RESEARCH ARTICLE

A Novel All-trans Retinoid Acid Derivative N-(3-trifluoromethyl-phenyl)- Retinamide Inhibits Lung Adenocarcinoma A549 Cell Migration through Down-regulating Expression of Myosin Light Chain Kinase

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Abstract

Aim: To observe the effects of a novel all-trans retinoid acid (ATRA) derivative, N-(3-trifluoromethyl-phenyl)-retinamide (ATPR), on lung adenocarcinoma A549 cells and to explore the potential mechanism of ATPR inhibiting of A549 cell migration. Materials and Methods: The cytotoxicity of ATRA and ATPR on A549 cells was assessed using MTT assay. Wound healing assays were used to analyze the influences of ATRA, ATPR, ML-7 (a highly selective inhibitor of myosin light chain kinase (MLCK)), PMA (an activator of MAPKs) and PD98059 (a selective inhibitor of ERK1/2) on the migration of A549 cells. Expression of MLCK and phosphorylation of myosin light chain (MLC) were assessed by Western blotting. Results: ATRA and ATPR inhibited the proliferation of A549 cells in a dose- and time-dependent manner, and the effect of ATPR was much more remarkable compared with ATRA. Relative migration rate and migration distance of A549 cells both decreased significantly after treatment with ATPR or ML-7. The effect on cell migration of PD98059 combining ATPR treatment was more notable than that of ATPR alone. Moreover, compared with control groups, the expression levels of MLCK and phosphorylated MLC in A549 cells were both clearly reduced in ATRA and ATPR groups. Conclusions: ATPR could suppress the migration and invasion of A549 cells, and the mechanism might be concerned with down-regulating the expression of MLCK in the ERK-MAPK signaling pathway, pointing to therapeutic prospects in lung cancer.

Keywords: Lung adenocarcinoma A549 cells - N-(3-trifluoromethyl-phenyl)-retinamide - MLCK - migration

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Introduction

Malignant tumors are part and parcel of primary causes leading to death of human beings in nowadays. The invasion and forming metastasis of cancer cells are responsible for worsening condition and mortality. Due to the difficulty of early diagnosis and the poor effects of traditional therapy, including surgical operation and chemotherapy, cancer curing is still a worldwide challenge. Therefore, to explore a novel effective therapeutic method is extremely urgent.

All-trans retinoid acid (ATRA) is a major metabolite of vitamin A, which plays an important role in growth, development and cell differentiation of vertebrate, especially during the embryo formation (Duester, 2008; Arisi et al., 2014). Tracking back to twenty years ago, Chinese scientists were the pioneer to use ATRA to induce cell differentiation of acute promyelocytic leukemia (APL) (Huang et al., 1988) and acquired complete remission (Chen et al., 1992b; Wang et al., 1999e). Subsequently, a series of studies of ATRA on APL and solid tumors were developed at full blast. Vast researches indicated that ATRA could induce cell differentiation and inhibit invasion and migration of multiple solid tumor cells, including breast cancer, gastric carcinoma and colon cancer, and so on (Bengtsson et al., 2013; Hu et al., 2012a; 2014b Wang et al., 2013a; Zhang et al., 2013b; 2014a; Garattini et al., 2014; Tang et al., 2014). But its application was largely limited because of its poor solubility and high toxicity (Gui et al., 2011; Dhar et al., 2012; Clemens et al., 2013; Wang et al., 2013a; 2013b). Hence, it is requisite to modify the structure and properties of ATRA to overcome

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its drawbacks. N- (3-trifluoromethyl-phenyl)-retinamide (ATPR), synthesized by Pharmacy School of Anhui Medical University, is a novel ATRA derivative and has lower toxicity and more powerful effects on both growth and differentiation of A549 cells than ATRA (Chew et al., 2002; Gui et al., 2011; Clemens et al., 2013; Wang et al., 2013c). This may give us a new treatment strategy.

