Zoledronate inhibits fibroblasts’ proliferation and activation via targeting TGF-β signaling pathway

Background: Previous preclinical and clinical studies have demonstrated that zoledronate might inhibit neointimal hyperplasia at least partly by inhibiting the proliferation, adhesion and migration of vascular smooth muscle cells (VSMCs). However, whether zoledronate influences fibroblasts’ proliferation and activation, which also play a key role in neointimal hyperplasia and vascular remodeling, remains largely unknown. In the present study, the effect of zoledronate on fibroblasts was investigated and the underlying molecular mechanisms were examined.

Methods: After treatment with zoledronate, changes in biological behaviors, including the morphology, proliferation, cell-cycle distribution and migration of fibroblasts (NIH3T3 cells), were observed. The expression of α-SMA, TGF-β1 and TGF-β2 and the level of Smad2/3 phosphorylation in cultured fibroblasts were examined by Western blot. In vivo expression of α-SMA and TGF-β1 was assessed by immunohistochemical staining.

Results: It was shown that the typical fibroblast cell morphology was altered after zoledronate exposure. Cultured fibroblasts treated with zoledronate displayed dose-dependent inhibition of cell proliferation due to cell-cycle arrest in the S phase. Cell migration activities were also dose dependently suppressed by zoledronate treatment. Expression of α-SMA in cultured fibroblasts was significantly reduced by zoledronate treatment. Further analysis showed decreased expression of TGF-β1 and α-SMA by periadventitial delivery of zoledronate in the rat carotid balloon-injury model. The expression of TGF-β1 and TGF-β2 and the phosphorylation of Smad2/3 in cultured fibroblasts were significantly inhibited by zoledronate treatment.

Conclusion: Our findings demonstrated that zoledronate can inhibit the proliferation, migration and activation of fibroblasts via the TGF-β signaling pathway and revealed a novel mechanism of zoledronate action against neointimal hyperplasia.

Keywords: zoledronate, fibroblasts, neointimal hyperplasia, TGF-β pathway

Introduction

Bisphosphonates, which are stable analogs of inorganic pyrophosphate, have been widely used in the treatment of bone metastasis and osteoporosis. They have been reported to inhibit the development of experimental neointimal hyperplasia due to reduced local inflammation by transient systemic inactivation of monocytes and macrophages. However, our previous study demonstrated that zoledronate, a third-generation bisphosphonate, could directly inhibit the proliferation, adhesion and migration of vascular smooth muscle cells (VSMCs). We further discovered that both systemic and periadventitial delivery of zoledronate resulted in a significant inhibition of neointimal hyperplasia in the rat carotid balloon-injury model.

As the predominant resident population of the adventitia, fibroblasts are able to proliferate, migrate, and deposit abundant collagen fibrils and secrete inflammatory cytokines and chemokines when activated. The activated adventitial fibroblasts, also...
known as myofibroblasts, can be identified by increased expression of contractile proteins, such as \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA). Abundant evidence indicates that these resident fibroblasts from the adventitia contribute to neointima formation and vascular remodeling.\(^6,7\)

Since bisphosphonates have been reported to concentrate in adventitia and periadventitial delivery of zoledronate remarkably inhibits neointimal growth in the rat carotid balloon-injury model,\(^5,8\) we hypothesized that the effect of zoledronate on neointimal hyperplasia may result from direct inhibition of fibroblasts. Therefore, in this investigation, we analyzed the impact of zoledronate on fibroblasts and examined possible mechanisms.

Materials and methods

Materials

Zoledronate was kindly provided by Novartis Pharma Stein AG (Stein, Switzerland). Antibodies to glycyraldehyde 3-phosphate dehydrogenase (GAPDH), TGF-\( \beta \)-1, TGF-\( \beta \)-2 and \( \alpha \)-SMA were purchased from Proteintech Group (Wuhan, China). Antibodies to Smad2, Smad3, p-Smad2, p-Smad3 and \( \alpha \)-SMA (Alexa Fluor \( ^6 \) 555) were obtained from Abcam (Shanghai, China). Recombinant human TGF-\( \beta \)-1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). FBS, DMEM, DAPI, Super Signal West Pico Chemiluminescent Substrate and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The 5-ethynyl-2-deoxyuridine (EdU) cell proliferation kit was purchased from Guangzhou Ribobo (Guangzhou, China). Propidium iodide reagent was obtained from Tianjin Sungene Biotech (Tianjin, China). Cell culture plates and transwell plates with 8-\( \mu \)m pore polycarbonate membranes were purchased from Corning Incorporated (Corning, NY, USA). Rat tail tendon collagen type I (5 mg/mL) in 0.006 N acetic acid was purchased from Shengyou Biotechnology Co., Ltd. (Hangzhou, China).

