Vandetanib, an Inhibitor of VEGF Receptor-2 and EGF Receptor, Suppresses Tumor Development and Improves Prognosis of Liver Cancer in Mice

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Abstract

Purpose: VEGF, EGF, and TGF-α are expressed in hepatocellular carcinomas (HCC) and play a role in its growth. Vandetanib, a multikinase inhibitor, suppresses the phosphorylation of VEGF receptor 2 (VEGFR-2) and EGF receptor (EGFR). The aim of this study was to clarify the antitumor effect of vandetanib in mouse HCCs.

Experimental Design: We evaluated the effects of vandetanib on proliferation of human umbilical vein endothelial cells (HUVEC) and three hepatoma cell lines, as well as the phosphorylation of VEGFR-2 and EGFR in these cells. Mice were implanted with hepatoma cells subcutaneously or orthotopically in the liver and treated with 50 or 75 mg/kg vandetanib. We analyzed the effects of treatment on tumor cell proliferation and apoptosis, vessel density, phosphorylation of VEGFR-2 and EGFR, and production of VEGF, TGF-α, and EGF in tumor tissues. Adverse events on vandetanib administration were also investigated.

Results: Vandetanib suppressed phosphorylation of VEGFR-2 in HUVECs and EGFR in hepatoma cells and inhibited cell proliferation. In tumor-bearing mice, vandetanib suppressed phosphorylation of VEGFR-2 and EGFR in tumor tissues, significantly reduced tumor vessel density, enhanced tumor cell apoptosis, suppressed tumor growth, improved survival, reduced number of intrahepatic metastases, and upregulated VEGF, TGF-α, and EGF in tumor tissues. Treatment with vandetanib was not associated with serious adverse events, including alanine aminotransferase abnormality, bone marrow suppression, or body weight loss.

Conclusions: The antitumor effects of vandetanib in mice suggest that it is a potentially suitable and safe chemotherapeutic agent for HCCs.

Clin Cancer Res; 18(14); 3924–33. ©2012 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the tropics and the Far East, including Japan (1). Recently, in the SHARP trial, a phase III, randomized, placebo-controlled trial established the efficacy of sorafenib, a multikinase inhibitor, in patients with advanced HCCs (2, 3). It is known to suppress the activities of the Raf/mitogen-activated protein kinase/extracellular signal–regulated kinase (Raf/MEK/ERK) signaling pathway, tyrosine kinase VEGF receptor (VEGFR)-2, VEGFR-3, and platelet-derived growth factor receptor-β (PDGFR-β), in patients with advanced HCCs (4–6). However, the benefits of sorafenib are limited to prolongation of survival for only 3 months. Thus, other molecular target agents are required for the treatment of advanced HCCs.

Tumor growth and formation of metastases are dependent on the existence of adequate blood supply (7, 8). In HCCs, the tumor tissue is supplied by blood from new arteries and the development of rich vasculature occurs in parallel with tumor development (9). VEGF plays a critical role in the process of angiogenesis (10, 11). VEGF is produced by hepatoma cells, hepatic stellate cells, and endothelial cells, and its expression level correlates with tumor growth (12). Hepatoma cells also produce EGF and TGF-α and express EGF receptor (EGFR; ref. 13). Increasing evidence has highlighted the importance of EGFR and its ligands EGF and TGF-α in hepatocarcinogenesis (14, 15).

Vandetanib (Zactima; ZD6474) is an orally bioavailable, small-molecule VEGFR-tyrosine kinase inhibitor with additional activity against EGFR-tyrosine kinase and RET receptor tyrosine kinase (16). Therefore, vandetanib, in addition to inhibiting endothelial cell proliferation through the...
blockade of VEGF-induced signaling can suppress tumor cell growth more directly through the blockade of EGFR autocrine signaling (17).

The present study was designed to assess the antitumor effects and adverse effects of vandetanib in mouse HCC models.

