 6-cm culture plates at an appropriate density (approximately $5.3x10^5$) and on the second day, various concentrations (0, 0.5 and 1.0 $\mu$M) of tanespimycin were added and the Calu-1 cells were cultured for the indicated time period (48 h). The Calu-1 cells were then observed under inverted phase contrast microscope (Nikon TS100) at x200 magnification.

*Statistical analysis*. Data from the siRNA experiments of Hsp90, VDAC1 and αTAT1 were compared with a multiple t-test (Holm-Sidak correction was applied to the P-values) and the comparison of interest is the relative protein in Cki (-) treatment. Comparisons between groups in IP were carried out with an unpaired Student's t-test. The data are expressed as the means ± standard deviation (SD) and error bars in the figures represent the SD values. Data from cell survival assay were analyzed by an unpaired Student's t-test and the analysis results were the means of 4 independent experiments. Data from Calu-1 apoptotic cells were calculated using ImageJ software and comparisons between groups were carried out with repeated measures (RM) one-way ANOVA and a Geisser-Greenhouse correction was applied to the P-values obtained from RM one-way ANOVA to control for comparisons. The analysis results are the means of 4 replicate determinations and bars standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc.). P-values of $<0.05$ were considered to indicate statistically significant differences.

## Results

*The molecular chaperone, Hsp90, mediates the expression of VDAC1*. Tanespimycin, an inhibitor of Hsp90, may disrupt the molecular chaperone activity of Hsp90 and influence cell survival. It has been found that geldanamycin and its derivative, 17-AAG (also known as tanespimycin), associate with the mitochondria, specifically VDAC1, via a hydrophobic interaction (19). The present study revealed that tanespimycin induced VDAC1 upregulation. The present study wished to determine whether an association exists the expression of Hsp90 and that of VDAC1, and whether Hsp90 plays a role in modulating VDAC expression and function to induce cell apoptosis. For this purpose, Hsp90α/β was silenced by siRNA to decrease the expression level of Hsp90. Hsp90α/β was knocked down in Calu-1 cells, and the cells were treated with tanespimycin for 48 h (Fig. 1A). The results of western blot analysis and GraphPad software statistical analyses revealed that the expression level of VDAC1 was upregulated in both the untreated cells in which Hsp90α/β was knocked down and in the cells in which Hsp90α/β was knocked down and treated with tanespimycin compared with the control cells (Fig. 1), suggesting that a reduction in Hsp90 expression or the loss of its activity may lead to an increase in VDAC1 expression. Thus, Hsp90 may mediate the expression level of VDAC or Hsp90 and VDAC in the mitochondrial outer membrane and this may be regulated in parallel or in a coordinated manner.

*Upregulation of VDAC1 is involved in mitochondria-dependent apoptosis*. To further elucidate the roles of VDAC1 in lung cancer cell apoptosis, VDAC1 was silenced by siRNA in Calu-1 cells, and the cells were then treated with 1.0 $\mu$M tanespimycin for 48 h. The results of western blot analysis revealed that when VDAC1 protein was knocked down, the activation of caspase-8, particularly a distinct anti-caspase-8-p18 protein band, was clearly inhibited, and PARP expression was decreased (Fig. 2A and B). VDAC1 is reportedly required for intrinsic apoptosis through its ability to process procaspase-8 into its active p18 form (25). In the present study, in the inverted phase contrast microscopy images, Calu-1 cell apoptosis was evident ($P<0.001$) and this was also confirmed by statistical analyses (Fig. 2C and D). Therefore, the present study demonstrated that VDAC1 upregulation was involved in mitochondria-dependent apoptosis induced by tanespimycin in lung cancer cells.

*Expression of Ac-α-tubulin induced by tanespimycin is elevated in apoptosis*. Microtubules consist of α/β-tubulin dimers and exert significant effects on cell growth state and