Cell motility is not only responsible for the invasion and migration of malignant cells, but also as a prerequisite for metastasis (Shin et al., 2009). The myosin light chain kinase (MLCK) is one kind of protein kinases which is Ca^{2+}/Calmodulin-dependent (Zhou et al., 2008). The activation and increased expression of MLCK are crucial to trigger smooth muscle cell and non-muscle cell motility (Sun et al., 2011; Zhu et al., 2012b). Some researches have reported that the overexpression of MLCK was occurred in many cancer cells (Kucharczak et al., 2001; Mills et al., 2011; Wang et al., 2013d). Recent researches and preliminary studies in our lab demonstrated that ATRA and its derivatives could decrease tumor cells growth and migration (Gui et al., 2011; Bengtsson et al., 2013; Wang et al., 2013a; Zhang et al., 2013b; 2014a; Garattini et al., 2014; Hu et al., 2014b). In our previous studies, it was explored that ATPR might inhibit tumor cells migration by down-regulating the expression of MLCK involving p38 signaling pathway (Wang et al., 2013a), one of MAPKs pathway cascade. The mitogen-activated protein kinases (MAPKs) cascades include three classical signal pathways: p38, ERK1/2 and JNK, which play crucial roles in different physiological processes, such as cell growth, apoptosis and inflammatory reaction (Zhu et al., 2012b; 2014a; Wang et al., 2013c). It was also reported that the elevation of ERK1/2 might activate the MLCK along with a series of downstream pathways activation (Srinivas et al., 2004; Zhou et al., 2008; Muralidharan-Chari et al., 2009; Zhu et al., 2012b; 2014a; Harrison et al., 2013). Therefore, we speculated whether ATRA and ATPR suppressed cell migration concerned with MLCK and ERK1/2. Here, we observed the influences of ATPR on A549 cells migration. Besides, the underlying mechanisms were explored.

Materials and Methods

Cell culture

Lung adenocarcinoma cell line (A549) was purchased from ATCC and routinely maintained in a 5% CO_2, 37°C incubator with tissue culture medium Dulbecco`s Modified Eagle`s medium (DMEM) supplemented with 10% calf serum (TBD Science, Tianjin, China), 100 U/ml penicillin, 100 U/ml streptomycin (Ameresco, USA) and 2 mM L-glutamine.

Assessment of cytotoxicity by MTT assay

The A549 cells, added in 96-well plates at a density of 1×10^4 cells/well, were treated with ATRA (10 μg/ml) or different concentrations (1, 2.5, 5, 7.5, 10 μg/ml) of ATPR, respectively. After incubation for different times (1, 2, 5 or 7 days), the culture medium was replaced by MTT solution. Then dimethyl sulfoxide (DMSO) was added to resolve the formazan generated from MTT after another 4h of incubation at 37°C. The absorption (A) of each well was recorded on a microplate reader (Bio-Tek, ELX800, USA) at the wavelength of 570 nm. The cytotoxicity of ATRA and ATPR were mirrored by relative inhibition ratio, which was figured out by the formula: relative inhibition ratio=$[1- (A_t-A_b)/ (A_c-A_b)]\times100\%$.

Migration assay

The migration of A549 cells was measured by wound healing assay. Cells were plated onto 24-well plates at a density of 5×10^6 cells/L. At confluence, the cell monolayer was scraped with a 20 μl plastic pipette tip to generate scratch wounds. Then cells with scratch-wounds were allowed to heal in a tissue culture incubator for 24 h, containing different concentrations of ATRA (1, 10 μg/ml), ATPR (1, 7.5 μg/ml) with or without ML-7 (1μM), PMA (1μM), PD98059 (1μM). At the 0 h and 24 h time point, A549 cells with scatch-wounds were observed by a inverted microscope. The relative distance of cells migration between 0 h and 24 h was calculated as a percentage of wound closure.

Western blot analysis

After treatment, A549 cells were washed with PBS for three times and lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 1 mM PMSF and 5 mg/ml leupeptin). The lysates were centrifuged at 14, 000 rpm for 30 min at 4°C, and the supernatant was transferred to a fresh tube. The protein concentrations were measured by a BCA kit (Pierce, Rockford, IL, USA). And the equal concentration of proteins were mixed with SDS sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes The membranes were blocked with 5% fat-free dry milk in TBST for 2h at room temperature, and then incubated overnight at 4°C with appropriate dilutions of primary antibodies (MLC, MLCK, *p*-MLC and β-actin). After that, the membranes were washed with TBST three times, incubated with horseradish peroxidase (HRP)-conjμgated secondary antibodies (Millipore, USA) for 2h at room temperature, and then detected by enhanced chemiluminescence (ECL-Plus kit, Beyotime, China).

Statistical analysis

Data analysis was performed using software SPSS 16.0. Statistical significance of the differences between groups was assessed by one-way analysis of variance (ANOVA) followed by LSD-test. $p<0.05$ was considered significant.

