Suberoylanilide hydroxamic acid upregulates histone acetylation and activates endoplasmic reticulum stress to induce apoptosis in HepG2 liver cancer cells

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Abstract. Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase inhibitor that has demonstrated clinical activity against various solid tumors. The aim of the present study was to explore the effects of SAHA on the apoptosis of HepG2 liver cancer cells, as well as the potential mechanisms involved in histone acetylation and endoplasmic reticulum (ER) stress. HepG2 cells were treated with various doses of SAHA (0, 1, 6 and 12 µM), and apoptosis was measured by flow cytometry. The levels of ER stress-associated molecules, including 78 kDa glucose-regulated protein (GRP78), PRKR-like endoplasmic reticulum kinase (PERK), phosphorylated (p)-PERK, activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP), were quantitated by western blot analysis and reverse transcription-quantitative PCR assay. The expression levels of acetylated histone H4 (acH4, acH4 lysine (K)5 and acH4K12) were detected by western blot analysis and reverse transcription-quantitative PCR assay. The expression levels of acetylated histone H4 in the promoter regions of GRP78, ATF4 and CHOP were evaluated by chromatin immunoprecipitation assays. Following treatment with higher doses of SAHA (6 and 12 µM) for 48 h, the proliferation of HepG2 cells was significantly suppressed. SAHA induced dose-dependent apoptosis and increased both protein and mRNA expression levels of GRP78, ATF4 and CHOP in HepG2 cells. The protein expression of PERK was markedly decreased by treatment with SAHA, whereas the p-PERK expression level was notably increased, which resulted in increased p-PERK/PERK ratio. Furthermore, the acetylation levels of H4 in the promoter regions of GRP78, ATF4 and CHOP were significantly increased in HepG2 cells exposed to 6 µM SAHA for 36 h. Thus, SAHA induces apoptosis in HepG2 cells by activating the ER stress-mediated apoptotic signaling pathway, at least partially by enhancing the acetylation of histone H4 on the promoter regions of ER-stress associated genes, including GRP78, ATF4 and CHOP.

Introduction

Liver cancer is among the most prevalent malignancies in the world (1); it has a high degree of malignancy and a poor prognosis. However, relatively limited treatment approaches are available in the clinic. Therefore, there is an urgent need to identify novel therapeutic targets and to develop effective strategies for liver cancer therapy. Emerging evidence has demonstrated a key role for epigenetics in tumorigenesis (2-4). The occurrence and development of liver cancer is also dependent on epigenetics-related disorders of various signal transduction pathways (5). Epigenetic regulation is defined as genomic modifications of heritable factors that do not involve alterations in the DNA sequence and affect the transcriptional activity of genes instead (6). Several mechanisms of epigenetic regulation, including histone modification, noncoding RNAs and DNA methylation, have been extensively studied (7). Among these mechanisms, histone modification is one of the important modes of epigenetic regulation in liver cancer (8).

Histones are well-conserved and highly alkaline proteins, which appear as an octameric core and help to package and order DNA into nucleosomes. The N-terminal tails of histones extend beyond the nucleosome and undergo a variety of post-translational modifications, such as acetylation, methylation, ubiquitination and phosphorylation (9). Acetylation of histones is mediated through histone acetylases (HATs) and...
histone deacetylases (HDACs) (10-12). Some studies have unveiled the importance of the balance of HATs and HDACs for the expression of several key genes, including p21, Fas, Bcl-2 and Bax (13). Once this balance is broken, an imbalance of gene transcription occurs, which may lead to tumorigenesis or abnormal cell proliferation. Moreover, the high expression of HDAC family members and the downregulation of histone acetylation in liver cancer tissues have been confirmed in certain studies (14,15). Therefore, the inhibition of HDAC activity by an HDAC inhibitor (HDACi) is expected to be an effective approach for the treatment of liver cancer.

