Original Article

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Expression, purification, and *S*-nitrosylation of recombinant histone deacetylase 8 in *Escherichia coli*

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Summary **Histone deacetylase (HDAC) 8 is a zinc ion dependent enzyme involved in removing the acetyl group from the core histones and other proteins which belong to Class I HDACs. It was reported that nitric oxide (NO) is a key regulator of HDAC function and *S*-nitrosylation of HDAC2 induces chromatin remodelling in neurons. This work reports the successful recombinant expression of human HDAC8 in *Escherichia coli* with two plasmids and the purification and *S*-nitrosylation *in vitro*. It was found that HDAC8 can be *S*-nitrosylated by the NO donor *S*-nitrosoglutathione (GSNO) *in vitro,* and the activity of HDAC8 was significantly inhibited when incubated with GSNO and *S*-nitrosocysteine in a time- and dosage-dependent manner, but sodium nitroprusside (SNP), and dithiothreitol cannot reverse this inhibition. These observations support and extend the concept that NO may regulate HDAC8 function by *S*-nitrosylation.**

Keywords: Histone deacetylase 8, *S*-nitrosylation, nitric oxide, *S*-nitrosoglutathione, *S*-nitrosocysteine

1. Introduction

Histone deacetylases (HDACs) which remove the acetyl group of ε-amino groups of lysine residues in the *N*-terminal extension of core histones play an important role in many biological processes such as: DNA replication, transcription, differentiation, and apoptosis. HDACs comprise a family of 18 members in humans and are divided into four classes according to amino acid sequence and number of catalytic active sites (*1*). Class I includes HDAC1, HDAC2, HDAC3, and HDAC8, which are expressed ubiquitously, localized predominantly to the nucleus, and display high enzymatic activity toward histone substrates. They possess relatively simple structures, consisting of a conserved deacetylase domain with short amino- and carboxy-terminal extensions (*2*). HDAC1 and HDAC2 are nearly identical and are generally found together in repressive complexes such as the Sin3, NuRD, CoResT, and PRC2 complexes (*3*). HDAC3 is found in distinct complexes such as the N-CoR–smRT complex, whereas no complex has been described for HDAC8 (*4*). Generally, the Zn^{2+}-dependent HDACs are more important to modulate transcription and gene expression, and most HDAC inhibitors are designed to act on these HDACs (*5*). The zinc-dependent HDAC8 catalyzes the removal of acetyl moieties from histone tails, and is critically involved in regulating chromatin structure and gene expression (*6*). Recently, it was reported that HDAC8-selective inhibitors are available, and HDAC8 may be a potential drug target for neuroblastoma differentiation therapy using selective inhibitors, avoiding nonspecific side effects (*7*). The activity evaluation of HDAC inhibitors needs pure HDACs, so the expression, purification, and characterization of HDACs are very necessary.

Nitric oxide (NO) is a free-radical product of mammalian cell metabolism that plays diverse and important roles in the regulation of cell function. Protein *S*-nitrosylation refers to the reversible attachment of the NO moiety to specific cysteine residue(s) on selected proteins, producing labile *S*-nitrosothiol structure and functional alterations (*8,9*). *S*-nitrosylation is involved in the regulation of a diverse array of

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protein functions and plays a dominant role in many signaling pathways. It is increasingly accepted that *S*-nitrosylation of proteins serves as a critical cellular regulation mechanism similar to *O*-phosphorylation (*10*). Besides the physiological importance, dysfunction in maintaining the homeostasis of *S*-nitrosylation has been linked to many disease states (*11*). Recently, it was reported that *S*-nitrosylation of HDAC2 induces chromatin remodelling in neurons (*12*). Moreover, NO donors markedly reduced the enzymatic activity of HDAC2, minimally influenced that of HDAC1, and were ineffective on HDAC3. NO regulates the enzymatic activity of HDAC2 by *S*-nitrosylation, but not that of HDAC1 and HDAC3, as was detected in C2C12 myoblasts exposed to NO donors (*13*). As HDAC8 also belongs to the Class I HDACs group, whether NO can affect the activity of HDAC8 was not yet clear. In this study, we cloned, expressed, and purified recombinant human HDAC8, and examined whether NO can affect the activity of human HDAC8 by *S*-nitrosylation.