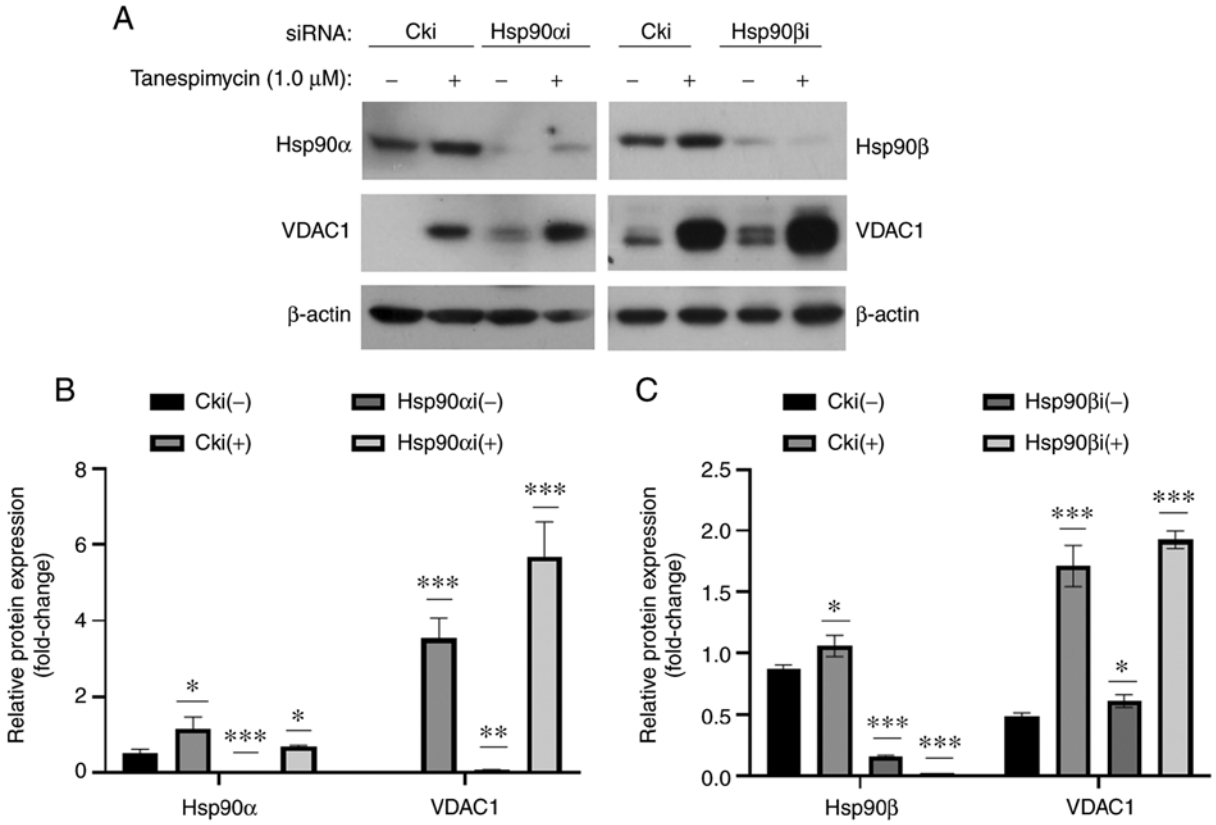


Figure 1. Hsp90α/β knockdown mediates VDAC1 expression. (A) Calu-1 cells were cultured in 6-well plates and on the 2nd day they were transfected with control siRNA (Cki) or Hsp90α/β siRNA (Hsp90αi/βi). At 24 h following transfection, cells were reseeded in a 6-well plate and treated with 1.0 $\mu$M tanespimycin for 48 h and the cells were then harvested for the preparation of whole-cell protein lysates for western blot analysis, and the indicated proteins Hsp90α/β, VDAC1 and β-actin were analysed. β-actin was used as the loading control (Hsp90α, 90 kDa; Hsp90β, 90 kDa; VDAC1, 32 kDa; β-actin, 45 kDa). (B and C) The above western blot analysis results were analyzed by ImageJ software and GraphPad Prism 8 software, and the analysis results are the means of 3 independent experiments. The western blot shown is representative of 3 independent experiments. Comparisons between groups were carried out with the Multiple t-test (Holm-Sidak correction was applied to the P-values of multiple comparison) and the comparison of interest is the relative protein in Cki(-) treatment. Values of $P<0.05$ were considered to indicate a statistically significant difference; $^{*}P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ compared with the untreated cells. Error bars in the figures represent the SD values. Hsp, heat shock protein; VDAC1, voltage-dependent anion channel 1.

signal transduction. When the levels of acetylated α/β-tubulin dimers or acetylated microtubules are increased, their cellular activities are altered (26). The authors and other researchers have reported that tanespimycin (17-AAG) induces cell death via the downregulation of c-FLIP$_L$ in lung cancer cells (20,27). In the present study, it was simultaneously found that when the Calu-1 cells were exposed to tanespimycin (1.0 $\mu$M for 48 h), the expression of Ac-α-tubulin induced by tanespimycin evidently increased in a dose-dependent manner, as detected by western blot analysis (Fig. 3A), and that the cell survival rate was reduced to approximately 30% of the control level, as determined by SRB assay (Fig. 3B). To further determine the functions of tanespimycin-induced Ac-α-tubulin in apoptosis, Calu-1 cells were treated for 48 h with a combination of tanespimycin (0, 0.5 and 1.0 $\mu$M) and docetaxel (0.5 nM). As a microtubule stabilizer, docetaxel potentiated the expression of Ac-α-tubulin and cellular apoptosis induced by tanespimycin monotherapy, as determined by western blot analysis and SRB assay, in which the cell survival rate was reduced to 23% of the control level (Fig. 3A and B). According to these combination experiments, cellular apoptosis was induced, as evidenced by a higher degree of caspase-9 and PARP activation, and the expression of Ac-α-tubulin, VDAC and Bax was elevated (Fig. 3A). Furthermore, the expression of Ac-α-tubulin was positively associated with apoptosis, suggesting that the acetylation of α-tubulin plays important roles in the induction of the apoptosis of non-small cell lung cancer (NSCLC) cells.

