

## Original Article

# Soluble epoxide hydrolase inhibitor, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, represses human aortic smooth muscle cell proliferation and migration by regulating cell death pathways via the mTOR signaling

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**Abstract:** The soluble epoxide hydrolase (sEH) is a molecule necessary for the metabolism of endogenous constituents implicated in blood pressure regulation and vascular inflammation. Scientific evidences indicate that sEH inhibitors such as 12-(3-Adamantan-1-yl-ureido)-dodecanoic acid (AUDA) could be a possible therapeutic option for cardiovascular diseases such as restenosis and atherosclerosis. However, the nature of the biological effects of AUDA still remains unclear. Herein, we intended to scrutinize the influence of AUDA on proliferation and migration of TNF- $\alpha$ -induced human aortic smooth muscle cells (HASMCs) and the underlying molecular mechanism. Pretreatment with AUDA (0.5-8  $\mu$ M) dose-dependently inhibited TNF- $\alpha$ -induced proliferation of HASMCs as revealed by the MTT assay and the decreased expression of Cyclin D1 and  $\beta$ -tubulin. Transwell analyses showed that AUDA equally suppressed TNF- $\alpha$ -induced migration of HASMCs. Moreover, AUDA induced the expression of apoptotic proteins (Caspase 3, PARP) and inhibited the expression of autophagy related markers (LC3-II and Beclin 1). More interestingly, AUDA inhibited TNF- $\alpha$ -induced phosphorylation of mTOR, the silencing of which abolished the inhibitory effects of AUDA on TNF- $\alpha$ -induced HASMCs. The present results point toward an inhibitory effect of AUDA on the proliferation and migration of TNF- $\alpha$ -induced HASMCs by regulation of cell death related signaling pathways via downregulation of the mTOR signaling. Thus, AUDA may be an important regulator of inflammation in the atherosclerotic lesion and a novel therapeutic drug for the treatment of atherosclerosis, restenosis and other cardiovascular diseases.

**Keywords:** Soluble epoxide hydrolase, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, vascular smooth muscle cell, proliferation, migration

## Introduction

Cardiovascular diseases, including atherosclerosis and stenosis, are prevalent and constitute the primary cause of death worldwide [1, 2]. Despite the improvement of the therapeutic landscape for these disorders, their pathogenesis is still poorly understood and requires in depth-investigations.

A series of investigations have indicated that the proliferation and migration of smooth muscle cells (SMCs) are critical events that are partly involved in the pathogenesis of atherosclerosis [3-5], restenosis after coronary angioplasty [5, 6] and late vein graft failure [7, 8]. Therefore scientists are attempting to elucidate the mo-

lecular mechanisms of SMC involvement in the pathogenesis of vascular diseases in order to develop gene or pharmacological-based targeted therapies.

In the vascular endothelium and vascular smooth muscle, the normal content in molecules such as epoxyeicosatrienoic acids (EETs) is the plinth that maintains vascular tone and homeostasis [9-12]. Especially, established scientific data suggest that that EETs can exhibit anti-inflammatory effects and control angiogenesis and/or blood vessel formation [9, 13] by mechanisms that are still not fully elucidated. Studies have also indicated that the biological actions of EETs can be interrupted by their conversion to dihydroxyeicosatrienoic acids (DHETs) [14,

15], which conversion is the consequence of soluble epoxide hydrolase (sEH) activity [16]. Numerous studies have evoked that inhibition of sEH, with an ensuing decrease in EET degradation, may attenuate the development of cardiovascular diseases [17]. Up till now, sEH inhibitors have proven to reduce blood pressure in hypertension patients, lessen hypertension-induced renal damage, decrease vascular smooth muscle cell proliferation, and attenuate tissue injury associated with lipopolysaccharide-induced systemic inflammation [17, 18]. Among sEH inhibitors, 2-(3-Adamantan-1-yl-ureido)-dodecanoic acid (AUDA) is an N'-carboxylic acid substitution that increases water solubility without an appreciable reduction in the potency of sEH inhibition. Although several research works reported that AUDA exerts some vasculo- and cardio-protective effects [19, 20], very little is known about the role of AUDA in the proliferation and migration of human aortic smooth muscle cells (HASMCs) and the underlying signaling pathways.

