Tryptanthrin Protects Hepatocytes against Oxidative Stress via Activation of the Extracellular Signal-Regulated Kinase/NF-E2-Related Factor 2 Pathway

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Tryptanthrin [6,12-dihydro-6,12-dioxoindolo-(2,1-β)-quinazoline], originally isolated from Isatidis radix, has been characterized as having anti-microbial and anti-tumor activities. It is well-known that excess oxidative stress is one of the major factors causing cell damage in the liver. This study investigated the cytoprotective effects and molecular mechanism of tryptanthrin against tert-butyl hydroperoxide (tBHP)-induced oxidative stress in human hepatocyte-derived HepG2 cells. Tryptanthrin pre-treatment blocked the reactive oxygen species production, mitochondrial dysfunction, and cell death induced by tBHP. Moreover, tryptanthrin reversed tBHP-induced GSH reduction. This study also confirmed the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by tryptanthrin as a plausible molecular mechanism for its cytoprotective effects. Specifically, tryptanthrin treatment induced nuclear translocation and transactivation of Nrf2 as well as phosphorylation of extracellular signal-regulated kinase (ERK), a potential upstream kinase of Nrf2. Tryptanthrin also up-regulated the expression of the enzymes catalyzing the detoxification of reactive oxygen species, which are representative target genes of Nrf2. Moreover, inhibitor of ERK was used to verify the important role of the ERK-Nrf2 pathway in the hepatoprotective effects of tryptanthrin. In conclusion, this study demonstrated that tryptanthrin protects hepatocytes against oxidative stress through the activation of the ERK/Nrf2 pathway in HepG2 cells.

Key words tryptanthrin; tert-butyl hydroperoxide; oxidative stress; mitochondria; nuclear factor erythroid 2-related factor 2 (Nrf2); extracellular signal-regulated kinase

The liver is the largest organ as well as central to the regulation of several key aspects of lipid metabolism. Impairment of the response to hepatic insult with age is characteristic of the liver, which increases the incidence of liver disease in the population of older people. Moreover, the liver has various functions related to metabolism, detoxification, synthesis of plasma proteins, and bile production.1,2 tert-Butyl hydroperoxide (tBHP) has been shown to induce cell death via apoptosis.3 tBHP also induces oxidative stress, which may cause a significant increase in the reactive oxygen species (ROS) formation, which are associated with cell damage and the development of chronic diseases.3 Therefore, antioxidant compounds may be useful for preventing oxidative stress-mediated diseases.3

Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor necessary for the induction of antioxidant enzymes, is related to liver regeneration and detoxification.4 Nrf2 is associated with the oxidative stress response in the liver as well as homocysteine-mediated induction of the glutamate-cysteine ligase catalytic subunit (GCLC),7 which is mediated through stimulation of Nrf2 nuclear translocation and protein–DNA binding at antioxidant-responsive elements (AREs).8 AREs are found in the promoters of antioxidant and detoxifying enzyme genes, including heme oxygenase 1 (HO-1), GCLC, and glutathione S-transferases (GSTs).9

Tryptanthrin (6,12-dihydro-6,12-dioxoindolo-(2,1-b)-quinazoline), originally isolated from Isatidis radix, has been shown to have anti-microbial and anti-tumor properties. Tryptanthrin was reported as having anti-inflammatory effects on murine macrophages in vitro, as assessed by reduced synthesis of both nitric oxide and prostaglandin E2.10 Moreover, it was shown to be effective in reducing the incidence of azoxymethane-induced colon cancer in rats.11 This study investigated whether or not tryptanthrin has antioxidant effects against oxidative stress during tBHP-induced oxidative injury in vitro.

