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**Original Research Article**

# Atorvastatin suppressed proliferation and facilitated apoptosis of A549 cells through mediating recruitment of Fas and CD59 in lipid raft

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## Abstract

***Purpose:*** *Lipid raft facilitated progression of NSCLC and atorvastatin could break cholesterol. The purpose of this study was to determine the potential mechanism of atorvastatin through lipid raft mediation in NSCLC.*
***Methods:*** *A549 cells were first grouped as NC, methyl-β-cyclodextrin (MβCD, lipid raft inhibitor, 5mM and 10mM), atorvastatin (0, 5mM, 10mM and 15mM) and MβCD (10mM) with atorvastatin (15mM). Later 10mM MβCD treated A549 cells were divided into three groups: NC, 3BDO (mTOR agonist) (60μM) and 3BDO (60μM) with atorvastatin (15mM) group. Thereafter, FLOT-2, SLP-2 expressions were assessed with RT-qPCR, mTOR proteins were measured by western blot and cell viability by CCK-8 method. Meanwhile, apoptosis was analyzed by flow cytometry. Moreover, lipid raft isolation was performed for acquiring Fas and CD59 and concentrations were detected by ELISA.*
***Results:*** *MβCD treatment significantly inhibited FLOT-2 and SLP-2 RNA expressions and cell viability of A549 cells but up-regulated apoptosis. Besides that, Fas protein level was promoted and CD59 was suppressed. Atorvastatin also repressed FLOT-2 and SLP-2 RNA levels. Meanwhile, atorvastatin downregulated cell viability and accelerated apoptosis. Moreover, Fas was increased and CD59 was inhibited by atorvastatin and MβCD enhanced functions of atorvastatin. MβCD inhibited mTOR RNA expression in A549 cells was increased by 3BDO but atorvastatin restored 3BDO caused up-regulation of mTOR. Furthermore, up regulated cell viability of A549 by 3BDO was declined with atorvastatin and decreased apoptosis by 3BDO was reversed through atorvastatin. Fas suppressed by 3BDO and promotion of CD59 were also resumed by atorvastatin.*
***Conclusion:*** *Atorvastatin suppressed FLOT-2 and SLP-2 and mediated recruitments of Fas and CD59 with suppressing cell viability and facilitating apoptosis of A549 cells via mTOR signaling pathway.*

***Keywords:*** *Atorvastatin, lipid raft, Fas, CD59, NSCLC*



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## INTRODUCTION

Lung cancer is one of the most malignant cancers worldwide with the 5-year overall survival rate low to 16.6% [1,2]. Lung cancer contains small cell lung cancer (SCLC) and non-

small cell lung cancer (NSCLC). The former on takes about 15% of all kinds of lung cancer and NSCLC occupies approximately 85% [3]. Although diagnosis of lung cancer has made huge progress, it is still unfortunate that most patients are diagnosed at advanced stage[4]. Based on analysis of NSCLC, its poor prognosis has correlation with lacking diagnostic biomarkers in early stage. Therefore, it is urgently needed to Figure out potential mechanism of occurrence and progression of NSCLC.

Lipid raft is a dynamically formed by lipid and protein with many kinds of regulatory molecules and receptors, which is regarded as a platform for signaling pathways [5]. Lipid raft was also found to play important role in cell activation, polarization and signal transduction [6]. Moreover, cancers can be occurred and processed according to those signaling pathways [7,8]. Previous studies have proven that lipid raft plays important role in many kinds of cancers. Merkel cell polyomavirus (MCPyV) entered cells through endocytosis of lipid raft, resulting in Merkel cell carcinoma (MCC), a skin cancer [9]. Besides that, lipid raft was gathered with migrated gastric cancer cells by RANKL [10]. Under ultraviolet light, cholesterol in lipid raft was increased and Fas receptor was congregated, leading to apoptosis of tumor cells in skin cancer [11]. Flotillin (Flot) is a protein family in lipid raft that can mediate cell survival, proliferation and apoptosis [12,13]. FLOT2 is one of the homologous types of Flot family, which was proved as a target of miR-133 in adenocarcinoma of lung [14]. Stomatin-like Protein 2 (SLP-2) was discovered to be highly expressed and accelerated proliferation in NCSLC through mediating surviving expression via β-catenin signaling pathway [15]. In addition to that, SLP-2 suppression blocked response of macrophage to TLR ligands through reducing total protein mass in rafts [16]. Hence, we selected these two biomarkers for measuring functions of lipid raft in NSCLC. Moreover, Lipid raft can also gather Fas to transmit apoptosis [17]. Besides that, CD59 can promote cancer cell progression in binding proteins in lipid raft[18]. Based on those detections, Fas and CD59 was also chosen to measure mechanism of lipid raft in progression of NSCLC.

