RESEARCH ARTICLE

New Insights into 4-Amino-2-tri-fluoromethyl-phenyl Ester Inhibition of Cell Growth and Migration in the A549 Lung Adenocarcinoma Cell Line

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Abstract

  Objective: The present study was designed to investigate the probable mechanisms of synthetic retinoid 4-amino-2-tri-fluoromethyl-phenyl ester (ATPR) inhibition of the proliferation and migration of A549 human lung carcinoma cells. Materials and Methods: After the A549 cells were treated with different concentrations of ATPR or all-trans retinoic acid (ATRA) for 72 h, scratch-wound assays were performed to assess migration. Immunofluorescence was used to determine the distribution of CAV1 and RXRα, while expression of CAV1, MLCK, MLC, P38, and phosphorylation of MLC and P38 were detected by Western blotting. Results: ATPR could block the migration of A549 cells. The relative migration rate of ML-7 group had significantly decreased compared with control group. In addition, ATPR decreased the expression of a migration related proteins, MLCK, and phosphorylation of MLC and P38. ATPR could also influence the expression of RARs or RXRs. At the same time, CAV1 accumulated at cell membranes, and RXRα relocated to the nucleus after ATPR treatment. Conclusions: Caveolae may be implicate in the transport of ATPR to the nucleus. Change in the expression and distribution of RXRα may be implicated in ATPR inhibition of A549 cell proliferation. The mechanisms of ATPR reduction in A549 cell migration may be associated with expression of MLCK and phosphorylation of MLC and P38.

Keywords: Retinoid ATPR - all-trans retinoic acid - A549 cells - migration - proliferation - myosin light chain kinase

Introduction

  ATRA is an active metabolism of retinal, which can inhibit the cancer cells proliferation and migration (Chen et al., 2012). Additionally, it could induce the differentiation of tumor cells (Watters et al., 2013). Originally, ATRA was regarded as an effective inducer for attaining complete remission in acute promyelocytic leukemia (APL) (Huang et al., 1988). Various tumors, for instance, human hepatoma, breast cancer and lung cancer can be inhibited by ATRA, which was discovered recently (Lin et al., 2005). However, its poor solubility and toxicity limit its clinical use. Therefore, novel ATRA derivatives are desperately in need. ATPR, a gift from college of Pharmacy, Anhui medical university, is an ATRA derivate with low mammalian toxicity (Gui et al., 2011). Retinoic acids (RA) induce differentiation and metabolism by activating retinoid acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ) which interact with specific DNA response element retinoic acid response element (RARE). The expression and distribution of RXRα are closely related to the

progression of neoplasm, such as, cervical carcinoma, prostate cancer and non-small cell lung cancer (Pandey et al., 2003; Brabender et al., 2005; Ocadiz-Delgado et al., 2012). However, it has not been examined whether ATRA and RAs can regulate the subcellular location of RXRα in addition to decreasing RXRα expression in A549 cells.

  After binding ATRA or RAs, RXRs and RARs can form heterodimers (RXR/RAR) or homodimers (RXR/RXR) and exert transcriptional regulation of target gene (Stunnenberg, 1993). ATRA and ATPR trafficking from cell membranes into the nucleus may involve caveolae. About more than 50 years ago, caveolae which exists most abundantly in terminally differentiated cells was identified, named after their flask-like, invaginated appearance in the plasma membrane (Yamada, 1955). Caveolae has been proposed to regulate various cellular biology functions such as vesicular transportation, cell cycle, cell migration (Mercier et al., 2009). CAV1 is an integral membrane protein which is essential for the formation of caveolae. It is still controversial about the role of CAV1 in tumor progression, especially for cell migration. In non-metastatic cells, for instance, fibroblasts,

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neurons and endothelial cells, CAV1 accumulates at the rear of the migrating cells (Beardsley et al., 2005; Sun et al., 2007; Lentini et al., 2008), while in metastatic cells, such as MDA-MB-231 human breast cancer cells and B16-F10 mouse melanoma, CAV1 does not accumulate at the edge during migration (Urra et al., 2012).