Therefore, this study was designed to survey the effects of AUDA HASMC proliferation and migration. We also aimed to investigate the regulatory mechanism associated with these effects. Our findings demonstrated that AUDA inhibited TNF-induced proliferation and apoptosis. Moreover, we showed that silencing of mTOR abolished the inhibitory effects of AUDA.

### Material and methods

#### Materials

12-[[[tricyclo[3.3.1.1.3,7]dec-1-ylamino]carbonyl]amino]-dodecanoic acid (AUDA, SML0177 SIGMA) with a HPLC purity of more than 98% was purchased from Sigma-Aldrich (St. Louis, MO). AUDA was dissolved in saline containing 0.1% alcohol which served as vehicle. Recombinant Human TNF-alpha Protein (cat# 210-TA) was purchased from R&D Systems. Antibodies for cyclin D1,  $\beta$ -tubulin, GAPDH, mTOR, p-mTOR, LC3-II, Beclin 1, PARP, Caspase 3 and cleaved-caspase 3 were provided by Abcam (Cambridge, UK). The RIPA Lysis and Extraction Buffer and the Pierce BCA Protein Assay were purchased from Thermo Fisher Scientific Company. The mTOR siRNA was provided by Cell Signaling (Danvers, MA). Soluble Epoxide Hydrolase Cell-Based Assay Kit (Item No 600090) was bought from the Cayman Chemical Company. Human aortic smooth muscle cells (HASMC)

were bought from Clonetics Corporation (San Diego, CA).

#### Cell culture

HASMCs were seeded in Smooth Muscle Growth Medium-2 (SmGM-2) supplemented with 5% FBS, growth factors and 50  $\mu$ g/mL gentamicin at a density of 3,500 cells/cm<sup>2</sup> at 37°C in a 5% CO<sub>2</sub> and humidified incubator. The cells were subcultured when they were 70-80% confluence following the "Clonetics™ human smooth muscle cell systems" user instructions.

#### siRNA transfection

HASMCs were transfected with 10 nM of mTOR siRNA or siRNA control using the procedure provided with Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY). After 48 hours of transfection, 10% FBS was added to the cells, further incubated for 48 hours and stored for subsequent analysis.