Results

ATPR inhibited cell growth in A549 cells

The cytotoxicity of ATRA and ATPR to A549 cells was evaluated by the MTT assay. A549 cells were treated with ATRA (10 μg/ml) or different concentrations of ATPR (1, 2.5, 5, 7.5, 10 μg/ml) for 48h. Then we chose 10 μg/ml of ATRA and 7.5 μg/ml of ATPR to treat A549 cells and observed them in 1, 2, 5, 7 days, respectively. Fig.1 showed that ATRA (10 μg/ml) and ATPR does-dependently suppressed cell viability. Among the results, 7.5 μg/ml and 10 μg/ml of ATPR significantly suppressed

cells growth. Figure 2 showed that ATRA and ATPR could inhibit cell growth time-dependently, while the growth inhibition of ATPR was much more remarkable than that of ATRA.

ATPR suppressed migration in A549 cells

The wound healing assay was used to verify whether ATRA or ATPR could inhibit A549 cells migration and further to reveal the potential mechanism. We could intuitively get the information that the relative migration ratios of A549 cells treated with 10 μg/ml ATRA , 1 μg/ml ATPR or 7.5 μg/ml ATPR, were shorter than control groups which were confirmed further by subsequent statistical chart (Figure 3A). Figure 3B not only showed the similar outcomes of ATRA and ATPR groups, but also unveiled that the effect of ATPR (7.5 μg/ml) combining PD98059 (1μM) on inhibiting cell migration was more remarkable than that of ATPR (7.5 μg/ml) alone. While the promotion of ATPR (7.5 μg/ml) combining PMA (1μM) on cell migration was not more notable than ATPR (7.5 μg/ml) alone. Obviously, ATPR induced A549 cells migration significantly compared with ATRA and control groups. At the same concentration, the effect of ATRA on migration was not as much as that of ATPR.

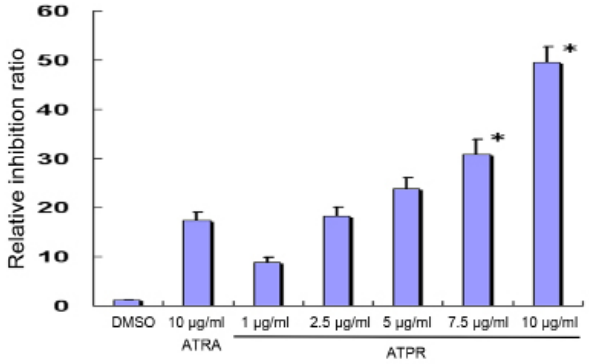

Figure 1. Dose-dependent of ATPR After A549 Cells Were Treated with 10 μg/ml ATRA or Different Concentrations of ATPR for 48 h, MTT was Applied to Determine Cell Viability. Relative inhibition ratio=[1-(A_t-A_b)/(A_c-A_b)]×100%. A_t: the absorption of treatment groups; A_b: the absorption of blank group; A_c: the absorption of control group. *p<0.05 vs the ATRA group (10 μg/ml)

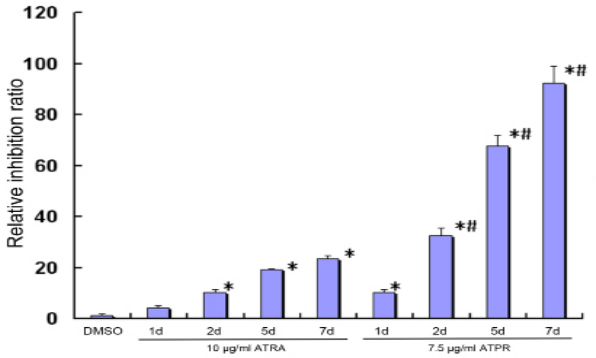

Figure 2. Time-dependent of ATPR After A549 Cells were Incubated with 10 μg/ml ATRA or 7.5 μg/ml ATPR for 1, 2, 5 or 7 Days, MTT was Applied to Determine Cell Viability. *p<0.05 vs the DMSO group; #p<0.05 vs the ATRA group (10 μg/ml) of the same time