Not only CAV1 but also MLCK is associated with the cells migration. Adhesion and migration of cancer cells are essential for cancer metastasis. First, protrusions are extended from cell surface, and then, new anchor points are formed; next, cell adhesion points move forward as fulcrums; finally, the attachment points which stay at the rear of cells break away from matrix, and then cell movement. The polymerization of actin promotes cell migration. Though impacting phosphorylation of myosin light chains (MLC), MLCK can regulate cell migration (Chew et al., 2002), a lot of studies have shown that MLCK assemble in protrusive during cell migration (Kolega, 2003).

Materials and Methods

Reagents

ATRA was purchased from Sigma Chemical (USA). ATPR was a gift from school of pharmacy, Anhui Medical University (Anhui, China). Fetal bovine serum (FBS) was obtained from the Zhejiang Tianhang Biological Technology, Co, Ltd (China). Dulbeccos modified Eagles medium (DMEM, low glucose) was purchased from Gibco BRL life Technologies (USA). Anti-RARα, anti-RARβ, anti-RARγ, anti-RXRα, anti-RXRβ, anti-RXRγ, anti-CAV1, anti-MLCK, anti–p-MLC, anti-MLC, anti-β-actin, antibodies were obtained from Santa Cruz Biotechnology (USA). The BCA protein determination kit was purchased from the Beyotime Institute of Biotechnology (China). Rhodamine (TRITC)-Conjugated AffiniPure Goat Anti-Rabbit IgG(H+L), Fluorescein(FITC)-conjugated AffiniPure Goat Anti-mouse IgG(H+L) and DAPI were obtained from ZSGB-BIO (China). ATRA, ATPR were dissolved in dimethyl sulfoxide (DMSO) at concentration of 10 mg/ml, which was stored at -20 °C.

Cell culture

Human lung adenocarcinoma cell line A549 cells were purchased from American Type Culture collection (ATCC, USA). They were cultured in DMEM medium with low glucose, supplemented with 10%FCS, 1 mmol/l glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin in 5%CO_2 and humidified air at 37 °C. In our laboratory previous studies (Wang et al., 2012), 5 mg/L, 10 mg/L of ATRA and ATPR were chosen as the treating concentration.

Scratch-wound assay

The cell migration was measured by scratch-wound assay. While monolayer A549 cells were grown to confluence in 24-well plate. A 20 μl plastic pipette tip was used to create a linear mechanical scratch wound. After the cells detached removing by PBS, the rest of the cells were incubated in medium containing ATRA (5, 10 mg/L) and

ATPR (5, 10 mg/L). The cells exposed to normal medium were used as controls. The rate of migration was measured by quantifying the total distance that the cells moved from the edge of the scratch toward the center of the scratch under Leica microscopy. The results were recorded at 0 h, 72 h and expressed as the average percent of wound closure by comparing the zero time.

Immunofluorescence

Cells were grown on 12 mm glass coverslips with DMEM which contains a variety of concentration of ATRA (5, 10 mg/L) or ATPR (5, 10 mg/L) for 72 h. Cells were then fixed with 4% paraformaldehyde for 20 min. 0.1% Triton-X100 was used to permeabilize cells. In order to decrease nonspecific binding of antibody, cells were pre-incubated in 5% bovine serum albumin (BSA) for 2 h, at room temperature. Cells were incubated with primary antibodies (1:50 dilution in 1%BSA, over night at 4 °C), and then incubated with TRITC and FITC conjugated second antibodies (1:100 dilution in PBS, 2 h, at room temperature). DAPI was used to stain nuclei and then were visualized using Leica Immunofluorescence microscopy.

Western blot analysis

A549 cells, treated with ATRA (5, 10 mg/L) or ATPR (5, 10 mg/L) for 72 h, were washed in PBS for three times. For extraction of cytoplasmic protein, The cells lysed in RIPA buffer (0.1%SDS, 1%NBonidet P-40, 1%sodium deoxycholate, sodium phosphate buffer 10 nM, Nacl 150 mM, EDTA 2 mM, PMSF 2 mM, leupeptin 10 mg/ml, aprotinin 10 mg/ml, sodium orthovanadate 2 mM, sodium fluoride 20 mM, sodium pyrophosphate 10 mM, PH7.2). The lysates were centrifuged at 15 000 g for 30 min, at 4 °C. Protein concentrations of the sample were measured by Micro-BCA protein Assay Reagent Kit. Membrane protein, to make sure total cells in each group are approximately equal, the same amount of cells were counted in each group. Membrane protein extraction kit (Sanggon Biotech, Shanghai, Co, Ltd) was used to extracted membrane protein. To obtain total protein (cytoplasmic and membrane protein), total protein extraction kit (Sanggon Biotech, Shanghai, Co, Ltd) was utilized to obtain total protein. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). And then, separated protein was transferred to PVDF membrane. 5% non-fat milk in TPBS (PBS contained 0.05% Tween20) was then used for 2 h to block the non-specific binding sites. The PVDF membranes were incubated with primary anti-bodies over night at 4 °C. After washing 3 times (10 min each time) with TPBS, PVDF membranes were then incubated at room temperature for 2 h with a horseradish peroxidase-conjugated secondary antibodies and stained with enhanced chemiluminescence reagents (Pierce, USA).

