Copper Chelation Enhances Antitumor Efficacy and Systemic Delivery of Oncolytic HSV

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Abstract

**Purpose:** Copper in serum supports angiogenesis and inhibits replication of wild-type HSV-1. Copper chelation is currently being investigated as an antiangiogenic and antineoplastic agent in patients diagnosed with cancer. Herpes simplex virus–derived oncolytic viruses (oHSV) are being evaluated for safety and efficacy in patients, but several host barriers limit their efficacy. Here, we tested whether copper inhibits oHSV infection and replication and whether copper chelation would augment therapeutic efficacy of oHSV.

**Experimental Design:** Subcutaneous and intracranial tumor-bearing mice were treated with oHSV ± ATN-224 to evaluate tumor burden and survival. Virus replication and cell killing was measured in the presence or absence of the copper chelating agent ATN-224 and in the presence or absence of copper in vitro. Microvessel density and changes in perfusion were evaluated by immunohistochemistry and dynamic contrast enhanced MRI (DCE-MRI). Serum stability of oHSV was measured in mice fed with ATN-224. Tumor-bearing mice were injected intravenously with oHSV; tumor burden and amount of virus in tumor tissue were evaluated.

**Results:** Combination of systemic ATN-224 and oHSV significantly reduced tumor growth and prolonged animal survival. Immunohistochemistry and DCE-MRI imaging confirmed that ATN-224 reduced oHSV-induced blood vessel density and vascular leakage. Copper at physiologically relevant concentrations inhibited oHSV replication and glioma cell killing, and this effect was rescued by ATN-224. ATN-224 increased serum stability of oHSV and enhanced the efficacy of systemic delivery.

**Conclusion:** This study shows that combining ATN-224 with oHSV significantly increased serum stability of oHSV and greatly enhanced its replication and antitumor efficacy. Clin Cancer Res; 1–11. ©2012 AACR.

Introduction

Oncolytic virus therapy is emerging as a novel strategy for the treatment of patients with a diverse range of cancers, including glioma (1). Oncolytic viruses derived from Herpes Simplex Virus-1 (oHSV) have been tested in several clinical trials for the treatment of malignant glioma and have shown low toxicity with encouraging indications of efficacy (2). However, efficient oncolysis in vivo is impeded by several tumor and/or host barriers, and an effort to understand and overcome these barriers remains critically important to improve therapeutic efficacy (3).

Changes in tumor microenvironment following viral oncolysis result in increased angiogenesis in residual tumor after viral clearance (4–5). Consistent with this, therapy that combines oHSV with antiangiogenic agents has been shown to improve antitumor efficacy significantly (5–11). Copper functions as an important cofactor for several angiogenic growth factors, including VEGF and angiogenin, and is also required for the secretion of several angiogenic factors by tumor cells (12, 13). An elevation of serum copper levels found in many human tumors correlates with an increased tumor burden and a worsened prognosis (14). Apart from supporting angiogenesis, copper found in serum has been shown to inhibit wild-type HSV infection, and its topical use is currently being evaluated as an anti-herpetic agent in patients with herpes skin lesions (15–17).
Translational Relevance

Herpes simplex virus-1-derived oncolytic viruses (oHSV) represent a promising biologic therapy that is currently being evaluated in cancer patients for safety and efficacy. A better understanding of the barriers faced by oHSVs can lead to the design of rational drug combinations that synergize with oncolytic viral therapy to improve patient outcome. Serum copper can support tumoral angiogenesis and can also inhibit wild-type HSV-1 infection. ATN-224 is a second-generation copper chelator that is currently being evaluated as an antineoplastic agent in patients. Here we show for the first time that ATN-224 enhances therapeutic efficacy of oHSV by increasing its serum stability and also permitting systemic delivery. Physiologic concentration of copper directly inhibited oHSV replication and copper chelation by ATN-224 rescued the inhibitory effect of copper in vitro, ex vivo, and in vivo. Our observation is a novel finding with global implications for enhancing the clinical efficacy of oHSV when delivered systemically in patients.