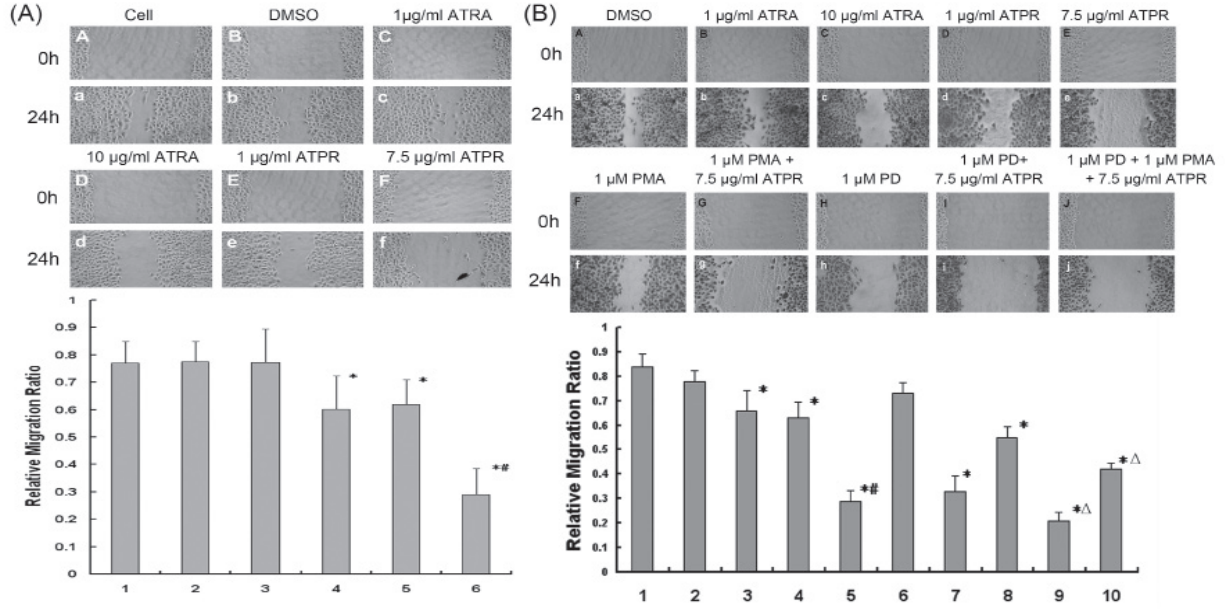

Figure 3. Effects of ATPR, PMA and PD98059 on the Migration of A549 Cells. A) Wound healing assay was to observe the effects of ATRA and ATPR on cell migration, which is mirrored by relative migration ratio. Calculating method: Relative migration ratio=(Start distance–End distance)/Start distance. 1: A549 cell, 2: A549 cell/DMSO, 3: A549 cell/1 μg/ml ATRA, 4: A549 cell/10 μg/ml ATRA, 5: A549 cell/1 μg/ml ATPR, 6: A549 cell/7.5 μg/ml ATPR. *p<0.05 vs the control group; #p<0.05 vs the ATRA group (10 μg/ml). **B)** wound healing assay was used to explore the effects of ATPR with or without PMA and PD98059 on A549 cells migration. 1: A549 cell/DMSO, 2: A549 cell/1 μg/ml ATRA, 3: A549 cell/10 μg/ml ATRA, 4: A549 cell/1 μg/ml ATPR, 5: A549 cell/7.5 μg/ml ATPR, 6: A549 cell/1 μM PMA, 7: A549 cell/1 μM PMA+7.5 μg/ml ATPR, 8: A549 cell/1 μM PD98059, 9: A549 cell/1 μM PD98059+7.5 μg/ml ATPR, 10: A549 cell/1 μM PMA+1 μM PD98059+7.5 μg/ml ATPR. *p<0.05 vs the DMSO group; #p<0.05 vs the ATRA group (10 μg/ml); Δp<0.05 vs the ATPR group (7.5 μg/ml)