Statistical analysis

Statistical analyses were performed using SPSS 19.0. One-way analysis of variance (ANOVA) was used to assess the statistical significance of the differences between multiple groups. Results are represented as means ± SEM.
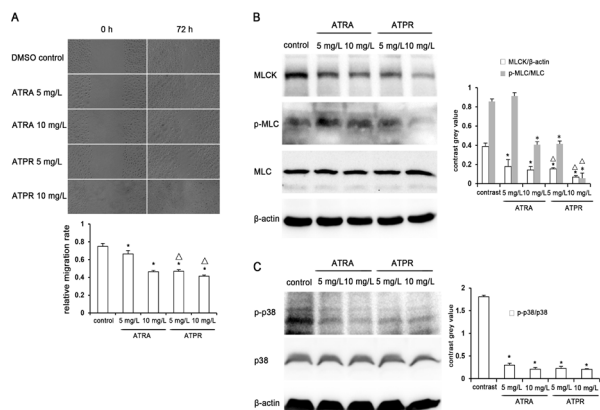
Figure 1. ATPR could inhibit A549 cell migration and the Effect of ATPR on the migration and migration proteins in A549 cells. (A) The relative migration rate of A549 cells treated with ATPR reached 40% at 72 h, while the ATRA group is over 66%. (B) After treating A549 cells with ATRA or ATPR for 72 h, the expression of MLCK and phosphorylation of MLC in A549 cells were decreased. (C) At the same time, the phosphorylation of P38 was significantly decreased. *$P<0.05$ relative to control; △$P<0.05$ relative to ATRA
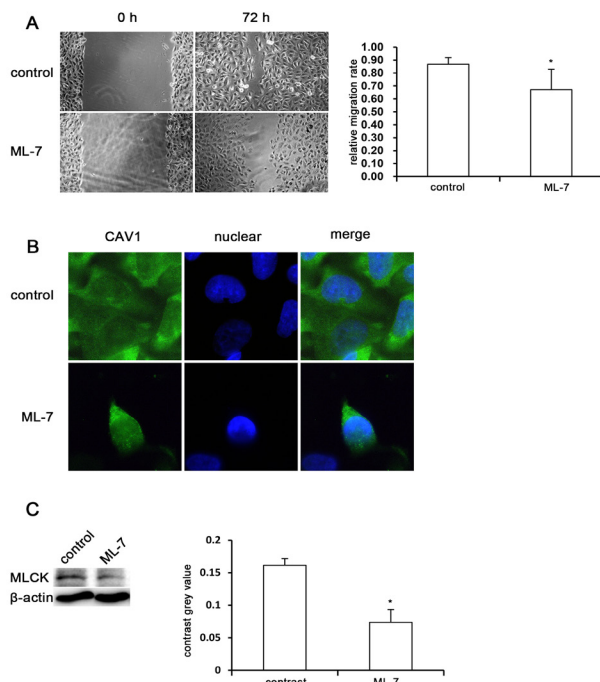

Figure 2. Effect of ML-7 on the migration and migration proteins in A549 cells. (A) ML-7, a selective inhibitor of MLCK, could inhibit A549 cell migration. (B, C) At the same time, after treating A549 cells with ML-7 for 72 h, CAV1 was localized to punctate spots at the cell edge and the expression of MLCK was decreased. *$P<0.05$ relative to control