Cell viability and viral replication assays

For cell viability assay, the indicated cells were infected with oHSV [multiplicity of infection (MOI) of 0.1], pre-incubated with ascorbate buffer ± copper (1 mg/L) ± ATN-224 (32 μmol/L) for 30 minutes at room temperature. Forty-eight hours post infection, the cells were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet. After washing, the crystals were dissolved in Sorenson’s buffer (0.025 mol/L sodium citrate, 0.025 mol/L citric acid in 50% ethanol), and plates were then read on a microplate reader at 590 nm. All assays were carried out in triplicate. For replication assay, the indicated cells were harvested 48 hours post oHSV infection, and the amount of infectious virus particles in cells and media was determined by carrying out a standard pfu assay on Vero cells, as previously described (10).

Animal surgery

All mice experiments were housed and handled in accordance with the Subcommittee on Research Animal Care of the Ohio State University guidelines and have been approved by the Institutional Review Board. Female athymic nu/nu mice (Charles River Laboratories), 4- to 5-week-old for subcutaneous tumor model and 6- to 8-week-old for intracranial tumor model, were used for all studies. For subcutaneous tumor studies, glioma cells (U251T3, 1.5 × 10^7) were implanted subcutaneously into the rear flank of female athymic nude mice. When tumors reached an average size of 100 mm^3, mice were randomized and treated with PBS or ATN-224 (0.7 mg/d dissolved in 100 μL PBS) by daily gavage (n = 10). Thirteen days later, PBS or rQnestin34.5 was administered intratumorally (1 × 10^5 pfu). Tumor volume was calculated using the following formula: volume = 0.5LW^2 as described (27). For intracranial tumor studies, anesthetized nude mice were fixed in a stereotactic apparatus, and a burr hole was drilled 2 mm lateral to the bregma. U87ΔEGFR cells (1 × 10^5) were then implanted at a depth of 3 mm. Three days post tumor implantation, mice were randomized to be fed...
with PBS or ATN-224 (0.7 mg/d dissolved in 100 μL PBS) by daily gavage. Ten days following tumor cell implantation, the mice were anesthetized again and injected intratumorally with PBS or 5 × 10^4 pfu of rQnestin34.5 at the same location. Animals were observed daily and were euthanized at the indicated time points when they become moribund, lethargic, anorexic, dehydrated, or distressed.

For *ex vivo* serum rescue assays, female nude mice were fed with PBS/ATN-224 by daily gavage for 10 days. Blood was collected from submandibular vein and serum was harvested as described (28). Twenty microliters of serum diluted with 20 μL of Hank’s buffered salt solution (HBSS) was incubated with rQnestin34.5 (2 × 10^3 pfu) for 1 hour at 37°C, and the amount of virus particles was measured by a standard plaque assay. For *in vivo* serum rescue assay, mice were fed with PBS/ATN-224 for 7 days and then hrR3 (2 × 10^7 pfu) was administered by tail vein injection. Twenty minutes post virus injection, serum was harvested and the number of infectious virus particles present was determined.

**PCR analysis**
To measure viral gene copy in *vivo*, tumor-bearing mice were sacrificed, and total DNA from the tumors was purified (Master PureTM Complete DNA & RNA Purification Kit, Epicentre Biotechnologies), as per manufacturer’s instruction. Viral gene copy present in the tumors was measured by determining the total number of copies of the HSV-specific ICp4 gene using quantitative real-time PCR (qPCR) analysis. ICp4 primers used were sense, 5’-CGACACGGATC-CACGACC-3’ and antisense, 5’- TATCCCCCTCCCGGCTTCGTCCG-3’. Total oHSV gene copy was determined by generating a linear regression curve using a plasmid containing the ICp4 of HSV-1 viral gene (Dr. Deborah Parris at The Ohio State University, Columbus, OH). To measure the changes in angiogenic gene expression, subcutaneous glioma models. Mice bearing subcutaneous U251T3 tumors (100 mm^3 in volume) were randomized and systemically administered ATN-224 (0.7 mg/d) or PBS via daily gavage (*n* = 10) and were then treated intratumorally with either oHSV (rQnestin34.5) or PBS. PBS-treated mice showed rapid tumor growth, whereas mice treated with rQnestin34.5 or ATN-224 alone showed a significant reduction of tumor growth (Fig. 1A). When mice were treated with both ATN-224 and rQnestin34.5, there was an even greater reduction in tumor growth with 7 of 10 mice when treated with both agents alone. On day 25, the mean tumor volumes in mice treated with ATN-224 alone, rQnestin34.5 alone, and ATN-224 plus rQnestin34.5 were 250.1 ± 106.7, 153.9 ± 50, and 21.5 ± 15.3, respectively. These data correlated to 49.7, 69, and 95.7% tumor growth inhibition as compared with PBS control (Fig. 1A).