Suberoylanilide hydroxamic acid (SAHA), the first HDACi approved for clinical use by the US Food and Drug Administration, has been widely acknowledged for its anti-tumor activity (16). It has been reported that SAHA decreases proliferation, induces differentiation and elicits apoptosis in many tumor cells during in vitro culturing (17-19). Additionally, SAHA has been demonstrated to be a potential inducer of endoplasmic reticulum (ER) stress that led to induction of apoptosis in lung cancer cells, mediated by the activation of the ER stress-mediated apoptotic signaling pathway (20). However, the effects of SAHA in liver cancer and the mechanism of SAHA in regulating ER stress are largely unknown.

In the present study, the effects of SAHA on apoptosis in the HepG2 liver cancer cells and the potential mechanisms involved in histone acetylation and ER stress were explored. Following treatment with various doses of SAHA, HepG2 cells were subjected to an apoptosis assay. Furthermore, the levels of the ER-stress related molecules 78 kDa glucose-regulated protein (GRP78), activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP), as well as the acetylation levels of the histone proteins H4 (acH4), H4-lysine 5 (H4K5) and H4-lysine 12 (H4K12) were quantitated. The effects of SAHA on acetylation of H4 in the promoter regions of GRP78, ATF4 and CHOP were evaluated by chromatin immunoprecipitation (ChIP) experiments.

Materials and methods

Cell culture. The HepG2 liver cancer cell line (cat. no. KCB 200507YJ) was purchased from the cell bank of the Type Culture Collection of the Chinese Academy of Sciences. The HepG2 cells were authenticated by short tandem repeat DNA profiling analysis and tested to be free of mycoplasma and other contaminants by the vendor. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (ScienCell Research Laboratories, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and grown in a humidified atmosphere with 5% CO₂ at 37°C. The cells were treated with SAHA (Abcam) or mock-treated with DMSO (Sigma-Aldrich; Merck KGaA), as indicated.

Real-time cellular analysis. Cell proliferation was monitored using an xCELLigence Real-Time Cell Analysis instrument (xCELLigence DP System; ACEA Biosciences, Inc.), which can continuously monitor live cell proliferation, morphology and viability with a label-free assay. The experimental procedures used were as previously described (21,22). Briefly, HepG2 cells were seeded at a density of 1x10⁴ cells/well in a 16-well µl plate (E-plate 16), cultured in complete medium supplemented with various doses of SAHA (0, 0.1, 1, 6, 12, 25, 50 and 100 µM), and placed at 37°C in a humidified incubator containing 5% CO₂. The effects of the various doses of SAHA on the proliferation of HepG2 cells were continuously monitored for 90 h. The cell index is a dimensionless parameter that is translated from the electrical impedance measured to denote cell proliferation.

Apoptosis assay. HepG2 cells (1x10⁶) were seeded onto 10-cm diameter dishes and treated with various doses of SAHA (0, 1, 6 and 12 µM) for 48 h. The cells were harvested and subjected to an apoptosis assay using an annexin V/propidium iodide (PI) cell apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd.), according to the manufacturer's instructions. Briefly, the cells were washed with cold phosphate-buffered saline (PBS). After centrifugation at 110 g for 5 min, the cell pellet was resuspended in 1 ml PBS (final concentration ~1-5x10⁵ cells/ml). After washing with 1X annexin V binding solution, the cells were stained with 5 µl of fluorescein isothiocyanate (FITC)-conjugated annexin V and 10 µl PI staining solution at room temperature for 20 min. After washing once with the 1X annexin V binding solution, the labeled cells (1x10⁴ cells) were detected immediately by a flow cytometer (FACSCalibur™; BD Biosciences). The data were analyzed by BD CellQuest Pro software (version 1.0; BD Biosciences). The apoptotic rate was defined as the percentage of FITC-annexin V single positive cells (early apoptotic cells) among total cells.