2. Materials and Methods

2.1. *Materials*

hHDAC8 gene (1-377 residues, NCBI accession No. NM_018486.1) was purchased from FulenGen (Guangzhou, China). Plasmids pGEX-6p-1 and pET21b were purchased from Invitrogen (Carlsbad, CA, USA). All enzymes, restriction endonucleases, and DNA markers were from Takara Biotechnology (Dalian, China). The plasmid purification kit and gel extraction kit were from Tiangen Biotech (Beijing, China). Sequencing primers were synthesized by Invitrogen China (Shanghai, China). Novagen® competent BL21 (DE3) *Escherichia coli* cells were from Merck (Darmstadt, Germany). *N*-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP) were from Thermo Scientific Pierce Protein Research Products (Rockford, IL, USA). Boc-Lys (acetyl)-AMC and AMC were purchased from Bachem AG (Bubendorf, Switzerland). Streptavidin-peroxidase polymer ultrasensitive, glutathione (GSH), cysteine (Cys), Sodium nitroprusside (SNP), agarose and all other chemicals were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. *Construction of plasmid pGEX-6p-1–hHDAC8, expression of glutathione S-transferase (GST)-hHDAC8 fusion protein and purification of hHDAC8*

HDAC8 gene (1-377 residues, GenBank accession No. AF230097) was amplified by using a polymerase chain reaction (PCR) with a forward primer 5'-TCAG GATCCATGGAGGAGCCGGAGGAACCG-3' and a reverse primer 5'-TTACTCGAGCTAGACCACATG CTTCAGATTCCC-3', the products were subcloned into pGEX-6p-1 plasmid (Invitrogen). The constructs were used to transform *Escherichia coli* cells, the transformed cells were streaked on a Luria-Bertani (LB) agar plate containing 100 μg/mL ampicillin. Positive clones were selected by colony PCR and DNA sequencing. The recombinant human HDAC8 proteins with an *N*-terminal GST-tag were overexpressed in *Escherichia coli* strain BL21 (DE3). The full-length HDAC8 proteins were purified using a GST column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) as described previously (*14*). Source Q and Superdex-200 columns (GE Healthcare Bio-Sciences) were used for further purification of human HDAC8.

2.3. *Construction of plasmid pET21b–hHDAC8, expression, and purification of hHDAC8*

Construction and expression of plasmid pET21b–*h*HDAC8 were similar to those of plasmid pGEX-6p-1–*h*HDAC8 just with a forward primer 5'-ATACATATG ATGGAGGAGCCGGAGGAACCG-3' and a reverse primer 5'-TTACTCGAGGACCACATGCTTCAGATTC CC-3', which contained Nde I and Xho I restriction sites. The constructs were transformed into *Escherichia coli* cells and the ampicillin-resistant colonies were selected by colony PCR and DNA sequencing. The recombinant human HDAC8 proteins with an *N*-terminal six His-tag were overexpressed in *Escherichia coli* strain BL21 (DE3). The *h*HDAC8 were first purified using a Ni-NTA resin column (GE Healthcare Bio-Sciences), then Source Q and Surperdex 200 columns were used for further purification.

2.4. *hHDAC8 activity assay*

Fluorescence analysis was used for the *h*HDAC8 activity assay (*15*). HDAC8 releases the acetate moiety from ε-acetylated lysine residues of the substrate peptides, the deacetylated peptides containing unprotected lysine residues are recognized by trypsin and subsequently cleaved to release 7-amino-4-methylcoumarin (AMC). The fluorescence measurement was done at an excitation of 390 nm and emission of 460 nm by fluorometry (Varioskan; Thermo Fisher Scientific, Waltham, MA, USA). A standard curve of the cleavage product AMC was used to convert the fluorescence readings into micromolar products and calculate the specific activity of HDAC8 which was purified using two different recombinant plasmids (*pGEX-6p-1–hHDAC8* and *pET21b–hHDAC8*).

