

## The Mechanism Involved in the Inhibitory Action of Tranilast on Collagen Biosynthesis of Keloid Fibroblasts

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**ABSTRACT**—Tranilast, an anti-allergic drug inhibiting the release of substances such as histamine and prostaglandins from mast cells, was previously reported to suppress collagen synthesis of fibroblasts derived from keloid tissues. However, the inhibitory mechanism on collagen synthesis is unknown. We studied its inhibitory mechanism on collagen synthesis by culturing fibroblasts from keloid and hypertrophic scar tissues of humans. Collagen synthesis of fibroblasts from keloid and hypertrophic scar tissue is greater than that from healthy human skin. Tranilast (3–100  $\mu$ M) did not inhibit prolyl hydroxylase (the rate-limiting enzyme in collagen synthesis) activity. Tranilast (3–300  $\mu$ M) suppressed the collagen synthesis of fibroblasts from keloid and hypertrophic scar tissue but not healthy skin fibroblasts. Tranilast (30–300  $\mu$ M) inhibited the release of transforming growth factor (TGF)- $\beta_1$  from keloid fibroblasts, which enhances the collagen synthesis of keloid fibroblasts. Anti-TGF- $\beta_1$  antibody (50  $\mu$ l/ml) inhibited the collagen synthesis, although diphenhydramine (10  $\mu$ M) and indomethacin (10  $\mu$ M) did not show any inhibition. These results suggest that tranilast inhibits collagen synthesis of fibroblasts from keloid and hypertrophic scar tissue through suppressing the release of TGF- $\beta_1$  from the fibroblasts themselves.

**Keywords:** Tranilast, Collagen synthesis, Keloid fibroblast, Transforming growth factor (TGF)- $\beta_1$ , Cytokine

Hypertrophic scar and keloid are clinically intractable diseases that cause disfigurement, itching and pain. The etiology of these diseases is not known with certainty. During the granulation period in the process of healing at injured sites such as surgical wounds, burns and so on, abnormal proliferation of fibroblasts and production of collagen result in keloids with the accumulation of excessive collagen (1–3).

It is well-known that tranilast is a useful drug for improving bronchial asthma, atopic dermatitis and allergic rhinitis. Several reports have shown that tranilast can prevent or act to improve keloid and hypertrophic scars (4, 5). Tranilast decreases the weight of granulation and inhibits the collagen synthesis by human keloid tissues transplanted into the backs of mice and by carrageenin-induced granulation-tissues in rats (6–8). Tranilast specifically suppresses the collagen synthesis rather than the cell proliferation by cultured fibroblasts derived from human keloid tissues (7). On the other hand, triamcinolone, a synthetic steroid, can suppress

the cell proliferation and collagen synthesis of fibroblasts (8, 9). For these reasons, attention has been paid to the fact that the inhibitory mechanisms of tranilast differs from that of triamcinolone. In general, the inhibition of protein synthesis by corticosteroids has been considered the reason for decreased collagen synthesis in dermal tissue. However, the precise mechanism for the action of tranilast on the collagen synthesis is yet unknown.

In the present study, we studied the suppressive mechanism of tranilast on the collagen synthesis by cultured fibroblasts from keloid tissues, and we estimated the effect of tranilast on prolyl hydroxylase (the rate-limiting enzyme in collagen synthesis) activity.

### MATERIALS AND METHODS

#### Drugs

Tranilast (*N*-(3,4-dimethoxycinnamoyl) anthranilic acid) was synthesized at the Kissei Pharmaceutical

Company (Matsumoto). Triamcinolone acetonide and diphenhydramine hydrochloride were obtained from Wako Pure Chemicals (Osaka); ethyl-3,4-dihydroxybenzoate, 2,2-bipyridyl and indomethacin were from Sigma Company (St. Louis, MO, U.S.A.); and anti-transforming growth factor (TGF)- $\beta_1$  antibody was from King Brewing (Kakogawa).

Tranilast was dissolved in 1% NaHCO<sub>3</sub>, and the other drugs were dissolved in 99.8% ethanol. Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin (BSA) (0.1% BSA/DMEM) or DMEM containing 10% fetal bovine serum (FBS) (10% FBS/DMEM). All test drugs were diluted to the desired concentration and sterilized through a membrane filter. The final concentration of ethanol was less than 1%, which did not affect the proliferation and collagen synthesis of the fibroblasts.