Western blot analysis. HepG2 cells (1x10⁶) were seeded onto 10-cm diameter dishes and treated at various doses of SAHA (0, 1, 6 and 12 µM) for 48 h. The cells were then lysed with 0.2 ml of radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). Total protein samples (60 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels), transferred to a polyvinylidene difluoride membrane and blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 60 min at room temperature. The membrane was incubated overnight at 4°C with the following primary antibodies: acH4 (1:1,000), acH4K5 (1:5,000), acH4K12 (1:1,000), GRP78 (1:1,500), PERK (1:1,500), phosphorylated (p)-PERK (1:1,500), ATF4 (1:1,500) and CHOP (1:1,500). After washing with TBST buffer, the membrane was incubated with the peroxidase-conjugated anti-rabbit secondary antibody (1:4,000; Abcam; cat. no. ab205718) for 90 min at room temperature. Following treatment with enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.), the Image Lab software (version 4.1; Bio-Rad Laboratories, Inc.) was used to quantitate the band intensities. The antibodies against β-actin (cat. no. ab8227), GRP78 (cat. no. ab21685), ATF4 (cat. no. ab184909), CHOP (cat. no. ab10444), PERK (cat. no. ab65142), acH4K5 (cat. no. ab51997) and acH4K12 (cat. no. ab46983) were purchased from Abcam. The antibody against acH4 (cat. no. 39925) was purchased from Active Motif, Inc. the antibody against p-PERK (cat. no. AF4499) was purchased from Affinity Biosciences.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from HepG2 cells using a TRIzol RNA...
isolation kit (Thermo Fisher Scientific, Inc.) and the purity and concentration of the RNA samples were determined by a DN2000 ultramicro nucleic acid analyzer (Thermo Fisher Scientific, Inc.). The total RNA was reverse transcribed into cDNA using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc), according to the manufacturer's instructions. RT-qPCR reactions were performed as follows: 25°C for 10 min, 48°C for 60 min and 95°C for 5 min. The qPCR was performed using SYBR green mix from Thermo Fisher Scientific, Inc. The reaction conditions included an initial pre-denaturation step at 95°C for 30 sec, followed by 40 cycles of thermal steps consisting of 95°C for 5 sec and 60°C for 30 sec. β-Actin was used as an internal control. The fold-change was calculated by the 2-ΔΔCq method (23). The primer sequences used for each gene are shown in Table I.

ChIP-qPCR. HepG2 cells (1 x 10^6) were seeded onto 10-cm diameter dishes and were mock-treated with DMSO (0 µM SAHA) or treated with 6 µM SAHA for 36 h. Following treatment, the cells were subjected to a ChIP assay using a ChIP kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer’s instructions. The HepG2 cells were cross-linked with 1% formaldehyde and lysed with 600 µl of radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.), and the lysates were sonicated to fragment the DNA into lengths between 200-1,000 base pairs. Immunoprecipitation was performed with the following antibodies: Anti-acH4 (1:50; Active Motif, Inc.; cat. no. 39925), rabbit IgG (1:50; Abcam; cat. no. ab205718) and anti-RNA Polymerase II (1:50; Abcam; cat. no. ab51462) at 4°C overnight. Protein G-sepharose beads were used for immunoprecipitation, and the immunoprecipitated DNA was recovered by reversing the cross-linking, and were purified and dissolved in distilled water. A corresponding sample handled without the addition of any antibody served as an input control. The ChIP DNA and input DNA were analyzed by qPCR. The abundance of the immunoprecipitated target DNA was expressed as a percentage of the input chromatin DNA. The primers used for detecting the promoter regions of the GRP78, ATF4 and CHOP genes are listed in Table I.