2.5. *Synthesis of S-nitrosoglutathione (GSNO) and S-nitrosocysteine (Cys-NO) and detection of their effect on HDAC8*

GSNO was synthesized by combining 200 mM $NaNO_2$

and 200 mM reduced glutathione in 0.5 M HCl in the dark at room temperature for 10 min. The solution was neutralized to pH 7.2 with 1 M NaOH, and its concentration determined by absorbance at 334 nm using an extinction coefficient of 900 M^{-1}•cm^{-1} (*16*). The synthesis of Cys-NO was similar to GSNO using cysteine to replace the glutathione. The recombinant HDAC8 was incubated with GSNO, Cys-NO, or SNP at a concentration gradient range for 30, 60, and 90 min at room temperature. Samples were desalted with Bio-Spin columns to remove excessive GSNO, Cys-NO, or SNP. To half of the samples at the 60 min incubation time, 1 mM dithiothreitol (DTT) was added for 15 min. The assay of HDAC8 activity was done as described above.

2.6. *Western blot to detect the S-nitrosylation of HDAC8 in vitro*

To induce *S*-nitrosylation *in vitro*, the recombinant HDAC8 proteins were incubated with freshly prepared GSNO (100 μM) in the dark for 30 min. Acetone precipitation was used to remove excessive GSNO. The Biotin-switch method was carried out essentially in the dark as detailed previously (*17*). Briefly, free protein thiols were blocked with 4 volumes of HEN buffer (250 mM Hepes-NaOH pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) containing 2.5% SDS and 20 mM methyl methanethiol sulfonate at 50°C for 30 min. Proteins were precipitated with cold acetone and suspended in HEN buffer containing 1% SDS. Ascorbate (1 mM) was added to specifically reduce the SNO bond to free protein thiols, which were then labeled with biotin-HPDP (4 mM) for 1 h at room temperature. Proteins were added with SDS-PAGE sample buffer without boiling and DTT, and SDS-PAGE was performed. After transferring to a poly(vinylidene fluoride) membtane for immunoblotting, biotinylated proteins were detected with anti-biotin mouse monoclonal antibody.

3. Results

3.1. *Clone, expression, and purification of human HDAC8*

The expressed *h*HDAC8 was about 42 kDa and the expression level was approximately 1.2% of the total bacteria proteins for the pGEX-6p-1–*h*HDAC8 plasmid (Figure 1A), and approximately 0.5% of the total bacteria proteins for the pET21b–*h*HDAC8 plasmid (Figure 1B). SDS-PAGE analysis revealed that most target proteins existed in insoluble fractions. This indicated that the target proteins were mainly expressed as inclusion bodies in *Escherichia coli*. PreScission Protease (PPase) which specifically recognized LeuGluValLeuPheGln/GlyPro was used to resection the GST-tag from the *h*HDAC8 fused with GST and combined to the GST-column. His-*h*HDAC8 combined to the Ni-NTA resin column was eluted with 250 mM imidazole. After affinity purification, the purity of *h*HDAC8 and His-*h*HDAC8 was over 90% (Figure 1). After further ion chromatography using a Source Q column and a final molecular sieve chromatography with Superdex-200, the purity of *h*HDAC8 was above 95% on SDS-PAGE stained with Coomassie brilliant blue. The HDAC8 purified from these two recombinant plasmids were all active proteins, and the specific activity of *h*HDAC8 from pGEX-6p-1-*h*HDAC8 plasmid was 0.73 ± 0.23 U/mg and His-*h*HDAC8 from pET21b-*h*HDAC8 plasmid was 1.39 ± 0.44 U/mg. The higher specific activity of His-*h*HDAC8 may indicate that the *N*-terminal six his tag may stabilize the structure of HDAC8, since the already known crystal structures of *h*HDAC8 were mostly purified from pET21b plasmid in *Escherichia coli* (*18*).