*Acetylation level of α-tubulin is modulated by the acetyltransferase, αTAT1.* The acetyltransferase, αTAT1, has been reported to catalyse α-tubulin acetylation at Lys40 inside the microtubule lumen to alter microtubule-based processes (28). The acetylation level of microtubules (MTs) influences their stability and can even render MTs inflexible, brittle, and more inclined to break under stress (29). Thus, the present study wished to determine whether Ac-α-tubulin induced by tanespimycin resulted from the breakage of acetylated MTs or the acetylation of free α/β-tubulin and to identify the association between α-tubulin acetylation and the acetyltransferase, αTAT1. To determine whether the acetyltransferase αTAT1 is the enzyme that catalyses the tanespimycin-induced acetylation of α-tubulin in Calu-1 lung cancer cells, a time-course experiment and a siRNA experiment were performed. In the time-course experiment, the expression levels of αTAT1 were consistent with the increasing levels of Ac-α-tubulin induced by tanespimycin (Fig. 4A). When αTAT1 was knocked down, the expression level of Ac-α-tubulin decreased simultaneously compared with the control treated with tanespimycin,

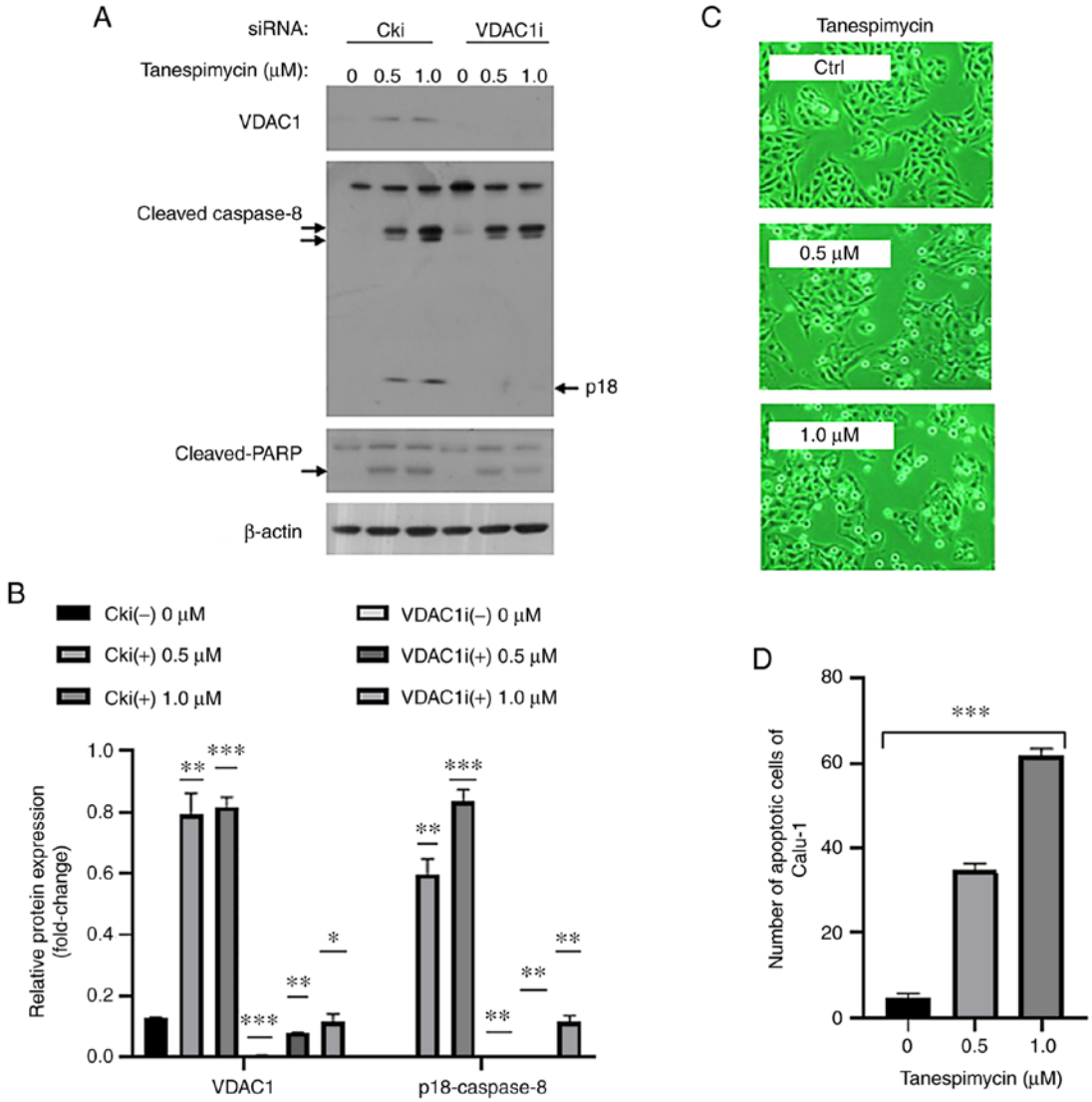


Figure 2. VDAC1 upregulation induced by tanespimycin is involved in cell apoptosis. (A) Calu-1 cells were cultured in 6-well plate and on the 2nd day they were transfected with control siRNA (Cki) or VDAC1 siRNA (VDAC1i). At 24 h following transfection, cells were reseeded in 6-well plate and treated with tanespimycin (0, 0.5 and 1.0 $\mu$M) for 48 h. The cells were then harvested for the preparation of whole-cell protein lysates for following western blot analysis to detect VDAC1, Caspase-8, PARP and β-actin levels. β-actin was used as the loading control (VDAC1, 32 kDa; caspase-8, 18, 43, 57 kDa; PARP, 89, 116 kDa; β-actin, 45 kDa). (B) The above western blot analysis results of VDAC1 and p18-caspase8 were analyzed by ImageJ software and GraphPad Prism 8 software and the analysis results were the mean of 3 independent experiments. The western blot shown is representative of 3 independent experiments. Comparison betweens groups were carried out with the Multiple t-test (Holm-Sidak correction was applied to the P-values of