The enhanced antitumor efficacy of oHSV combined with ATN-224 was also observed in mice bearing intracranial glioma (Fig. 1B). Mice bearing orthotropic U87ΔEGFR human glioma were treated with ATN-224 or PBS for 7 days before being treated with a single dose of rQnestin34.5 (5 × 10^7 pfu) or PBS by direct intratumoral injection. The survival of mice in each group (*n* = 8 per group) was
analyzed by Kaplan–Meier curves. These results showed a significant improvement in median survival of mice treated with ATN-224 plus rQnestin34.5 over that of single agent alone (median survivals: 19, 25, and 40 days for mice treated with ATN-224, rQnestin34.5, and ATN-224 + rQnestin34.5, respectively).

**Combination of ATN-224 and oHSV decreases blood vessel formation and vascular hyperpermeability in tumors**

To evaluate the impact of ATN-224 on glioma angiogenesis, we evaluated tumor microvessel density in subcutaneous and intracranial tumors. Figure 2A shows photomicrographs of U251T3 subcutaneous tumor sections immunostained for CD31 to highlight endothelial cells. Quantification of CD31 staining showed a statistically significant reduction in microvessel density in tumors treated with ATN-224 (Fig. 2B). More importantly, a significant inhibition in vessel density was also observed in tumors derived from mice treated with both ATN-224 and rQnestin34.5 compared with mice treated with rQnestin34.5 alone. ATN-224 has been previously shown to reduce the expression of angiogenic cytokines (29). To evaluate whether ATN-224 reduced the expression of tumoral and host angiogenic cytokines in glioma-bearing mice, we measured changes in both mouse and human angiogenic factors, VEGF and IL-8. Figure 2C shows a significant downregulation of both tumoral and host VEGF and IL-8 factors when mice are treated with ATN-224 in addition to rQnestin34.5, as compared with mice treated with rQnestin34.5 alone.

To evaluate the physiologic consequence of ATN-224 on perfusion in oHSV-treated brain tumors, we carried out dynamic contrast enhanced MRI (DCE-MRI) of intracranial glioma-bearing mice. Figure 3A shows representative images of coronal brain sections of mice treated with rQnestin34.5 or ATN-224 + rQnestin34.5. The top panel shows contrast enhancement of tumor-bearing mice imaged 3 days after the oHSV treatment and immediately following administration of gadolinium (Gd) diethylene triamine penta acetic acid. Contrast enhancement at the site of oHSV injection was evident in all of the oHSV-treated mice. The middle and bottom panels are parametric images showing changes in $K_{trans}$ (blood-to-tissue transfer constant or vessel leakiness) and $v_e$ (volume of contrast in the extravascular and extracellular space per unit volume of tissue) between PBS- and ATN-224–fed animals treated with rQnestin34.5 alone and ATN-224 + rQnestin34.5–treated animals compared with rQnestin34.5–treated animals (Fig. 3A and B). Interestingly in mice that were not treated with rQnestin34.5, ATN-224 did not alter $K_{trans}$ or $v_e$ (Supplementary Fig. S1). The reduction in vascular perfusion and leakiness indicated by MRI analysis was further investigated histologically. Figure 3C are representative hematoxylin and eosin (H&E)- and CD31-stained images of tumor-bearing brain sections, showing increased necrosis (asterix) and reduced vessel formation in mice treated with ATN-224 in addition to rQnestin34.5, as compared with mice treated with rQnestin34.5 alone. Quantification of CD31 staining...
revealed the reduced vessel formation to be statistically significant (Fig. 3D).