Cell culture

NIH3T3 mouse fibroblasts were purchased from KeyGen Biotech (Nanjing, China). The cells were cultured in complete DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 \( \mu \)g/mL streptomycin. Cultures were maintained at 37\( ^\circ \)C in a humidified 95% air and 5% CO\(_2\) atmosphere. The cells were seeded into the indicated plates for different assays when the cells had attained 80% confluence.

Cell morphology analysis

Approximately 2\( \times \)10\(^4\) NIH3T3 cells were seeded in each well of a 24-well plate and incubated in DMEM containing 10% FBS with increasing concentrations (0–20 \( \mu \)M) of zoledronate for 48 hours. The morphologic changes in fibroblasts were analyzed using an inverted phase-contrast microscope (Olympus IX81; Olympus Corporation, Tokyo, Japan). Cells grown in DMEM with 10% FBS alone served as controls.

Cell proliferation assay

EdU assays were used to assess cell proliferation ability according to the methods we previously described.\(^9\) Briefly, 6\( \times \)10\(^3\) NIH3T3 cells were seeded into each well of 96-well plates and incubated for 8 hours before the experiment. The cells were incubated with different concentrations of zoledronate (0–20 \( \mu \)M) for 48 hours. The culture medium was then removed, and 100 \( \mu \)L of fresh DMEM containing EdU (100 \( \mu \)M) was added to the wells. After a 1.5-hour incubation, the EdU medium was discarded and the fibroblasts were fixed with 4% paraformaldehyde for 20 minutes. The cells were washed twice with glycine for 5 minutes per wash and then stained with Apollo fluorescent azide for 30 minutes and Hoechst stain for 15 minutes. Finally, images were captured using the Olympus IX81 microscope.

Cell-cycle distribution analysis

Approximately 4\( \times \)10\(^4\) NIH3T3 cells were seeded into each well of a six-well plate and incubated for 8 hours. The culture medium was replaced with DMEM without FBS to starve the cells for 12 hours. The cells were then incubated with different concentrations of zoledronate (0–20 \( \mu \)M) for 48 hours before the experiment. The cells were collected and fixed using 70% ethanol at 4\( ^\circ \)C for 10 hours. The ethanol was removed by centrifugation, and the cells were washed twice with PBS before staining. Then, the cells were resuspended in the propidium iodide solution and incubated for 20 minutes in the dark. We analyzed the samples using a flow cytometer (Cell Lab Quanta SC; Beckman Coulter Inc. Brea, CA, USA). The acquired data were further analyzed using ModFit software.

Cell migration assay

The effect of zoledronate on fibroblast migration was measured as described previously using transwell chambers.\(^4\) Briefly, NIH3T3 cells were starved overnight. The cells were then digested and resuspended in 0.5% FBS medium containing different concentrations of zoledronate. Approximately 10\(^4\) cells were seeded into the upper chamber of transwell units, which were placed in 24-well plates, with each well filled with 750 \( \mu \)L cell culture medium with 1% FBS. After incubation for 12 hours, the transwell units were fixed with 4% paraformaldehyde for 20 minutes. Non-migrating cells on the upper surface were gently scraped...
away, and cells that had migrated to the lower surface of the membrane were stained with 0.1% crystal violet dye for 15 minutes at room temperature. Finally, images of the migrated cells were captured using the Olympus IX81 microscope.

**Immunofluorescence assay**

NIH3T3 cells were seeded on cover glasses and incubated in the culture medium containing increasing concentrations (0–20 µM) of zoledronate for 48 hours. The cells were washed with PBS once and fixed with 4% paraformaldehyde for 20 minutes at room temperature. Cells were then treated with 0.1% Triton X-100 for 20 minutes and blocked with 5% BSA for 1 hour. The cells were incubated with α-SMA antibody for 1 hour and DAPI for 20 minutes. Finally, images were obtained using the Olympus IX81 microscope.