multiple comparison). The comparison of interest is the relative protein in Cki (-) treatment; $^{*}$P<0.05, $^{**}$P<0.005 and $^{***}$P<0.001. Error bars in the figures represent the SD values. (C) Calu-1 cells were treated with tanespimycin (0, 0.5 and 1.0 $\mu$M), and then the cells were subjected to take photos by inverted phase contrast microscope (x200 magnification) to observe the cell apoptotic state. (D) The number of Calu-1 apoptotic cells were calculated by ImageJ software. Comparison between groups was carried out with the RM one-way ANOVA method (a Geisser-Greenhouse correction was applied to the P-values). $^{***}$P<0.001. The analysis results are the means of 4 replicate determinations; bars represent SD. P<0.001. VDAC1, voltage-dependent anion channel 1.

as shown by western blot analysis and GraphPad software analyses (Fig. 4B and C). These results indicate that αTAT1 is the acetyltransferase of α-tubulin in lung cancer cells and that it may regulate the levels of acetylated α-tubulin.

*Acetylation of α-tubulin promotes VDAC1 permeability in mitochondria-mediated apoptosis*. In the above studies, we also observed that VDAC1 expression was elevated in both the time-course experiment and in the drug combination experiments and was accompanied by an increase in Ac-α-tubulin (Figs. 3A and 4A). The present study then wished to determine the functions of both proteins in apoptosis. In the tubulin-VDAC1 interaction model, the anionic C-terminal tail of tubulin penetrates into the VDAC1 cationic channel lumen and interacts with VDAC1, thus blocking channel conductance (17). In the present study, the acetylation of α-tubulin was markedly induced, and this change was accompanied by lung cancer cell apoptosis. Therefore, it was hypothesized that the acetylation of α-tubulin in microtubules, particularly the acetylation of α/β dimers, would decrease the effective level of free tubulin in the MOM and that the density of the C-terminal tail of α-tubulin, which interacts with VDAC1, would be reduced in the interior β-barrel of VDAC1. To examine this hypothesis, Calu-1 cells were treated with 1.0 $\mu$M tanespimycin for 32 h (not for 48 h as treatment for this duration induces apoptosis that is overly severe), and an IP experiment was then

