

# The Different Effect of R and S Albuterol on Proliferation and Migration of Airway Smooth Muscle Cells

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## ABSTRACT

Previous studies have shown that the R- and S-enantiomers of racemic albuterol, a  $\beta_2$ -adrenergic receptor agonist used in asthma treatment, have differential effects on the contractile properties of airway smooth muscle. However, the effect of albuterol on the proliferation and migration has never been tested. Since (R)-, but not (S)-albuterol enhances bronchodilation, we expect the two racemic isomers would also affect proliferation and migration of tracheal cells differentially. By monitoring migratory properties of airway smooth muscle cells in the presence of albuterol isomers, the different effect of albuterol on proliferation and migration of airway smooth muscle cells is probed. The results show Both of R- and S-albuterol could inhibit the proliferation of smooth muscle cells and the inhibition ratio of these two isomers had no significant difference; R-albuterol, but not S-albuterol, inhibited cell migration.

## 1. INTRODUCTION

Previous studies have shown spasm of bronchial smooth muscle is the main cause of asthma; thereby  $\beta_2$ -adrenergic receptor agonists are widely used for asthma treatment [1]. Albuterol is one of such medicines. By binding to  $\beta_2$ -adrenergic receptor, albuterol could activate cyclic adenosine monophosphate kinase, and increase concentration of cAMP and calcium in cells, resulting in the relaxation of bronchial smooth muscle [2]. Albuterol has two isomers, in terms of R- and S-albuterol [3, 4]. It has been proven that these two isomers have differential effects on the contractile properties of airway smooth muscle [5]. However, the different effects of R- and S-albuterol on smooth muscle cells proliferation and migration have never been tested.

In the pathological process of asthma, the airway smooth muscle cells could differentiate from contractile phenotype to proliferation and actively release several cytokines, chemokines, growth factors and adhesion molecules to facilitate the inflammation and remodeling of airway, which might lead to airway

hyperplasia and hypertrophy, resulting in promotion of pathological process of asthma [6-9]. More important, people found that, during the pathogenesis of asthma, smooth muscle cells could differentiate into the migration phenotype. With the assistance of several growth factors and chemokines, smooth muscle cells could migrate into the inner wall of airway, which might facilitate the tracheal subepithelial fibrosis [10, 11]. Furthermore, in a rat model of chronic asthma, it has been shown the migration activities of smooth muscle cells were significantly increased, compared with normal rat smooth muscle cells, and it also pointed out that this uncontrolled increase of migration of smooth muscle cells might be one of the critical factors for aggravation of asthma [12]. As a kind of well mature pharmaceutical for asthma treatment, it would be very important to distinguish the effect of two different isomers of albuterol on proliferation and migration activities of airway smooth muscle cells.

In this study, first we probed the different effect of R- and S-albuterol on proliferation of primary rat airway smooth muscle cells; second, the migration activities of primary rat airway smooth muscle cells were measured by migration assay and wound-healing assay with R- and S-albuterol; third, whole tissue culture was also performed to further demonstrate the different effect of R- and S-albuterol on migration of airway smooth muscle cells.

## 2. MATERIALS AND METHODS

### 2.1. Cell Culture

Primary culture of rat airway smooth muscle cells was performed by enzyme digestion. Briefly, adult SD rats (Ace Animals Inc.) were anesthetized by 0.3% Pentobarbital and tracheas were taken out immediately. After removal of blood vessels, connective tissues and cartilage, pure airway smooth muscle tissue was rinsed with PBS twice and cut into small pieces. The whole tissue was digested by 2 ml digestion buffer, including 2 mg/ml collagenase, 1 mg/ml elastase (Sigma) for 2 h, before centrifuge 1000 r/min for 5 min. After rinse with PBS, smooth muscle cell culture media (Cell Application Inc) was mixed and then seeded into 12 well culture plates coating with fibronectin (R & D, Minneapolis). Cells were incubated at 37°C, 5% CO<sub>2</sub> for 10 days and then identified with  $\alpha$ -actin mono-antibody (Sigma).