**Western blot**

Total protein was extracted using a modified buffer with 0.5% sodium dodecyl sulfate (SDS) containing a protease inhibitor cocktail (Sigma-Aldrich Co., St Louis, MO, USA). Approximately 20 µg of protein were electrophoresed using 10% SDS/polyacrylamide gel electrophoresis (PAGE) gels. The separated proteins were transferred to 0.22 µm polyvinylidene fluoride membranes at 4°C. Nonfat milk (5%) was used to block nonspecific sites for 2 hours at room temperature. The membranes were then incubated with antibodies against GAPDH, α-SMA, TGF-β1, TGF-β2, Smad2, Smad3, p-Smad2 and p-Smad3 at 4°C overnight, followed by HRP-conjugated secondary antibodies for 2 hours. The membranes were washed three times in TBS-T, and bands were identified using enhanced chemiluminescence kits and visualized using the GeneGnome HR Image Capture System (Syngene, Frederick, MD, USA).

**Animals**

Sprague Dawley rats (male, 300–350 g) were purchased from the Shanghai Laboratory Animal Centre of the Chinese Academy of Sciences. Animals were fed in standard pathogen-free conditions. All surgeries were performed after the rats were anesthetized intraperitoneally with 10% chloral hydrate (3 mL/kg). All animal experiments were performed in agreement with the guidelines of Tongji University School of Medicine on the ethical use of animals and were approved by the Biomedical Research Ethics Committee of Tongji University.

**Rat balloon-injury model**

Balloon injury of the right common carotid arterial endothelium was performed as described previously. Briefly, rats were anesthetized intraperitoneally with 10% chloral hydrate (3 mL/kg). A midline incision was made in the neck, and the right common carotid artery was exposed. A small cut was then made in the external carotid artery, and an embolectomy catheter (Fogarty 2F; Edwards Lifesciences, Irvine, CA, USA) was passed into the common carotid artery. The balloon was inflated with water until slight resistance was met to expand the common carotid artery and then slowly twisted back to the carotid bifurcation. This procedure was done three times to achieve complete denudation of the endothelium, including a mild-to-moderate injury of the inner layers of the media smooth muscle cells. Finally, we removed the balloon and ligated the external carotid artery.

**Therapy**

A stock solution (10 mM) of zoledronate was prepared in calcium-free PBS as follows. A 1 mL solution of collagen (4.9 mg/mL) and zoledronate (100 µM) was mixed with 980 µL of 5 mg/mL collagen, 10 µL of 10 mM zoledronate and 6 µL of 1N NaOH. The mixture was kept on ice until ready for use.

Twelve rats were divided into a non-treated group and a local zoledronate-treated group. All rats underwent balloon injury on the right carotid artery. The left uninjured carotid arteries from the non-treated group were considered as normal artery samples. For the local zoledronate-treated group, 200 µL of a mixture of rat tail collagen (4.9 mg/mL) and zoledronate (100 µM) was coated around the adventitia of the injured carotid artery.

**Immunohistochemical staining**

After 21 days of arterial injury, the rats were euthanized after anesthesia with chloral hydrate. The right (injured) and left (uninjured) segments of the common carotid arteries were removed and fixed in 10% buffered formalin. At 24 hours post fixation, the arterial segments were dehydrated, embedded in paraffin and cut into longitudinal sections. The sections were stained with H&E for morphology and TGF-β1 and α-SMA for immunohistochemistry. Histologic changes were evaluated using an Olympus IX81 microscope.

**Statistical analyses**

Data are shown as mean±SD values. Statistical significance was determined by one-way ANOVA and Bonferroni’s post hoc test. P<0.05 was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).
Results

Effect of zoledronate on fibroblast morphology
The morphology of untreated control fibroblasts and fibroblasts exposed to zoledronate at 10 µM or 20 µM was observed under a phase-contrast microscope. Untreated cells were more flattened and spread out with variable cytoplasmic shapes. In contrast, zoledronate-treated cells retracted from the substratum and lost contact with neighboring cells and were more spindle shaped with scant cytoplasm extending into two or more long slender cytoplasmic processes (Figure 1).