Materials and Methods

Reagents, cells, and animals

We used 3 hepatoma cell lines, HAK1-B, HuH-7, and KYN-2. Human umbilical vein endothelial cells (HUVEC) and HuH-7 were obtained from CAMBREX Bio Science Walkersville Inc. KYN-2 (18) and HAK1-B (19) were obtained from the Department of Pathology of our University. Male 5-week-old nude mice (BALB/c nu/nu; Kyudo KK.) and mice with severe-combined immunodeficiency (SCID; CB-7/1cr, Kyudo KK.) were acclimatized and placed in separate cages. All animals received humane care according to the guideline of the NIH for the Policy on Humane Care and Use of Laboratory Animals. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of Kurume University (Fukuoka, Japan).

In vitro inhibition of cell proliferation by vandetanib

Approximately 1,000 HUVECs in 200 μL of endothelial cell growth medium-2 (EGM-2) medium (EGM-2 Bullet Kit; Clonetics) supplemented with 5% FBS were added to each well of 96-well plastic dishes and incubated at 37°C for 24 hours. The medium was replaced with 200 μL of medium containing various concentrations of vandetanib (0, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 μmol/L). After incubation for 30 minutes, VEGF (3 ng/mL) was added. In another experiment, approximately 1,000 hepatoma cells in 200 μL of Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco Invitrogen Cell Culture) supplemented with 10% FBS were added to each well of 96-well plastic dishes and incubated at 37°C for 24 hours. The medium was replaced with 200 μL of medium containing various concentrations of vandetanib (0, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 μmol/L). After incubation for 72 hours, cell proliferation was measured by a tetrazolium-based assay (Cell Count Reagent SF; Nacalai Tesque Inc.). Then, we conducted cell-cycle analysis of 3 hepatoma cell lines by flow cytometry. After incubation for 72 hours with vandetanib (0, 10 μmol/L), the floating and attached cells were harvested and washed with PBS. The DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACSCalibur (Becton Dickinson). The percentage of cells in the sub-G0/G1 population was determined using CellQuest software (BD).

Western blotting

HUVECs were cultured in serum-free and VEGF-free medium, and hepatoma cells were cultured in serum-free medium for 12 hours. HUVECs were treated with various concentrations of vandetanib (0, 1.0, 5.0, 10.0 μmol/L) for 60 minutes and then incubated with VEGF (0, 50 ng/mL) for 5 minutes. Hepatoma cells were treated with vandetanib (0, 1.0, 5.0, 10.0 μmol/L) for 60 minutes and then incubated with EGF (0, 100 ng/mL) for 5 minutes. Total cell protein (50 μg) and tissue lysates were run on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with rabbit anti-phosphorylated VEGFR-2 antibody (Ty1175; Cell Signaling Technology Inc.), rabbit anti-VEGFR-2 antibody (Calbiochem-Novabiochem Corporation), rabbit anti-phosphorylated EGFR (Ty1173; Cell Signaling Technology Inc.), rabbit anti-EGFR (Cell Signaling Technology), rabbit anti-VEGF antibody (Abcam Japan), rat anti-EGF antibody (Monosan), rabbit anti-TGF-α antibody (Abcam Japan), and mouse anti-actin antibody (Sigma-Aldrich, Inc.). Each antibody was diluted 500-fold. After incubation with secondary donkey anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (dilution, 1:10,000 dilution; GE Healthcare Bio-Sciences GK), anti-rat HRP-conjugated mouse IgM (dilution, 1:2,000; Zymed Laboratories), or donkey anti-mouse HRP-conjugated IgG (dilution, 1:5,000; GE Healthcare Bio-Sciences GK) for 1 hour, immunoreactive bands were stained by an enhanced chemiluminescence Western blot analysis system (Amersham Pharmacia Biotech).

Protocols of tumor growth studies of subcutaneous tumor models

Tumor cells (5 × 10^6) were injected subcutaneously into the dorsal side in nude mice. The tumor-bearing mice were randomly divided into PBS-treated group (n = 6) and vandetanib-treated groups (n = 6). Treatment was initiated when the average size of the tumor reached 50 to 100 mm^3; the tumor-bearing mice were orally administered PBS or vandetanib (50 or 75 mg/kg) every day. To evaluate the
antitumor effect of vandetanib in mice bearing large tumors of HuH-7 (>500 mm³), the mice received PBS (n = 6) or vandetanib (75 mg/kg; n = 6). Two dimensions of the tumor were measured by calipers every 3 days, and the tumor volume was calculated by the equation: length × width² × 0.52.