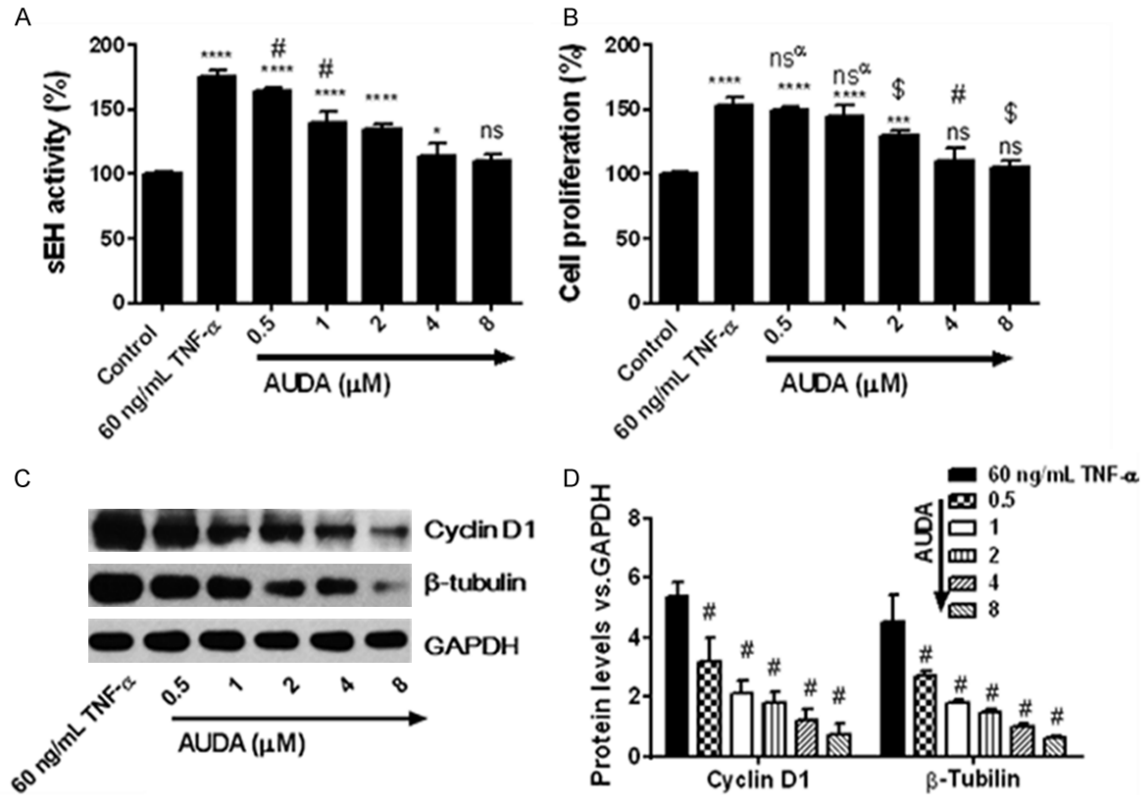
#### Cell viability assay

Freshly collected HASMCs (passage 5) were cultured in a 96-well plate to a final density of 3500 cells/well containing 200  $\mu$ l of the SmGM-2 medium. Cells were first incubated for 24 h to permit cell attachment. Then, 0.1  $\mu$ l of DMSO (vehicle) or serial concentrations of TNF- $\alpha$  and/or AUDA were added for an additional 48 h culture. The viability of HASMCs was measured using the MTT assay. Briefly, after 48 h culture, 5 mg/mL MTT reagent was added and further incubated for 4 h. After that, the culture medium in each well was replaced by 200  $\mu$ l DMSO and cell viability assessed by spectrophotometric approach at 490 nm using an ELISA reader. The experiments were performed in quadruplets and the data were expressed as the mean  $\pm$  SD.

#### Cell migration assay

The migration of HASMCs was examined in Transwell cell culture chambers using 8-micron Transwell filters. Cells were pretreated with 0.1  $\mu$ l of DMSO (vehicle) or serial concentrations of AUDA for 30 minutes at 37°C and subsequently placed in the upper chamber at a density of 3500 cells/well. Migration of HASMCs was induced by adjunction of different concentrations of TNF- $\alpha$  (50 to 400 U/mL) to the lower compartment. After a 6-hour migration period, cells that had migrated through the membrane

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**Figure 1.** AUDA inhibits soluble epoxide hydrolase (sEH) activity and human aortic smooth muscle cell proliferation. A. The measurement of sEH activity expressed as the percentage (%) of the activity measured in the control group (untreated HASMCs) showed that AUDA inhibited TNF- $\alpha$ -induced sEH activity. B. The MTT assay showed that AUDA inhibited TNF- $\alpha$ -induced HASMC proliferation. C. Western blot analysis showed that AUDA inhibited TNF- $\alpha$ -induced expression of Cyclin D1 and  $\beta$ -tubulin in HASMCs. Only representative bands were presented. D. Densitometry analysis of bands obtained from the western blot analysis. The quantification was carried out using the bands obtained from three different independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns = non-significant when compared with the control group; \$ $P < 0.01$ , # $P < 0.0001$ , ns $^{\alpha}$  = non-significant when compared with the group of TNF- $\alpha$ -stimulated cells.

were stained, counted using an Axiovert 135 microscope and photographed. Experiments were done in triplicate and were repeated four times.