Results

ATPR blocks A549 cells migration

To exam whether ATPR is advantageous for blocking migration of A549 cells compared to ATRA, scratch-wound assay was performed. At the same time, vehicle control was settled. Results showed that (5, 10 mg/L) of ATRA or ATPR could block the migration of A549 cells. With the same concentration, the blocking effect of ATPR was more effective than that of ATRA (Figure 1A). In order
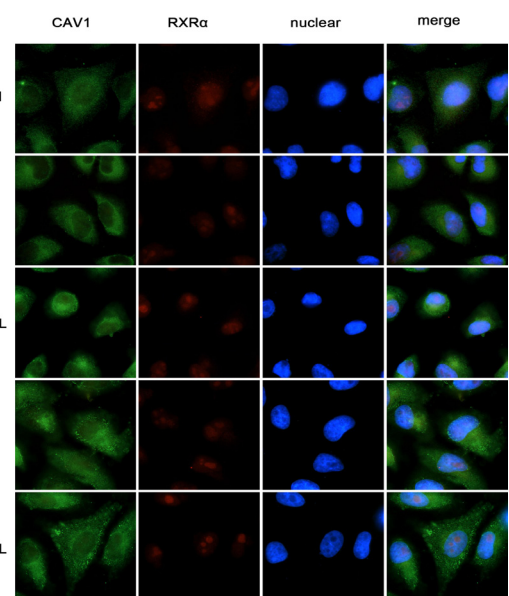

Figure 3. Immunofluorescent location of CAV1 and RXRα in A549 cells. After treating A549 cells with ATRA or ATPR for 72 h, RXRα was more gathered in the nucleus, CAV1 was localized to punctate spots. Not only cell membrane but also nucleus membrane could observe the punctate spots

to investigate the mechanisms that ATRA and ATPR block A549 cells migration, ML-7 which is the specific inhibitor of MLCK was also used to treat A549 cells. As shown in Figure 2A, ML-7 could evidently inhibit the migration of A549 cells compared to the vehicle control.

CAV1 translocate to the membrane after being treated with ATRA, ATPR and ML-7

As shown in Figure 2B, after treating A549 cells with ML-7, CAV1 was localized to punctate spots at the cell edge. While caveolae is familiar to be as petty as 50 nm in diameter, well below the limit of resolution of the Immunofluorescence, these punctate spots were consistently observed. The same punctate spots have been reported before (Je et al., 2004). It has already been proved to be clustering of caveolae in electron micrographs (Isshiki et al., 2002). The punctate spots were consistent with ATRA or ATPR that treated A549 cells (Figure 3). Not only cell membrane but also nucleus membrane could observe the punctate spots. Meanwhile, we performed western blot (Figure 4) for detecting the distribution of CAV1 in A549 cells which treated with ATRA (5, 10 mg/L) or ATPR (5, 10 mg/L). After being treated with ATRA or ATPR the expression of membrane CAV1 protein was significantly elevated, while total and cytoplasm CAV1 protein were decreased.

ATPR exert the location and expression of RXRα

It revealed that RXRα distributed evenly in the control group, however, in A549 cells treated with ATRA or ATPR (5, 10 mg/L), RXRα was more gathered in the nucleus and less in the cytoplasm (Figure 3). In order to detect the expression of RARs and RXRs in A549 cells which were treated with ATRA or ATPR (5, 10 mg/L), western blot was performed and showed in Figure 5. Compared to the vehicle control, the expression of RARα and RARγ were
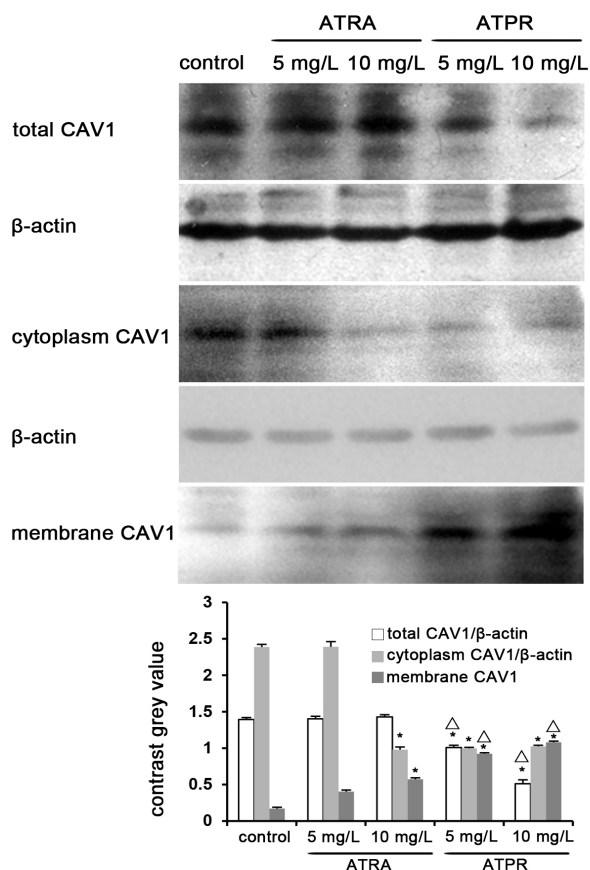
Figure 4. Effect of ATPR on the distribution of total, membrane, and cytoplasm CAV1 in A549 cells. After being treated with ATRA or ATPR for 72 h, the expression of membrane CAV1 was increased, while total and cytoplasm CAV1 were decreased. *P<0.05 relative to control; △p<0.05 relative to ATRA