Protocols of growth, survival, and intrahepatic metastases studies of liver tumor models

For tumor growth studies, nude mice were injected with KYN-2 into the liver. The mice were randomly divided into PBS-treated group (n = 6) and vandetanib-treated groups (50 mg/kg, n = 6; 75 mg/kg, n = 6). After 7 days, the mice were treated orally with vandetanib every day for 3 weeks. They were subsequently sacrificed at day 28, and tumor volume was evaluated.

For survival studies, KYN-2 cells were implanted into another group of 12 nude mice, which were then randomly divided into PBS-treated group (n = 6) and vandetanib-treated group (75 mg/kg; n = 6). Mice were sacrificed according to the clinical signs of weakness, anorexia, or more than 20% weight loss.

To evaluate intrahepatic metastasis, 2 × 10⁶ KYN-2 cells were implanted into the liver of SCID mice. The mice were then randomly divided into PBS-treated group (n = 6) and vandetanib-treated group (75 mg/kg; n = 6). Administration of vandetanib for 3 weeks was followed by counting the number of intrahepatic nodules.

Assessment of vascular density, proliferation activity, and apoptotic index in tumor tissues of liver tumor model

The sections of liver tumor tissues were incubated with rabbit anti-mouse CD31 antibody (dilution, 1:100; Abcam Japan) and rabbit anti-PCNA antibody (dilution, 1:100; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Then, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated (dilution, 1:100) or EnVision+System-HRP-labeled polymer anti-rabbit (Dako Japan). The sections were also examined for apoptosis of tumor cells by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining with In Situ Apoptosis detection kit (Oncor). The numbers of CD31-positive blood vessels in tumor tissues were counted in 50 blindly selected random fields (z-series, ×63 oil magnification). Proliferating cell nuclear antigen (PCNA)- and TUNEL-positive cells among 1,000 hepatoma cells were counted in 28 blindly selected random fields.

Measurement of serum levels of α-fetoprotein, vandetanib, alanine aminotransferase, bone marrow functions, and body weight

Serum α-fetoprotein (AFP) levels were measured at the time of sacrifice in tumor-bearing mice. Serum levels of vandetanib were measured by high-performance liquid chromatography (HPLC) at the time of sacrifice. We also determined leukocyte and platelet counts, hemoglobin (Hb) levels, and serum alanine aminotransferase (ALT) levels. Body weight was evaluated at the start of treatment and at sacrifice.

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Mann–Whitney U test, the Kruskal–Wallis rank test, and the log-rank test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Vandetanib inhibits endothelial cell and hepatoma cell proliferation

Vandetanib suppressed cell proliferation of HUVECs (IC₅₀ = 7.1 μmol/L) from 0.01 μmol/L of vandetanib in a dose-dependent manner (Fig. 1A). It also suppressed cell proliferation of the human hepatoma cell line (HAK1-B; IC₅₀ = 10.0 μmol/L) in a dose-dependent manner from 0.05 μmol/L of vandetanib (Supplementary Fig. S1A). Vandetanib also suppressed the proliferation of KYN-2 cells (IC₅₀ = 8.1 μmol/L) and HuH-7 cells (IC₅₀ = 9.4 μmol/L) from 5 to 10 μmol/L of vandetanib (Supplementary Fig. S1B and S1C). The rates of apoptosis of vandetanib-treated hepatoma cells (HAK1-B, 74.3%; KYN-2, 41.8%; HuH-7, 62.5%) were higher than those of nontreated hepatoma cells (HAK1-B, 20.4%; KYN-2, 10.7%; HuH-7, 31.3%; Fig. 1B).

