Suberoylanilide Hydroxamic Acid, an Inhibitor of Histone Deacetylases, Suppresses the Growth of Prostate Cancer Cells in Vitro and in Vivo

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ABSTRACT

Suberoylanilide hydroxamic acid (SAHA) is the prototype of a family of hybrid polar compounds that induce growth arrest in transformed cells and show promise for the treatment of cancer. SAHA induces differentiation and/or apoptosis in certain transformed cells in culture and is a potent inhibitor of histone deacetylases. In this study, we examined the effects of SAHA on the growth of human prostate cancer cells in culture and on the growth of the CWR22 human prostate xenograft in nude mice. SAHA suppressed the growth of the LNCaP, PC-3, and TSU-Pr1 cell lines at micromolar concentrations (2.5–7.5 μM). SAHA induced dose-dependent cell death in the LNCaP cells. In mice with transplanted CWR22 human prostate tumors, SAHA (25, 50, and 100 mg/kg/day) caused significant suppression of tumor growth compared with mice receiving vehicle alone; treatment with 50 mg/kg/day resulted in a 97% reduction in the mean final tumor volume compared with controls. At this dose, there was no detectable toxicity as evaluated by weight gain and necropsy examination. Increased accumulation of acetylated core histones was detected in the CWR22 tumors within 6 h of SAHA administration. SAHA induced prostate-specific antigen mRNA expression in CWR22 prostate cancer cells, resulting in higher levels of serum prostate-specific antigen than predicted from tumor volume alone. The results suggest that hydroxamic acid-based hybrid polar compounds inhibit prostate cancer cell growth and may be useful, relatively nontoxic agents for the treatment of prostate carcinoma.

INTRODUCTION

Agents that induce differentiation and/or apoptosis of transformed cells have potential for the treatment of cancer (1, 2). SAHA is the prototype of a series of hydroxamic acid-based HPCs that are potent inducers of differentiation and/or apoptosis in murine erythroleukemia, bladder, and myeloma cell lines (3, 4) at micromolar concentrations. SAHA has growth-inhibitory effects on T/5 rat prostate cancer cells (5). SAHA administered p.o. inhibits the development of N-methylN-nitroso-N-methylurea-induced mammary tumors in rats (6) and lung tumors in mice induced by 4-(methylnitros-amino)-1-(3-pyridyl)-1-butanone, the carcinogen found in tobacco smoke (7).

SAHA and other hydroxamic acid-based HPCs are potent inhibitors of HDACs (4, 8). We have shown previously that SAHA inhibits partially purified HDACs 1 and 3 at concentrations as low as 100 nm and causes an accumulation of acetylated histones in cells in culture at low micromolar concentrations (4). The active catalytic site in HDACs is evolutionarily conserved and consists of a core pocket with a zinc atom at the base of the pocket (8). Co-crystals of SAHA and a HDAC-like protein have shown that SAHA fits into the core pocket of HDAC-like protein, and its hydroxamic acid moiety interacts directly with the zinc atom in the catalytic site (8). The acetylation of nucleosomal histones is regulated by the opposing activities of HDACs and histone acetyltransferases such as cAMP-responsive element binding protein-binding protein/p300, which are components of multiprotein complexes associated with gene promoters. It has been postulated that hypoacetylated histones maintain the repressed state of a gene by condensing the associated chromatin and restricting the access of transcription factors to the DNA, whereas acetylation of histones is generally associated with activation of gene expression (9, 10). Inhibition of HDACs by SAHA may contribute to the induction of differentiation or apoptosis in transformed cells by activating transcription of target genes.

Cancer of the prostate is a major health problem and is the second highest cause of deaths from cancer each year in males in the United States. The current treatment for most prostate cancers that have spread beyond the prostate gland is surgical or chemical castration, which causes regression of the tumor due to the dependence of prostatic epithelial cells on physiological levels of circulating androgens for growth and survival. Whereas this treatment may be initially successful, most tumors eventually recur due to the expansion of an androgen-insensitive population of tumor cells. Treatment of androgen-independent tumors with cytotoxic agents is generally unsatisfactory, and, at this stage, the disease is usually fatal to the patient (11).

