Patuletin induces apoptosis of human breast cancer SK-BR-3 cell line via inhibiting fatty acid synthase gene expression and activity

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Abstract. Fatty acid synthase (FASN) is a key enzyme involved in fatty acid biosynthesis and serves an important role in breast cancer development. The aim of the present study was to investigate the effects of patuletin on the gene expression and activity of FASN in the human breast cancer SK-BR-3 cell line, and the apoptotic effects of patuletin to breast cancer cells. Quantitative reverse transcription polymerase chain reaction, western blotting and intracellular FASN activity assays were used to evaluate FASN gene expression, protein expression and activity in patuletin-treated SK-BR-3 cells. MTT assays and flow cytometry were used to measure cell growth and cell apoptosis, respectively, following patuletin treatment. As a result, it was demonstrated that patuletin dose-dependently reduces FASN expression and intracellular activity in human breast cancer cells, and induces apoptosis in FASN over-expressing SK-BR-3 cells. Notably, apoptosis is associated with the reduction of intracellular FASN activity. The present study demonstrates that patuletin may be considered as a novel natural inhibitor of FASN, may induce anti-proliferative and pro-apoptotic effects in certain human breast cancer cells and may be useful for preventing and/or treating human breast cancer.

Introduction

Fatty acid synthase (FASN) is a multi-enzyme that catalyzes the *de novo* synthesis of palmitate (C16:0, a long-chain saturated fatty acid) from acetyl-CoA and malonyl-CoA, in the presence of NADPH (1). FASN is not only a key factor in the role of fatty acid biosynthesis for energy storage (2,3), but also its expression level increases significantly in adipose tissues and a variety of human carcinomas, including liver, breast, prostate, lung, endometrium, ovary, colon and pancreatic cancer (4-13). This prominent difference of FASN expression between normal and neoplastic tissues makes FASN a potential diagnostic tumor marker (14).

Breast cancer is the most common type of cancer and a leading cause of cancer-associated mortalities among females, with a common feature of abnormal cell apoptosis in its development (15). In addition, high levels of FASN expression has been demonstrated to be associated with poor clinical outcome in breast carcinomas, suggesting that FASN expression and tumor aggressiveness are closely associated (4,16). It was identified that obesity may serve a crucial role in the incidence and progression of breast cancer (17). According to the close association between FASN, obesity and breast cancer, the studies of FASN inhibitors have indicated their role as targets for chemotherapy in breast cancer and a novel strategy for antineoplastic intervention (18). In fact, previous studies demonstrated that certain synthetic and natural FASN inhibitors, including C75, desoxyrhaponticin, rhaponticin and α-mangostin may lead to selective cytotoxicity in FASN over-expressing cancer cell lines (18-20). This result suggested again that the pharmacological inhibition of FASN may represent a potential target for drug development.

In previous studies, a number of dietary polyphenols, including α-mangostin, resveratrol, curcumin and quercetin exhibited high inhibitory activity against FASN (21-30). Although the detailed mechanism of the inhibitory effect of polyphenols on FASN was not fully understood, the structure activity association analysis demonstrated that the flavonoids containing two hydroxyl groups in the B ring and 5, 7-hydroxyl groups in the A ring with C-2, 3 double bond were the most potent inhibitors on FASN (31). Patuletin (3,5,7,3',4'-penta-hydroxy-6-methoxy-flavone) (Fig. 1A), a natural flavonoid primarily present in the genus *Eriocaulon* (32), exhibits anti-inflammatory, anti-oxidant and anti-bacterial properties (33-36). However, no anti-neoplastic effects of patuletin have been identified at present. Increased expression of FASN

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has previously been demonstrated in different breast cancer cells (37). Among them, human breast cancer SK-BR-3 cells exhibited higher expression levels of FASN compared with other breast cancer cells, including MCF-7 and MDA-MB-231 cells. The present study aimed to identify for the first time that patuletin induces apoptosis in FASN over-expressing human breast cancer SK-BR-3 cells.

