Echinatin mitigates H$_2$O$_2$-induced oxidative damage and apoptosis in lens epithelial cells via the Nrf2/HO-1 pathway

Haijun Ran$^{1,B,D}$, Han Liu$^{2,B,C}$, Ping Wu$^{3,A,E,F}$

$^1$ Nanchong Aier Mega Eye Hospital, China
$^2$ Department of Ophthalmology, Jiangjin Central Hospital of Chongqing, China
$^3$ Chongqing Aier Eye Hospital, China

A — research concept and design; B — collection and/or assembly of data; C — data analysis and interpretation; D — writing the article; E — critical revision of the article; F — final approval of the article

Abstract

Background. Oxidative stress has been reported to be an early factor in the development of cataracts. Echinatin (Ech) is a active ingredient of licorice that exhibits antioxidant effects.

Objectives. To investigate the effects of Ech on oxidative stress–induced lens epithelial cell (LEC) damage.

Materials and methods. Human lens epithelial B3 cells (HLECs) were exposed to hydrogen peroxide (H$_2$O$_2$) and were pretreated with or without Ech. For rescue experiments, ML385, an inhibitor of the Nrf2 pathway, was added into the medium.

Results. Echinatin reversed the H$_2$O$_2$–induced reduction of cell viability in B3 cells. Additionally, H$_2$O$_2$ induced oxidative stress, evidenced by an increase of reactive oxygen species (ROS) and malondialdehyde (MDA) levels, and a decrease in superoxide dismutase (SOD) and catalase (CAT) levels, which could be abolished by Ech. Echinatin treatment also reduced HLEC apoptosis induced by H$_2$O$_2$. In addition, Ech pretreatment promoted Bcl-2 expression, and suppressed Bax and caspase-3 expression levels, in H$_2$O$_2$-treated B3 cells. Moreover, H$_2$O$_2$ significantly reduced Nrf2 nuclear localization, as well as HO-1 and NQO1 expression, which could be reversed by Ech. Inhibition of Nrf2 by ML385 aggravated H$_2$O$_2$–induced oxidative damage and apoptosis in HLECs, and the protective effects of Ech on H$_2$O$_2$–induced oxidative damage and apoptosis could be restored by ML385.

Conclusions. Echinatin mitigates H$_2$O$_2$–induced oxidative damage and apoptosis in HLECs via the Nrf2/HO-1 pathway, suggesting that Ech may be a potential drug for the treatment of cataracts.

Key words: cataract, lens epithelial cells, apoptosis, oxidative stress, echinatin
Background

Cataracts have become the main cause of loss of useful vision worldwide. It is currently believed that, with the exception of congenital cataracts, the apoptosis of lens epithelial cells (LECs) is the cytological basis for the formation of various types of cataracts. Due to long-term exposure to light, the lens is continuously damaged by reactive oxygen species (ROS), which is considered to be a key factor in the development of cataracts. Studies have confirmed that cataracts are directly related to the apoptosis of LECs caused by oxygen free radicals. Thus, it is important to explore antioxidant drugs that can prevent the formation and development of cataracts.

The process of oxidative stress mainly involves a variety of stress-sensitive signaling pathways. As one of the main cellular defense mechanisms against oxidative stress, Nrf2 is crucial in resisting cell damage caused by endogenous and exogenous stress. As the main regulator of the antioxidant response, Nrf2 can induce the expression of target genes, such as NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1) and catalase (CAT). The dysfunction of Nrf2 is inseparable from the development of cataract. Studies have shown that the protein and gene expression of Nrf2 in the lens significantly decreases with age. The decreased activity of Nrf2 limits the transcription of its downstream antioxidant enzymes and causes the antioxidant system to fail, which ultimately leads to age-related cataracts. Targeted activation of Nrf2 signaling can protect LECs from damage induced by oxidative stress.