Effect of zoledronate on fibroblast proliferation
The effect of increasing concentrations (0–20 µM) of zoledronate on the proliferation of fibroblasts was assessed using EdU cell proliferation assays. There were fewer EdU-positive cells in both zoledronate-treated groups compared with those in the untreated group (Figure 2A). Quantitative analysis showed that 10 and 20 µM of zoledronate decreased EdU-positive fibroblasts by 89.7% and 95.0%, respectively, compared with the control group (Figure 2B).

Effect of zoledronate on fibroblast cell-cycle distribution
To further examine whether the decrease in proliferation of fibroblasts reflected cell-cycle arrest, cell-cycle progression was analyzed by propidium iodide staining and flow cytometry. The control fibroblasts revealed a cell-cycle distribution typical of fast proliferating cells (Figure 3A and D), with an average of 53.6% of cells exhibiting a 2n DNA content (G1 phase), 21.3% of cells featuring a 4n DNA content (G2/M phase) and 25.1% of cells revealing a DNA content between 2 and 4n (S phase). After treatment with 10 or 20 µM zoledronate for 48 hours, the G1 phase occupied only 40.06% and 26.50% of the cell cycle, respectively, while cells in the S phase increased to 32.16% and 48.76%, respectively (Figure 3B–D). The results indicated that zoledronate downmodulated fibroblast proliferation via cell-cycle arrest at the S phase.

Effect of zoledronate on fibroblast migration
The effect of zoledronate on the migration of fibroblasts was measured using transwell migration assays. Treatment with increasing concentrations of zoledronate resulted in a dose-dependent inhibition of fibroblast migration (Figure 4A–C). Quantitative data showed that 10 and 20 µM zoledronate suppressed the migration of fibroblasts by ~47% and ~67%, respectively, compared with the untreated group (Figure 4D).

Effect of zoledronate on fibroblast expression of α-SMA in vitro
Activated fibroblasts accelerate the neointimal hyperplasia process, and increased expression of α-SMA is a major marker of these cells. The data from immunofluorescence assays showed that zoledronate inhibited fibroblast expression of α-SMA in a dose-dependent manner (Figure 5A), which was confirmed using Western blot (Figure 5B and C).

Figure 1  Effect of zoledronate on fibroblast morphology.
Notes: NIH3T3 cells were treated with 0, 10 and 20 µM zoledronate for 48 hours. Morphologic changes in the cells were examined using an inverted phase-contrast microscope. Scale bar: 100 µm.
These in vitro data indicated that zoledronate could inhibit the activation of fibroblasts.

**Effect of zoledronate on fibroblast expression of TGF-β1 and α-SMA in vivo**

The data from the current investigation were consistent with our previous report that zoledronate could improve the histopathological changes and decrease neointima formation in injured arteries. In the non-treated group, the expression of TGF-β1 and α-SMA was significantly higher in injured arteries than in normal artery samples, and periadventitial delivery of zoledronate inhibited the expression of TGF-β1 and α-SMA (Figure 6A). Furthermore, we used Western blot to detect the expression of TGF-β1 and α-SMA. The data were consistent with those of immunohistochemical staining and showed that zoledronate inhibited the expression of TGF-β1 and α-SMA (Figure 6B and C). Thus, zoledronate was associated with a decreased expression of TGF-β1 and α-SMA in injured arteries, consistent with histopathological changes in neointima.

**Effect of zoledronate on TGF-β expression and the TGF-β signaling pathway**

The TGF-β pathway is one of the major signaling pathways that regulate cell growth, differentiation and morphogenesis, which is usually mediated by the Smad pathway. In order to examine the impact of zoledronate on TGF-β expression levels and phosphorylation of Smads in fibroblasts, we analyzed the protein expression levels of TGF-β1, TGF-β2 and p-Smad2/3 protein as well as total levels of Smad2/3 in fibroblasts by Western blot. As shown in Figure 7A and B, protein levels of TGF-β1 and TGF-β2 were downregulated dose dependently in fibroblasts, and phosphorylated forms of Smad2/3 were significantly decreased by zoledronate. Further data showed that zoledronate inhibited the phosphorylation of Smad2/3 induced by TGF-β1 (Figure 7C and D). These data suggested that zoledronate could suppress fibroblast proliferation and activation by inhibiting TGF-β expression levels and the TGF-β/Smad pathway.
Figure 3 Zoledronate induces cell-cycle arrest at S phase.