Statistical analysis. SPSS version 20 statistical software (IBM Corp.) was used for statistical analysis. Data were expressed as the mean ± SD. The one-way analysis of variance method was used for the multivariate comparison, and the least significant difference method was used as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

SAHA inhibits the proliferation of HepG2 cells in a dose- and time-dependent manner. Firstly, the effect of SAHA on the proliferation of HepG2 cells was determined by real-time cellular analysis. This involved live monitoring of cell proliferation in a label-free manner. The electrical impedance of adherent cells was detected by the electrode at the bottom of an E-plate 16, and the cell index is a dimensionless parameter that was translated from the measured electrical impedance to denote cell proliferation. After HepG2 cells were incubated for 10 h to allow cell adhesion, SAHA was added at various doses in the culture medium to treat cells. This resulted in a rapid decrease in HepG2 cell proliferation at 10 h (Fig. 1), which was probably due to the transient effect of SAHA vehicle DMSO on cell proliferation. Compared with the mock-treated group (0 µM SAHA), treatment with SAHA at concentrations >1 µM resulted in a notable inhibitory effect on the proliferation of HepG2 cells (Fig. 1). Furthermore, SAHA at 6 µM resulted in significantly (P<0.01) decreased normalized cell index, by >70%, at 50 h after treatment. A significant (P<0.01) suppression of proliferation was observed in HepG2 cells treated with higher doses of SAHA (12, 25 and 50 µM) for longer periods of time. Thus, the antiproliferative effect of SAHA on HepG2 cells occurs in a dose- and time-dependent manner.

SAHA induces apoptosis in HepG2 cells in a dose-dependent manner. The effect of SAHA on apoptosis in HepG2 cells was investigated by annexin V-FITC and PI staining. Following
treatment with SAHA for 48 h, the apoptotic rates were significantly elevated in HepG2 cells treated with 1, 6 or 12 µM SAHA, compared with the untreated cells (Fig. 2). While the control group (0 µM SAHA) had an average apoptotic rate of ~1%, 12 µM SAHA led to an increased apoptotic cell population to ~10%. These results indicate that SAHA could significantly promote apoptosis in HepG2 cells in a dose-dependent manner.

SAHA induces ER stress-mediated apoptotic signaling pathway in HepG2 cells. Since SAHA was revealed to be a potential inducer of ER stress, the effect of SAHA on ER stress in HepG2 cells was determined by measuring the levels of ER stress-associated molecules, including GRP78, PERK, p-PERK, ATF4 and CHOP. No significant changes were observed in the expression of GRP78 at either the mRNA (Fig. 3A) or protein level (Fig. 3B and C) in cells treated with 1 µM SAHA, whereas cells treated with 6 and 12 µM SAHA had significantly increased levels of GRP78 mRNA and protein compared with control cells (0 µM SAHA). No significant changes in the level of PERK mRNA were observed following treatment with any of the tested doses of SAHA (Fig. 3A). However, the expression of PERK protein was significantly decreased in the HepG2 cells treated with different concentrations of SAHA, while the level of p-PERK protein was significantly increased (Fig. 3B and C), which resulted in an increased ratio of p-PERK/PERK following SAHA treatments (Fig. 3D). Notably, following treatment with SAHA, the mRNA and protein expression levels of ATF4 and CHOP were significantly elevated in a dose-dependent manner (Fig. 3A and C). Moreover, the protein levels of ATF4 and CHOP in the HepG2 cells were significantly upregulated by over 3-fold following treatment with 12 µM SAHA.

SAHA significantly upregulates the level of acH4 in HepG2 cells. The role of SAHA, as an HDACi, in the acetylation of histone H4 was determined by western blot analysis. The protein levels of total acH4, acH4K5 and acH4K12 were detected in HepG2 cells treated at various doses of SAHA for 48 h (Fig. 4). Compared with the control group (0 µM SAHA), the levels of acH4 and acH4K12 were not significantly increased in the HepG2 cells treated with 1 µM SAHA, whereas the level of acH4K5 was significantly increased; these findings were likely due to the varied sensitivities of histone deacetylases to inhibition by SAHA. In cells treated with 6 and 12 µM SAHA, the levels of total acH4, acH4K5 and acH4K12 were all significantly higher than in those treated with 0 and 1 µM SAHA. These results suggest that 6 µM SAHA was sufficient to markedly elevate the acetylation of histone H4.