Echinatin (Ech; 4,4'-dihydroxy-2-methoxychalcone; Fig. 1A), a retrochalcone, is an active ingredient of licorice and the main active form with pharmacokinetic function. Studies have shown that Ech has a wide range of biological properties, including anti-inflammatory and anti-tumor effects. Importantly, Liang et al. confirmed that Ech may undergo electron transfer and proton transfer to cause antioxidant effects. However, to date, the effects of Ech on oxidative stress-induced LEC damage have not been reported, and its molecular mechanism is largely unclear. Hence, the present study attempted to investigate the potential role of Ech as an agent for controlling cataract progression against H2O2-induced oxidative stress and apoptosis in human B3 cells.

Objectives

Oxidative stress has been shown to be an early factor in the development of cataracts. Echinatin is the active ingredient of licorice, and its pharmacological effects are closely related to antioxidants. Thus, the aim of the current study is to investigate the effects of Ech on oxidative stress-induced LEC damage.

Materials and methods

All in vitro experiments in the current study were carried out using human LECs (HLECs). This study does not contain any experiments using human participants or animal subjects.

Cell culture and treatment

The human lens epithelial cell (HLEC) line B3 cells were purchased from American Type Culture Collection (CRL-11421; ATCC, Manassas, USA). B3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO2 at 37°C. B3 cells at 80% confluence were treated with different concentrations of H2O2 (Sigma–Aldrich, Seelze, Germany; 0, 50, 100, 200, 400, and 800 µM) for 24 h. To investigate the role of Ech, B3 cells were pretreated with different concentrations of Ech (0, 5, 10, 20, 50, and 100 µM) for 12 h before the H2O2 treatment. For rescue experiments, B3 cells were pretreated with ML385 (5 µM; Sigma–Aldrich), a specific inhibitor of Nrf2, for 12 h in the presence or absence of Ech (50 µM), followed by exposure to H2O2. Echinatin (C16H14O4; CAS No. 34221-41-5, M.W. 270.2, purity 99%) was purchased from Chengdu Alfa Biotechnology Co. Ltd. (Chengdu, China).
MTT assay

The cells (2 × 10^5) were seeded in a 96-well culture plate and incubated with H_2O_2 (0, 50, 100, 200, 400, and 800 µM) for 24 h alone or after pretreatment with Ech (0, 5, 10, 20, 50, and 100 µM) for 12 h. The cells were then incubated with 5 mg/mL MTT solution (Beyotime, Shanghai, China) for 4 h at 37°C. The supernatant was aspirated and dimethyl sulfoxide (DMSO) was added to the cells. The optical density at 490 nm was observed using a microplate reader (SpectraMax iD3; Molecular Devices, San Jose, USA).

Determination of intracellular ROS levels

The ROS level was gauged using 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (DCHFDA; Beyotime) according to the manufacturer's protocol. Briefly, after exposure to corresponding treatment, the culture medium was removed and B3 cells were incubated with 25 µM of DCHFDA mix for 45 min at 37°C. The absorbance was detected at a wavelength of 488 nm.

Measurement of MDA, SOD and CAT

After centrifugation of cell lysates, the supernatants were collected. The protein concentration was measured according to the BCA Assay Kit instructions (Beyotime). Malondialdehyde (MDA) was measured using a lipid peroxidation MDA assay kit (Beyotime). The test working solution was added to the sample and mixed. The mixture was heated at 100°C for 15 min. After centrifugation, the supernatants were collected. The absorbance was detected at a wavelength of 532 nm and the results were expressed as nmol/mg protein.

Total superoxide dismutase (SOD) content was determined using a SOD assay kit (Beyotime), and the results were expressed as U/mg protein. A Cu/Zn-SOD inhibitor was added to the samples to inhibit the activity of the Cu/Zn-SOD enzyme, followed by incubation with the WST-8/enzyme working solution at 37°C for 30 min. The absorbance was detected at a wavelength of 450 nm. One SOD enzymatic activity unit (U) was defined as the amount of sample needed to achieve a 50% inhibition rate of WST-8 formazan dye.