Vandetanib inhibits phosphorylation of VEGFR-2 and EGFR

Vandetanib (at both 5 and 10 μmol/L) significantly inhibited VEGFR-2 phosphorylation in HUVECs (Fig. 1C). Although vandetanib (at 1–10 μmol/L) suppressed EGFR phosphorylation in the 3 hepatoma cell lines (Fig. 1D; Supplementary Fig. S2A and S2B), it did not affect the expression of total VEGFR-2 and EGFR in the same cells.

Vandetanib inhibits tumor growth of hepatoma cells in subcutaneous tumor model

In the HuH-7 xenograft model, the tumor volumes at baseline of the PBS group and 50 and 75 mg/kg vandetanib groups were 63.5 ± 11.9, 70.3 ± 16.2, and 72.3 ± 11.6 mm³, respectively. After 3 weeks of treatment, the respective tumor volumes were 4,704.7 ± 2,205.4, 773.4 ± 458.5, and 279.4 ± 91.9 mm³, respectively (Fig. 2A). In another experiment, treatment commenced when tumor volume was more than 500 mm³. Before treatment, the tumor volumes were 570.0 ± 95.6 mm³ and 614.5 ± 169.2 mm³ in the PBS and 75 mg/kg vandetanib groups, respectively. After 15 days of treatment, the respective tumor volumes were 2,491.1 ± 1,451.9 and 572.2 ± 441.5 mm³ (Fig. 2B).

In the HAK1-B xenograft model, the tumor volumes before treatment of the PBS and 50 mg/kg vandetanib groups were 68.8 ± 12.2 and 75.6 ± 15.4 mm³, respectively.
After 3 weeks, the respective tumor volumes were 461.4 ± 134.9 and 169.8 ± 37.8 mm³ (Fig. 2C). In the KYN-2 xenograft model, the tumor volumes before treatment of the PBS and 50 mg/kg vandetanib groups were 56.7 ± 12.0 and 62.6 ± 13.3 mm³, respectively, which increased at 3 weeks after the treatment to 10,092.9 ± 7,795.3 and 1,434.4 ± 903.1 mm³, respectively (Fig. 2D).

Vandetanib inhibits tumor growth and phosphorylation of VEGFR-2 and EGFR

In mice implanted with KYN-2 cells, tumor volume showed a significant inverse relationship with the dose of administered vandetanib (PBS group, 2,137.4 ± 873.3 mm³; 50 mg/kg vandetanib group, 928.9 ± 515.5 mm³; 75 mg/kg vandetanib group, 295.5 ± 427.6 mm³; Fig. 3A; Supplementary Fig. S3A). Serum AFP levels were 50,567 ± 11,300 and 16,540 ± 14,297 ng/mL in the PBS and 75 mg/kg vandetanib groups, respectively (Supplementary Fig. S3B). In tumor-bearing mice treated with vandetanib, tumor tissues showed significant suppression of VEGFR-2 and EGFR phosphorylation (Fig. 3B and C). The production levels of VEGF, TGF-α, and EGF were significantly upregulated in the 50 and 75 mg/kg vandetanib-treated groups compared with the PBS-treated group (Fig. 3D).

Vandetanib prolongs survival of tumor-bearing mice

The survival time ranged from 55 to 75 days (mean, 66.4 ± 8.6 days) in mice bearing tumors of KYN-2 treated with 75 mg/kg vandetanib. This was significantly longer than that of PBS-treated mice (range, 28–62 days; mean, 40.5 ± 11.7 days; Fig. 4A). However, all tumor-bearing mice ultimately died of tumor growth.

Vandetanib inhibits intrahepatic tumor metastasis

In the PBS group, the number of tumor nodules in livers implanted with KYN-2 cells ranged from 4 to 16 (mean, 7.7 ± 4.5). Treatment with 75 mg/kg vandetanib significantly reduced the number of tumor nodules (range, 3–4; mean, 3.2 ± 0.4; Fig. 4B).