MATERIALS AND METHODS

Materials Tryptanthrin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2’,7’-di-chlorofluorescin diacetate (DCFH-DA) and β-actin antibody were supplied from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM) and antibiotics were supplied from Gibco(BRL (Eggenstein, Germany). Nrf2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and GCLC antibody was purchased from Abcam (Cambridge, MA, U.S.A.). Phosphorylated extracellular signal-regulated kinase (p-ERK), B-cell lymphoma-extra large (Bcl-XL), caspase-3, poly(ADP-ribose) polymerase (PARP), HO-1 and lamin A/C antibodies were supplied from Cell Signaling Technology (Danvers, MA, U.S.A.). PD98059 was purchased from EMD Millipore (Billerica, MA, U.S.A.).

Cell Culture HepG2 (human hepatoma cell line) and H4IE (rat hepatoma cell line) cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were maintained in DMEM containing 10% FBS, 50 U/mL penicillin, the cells were grown to 80–90% confluency, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2’,7’-di-chlorofluorescin diacetate (DCFH-DA) and β-actin antibody were supplied from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM) and antibiotics were supplied from Gibco/BRL (Eggenstein, Germany). Nrf2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and GCLC antibody was purchased from Abcam (Cambridge, MA, U.S.A.). Phosphorylated extracellular signal-regulated kinase (p-ERK), B-cell lymphoma-extra large (Bcl-XL), caspase-3, poly(ADP-ribose) polymerase (PARP), HO-1 and lamin A/C antibodies were supplied from Cell Signaling Technology (Danvers, MA, U.S.A.). PD98059 was purchased from EMD Millipore (Billerica, MA, U.S.A.).

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and were subjected to no more than 20 cell passages. To assess the effects of tryptanthrin, the cells were incubated in the medium without FBS for 12 h and then exposed to tBHP in the presence or absence of tryptanthrin for the indicated time periods. Tryptanthrin dissolved in dimethylsulfoxide (DMSO) was added to the incubation medium 1 h prior to the addition of tBHP.

**MTT Assay** The cells were plated at a density of $5 \times 10^4$ cells per well in a 24 well plate to determine cytoprotective activity of tryptanthrin. Cells were serum-starved for 12 h, and then treated with tryptanthrin 1 h prior to the addition of tBHP and the cells were further incubated for the next 12 h. After incubation of the cells, viable cells were stained with MTT (0.1 $\mu$g/mL, 4 h). The media were then removed, and produced formazan crystals in the wells were dissolved by addition of 300 $\mu$L of DMSO per well. Absorbance was measured at 570 nm using a Titertek Multiskan automatic multimode reader (Model Infinite 200 PRO). Cell viability was defined relative to control cells, [i.e., viability (% of control) = (absorbance of treated sample)/(absorbance of control)] x 100.

![Fig. 1. The Effect of Tryptanthrin on Hepatocyte Viability](image)

The effects of tryptanthrin on tBHP-induced cytotoxicity were assessed with a MTT assay. HepG2 (A) and H4IIE (B) cells were treated with 0.03–1 $\mu$M of tryptanthrin for 1 h prior to the addition of tBHP (100 $\mu$M), and cell was further incubated for 12 h. The data represent the mean±S.D. of three separate experiments (significant as compared with vehicle-treated control, **p < 0.01; significant as compared with tBHP alone, #p < 0.05, ##p < 0.01). (C) TUNEL assay. HepG2 cells were treated with tryptanthrin (1 $\mu$M) for 1 h and were continuously incubated with tBHP (100 $\mu$M) for 12 h. TUNEL assay was conducted as described in Materials and Methods. (D) Effects of tryptanthrin on the expression of apoptosis-related proteins. Immunoblot analyses were performed on HepG2 cell lysates. The levels of apoptotic marker proteins were monitored 12 h after treatment of cells with tBHP (100 $\mu$M), with or without tryptanthrin (1 $\mu$M) pretreatment (i.e., 1 h before tBHP). The $\beta$-actin was used as a loading control.