increased, RXRα and RXRγ were decreased, while the levels of RARβ and RXRβ were not altered obviously.

ATPR decreased the expression of migration associated proteins

After being treated with ATPR (5, 10 mg/L), the expression of MLCK in A549 cells was decreased (Figure 1B). The expression of MLCK in ATPR group decreased sharper than ATRA group. MLC can be phosphorylated by MLCK at 19 serine amino-acid residue. The result in Figure 1B also showed that ATPR most potently decrease the level of phosphorylated MLC. The same result was observed when the A549 cells were treated with ML-7 (Figure 2C). In the interest of investigating the mechanism of blocking migration of A549 cell caused by ATPR, p-P38 were also detected (Figure 1C). The phosphorylation of P38 was significantly decreased when cells incubated with ATPR.

Discussion

Lung cancer has the highest mortality rate among all kinds of cancers, with 10~20% 5-years survival rate (Jemal et al., 2008). The non-small cell lung cancer (NSCLC) takes up about 80% of lung cancers, which contains three histological subtypes, squamous cell carcinoma, large cell carcinoma and adenocarcinoma. It often already developed
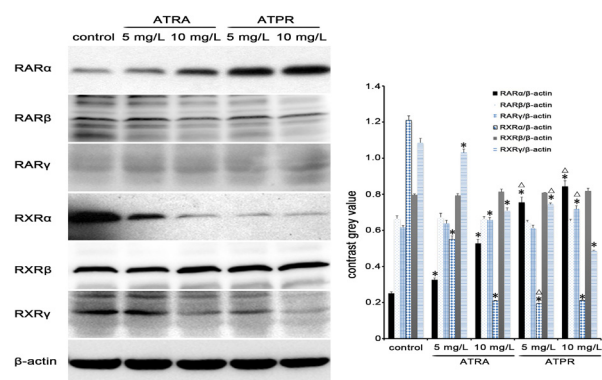

Figure 5. Effect of ATPR on the expression levels of RXRs and RARs in A549 cells. After treating A549 cells with ATRA or ATPR for 72 h, compared with the vehicle control, the expression of RARα and RARγ were increased, RXRα and RXRγ were decreased, while the levels of RARβ and RXRβ were not altered obviously. *P<0.05 relative to control; △p<0.05 relative to ATRA

into advanced stages when NSCLC is detected (Fei et al., 2013), so the early detection and treatment of lung cancer is very important. The chemotherapeutic agents currently in use for lung cancer are unsatisfactory due to associated lack of efficacy, drug resistance, and co-lateral toxicity (Arafat et al., 2013). Induction differentiation therapy is a new treatment strategy emerging in recent years. RAs are commonly used as differentiation inducer (Tsiftsoglou et al., 2009). However, the major limitation in using ATRA compounds for therapeutic purposes include high lipophilicity, short biological half-life, adverse toxicities and appearance of resistant cells during therapy (Vyas et al., 2012). In order to discover agents with lower toxicity and higher efficacy, researchers have continued to alter the structure of ATRA (Gui et al., 2011), such as ATPR. However, ATRA and its derivatives anti-tumor mechanisms are still unclear.