### 2.2. Immunofluorescence Staining

Cells were seeded on cover-slips coating with human fibronectin (Becton Dickinson), then incubated for 24 h till full spread. After rinse with PBS twice, cells were fixed for 15 min in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 for 5 min. Then cells were blocked by 10% BSA (Sigma) for 1 h and incubated with  $\alpha$ -actin mono-antibody 1 h at 37°C. After rinse with PBS 5 × 5 min, the secondary goat anti-rat antibody was added and incubated for 1h at room temperature. The cover-slips were rinsed with PBS 5 × 5 min and mounted with Mowiol (Sigma). All images were obtained with BioRad Radiance 2000 confocal system and processed with Laser Sharp 2000 BioRad software.

### 2.3. Cell Proliferation Assay

Cell proliferation was measured with CyQUANT<sup>®</sup> Cell Proliferation Assay Kit (Invitrogen). Cells were seeded in human fibronectin coated 96-well plates (Becton Dickinson) with 10<sup>4</sup> cell/well and incubated in full media at 37°C, 5% CO<sub>2</sub> for 48 h. Then, cells were starved with DMEM culture media for 24 h before changing full culture media containing 100 ug/ ml R, S-albuterol (Sepracor Inc., Marlborough, MA) and then incubated for 24 h, 36 h, 48 h, 60 h respectively. 100  $\mu$ l CyQUANT<sup>®</sup> GR dye/cell-lysis buffer was added into each well with 5 min incubation at room temperature. The relative intensity of fluorescence in each well was measured with fluorophotometer (VARIAN Inc) at 480 nm excitation wavelength and 520 nm scattering wavelength respectively.

### 2.4. Transwell Assay

Cell migration was conducted via 24-well transwell plates with 8  $\mu$ m membrane (Becton Dickinson).

The inner and outer chamber membranes were coated with 5 ug/ml human fibronectin. Cells were incubated with 100 ug/ml R, S-albuterol for 48 h, and then seeded in the inner chamber with concentration of  $2 \times 10^4$  cells/well, 800 ul full culture media was added the outer chamber. After incubation for 8 h, the number of total migrated cells was counted in fields randomly chosen from 9 equally divided zone under the microscope.

## 2.5. Wound-Healing Assay

Cells were seeded in 24-well plates till the formation of confluent monolayer. The cell monolayer was scraped in a straight line to create a “scratch” with a 200 ul pipet tip. Debris was removed by washing the cells once with culture medium. Cells were incubated with 100ug/ ml R, S-albuterol respectively. After incubation, the plates were placed under a phase-contrast microscope to acquire the images of the scratch at different time point. All images were processed with photoshop CS4 and the relative migration distance was presented as pixel.

## 2.6. 3D-Tissue Culture Migration Assay

3D-tissue culture migration was measured with Cellmatrix<sup>®</sup> kit (Nitta Gelatin Inc, Japan). Briefly, pure smooth muscle tissue and whole rat airway were cut into small pieces around 1 mm<sup>2</sup>, and embedded into matrigel. All tissue-containing gels were incubated in full culture media with 100 ug/ml R, S-albuterol respectively. After 6 days incubation, the number of migrated cells from the tissue was counted under a phase-contrast microscope.

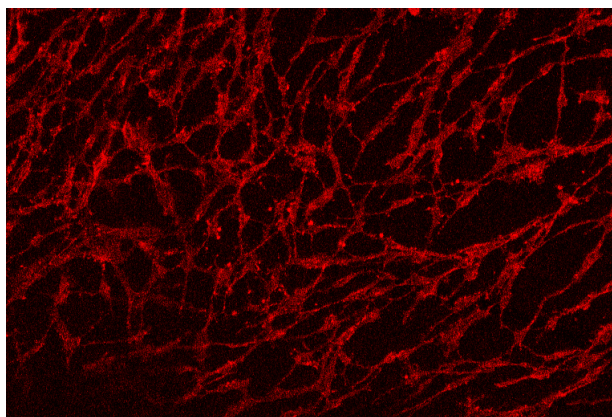
## 2.7. Statistical Analysis

Data were expressed as the mean  $\pm$  SD. Statistical difference between groups was examined using the ANOVA and Student's t-test.  $P < 0.05$  was considered as statistically significant.