## EXPERIMENTAL

### Cell culture and treatment

Human non-small cell lung cancer cell line, A549 was acquired from American Type Culture Collection (ATCC, USA). Thereafter, A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% FBS (Gibco, USA) at 37℃, 5%$CO_2$. Then, A549 cells were grouped as NC group, MβCD (lipid raft inhibitor, 5mM and 10mM) group[19], atorvastatin (0, 5mM, 10mM and 15mM) group, MβCD (10 mM)+atorvastatin (15mM) group, 3BDO (60μM)+ MβCD (10mM) group and 3BDO (60μM)+atorvastatin (15mM)+ MβCD (10mM) group. Cells in log phase were used for following experiments.

### RT-qPCR

In accordance with manufacturer's instruction of TRIzol Reagent (Invitrogen, USA), total RNA was separated and extracted from cells. Later, cDNA was compounded through reverse transcription of RNA by using High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (USA). Thereafter, 7500 Fast Food Safety Real-Time PCR System (Applied Biosystems, USA) was applied for amplification. Sequences of primers in this study were listed as follows: FLOT2: forward, 5'-CCCCAGATTGCTGCCAAA-3' and reverse, 5'—TCCACTGAGGACCACAATCTCA-3'[20];
SLP-2: forward, 5'-CTGGAGCCTGGTTTGAA CAT-3' and reverse, 5'-AGGATCTGGGCCTG TTTCTT-3' [21], mTOR: forward, and GAPDH: forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3' [20]. GAPDH was considered as internal reference and expressions of FLOT and SLP-2 were calculated by $2^{-\triangle\triangle Ct}$ method [22]. As for PCR condition, predenaturation was first performed at 95℃ for 15min. Following, denaturation was at 95℃ for 45s and annealing was at 60℃ for 30s. Finally, extension was applied for 1min at 72℃. The program contained a total of 30 cycles.

### CCK-8

A549 cells treated with NC, MβCD (5mM and 10mM); NC, atorvastatin (0, 5mM, 10mM and 15mM); NC, atorvastatin (15mM), MβCD (10mM) + atorvastatin (15mM); NC, 3BDO (60μM) and 3BDO (60 μM)+atorvastatin (15mM) were seeded into 95-well plate with $5\times10^3$ cells per well and incubated at 37℃, 5% $CO_2$. For cell proliferation detection, 10μl CCK-8 (Beyotime, Shanghai, China) was added into wells at 24h, 48h and 72h. After CCK-8 was mixed, cells were incubated for another 3h. Finally, Varioskan™ LUX Microplate Reader (Thermo Scientific, USA) was used to measure optical density (OD) values at 450nm wavelength. In cytotoxicity test, 10μl different concentrations of atorvastatin (0, 5mM, 10mM and 15mM) was added into A549 cells in 96-well plate and cultured for 72h. Later, 10μl

CCK-8 was added and incubated for 2h. OD values of cells were measured at 450 nm wavelength.

**Western blot method**

Cells were collected and protein was extracted using RIPA. The BCA kit was applied to determine the protein concentrations (Bioss, Beijing, China). Furthermore, proteins were separated by SDS-PAGE method and then transferred to PVDF membranes (Millipore, Shanghai, China). The primary antibody against mTOR(bs-1992R, 1:500, Bioss) was added to incubate the membranes at 4ºC overnight, with β-actin(bs-0061R, 1:500, Bioss) as the internal reference. On the second day, the membranes were incubated in secondary antibody after washing using TBST for thrice. ECL kit (Chuanshi Biotech, Tianjin, China) was added onto the membranes. The blotting bands were imaged and the protein expression was analyzed using ImagePro (MD, USA).