**Terminal Deoxynucleotidyl Transferase Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay** TUNEL assay was performed using the In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Indianapolis, IN, U.S.A.), according to the manufacturer’s instruction. Briefly, HepG2 cells were fixed at room temperature for 30 min. In incubation with 3% H$_2$O$_2$ at 4°C for 1 h and were permeabilized with 0.1% Triton X-100 for 5 min. After washing with PBS, each sample was incubated with POD 50 $\mu$L equilibration buffer at 37°C for 1 h. After washing with PBS, the reaction was stopped by immersing the sample in 50 $\mu$L converter-POD buffer for 30 min. Finally, the samples were developed using the diaminobenzidine substrate for 10 min. The samples were washed and examined under optical microscope (Olympus, Tokyo, Japan).

**Determination of Reduced Glutathione (GSH) Content** Reduced GSH in the cells was quantified using a commercial GSH determination kit (Oxis International, Portland, OR, U.S.A.). Absorbance was measured at 540 nm using a Titertek Multiskan automatic multimode reader (Model Infinite 200 PRO).
Measurement of ROS Intracellular ROS accumulation was monitored in HepG2 cells by adding the DCFH-DA. In brief, 1×10⁴ cells per well in a 96 well black plate. The medium was replaced and HepG2 cells were treated with tryptanthrin (1 µM) with or without tBHP (100 µM) for 5 h. And then the cells were followed by incubation with DCFH-DA (10 µM) for 1 h. Fluorescence was assessed at excitation and emission wavelengths of 485 and 530 nm using a Titertek Multiskan Automatic multimode reader (Model Infinite 200 PRO).

Mitochondrial Membrane Potential (MMP) Analysis MMP was measured with rhodamine 123, a membrane-permeable cationic fluorescent dye using a fluorescence-activated cell sorter (FACS). HepG2 cells were treated according to the individual experiment, stained with 0.05 µg/mL of Rhodamine for 40 min, and harvested by trypsinization. After washing with PBS containing 1% FBS, the change in MMP of cells was monitored using a Partec GmbH FACS Calibur flow cytometer (Münster, Germany).

Preparation of Whole Cell Lysates and Nuclear Extracts Cells were lysed in the lysis buffer. Cell lysates were centrifuged at 15000×g for 30 min to remove debris. Nuclear and cytosolic fractions were prepared by nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, U.S.A.), as previously described. Nuclear proteins were extracted in NER buffer supplemented with protease inhibitors according to the manufacturer’s instructions.

Immunoblot Analysis Immunoblot analyses were performed according to previously published. Proteins were separated by gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with antibodies overnight and was incubated with hors eradish peroxidase-conjugated secondary antibodies. Scanning densitometry of the immunoblot was performed with Image Processing and Analysis in Java program. Repeated experiments were performed. Equal loading of proteins was verified by immunoblot for β-actin or lamin A/C protein immunoblotting.

Generation of Stable Cell and Reporter Gene Assays ARE-driven reporter gene construct, pGL4.37 [luc2P/ARE/Hygro] was obtained from promega (Madison, WI, U.S.A.). To generate GSTA2 promoter-driven luciferase construct, pGL415-rGSTA2-1128, rGSTA2 promoter region from -1128 to -1 bp was amplified using pGL3-rGSTA2-1128 as a template, and ligated into KpnI/BglII sites of pGL4.15 (Promega). The nucleotide sequence of the construct was verified by sequence analysis using an ABI7700 DNA cycle sequencer. HepG2 cells were stably transfected with pGL4.37 or pGL415-rGSTA2-1128 plasmid using Fugene HD (Promega) according to manufacturer’s instruction and 80 µg/mL hygromycin was added to select the resistant colonies. The resistant colonies were pooled and used for reporter gene analysis. To determine luciferase activity, stably transfected cells (5×10⁴ cells/well) were replated in 12-well plates overnight, serum starved for 12 h, and exposed to 0.03–1 µM tryptanthrin for 24 h. Luciferase activities in cell lysates were measured by adding luciferase assay reagent (Promega) using Titered Multiskan automatic multimode reader (Model Infinite 200 PRO). The relative luciferase activity was calculated as the relative change of protein content determined by bichinchoninic acid (BCA) method.