# 3. RESULTS

## 3.1. The Effect of R, S-Albuterol on Proliferation of Airway Smooth Muscle Cells

The primary culture cells from rat airway tissue were identified with  $\alpha$ -actin, which is the specific marker of smooth muscle cells. The confocal images showed more than 90% primary culture cells were smooth muscle cells [Figure 1].



**Figure 1.** Identification of primary culture cells with  $\alpha$ -actin. Cells were blocked by 10% BSA for 1 h and incubated with  $\alpha$ -actin mono-antibody 1 h at 37°C and then incubated with the secondary goat anti-rat antibody conjuncting with red fluorescence. Confocal images showed that more 90% cells presented red fluorescence.

Cell proliferation was measured with CyQUANT® Cell Proliferation Assay Kit and the relative intensity of fluorescence in each well presented the actual proliferation activities of the cells. The results showed that both of R, S-albuterol could inhibit the proliferation of smooth muscle cells, compared with the control cells. But, there was no significant difference between R, S-albuterol on inhibition of the cell proliferation [Figure 2].

### 3.2. The Effect of R, S-Albuterol on Migration of Airway Smooth Muscle Cells via Transwell Assay

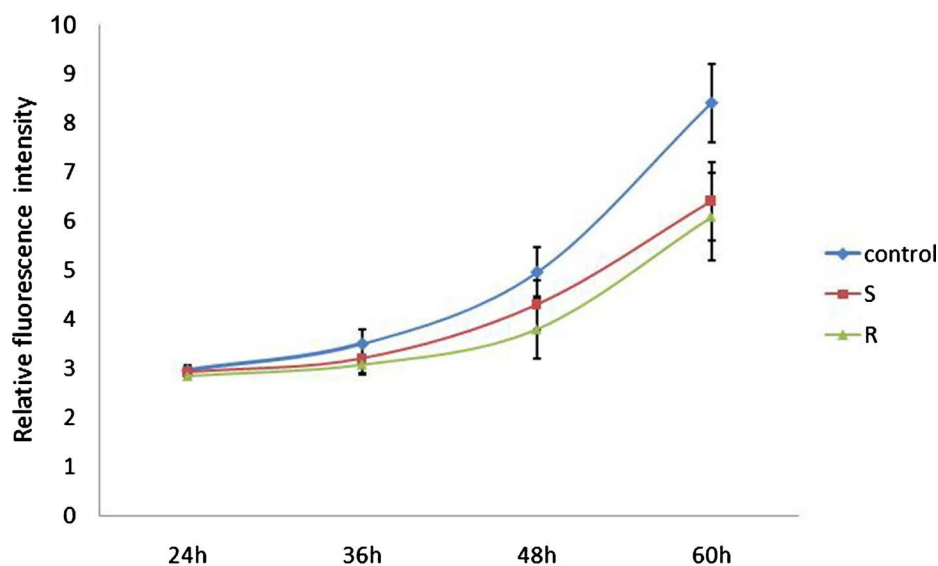
The effect of R, S-albuterol on migration of smooth muscle cells was first measured with transwell assays containing 8  $\mu$ m membranes. The number of migrated cells in the outer chambers presented the relative migration activities, cells without albuterol were set as control. The results showed that there were much less migrated cells on the membrane of R-albuterol chamber [Figure 3(a)] and actual number of migrated cells was significantly lower than S-albuterol and control [Figure 3(b)], S-albuterol showed no significant inhibition on migration of the cells, compared with the control.