SAHA treatment enhances the acetylation level of histone H4 in the promoter regions of the GRP78, ATF4 and CHOP genes in HepG2 cells. In order to confirm whether the regulation of transcription of GRP78, ATF4 and CHOP genes by SAHA is mediated by the upregulation of acH4, ChIP assays were conducted. This involved determination and quantitation of the acH4-associated promoter regions of these genes in HepG2 cells (Fig. 5). Following treatment with DMSO (0 µM SAHA) or 6 µM SAHA for 36 h, the HepG2 cells were lysed and immunoprecipitated with specific anti-acH4 antibody. qPCR results demonstrated significant increase in the acH4-associated promoter regions of the GRP78, ATF4 and CHOP genes in HepG2 cells treated with 6 µM SAHA. The promoter regions of ATF4 and CHOP were increased by 2-fold and 3-fold, respectively, the promoter region of GRP78 was enriched by ~4-fold (the output to input ratio increased from ~15 to ~60%). These results confirmed that SAHA induces the expression of ER stress-associated molecules by increasing their transcription, at least partially through enhancing the acetylation of histone H4 in the promoter regions of these genes.

Discussion

Epigenetic therapy using an HDACi, alone or in combination with other treatments, has shown therapeutic potential in clinical trials for the treatment of several types of cancer, including non-small cell lung cancer and breast cancer (24,25). As an HDACi, SAHA specifically induces tumor cells to undergo apoptosis by regulating the expression of key genes involved in apoptotic signaling.
pathways (26). The present study revealed that SAHA induced apoptosis in HepG2 liver cancer cells by activating the ER stress-mediated apoptotic signaling pathway, at least partially through enhancing the acetylation of histone H4 on the promoter regions of ER-stress associated genes, including GRP78, ATF4 and CHOP. This suggests that ER stress-mediated apoptotic signaling may play a central role in HDACi-induced apoptosis of HepG2 cells and that SAHA alone or in combination with inducers of ER stress can potentially be applied in patients with liver cancer.

SAHA-induced apoptosis has been demonstrated to be associated with activation of the intrinsic apoptotic pathways (27). It can be concluded that in tumor cells exposed to SAHA, proapoptotic genes (such as Bax and Bim) are upregulated, whereas the expression levels of antiapoptotic genes (such as Bcl-2 and Bcl-XL) are suppressed (28). SAHA has also been shown to influence the expression of death receptors (such as Fas and TNF-related apoptosis-inducing ligand receptor) and death receptor ligands in leukemia, which are responsible for the extrinsic apoptotic pathways (29). SAHA was also demonstrated to activate the ER stress-associated apoptotic signaling pathway. Kim et al (30) demonstrated increased expression levels of p-PERK, ATF4 and CHOP in papillary thyroid cancer and anaplastic thyroid cancer cells treated with SAHA, and increased apoptotic rates of these cells. However, the mechanism of SAHA on regulating the expression of the ER stress signaling pathway-related molecules remains unclear.