RAs mediated cancer cells differentiation and apoptosis through binding to nuclear receptors (RARs, RXRs). After combing with RAREs, RAs can regulate the target genes transcription (Nomoto et al., 2012). The way of RAs translocation to the nuclears is very important. In this study, we showed that caveolae, as a vector, could transport RAs from membrane to the nuclear. Caveolae have been implicated in various vesicular transport, such as transcytosis, potocytosis and endocytosis (Parton et al., 2007). In Figure 2B and Figure 3, we present caveolae assembled in the cell membrane and nuclear membrane. So we suppose caveolae can transport RAs to nuclear. However, it is still controversial about the expression and distribution of CAV1 in cell migration. CAV1 preferentially accumulates at the cell front in transmigrating endothelial cells and neurons, but at the rear of migrating fibroblasts neurons and endothelial cell (Parat et al., 2003; Sun et al., 2007; Lentini et al., 2008). Unlike for the cells we have mentioned, polarization and relocation to the trailing edge of CAV1 were not observed in migrating metastatic cells (Urra et al., 2012). In this article we showed that when treated A549 cells with ATRA or ATPR, CAV1 relocated to the membrane. Probably it is a mechanism in which ATRA or ATPR restrain A549

cells migration, but further studies is required.

Previous research has shown that cellular expression and location of protein is associated with diverse signaling transduction pathways and the resultant physiological process (Ye et al., 2004). ATRA could induce the translocation of RXRα from a cytoplasmic to the nucleus in MCF10A cells (Luo et al., 2006). Recent studies have suggested that transfection with the RXRα gene followed by RXRα assembled in the nuclear can inhibit the proliferation of prostate cancer cells (Zhong et al., 2003). RAs can regulate the expression of RARs and RXRs (Wan et al., 1998). The expression of RXRα is also a critical determinant for cell proliferation. In hep3B cells, RAs can inhibit cell growth by down regulating the expression of RXRα (Wan et al., 2000). Previous studies also suggest that RARα and RARγ enhance growth suppression and apoptosis of neoplastic epidermal keratinocytes (Hatoum et al., 2001). In our laboratory previous studies (Wang et al., 2012), ATRA and ATPR could inhibit A549 cells proliferation in a does-dependent manner. Our experiments showed that after combating A549 cells with ATRA or ATPR, the expression of RXRα declined, while RARα and RARγ enhanced (Figure 5), at the same time, the RXRα accumulated in the nuclear (Figure 3). So we can conclude that ATPR can inhibit cell proliferation partly by decreasing the expression of RXRα, increasing the expression of RARα and RARγ and accumulating RXRα in the nuclear.

Cancer progression is associated with cell migration and invasion (Arafat et al., 2013; Pitchakarn et al., 2013), ATPR has a better efficiency in blocking A549 cells migration, however, the mechanisms is still unclear. The research between tumor cells migration and MLCK were still seldom. MLCK plays an important role in cell motility and smooth muscle contration (Schmidt et al., 2002). Phosphorylation of MLC by MLCK in non-muscle cells has been proved to play an vital physiological function (Wadgaonkar et al., 2003). In our laboratory previous study of breast cancer cell lines MDA-MB-231, ATPR could inhibit the migration of MDA-MB-231 cells via MLCK involving p38-MAPK pathway (Wang et al., 2013). Interestingly, we also find that ATRA could depression the A549 cells migration by reducing the expression of MLCK. In our studies, the combination treatment of A549 cells with ATRA or ATPR could decrease the expression of MLCK, at the same time, down regulation of the Phosphorylation of MLC and p38 (Figure 1B, 1C). ML-7, a selective inhibitor of MLCK, was also used to treat A549 cells. We found the same phenomenon, the migration of A549 cells were inhibited and the expression of MLCK was also reduced (Figure 2C). So we supposed ATRA and ATPR could inhibit the migration of A549 cells partly though decreasing the expression of MLCK and p38-MAPK pathway.

In summary, our results showed that Caveolae can transport RAs to the nuclear, the mechanism of ATPR inhibiting A549 cells proliferation is partly through influencing the expression of RARs, RXRs and the distribution of RXRα. Impacting the expression of MLCK and the phosphorylation of MLC, maybe one of the mechanisms of ATPR blocking A549 cells migration.

Acknowledgements

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