ATN-224 increases oHSV propagation in tumors

Interestingly, even though ATN-224 was found to be antiangiogenic in intracranial U87\textsuperscript{ΔEGFR} tumors, ATN-224 treatment alone did not increase survival of these mice (Fig. 1B). To evaluate whether increased efficacy was also because of increased oHSV propagation \textit{in vivo}, we compared viral distribution of rQnestin34.5 in mice fed with PBS to mice fed with ATN-224. Figure 4A shows representative microphotographs of HSV-1 immunostained brain sections of U87\textsuperscript{ΔEGFR} intracranial tumor-bearing mice. Although tumors from mice treated with rQnestin34.5 showed some staining for virus in tumors, the entire tumor section was highly positive for HSV-1 staining in tumors when mice were fed ATN-224 (Fig. 4A). Similarly, HSV-1 staining of U251T3 subcutaneous tumors showed enhanced virus propagation when mice were fed ATN-224 compared with mice fed with PBS (Fig. 4B). No staining was observed in tumors injected with PBS instead of oHSV (Supplementary Fig. S2). Consistent with increased HSV-1 staining, we found that the total viral yield in tumors of animals treated with ATN-224 plus rQnestin34.5 was about 3.31-fold higher than in mice treated with rQnestin34.5 alone ($P = 0.018$; Fig. 4C).

ATN-224 rescues copper-mediated inhibition of oHSV replication and cytotoxicity \textit{in vitro} and \textit{in vivo}

Apart from increasing angiogenesis, copper (Copper) has been shown to inhibit wild-type HSV-1 replication in cultured cells \textit{in vitro} (16). Thus, we tested whether the ability of ATN-224 to chelate copper directly increases oHSV propagation. We first tested the effect of Copper on the ability of oHSV to replicate \textit{in vitro}. Stable glioma cell lines, U251T3 and GLI36\textsuperscript{ΔEGFR}, and a patient-derived glioma cell, X12-V2, were infected with rQnestin34.5 or HSVQ1 at an MOI of 0.1 ± Copper. Uninfected cells were used as negative control. As both viruses encode for GFP, fluorescent microscopy was used to visualize oHSV-infected cells. A significant reduction in GFP-positive cells was apparent when the addition of copper (Fig. 5A and Supplementary Fig. S3A). Along with reduced infection, both rQnestin 34.5 and rHSVQ1 showed significantly reduced glioma cell killing when the viruses were preincubated with copper. Figure 5B, and Supplementary Fig. S3B shows increased cell survival when cells are treated with rQnestin 34.5 or rHSVQ1,
respectively, in the presence of Copper. Next, we investigated the ability of ATN-224 to rescue copper-mediated inhibition of oHSV by fluorescent microscopy and by measuring viral replication and cytolysis (Fig. 5C–E). Fluorescent microscopy of GFP-positive cells showed increased number of GFP-positive infected cells with ATN-224 + Cu(II) compared with virus incubated with Cu(II) alone (comparing middle panel to the right panel; Fig. 5C). More significantly, ATN-224 treatment completely reversed the Copper-mediated inhibition of oHSV replication and oncolysis (Fig. 5D and E). ATN-224 treatment in the absence of Copper has no significant effect on the proliferation of glioma cells in vitro or the ability of rQnestin34.5 to kill glioma cells (Supplementary Fig. S4).

To examine the physiologic relevance of these results, we investigated the effect of ATN-224 on oHSV replication in ex vivo and in vivo models. Serum from mice fed with ATN-224 or PBS was incubated with rQnestin34.5 for 30 minutes and the ability of the virus to form plaques on vero cells was measured by a standard plaque assay. Bright field and fluorescent microscopy images showed an increase in GFP-positive infected cells treated with virus incubated with serum obtained from ATN-224–treated mice compared with control mice (Fig. 6A and Supplementary Fig. S5).
Consistent with this, quantification of oHSV showed a significant increase in survived infectious virus particles in samples treated with serum obtained from ATN-224–fed mice compared with control mice (Fig. 6B). To examine whether the serum stability of oHSV was increased in vivo, mice fed with ATN-224 or PBS were injected with a single dose of oHSV (hrR3; 2 × 10^7) via tail vein. Twenty minutes post viral injection, mouse serum was harvested to evaluate the amount of infectious virus particles. Figure 6C shows a significant increase in the number of virus particles present in serum from ATN-224–treated mice compared with that from PBS-treated control mice (P < 0.01). Collectively, these results suggested that ATN-224 treatment increased the serum stability of oHSV in vivo.