### Soluble epoxide hydrolase assay

The activity of sEH was assessed using the Soluble Epoxide Hydrolase Cell-Based Assay Kit (Item № 600090) purchased from the Cayman Chemical Company following the manufacturer's instructions.

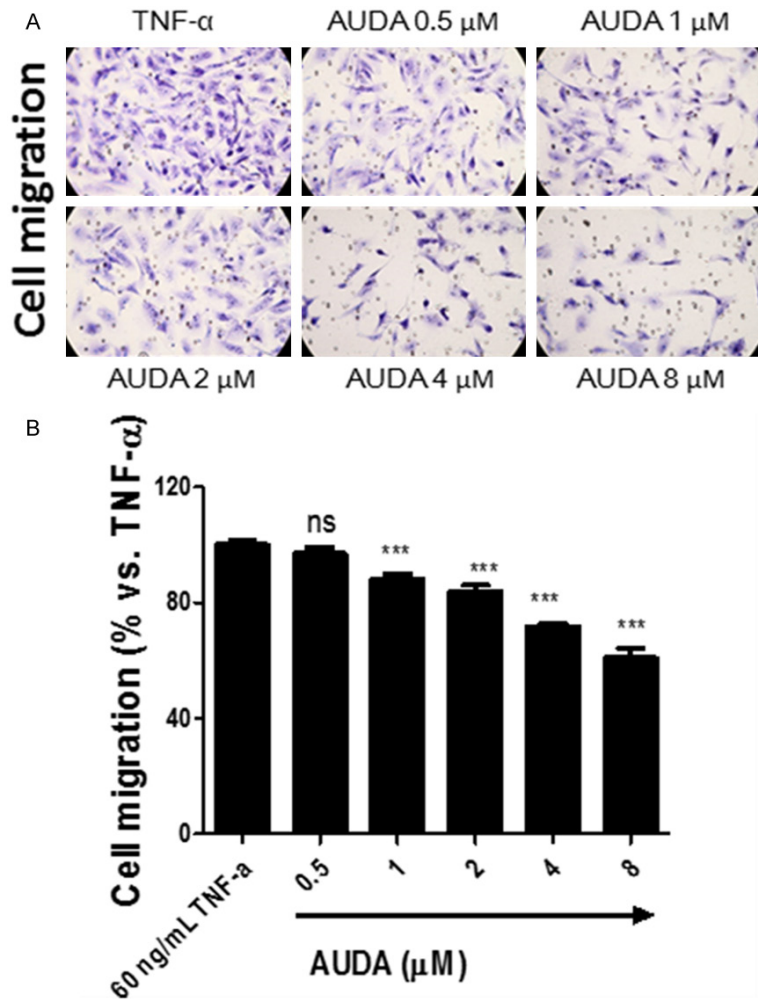
### Western blot analysis

HASMCs were lysed using a RIPA Lysis and Extraction Buffer and quantified with a BCA assay kit (Thermo Fisher Scientific) following the vendor's guidelines. After purification by

SDS-PAGE, protein aliquots were transferred to PVD membranes and blocked with 5% defatted milk. Next, membranes were incubated with primary antibody against GAPDH (loading sample control), cyclin D1,  $\beta$ -tubulin, mTOR, p-mTOR, LC3-II, Beclin 1, PARP, Caspase 3 and cleaved-caspase 3 overnight at 4°C. After washings with PBS/0.1% Tween 20, the membrane was incubated for 1 h with a goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody. Signals were detected with Amersham ECLTM western blot revelation reagents (GE Healthcare, UK). Densitometry analysis of bands was analyzed by Image J software.