### 3.3. The Effect of R, S-Albuterol on Migration of Airway Smooth Muscle Cells via Wound-Healing

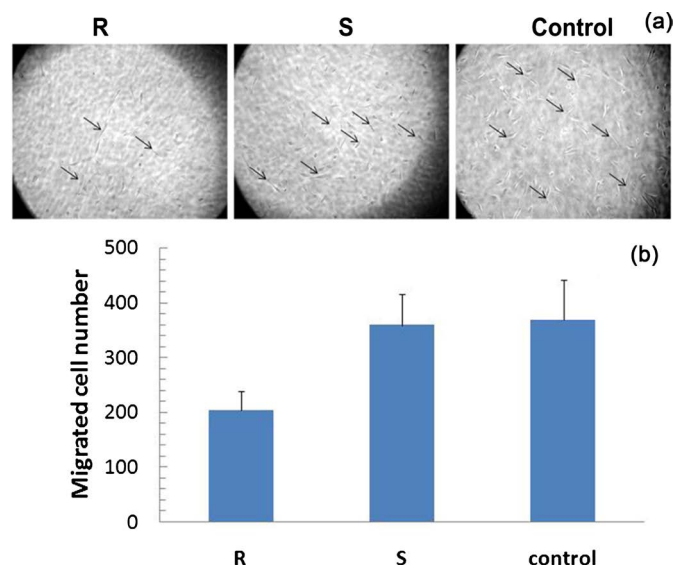
The wound-healing assays were used to further probe the effect of R, S-albuterol on migration of smooth muscle cells. The width of the scratch on the mono-layer of the cells demonstrated the migration activities of cells in presence of R, S-albuterol. Cells without albuterol were set as control. The results showed that cells with R-albuterol migrated much slower than control and S-albuterol seemed to have no difference with control [Figure 4].

### 3.4. The Effect of R, S-Albuterol on Migration of Airway Smooth Muscle Cells via 3D-Tissue Culture Migration Assay

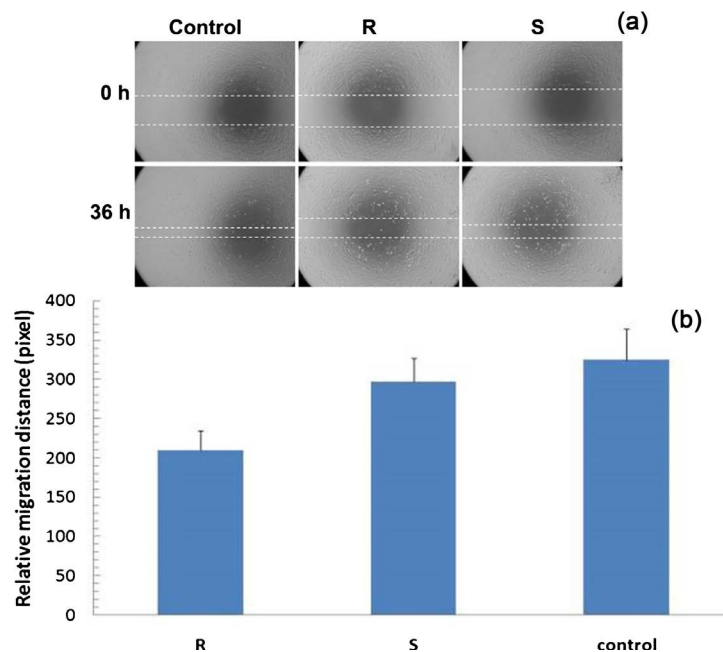
To probe the effect of R, S-albuterol on migration of smooth muscle cells in airway tissues, 3D-tissue culture migration assays were conducted in presence of R, S-albuterol. Pure smooth muscle tissue and whole rat airway were cut into small pieces and incubated with R, S-albuterol. After 6 days incubation, the



**Figure 2.** The effect of R, S-albuterol on proliferation of smooth muscle cells. Cells were starved for 24 h, and then changed into full culture media containing 100  $\mu$ g/ml R, S-albuterol, the control was normal culture cells without albuterol. The proliferation activities were measured at 24 h, 36 h, 48 h and 60 h. The results showed that both R, S-albuterol could inhibit the proliferation of smooth muscle cells, but the inhibition ratio of R, S-albuterol had no significant difference.