Catalase (CAT) content was tested using a CAT assay kit (Beyotime). After the protein expression in whole cell lysates or nuclear extracts was analyzed using western blot analysis. The radioactive immunoprecipitation assay (RIPA) lysis buffer (Beyotime) was used to extract total protein from B3 cells. Next, a BCA protein assay kit (Beyotime) was used to quantify the proteins. Then, 30-µg protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The membranes were then blocked using 5% skimmed milk for 1 h at room temperature, followed by incubation with primary antibodies against Bcl-2 (Abcam, Cambridge, USA), Bax (Abcam), Nrf2 (Cell Signaling Technology, Beverly, USA), HO-1 (Abcam), NQO1 (Cell Signaling Technology), and GAPDH (Cell Signaling Technology) at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Abcam) for 1 h at room temperature, followed by exposure to the enhanced chemiluminescent reagent (Pierce, Rockford, USA). The intensity of proteins signals was quantified using Quantity One software v. 4.1.1 (Bio-Rad Laboratories, Hercules, USA).

Measurement of apoptosis

Cells (1 × 10^5) were seeded and pretreated with or without Ech for 12 h, followed by exposure to 100 µM H_2O_2 for 24 h. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The apoptosis of B3 cells was measured using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (Cell Death Detection Kit; Beyotime), according to the instructions of the manufacturer. The cells were visualized under a fluorescent microscope (Olympus Corp., Tokyo, Japan). Data were expressed as the ratio of TUNEL-positive cells to total cells.

Western blot assay

The protein expression in whole cell lysates or nuclear extracts was analyzed using western blot analysis. The radioactive immunoprecipitation assay (RIPA) lysis buffer (Beyotime) was used to extract total protein from B3 cells. Next, a BCA protein assay kit (Beyotime) was used to quantify the proteins. Then, 30-µg protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The membranes were then blocked using 5% skimmed milk for 1 h at room temperature, followed by incubation with primary antibodies against Bcl-2 (Abcam, Cambridge, USA), Bax (Abcam), Nrf2 (Cell Signaling Technology, Beverly, USA), HO-1 (Abcam), NQO1 (Cell Signaling Technology), and GAPDH (Cell Signaling Technology) at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Abcam) for 1 h at room temperature, followed by exposure to the enhanced chemiluminescent reagent (Pierce, Rockford, USA). The intensity of proteins signals was quantified using Quantity One software v. 4.1.1 (Bio-Rad Laboratories, Hercules, USA).

Measurement of caspase-3 activity

As described previously,18 caspase-3 activity was measured using a caspase-3 activity assay kit (Beyotime) according to the manufacturer’s protocol. For each sample, an equal amount of protein (200 µg) was mixed with reaction buffer (50 µL) and caspase-3 substrate (5 µL) in the dark at 37°C for 30 min. The absorbance at 485 nm was measured using a microplate reader.

Statistical analyses

Data are expressed as mean ± standard deviation (SD) from at least 3 independent experiments, and the results were analyzed using GraphPad Prism v. 8.0 (GraphPad Software, San Diego, USA). Data from individual groups were confirmed to follow a normal distribution using
the Kolmogorov–Smirnov test. Comparisons between 2 groups were analyzed using Student’s t-tests (Welch’s correction was used in cases of unequal variance), and comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc tests. A p-value of less than 0.05 was considered statistically significant.

**Results**

**Echinatin attenuates the effects of H$_2$O$_2$ on viability of HLECs**

We performed a MTT assay to evaluate the effects of H$_2$O$_2$ and Ech on the viability of B3 cells. The B3 cells were exposed to H$_2$O$_2$ (0–800 µM) for 24 h and the results showed that higher concentrations of H$_2$O$_2$ (100–800 µM) restrained cell viability in a dose-dependent manner (Fig. 1B). In view of the results that the treatment with 100 µM H$_2$O$_2$ for 24 h could reduce cell viability to approx. 50% compared to the control group, this concentration was chosen for subsequent experiments. Additionally, Ech (0–50 µM) did not affect the viability of HLECs (Fig. 1C). Moreover, the pretreatment with Ech (10–50 µM) illustrated a protective effect against H$_2$O$_2$-induced damage in a dose-dependent manner (Fig. 1D).