Notes: Fibroblasts were treated with zoledronate for 48 hours, and cell-cycle distribution was analyzed using flow cytometry. Representative images of cell-cycle distribution in (A) 0 µM, (B) 10 µM and (C) 20 µM groups. (D) The analysis of cell-cycle distribution. n=3. **P<0.001 and ***P<0.0001 vs 0 µM.

Discussion

In the present study, we provide evidence that zoledronate can inhibit the proliferation, migration and activation of fibroblasts via the intervention of the TGF-β signaling pathway. We found that zoledronate induced dramatic morphologic changes in fibroblasts. After 48 hours of zoledronate treatment, cell bodies retracted from the substratum and lost contact with adjacent cells, which appeared to become more spindle shaped. These results confirmed previous published studies showing that zoledronate caused morphologic changes in VSMCs and some cancer cells. These morphological changes might be explained by the fact that zoledronate prevents protein prenylation of a wide variety of small G-proteins, including Ras, Rac, Rab and Rho, which are associated with cytoskeleton organization.

In our study, zoledronate induced significant antiproliferative effects in the range of 10–20 µM. Similar concentrations (typically 10–50 µM) of zoledronate have been utilized elsewhere to produce antiproliferative effects against VSMCs and some cancer cells. However, the peak serum concentration of zoledronate following administration of a 4 mg dose ranges only from 1 to 3 µM. The primary concern is that the doses required to inhibit cell growth may not be achieved in vivo using current treatment regimens. This concern has led us to the concept of local drug delivery where higher tissue concentrations of a drug could be achieved while minimizing the likelihood of systemic side effects. Our previous work demonstrated that periadventitial delivery of zoledronate could remarkably improve histopathological changes and decrease neointima formation in injured arteries.
To further examine whether the decrease in cell proliferation reflected cell-cycle arrest, cell-cycle progression was analyzed by flow cytometry. The results revealed an accumulation of cells in the S phase after zolendronate treatment, which indicated that zolendronate inhibited fibroblast proliferation, at least in part, by arresting the cell cycle at the S phase. This effect has also been observed in previous studies on several tumor cells and oral keratinocytes after zolendronate exposure.\(^{18-20}\) Thus, cell-cycle arrest at the S phase may be a common cellular response induced by zolendronate. However, the detailed mechanism underlying regulation of the cell cycle at the S phase remains to be explored.

It is well known that \(\alpha\)-SMA is the marker of activated myofibroblasts, which can proliferate, migrate, deposit extracellular matrix and secrete inflammatory cytokines. To evaluate the effect of zolendronate on fibroblast activation and differentiation, we assessed the expression of \(\alpha\)-SMA by immunofluorescence staining and Western blot. The results showed that zolendronate suppressed \(\alpha\)-SMA protein levels dose dependently. Furthermore, \(\alpha\)-SMA expression in balloon-injured arteries after 21 days decreased significantly with the periadventitial delivery of zolendronate in rats. Therefore, our in vitro and in vivo results indicate that zolendronate may reduce neointima formation by inhibiting the activation and differentiation of fibroblasts.

Transwell assay results showed that zolendronate inhibited the migration of fibroblasts in a dose-dependent manner. Previous studies have similarly shown the inhibitory effects of zolendronate on the migration of various cell types.\(^{4,21-23}\) As mentioned earlier, nitrogen-containing bisphosphonates can prevent protein prenylation of a wide variety of small G-proteins, including Ras, Rac, Rab and Rho, which are associated with cell migration. Our previous study and other studies showed that zolendronate inhibited cell migration by decreasing phosphorylation of focal adhesion kinase,\(^4\) suppressing prenylation-dependent RhoA/ROCK signaling pathway\(^{21}\) and downregulating expressions of vascular endothelial growth factor and matrix metalloproteinases.\(^{22}\) In agreement with our results, zolendronate was also indicated to inhibit TGF-\(\beta\)1-induced
cell migration through the Smad pathway in human gingival fibroblasts.23