#### *Collagen synthesis of fibroblasts*

**Cell culture of fibroblasts:** Keloid, hypertrophic scar and healthy skin in the vicinity of keloid tissues from each human subject were obtained surgically and aseptically sliced into fine pieces with ophthalmic scissors. Four or five slices were left on a 35-mm plastic plate (Becton Dickinson Co., Ltd., Lincoln Park, NJ, U.S.A.). The primary culture of fibroblasts was performed at 37°C in the 20% FBS/DMEM, penicillin (50 IU/ml), streptomycin (50  $\mu$ g/ml) and kanamycin (100  $\mu$ g/ml) under a humidified atmosphere of 5% CO<sub>2</sub> in air. The culture medium was renewed every 3–4 days. Confluent cells were detached from the plates by the addition of 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). No difference in morphologic features and purity of cells was observed among the cultured fibroblasts of keloid, hypertrophic scar and healthy skin. The cultured cells from the 6th to 8th passage in 10% FBS/DMEM were used for the following experiments.

**Collagen synthesis of fibroblasts in culture:** Fibroblasts ( $4 \times 10^5$  cells) derived from keloid, hypertrophic scar and healthy skin were disseminated into 25-cm<sup>2</sup> tissue culture flasks (Costar, Cambridge, MA, U.S.A.) and cultured for 6 days in 10% FBS/DMEM. After they reached confluence, the cells were rinsed three times with calcium- and magnesium-free phosphate-buffered saline (PBS), and the medium was replaced with 0.1% BSA/DMEM. Collagen synthesis of keloid fibroblasts was induced in the presence of 0.1% BSA/DMEM, tranilast, triamcinolone, anti-TGF- $\beta_1$  antibody, diphenhydramine or indomethacin. After the addition of *l*-ascorbic acid (50  $\mu$ g/ml), the incubation was continued for 1 hr. The culture medium was replaced with 0.1% BSA/DMEM containing  $\beta$ -aminopropionitrile (100

$\mu$ g/ml) and 37 kBq/ml of <sup>3</sup>H-proline (NEN), and the cultivation was maintained for 6 hr. Ten percent trichloroacetic acid and 0.5% tannic acid were added to the culture medium or to the cell homogenate, and the mixture was allowed to stand for 10 min. According to the bacterial collagenase digestion method of Peterkofsky and Diegelmann (10), the radioactivities of collagenase-sensitive protein (collagen) and collagenase-insensitive protein (non-collagen) were measured. The relative synthetic activity of collagen was calculated by the following formula:

$$\text{Relative synthetic activity of collagen (\%)} = \frac{\text{collagen (dpm/\mu g cell protein)}}{\text{collagen (dpm/\mu g cell protein)} + 5.4 \times \text{non-collagen (dpm/\mu g cell protein)}} \times 100$$

The amount of protein in the cell homogenate was determined by the Bio-Rad protein assay.

**Proliferation of fibroblasts:** Fibroblasts ( $10^5$  cells) derived from keloid, hypertrophic scar and healthy skin were suspended in 10% FBS/DMEM and disseminated into a 25-cm<sup>2</sup> culture flask (Costar). After the culture for 24 hr at 37°C, the medium was replaced with 10% FBS/DMEM containing tranilast or triamcinolone at various concentrations, and cultivation was continued for 24 hr. Then, the cells were treated with 0.25% trypsin–0.02% EDTA, and the number of cells was counted under a microscope after staining with 0.3% trypan blue. Cell viability was 94% and more.

#### *Assay of TGF- $\beta_1$ release from keloid fibroblasts in culture*

Fibroblasts ( $4 \times 10^5$  cells) of the 6th to 8th-passages were disseminated into a 25-cm<sup>2</sup> culture flask (Costar) and cultured for 6 days. When the cultured fibroblasts were brought to confluence, 0.1% BSA/DMEM containing the drug tested was added to the culture flask. The supernatant was collected after culturing for 48 hr. TGF- $\beta_1$  in the supernatant was determined by the enzyme-immunoassay. The amount of protein in the cell homogenates was determined by Bio-Rad protein assay.