**Flow cytometry**

Flow cytometry was applied to measure apoptosis rate of A549 cells, NC, MβCD (5mM and 10mM); NC, atorvastatin (15 mM), MβCD (10mM)+atorvastatin (15mM); NC, 3BDO (60μM), 3BDO (60μM)+atorvastatin (15mM) were used to treat A549 cells. After treatment, cells were rinsed by PBS. Then, Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) was used for apoptosis detection. A549 cells were resuspended by Annexin V-FITC binding buffer and following, 5μl Annexin V-FITC (20μg/ml) and 10μl PI (50μg/ml) were added and incubated at room temperature for 15min without light. Finally, Agilent NovoCyte Advanteon Flow Cytometer (Agilent, USA) was performed to measure apoptosis rate of A549 cells.

**ELISA**

Human Fas/TNFRSF6/CD95 Quantikine ELISA Kit (R&D Systems, Minnesota, USA) and Human CD59 ELISA Kit (ab263893) (Abcam, UK) were performed to measure protein levels of Fas and CD59. Wash buffer RT (ab206977) was used to coat capture antibodies of Fas and CD59 in SimpleStep 96-well plate (ab206978). Thereafter, 50μl of sample was mixed with 50μl capture and detector antibodies of Fas and CD59 and cultured for 1h at room temperature. Next, mixture was washed with wash buffer (ab206977) and 100μl stop solution was added to stop experiment. Protein levels of CD59 and Fas were detected at 450nm wavelength with Varioskan™ LUX Microplate Reader (Thermo Scientific, USA).

**Lipid raft isolation [23]**

Isolation of lipid raft was performed on ice. A549 cells were seeded onto 100mm plate and cultured for 72h in DMEM medium. Thereafter, cells were gathered using ealkaline buffer (20mM Tris-HCl, pH7.8, 200mM sucrose, 1mM $Mgcl_2$, 1mM $CaCL_2$ and 100μM sodium orthovanadate)[24]. Later, cells were lysed with 22 g×3'' needle and centrifuged for 10min at 1000g to gather liquid supernatant. Next, cells were lysed again with adding 1ml base buffer containing Leupeptin Protease Inhibitor (Thermo Scientific, USA) and centrifuged at 1000g for 10min. Gathered liquid supernatant was merged with former one. After that, isometric 50% OptiPrep (Sigma-Aldrich, Germany) was added into merged liquid supernatant and 0-20% gradient of OptiPrep were mixed. Then, mixed liquor was centrifuged at 52000xg for 90min and fractionated. Then, ELISA was used to measure proteins.

**Statistical analysis**

All data were shown as mean±SD and analyzed by Graph Pad Prism 7.0 (USA) and SPSS19.0 (USA). Experiments were all run in a triplicate. Student's t-test and one way ANOVA were used to measure comparisons of groups. $P<0.05$ was considered to have meanings.

## RESULTS

**MβCD treatment suppressed FLOT-2 and SLP-2 expressions and mediated cell viability, apoptosis and expressions of Fas and CD59**

In order to analyze function of lipid raft in progression of NSCLC, MβCD was performed to suppress lipid raft. After different concentrations of MβCD (5mM and 10mM) were added, both FLOT-2 and SLP-2 RNA expressions were significantly downregulated in a dose dependent manner (Figure 1A). Meanwhile, viability of A549 cells was detected revealing that cell viability was markedly reduced as MβCD concentration increased (Figure 1B). Apoptosis of A549 cells was analyzed later, indicating that MβCD noticeably promoted apoptosis of A549 cells (Figure 1C). Then, Fas and CD59 protein levels were evaluated, revealing that Fas protein expression was remarkably increased with up-regulated concentrations of MβCD while CD59 was significantly declined (Figure 1D).

