Vaticaffinol, a resveratrol tetramer, exerts more preferable immunosuppressive activity than its precursor in vitro and in vivo through multiple aspects against activated T lymphocytes

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A B S T R A C T

In the present study, we aimed to investigate the immunosuppressive activity of vaticaffinol, a resveratrol tetramer isolated from Vatica mangachapoi, on T lymphocytes both in vitro and in vivo, and further explored its potential molecular mechanism. Resveratrol had a wide spectrum of healthy beneficial effects with multiple targets. Interestingly, its tetramer, vaticaffinol, exerted more intensive immunosuppressive activity than resveratrol. Vaticaffinol significantly inhibited T cells proliferation activated by concanavalin A (Con A) or anti-CD3 plus anti-CD28 in a dose- and time-dependent manner. It also induced Con A-activated T cells undergoing apoptosis through mitochondrial pathway. Moreover, this compound prevented cells from entering S phase and G2/M phase during T cells activation. In addition, vaticaffinol inhibited ERK and AKT signaling pathways in Con A-activated T cells. Furthermore, vaticaffinol significantly ameliorated ear swelling in a mouse model of picryl chloride-induced ear contact dermatitis in vivo. In most of the aforementioned experiments, however, resveratrol had only slight effects on the inhibition of T lymphocytes compared with vaticaffinol. Taken together, our findings suggest that vaticaffinol exerts more preferable immunosuppressive activity than its precursor resveratrol both in vitro and in vivo by affecting multiple targets against activated T cells.

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Introduction

Immunosuppressants are usually used for the treatment of a variety of immunologic disorders such as transplant rejection, rheumatic arthritis, systemic lupus erythematosus and multiple sclerosis. However, most of the immunosuppressive agents currently available, including glucocorticoids, cyclophosphamide and even cyclosporine A, have been reported to inevitably possess severe side effects primarily owing to poor selectivity (Hackstein and Thomson, 2004; Halloran, 2004; Kahan, 2003). Therefore, there is an urgent need for new potent immunosuppressive agents with negligible or acceptable toxicity.

Plant-derived natural products occupy a very important position in the area of drug discovery. Molecules such as paclitaxel, camptothecin, aristemisinin, triptolide and curcumin are invaluable contributions of nature to modern medicine (Corson and Crews, 2007; Gautam and Jachak, 2009; Li and Vederas, 2009). However, the quest to find out novel effective natural remedies for the treatment of diseases is a never-ending venture. Recently, some substantially immunosuppressive and nontoxic polyphenols have received increasing attention. For example, dalesconols A and B were identified as novel immunosuppressive polyphenols with higher selectivity (Zhang et al., 2008). Astilbin isolated from the Smilax glabra rhizome was proved to be an immunosuppressive flavonoid unique in its selective inhibition on activated T lymphocytes (Fei et al., 2005). Those findings encouraged us to search for promising immunosuppressants from the Dipterocarpaceae family, which were shown to be abundant in bioactive polyphenols (Ge et al., 2006, 2008, 2009, 2010). Dipterocarpaceous plants are well known as rich sources of various resveratrol (3,5,4′-trihydroxystilbene) oligomers (Shen et al., 2009), many of which exhibit remarkable bioactivities such as antioxidant (Ge et al., 2009), antitumor (Ge et al., 2006), immunosuppressive (Ge et al., 2010) and acetylcholinesterase inhibitory (Ge et al., 2008) effects. Previously, it was reported that resveratrol, a well-known polyphenol, exhibited anticancer, antioxidant, anti-aging, chemopreventive, cardioprotective, neuroprotective, anti-inflammatory and immunomodulatory activities (Albani et al., 2010; Bereswill et al., 2010; Das and Das, 2010; Juan et al., 2012; Kang et al., 2009; Ungvari et al., 2010). In the present study, we found that vaticaffinol, a resveratrol tetramer isolated from an ethyl acetate extract of the branches and twigs of V. mangachapoi (Qin et al., 2011), exerted a more potent immunosuppressive activity than resveratrol both in vitro and in vivo. The mechanism of vaticaffinol’s immunosuppressive effect on T cells involves cell cycle arrest, mitochondrial apoptosis and inhibition of ERK/AKT signalings.
Materials and methods