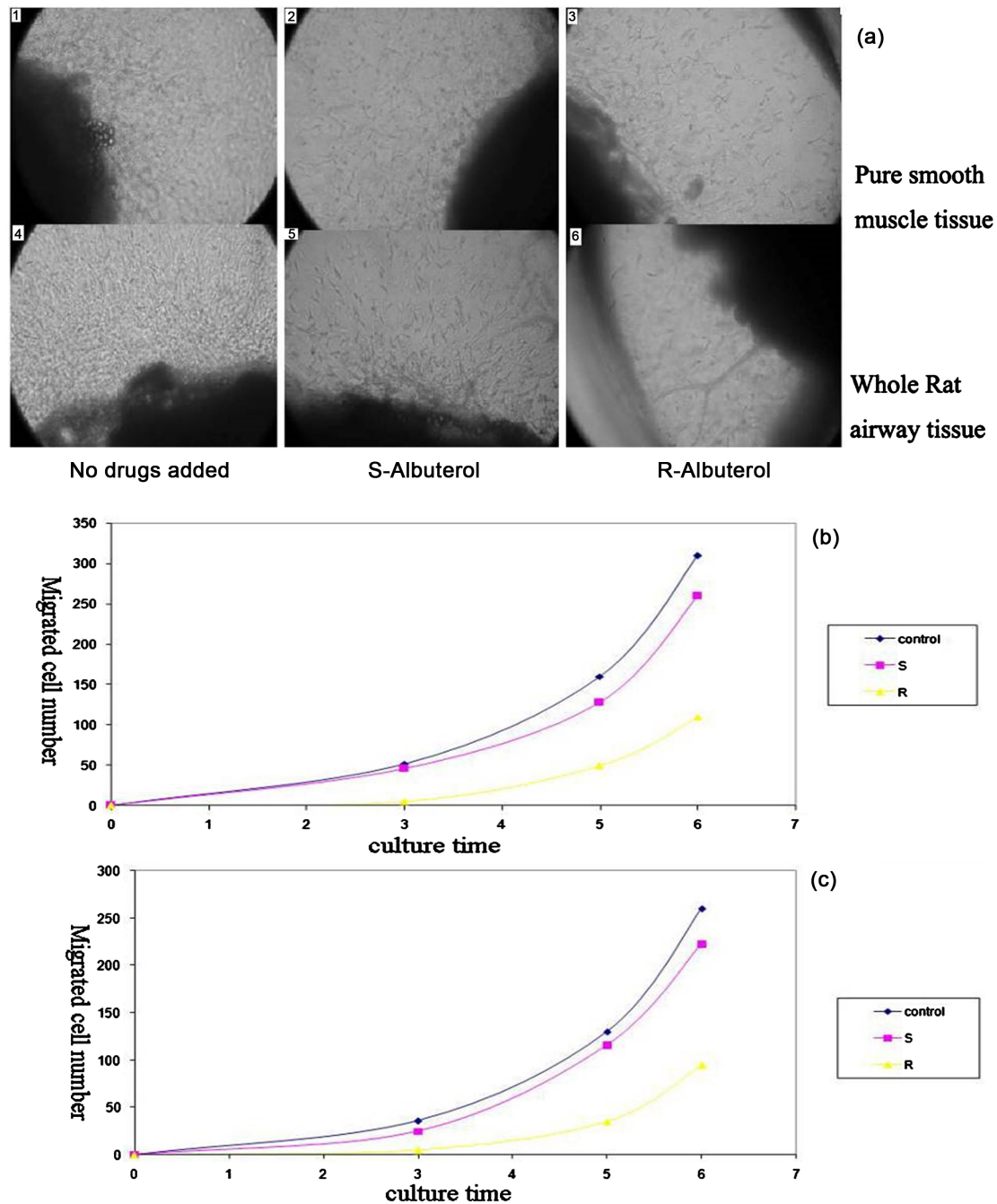
**Figure 3.** The effect of R, S-albuterol on migration of smooth muscle cells via transwell assays. (a) the migrated cells under phase-contrast microscope. there were much less cells on the membrane of R-albuterol chambers compared with S-albuterol and control; (b) the number of migrated cells in presence of R, S-albuterol. R-albuterol showed significant inhibition on migration of smooth muscle cells and the relative migration ratio decreased to 50% of the control cells. S-albuterol showed on significant inhibition of cell migration, compared with control. Black arrows representing the trans-membrane migrated cells.