Certain studies have shown the inhibitory effect of SAHA on the proliferation of liver cancer cells and the induction of apoptosis (31,32). Furthermore, another study demonstrated significant inhibition of proliferation of MHCC97L hepatocellular carcinoma cells in vitro by SAHA (Cai et al, unpublished data). In the present study, SAHA was demonstrated to suppress cell proliferation and induce apoptosis in a dose-dependent manner in HepG2 cells. Notably, the expression of GRP78 at both the mRNA and protein levels in HepG2 cells was significantly increased following treatment with 6 or 12 µM SAHA. Since GRP78 is a protein marker of ER stress (33), its increased expression suggests that SAHA may induce ER stress in HepG2 cells. In addition, it was found that the mRNA level of PERK was unaffected by SAHA treatment, indicating that SAHA does not regulate the expression of PERK at the transcriptional level. Nevertheless, treatment with SAHA resulted in significantly decreased expression of PERK and increased expression of p-PERK in HepG2 cells at protein level. This may be because SAHA-induced HepG2 cells undergo ER stress, upon which GRP78 changes its binding preference towards unfolded/misfolded proteins accumulated in the ER, resulting in the release of PERK (34).
Following the release from GRP78, PERK may undergo self-phosphorylation and dimerization (35-37), which may lead to a decreased level of PERK protein and an increased level of PERK activity. This increase in PERK activity can result in the activation of transcription factors such as ATF4 and CHOP, which play important roles in the regulation of protein synthesis and cell death. It has been shown that SAHA, a histone deacetylase inhibitor, can increase the acetylation levels of histone H4 at the promoter regions of GRP78, ATF4, and CHOP genes in HepG2 cells. This increase in acetylation levels can lead to the release of histone H4 from the promoter regions, allowing for the transcription of these genes and consequently initiating the ER stress response. The increase in acetylation levels is associated with an increase in the relative protein expression levels of GRP78, PERK, p-PERK, ATF4, and CHOP. The increased expression of these genes can lead to the activation of the unfolded protein response (UPR) and the induction of apoptosis. The results of the study suggest that SAHA-induced ER stress is a potential mechanism for the induction of apoptosis in HepG2 cells.
of p-PERK protein. Studies have demonstrated that p-PERK can upregulate the expression levels of ATF4 and CHOP in cells and activate the ER stress-induced apoptotic signaling pathway (38). In the present study, the increased expression of ATF4 and CHOP, at both the mRNA and protein levels, and augmented apoptosis were observed in HepG2 cells. These results suggest that induction of apoptosis in HepG2 cells by SAHA can be partially attributed to the activation of the PERK-ATF4-CHOP signaling pathway. However, it is worth noting that 12 µM SAHA resulted in significantly decreased proliferation of HepG2 cells (Fig. 1), however apoptosis was only induced in 10% of HepG2 cells treated for 48 h (Fig. 2). This suggests that SAHA may also induce cell death in other ways to inhibit tumor cell proliferation. For example, another study demonstrated that SAHA induced autophagic death of HepG2 cells in vitro (Cai et al, unpublished data).

The present study also unveiled an unappreciated mechanism of SAHA-mediated activation of the PERK-ATF4-CHOP signaling pathway in HepG2 cells. It was inferred that this may be associated with the essential role of SAHA, as an HDACi, in the upregulation of histone acetylation. Changes in histone acetylation influence chromatin condensation, and these alterations affect gene transcription (39). In previous studies, it was found that SAHA significantly upregulated the acetylation levels of histones H3K9 and H3K27 in HepG2 cells (40). In the present study, SAHA was identified to be able to upregulate the acetylation levels of histones H4, H4K5 and H4K12, at concentrations of 6 and 12 µM. In addition, it was reported that the acetylation levels of histone H3 in the promoter region of the GRP78 gene was significantly increased, following treatment with an HDACi and thereby promoting the genetic transcription of GRP78 (41). However, it was not clear whether the increased acetylation level of H4 regulates the transcription of genes associated with the ER stress-mediated apoptotic signaling pathway. The ChIP-qPCR results in the present study suggest that the acetylation levels of histone H4 in the gene promoter regions of GRP78, ATF4 and CHOP were all significantly increased, which was associated with significantly increased mRNA levels of these genes.

In conclusion, the HDACi SAHA induces apoptosis in HepG2 cells by activating the ER stress-mediated apoptotic pathway, at least partially through upregulation of the acH4 level on the promoter regions of the ER stress-associated molecules GRP78, ATF4 and CHOP. Further studies will be conducted to investigate the effect of acetylation modification at different sites of H4 on the expression of molecules associated with the ER stress-mediated apoptotic pathway. In addition, a study that aims to demonstrate whether SAHA has the same inhibitory effect in a mouse xenograft model in vivo will also be conducted. Overall, the present study underscores the critical roles of ER stress in mediating apoptosis in HepG2 cells and also suggests the potential application of SAHA and other inducers of ER stress for the treatment of patients with liver cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LY, TT, LZ and LT performed the majority of the experiments. BH, SC, ZM and TY provided analytical tools and performed the statistical analysis. RX and QY designed the study. RX and BH provided financial support for this work and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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