### Statistical analysis

One-way ANOVA followed by Dunnett's post-test or two-way ANOVA followed by Bonferroni mul-



**Figure 2.** AUDA inhibits human aortic smooth muscle cell migration. A. The Transwell assay was used to evaluate the migration ability of HASMCs. Only representative photographs were presented. Increasing concentration of AUDA gradually inhibited the TNF- $\alpha$ -induced HASMC migration. B. Quantitative analysis of photographs obtained from the Transwell assay. The quantification was carried out using the photographs obtained from three different independent experiments. \*\*\* $P < 0.001$ , ns = non-significant when compared with the group of TNF- $\alpha$ -stimulated cells.

tiple comparisons test was achieved using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, ([www.graphpad.com](http://www.graphpad.com)). Statistical significance was set at  $p$ -value cutoff of 0.05.

## Results

### *AUDA inhibits the proliferation of TNF- $\alpha$ -induced HASMCs*

Prior to investigating the effect of AUDA on cell proliferation, we first tested its effect on sEH activity. As shown in **Figure 1A**, increasing con-

centration of AUDA led to effective inhibition of TNF- $\alpha$ -induced epoxide hydrolase (EH). The MTT assay showed that AUDA caused a dose-dependent inhibition of TNF- $\alpha$ -induced proliferation of HASMCs (**Figure 1B**). To further check the adverse action of AUDA on cell proliferation, we evaluated the levels of Cyclin D1 and  $\beta$ -tubulin using western blotting. The results (**Figure 1C** and **1D**) showed that AUDA inhibited the expression of Cyclin D1 and  $\beta$ -tubulin in TNF- $\alpha$ -induced HASMCs, suggesting that the anti-proliferative effect of AUDA may be exerted by the negative regulation of cell cycle.

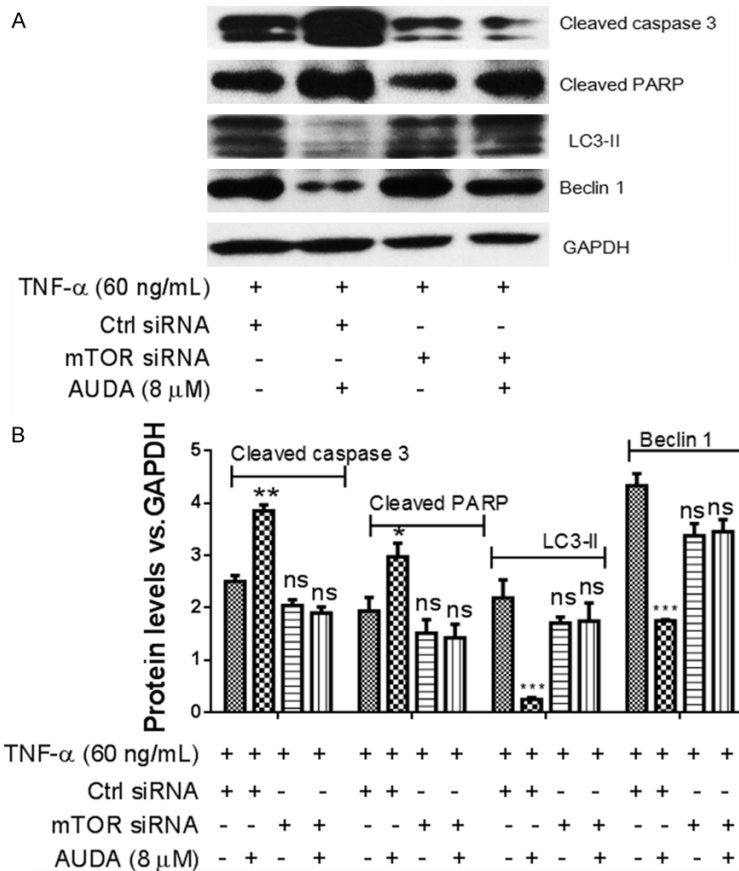
### *AUDA inhibits the migration of TNF- $\alpha$ -induced HASMCs*

To analyze the effect of AUDA on HASMCs migration, we first stimulated cell migration by an effective dose (60 ng/mL) of TNF- $\alpha$ . TNF- $\alpha$  significantly induced the migration of HASMCs compared to the control group. The results also indicated that AUDA dose-dependently inhibited the migration (**Figure 2A** and **2B**) of TNF- $\alpha$ -induced HASMCs compared to cells treated uniquely with TNF- $\alpha$ .