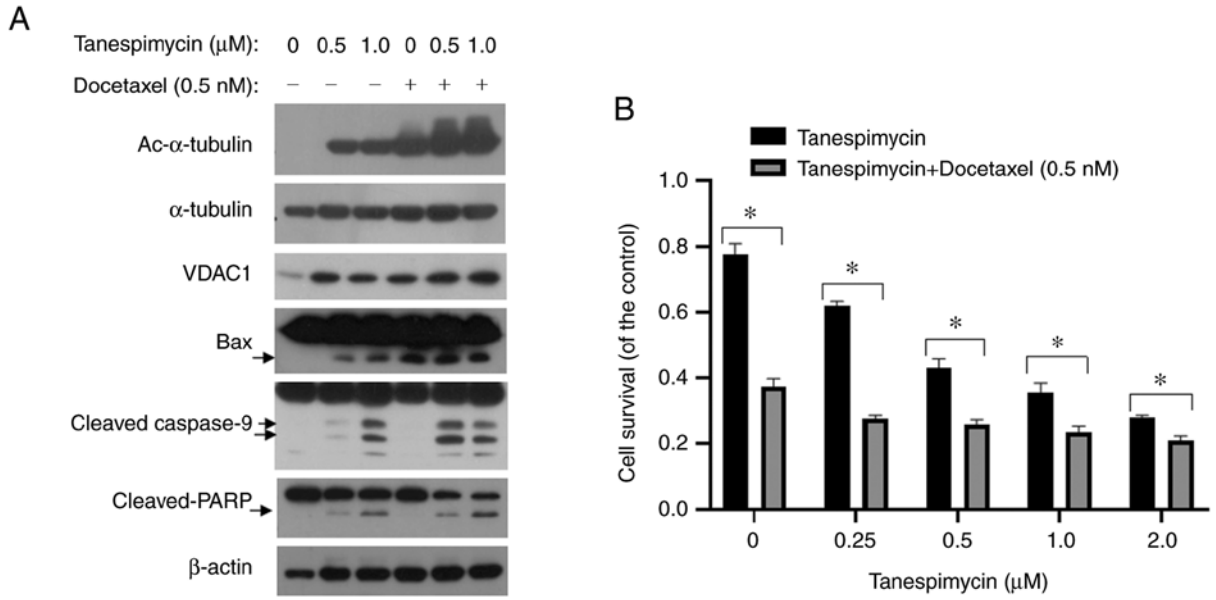


Figure 3. Docetaxel enhances the upregulation of VDAC1, Ac-α-tubulin and cellular apoptosis induced by tanespimycin. (A) Calu-1 cells were treated with the given concentrations of tanespimycin (0, 0.5 and 1.0 $\mu$M) or in combination with docetaxel (0.5 nM) for 48 h, and the the cells were then subjected to the preparation of whole-cell protein lysates and the given proteins Ac-α-tubulin, α-tubulin, VDAC1, Bax, caspase-9, PARP and β-actin were detected by western blot analysis. β-actin was used as the loading control (Ac-α-tubulin, 52 kDa; α-tubulin, 52 kDa; VDAC1, 32 kDa; Bax, 20 kDa; caspase-3, 17, 19, 35 kDa; PARP, 89, 116 kDa; β-actin, 45 kDa). (B) Calu-1 cells were seeded in 96-well plate and on the 2nd day treated with the given concentrations of tanespimycin (0, 0.25, 0.5, 1.0 and 2.0 $\mu$M) and docetaxel (0.5 nM) for 48 h. Cell number was estimated by SRB assay for calculation of cell survival. Comparison between groups was carried out with an unpaired Student's t-test. The analysis results were the mean of 4 replicate determinations; bars, SD. *P<0.05. VDAC1, voltage-dependent anion channel 1; Ac-α-tubulin, acetylated α-tubulin.

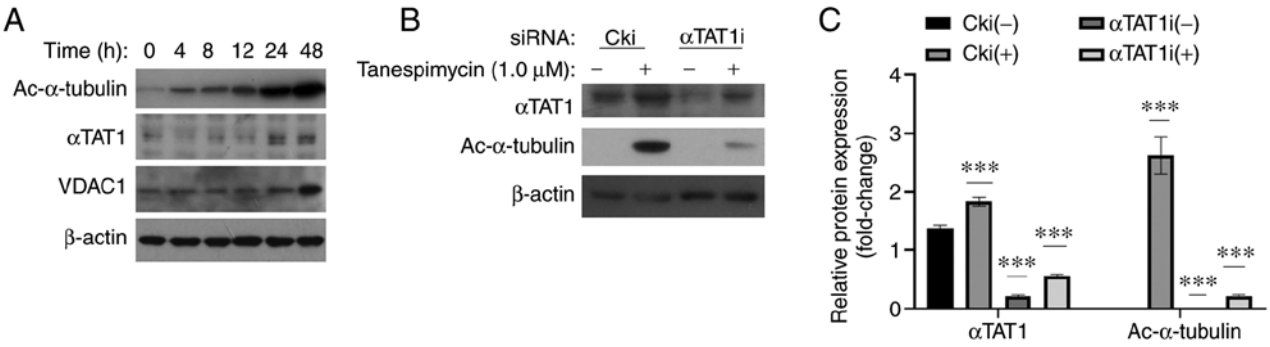


Figure 4. Expression level of Ac-α-tubulin is modulated by the acetyltransferase, αTAT1. (A) Calu-1 cells were treated with 1.0 $\mu$M tanespimycin for the indicated periods of time (0, 4, 8, 12, 24 and 48 h); the cells were then subjected to the preparation of whole-cell protein lysates and the given proteins Ac-α-tubulin, αTAT1, VDAC1 and β-actin were detected by western blot analysis. (B) Calu-1 cells were cultured in 6-well plate and on the 2nd day they were transfected with control siRNA (Cki) or αTAT1 siRNA (αTAT1i). At 24 h following transfection, cells were treated with 1.0 $\mu$M tanespimycin for 48 h. The cells were then harvested for preparation of whole-cell protein lysates for following western blot analysis to detect the expression levels of the given proteins Ac-α-tubulin, αTAT1 and β-actin. β-actin was used as the loading control (αTAT1, 43 kDa; Ac-α-tubulin, 52 kDa; VDAC1, 32 kDa; β-actin, 45 kDa). (C) The above western blot results of αTAT1 and Ac-α-tubulin were analyzed by ImageJ software and GraphPad Prism 8 software and the analysis results are the means of 3 independent experiments. The western blot shown is representative of 3 independent experiments. Comparisons between groups were carried out with Multiple t-test (Holm-Sidak correction was applied to the P-values of multiple comparison) and the comparison of interest is the relative protein in Cki (-) treatment. ***P<0.001 compared with the untreated cells. Error bars in the figures represent the SD values. VDAC1, voltage-dependent anion channel 1; Ac-α-tubulin, acetylated α-tubulin; αTAT1, α-tubulin acetyltransferase 1.