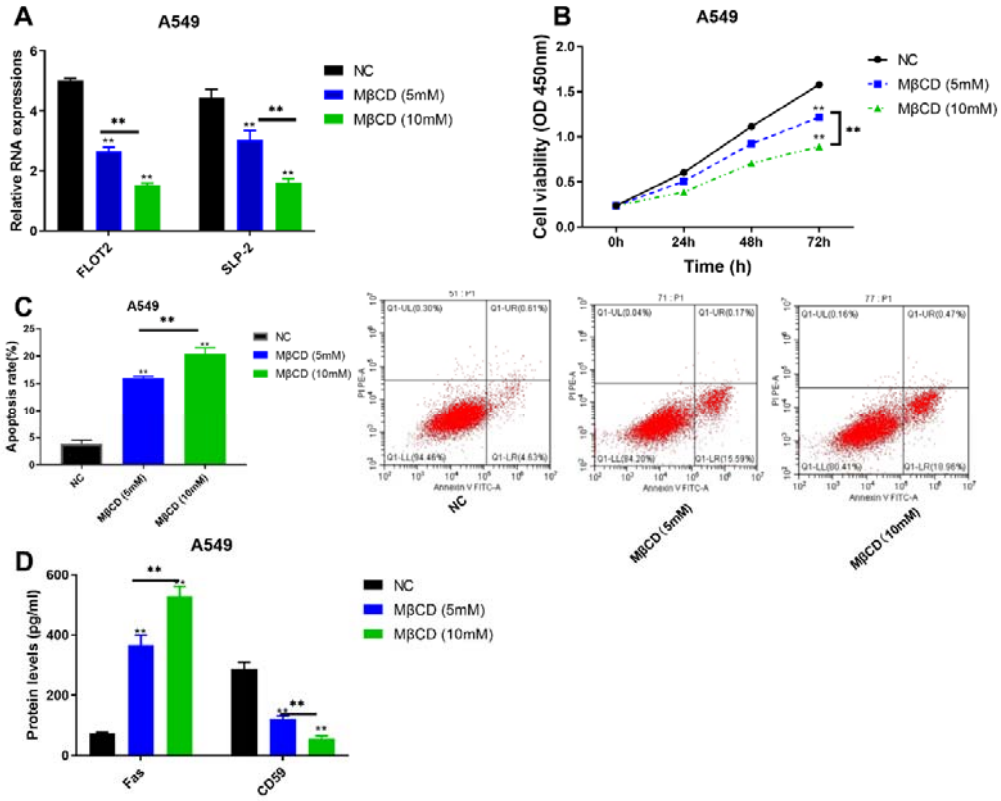


**Figure 1** MβCD treatment suppressed FLOT-2 and SLP-2 expressions and mediated cell viability, apoptosis and recruitments of Fas and CD59 A: FLOT-2 and SLP-2 RNA expressions were assessed by RT-qPCR in A549 cells treated with MβCD (5mM and 10mM), P<0.05. B: Cell viability of A549 cells with different concentrations of MβCD (5mM and 10mM) were measured by CCK-8, P<0.05. C: Flow cytometry was applied to analyze apoptosis rate of A549 cells after treated by MβCD (5mM and 10mM), P<0.05. D: Protein levels of Fas and CD59 after MβCD treatment (5mM and 10mM) in lipid raft of A549 cells were measured by ELISA, P<0.05. Experiments were all repeated three times

Figure 2A). Then, FLOT-2 and SLP-2 RNA expressions with atorvastatin were detected, revealing that atorvastatin significantly reduced RNA levels of FLOT-2 and SLP-2. Moreover, combination of MβCD and atorvastatin resulted in the lowest levels of FLOT-2 and SLP-2 (Figure 2B). Cell viability was then analyzed, indicating that atorvastatin caused much lower viability of A549 cells and compounds of MβCD and atorvastatin obviously reduced cell viability of A549 compared to atorvastatin only (Figure 2C). Meanwhile, detection of apoptosis indicated that atorvastatin resulted in markedly higher apoptosis rate of A549 cells and added MβCD sharply up-regulated apoptosis of A549 cells on the basis of atorvastatin treatment (Figure 2D). Furthermore, Fas and CD59 protein levels were analyzed, showing that atorvastatin treatment significantly up-regulated Fas protein expression and declined CD59 protein levels. Moreover, combination of MβCD and atorvastatin remarkably magnified up-regulation of Fas and inhibition of CD59 in A549 cells (Figure 2E).