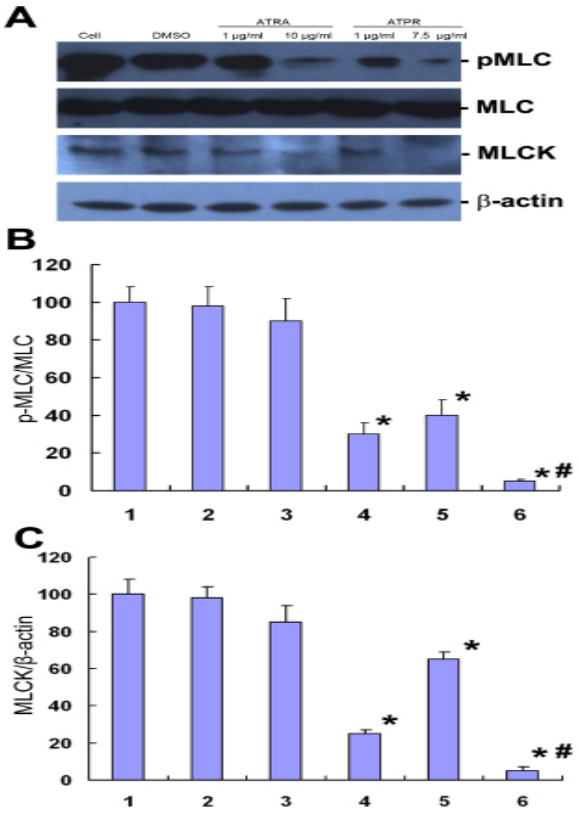

Figure 4. Effects of ATPR on the Expression of MLCK and Phosphorylation of MLC in Lung Adenocarcinoma A549 Cells Forty-eight Hours After Incubation with ATRA or ATPR, Representative Western Blot. A) showed specific bands for markers involved in migration and densitometric analysis of *p*-MLC; **B)** and MLCK; **C)** relative to internal control were shown in arbitrary units. 1. A549 cells, 2. A549 cell/DMSO, 3. A549 cell/1 μg/ml ATRA, 4. A549 cell/10 μg/ml ATRA, 5. A549 cell/1 μg/ml ATPR, 6. A549 cell/7.5μg/ml ATPR. **p*<0.05 vs the DMSO group; #*p*<0.05 vs the ATRA group (10 μg/ml)

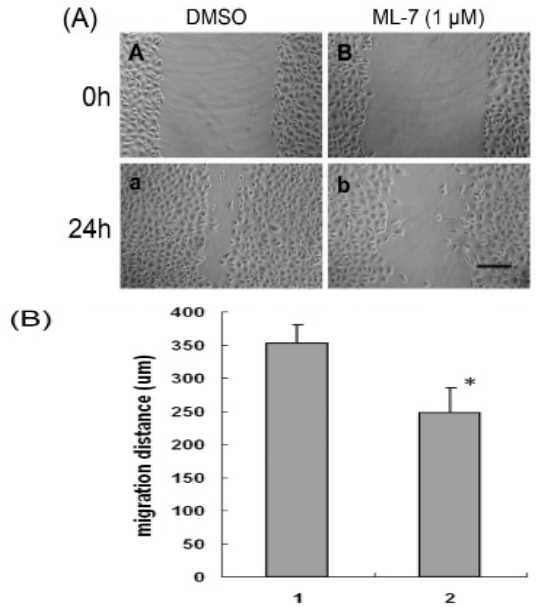

Figure 5. Effect of ML-7 on the Migration of A549 Cells. To further testify the relationship between cell migration and MLCK, wound healing assay was performed in A549 cells with ML-7 application. 1: A549 cell/DMSO, 2: A549 cell/1 μM ML-7. **p*<0.05 vs the control group

ATPR decreased expression of MLCK and phosphorylation of MLC in A549 cells

Western blot was used to testify the effects of ATPR on the expression of proteins related to migration. In Figure 4A, we could clearly distinguish that ATRA (10μg/ml) and ATPR (7.5μg/ml) largely suppressed the expression of MLCK and phosphorylation of MLC (pMLC). Moreover, by calculating the contrast gray value of pMLC/MLC and MLCK/β-actin, the similar results could be drawn (Figure 4B).

ML-7 inhibited migration in A549 cells

To ulteriorly verify the correlation between cell migration and MLCK, we used ML-7 to observe the would healing of A549 cells. ML-7 is a specific inhibitor of MLCK, which can specifically inhibit MLCK phosphorylating MLC. After being treated with ML-7 (1μM) for 48h, the migration distance of A549 cells was about 245 μm, while that of control group was about 350 μm (Figure 5).