conducted. It was observed that α-tubulin and Ac-α-tubulin were co-precipitated in the IP complexes, as detected by western blot analysis (Fig. 5A). However, in the IP complexes, 2 distinct (136 and 185 kDa) protein bands were clearly detected by an anti-VDAC1 antibody and were found to correspond to a tetramer and hexamer of VDAC1. However, there were no bands that represented VDAC1 monomers (32 kDa). The level of VDAC1 was not apparently increased in either the lysate or the IP complex between the tanespimycin-treated cells and the control cells (not treated with tanespimycin) (Fig. 5A), while the level of α-tubulin was slightly decreased in either the lysate or the IP complex in the treated cells compared to the untreated cells. Based on the results of IP analyzed by GraphPad software (Fig. 5B), following treatment with tanespimycin for 32 h, the level of VDAC1 in the IP complex was not increased. This may have been because 32 h pf tanespimycin treatment was not sufficient to induce VDAC1 upregulation, although it was sufficient to cause a shift from the monomeric form to the

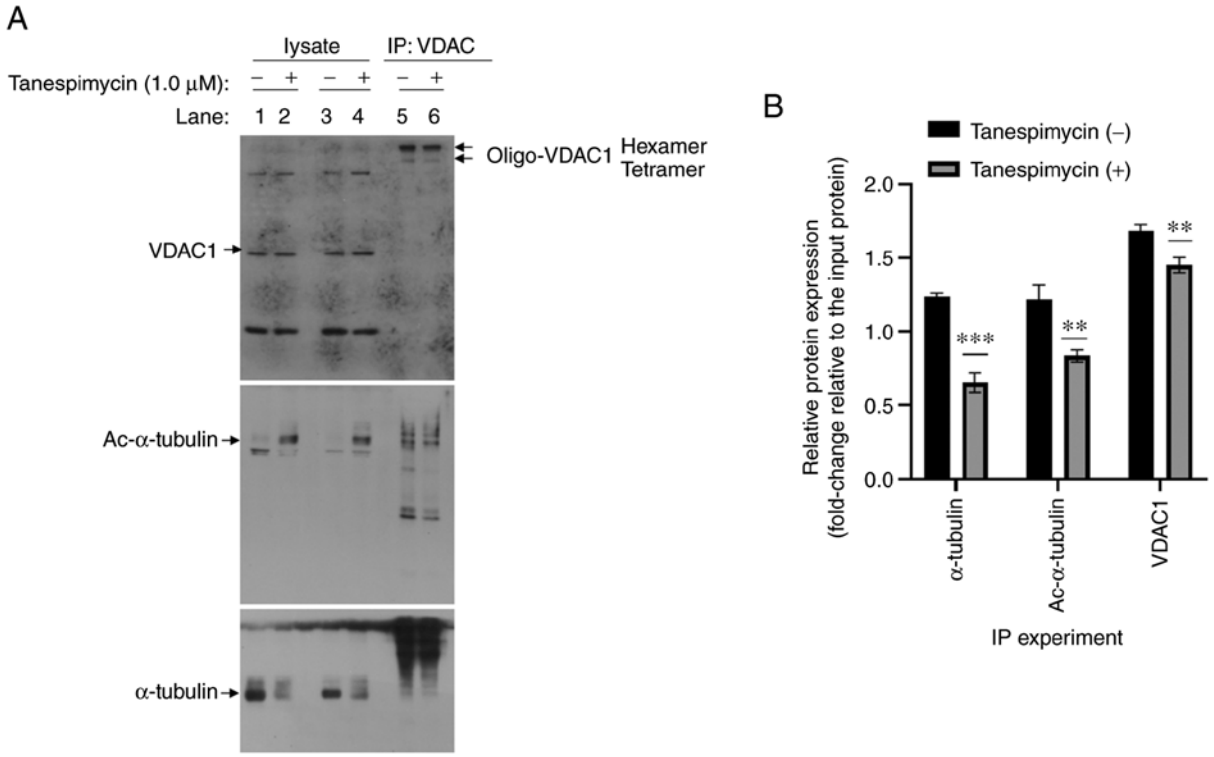


Figure 5. α-tubulin interacts with VDAC1 in lung cancer apoptosis induced by tanespimycin. (A) Calu-1 cells were treated with 1.0 $\mu$M tanespimycin for 32 h and cell lysates were then subjected to immunoprecipitation with anti-VDAC1 antibodies and Protein-A/G Agarose (1:1) and the complexes were incubated at 4˚C overnight. The precipitated proteins and the whole-cell protein lysates were isolated by SDS-PAGE and detected by western blot analysis with anti-α-tubulin, anti-Ac-α-tubulin and anti-VDAC1 antibodies (lanes 1, 2, 3, 4 represent the whole cell lysate samples; lane 5 and 6 represent the IP samples; lanes 2, 4 and 6 represent samples treated with by 1.0 $\mu$M tanespimycin for 32 h; Ac-α-tubulin, 52 kDa; α-tubulin, 52 kDa; VDAC1, 32 kDa). (B) The above western blot analysis results of α-tubulin, Ac-α-tubulin and VDAC1 in the IP experiments were analyzed respectively using ImageJ software and GraphPad Prism 8 software and the analysis results are the means of 3 independent experiments. Comparisons between groups was carried out with an unpaired Student's t-test. $^{**}$P<0.005 and $^{***}$P<0.001 compared with untreated cells. Error bars in the figures represent the SD values. VDAC1, voltage-dependent anion channel 1; Ac-α-tubulin, acetylated α-tubulin.

oligomeric form and to induce VDAC1 permeability and cell apoptosis (Fig. 5A and B).