TGF-β is regarded as a key fibrogenic cytokine and can drive fibroblasts to myofibroblast differentiation.24,25 The TGF-β pathway is one of the major signaling pathways that regulates cell growth, differentiation, migration and extracellular matrix production,26,27 which is usually mediated through its transmembrane receptor and subsequent activation of cytoplasmic Smad proteins.28 Meanwhile, the expression of α-SMA is regulated by the TGF-β pathway.29,30 In order to examine the impact of zoledronate on TGF-β expression levels and TGF-β downstream signaling

Figure 5 Effect of zoledronate on the expression of α-SMA in vitro.
Notes: Fibroblasts were treated with zoledronate for 48 hours, and the expression of α-SMA was examined using immunofluorescence staining and Western blot. (A) Representative images of the expression of α-SMA in different groups by immunofluorescence staining. Scale bar: 100 µm. (B) Representative image of α-SMA expression by Western blot. (C) Statistical analysis of α-SMA protein expression. *P<0.05 and ***P<0.001 vs 0 µM.

Figure 6 (Continued)
**Figure 6** Effect of zoledronate on the expression of TGF-β1 and α-SMA in vivo.

Notes: The histopathological changes and expression of TGF-β1 and α-SMA were examined by immunohistochemical staining 21 days after balloon injury. Periadventitial delivery of zoledronate improved the histopathological changes and decreased neointima formation in injured arteries. The expression values of TGF-β1 and α-SMA were both significantly inhibited by local delivery of zoledronate. (A) Representative images of TGF-β1 and α-SMA expression in different groups. Scale bar: 200 µm. (B) Expression of TGF-β1 and α-SMA in different groups detected using Western blot. (C) Statistical analysis of the results of Western blots. n=3. *P<0.05 and **P<0.01 vs control.

**Figure 7** Effects of zoledronate on TGF-β expression and the TGF-β signaling pathway.

Notes: Fibroblasts were treated with zoledronate for 48 hours, and protein expression levels were detected using Western blot. The protein levels of TGF-β1 and TGF-β2 were downregulated in fibroblasts dose dependently, and the phosphorylated forms of Smad2 and Smad3 were significantly decreased by zoledronate. Meanwhile, fibroblasts were stimulated using TGF-β1 (5 ng/mL) for 30 or 60 minutes with or without zoledronate, respectively. The phosphorylated forms of Smad2 and Smad3 were significantly decreased in the zoledronate-treated group. (A) Representative images of expression of TGF-β1, TGF-β2, Smad2, Smad3, p-Smad2 and p-Smad3 in different groups. Scale bar: 200 µm. (B) Statistical analysis of the results of Western blots. n=3. *P<0.05 and **P<0.01 vs control.
molecules in fibroblasts, we analyzed the protein expression levels of TGF-β1 and TGF-β2 and the level of Smad2/3 phosphorylation in fibroblasts by Western blot. The results showed that protein levels of TGF-β1 and TGF-β2 were downregulated in fibroblasts dose dependently, and the phosphorylation of Smad2/3 was significantly inhibited by zoledronate. These data suggest that zoledronate may suppress fibroblast proliferation, migration and activation by inhibiting TGF-β expression levels and the TGF-β/Smad signaling pathway.

In this work, several aspects of the molecular mechanism of zoledronate action on fibroblasts have been explored. However, in addition to the TGF-β/Smad signaling pathway, other signaling pathways including the MAPK, PI3K and JNK pathways are also involved in fibroblast activation and differentiation. Therefore, it will be necessary to evaluate the influence of zoledronate on these pathways as well.

Conclusion

Our findings demonstrate that zoledronate can change the morphology of fibroblasts and inhibit their proliferation, migration and activation via inhibition of TGF-β expression levels and intervention in the TGF-β signaling pathway. These results reveal a novel mechanism of bisphosphonates against neointimal hyperplasia and shed new light on the therapeutic role of zoledronate in the treatment of neointimal hyperplasia.

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Disclosure

The authors report no conflicts of interest in this work.

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