Discussion

With the fast-growing morbidity and mortality, lung cancer is one of the most serious malignant tumors which gravely threaten human beings' health and lives (Chiara et al., 2014). The majority of lung cancer patients have formed distant metastases before being diagnosed, which are mostly located in bone and brain (Chiara et al., 2014; Rafal et al., 2014). Then, chemotherapy and radiotherapy occupy the main position in lung cancer treatment field. Nevertheless, because of the drug resistance and side-effects of chemotherapy, the radiation damage and constricted function of radiotherapy, the therapy actuality is still unsatisfied (Arafat et al., 2013; Chiara et al., 2014; Lombardi et al., 2014; Pashha et al., 2014; Rafal et al., 2014; Zhuang et al., 2014). In this case, inducing tumor cells differentiation as a potential new therapeutic method , is attracting more and more attention. In the current study, we took A549 cell line, which was initially got through explant culture of lung adenocarcinoma tissue from a 58-year-old Caucasian male, as a model to investigate lung cancer. ATRA, an activated metabolite of vitamin A (Clemens et al., 2013), can influence the proliferation and differentiation of both normal and cancer cells (Chen et al., 2012a). Several researches indicated that ATRA was able to induce morphological differentiation and control cells invasion and migration of many types of tumors (Garattini et al., 2014; Hu et al., 2014b; Zhang et al., 2014a), making it the foremost inducing differentiation therapeutic agent. However, because of its instability and toxicity (Gui et al., 2011; Wang et al., 2013c), ATRA was not applied extensively in clinical except for dermatosis. In the study of our lab, ATPR, a novel derivative of ATRA, with the advantage of lower toxicity and better stability, showed more intense pharmacological action on biological behavior of A549 cells. But the definite mechanisms remain indeterminate. What's more, it was reported that the treatment of ATRA combining cisplatin was significantly more effective on liver cancer cells than cisplatin alone (Zhang et al., 2013b). The mechanism may

be associated with that ATRA could enhance the toxicity of cisplatin by inducing the differentiation of tumor initiating cells (Zhang et al., 2013b). This may present a novel thought on clinical therapy of other neoplastic diseases, including lung cancer.

Cell migration is intimately connected with situation exacerbation and metastasis. Meanwhile, cell motility is the key element of cell migration (Zhou et al., 2008). It is known to us that MLCK plays a crucial role in cell motility thus is the key point of cell migration (Sun et al., 2011). As long as the intracellular Ca^{2+} increase, MLCK is subsequently activated, which will increase the level of phosphorylated MLC. Next the phosphorylated MLC promotes myosin interacting with actin to drive cell moving on (Shin et al., 2009; Sun et al., 2011). Studies have shown that the phosphorylation of ERK1/2 promoted the activation of MLCK to trigger cell migration in smooth muscle cells, vascular endothelial cells, and breast cancer cells (Srinivas et al., 2004; Zhu et al., 2012b; 2014a; Harrison et al., 2013). Besides, there exist other studies revealing the crosstalk between MLCK and ERK1/2 in the process of cell migration (Zhou et al., 2008). ERK1/2, one of MAPKs signal cascade, is known as a prominent signal pathway in cell proliferation and differentiation (Pan et al, 2014; Zhu et al., 2014a). Whereas the detailed mechanisms of ERK1/2 together with MLCK in cell migration are unclear. As for the lung adenocarcinoma A549 cells, the studies about ERK1/2 and MLCK in migration were scanty. In our study, we tentatively probed the role of ERK1/2 in A549 cells migration using wound healing assay. When A549 cells with scratch-wounds were treated with PD98059 combining ATPR, the migration was inhibited more markedly than that of ATPR (7.5 μg/ml) alone. While the increased migration of PMA (1 μM) combining ATPR (7.5 μg/ml) had no statistics significance compared with ATPR (7.5 μg/ml) alone. It was also revealed that 7.5 μg/ml ATPR blocked A549 migration dramatically than ATRA (10 μg/ml) and control group (p<0.05). And western blot assay indicated that 7.5 μg/ml ATPR and 10 μg/ml ATRA down-regulated the expression of MLCK and phosphorylation of MLC brilliantly. Moreover, ML-7, a selective inhibitor of MLCK, was used to treat A549 cells and the similar result that ML-7 could suppress A549 cells migration revealed that ATPR suppressed A549 cells migration by down-regulating the expression of MLCK. Furthermore, PD98059, a selective inhibitor of ERK1/2, strengthened the effects of migration inhibition of ATPR when combined with ATPR. So we speculated that the migration of A549 cells might be achieved by activating MLCK as well as ERK1/2 signal pathway. Of course, more experiments are needed to further check the assumption. This study may lay the foundation of exploring the application of ATRA derivatives and MLCK and ERK inhibitor in the therapy for lung cancer.

In summary, in our study, we observed that new synthetic derivative of retinoic acid, ATPR, more notably inhibited A549 cells invasion and migration than ATRA. And the migration inhibitory effect may be achieved through MLCK along with ERK1/2 signal pathway.

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