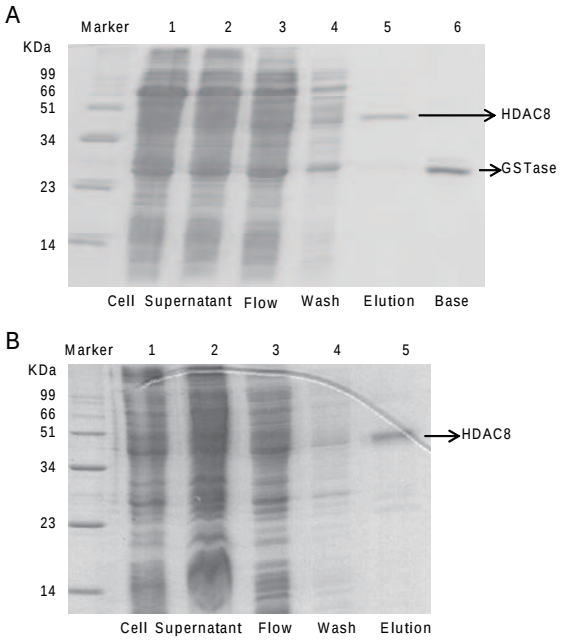

Figure 1. Expression and purification of *h*HDAC8. Ten microliters of proteins from each step were loaded. **(A)** Expression and purification of *h*HDAC8 from pGEX-6p-1–*h*HDAC8 plasmid. Lane 1, Isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cells (arrow indicates GSTase–*h*HDAC8); lane 2, soluble lysate from induced cells; lane 3, soluble lysate after GST column flow-through; lane 4, proteins after GST column flow-through with wash buffer (25 mM Tris-HCl, pH 8.0, containing 150 mM NaCl); lane 5, *h*HDAC8 eluted from GST column by using prescission protease (PPase) to resection the GST-tag; lane 6, GSTase left on GST column base. **(B)** Expression and purification of *h*HDAC8 from pET21b–*h*HDAC8 plasmid. Lane 1, IPTG-induced cells (arrow indicates His-*h*HDAC8); lane 2, soluble lysate from induced cells; lane 3, soluble lysate after Ni-NTA resin column flow-through; lane 4, proteins after Ni-NTA resin column flow-through with wash buffer (25 mM Tris-HCl, pH 8.0, containing 5 mM imidazole and 150 mM NaCl); lane 5, His-*h*HDAC8 eluted from Ni-NTA resin column by 25 mM Tris-HCl, pH 8.0, containing 250 mM imidazole, 150 mM NaCl.

3.2. *The activity of hHDAC8 can be inhibited by GSNO, Cys-NO but not SNP*

It was reported that Class I HDACs which include HDAC1, HDAC2, and HDAC3 can be the targets for *S*-nitrosylation and that NO donors significantly reduced the enzymatic activity of HDAC2, minimally influenced that of HDAC1, and were ineffective on HDAC3 (*13*). However, the effect of NO donors on HDAC8 has not been reported. In this study, we tested whether NO donors caused functional changes in HDAC8. As shown in Figure 2, GSNO (50-1,000 μM) caused concentration- and time-dependent inhibition of *h*HDAC8 activity, and the same inhibition also occurred with Cys-NO (75-800 μM), but not SNP. From our synthesis of GSNO and Cys-NO, the GSNO we used might contain a small amount of GSH, $NaNO_2$, and oxidized glutathione disulfide (GSSG), while the Cys-NO might contain a small amount of cysteine and $NaNO_2$ (*19*). We tested whether these compounds could cause HDAC8 inhibition. We found that GSH, cysteine, $NaNO_2$, and GSSG at concentrations of 50-1,000 μM had no detectable effects on HDAC8 activity (data not shown), suggesting that the contaminating compounds did not contribute to the inhibitory effects of GSNO and Cys-NO. When DTT, a reagent that effectively decomposes *S*-nitrosothiol, was added to the GSNO or Cys-NO treated samples, HDAC8 activity was recovered completely to the original level (Figure 2), further supporting the hypothesis that *S*-nitrosylation plays a role in controlling HDAC8 activity.

3.3. *HDAC8 can be S-nitrosylated in vitro*

S-nitrosylation is involved in the regulation of a diverse array of protein functions and plays a dominant role in many signaling pathways. It was reported that *S*-nitrosylation of HDAC2 induced chromatin remodeling and emerged as an important regulatory mechanism for signal-dependent control of gene expression (*12*). In this study, we found the activity of *h*HDAC8 can be inhibited by GSNO and Cys-NO which existed in physiological conditions but not SNP. Therefore we used the biotin switch method to detect protein *S*-nitrosylation *in vitro*. As shown in Figure 3, HDAC8 obtained from two plasmids can be *S*-nitrosylated *in vitro* by incubation with 100 μM GSNO. These results indicated that HDAC8 can be *S*-nitrosylated *in vitro*, and NO may regulate HDAC8 function by protein *S*-nitrosylation.