**ATN-224 treatment increases systemic delivery of oHSV**

Next we tested whether treatment with ATN-224 could be used to improve systemic delivery of oHSV to tumors. U251T3 subcutaneous tumor-bearing mice were treated with PBS or ATN-224, and oHSV (hrR3 at 1 × 10^7 pfu) was injected intravenously via tail vein on day 7 post-commencement of ATN-224 or PBS treatment. Three days post-oHSV treatment, tumors were harvested and total DNA was analyzed for viral gene copy. Figure 6D shows a 2.21-fold increase in the number of oHSV particles detected in the tumor tissues from mice treated with ATN-224 compared with mice treated with PBS. Immunostained sections derived from these tumors for HSV-1 showed increased oHSV propagation when mice were fed ATN-224 compared with PBS (Fig. 6E).

As ATN-224 treatment increased virus stability in blood and increased virus presence in tumor tissue after systemic delivery, we assessed the effect of ATN-224 on the antitumor efficacy of intravenous oHSV administration. Mice bearing subcutaneous U251T3 tumors (150 mm^3) were fed ATN-224 or PBS and then injected intravenously with oHSV (rQnestin34.5 at 1 × 10^7), and tumor growth was monitored. Figure 6F shows an enhancement of the antitumor efficacy of oHSV in mice fed with ATN-224. Importantly, 5 of 8 mice treated with ATN-224 and rQnestin34.5 in combination achieved complete regression. These results suggested that ATN-224 can improve the efficacy of oHSV and also enhance its systemic delivery.

**Discussion**

Oncolytic virus therapy, including oncolytic herpes simplex virus (oHSV), is a promising biologic approach for the treatment of malignant tumors, and oncolytic viruses are currently under evaluation for their safety and efficacy in human patients (1, 30). Although recent phase I/II trials have shown acceptable safety profiles in patients, several barriers within the host limit oncolytic virus efficacy (3, 31).
Thus, efforts to develop innovative strategies to enhance the therapeutic efficacy of these agents are in high demand. Here we tested the effect of ATN-224, an antiangiogenic and novel copper chelator, on oHSV therapy. Our results show that combining ATN-224 with oHSV for the treatment of glioma remarkably enhances its antitumor efficacy compared with oHSV treatment alone due to both the inhibition of angiogenesis and the direct rescue of copper-mediated inhibition of oHSV.

Copper has been shown to increase endothelial cell proliferation in vitro and increase angiogenesis in vivo (12, 32). The angiogenic effects of copper have been attributed to an increased stabilization of HIF1α and an increased production and secretion of several angiogenic factors, including FGF, IL-8, and VEGF (32–35). In addition, increased levels of copper have been found accumulated within the malignant tissues of metastatic carcinoma and malignant glioma (36). On the basis of these results, copper depletion has been tested as an antiangiogenic strategy in preclinical animals and patients. Specifically, it has been shown that animals fed with a copper-depleted diet have a significant reduction in serum copper levels, decreased copper staining of tumor cell nuclei, reduction in microvascular density, reduction in tumor volume, and reduction in endothelial cell turnover. Because reduction of brain tumor copper was observed even in CDPT rabbits, we believe it may not be necessary for ATN-224 to cross the blood–brain barrier (BBB) to reduce brain tumor copper levels. Interestingly, however, despite reduced tumor burden and angiogenesis, these animals also showed an increase in vascular permeability (breakdown of the BBB) as well as peritumoral brain edema and thus showed no
significant improvement in survival (37). Consistent with these results, reduction in serum copper levels achieved by diet did not correlate with increased survival in patients diagnosed with glioblastoma multiforme (38). ATN-224, bis (2-hydroxyethyl) trimethylammonium, is a second-generation analog of ammonium terathiomolybdate that is U.S. Food and Drug Administration approved for Wilson disease. Apart from copper chelation, ATN-224 has been shown to have copper independent antiangiogenic effects (39, 40), to directly inhibit tumor cell invasion and to induce cancer cell anoikis (19). On the basis of promising preclinical data, it is currently being investigated as an anticancer agent in several clinical trials (NCT00383851, NCT00405574; ref. 41). Here we show that ATN-224 treatment in combination with oHSV significantly improves antitumor efficacy of oHSV. Consistent with previous studies on animals fed with a copper-depleted diet, we found ATN-224 treatment of tumor-bearing mice reduced tumoral angiogenesis and slowed down tumor growth of animals bearing subcutaneous tumors.