### *AUDA inhibits autophagy and induces apoptosis of TNF- $\alpha$ -induced HASMCs via regulation of mTOR signaling*

Due to the anti-proliferative effect of AUDA on the TNF- $\alpha$ -induced HASMCs, we hypothesized that AUDA could interfere in cell apoptosis and autophagy. To verify this hypothesis, we determined the effect of AUDA on the expression of apoptotic and autophagic markers in TNF- $\alpha$ -induced HASMCs. The western blot analysis (**Figure 3**) showed that following AUDA treatment, the expression of autophagic markers (Beclin 1, LC3-2) was significantly downregulat-

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**Figure 3.** AUDA inhibits autophagy and induces apoptosis of human aortic smooth muscle cells via regulation of mTOR signaling. **A.** Western blot analysis showed that AUDA induced the expression of apoptotic markers and inhibited autophagic markers in HASMCs and silencing of mTOR using mTOR siRNA abolished these effects. HASMCs in Control siRNA, AUDA, mTOR siRNA and mTOR siRNA + AUDA were all treated with TNF- $\alpha$ . Only representative bands were presented. **B.** Densitometry analysis of bands obtained from the western blot analysis. The quantification was carried out using the bands obtained from three different independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$  when compared with the control siRNA group; ns = non-significant.

ed when compared with the control group. On the contrary, pro-apoptotic markers, Caspase 3 and PARP, were activated by the AUDA treatment. These observations indicated that AUDA inhibits the proliferation of TNF- $\alpha$ -induced HASMCs by inhibiting autophagy and inducing cell apoptosis. Meanwhile, we also investigated the role of mTOR signaling in AUDA-mediated induction of cell apoptosis and autophagy inhibition. To this purpose, HASMCs were transiently transfected with mTOR siRNA and the expression of Beclin 1, LC3-2, Caspase 3 and PARP was measured. As shown in **Figure 3**, transfection with mTOR siRNA significantly inhibited the expression of autophagic markers while promoting the expression of apoptotic markers. This

implies that AUDA induces apoptosis and inhibits autophagy of TNF- $\alpha$ -induced HASMCs via negative regulation of the mTOR pathway.

*AUDA inhibits the proliferation and migration of TNF- $\alpha$ -induced HASMCs via mTOR signaling pathway*