## Atorvastatin repressed FLOT-2 and SLP-2 in lipid raft and mediated viability and apoptosis of A549 cells via mTOR signaling pathway

After correlation between MβCD and atorvastatin was Figure out, deep mechanism of atorvastatin mediating lipid raft in A549 cells was measured. In normal A549 cells and MβCD treated A549 cells, mTOR protein was significantly downregulated in A549 cells after MβCD treatment compared to normal A549 cells (Figure 3A). Therefore, A549 cell with MβCD (10mM) treatment was chosen or getting connections among MβCD, mTOR and atorvastatin. As 3BDO (mTOR agonist) added, mTOR was remarkably promoted while atorvastatin treatment restored up-regulation of mTOR caused by 3BDO (Figure 3B). Besides that, cell viability was analyzed, indicating that 3BDO treatment obviously up-regulated viability of A549 cells but added atorvastatin resumed up-regulation of 3BDO resulting in low cell viability (Figure 3C). Meanwhile, 3BDO suppressed apoptosis rate was also reversed to be up-regulated with

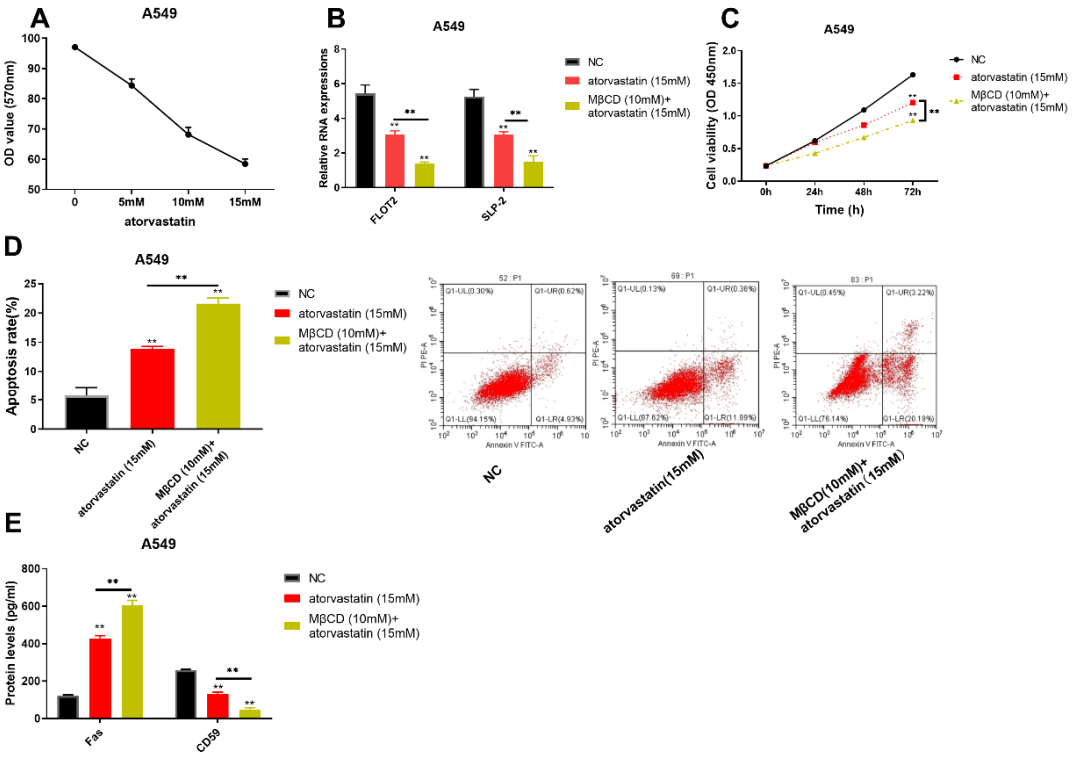


**Figure 2**: Combination of atorvastatin and MβCD downregulated FLOT-2 and SLP-2 expressions and mediated viability, apoptosis and aggregation of Fas and CD59 A: Cell toxicity of A549 with atorvastatin (0, 5mM, 10mM and 15mM) were evaluated by CCK-8. B: FLOT2 and SLP-2 RNA levels in A549 cells with atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM) were examined by RT-qPCR, P<0.05. C: CCK-8 was applied to measure cell viability of A549 cells after treated by atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM), P<0.05. D: Apoptosis rate of A549 cells were detected through flow cytometry in A549 cells with atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM) treatment, P<0.05. E: Fas and CD59 protein levels were evaluated after atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM) treatment in A549 cells through ELISA, P<0.05. Each