RESULTS

Inhibition of tBHP-Induced Cell Death by Tryptanthrin tBHP, an analog of lipid hydroperoxide, is frequently used as an oxidative stress inducer to screen antioxidant drug candidates. Therefore, the effects of tryptanthrin on tBHP-induced cytotoxicity were examined in this study. MTT assay was conducted to test whether or not tryptanthrin prevents tBHP-mediated cytotoxicity. Cells exposed to 100 µM tBHP reduced about 75% in cell viability compared to the control. However, tryptanthrin pre-treatment reversed the effects of tBHP in HepG2 and H4II2 cells by increasing cell viability in a concentration-dependent manner. The maximal cytoprotective effect of tryptanthrin was observed at 1 µM (Figs. 1A, B). Therefore, 1 µM tryptanthrin was used in subsequent experiments. Previous studies have shown that tBHP treatment induces severe oxidative stress and apoptosis in HepG2 cells (16,17). Therefore, we confirmed the protective effects of tryptanthrin against tBHP-induced toxicity using TUNEL assay. As shown in Fig. 1C, tBHP induced apoptotic cell death, whereas tryptanthrin protected cells against tBHP in HepG2 cells. Consistent with this result, tBHP treatment also reduced expression levels of full-length PARP (116 kDa), procaspase-3, and Bcl-XL: an anti-apoptotic member of the Bcl-2 family, confirming that exposure of cells to tBHP induced apoptosis. As expected, tryptanthrin treatment inhibited
altered apoptosis-related proteins (Fig. 1D).

Inhibition of tBHP-Inducible ROS Production and GSH Depletion by Tryptanthrin  tBHP has shown to cause significant elevation of ROS such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. To investigate the protective effects of tryptanthrin against tBHP-induced oxidative stress, production of intracellular ROS in HepG2 cells was assayed using DCFH-DA. As expected, tBHP significantly induced DCFH-DA oxidation, which was attenuated by tryptanthrin treatment. In addition, treatment with tryptanthrin alone had no effect on ROS production (Fig. 2A). Next, to determine the antioxidant effect of tryptanthrin, GSH contents in cells treated with tBHP were measured in the presence or absence of tryptanthrin. Tryptanthrin treatment alone significantly increased cellular GSH levels compared to control, whereas tBHP treatment considerably reduced GSH basal levels. As expected, GSH depletion caused by tBHP was restored in cells treated concomitantly with tryptanthrin (Fig. 2B).

Inhibition of Mitochondrial Dysfunction by Tryptanthrin  tBHP treatment has been shown to induce loss of mitochondrial membrane potential (MMP), which is directly associated with apoptosis. To determine whether or not tryptanthrin protects against mitochondrial dysfunction induced by tBHP, MMP was measured using FACS analysis after cell staining with rhodamine, a membrane-permeable cationic fluorescent dye. tBHP treatment significantly increased the number of rhodamine-negative cells (R1 fraction), indicating mitochondrial damage and dysfunction (Fig. 3). In contrast, tryptanthrin treatment inhibited all changes in MMP induced by tBHP. Further, tryptanthrin pre-treatment alone had no effect on MMP. These results suggest that tryptanthrin treatment inhibited MMP dysfunction induced by tBHP.

Nrf2 Activation and Induction of Antioxidant Enzymes by Tryptanthrin  Nrf2 is a key transcriptional regulator that protects cells against oxidative stress and enhances cellular defense systems through induction of antioxidant and detoxification phase II enzymes. Therefore, we examined whether or not Nrf2 activation is associated with tryptanthrin-mediated cytoprotection. First, we monitored the concentration- and time-response profiles of tryptanthrin with respect to nuclear accumulation of Nrf2. Treatment of cells with tryptanthrin for 24h markedly increased nuclear Nrf2 accumulation in a concentration-dependent manner (Fig. 4A). Moreover, maximal elevation of nuclear Nrf2 expression occurred at 6h after tryptanthrin treatment (Fig. 4B). Next, to confirm whether or not accumulated Nrf2 in the nucleus binds to ARE and

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**Fig. 3. Inhibition of Mitochondrial Dysfunction by Tryptanthrin**