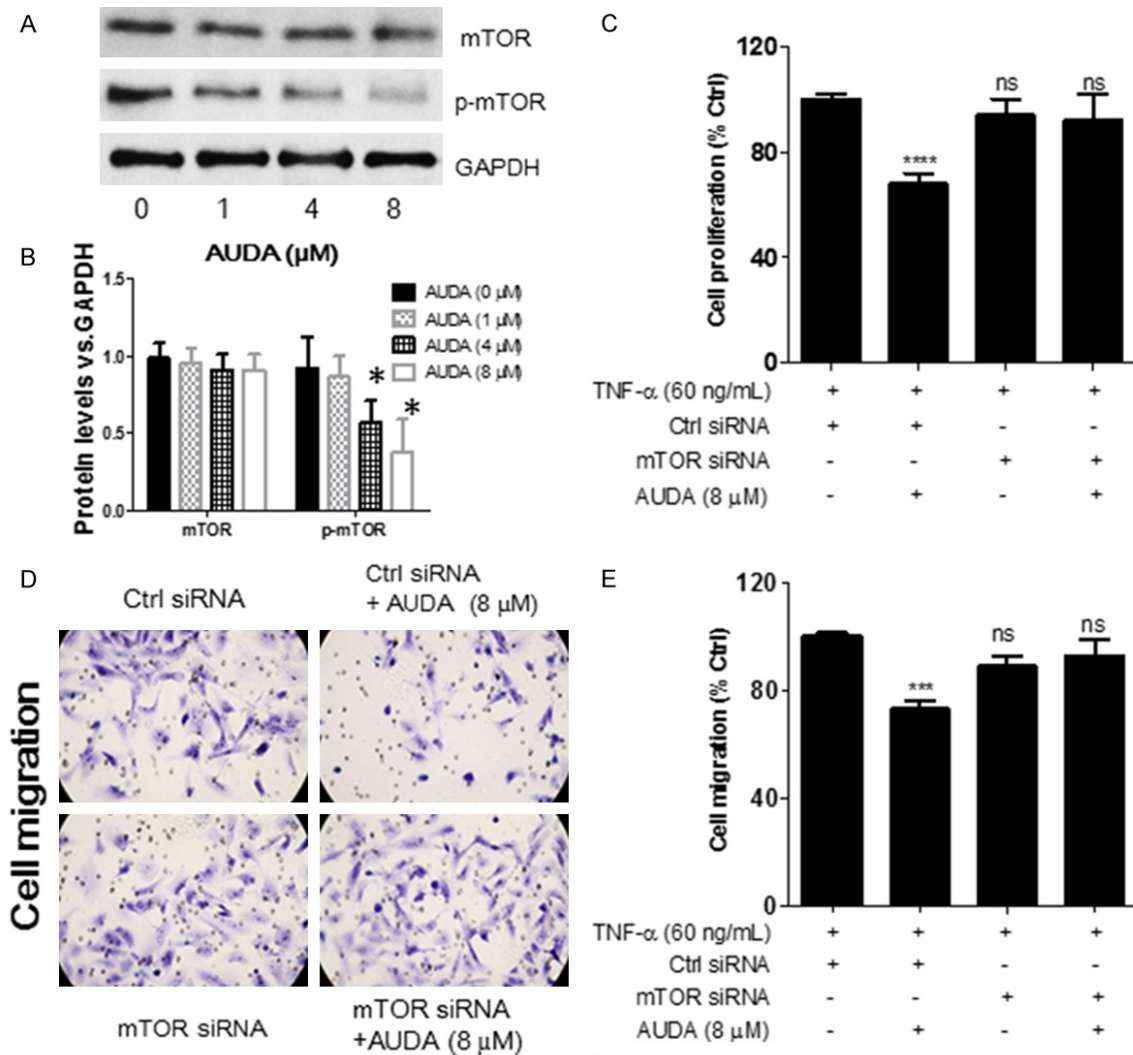
The mTOR signaling pathway is an important cascade that translates extracellular signals into intracellular activities. In order to determine whether the mTOR signaling pathway was involved in AUDA's inhibition of cell proliferation and migration, we determined the expression of mTOR and its phosphorylated form (p-mTOR) by Western blotting. The results (**Figure 4A** and **4B**) showed that treatment with different concentrations of AUDA did not significantly affect the expression of mTOR. On the contrary, the expression of p-mTOR was significantly inhibited. To further verify the functional role of mTOR signaling, we measured the proliferation and migration of HASMCs transfected with mTOR siRNA and stimulated with TNF- $\alpha$ . The results indicated that the transfection with mTOR siRNA sig-

nificantly suppressed the proliferative (**Figure 4C**) and migratory (**Figure 4D** and **4E**) properties of TNF- $\alpha$ -induced HASMCs. For TNF- $\alpha$ -induced HASMCs that were treated with both mTOR siRNA and AUDA, the inhibitory effects were more pronounced. This result demonstrated that AUDA represses the proliferation and migration of TNF- $\alpha$ -induced HASMCs by regulating cell death pathways via, in part, the inhibition of the mTOR signaling.