experiment was run in a triplicate

atorvastatin treatment in A549 cells (Figure 3D). Fas and CD59 protein expression were analyzed as well, revealing that Fas protein level was elevated after 3BDO treatment and decreased with atorvastatin usage while CD59 was inhibited by 3BDO and then rescued by atorvastatin (Figure 3E).

## DISCUSSION

Statins are the most common drugs for preventing cardiovascular diseases and reducing mortality and morbidity through downregulating plasma cholesterol levels[25]. According to previous studies, statins could also anti-tumors via inducing apoptosis and inhibiting cancer cell growth and angiogenesis[26, 27].Atorvastatin, an antilipemic agent, was found to suppress progression of NSCLC through promoting expressions of cleaved caspase-3 and PARP and repressing autophagy[28]. Besides that, atorvastatin was discovered to be a lipid raft inhibitor by breaking cholesterol[29]. Moreover, atorvastatin attenuated TLR4 recruitment into lipid raft to affect anti-inflammatory response[30]. Though atorvastatin has been detected to be an inhibitor in NSCLC, its correlation with lipid raft in NSCLC was never mentioned. Therefore, in this study, we have analyzed correlation between atorvastatin and lipid raft and mechanisms. At first, we have proven that lipid raft facilitated FLOT-2 and SLP-2 expression in NSCLC while suppression of lipid raft through MβCD retarded FLOT-2 and SLP-2 RNA expressions, viability of A549 cells and promoted apoptosis with up-regulating aggregation of Fas and reducing CD59 in lipid raft. Based on these detections, we primarily clarified that lipid raft accelerated progression of NSCLC through up-regulating recruitments of FLOT-2, SLP-2 and CD59 and MβCD blocked functions of lipid raft.

Moreover, functions of atorvastatin were examined later. With concentrations of atorvastatin up-regulating, cell toxicity was increased. Meanwhile, atorvastatin treatment