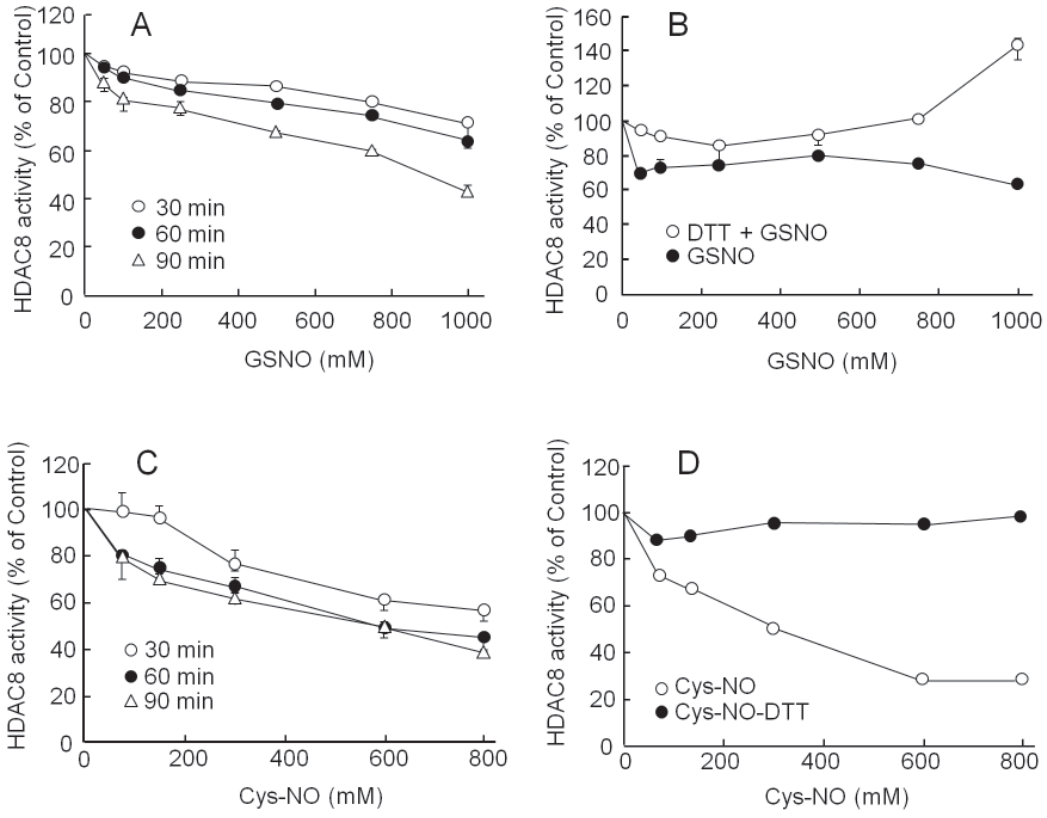

Figure 2. The activity of *h*HDAC8 was inhibited by GSNO and Cys-NO, and the inhibition was reversed by adding DTT. (A) HDAC8 activity was significantly inhibited by incubating with GSNO at final concentrations of 0, 50, 100, 250, 500, 750, and 1,000 μM for 30, 60, and 90 min. **(B)** 1 mM DTT was added to the sample HDAC8 incubated with GSNO for 60 min. **(C)** HDAC8 activity was significantly inhibited by incubating with Cys-NO at final concentration of 0, 75, 150, 300, 600, and 800 μM for 30, 60, and 90 min. **(D)** 1 mM DTT was added to the sample HDAC8 incubated with Cys-NO for 60 min. The result is representative of three different experiments.