Mice. Female BALB/c mice (6–8 weeks old, 18–22 g) were obtained from the Experimental Animal Center of Yangzhou University (Yangzhou, China). They were maintained with free access to pellet food and water in plastic cages 21 ± 2 °C and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals’ suffering and to reduce the number of animals used.

Cells and reagents. Mouse CD3+ T cells from lymph nodes of BALB/c mice were purified using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) via magnetic cell separation with more than 98% purity. CD3+ cells were incubated in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO2 atmosphere at 37 °C. The following drugs and reagents were used: vaticafillin, 5,5′-D-glucopyranosyl-2-N-(2′-hydroxy-18-β-D-glucopyranosyl-2′-α-alkenyl)-3-hydroxy-9-methyl-4,8-sphingadienine, 99.5% of purity, isolated and identified as reported previously (Qin et al., 2011). Resveratrol, concanavalin A (Con A), ionomycin, 3-(4, 5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and carboxyfluorescein diacetate succinimidyl ester (CFSE) were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A (CsA) was purchased from Sandoz Ltd. (Basel, Switzerland). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-benizmid-3H-carbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). Picryl chloride (PCI) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Purified anti-mouse CD3 (145-2C11) and purified anti-mouse CD28 (37.51) were purchased from BD Pharmingen (San Diego, CA). Annexin V-FITC/PI kit was purchased from BD Biosciences (San Jose, CA). Antibodies against PARP, caspase-3, Bcl-2, ERK, phospho-ERK (Thr 202/Tyr 204), AKT, phospho-AKT (Thr 308), PI3K, BAD, and p-BAD (Ser136) were purchased from Cell Signal Technology (Beverly, MA). Antibodies against Tubulin and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell proliferation assay. Lymph nodes cells were cultured in 96-well plates at a density of 3 × 10^5 cells/well in RPMI 1640 medium (0.2 ml) and treated with 1, 3, 10 μM vaticafillin or 10 μM resveratrol for 24, 48 and 72 h in the presence of Con A (5 μg/ml) or anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml). MITT (4 mg/ml in PBS, 20 μl per well) was added to each well 4 h before the end of incubation. Then culture media was removed and 200 μl DMSO was added to dissolve the crystals. The absorption values at 540 nm were measured. In some cases, cell proliferation was also determined by carboxyfluorescein diacetate succinimidyl ester (CFSE) assay as we previously reported (Sun et al., 2010). Briefly, purified T cells (1 × 10^5 cells per ml) from lymph nodes of BALB/c mice were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin and labeled with 2.5 μM CFSE for 10 min at 37 °C. After labeling, the cells were washed three times in 15% fetal bovine serum medium and resuspended in RPMI 1640 medium (1 × 10^5 cells per ml). The CFSE-labeled cells were cultured with 1, 3, 10 μM vaticafillin or 10 μM resveratrol for 48 h in the presence or absence of 5 μg/ml Con A.

Cell mitochondrial membrane potential assay. Cells were seeded in 12-well plates at a density of 1 × 10^5 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticafillin or 10 μM resveratrol for 48 h in the presence of Con A (5 μg/ml). Disruption of mitochondrial membrane potential was measured using JC-1 staining (10 μg/ml) as previously reported (Sun et al., 2009).

Subcellular fractionation. In some experiments, cells were separated into cytosolic and mitochondrial fractions using the ProteoExtract Cytosol/Mitochondria Fractionation Kit (Merck Bioscience, Bad Soden, Germany) according to the procedures provided by the manufacturer.