Serum vandetanib levels and inhibition of tumor vascularization

Serum vandetanib levels in mice treated with 50 and 75 mg/kg ranged from 2.5 to 14.1 μmol/L (mean, 7.3 ± 4.6).
and 3.8 to 12.7 μmol/L (mean, 8.5 ± 3.0) at the time of sacrifice, respectively. The mean number of vessels in tumor tissues of the PBS, 50 and 75 mg/kg vandetanib groups were 15.6 ± 7.4 per high-power field (HPF), 9.3 ± 2.9 per HPF, and 6.0 ± 2.4 per HPF, respectively. Vandetanib suppressed vascular development in a dose-dependent manner (Fig. 5A and B). The vascular density in these tumors correlated with tumor volume (data not shown).

Effects of vandetanib on cell proliferation and apoptosis in tumor tissues
Vandetanib had not effect on cell proliferation of hepatoma cells (data not shown), but it increased the apoptotic index in tumor tissues from 1.2% ± 0.7% in the PBS to 2.5% ± 0.7% and 3.1% ± 0.9% in the 50 and 75 mg/kg treatment groups, respectively. The effect of vandetanib on apoptosis was dose-dependent.

Effects of vandetanib on serum ALT, body weight, and bone marrow function
There was no significant difference in body weight between the start and end of treatment in the PBS group, 50, and 75 mg/kg vandetanib groups. There were also no significant differences in body weight of the 3 groups at the start of treatment and at sacrifice (Fig. 6A). There were also no significant differences of serum ALT levels, leukocyte count, platelet count, and Hb levels among the 3 groups at sacrifice (Fig. 6B and C).
Figure 3. Vandetanib inhibits tumor growth in the liver in nude mice. A, comparison of tumor volume. Tumor volumes are expressed as mean ± SD (n = 6 per group). * P < 0.05, by Kruskal-Wallis test; ** P < 0.05, by Mann-Whitney U test compared with PBS-treated mice. B, expression of pVEGFR-2 and VEGFR-2. C, Expression of pEGFR and EGFR. D, expression of VEGF, TGF-α, and EGF. Tissue lysate protein (50 μg) was run on 10% SDS-PAGE. pEGFR, phosphorylated EGFR; pVEGFR-2; phosphorylated VEGFR-2.
Discussion

In general, signal transduction through VEGFR-2 participates in endothelial cell proliferation much more than VEGFR-1 (20). Gule and colleagues (16) reported that the antitumor effects of vandetanib were mediated through inhibition of VEGF signaling and antiangiogenesis rather than through direct antiproliferative effects on tumor cells. In our in vivo study, vandetanib dose dependently suppressed the phosphorylation of VEGFR-2 and microvascular development. Furthermore, vandetanib also induced apoptosis of hepatoma cells in vivo, although it did not suppress the proliferation of hepatoma cells. O’Reilly and colleagues (21) reported that antiangiogenic therapy upregulated the apoptotic index of tumor cells but did not reduce the proliferation of tumor cells. The above results suggest that vandetanib mainly suppresses tumor growth through its tumor antiangiogenic effect by inhibition of VEGF signaling rather than suppressing the proliferation of tumor cells.

In our study, however, vandetanib at relatively high concentrations suppressed cell proliferation and increased apoptosis of hepatoma cells in vitro. In addition to EGF, fibroblast growth factor (FGF), and PDGF also participate in hepatoma cell proliferation (22, 23). At relatively high concentrations, vandetanib inhibits FGFR and PDGFR kinases (24). Because serum vandetanib levels were relatively high, high rate of apoptosis in vivo might be induced through inhibition of EGF and PDGF signaling, as well as FGF signaling. Thus, the inhibition of these signaling pathways seems important for the effects of vandetanib in the mouse HCC model, in addition to the inhibition of VEGF signaling.

In the orthotopic liver tumor xenograft model, which mirrors the clinical course of hepatoma more accurately than the subcutaneous xenograft model, serum vandetanib levels in 50- and 75 mg/kg–treated mice were not significantly different. However, tumor volume was significantly suppressed in a dose-dependent manner. In addition, vandetanib prolonged the survival time of tumor-bearing mice. It also suppressed the growth of larger HuH-7 xenografts. These findings suggest that vandetanib is potentially useful for patients with advanced HCCs. However, our study did
not provide answers to why the antitumor effect of 75 mg/kg vandetanib was superior to that of 50 mg/kg even though the serum levels were not significantly different. Further studies of pharmacodynamics of vandetanib are needed.