### Discussion

SMCs proliferation is regarded as a pivotal risk factor for cardiovascular morbidity and mortality, and multiple related pathological conditions

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**Figure 4.** AUDA inhibits the TNF- $\alpha$ -induced mTOR phosphorylation and silencing of mTOR does not affect but abolishes the effect of AUDA on TNF- $\alpha$ -stimulated HASMCs. **A.** Western blot analysis showed that AUDA inhibited the phosphorylation of mTOR in HASMCs. **B.** Densitometry analysis of bands obtained from the western blot analysis. The quantification was carried out using the bands obtained from three different independent experiments. **C.** HASMCs in Control siRNA, AUDA, mTOR siRNA and mTOR siRNA + AUDA were all treated with TNF- $\alpha$  and cell proliferation measured. mTOR siRNA abolished the anti-proliferative effect of AUDA. **D, E.** HASMCs in Control siRNA, AUDA, mTOR siRNA and mTOR siRNA + AUDA were all treated with TNF- $\alpha$  and cell migration was evaluated using Transwell assay. Only representative images were presented. mTOR siRNA mimicked the anti-migratory effect of AUDA on HASMCs. \*\*\* $P < 0.001$  when compared with the control siRNA group treated with TNF- $\alpha$ .

such as atherosclerosis and restenosis after vascular injury are related to HASMCs proliferation and migration [21]. Soluble epoxide hydrolase (sEH) has been reported to promote the atherosclerotic progression and vascular remodeling after injury by regulating smooth muscle cell migration [9, 22]. This observation suggested that blocking of sEH activity could possibly be an effective therapeutic approach for improving cardiovascular diseases, particularly atherosclerosis and restenosis. Thus, scientists are

actively scrutinizing the potential of sEH inhibitors in the treatment of several cardiac and vascular diseases [23-27]. For example, *in vivo* studies in ApoE<sup>-/-</sup> mouse indicated that AUDA exerts therapeutic effects on atherosclerotic diseases and protected the cardiovascular system by downregulating TLR4 and NF $\kappa$ B in aortas [27]. However, the effects of AUDA on HASMCs is still unclear. In this study, we found that AUDA reduced proliferation and migration of TNF- $\alpha$ -induced HASMCs, inhibited autophagy,

induced apoptosis and downregulated the expression of mTOR. In TNF- $\alpha$ -induced HASMCs, mTOR expression was markedly upregulated while cell proliferation and migration were highly increased. These observations were in conformity with previous findings that mTOR signaling promotes SMCs proliferation and migration [28-31]. In the present study, we found that AUDA treatment inhibited cell autophagy and induced apoptosis. This result was contrary to previous findings suggesting that direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death [32]. However, the above observation was supported by studies indicating that autophagy inhibition improves therapy-induced apoptosis in a Myc-induced model of lymphoma [33] and that inhibition of macroautophagy triggers apoptosis [34]. This suggested that AUDA might regulate cellular processes of HASMCs via macroautophagy.

We equally found that via mTOR silencing, the autophagic markers LC3-II and Beclin1 were significantly upregulated whereas the expression of pro-apoptotic markers Caspase 3 and PARP were found downregulated. More importantly, we found that the silencing of mTOR reversed the effect of AUDA on cell autophagy as well as apoptosis, which indicates the vital role of mTOR signaling in AUDA regulation of cellular processes. More specifically, mTOR silencing abolished the inhibitory effects of AUDA on the migration and proliferation of HASMCs. These observations implied that AUDA represses HASMC proliferation and migration by regulating cell death pathways via the mTOR signaling.

### Conclusions

In summary, our findings showed that AUDA exerted anti-proliferative effects against TNF- $\alpha$ -induced HASMC proliferation and migration by the downregulation of autophagic pathways and the upregulation of apoptotic pathways via inhibition of the mTOR signaling. These results implies that AUDA can be applied for pharmacological cure of cardiovascular maladies including atherosclerosis and restenosis. However, further in-depth surveys including *in vivo* studies are needed to elucidate and validate the clinical use of AUDA for the treatment of these disorders.

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### Disclosure of conflict of interest

None.

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