#### *Assay of prolyl hydroxylase activity*

Prolyl hydroxylase was extracted from 13th-day chicken embryos according to the method of Tuderman et al. (11). The enzyme was partially purified by means of Sepharose-4B-poly-L-proline affinity column chromatography. Prolyl hydroxylase activity was measured by the method of Rhoads and Udenfriend (12). Prolyl hydroxylase solution (100  $\mu$ l) and the drug tested were added to 6 mM Tris-HCl buffer (pH 7.5) containing 2-oxo [1-<sup>14</sup>C]-glutarate ferrous sulfate (20  $\mu$ M), ascorbic

acid (200  $\mu$ M), dithiothreitol (10  $\mu$ M), catalase (2 mg/ml) and BSA (2 mg/ml). The mixtures were incubated for 30 min at 37°C with bubbling mixed gas of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The reaction was stopped by adding 10% HClO<sub>4</sub>, and the mixture was allowed to stand for 1 hr in order to ensure the complete production of [<sup>14</sup>C]-CO<sub>2</sub>. The activity of prolyl hydroxylase was estimated from the amount of [<sup>14</sup>C]-CO<sub>2</sub> produced.

#### Statistical analysis

The results obtained were expressed as the mean  $\pm$  S.E. The Student's *t*-test for paired observations was used to test for statistical significance.

## RESULTS

#### Effect on collagen synthesis of keloid, hypertrophic scar and normal skin fibroblasts

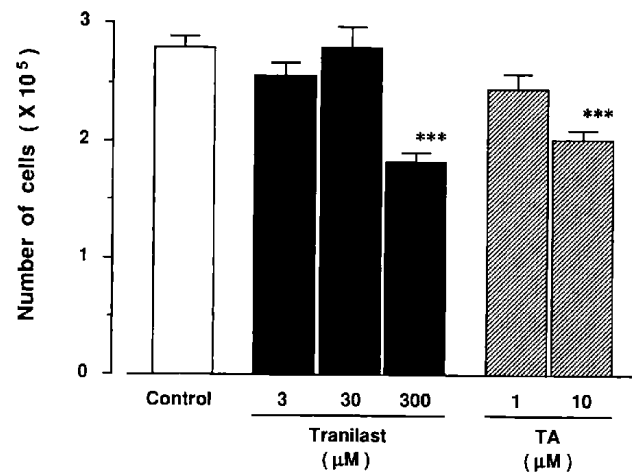
Tranilast (3–300  $\mu$ M) suppressed the relative synthetic activity of collagen of keloid and hypertrophic scar fibroblasts, while the agent did not affect the synthesis by normal skin fibroblasts (Table 1). On the other hand, triamcinolone inhibited the collagen synthesis by fibroblasts derived from keloids, hypertrophic scars and normal skin.

Triamcinolone suppressed the cell proliferation of fibroblasts. However, tranilast exhibited the inhibition of cell proliferation at the highest concentration of 300  $\mu$ M (Fig. 1).

The collagen synthesis by keloid fibroblasts in culture was suppressed by anti-TGF- $\beta$ <sub>1</sub> antibody. However, diphenhydramine and indomethacin showed no effect on the collagen synthesis of keloid fibroblasts (Fig. 2).

#### Effect on chemical mediator release from keloid fibroblasts

To evaluate whether keloid and hypertrophic scar fibroblasts produce chemical mediators that accelerate the collagen synthesis by fibroblasts, the supernatant of cultured keloid fibroblasts was added to fibroblasts derived from healthy human skin. As shown in Fig. 3, the amount of collagen synthesis of healthy fibroblasts was increased by the addition of the medium supernatant from cultured keloid fibroblasts. The collagen synthesis by the supernatant was inhibited when the keloid fibroblasts had been incubated with tranilast (30–300  $\mu$ M); the inhibition by tranilast was dose-dependent.



**Fig. 1.** Effect of tranilast and triamcinolone acetate (TA) on the cell proliferation of hypertrophic scar fibroblasts in culture. The cell counts of fibroblasts were determined after the incubation for 24 hr. Each column expresses the mean  $\pm$  S.E. of 5–7 experiments. \*\*\*: Significantly different from the control at  $P < 0.001$ .