Agents that induce differentiation and/or apoptosis of prostate cancer cells may provide an alternative or additional approach to the treatment of this disease. Several classes of differentiating agents, including SAHA (5), butyrate and its analogues (12–15), retinoids (16–18), and vitamin D analogues (17–22), are reported to inhibit the growth of prostate cancer cells in culture and cause a shift in cellular phenotype toward a more differentiated epithelioid morphology. In this study, we tested the activity of SAHA in three prostate cancer cell lines (LNCaP, PC-3, and TSU-Pr1) and evaluated the antitumor effects of SAHA in vivo using the CWR22 human prostate cancer xenograft model in nude mice. CWR22 is an androgen-dependent tumor derived from a patient with metastatic prostate cancer (23, 24) that can be grown s.c. in nude mice. The tumors regress significantly after castration of the host mice, and this regression is preceded by a several thousand-fold reduction in serum PSA levels. The reduction in tumor volume is maintained for 80–400 days, after which androgen-independent CWR22 tumors emerge in the castrated hosts and grow until the animal must be sacrificed (25, 26). The initial response of the primary CWR22 tumors to androgen ablation and the later growth of androgen-independent tumors mimic the clinical events of human prostate cancer, making this an attractive model for the evaluation of the efficacy of SAHA in vivo. Furthermore, in vivo histocultures, these tumors have gene expression profiles and changes in PSA expression and sensitivity to cytotoxic drugs similar to those seen in human prostate tumors (27). Here, we show that SAHA inhibits the proliferation of prostate cancer cell lines in culture and markedly

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2 To whom requests for reprints should be addressed, at Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-6573; Fax: (212) 639-2861; E-mail: v-richon@ski.mskcc.org.

3 The abbreviations used are: SAHA, suberoylanilide hydroxamic acid; HDAC, histone deacetylase; HPC, hybrid polar compound; PSA, prostate-specific antigen.
suppresses the growth of CWR22 tumors in nude mice at doses that cause little evident toxicity.

MATERIALS AND METHODS

Cell Culture and Proliferation Assay. The LNCaP and PC-3 cell lines were purchased from the American Type Culture Collection. TSU-Pr1 prostate carcinoma cell lines were kindly provided by Dr. Neal Rosen (Memorial Sloan-Kettering Cancer Center, New York, NY). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

SAHA was synthesized as described previously (3) and dissolved and diluted in DMSO. The effect of SAHA on the viability of prostate cancer cells was determined by cell counting using a hemocytometer. Cells were seeded in triplicate in 24-well dishes at a density of 3 × 10^4 cells/well. Cells were allowed to attach overnight, and then the medium was replaced with medium containing the appropriate concentration of SAHA (0.25, 5, or 7.5 μM). Cells were counted at various times after the start of SAHA treatment, and cell viability was assessed by trypan blue dye exclusion.

Preparation of the CWR22 Prostate Xenografts. The CWR22 human prostate cancer xenograft was kindly provided by Dr. Thomas G. Pretlow (Case Western Reserve University, Cleveland, OH). Male BALB/c nude (nu/nu) mice were purchased at 4–6 weeks of age from the National Cancer Institute-Frederick Cancer Research & Development Center. Mice were housed under barrier conditions and maintained on a 12-h light/12-h dark cycle, with food and water supplied ad libitum. Two days before inoculation of the mice with tumor cells, a 90-day sustained-release 12.5-mg testosterone pellet (Innovative Research of America, Sarasota, FL) was implanted in the left flank. To inoculate mice with the CWR22 tumor cells, an established tumor was removed from a host mouse, minced finely through a mesh support, and suspended in RPMI 1640. An equal volume of the tumor cell suspension was removed and divided into two pieces, one of which was snap frozen in liquid nitrogen.