Materials and methods

Reagents. Acetyl-Coenzyme A (CoA), Malonyl-CoA, dexamethasone, NADPH, ethyl acetate (EtOAc), chloroform, methanol, MTT, 3-isobutyl-1-methylxanthine (IBMX), EDTA and DTT were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit was purchased from BD Biosciences (San Jose, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, PBS, penicillin-streptomycin, trypsin-EDTA, dimethyl sulfoxide (DMSO), TRIzol, SuperScript III First-Strand Synthesis system were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Rabbit antibodies against FASN (cat no. 3180) and β-actin (cat no. 4967) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Extraction and isolation of patuletin. The whole plants of *Eriocaulon buergerianum* were collected in Zhejiang (China) by the research group. Patuletin from air-dried whole plants of *E. buergerianum* (3.0 kg) was extracted with 95% ethanol at room temperature. The concentrated crude extract was dissolved in H_2O and partitioned with EtOAc. The EtOAc portion (225 g) was chromatographed on a silica gel column eluting with a chloroform-methanol gradient system to yield patuletin (300 mg). Isolated patuletin was ≥98% pure as determined by HPLC-UV (Agilent Technologies, Inc., Santa Clara, CA, USA).

Cell culture. The human breast cancer SK-BR-3 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated at 5% CO_2 and 37°C in a medium containing 89% DMEM (high glucose), 10% bovine fetal serum and 100 U/ml penicillin-streptomycin. For passage, the cells were digested by 0.25% trypsin-EDTA every 4 days.

Cell viability assay. SK-BR-3 cells were seeded in 96-well plate firstly, at a density about $5x10^3$ cells/well and then treated with purified patuletin in different concentrations (5, 10, 20, 40, 80 or 160 μM) for 24 h. Thereafter, 5 mg/ml MTT solution was added into each well and incubated for 4 h at 37°C. Then, the medium with MTT was aspirated, 200 μl DMSO/well was added to the wells and the cells were incubated for 15 min. Finally, the concentration was measured at 492 nm by a microplate spectrophotometer (BioTek China, Beijing, China). PBS was used as blank control, and cells without patuletin treatment were used as negative control.

Cell lysis and immunoblotting. Cells were lysed as previously described (38). Protein concentration of cell lysates was measured by the Pierce BCA protein assay kit using bovine

serum albumin as a standard control. 50 μg protein was loaded per lane, separated by SDS-PAGE (12% gel), and then electrophoretically transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Then the protein samples were blocked with 5% skimmed milk for 1-2 h at room temperature to prevent nonspecific antibody binding, and probed with primary antibodies against FASN and β-actin at a dilution of 1:1,000 overnight at 4°C. Subsequently, membranes were washed twice with TBST (10 mM Tris, 10 mM NaCl, 0.1% Tween-20), and incubated 1 h at room temperature with corresponding peroxidase conjugated secondary antibody (cat no. 7074) and developed with a commercial enhanced chemiluminescence kit (West Pico chemiluminescent substrate; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) according to the manufacturer's protocol. Blots were probed with an antibody against β-actin as the control.

Cell apoptosis assay. Cell apoptosis detection was performed using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer's protocol. First, cells were collected after 24 h treatment with patuletin at different concentrations (20, 40 and 80 μM). Then, cells ($1x10^6$ cells/tube) were washed twice with cold PBS and resuspended in 100 μl 1X binding buffer (Biomiga Inc., San Diego, CA, USA). Cell suspension was incubated with 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) for 15 min at room temperature and kept in a dark place. Immediately following that, 400 μl 1X binding buffer was added and the cells were analyzed by a CellQuest Pro software (FACSstation 6.0; BD Biosciences) in a BD FACSCalibur™ flow cytometer (BD Biosciences) within 1 h. Those cells stained with Annexin V^+/PI^- were early apoptotic cells and those stained with Annexin V^+/PI^+ were late apoptotic cells.