**Table 1.** Effect of tranilast and triamcinolone acetate (TA) on relative collagen synthetic activity of keloid, hypertrophic scar and healthy skin fibroblasts in culture

Drugs	Concentration ( $\mu$ M)	Relative synthetic activity of collagen <sup>a)</sup>		
		keloid	Fibroblasts of hypertrophic scar	healthy skin
Control	—	10.54 $\pm$ 1.60	10.53 $\pm$ 0.23	3.95 $\pm$ 0.22
Tranilast	3	6.63 $\pm$ 0.48*	9.08 $\pm$ 0.55*	3.69 $\pm$ 0.04
	30	6.25 $\pm$ 0.29*	8.50 $\pm$ 0.30***	3.57 $\pm$ 0.21
	300	6.37 $\pm$ 0.25*	7.92 $\pm$ 0.14***	4.54 $\pm$ 0.26
	300	6.37 $\pm$ 0.25*	7.92 $\pm$ 0.14***	4.54 $\pm$ 0.26
TA	1	5.16 $\pm$ 0.15**	8.78 $\pm$ 0.64*	2.91 $\pm$ 0.24*
	10	5.97 $\pm$ 0.48*	8.17 $\pm$ 0.53**	3.13 $\pm$ 0.19*

Each value indicates the mean  $\pm$  S.E. of 3–5 experiments. <sup>a)</sup>: Collagen/Total protein (%). \*, \*\* and \*\*\*: Significantly different from the control at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

### Effect on the release of TGF- $\beta_1$ from fibroblasts

As shown in Fig. 4, the effect of tranilast and triamcinolone on the release of TGF- $\beta_1$  from keloid fibroblasts in vitro was examined. Tranilast at 30–300  $\mu$ M suppressed TGF- $\beta_1$  release. On the other hand, triamcinolone showed a weak inhibition of TGF- $\beta_1$  release.

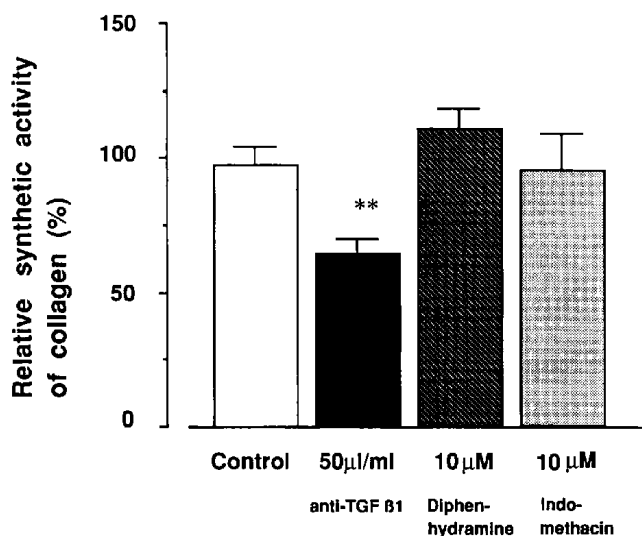


Fig. 2. Effect of anti-TGF- $\beta_1$  antibody, diphenhydramine and indomethacin on collagen synthesis of keloid fibroblasts in culture. Each column expresses the mean  $\pm$  S.E. for 5–11 experiments. \*\*: Significantly different from the control at  $P < 0.01$ .

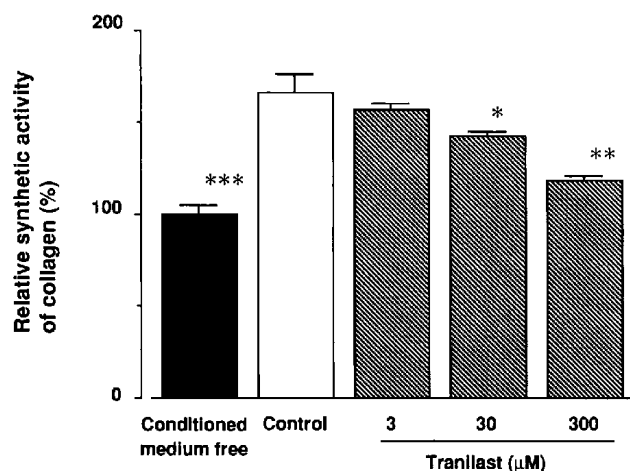


Fig. 3. Effect of tranilast on the release of chemical mediator enhancing collagen synthesis of healthy skin fibroblasts from keloid fibroblasts in culture. Each column indicates the percent of collagen synthesis of healthy skin fibroblasts and the mean  $\pm$  S.E. for 3–5 experiments. \*, \*\* and \*\*\*: Significantly different from the control at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