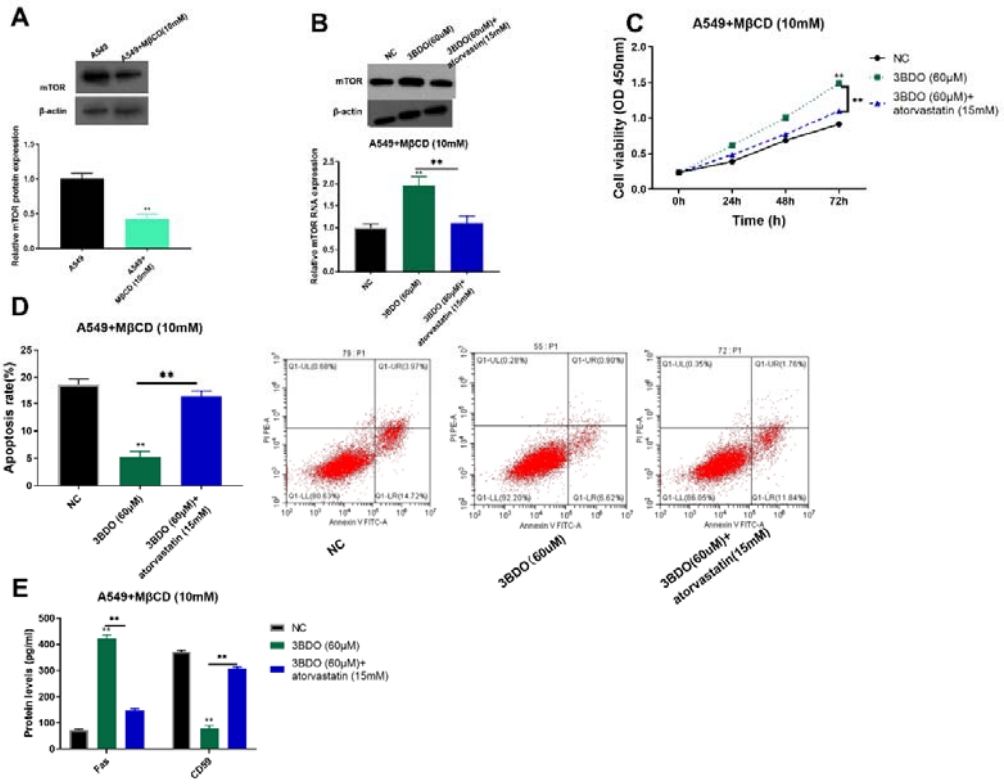


**Figure 3:** Atorvastatin repressed FLOT-2 and SLP-2 in lipid raft and mediated viability and apoptosis of A549 cells via mTOR signaling pathway A: Protein expression of mTOR in A549 cells and MβCD (10mM) treated A549 cells were assessed by western blot, P<0.05. B: Protein levels of mTOR in MβCD (10mM) treated A549 cells were measured by western blot after 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM) treatment, P<0.05. C: CCK-8 was performed to measure cell viability of MβCD (10mM) treated A549 cells under 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM) treatment, P<0.05. D: Apoptosis rate of MβCD (10mM) treated A549 cells was detected by flow cytometry after treated by 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM), P<0.05. E: Protein levels of Fas and CD59 were measured by ELISA in MβCD (10mM) treated A549 cells after 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM) treatment, P<0.05. All experiments were repeated three times

significantly decreased recruitments of FLOT-2 and SLP-2 in lipid raft and combination of MβCD with atorvastatin caused lower levels of FLOT-2 and SLP-2 than atorvastatin only. As for cell viability detection, added atorvastatin obviously declined viability of A549 cells and mixed MβCDenhanced suppression of atorvastatin while apoptosis was significantly up-regulated. Recruitments of Fas and CD59 were checked later, revealing that atorvastatin treatment markedly increased protein level of Fas and repressed CD59 and added MβCD magnified regulation of atorvastatin in promoting Fas and reducing CD59. After functions of atorvastatin were measured, deeper mechanism of atorvastatin in mediating lipid raft was examined. Atorvastatin was proved to retard inflammation in RAW264.7 with suppressing phosphorylation of mTOR[31, 32]. Lipid raft was discovered to mediate activation of mTOR signaling pathway in cancers[33]. Based on these studies, we measured role of mTOR in progression of NSCLC with lipid raft and atorvastatin. With MβCD treatment, mTOR protein expression was obviously reduced. After 3BDO treatment, mTOR was promoted while atorvastatin treatment reversed function of 3BDO and downregulated mTOR. Moreover, increased cell viability of 3BDO was also restrained with atorvastatin but apoptosis suppressed by 3BDO was promoted after atorvastatin added. Furthermore, Fas was promoted by 3BDO in lipid raft and then reversed by atorvastatin while CD59 inhibited by 3BDO was recovered by atorvastatin. Results reminded that atorvastatin retard functions of lipid raft in promoting progression of NSCLC through blocking mTOR signaling pathway.

## CONCLUSION

Atorvastatin inhibited FLOT-2 and SLP-2 RNA expressions and cell viability but promoted apoptosis of NSCLC cells and mediated recruitments of CD59 and Fas in lipid raft via mTOR signaling pathway, suggesting

atorvastatin could be used for blocking functions of lipid raft in NSCLC. Nevertheless, further detections in vivo and in clinical stage are needed for getting more knowledge.

## DECLARATIONS

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Jiahuan Ludesigned the work and carried out the literature search, data analysis, manuscript preparation and review. Huiping Qiang conceptualized the work, and was involved in the design, data acquisition and manuscript editing. Tianqing Chu was also involved in literature search and definition of intellectual content.

### *Open Access*



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