FASN gene expression analysis. FASN gene expression analysis was performed in SK-BR-3 cells treated with patuletin at different concentrations (5, 10, 20, 40, 80 and 160 μM) for 24 h. Cells were washed with PBS twice for RNA extraction. Total RNA was isolated from SK-BR-3 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. A total of ~2 μg RNA was reverse transcribed into complementary DNA (cDNA) using SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific, Inc.), from the control and treated cells. Polymerase chain reaction (PCR) was performed in 20 μl of the final volume, using primers for analyses of the FASN and β-actin genes. The conditions for PCR were as follows: Initial denaturation at 95°C for 5 min and followed by 45 cycles (95°C for 15 sec, 55°C for 15 sec, 72°C for 20 sec). (FASN sense, 5'-TATGCTTCT TCGTGCAGCAGTT-3' and antisense, 5'-GCTGCCACACGC TCCTCTAG-3'; β-actin sense, 5'-AAAGACCTGTACGCC AACACAGTGCTGTCTGG-3' and antisense, 5'-CGTCAT ACTCCTGCTTGCTGATCCACATCTGC-3') The β-actin gene, which is a housekeeping gene, was used as an internal control, and samples without reverse transcription were used as negative control. Quantitative PCR was performed in 25 μl final volume containing 2 μl cDNA, SYBR Green Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). FASN gene and β-actin gene expression levels
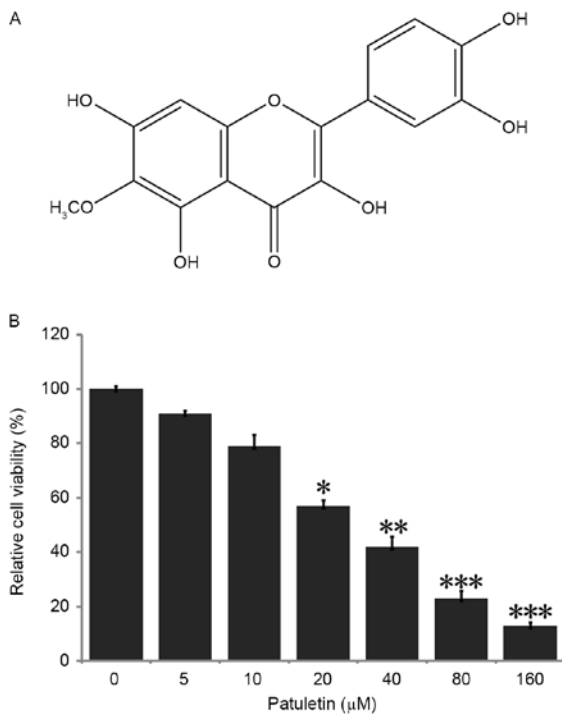
Figure 1. Patuletin dose-dependently inhibits the survival of SK-BR-3 cells. (A) Chemical structure of patuletin. (B) Cell survival rate was determined by MTT assay. SK-BR-3 cells were cultured with patuletin for 24 h at different concentrations of 5, 10, 20, 40, 80 and 160 μM. Bars represent means ± standard deviation. P-values were measured by one-way analysis with Dunnett's post-hoc test. *P<0.05 vs. 0 μM patuletin-treated group; **P<0.01 vs. 0 μM patuletin-treated group; ***P<0.001 vs. 0 μM patuletin-treated group.
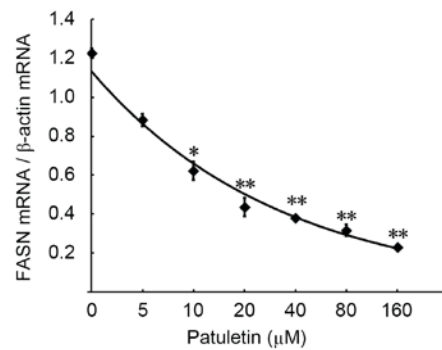

Figure 2. Inhibitory effects of patuletin on FASN mRNA levels in SK-BR-3 cells. The effect of patuletin on FASN gene expression in SK-BR-3 cells was measured by quantitative reverse transcription PCR. Increasing concentrations of patuletin, incubated with SK-BR-3 cells for 24 h, resulted in a concentration-associated reduction on FASN gene expression. P-values were measured by one-way analysis with Dunnett's post-hoc test. *P<0.05 vs. 0 μM patuletin-treated group; **P<0.01 vs. 0 μM patuletin-treated group. FASN, fatty acid synthase; PCR, polymerase chain reaction.

were determined with the comparative C_q method in triplicate experiments (39).