HepG2 cells were treated with tryptanthrin (1 µM) with/without tBHP (100 µM) for 12h, and stained with rhodamine 123 for 40min. Proportion of cell population with low rhodamine 123 intensity (R1 fraction). The data represent the mean±S.D. of three separate experiments (significant as compared with vehicle-treated control, **p<0.01; significant as compared with tBHP alone, ##p<0.01).**
induces target gene expression, we measured ARE-driven luciferase activity in HepG2 cells. As shown in Fig. 4C, tryptanthrin significantly up-regulated ARE-driven luciferase activities in a concentration-dependent manner. Especially, 1 µM tryptanthrin induced the highest luciferase activity (about 6-fold that of the control). Furthermore, GSTA2, another target gene of Nrf2 activation, was transactivated by tryptanthrin treatment in a concentration-dependent manner, as indicated by increases of GSTA2 promoter reporter gene (Fig. 4D). To verify whether or not luciferase activation by tryptanthrin in HepG2 cells corresponds with expression of Nrf2-mediated antioxidant enzymes, such as HO-1 and GCLC, we performed immunoblot analyses. As expected, tryptanthrin significantly up-regulated HO-1 and GCLC protein expression by about 3.7- and 4-fold, respectively (Fig. 4E).

Role of ERK1/2 in Tryptanthrin-Induced Nrf2-ARE Activation Nrf2 activation is regulated by protein kinase C (PKC), phosphoinositide 3-kinase (PI3K)/Akt, and mitogen-
activated protein kinase (MAPK). As such, we attempted to identify the upstream kinase involved in tryptanthrin-mediated Nrf2 activation and cytoprotection. Immunoblots were performed on lysates of cells treated with tryptanthrin from 10 min to 6 h. Tryptanthrin treatment increased Nrf2 phosphorylation in a time-dependent manner. Specifically, Nrf2 phosphorylation occurred initially at 1 h after treatment, after which it declined at 6 h after treatment (Fig. 5A). Tryptanthrin treatment also induced ERK phosphorylation in a time-dependent manner matching that of Nrf2 nuclear accumulation. ERK phosphorylation occurred at 0.5 h after treatment, after which it declined at 6 h after treatment (Fig. 5A). Tryptanthrin-mediated Nrf2 phosphorylation and ARE reporter gene induction were blocked by PD98059, an inhibitor of ERK, indicating that ERK was the responsible upstream kinase for Nrf2 phosphorylation (Fig. 5B).

To determine whether or not ERK phosphorylation contributes to tryptanthrin-mediated cytoprotection against tBHP, the effects of PD98059 treatment on MMP were examined. The ability of tryptanthrin to prevent elevation of the RNI1 fraction by tBHP was antagonized by PD98059 pre-treatment. These results provide evidence that the antioxidant effects of tryptanthrin were mediated by Nrf2 activation in association with ERK phosphorylation (Fig. 5C).

**DISCUSSION**

*Isatidis radix* has been shown to clear heat from the blood and relieve sore throat symptoms associated with the common cold. As such, *I. radix* is typically used for treating fever and headaches in Oriental herbal medicine. In addition, *I. radix* has been used clinically for the treatment of influenza and for the prevention of severe acute respiratory syndrome. It was also reported to possess antagonistic effects on endotoxin produced by *E. coli*. Tryptanthrin, a naturally occurring compound found in *I. radix*, has been reported as having anti-tumor activity toward a variety of human leukemia and cancer cells. However, there are few reports on the mechanism behind the hepatoprotective properties of tryptanthrin.