As noted above, although copper depletion has been shown to reduce vessel density, it has also been shown to increase vascular edema in tumor-bearing animals (37). To investigate this issue in our model, we used DCE-MRI to assess changes in $K_{trans}$ and $v_e$ in mice treated with ATN-224 compared with PBS. Interestingly, in the context of oHSV-treated tumors, ATN-224 treatment reduced both $K_{trans}$ and $v_e$, suggesting a reduced vascular leakage in oHSV-treated tumors. It will be

Figure 6. Increased oHSV viability (stability) in ATN-224–treated mice serum. A and B, ex vivo serum rescue assay. Serum from mice fed PBS or ATN-224 was collected and incubated with rQnestin34.5 for 1 hour at 37°C ($n = 4$ per group). A, bright field and fluorescent microscopy images of GFP-positive infected cells infected with rQnestin34.5 incubated with serum from animals fed PBS or ATN-224. B, quantification of infectious viral pfu after incubation with serum from mice fed PBS or ATN-224. Data shown are the mean viral titers obtained ± SD ($n = 4$ per group). *P < 0.05 compared with PBS. C, tumor-bearing mice fed PBS or ATN-224 by daily gavage were administered oHSV (hrR3) via tail vein injection. Total virus in serum was measured by a standard plaque assay. Data shown are the mean pfu/mL in serum of mice ± SD ($n = 6$ per group). *P < 0.05 compared with PBS. D, ATN-224 treatment increased systemic delivery of oHSV. Mice with subcutaneous U251T3 tumors were fed PBS or ATN-224 by daily gavage, hrR3 was administered intravenously and tumors were analyzed for viral gene copy. Data shown are mean HSV-1 gene copy/mg of tumor DNA ± SE. *P < 0.05 compared with PBS. E, representative photomicrographs of tumor sections stained with anti-HSV-1 antibody highlighting virus propagation in vivo. F, antitumor efficacy of oHSV is increased in mice bearing subcutaneous U251T3 tumors fed ATN-224 compared with PBS. *P < 0.05 compared with oHSV.
interesting to investigate how copper-independent antian- 
tigenic and antitumorigenic effects of ATN-224 may 
account for these differences.

Copper has also been shown to be a potent inhibitor of 
several enveloped and nonenveloped DNA and RNA viruses 
(42), possibly due to the inhibition of viral DNA replication 
and copper-mediated DNA damage (17, 26). On the basis 
of these observations, topical formulations containing cop- 
per are currently being evaluated as antiviral agents in 
patients with herpetic skin lesions (15). Here we show for 
the first time that serum-mediated inhibition of oHSV can 
be reduced by copper-chelating agent ATN-224, resulting in 
improved oHSV’s serum stability and therapeutic efficacy. 
Clinically, oHSV has been given by direct intratumoral 
junctures. These findings may facilitate its systemic delivery 
and provide a significant framework for clinical translation.

Disclosure of Potential Conflicts of Interest 
A. P. Mazar has significant equity and is a consultant for the company 
that makes ATN-224. T.N. Teknos is Co Chair of the task force on recurrent 
and metastatic disease at the National Cancer Institute. No potential conflicts 
of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Dr. Qintin Pan for helpful suggestions and advice for 
the use of ATN-224 in animals.

Grant Support
This work was supported by funding from the NIH (1R01NS064607 and 
1R01CA150153 to B. Kaur; 5R01CA119298-5 and P01 NS040923 to J.C. 
Glorioso).

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Received March 6, 2012; revised May 15, 2012; accepted June 7, 2012; 
published OnlineFirst July 2, 2012.


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Clin Cancer Res Published OnlineFirst July 2, 2012.

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doi:10.1158/1078-0432.CCR-12-0697

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