### Effect on prolyl hydroxylase

Tranilast at concentrations of 3–100  $\mu$ M had no effect on the partially purified prolyl hydroxylase activity, but it was inhibitory at 300  $\mu$ M. Ethyl-3,4-dihydroxybenzoate and 2,2-bipyridyl inhibited the enzyme activity (Table 2).

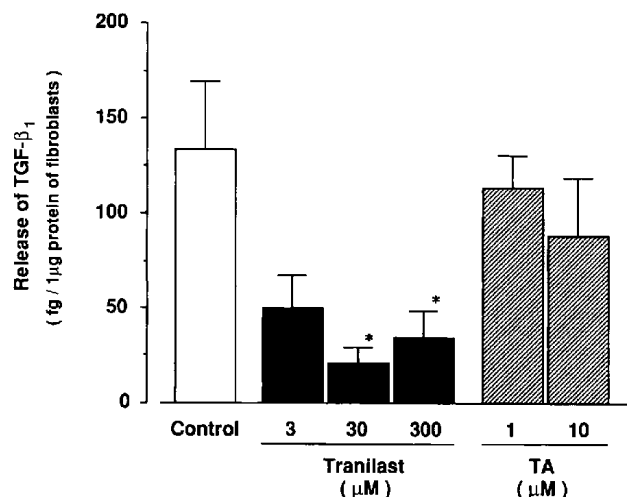


Fig. 4. Effect of tranilast and trimacinolone acetone (TA) on the release of TGF- $\beta_1$  from keloid fibroblasts in culture. Each column expresses the mean  $\pm$  S.E. for 4–5 experiments. \*: Significantly different from the control at  $P < 0.05$ .

Table 2. Effect of tranilast, ethyl-3,4-dihydroxybenzoate (DHB) and 2,2-bipyridyl on purified prolyl hydroxylase from chick embryos

Drugs	Concentration ( $\mu$ M)	Prolyl hydroxylase activity (%)
Control	—	100
Tranilast	3	104.7 $\pm$ 2.32
	30	103.8 $\pm$ 2.01
	100	104.2 $\pm$ 3.73
	300	66.4 $\pm$ 3.05**
	300	62.0 $\pm$ 0.73***
Ethyl-3,4-DHB	300	15.5 $\pm$ 0.47***
	300	37.9 $\pm$ 0.36***

Each value is expressed as the percent of activity in the control drug-free incubation and shows the mean  $\pm$  S.E. for 4 experiments. \*\* and \*\*\*: Significantly different from the control at  $P < 0.01$  and  $P < 0.001$ , respectively.

## DISCUSSION

Keloid and hypertrophic scar are caused by abnormal proliferation of fibroblasts and excessive accumulation of collagen during the process of wound healing. In this study, tranilast suppressed the collagen synthesis of cultured fibroblasts derived from keloid and hypertrophic scar; however, it had no effect on that of healthy skin fibroblasts. Cell proliferation of fibroblasts was inhibited by tranilast only at the highest concentration. On the other hand, triamcinolone, an inhibitor of protein synthesis, suppressed the collagen synthesis as well as the cell proliferation of fibroblasts derived from keloid, hypertrophic scar and healthy skin of humans. Furthermore, tranilast showed no effect on the production of non-collagen protein and glycosaminoglycan, which are components of the extracellular matrix in keloid and hypertrophic scar (7). These facts support that tranilast, unlike triamcinolone, shows specific inhibition of the collagen synthesis of fibroblasts derived from keloid and hypertrophic scar.