It has been shown that GSK3β can phosphorylate VDAC and induce VDAC opening. GSK3β and VDAC are two direct substrates of the kinase, AKT. Phosphorylated AKT (p-AKT) can promote GSK3β and VDAC phosphorylation; however, phosphorylated GSK3β does not catalyse VDAC phosphorylation, resulting in mitochondrial permeability blockade (14). To further examine the state of VDAC1 permeability, the present study performed dose-dependent experiments and it was found that the increase in GSK3β (total) levels paralleled the upregulation of acetylated α-tubulin and VDAC1. At the same time, it was observed that the p-AKT levels were decreased in Calu-1 cells (Fig. 6A).

To further elucidate the mechanisms underlying the role of VDAC1 in apoptosis, tanespimycin (0, 0.5 and 1.0 $\mu$M) was used in combination with rapamycin (50 nM) for 48 h, an mTOR inhibitor that has been reported to promote cell survival (30). The results revealed that the combination of tanespimycin and rapamycin markedly reduced the levels of Ac-α-tubulin and Bax, and partially inhibited caspase-3 activation (Fig. 6B). In the combination experiment, VDAC1 expression was not reduced, which suggests that VDAC expression is not affected by rapamycin treatment and that rapamycin has a greater influence on α-tubulin acetylation and cell death. That is, acetylation of α-tubulin is related to the mTOR signalling pathway. According to the above-mentioned results, an increase in Ac-α-tubulin expression induced by tanespimycin weakens the interaction between VDAC1 and tubulin, and results in an increase in MOM permeability and cellular apoptosis through the intrinsic apoptotic pathway.

## Discussion

The present study demonstrated that tanespimycin induced α-tubulin acetylation, VDAC1 upregulation and cell death, during which the conformational structure of VDAC1 may shift from the monomeric form to the oligomeric form. The quantity of acetylation of α-tubulin may decrease the level of free α-tubulin that interacts with VDAC1 and induce VDAC1 permeability to release proapoptotic materials from the mitochondria. It is well known that the mitochondria are cell organelles in which ATP and intermediates are produced for energy, and mitochondria play a crucial role in deciding cell fate for survival or apoptosis (1,31). The permeability of VDAC1, as a major protein in the mitochondrial outer membrane, is modulated by a variety of factors, and the switch of the VDAC1 channel is also influenced by its conformational structure (32-34). VDAC1 has been reported to be presented as a monomer or oligomer during apoptosis induction (6). This finding is consistent with the results of the present study. The overexpression of VDAC1 induced by tanespimycin may alter

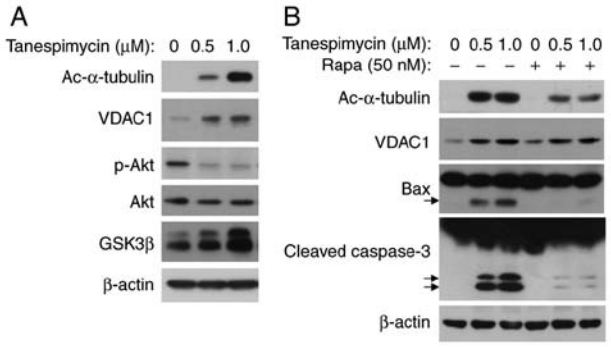


Figure 6. p-Akt/GSK3β signaling pathway modulates the phosphorylation of VDAC1. (A) Calu-1 cells were treated with the given concentrations of tanespimycin (0, 0.5 and 1.0 $\mu$M) for 48 h, and the cells were then harvested for the preparation of whole-cell protein lysates for following western blot analysis to detect the expression levels of the given proteins, Ac-α-tubulin, VDAC1, p-Akt, Akt, GSK3β and β-actin. (B) Calu-1 cell lines were treated with the given concentrations of tanespimycin (0, 0.5 and 1.0 $\mu$M) or in combination with rapamycin (50 nM) for 48 h and the cells were then subjected to the preparation of whole-cell protein lysates, and the given proteins Ac-α-tubulin, VDAC, Bax, caspase 3 and β-actin were detected by western blot analysis. β-actin was used as the loading control (Ac-α-tubulin, 52 kDa; VDAC1, 32 kDa; p-Akt, 60 kDa; Akt, 60 kDa; GSK3β, 46 kDa; Bax, 20 kDa; caspase-9, 35, 37, 47 kDa; PARP, 89, 116 kDa; β-actin, 45 kDa). VDAC1, voltage-dependent anion channel 1; Ac-α-tubulin, acetylated α-tubulin; GSK3β, glycogen synthase kinase 3β.

the balance between its monomeric and oligomeric forms, and is conducive to the formation of oligomers, as previously reported (35).