**Echinatin reduces H$_2$O$_2$-induced oxidative damage in HLECs**

Oxidative stress is considered to be an early factor in the development of cataracts. B3 cells were treated with H$_2$O$_2$ for 24 h and exhibited the onset of oxidative stress manifested by the enhanced levels of ROS (Fig. 2A) and MDA (Fig. 2B), and suppressed levels of SOD (Fig. 2C) and CAT (Fig. 2D), compared to control cells. The cells pretreated with Ech restored levels similar to those of control cells in a dose-dependent manner (Fig. 2A–D), indicating that Ech had a protective effect on oxidative damage in HLECs.

**Ech reduces cell apoptosis induced by H$_2$O$_2$ in HLECs**

Apoptosis of LECs is the main cytological basis for the formation of various types cataract. Here, the TUNEL assay was used to measure HLEC apoptosis after exposure to Ech and/or H$_2$O$_2$. The results illustrated that H$_2$O$_2$ exposure markedly induced apoptosis, compared to the control group (Fig. 3A,B). Additionally, Ech treatment could reduce HLEC apoptosis induced by H$_2$O$_2$ in a dose-dependent manner (Fig. 3A,B). Moreover, western blot analysis showed that the expression of the anti-apoptotic protein Bcl-2 was markedly suppressed, while the expression of the pro-apoptotic protein Bax was significantly enhanced after the exposure to H$_2$O$_2$, which could be reversed by Ech pretreatment (Fig. 3C,D). Furthermore, Ech pretreatment eliminated the increase in caspase-3 activity induced by H$_2$O$_2$ (Fig. 3E).

**Ech activates the Nrf2/HO-1 pathway in H$_2$O$_2$-treated HLECs**

To determine the effects of Nrf2 on H$_2$O$_2$-induced HLECs, the expression levels of Nrf2 and its downstream targets (HO-1 and NQO1) were analyzed using the western blot assay. Since Nrf2 nuclear translocation is an essential step for activation of the Nrf2 pathway, the nuclear localization of Nrf2 in B3 cells was also analyzed using the western blot assay. The results showed that H$_2$O$_2$ significantly reduced Nrf2 nuclear localization, which could be reversed with Ech pretreatment (Fig. 4A,B). Moreover, compared to the control group, the expression levels of HO-1 and NQO1 were decreased after the exposure to H$_2$O$_2$, which could be reversed with Ech pretreatment (Fig. 4C,D). These data demonstrate that Ech may activate the Nrf2/HO-1 pathway in H$_2$O$_2$-treated HLECs.

**Inhibition of Nrf2 by ML385 aggravates H$_2$O$_2$-induced oxidative damage and apoptosis in HLECs**

We assessed the effect of ML385 (5 µM) alone on the viability of B3 cells, and the results confirmed that ML385 had...
Fig. 4. Echinatin (Ech) activates the Nrf2/HO-1 pathway in H2O2-treated human lens epithelial B3 cells (HLECs)

B3 cells were pretreated with Ech (10 µM, 20 µM and 50 µM) for 12 h, followed by exposure to H2O2 (100 µM) for 24 h. A. The expression of nuclear and cytosolic Nrf2 was analyzed using the western blot assay; B. Quantitative results of Nrf2 levels; C. The expression of HO-1 and NQO1 was analyzed using the western blot assay; D. Quantitative results of HO-1 and NQO1 levels; ****p < 0.0001, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001.
no effect on the viability of B3 cells, but aggravated the inhibitory effect of H2O2 on cell viability (Fig. 5A). Compared with the control group, ML385 increased ROS and MDA levels, and decreased SOD and CAT levels in B3 cells. Simultaneously, ML385 enhanced the oxidative stress damage caused by H2O2 (Fig. 5B–E). There was no change in the apoptosis rate between the ML385 group and the control group. Importantly, inhibition of Nrf2 by ML385 also exacerbated H2O2-induced cell apoptosis in HLECs (Fig. 5F).