**Figure 4.** Wound-healing assays of smooth muscle cells in presence of R, S-albuterol. (a) Images of scratch on mono-layer of smooth muscle cells and the parallel dash lines presented the migration gap of the cells. After 36 h incubation, the scratch in R-albuterol was significantly wider than control. There was no significant difference between S-albuterol and control; (b) the relative migration distance of smooth muscle cells in presence of R, S-albuterol.



number of migrated cells from the tissue was counted under a phase-contrast microscope. The results showed that R-albuterol significantly attenuated cells migrating from both pure smooth muscle tissue and whole airway fragments, compared with control cells; S-albuterol slightly decreased the number of migrating cells with much lower inhibition ratio [Figure 5].



**Figure 5.** 3D-tissue culture migration assays of smooth muscle cells in presence of R, S-albuterol. (a) images of smooth muscle cells migrated from the tissues in matrigel. 1 - 3 images present pure smooth muscle tissues and 4 - 6 images present whole rat airway tissues; (b) the number of migrated cells out of pure smooth muscle tissue according to the incubation time; (c) the number of migrated cells out of whole rat airway tissues according to the incubation time.

## 4. RESULTS

Asthma, a chronic disease characterized by airway obstruction, induces substantial morbidity worldwide. Although precise mechanisms facilitating airway obstruction in asthma remain unclear, airway smooth muscle cells seem to play a critical role in disease pathogenesis [6-9]. Airway smooth muscle cell is commonly considered as a potential inflammation cell as well as airway remodeling factor. Recently, people found airway smooth muscle cells presented diverse phenotypes that might induce abnormal proliferation and migration of smooth muscle cells during the pathogenesis of asthma [10-12]. Albuterol is widely used medicine for asthma treatment, because its definite effect on relaxation of bronchial smooth muscle and inhibition on remodeling of airway. Since two isomers of albuterol have differential effects on the contractile properties of airway smooth muscle [5]. It would be interesting to elucidate the different effect of R- and S-albuterol on smooth muscle cells proliferation and migration.

When we probed the difference of R- and S-albuterol on proliferation of airway smooth muscle cells, we found both of two albuterol isomers showed significant inhibition on cell proliferation with no significant difference, which is consistent with previous studies [13]. This phenomenon might be because R- and S-albuterol have the similar effect of cAMP concentration, which would trigger the PKA-cAMP cascade inducing the apoptosis of smooth muscle cells. Abnormal migration of smooth muscle cells is believed to be a critical step in aggression of asthma. First, the transwell assays based on chemotaxis were used to check the difference of R- and S-albuterol on smooth muscle cells migration. The results showed that only R-albuterol presented inhibition on cell migration; Second, to eliminate the crossing-membrane effect, wound-healing assays were also used to distinguish the difference of R- and S-albuterol on migration of smooth muscle cells. The results were much similar with the transwell assays, R-albuterol significantly attenuated cell migration in plate culture condition; Considering the *in vitro* culture condition might facilitate the differentiation of primary smooth muscle cells, we further probed the effect of R- and S-albuterol on migration of smooth muscle cells in 3D tissue culture mode and tried to find out whether any differences between R- and S-albuterol to affect smooth muscle cells migrate out of the tissue. No surprise, only R-albuterol could inhibit smooth muscle cells migrating out of the tissue, including pure smooth muscle and whole airway tissues. It will be very interesting to probe the possible cascades via which albuterol affects the proliferation and migration of airway smooth muscle cells.

## 5. CONCLUSION

This study focused on distinguishing the differences of R- and S-albuterol on proliferation and migration of airway smooth muscle cells. Both of R- and S-albuterol could inhibit the proliferation of smooth muscle cells and the inhibition ratio of these two isomers had no significant difference; R-albuterol, but not S-albuterol, inhibited cell migration. Our findings thus not only shed new light into the mechanism of albuterol's *in vivo* effect, but also suggest potential use of this drug in other types of tissues.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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