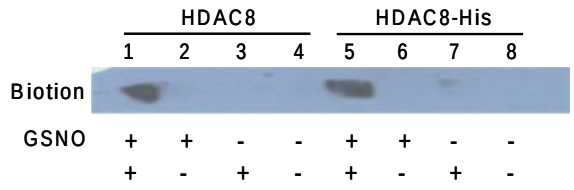

Figure 3. Western blot analysis of *S*-nitrosylated HDAC8. HDAC8 can be *S*-nitrosylated by incubation with GSNO (100 μM) *in vitro*. HDAC8 used on lines 1, 2, 3, and 4 was purified from pGEX-6p-1–*h*HDAC8 plasmid, while HDAC8 on lines 5, 6, 7, and 8 was purified from pET-21b–*h*HDAC8 plasmid. First, samples were divided into two parts, half incubated with 100 μM GSNO (lines 1, 2, 5, and 6), while the other with an equal volume of water (lines 3, 4, 7, and 8). Then, at the step of biotin-HPDP labelling on the SNO, samples were divided into two parts again, half was labeled with biotin-HPDP (lines 1, 3, 5, and 7), while the other half with solvent (lines 2, 4, 6, and 8). The result is representative of three different experiments.

4. Discussion

HDAC8, a eukaryotic zinc-dependent HDAC, is important for the growth of human tumor cell lines and has a distinct inhibition pattern that differs from that of HDAC1 and HDAC3, which both share 43% sequence identity with HDAC8 (*18*). It was reported that HDAC8 expression correlates with a poor outcome in neuroblastoma and selective HDAC8 inhibition induces cell differentiation (*7*), therefore, HDAC8 may be a potential drug target for neuroblastoma differentiation therapy using HDAC8-selective inhibitors. The cloning, expression and purification of human HDAC8 will help to screen for HDAC8 selective inhibitors.

Biological actions of NO arise as a direct consequence of chemical reactions between NO or NO-derived species and protein targets (*10*). NO exerts its action through addition to the transition metal ions which normally function as protein cofactors. However, more and more often direct structural modifications of peptidyl amino acid residues are being studied, such as the modifications of cysteine (*S*-nitrosylation) and tyrosine (nitration) residues, with respect to their putative signaling functions (*20*). It was reported that *S*-nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons (*13*). Moreover, NO decreases the enzymatic activity of a subgroup of neuronal HDACs *in vitro* and indicates that NO is a key regulator of HDAC function in mammalian neurons (*21*). In this study, we found NO donors markedly reduced the enzymatic activity of HDAC8 in a time and concentration dependent manner. The NO donors, GSNO and Cys-NO, which exist in the physiological state could inhibit the activity of HDAC8 and DTT can reverse this inhibition, but SNP has no effect. This may indicate that NO effects on HDAC8 needs a special structure to transfer the NO molecule, and this inhibition is reversible.

In this study, we further found HDAC8 can be *S*-nitrosylated when incubated with a NO donor (GSNO) at a physiological concentration. Herein, we only found that HDAC8 can be *S*-nitrosylated, but the cysteine modified site of HDAC8 was not determined. In general, there has been a delay in adequate appreciation of the role of *S*-nitrosylation in biological signaling by NO. This lag is attributed to a poor understanding of the basis for selective targeting of NO to particular thiols, and methodological limitations in accurately quantifying this modification. Of course recent breakthroughs in concepts and methods diminish these barriers, such as the SNO-SID method (*10,22*). There are nine cysteine residues in the HDAC8 sequence, therefore, the identification of cysteine *S*-nitrosylation sites is very necessary for further research. In addition, we only found *S*-nitrosylation of HDAC8 *in vitro* but did not detect *S*-nitrosylation of HDAC8 in physiological and pathological processes *in vivo*. Since HDAC8 associates with smooth muscle alpha-actin, is essential for smooth muscle cell contractility (*23*), and induces neuroblastoma cell differentiation (*7*), study of the *S*-nitrosylation level of HDAC8 in these processes may be helpful to understand the role of the molecular mechanisms.

In conclusion, we cloned, expressed, and purified active human HDAC8 from two plasmids in *Escherichia coli*, and first found NO may regulate the activity of HDAC8 by *S*-nitrosylation *in vitro*. These findings should give some insight into the further research of HDAC8 in disease.

Acknowledgements

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