**ML385 attenuates the protective effect of Ech on H2O2-induced oxidative damage and apoptosis in HLECs**

To confirm that Ech could resist H2O2-induced cell damage by activating the Nrf2 pathway, B3 cells were pretreated with Ech (50 µM) and ML385 (5 µM) for 12 h, followed by exposure to H2O2 (100 µM) for 24 h. The results showed that Ech restrained the enhancement of ROS and MDA, and weakened SOD and CAT induced by H2O2, whereas ML385 abolished the effects of Ech on oxidative stress (Fig. 6A–D). Similarly, the anti-apoptotic effects of Ech on H2O2-induced B3 cells also could be blocked by ML385 (Fig. 6E–G). These results reveal that Ech protected B3 cells against H2O2-induced oxidative injury and apoptosis via activation of the Nrf2 pathway (Fig. 7).

**Discussion**

Oxidative stress has been proven to be an early factor in the development of cataracts, and drugs that prevent this event are needed to resist adverse cellular reactions. Oxidative stress damage refers to the comprehensive effect of exogenous or endogenous ROS on the cell signal transduction system, or to damage to nucleic acids, proteins and lipid molecules. Hydrogen peroxide can induce the production of ROS in cells, leading to oxidative damage. Early studies confirmed that high concentrations of H2O2 in the lens and aqueous humor can cause cataracts. Exogenous H2O2 treatment is a simple and feasible cell model for studying LEC oxidative damage, which can effectively simulate the process of oxidative damage in LECs that results in cataracts. Therefore, in the present study, 100 µM H2O2 was selected as an inducer of oxidative damage in B3 cells.

Previous work has confirmed that the use of antioxidants and certain metabolic receptor agonists can delay the occurrence of cataracts. For instance, glycyrrhizin, a substance extracted from licorice, prevents sodium iodate-induced retinal pigment epithelium and retinal injury via the inhibition of ROS. Echinatin has been shown to prevent or treat cardiovascular disease, tumors and diabetic nephropathy. Recently, the antioxidant properties of Ech have been confirmed. Kwak et al. reported that Ech can exert anti-cancer effects by inducing ROS/endoplasmic reticulum stress (ERS)-dependent apoptosis. Tian et al. proposed that Ech improves myocardial injury caused by ischemia and reperfusion by reducing oxidative stress and apoptosis of cardiomyocytes. However, the effects of Ech on the development of cataracts remain unclear. In the present study, Ech significantly improved the viability of H2O2-treated LECs. Simultaneously, Ech pretreatment prevented the production of ROS and MDA, and enhanced the activity of SOD and CAT in LECs treated with H2O2. These data suggest that Ech can effectively prevent oxygen free radicals from
entering the lens, and has a protective effect on the oxidative damage of LECs induced by H\textsubscript{2}O\textsubscript{2} by enhancing cell viability.

Studies on the prevention and treatment of primary or subsequent cataracts mainly focus on how to control the apoptosis of LECs.\textsuperscript{29,30} Apoptosis is a type of programmed cell death different from cell necrosis,\textsuperscript{31} where the mitochondrial apoptotic pathway plays a key role. The \textit{Bcl-2} family and the caspase family play important roles in the mitochondrial pathway.\textsuperscript{32,33} \textit{Bcl-2} family members play a key role in maintaining the integrity of the outer mitochondrial membrane and regulate the release of cytochrome C, which determines the direction of apoptosis regulation in the apoptosis pathway.\textsuperscript{34} Caspase is a protease system that can lead to the disintegration of apoptotic cells, among which caspase-3 is the main executor of apoptosis.\textsuperscript{35,36} In addition to congenital cataracts, the apoptosis of LECs plays a vital role in the formation of other types of cataracts. Studies have shown that \textit{p53}-dependent LECs can be induced to undergo apoptosis by ultraviolet radiation, which further leads to the formation of cataracts.\textsuperscript{37,38} Tamada et al.\textsuperscript{39} found that apoptosis in selenite cataracts may be an early event, which is reflected in the increase of caspase-3 enzyme activity. Similar studies have found that high glucose can also induce the apoptosis of LECs, which is essential for the formation of cataracts.\textsuperscript{40,41} Moreover, cataracts are directly related to the apoptosis of LECs caused by oxygen free radicals.\textsuperscript{42,43}