SAHA Preparation and Administration. Mice with palpable CWR22 tumors were divided into four groups (eight or nine mice/group) for the treatment study. All mice in each treatment group had tumors of similar size (5 × 5 mm) at the start of treatment. For administration to mice, SAHA was dissolved and diluted in a vehicle of DMSO. Each group of mice received 25, 50, or 100 mg/kg SAHA daily by i.p. injection for 21 days. A control group of eight animals was injected with vehicle only (DMSO). The injection volume was kept constant at 1 μl/g body weight. The mice were weighed three times during the experimental period to assess toxicity of the treatments, and the tumors were measured twice weekly using calipers. Tumor volume was calculated from the two-dimensional caliper measurements using the following formula: tumor volume = length × (width)^2 × π/6.

The treatment period was completed after 21 days, when the vehicle-treated group of mice had large tumors, requiring that the animals be sacrificed. On the final day of the study, all mice were sacrificed by carbon dioxide inhalation, and blood was taken from each animal by cardiac puncture. The s.c. tumor was removed and divided into two pieces, one of which was snap frozen in liquid nitrogen, and one of which was fixed in formalin and embedded in paraffin. At the end of the experimental period, one animal from each dose group was submitted to an animal pathologist at the Research Animal Resource Center of Cornell University Medical College and Memorial Sloan-Kettering Cancer Center for a complete tissue necropsy and blood cell analysis. Necropsies were also performed on two mice that died during the study.

Statistical Analyses. Tumor growth curves are presented in terms of treatment group means and SEs. Statistical significance of treatment effect was assessed by repeated-measures ANOVA after applying a power transformation to equalize residual variances and linearize the tumor growth curves. We report Bonferroni-adjusted P values for all pairwise comparisons of growth curve slopes between SAHA dose groups.

Analysis of Serum PSA Levels. Levels of PSA protein were measured in the serum of all animals at three different time points during the treatment period. Blood samples (approximately 50 μl) were taken from the tail of each mouse at the start of treatment (day 0) and at day 9 of the protocol, and a cardiac bleed was performed on each mouse at the time of sacrifice (day 21). PSA was detected in the serum using a Tandem-R radioimmunoassay method (Hybritech, San Diego, CA).

Detection of PSA mRNA by Northern Blot. Expression of PSA mRNA by the CWR22 tumor cells was analyzed for two animals in each treatment group by Northern blotting. Total RNA was extracted from the excised tumors by the guanidine isothiocyanate/phenol/chloroform method (28). RNA (20 μg) was electrophoresed through a 1% agarose gel containing 1% formaldehyde. The RNA was blotted onto Hybrid-Bond N membranes (Amersham, Buckinghamshire, United Kingdom) using standard techniques, and the blots were hybridized with a 5166 bp 32P-labeled PSA cDNA probe.

Histone Acetylation in LNCaP Cells and CWR22 Tumors. Histones were isolated from LNCaP cells by rinsing dishes of 5 × 10^6 cells with ice-cold PBS and then scraping the cells in 10 ml of ice-cold PBS. Cells were centrifuged at 1000 rpm for 5 min, and the pellet was resuspended in 1 ml of histone lysis buffer [8.6% sucrose, 1% Triton X-100, 50 mM sodium bisulfite, 10 mM Tris-HCl (pH 6.5), and 10 mM MgCl2]. Dounce homogenized lysates were centrifuged at 700 rpm for 5 min, and the nuclear pellet was washed three times with the lysis buffer and once with 10 mM Tris-HCl (pH 7.4) and 13 mM EDTA. Histones were then acid extracted from the nuclear pellets as described previously (29). CWR22 tumors were homogenized directly in histone lysis buffer, and histones were isolated from the cell lysates as described above. Acetylation of core histones in the cells or tumors was determined by Western blotting using rabbit polyclonal antibodies against acetylated histone H3 or H4 (Upstate Biotechnology, Lake Placid, NY) and visualized using the Super Signal chemiluminescence system (Pierce, Rockford, IL), as described previously (30).