Collagen synthesis in keloid and hypertrophic scar is significantly greater than in healthy skin. In this study, we found that the collagen synthesis of cultured fibroblasts in keloid and hypertrophic scar was about three times greater than that of healthy skin fibroblasts. Biochemical differences have been found among keloid, abnormal scar, mature scar, and healthy skin (13). Prolyl hydroxylase participates in the collagen synthesis, and the activity correlates well with the rate of collagen synthesis. Prolyl hydroxylase activity is higher in keloid than in healthy skin, resulting in the accumulation of excess collagen in keloid tissues (14). In this study, tranilast showed a specific inhibition on the collagen synthesis of fibroblasts derived from keloid and hypertrophic scar. So, we made a preliminary investigation of whether or not tranilast prevented the prolyl hydroxylase activity. However, tranilast inhibited the activity of prolyl hydroxylase only at a higher concentration (300  $\mu$ M). Therefore, it is unlikely that tranilast suppresses the collagen synthesis of fibroblasts through the inhibition of the prolyl hydroxylase activity, the rate-limiting enzyme in collagen biosynthesis.

As the causes of keloid and hypertrophic scar, fibroblasts, in addition to other cells such as mast cells, lymphocytes, monocytes and macrophages are known to be involved in chronic inflammatory reactions. Various chemical mediators produced by these cells play important roles in regulating the acceleration or inhibition of chemotaxis, proliferation and collagen synthesis of fibroblasts (15). Tranilast suppresses the release of chemical mediators such as histamine, leukotrienes, prostaglandins and PAF, which are produced from mast cells

and basophils during antigen-antibody reactions (16–19). On the other hand, there are few reports on the inhibitory activity of tranilast on the production of cytokines. Yanagi et al. (20) reported that tranilast inhibits the production of IL-1 and IL-2, regulating cell proliferation from human macrophages and T-cells.

This study showed that a chemical mediator which promotes the collagen synthesis of fibroblasts was produced by cultured keloid fibroblasts themselves. Anti-TGF- $\beta_1$  antibody suppressed the collagen synthesis of keloid fibroblasts and TGF- $\beta_1$  was found in the supernatant of keloid fibroblasts. TGF- $\beta_1$  is found initially from blood platelets, but it is also produced from monocytes and macrophages and is known as a regulatory factor of cell proliferation (21). This study showed that TGF- $\beta_1$  was produced by the fibroblasts. It has been reported that, although TGF- $\beta_1$  enhances both fibroblast proliferation and collagen synthesis, the latter action is stronger (22, 23). Furthermore, we demonstrated that TGF- $\beta_1$  enhanced the collagen synthesis by fibroblasts from human skin (data not shown). These facts demonstrate that TGF- $\beta_1$  released from keloid fibroblasts themselves stimulates the collagen synthesis of keloid fibroblasts. Tranilast inhibited the release of TGF- $\beta_1$  from fibroblasts derived from keloids. If we consider this in connection with the facts described above, the inhibitory mechanism of tranilast on the collagen synthesis of keloid fibroblasts would be due to its suppression of the release of TGF- $\beta_1$  from the fibroblasts themselves. On the other hand, triamcinolone did not suppress the release of TGF- $\beta_1$  from fibroblasts. The difference between the inhibitory effect of tranilast and triamcinolone on TGF- $\beta_1$  release from fibroblasts would reflect their different mechanisms of their inhibitory action on collagen synthesis.

Prostaglandins are important chemical mediators released from various inflammatory cells and are known as factors regulating the proliferation of fibroblasts (24). Attention has been focused on the involvement of histamine released from mast cells, since mast cells are found in the tissues of hypertrophic scar and keloid, and histamine enhances the growth of fibroblasts (25–27). However, the collagen synthesis of keloid fibroblasts was not suppressed by diphenhydramine or indomethacin. From these results, there is no possibility that prostaglandins and histamine play roles in accelerating the collagen synthesis of keloid fibroblasts *in vitro*.

In conclusion, in this study, tranilast inhibited the collagen synthesis by fibroblasts from keloid and hypertrophic scar and the release of TGF- $\beta_1$  that enhances the collagen synthesis from keloid fibroblasts. From these facts, the inhibitory effect of tranilast on the col-

lagen synthesis by fibroblasts would be related to the inhibition of TGF- $\beta_1$  release from fibroblasts themselves.

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