VDAC1 can also form hetero-oligomers with Bax or Bak, which are two essential mediators of apoptosis (36,37). In the present study, it was also found that Bax expression was elevated along with cellular apoptosis. However, it is not known whether VDAC1 forms hetero-oligomers with Bax, and this warrants further investigation. Studies have confirmed that VDAC1 overexpression triggers the oligomerization of VDAC1 with itself or with other proteins, resulting in cellular apoptosis (38). When encountering apoptosis signals, Bax translocates to the mitochondria to increase the VDAC1 pore size, which leads to the permeabilization of the MOM (39,40). In the present study, tanespimycin not only inhibited Hsp90 biological activity, but also induced an increase in VDAC1 expression. Indeed, it was found that the upregulation of VDAC1 and Bax was associated with an increase in tanespimycin concentration. VDAC1, Bax and Bak are all localized in the mitochondrial outer membrane. Another study demonstrated that VDAC1 knockdown inhibited Bax activation, but not Bak activation in CDDP-induced cellular apoptosis (12). The present study also revealed that Bax (not Bak) was activated following elevation of VDAC1 expression (Fig. 3A). Therefore, it was hypothesized that VDAC1 and Bax may form homo-oligomers or hetero-oligomers to generate channels that are large enough to allow CytC release from the mitochondria, inducing cellular apoptosis.

The acetylation of α-tubulin during apoptosis is controversial at present as it is unclear whether α-tubulin acetylation is the cause or an effect of apoptosis. In the present study, the combination of tanespimycin and docetaxel further promoted α-tubulin acetylation accompanied by VDAC1 and Bax elevation, implying that apoptosis induced by tanespimycin is associated with both microtubules and mitochondria. Docetaxel is an anti-tumour drug that inhibits microtubule depolymerization by binding to tubulin, which leads to an apparent decrease in free tubulin and induces cancer cell apoptosis. To further evaluate the association between apoptosis and increases in α-tubulin acetylation and VDAC1, tanespimycin was used in combination with rapamycin. The results revealed that apoptosis was alleviated, as indicated by a marked reduction in cleaved-caspase-3 expression, and that α-tubulin acetylation was simultaneously decreased, although that the change in VDAC1 expression was minimal. These results demonstrate that there is a positive association between cellular apoptosis and α-tubulin acetylation induced by tanespimycin.

Protein acetylation is a reversible post-translational modification that is modulated by acetyltransferase and deacetylase. The present study demonstrated that the acetylation levels of α-tubulin are consistent with the expression of the acetyltransferase, αTAT1. The depletion of the tubulin acetyltransferase, αTAT1, has been reported to lead to an apparent increase in the frequency of microtubule disruption, which indicates that acetylation is necessary to maintain the persistence of long-lived microtubules (41,42). Surprisingly, in the present study, Hsp90 inhibition by tanespimycin induced the acetylation of α-tubulin acetylation (43), and also simultaneously caused cellular apoptosis and the enhanced expression of VDAC1 and Bax. However, the present study wished to determine the origin of this Ac-α-tubulin, namely to determine whether it is the result of acetylated microtubule breakage or the acetylation of free α-tubulin. It also needs to be determined whether the level of free α-tubulin that interacts with VDAC1 was influenced. Based on the results of the present study, it was concluded that Hsp90 may mediate the expression level of VDAC1 and that VDAC1 plays its normal physiological roles in the MOM under normal conditions. However, when Hsp90 activity is disrupted by tanespimycin or when Hsp90 levels are reduced, higher VDAC1 expression is induced, which may result in VDAC permeability through the formation of homo-oligomers or hetero-oligomers by VDAC and Bax. Based on the tubulin-VDAC interaction model and the current IP experiments, it was elucidated that abundant acetylation of α-tubulin may reduce the level of free α-tubulin or α/β tubulin dimers in the MOM and subsequently affect VDAC permeability. The level of free α-tubulin or α/β tubulin dimers (although the level of β tubulin was not determined) in the MOM affected VDAC1 permeability, as evidenced by a decrease in p-AKT levels, which indicates that this interaction may be mediated by the AKT/GSK3β signalling pathway.

Hexose kinase (HK) is an important protein that interacts with VDAC1, and HK-I and HK-II, which are overexpressed in many types of cancers, control the rates of tumour growth and migration. We suspect that tanespimycin can compete with HK to bind to VDAC1. This may be an interesting area of future study for identifying the association between mitochondria-mediated apoptosis and the Warburg effect.

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## Availability of data and materials

All data generated or analysed during this study are included in this published article.

## Authors' contributions

QW and XL designed the study. QW conducted the experiments, analysed the data and drafted the manuscript. XL was involved in the interpretation of the data. Both authors contributed to, and read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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