Intracellular fatty acids assay. SK-BR-3 cells were collected after 24 h treatment with patuletin at different concentrations (20, 40, 80 μM, respectively). Then, cells were washed twice with cold PBS and extracted by homogenization with pure chloroform containing 1% Triton X-100 (Sigma-Aldrich; Merck KGaA). The extract was centrifuged at 10,800 x g for 5-10 min at 4°C, to collect the organic phase. Next, the organic phase was air and vacuum dried to remove chloroform. The dissolved dried lipids were applied to detect the amount of intracellular fatty acid by Fatty Acid Assay kit (BioVision, Inc., Milpitas, CA, USA), following the manufacturer's protocol. The fatty acids concentration was measured at 570 nm by a microplate spectrophotometer.

Cell FASN activity assay. Intracellular FASN activity was assessed as described previously (40). SK-BR-3 cells were harvested and collected in cold assay buffer containing 100 mM potassium phosphate buffer, 1 mM EDTA, 0.6 mM PMSF and 1 mM dithiolthreitol (pH 7.0). Then, the cell suspension was centrifuged at 10,800 x g for 30 min at 4°C, and the supernatant was collected for the overall reaction assay. A total of 25 ml supernatant was added into the reaction mix containing 25 mM KH_2PO_4-K_2HPO_4 buffer, 0.25 mM EDTA, 0.25 mM dithiothreitol, 30 mM acetyl-CoA, 100 mM malonyl-CoA, 350 mM NADPH (pH 7.0) to a total volume of 200 ml. The protein content in the supernatant was determined

using a bicinchoninic acid (BCA) assay (Pierce; Thermo Fisher Scientific, Inc.) and results were expressed as the specific activity of FASN at the same protein concentration.

Quantification of fatty acid. Following treatment with patuletin at the corresponding concentrations (0, 20, 40 and 80 μM), cells were harvested using trypsin-EDTA, washed twice with PBS, and stored at -80°C. The amount of intracellular fatty acid was determined with a Free Fatty Acid Assay kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol.

Statistical analysis. All values are presented as mean ± standard deviation. To determine if differences between experimental and control groups existed in cancer cell viability, apoptosis, FASN gene expression and activity and in intracellular fatty acids concentration, the results were evaluated and analyzed by one-way analysis of variance and the Dunnett's post-hoc test using GraphPad software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory effects of patuletin on the viability of SK-BR-3 cells. To identify whether patuletin affected the viability of the breast cancer SK-BR-3 cell line, cells were treated with 0-160 μM patuletin for 24 h, and following this, the ability of cell survival was examined by MTT assay. As demonstrated in Fig. 1B, SK-BR-3 cell viability was reduced significantly subsequent to treatment with 20, 40, 80 and 160 μM patuletin. When compared with the negative control (0 μM patuletin), cell survival rate was markedly reduced to 13% following treatment with 160 μM patuletin. Patuletin demonstrated high inhibition of cell population in a dose-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) value of 24 μM.