Our results also showed that vandetanib significantly suppressed intrahepatic metastasis of KYN-2 cells. In their in vitro study, Giannelli and colleagues (25) reported that vandetanib blocked the proliferation, adhesion, migration, and invasion of hepatoma cells via inhibition of the EGFR pathway. Several studies have investigated tumor cell proliferation and metastasis (16, 25, 26), as well as the correlation between angiogenesis and tumor metastasis (27). What are the mechanisms of vandetanib-induced suppression of intrahepatic macrometastasis? While no direct mechanism was identified, our study showed 3 possible mechanisms. First, vandetanib suppresses tumor cell migration from the primary tumor by inhibiting primary tumor growth and expansion. Second, vandetanib inhibits the EGFR pathway and thus suppresses the adhesion, migration, and invasion of hepatoma cells (28), which are critical steps in the metastatic process. Third, vandetanib inhibits metastatic tumor enlargement ensuring inactivity of micrometastases (29).

Vandetanib administration did not reduce body weight, increase ALT, induce bone marrow suppression, or cause other serious adverse events. Recent clinical studies on the use of vandetanib in patients with lung cancer, the most common adverse events that resulted in discontinuation of vandetanib were diarrhea, rashes, and QTc prolongation (30). In the present study, we did not experience severe

Figure 6. Effects of vandetanib on body weight, serum ALT levels, and bone marrow function in tumor-bearing mice. A, body weight of tumor-bearing mice in the PBS- and vandetanib-treated groups (50 and 75 mg/kg) at the start of treatment and at sacrifice. There was no significant difference in body weight between start of treatment and at sacrifice in each group. B, serum ALT levels in mice treated with PBS and vandetanib (50 and 75 mg/kg). C, leukocyte count, Hb level, and platelet count in PBS- and vandetanib-treated mice (50 and 75 mg/kg). Vandetanib did not result in any significant change in all 4 parameters. n.s., not significant; WBC, white blood cells.
diabetes and skin rash in tumor-bearing nude mice. These differences could represent differences in species.

Several types of molecular-targeted agents are currently being investigated clinically. A recent study on advanced HCCs described the efficacy of the combination therapy of bevacizumab, a monoclonal antibody for VEGF-A, and erlotinib, which inhibits the phosphorylation of EGFR (31). The median survival period of patients on the combination therapy was 15.6 months and appeared favorable. The dual inhibition of VEGF and EGFR signaling may be more effective in the treatment of HCCs. Several reports indicated that a larger EGFR gene copy number and the presence of EGFR mutation enhanced the therapeutic efficacy of EGFR inhibitors in lung cancer and metastatic colorectal cancer (32, 33). If such predictive markers are proved to be useful in HCCs, it will be easier to select patients with HCCs who will benefit most from vandetanib.

In this study, we used xenograft HCC models and thus could not evaluate the influence of cirrhosis on treatment outcome. Such model might not precisely mirror the situation of human HCCs. Another investigation using HCC model with liver cirrhosis is required before any clinical application of vandetanib is possible.

In conclusion, we have shown in this study that vandetanib, a small-molecule tyrosine kinase inhibitor of VEGFR-2 and EGFR, significantly inhibited tumor growth and intrahepatic metastasis of hepatoma cells, had no serious adverse events, and prolonged the survival time of tumor-bearing mice.

Disclosure of Potential Conflicts of Interest
M. Sata or other entity (e.g., expert testimony) in MSD. No potential conflicts of interests were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Inoue, T. Torimura, T. Nakamura, H. Masuda, O. Hashimoto, H. Koga, T. Ueno, M. Sata
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Inoue, T. Torimura, T. Nakamura, H. Masuda, O. Hashimoto, H. Koga, T. Ueno, M. Sata
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doi:10.1158/1078-0432.CCR-11-2041

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