Cell apoptosis assay. Cells were seeded in 12-well plates at a density of 1 × 10^5 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticafillin or 10 μM resveratrol for 48 h in the presence of Con A (5 μg/ml). Cultured cells were stained with Annexin V-FITC and propidium iodide (PI) in the dark at 4 °C for 30 min and analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

Cell cycle assay. Cells were seeded in 12-well plates at a density of 1 × 10^5 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticafillin or 10 μM resveratrol for 48 h in the presence of Con A (5 μg/ml). Cell cycle was examined as we previously reported (Luo et al., 2011). Briefly, cultured cells were fixed in 75% ethanol at 4 °C overnight, and then stained with 50 μg/ml of PI containing 100 μg/ml of RNase A and 1% Triton-X-100 in the dark at room temperature for 45 min. DNA contents of stained cells were analyzed with Modfit software (Becton Dickinson, San Jose, CA, USA).

Western blot. Cells were seeded in 6-well plates at a density of 1 × 10^7 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticafillin or 10 μM resveratrol for 24 h in the presence of Con A (5 μg/ml). Proteins lysed from cultured cells were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). After blocked in 5% nonfat milk at room temperature for 1 h, membranes were incubated with aimed primary antibodies at 4 °C overnight, and then with a horseradish-coupled secondary antibody at room temperature for 1.5 h. Final detection was performed using a Lumiglo chemiluminescent substrate system (KPL, Guildford, UK).

Picryl chloride (PCI)-induced contact hypersensitivity. On the first day (day 0), female BALB/c mice were shaved and sensitized by painting 0.1 ml of 1% PCI in absolute ethanol on the naked skin of their abdomens. Five days later (day 5), vaticafillin (10, 30 mg/kg) and resveratrol (10 mg/kg) dissolved in olive oil were given i.p., and then mice were challenged by painting 30 μl of 1% PCI in olive oil on right ear lobe. Eighteen hours later, ear thickness of right against left was measured with a digimatic micrometer (0.001 mm, Mitutoyo Co., Tokyo, Japan). The control mice were run parallel with other groups except for intraperitoneal (i.p.) administration of same volume of olive oil (Luo et al., 2011).

Fig. 1. Chemical structures of resveratrol and its tetramer vaticafillin.
Statistical analysis. All results were expressed as mean ± SEM of three independent experiments with each experiment including triplicate sets in vitro, or of eight animals per group in vivo. One-way ANOVA analysis and Student’s t test were used to evaluate the differences between various experimental and control groups, or vaticafinol and resveratrol. The level of significance was set at a P-value of 0.05.

Results

Vaticafinol inhibited the proliferation of T cells in vitro

Vaticafinol isolated from Vatica mangachapoi was subjected to high-performance liquid chromatography (HPLC) analysis. Its purity
was confirmed to be 99.5%. The structure of vaticafinol (Fig. 1) was identified by mass spectrometry and nuclear magnetic resonance spectral analysis and compared with the reported data (Sultanbawa et al., 1981).

For the choice of dose used for in vitro experiments, various doses of vaticafinol were performed in the pre-experiments ranging from 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM to 30 μM. There is no change of effect between 10 μM and 30 μM on the activated T cells. In addition, vaticafinol has no effect at dose below 1 μM. So, 1 μM, 3 μM and 10 μM of vaticafinol are selected for in vitro experiments.

Con A is a classic T cell mitogen which is usually used to activate T cells for proliferation. In this study, vaticafinol significantly inhibited T cell proliferation induced by Con A in a dose- and time-related manner (Fig. 2A). Similar result was also observed in T cells activated by anti-CD3 plus anti-CD28 (Fig. 2C), a T cell receptor stimulator (Morris and Allen, 2012). In addition, vaticafinol had no effect on naïve T cells at the aforementioned concentrations and timings (Fig. 2B), indicating that the immunosuppressive activity of vaticafinol observed here was not due to its cytotoxicity. Notably, the inhibition of vaticafinol on activated T cells was significantly stronger than that of resveratrol. These results were further confirmed by CFSE assay (Fig. 2D). It should be noted that vaticafinol also inhibited the proliferation of B cells and macrophages induced by lipopolysaccharide (Supplementary Figs. S1 and S2).