Oxidative stress is a disruption in the balance between free radical synthesis and free radical scavenging by the endogenous antioxidant system. It is documented as being involved in the pathogenesis of several chronic ailments. Oxidative stress is also associated with the formation of ROS, which mainly consist of hydrogen peroxide, hydroxyl radicals, and superoxide anions. Excessive production of ROS induces cellular damage to components including lipids, proteins, and DNA. Free radical stress may also alter cell membrane permeability by non-specific interactions, which may cause...
development and progression of cellular damage. For this reason, prevention of ROS by antioxidant enzymes and detoxifying molecules is crucial for maintaining the balance between oxidants and antioxidants. tBHP is frequently employed as an oxidative stress inducer, which induces damage to lipids and DNA.\(^3,4\) In this study, tBHP caused apoptotic death in hepatocytes through dysfunction of MMP and ROS production. However, pre-treatment with tryptanthrin inhibited tBHP-induced cell death, alteration of MMP, and production of ROS.

GSH is an important intracellular antioxidant in mammals that is synthesized mainly in the liver.\(^30\) GSH is an endogenously synthesized tripeptide thiol that plays an essential role in maintaining redox status, extinction of free radicals, conjugation/detoxification reactions, apoptosis, and cell signaling.\(^31,32\) Increased ROS and decreased GSH levels contribute to loss of MMP, which is directly associated with apoptosis.\(^18\) GSH is also the most abundant thiol antioxidant in cells and is regulated by GCL.\(^33\) Here, tryptanthrin inhibited reduction of GSH production by tBHP.

Nrf2 is a master regulator for the expression of many genes, including HO-1, GCLC, and nicotinamide adenine dinucleotide phosphate (NAD(P)H) quinone oxidoreductase-1 (NQO1), that alleviate oxidative stress.\(^9\) Activated Nrf2 is released from its cytosolic repressor Keap1 and then translocates into the nucleus, whereupon it binds to AREs in the promoter regions of target antioxidant genes.\(^34,35\) ARE is a cis-acting element that regulates the transcription of antioxidant target genes and repairs proteins.\(^30\) In this study, Nrf2 was activated by tryptanthrin, as evidenced by increased nuclear accumulation of Nrf2, transactivation of Nrf2, and protein expression of target genes (GSTA2, HO-1, and GCLC).

Expression of Nrf2-mediated target genes promotes cell survival in oxidizing environments via enhancement of free radical metabolism, regulation of proteasome function, maintenance of glutathione homeostasis, inhibition of cytokine-mediated inflammation, and recognition of damaged DNA.\(^22\) HO-1 is a highly inducible enzyme that catalyzes the rate-limiting step of free heme degradation into Fe\(^{2+}\), carbon monoxide, and biliverdin.\(^35\) There is increasing evidence to suggest that induction of HO-1 expression protects against a wide variety of chronic diseases.\(^38–40\) GCLC, a heterodimeric protein comprising catalytic and modifier subunits expressed by certain genes, plays a role in maintaining cellular redox homeostasis and reducing oxidative stress damage by glutathione synthesis.\(^41–43\) GSTs comprise a family of enzymes that promote excretion of carcinogens by making them hydrophilic through conjugation with GSH. Among GST genes, the GSTA2 gene is regulated by Nrf2 activation and binding of activated Nrf2 to AREs.\(^44\) In this study, tryptanthrin was shown to significantly induce HO-1 and GCLC protein expression in HepG2 cells.

Nuclear translocation of Nrf2 can be induced by p38 mitogen-activated protein kinase (p38 MAPK), ERK, P3K, and PKC, which phosphorylate Nrf2.\(^21,45–48\) Among them, ERK is known as an upstream kinase of Nrf2 activation as well as a signaling enzyme determining cell survival against oxidative stress.\(^49,50\) Results of this study clearly indicate that tryptanthrin activated Nrf2 phosphorylation via ERK activation. In addition, blockade of the ERK pathway using an MEK1 chemical inhibitor attenuated tryptanthrin-mediated Nrf2 phosphorylation and protection against mitochondrial dysfunction.

In conclusion, our study results demonstrate for the first time that tryptanthrin effectively suppresses tBHP-induced oxidative damage, including production of ROS and alteration of MMP. This inhibition may be associated with up-regulation of HO-1 and GCLC expression through the ERK/Nrf2 pathway. These findings will help researchers understand the effects of tryptanthrin and suggest an herbal drug for the treatment of liver disease.

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