RESULTS

SAHA Suppresses the Growth of Prostate Cancer Cells in Vitro. The growth-inhibitory activity of SAHA was assessed in three prostate cancer cell lines (LNCaP, PC-3, and TSU-Pr1) in culture. SAHA caused a dose-dependent inhibition of proliferation in all three cell lines at micromolar concentrations (2.5–7.5 μM), with LNCaP cells being the most sensitive to SAHA treatment, and TSU-Pr1 cells being the least sensitive to SAHA treatment (Fig. 1A). Culture with 2.5 μM SAHA caused complete growth suppression of the androgen-sensitive LNCaP cells but little evident induction of cell death as assayed by trypan blue dye exclusion. At concentrations of 5 and 7.5 μM, SAHA induced cell death in the LNCaP cells within 48 h of culture (Fig. 1A). Culture with SAHA at the indicated concentrations caused growth suppression of the androgen-insensitive cell lines PC-3 and TSU-Pr1 with little detectable cell death (Fig. 1A).

The effect of SAHA on histone acetylation in prostate cancer cells was assessed in LNCaP cells treated with SAHA (0.005–5 μM) for 3 h in culture (Fig. 1B). Cells treated without or with 0.005 or 0.05 μM SAHA had similar baseline levels of acetylated histones H3 and H4 (Fig. 1B). An incremental accumulation of acetylated histones H3 and H4 was observed in cells cultured with 0.5 and 2 μM SAHA. No further increase was observed after culture with 5 μM SAHA.

Efficacy and Toxicity of SAHA in the CWR22 Prostate Xenograft Model. The activity of SAHA against the growth of prostate cancer cells in vivo was examined in the androgen-dependent CWR22 human prostate cancer xenograft, which was grown s.c. in nude mice. Daily administration of SAHA caused significant suppression of the growth of established CWR22 tumors, such that doses of 25, 50, and 100 mg/kg/day caused reductions of 78%, 97%, and 97%, respectively, in the mean final tumor volume compared with vehicle-treated control animals (Fig. 2). Tumor regression (a reduction in tumor size relative to the size of the tumor at the initiation of treatment) was observed in one of eight mice receiving 25 mg/kg/day SAHA and in five of nine mice receiving 50 mg/kg/day SAHA. In the five animals receiving 100 mg/kg/day SAHA that survived for at least 20 days of treatment, three showed tumor regression.

The toxicity of SAHA was assessed by monitoring the body weight and survival of the mice and by the necropsy of one mouse in each group at the conclusion of the study (after 21 days of treatment).
Necropsy included histological examination of at least 26 different tissues or organs and a complete blood count and differential. No deaths were observed among the vehicle-treated controls or the mice treated with 25 mg/kg/day SAHA, and, on average, these mice gained 1.5 and 1.7 grams in body weight, respectively, relative to their weights at the initiation of treatment (Table 1). One of the nine mice from the 50 mg/kg/day SAHA treatment group died on the penultimate day of the study but had no preceding weight loss, suggesting that death may have been due to an injection injury. The mice in this group gained on average 1.0 gram in body weight relative to their weight at the start of treatment (Table 1). Five of the nine mice receiving 100 mg/kg/day SAHA died during the study, and each of the mice that died experienced 1–2 days of rapid weight loss before death, suggesting that this is a toxic dose level. Surviving mice in this dose group had an average weight loss of 0.8 grams relative to their weight at the start of treatment.

Mice in all treatment groups, including the vehicle-treated control group, had evidence of inflammation and bacterial infection in the peritoneum, in the area of injection. The necropsied mouse that received vehicle alone showed no other abnormalities in the organs examined, and the hematology values were within normal limits. The necropsied mice receiving SAHA at each of the three dose levels had increased erythropoiesis in the spleen and bone marrow, but peripheral blood erythrocyte counts were within normal limits. The necropsied mice receiving SAHA at each of the three dose levels had increased erythropoiesis in the spleen and bone marrow, but peripheral blood erythrocyte counts were within normal limits. The necropsied mouse from the 50 mg/kg/day treatment group had evidence of mild lymphoid hyperplasia in the spleen and bone marrow, but peripheral blood erythrocyte counts were within normal limits. The necropsied mouse receiving 100 mg/kg/day also had mild lymphoid hyperplasia in the spleen and showed evidence of neutrophilic leukocytosis. No other gross or histological abnormalities were found at necropsy.