Vaticafinol induced apoptosis of activated T cells

Collapse of mitochondrial membrane potential is a landmark event in the early apoptosis. To examine whether vaticafinol can induce apoptosis of activated T cells, mitochondrial membrane potential was detected using JC-1 staining. We observed an obvious reduction in mitochondrial membrane potential in vaticafinol-treated cells (Figs. 3A and B).

Figure 4. Vaticafinol triggered caspase-dependent apoptosis in Con A-activated T cells. (A) Purified T cells from lymph nodes of BALB/c mice were treated with 1, 3, 10 μM vaticafinol or 10 μM resveratrol for 48 h in the presence of 5 μg/ml Con A. Then cells were stained with Annexin V/PI and assayed by flow cytometry for the percentages of apoptosis population. (B) Annexin V+/PI− and Annexin V+/PI+ cells of three different experiments were shown as a histogram of mean±SEM. (C) Purified T cells were treated with 1, 3, 10 μM vaticafinol or 10 μM resveratrol for 24 h in the presence of 5 μg/ml Con A. Protein expressions were examined by Western blot. Data represent one of three independent experiments with similar results. (D) Data summary of (C) are expressed as a histogram of mean±SEM of three independent experiments. *P<0.05, **P<0.01 vs Con A group; #P<0.05, ##P<0.01.
contrast, no significant change was seen in resveratrol-treated group. We also examined mitochondrial and cytosolic protein isolated from vaticaffinol- or resveratrol-treated activated T cells, respectively. As shown in Fig. 3C, vaticaffinol greatly increased the cytosolic cytochrome c.

Furthermore, vaticaffinol significantly triggered apoptosis of Con A-activated T cells by Annexin V/PI staining assay (Fig. 4A). There was a proportion of Annexin V+/PI+ cells under the treatment of vaticaffinol as well, suggesting that vaticaffinol also induced necrosis of Con A-activated T cells. Moreover, after 24 h of incubation with vaticaffinol in the presence of 5 μg/ml Con A, the levels of the anti-apoptotic proteins, Bcl-2 and p-BAD, were significantly down-regulated. We also observed an increase in cleaved caspase-3 and PARP (Figs. 4C and D). Interestingly, at the concentration of 10 μM, resveratrol had almost no effect on apoptosis (Figs. 4C and D). These results suggest that vaticaffinol induces apoptosis of activated T cells through mitochondrial pathway, which is even stronger than the effect of resveratrol.

Vaticaffinol caused G0/G1 phase arrest in activated T cells

Next we analyzed the effect of vaticaffinol on cell cycle progression of activated T cells. PI staining of vaticaffinol-treated T cells showed an increase in cell cycle arrest at G0/G1 phase. 33.8% of T cells entered into S and G2/M phase under Con A stimulation (control group) and this progression was efficiently reduced by 31.5% and 40.3% with the addition of vaticaffinol at 3 and 10 μM, respectively (Figs. 5A and B). Nonetheless, the reduction effect of resveratrol treatment was only 0.03%, which was markedly less than that of vaticaffinol.

Vaticaffinol inhibited both ERK signaling and AKT signaling

It is known that the ERK (extracellular-signal-regulated kinase) pathway and PI3K/AKT pathway are at the heart of signaling networks that control proliferation, differentiation and cell survival (Shen et al., 2012; Tan and Lam, 2010). To further explore the molecular mechanisms of vaticaffinol against T-cell-mediated immune response, we examined the effect of vaticaffinol on ERK and AKT signaling pathways. As shown in Fig. 6, vaticaffinol markedly inhibited the phosphorylations of ERK and AKT in Con A-activated T cells.