Patuletin reduces gene expression of FASN in SK-BR-3 cells. The effect of patuletin on FASN gene expression in SK-BR-3 cells was measured by PCR and reverse transcription-PCR. As demonstrated in Fig. 2, treatment of SK-BR-3 cells for 24 h
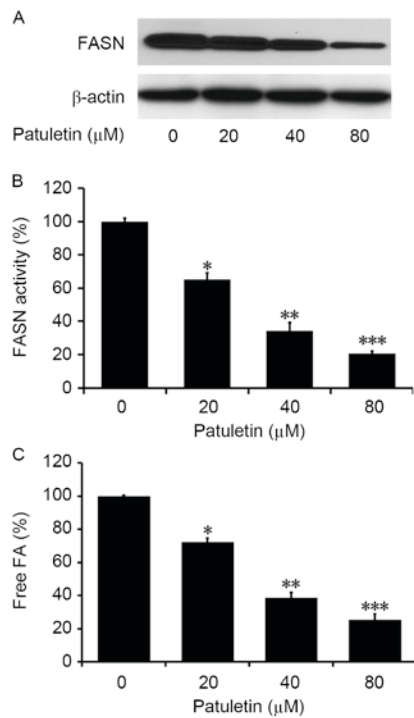
Figure 3. Inhibitory effects of patuletin on intracellular FASN expression and activity. SK-BR-3 cells were treated with 20, 40 and 80 μM patuletin for 24 h, and then analyzed by different methods. Data were normalized to control cells without patuletin (0 μM). (A) Effect of patuletin on FASN protein expression analyzed by western blotting. (B) Intracellular FASN activity assay. Relative FASN activity was presented as means ± SD. *$P<0.05$ vs. with 0 μM patuletin-treated group; **$P<0.01$ vs.0 μM patuletin-treated group; ***$P<0.001$ vs. 0 μM patuletin-treated group. (C) The amount of intracellular FA was measured by Fatty Acid Assay kit. Data was presented as means ± SD. (n=3). *$P<0.05$ vs. 0 μM patuletin-treated group; **$P<0.01$ vs. 0 μM patuletin-treated group; ***$P<0.001$ vs. 0 μM patuletin-treated group. FASN, fatty acid synthase; SD, standard deviation; FA, fatty acid.

with increasing concentrations of patuletin (from 5-160 μM) resulted in a significant reduction in FASN mRNA expression.

Patuletin inhibits intracellular FASN activity in SK-BR-3 cells. The effect of patuletin on the FASN activity in SK-BR-3 cells was measured by western blotting analysis. As demonstrated in Fig. 3A, SK-BR-3 cells treated with patuletin for 24 h exhibited much lower levels of FASN compared with the control. When SK-BR-3 cells were treated with patuletin at different

concentrations for 24 h, intracellular FASN activity was significantly reduced to 65.0, 34.3 and 20.6%, respectively ($P<0.05$, $P<0.01$ and $P<0.001$, respectively; Fig. 3B). This suggests that intracellular FASN activity was significantly suppressed by patuletin, and that the inhibition was dose-dependent.

Patuletin reduced intracellular fatty acids in SK-BR-3 cells. The levels of intracellular fatty acids in SK-BR-3 cells treated with 20, 40 and 80 μM patuletin was measured by Fatty Acids Assay kit. As demonstrated in Fig. 3C, compared with the control (0 μM patuletin), the levels of intracellular fatty acids in treated cells significantly decreased to 63.8, 52.4 and 31.2% ($P<0.05$, $P<0.01$ and $P<0.001$, respectively).

Patuletin induced SK-BR-3 cells apoptosis. The apoptotic rate of SK-BR-3 cells following 20, 40 and 80 μM patuletin treatment for 24 h was measured using a Annexin V-FITC Apoptosis Detection kit and analyzed by flow cytometry. As demonstrated in Fig. 4, patuletin markedly induced SK-BR-3 cell apoptosis in a dose-dependent manner.

Discussion

The present study focuses on the effects of patuletin on FASN gene expression and activity in human breast cancer SK-BR-3 cells. To the best of our knowledge, it was demonstrated for the first time that patuletin dose-dependently decreases the gene and protein expression levels of FASN and its activity in the human breast cancer SK-BR-3 cell line. In addition, this natural flavone markedly inhibits cell proliferation and induces apoptosis in SK-BR-3 cells.

Natural polyphenolic compounds include of a wide variety of biologically active compounds, a number of which have been suggested to exhibit antineoplastic properties (40-42). However, the anti-cancer activity of patuletin has not yet been examined, to the best of our knowledge. In the present study, the inhibition of FASN activity was associated with the apoptosis of cancer cells, which suggested that efficient FASN inhibitors may be potential target drugs for the treatment of cancer. It was also demonstrated that the natural polyphenolic compound patuletin may inhibit intracellular FASN activity, and therefore induce breast cancer cell apoptosis.