Necropsies were also performed on two of the animals that died in the 100 mg/kg/day SAHA treatment group. Both mice showed in-
SAHA suppresses prostate cancer cell growth

Tumor-bearing experimental groups before treatment (Fig. 3). In the animals receiving vehicle, PSA levels increased over time but did so at a lower rate (Fig. 3A). Northern analysis on RNA extracted from two tumors in each treatment group revealed a treatment dose-dependent increase in the levels of PSA mRNA in the SAHA-treated groups compared with vehicle-treated controls (Fig. 3B).

Acetylation of Histones in CWR22 Tumors. The effect of SAHA on the acetylation of histones in the CWR22 tumors in vivo was analyzed at two time points after the administration of the agent. Mice with established CWR22 tumors were injected with vehicle alone or with 25 or 50 mg/kg SAHA (four mice/group), and two mice from each group were sacrificed at 6 and 12 h after the injection. Tumors were removed from the animals, and histones were extracted for the detection of acetylated lysine residues by Western blotting (see “Materials and Methods”).

CWR22 tumors from mice treated with vehicle alone had low baseline levels of acetylated histones H3 and H4 (Fig. 4). At 6 h after injection of SAHA, an increased accumulation of acetylated histones H3 and H4 was present in tumors from one animal receiving 25 mg/kg SAHA and in both animals receiving 50 mg/kg SAHA (Fig. 4). By 12 h after injection of SAHA, the accumulation of acetylated histones

Table 1  Effect of SAHA administration on the body weights and survival of the CWR22-bearing mice

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<td>Vehicle (DMSO)</td>
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<td>25 mg/kg SAHA</td>
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<td>50 mg/kg SAHA</td>
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* All values are presented as the mean ± SE for the surviving animals (n) in each treatment group. Initial and final body weights refer to the weight of the mice at the start of SAHA treatment and the end of the experiment, respectively.

* Mice that died in this dose group showed marked weight loss for 1–2 days before death.

Fig. 3. Effect of SAHA treatment on the peripheral blood PSA levels in mice bearing CWR22 prostate xenografts and PSA mRNA expression in the tumors. A, levels of PSA protein in the serum (shown as mean ± SE) were measured in all mice treated daily with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. PSA was detected by RIA in serum taken from each mouse by tail-bleed on the first day of treatment (day 0), day 9 of treatment, and at the end of the experimental period (day 21). B, the expression of PSA mRNA was determined by Northern blot using total RNA extracted from two tumors in each treatment group. The top panel shows the PSA mRNA levels in the tumors detected using a 32P-labeled 510-bp cDNA probe corresponding to a region within the open reading frame of the human PSA protein. The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.

Fig. 2. s.c. growth of the CWR22 prostate cancer xenograft in nude mice treated with daily i.p. injection of vehicle alone or SAHA (25, 50, or 100 mg/kg). Data are presented as the mean tumor volume ± SE of the surviving animals in each treatment group. All groups contained eight or nine mice at each time point, except for the group receiving 100 mg/kg/day SAHA, in which five animals died by the end of the treatment period. Treatment commenced 7 days after implantation of the tumors, when palpable tumors (mean volume, 65 mm³) were evident in the animals (designated as day 0).

These mice also had suppressed erythropoiesis in the spleen and bone marrow.

The striking finding is that 50 mg/kg/day SAHA for 21 days caused marked to complete suppression of CWR22 prostate tumor growth without apparent toxicity. At 25 mg/kg/day, there was no apparent toxicity, but the suppression of tumor growth was less; whereas at 100 mg/kg/day, there was marked to complete suppression of tumor growth associated with evidence of toxicity.

Serum PSA Levels. PSA was measured in blood samples taken from each animal at days 0, 9, and 21 of the treatment period. There were no significant differences in the serum levels of PSA among the tumor-bearing experimental groups before treatment (Fig. 3A). In the animals receiving vehicle, PSA levels increased approximately 47-fold over the 21-day experimental period (Fig. 3A), in agreement with previous findings that serum PSA levels rise in proportion to the increasing tumor burden (24). In each of the SAHA-treated groups of mice, the serum PSA concentration also increased over time but did so at a lower rate (Fig. 3A). Northern analysis on RNA extracted from two tumors in each treatment group revealed a treatment dose-dependent increase in the levels of PSA mRNA in the SAHA-treated groups compared with vehicle-treated controls (Fig. 3B).