Vaticaffinol ameliorated PCI-induced contact hypersensitivity in mice

In our pre-experiment, we found that the drug concentration in the serum of mice with i.p. administered 10 mg/kg of vaticaffinol reached a peak at about 2.6 μM by a high performance liquid chromatography assay, which just fell within the range of the drug concentrations (1–10 μM) in vitro. So 10 and 30 mg/kg of vaticaffinol were selected for in vivo experiments. To assess the immunosuppressive property of vaticaffinol in vivo, we examined the effect of vaticaffinol on a mouse model of PCI-induced contact dermatitis. Administration with vaticaffinol significantly inhibited ear swelling while resveratrol showed only a slight inhibition (Fig. 7A). Histopathological analysis of ear sections revealed severe inflammatory cell infiltration (mostly monocyte-macrophages and neutrophils), vascular congestion and moderate edema in the dermis and subcutaneous tissue of control mice. Compared with control group, vaticaffinol-treated mice only showed a mild cellular infiltration and vasodilatation with no obvious edema (Figs. 7B and C).

Discussion

Crude herbs have been the basis of many traditional medicine systems throughout the world for thousands of years and continued to provide mankind with new remedies (Gautam and Jachak, 2009). In recent years, with the application of new technology, development of immunosuppressants from Chinese medicine has achieved a significant progress. For example, curcumin, a polyphenol derived from dietary spice turmeric, has been proved to possess wide-ranging anti-inflammatory and anticancer properties (Sharma et al., 2005; Wang et al., 2009). In addition, triptolide, the extract of Tripterygium wilfordii Hook F. has been proven to have anti-inflammatory and immunosuppressive activities in clinical trials for rheumatoid arthritis (Ma et al., 2007). In the present study, we identified the immunosuppressive activity of vaticaffinol for the first time both in vitro and in vivo. The bioactivity of vaticaffinol was attributed to its targeting against activated T cells through multiple aspects including inhibiting proliferation, inducing apoptosis, causing G0/G1 arrest and regulating ERK/AKT signaling pathway. Our findings may provide a novel candidate for the treatment of inflammatory and autoimmune diseases.

In this study, we found that vaticaffinol exerted more intensive immunosuppressive activity than its precursor resveratrol did. Our conclusions were based on the following observations: First, the inhibitory rate of 10 μM vaticaffinol on T cell proliferation induced by anti-CD3 plus anti-CD28 was up to 87.6% while resveratrol at the same concentration only exhibited an inhibitory rate of 5.1% (Fig. 2C). Similar results were observed in Con A-activated T cells by MTT assay (Fig. 2A) and CFSE assay (Fig. 2D). More importantly, vaticaffinol at the concentrations mentioned above did not affect T lymphocyte’s viability by MTT uptake assay (Fig. 2B), indicating that the inhibition of vaticaffinol on T cell proliferation, at a concentration up to 10 μM, was not due to a cytotoxic effect. Second, activated T cells treated with vaticaffinol had the following features: down-regulation of anti-apoptotic protein Bcl-2 and p-BAD, collapse of mitochondrial membrane potential, release of cytochrome c to cytosol, activation of caspase-3 and cleavage of