FASN is a key multi-enzyme that catalyzes fatty acid synthesis. The expression level of FASN is relatively low in the
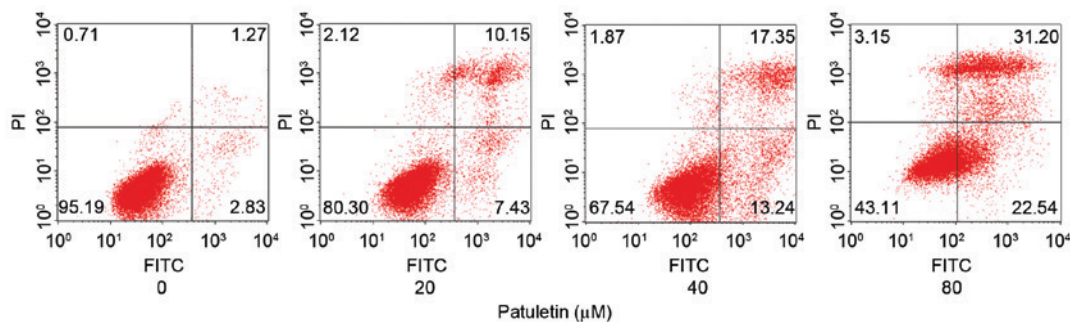

Figure 4. Apoptotic effect of patuletin on SK-BR-3 cells. SK-BR-3 cells were treated with 0, 20, 40 and 80 μM patuletin for 24 h, and then the apoptotic fraction of the SK-BR-3 cells was detected by Annexin V-FITC and PI double staining. The concentrations of patuletin in each part of the figure (left-right) were 0, 20, 40 and 80 μM, respectively. FITC, fluorescein isothiocyanate; PI propidium iodide.

majority of normal tissues, however; increased expression of FASN has been identified in human breast cancer cells, particularly in SK-BR-3 cells (37). According to previous studies on tumor proliferation, FASN may contribute to the generation of tumor cell membranes (43). Therefore, FASN inhibitors such as C75 and orlistat are promising potential anti-cancer drugs for the prevention and/or treatment of a variety of cancers such as cervical, prostate, leukemia and colon cancer (44-46). It is essential to identify more effective FASN inhibitors that may be applied practically as chemotherapeutic drugs.

The present study identified that patuletin not only downregulated mRNA and protein expression of FASN, but also demonstrated a high inhibitory activity on intracellular FASN. The decrease of intracellular FASN activity and fatty acids levels in SK-BR-3 cells revealed that patuletin acted on FASN as an inhibitory target. The intracellular activity of FASN directly affected the amount of intracellular fatty acids as FASN serves a key role in *de novo* fatty acid biosynthesis.

Like certain FASN inhibitors such as C75 and cerulenin (18), patuletin has been suggested to induce apoptosis of breast cancer cells. The results demonstrated that the apoptotic ratio of patuletin treated SK-BR-3 cells increased from 4.10% (control) to 53.74% (80 μM patuletin). The mechanism of cancer cell apoptosis through the inhibition of intracellular FASN expression may be explained by accumulating malonyl-CoA, which was considered as a trigger of cancer cell death and apoptosis (47,48). The present study also concluded that the signal pathways in cancer cell apoptosis exhibit close associations with the inhibition of FASN, therefore FASN inhibitors may be ideal drugs for the treatment of cancer.

In conclusion, patuletin induced apoptosis in breast cancer SK-BR-3 cells via inhibiting intracellular FASN activity and downregulating the mRNA and protein expression levels of FASN. As patuletin demonstrated a significant promotion of apoptosis in SK-BR-3 cells, it exhibits potential for application as an anti-cancer drug candidate for the treatment of human breast cancers.

Acknowledgements

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