The effect of SAHA on the acetylation of histones in the CWR22 tumors in vivo was

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was reduced to the level seen in vehicle-treated controls. These findings are consistent with the relatively short half-life of SAHA (15–20 min) that we have determined in a mouse, dogs, and humans (data not shown).

**DISCUSSION**

We have reported previously that hydroxamic acid-based HPCs, such as SAHA, are potent inducers of differentiation and/or apoptosis in transformed cells in culture (3). Here we demonstrate that SAHA can suppress the growth of both androgen-sensitive and -insensitive prostate cancer cells in culture at micromolar concentrations, and administration of the agent to nude mice bearing human androgen-dependent CWR22 prostate tumors can arrest tumor growth at doses that cause little detectable toxicity. A dose of 50 mg/kg/day caused a reduction of 97% in the mean final tumor volume compared with that of tumor-bearing mice receiving vehicle alone, with no detectable toxicity as evaluated by weight loss and extensive necropsy studies. Our findings suggest that this novel class of agents may be effective for the treatment of human prostate cancers and other neoplasms.

s.c. growth of the CWR22 xenograft in nude mice is usually associated with an increase in serum levels of PSA that correlates with tumor burden (24). We had similar findings in the prostate tumor-bearing animals not receiving SAHA. In animals treated with SAHA (50 mg/kg), despite an almost complete suppression of CWR22 tumor growth, serum PSA levels rose 12-fold. Northern blot analysis to evaluate gene expression in prostate tumors revealed that the rise in serum PSA levels in SAHA-treated mice was correlated with an induction of PSA mRNA. These findings indicate that serum PSA levels may not be a good surrogate marker of tumor burden or disease progression after treatment with SAHA. Induction of PSA expression in prostate cancer cells has also been observed with other agents that suppress the growth of prostate cell lines in vitro and/or in vivo, including butyrate analogues (14, 15), retinoids (17), and vitamin D3 analogues (17, 21).

One of the well-characterized biochemical effects of SAHA and other hydroxamic acid-based HPCs is increased accumulation of acetylated core histones caused by inhibition of HDAC activity (4, 8, 13, 14, 19). In the present study, accumulation of acetylated histones was demonstrated in LNCaP prostate cells treated with SAHA in culture and in CWR22 tumors from mice treated with SAHA. Within 3 h of culture with SAHA, an increased accumulation of acetylated histones was detected in LNCaP cells. Similarly, in vivo, within 6 h of SAHA administration to mice, increased accumulation of acetylated histones was observed in CWR22 tumors from one of two mice receiving 25 mg/kg SAHA and in two of two mice receiving 50 mg/kg SAHA. Observations in our laboratory and other laboratories indicate that histone acetylation increases in both normal and tumor cells after treatment with HDAC inhibitors (31). Nevertheless, the growth-suppressive and apoptotic activity of these agents appears to be confined to transformed cells. Treatment of normal human fibroblasts or melanocytes with the HPCs azelaic bishydroxamic acid or azelaic-1-hydroxamate-9-anilide causes no growth inhibition at doses that suppress the growth of transformed cell lines (31, 32), although both the normal and transformed cells show similar increases in histone acetylation (31). Furthermore, azelaic-1-hydroxamate-9-anilide, azelaic bishydroxamic acid, and SAHA each suppress the growth of tumor xenografts in vivo at doses that cause little or no apparent toxicity to the animals (31). The mechanism of this selectivity is not yet understood. Hyperacetylation of the histones in either normal or tumor cells may prove to be a useful intermediate marker for biological activity of the HDAC inhibitors in the design of preclinical and clinical trials of these agents.

**ACKNOWLEDGMENTS**

We thank Dr. Hai Nguyen for performing the animal necropsies during the study, Tom Tolentino for expert technical assistance, and William Fox for assistance with the animal database and helpful discussions during the study. The PSA cDNA probe was generated from a full-length construct provided by Drs. George Farmer and Len Freedman.

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