![Fig. 5. Vaticaffinol caused T cell arrest in G0/G1 phase of the cell cycle. (A) Purified T cells from lymph nodes of BALB/c mice were treated with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 48 h in the presence of 5 μg/ml Con A, and then stained with PI for cell cycle distribution analysis in flow cytometry. (B) Data summary of (A). All data shown here are representative of three different experiments with similar results.](image-url)
PARP (Figs. 3 and 4). These data suggested that vaticafinol induced apoptosis of T cells through mitochondrial pathway. It should be noted that vaticafinol also induced necrosis of Con A-activated T cells, suggesting that both necrosis and apoptosis contribute to the immunosuppressive activity of this compound. However, the apoptosis-inducing effect of resveratrol at the same concentration was very weak. These characteristics integrally indicated that vaticafinol, rather than resveratrol, initiated the caspase-dependent apoptotic cell death in T cells. Third, vaticafinol caused G0/G1 phase arrest (Fig. 5) and reduced the phosphorylation of ERK and AKT in activated T cells (Fig. 6), suggesting that cell cycle arrest and inhibition of ERK and AKT signalings contribute to the impairment of T cell response by vaticafinol. Finally, vaticafinol significantly inhibited ear swelling induced by PCI in mice while resveratrol only showed a slight inhibition (Fig. 7). Similarly, two resveratrol aneuploids have been reported to possess a better immunosuppressive activity than resveratrol did (Ge et al., 2010). Taken together, our results suggest that vaticafinol exerts more preferable immunosuppressive activity than its precursor resveratrol does both in vitro and in vivo through multiple aspects against activated T lymphocytes.

Fig. 6. Vaticafinol inhibited the activation of ERK and AKT in Con A-activated T cells. (A) Purified T cells from lymph nodes of BALB/c mice were treated with 1, 3, 10 μM vaticafinol or 10 μM resveratrol for 24 h in the presence of 5 μg/ml Con A. Then cells were harvested and the whole cell extracts were analyzed by Western blot for the phosphorylations of ERK1/2 and AKT. Data shown here are one of three independent experiments with similar results. (B) Data summary are expressed as a histogram of mean ± SEM of three independent experiments. *P<0.05, **P<0.01 vs Con A group; *P<0.05.

Fig. 7. Vaticafinol suppressed PCI-induced contact hypersensitivity in mice. Mice were sensitized by painting 0.1 ml of 1% PCI in ethanol on the shaved skin of their abdomens. On day 5, vaticafinol (10, 30 mg/kg) and resveratrol (10 mg/kg) were given i.p., and then mice were challenged by painting 30 μl of 1% PCI in olive oil on the right ear lobe. (A) Eighteen h after the challenge, thickness of right and left ears were measured. Ear swelling was presented as the increase in ear thickness. (B) Ear histologic scoring. (C) Hematoxylin and eosin staining of ear sections (original magnification 200 ×). Results are expressed as mean ± SEM, n = 8. *P<0.05, **P<0.01 vs PCI-CS control group; *P<0.05.
Delayed-type hypersensitivity (DTH) was typically used for evaluation of in vivo immunocompetency since it entirely depends on effects of T cells. Contact hypersensitivity is a T-cell-mediated immune response to cutaneous sensitization and subsequent challenge with haptons (Kaplan et al., 2012; Kimber et al., 2012). Apart from contact hypersensitivity, T cells also played a vital role in many other autoimmune-related diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. All these diseases have been known to involve the DTH mechanism. Currently, immunosuppressive drugs used to treat diseases mentioned above always have severe side effects since their inhibition on immunity response may lead to infections or other complications (Ge et al., 2010). So the future tendency will be the search for high selective immunosuppressants with low toxicity to minimize the risk of side effects (Cattaneo and Vinks, 2012).

In the present study, vaticafinol significantly inhibited the ear swelling in response to PCl, suggesting that vaticafinol mainly inhibits the function of the effector T cells involved in DTH reaction. On the contrary, resveratrol showed a slighter effect at the same dose. These results reveal that vaticafinol has potential for the treatment of T-cell-mediated disorders for its high activity in vivo.

In conclusion, vaticafinol, a resveratrol tetramer isolated from Vatica mangachapoi, exerts more intensive immunosuppressive activity than its precursor resveratrol does in vitro and in vivo. Its mechanism may involve multiple effects against activated T cells: regulation of signalings involved in cell proliferation, G0/G1 arrest of T cells, as well as an apoptosis induction in activated effector T cells. These results suggest that vaticafinol may be a novel candidate compound used for T-cell-mediated autoimmune diseases.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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