It has been reported that antioxidant genes and drugs can inhibit the oxidative damage caused by H\textsubscript{2}O\textsubscript{2} by reducing the activity of ROS.\textsuperscript{44,45} Lens epithelial cells initiate apoptosis-related signal transduction pathways under oxidative stress, which mediates the apoptosis of LECs and promotes the development of cataracts. Reactive oxygen species promote the entry of cytochrome C into
the cytoplasm by oxidizing the thiol group on the adenine nucleotide transporter, and induce the caspase cascade, which ultimately leads to irreversible cell apoptosis.46 Wu et al.47 reported that the ERS inhibitor, salubrin, reduces the H$_2$O$_2$-induced oxidative stress damage in HepG2 cells by inhibiting cell apoptosis. Here, we analyzed the effect of Ech on the apoptosis of LECs treated with H$_2$O$_2$. Similar to previous reports,48 this study showed that H$_2$O$_2$ induced the apoptosis of HLECs, which may be an important mechanism for the development of cataracts caused by oxidative stress. Additionally, Ech reduced the apoptosis of HLECs induced by H$_2$O$_2$ treatment. Echinatin also inhibited the levels of Bax and caspase-3, and promoted the expression of Bcl-2. These results indicate that Ech can protect HLECs from apoptosis caused by oxidative stress.

Nrf2 signal transduction is a pivotal mechanism to maintain oxidation and antioxidation homeostasis, and to reduce oxidative stress damage.49 Normally, Nrf2 is anchored in the cytoplasm through Keap1. Nrf2 is dissociated from Keap1 and transferred to the nucleus under the stimulation of oxidative stress, phosphorylation or electrophiles.50 HO-1 and NQO1 are the key downstream factors of Nrf2 signal transduction, which are very important in protecting cells from the oxidative damage.51,52 Recent studies have shown that trimetazidine can delay the formation of age-related cataracts by regulating the expression of Nrf2 and reducing the production of ROS.53 Whitson et al.54 found that LECs lacking glutathione (GSH) depend on the activation of the Nrf2 signaling pathway to trigger oxidative stress. Moreover, Nrf2 inhibitors may increase the oxidative stress of the lens, and Nrf2 inducers can prevent cataract formation by reducing oxidative stress. Therefore, Nrf2 pathway activation can be used as a target for the prevention and treatment of age-related cataracts induced by oxidative stress. In the present study, we found that Ech abolished the inhibitory effect of H$_2$O$_2$ on Nrf2 nuclear translocation in B3 cells, as well as the expression of HO-1 and NQO1. Furthermore, administration of the Nrf2 inhibitor ML385 could reverse the protective effect of Ech, suggesting that the potential antioxidant mechanism of Ech may include Nrf2 signal transduction. Importantly, it has been reported that Ech can inhibit activation of the NF-$\kappa$B pathway55 and the AKT/mTOR pathway.56 Therefore, the protective effect of Ech may also involve other signal pathways, a hypothesis that needs further study. In addition, because the potential toxicity and side effects of Ech and its derivatives are still unclear, there is still a lot of research to be performed before this drug can be applied in the clinic.

Limitations

The therapeutic effect of Ech should be further identified in an animal model of cataracts. In addition, more work needs to be done to elucidate its underlying molecular mechanisms.

Conclusions

As far as we know, the present study is the first demonstration that Ech can protect HLECs from the oxidative stress damage caused by the exposure to H$_2$O$_2$. More importantly, Ech pretreatment reduced cell apoptosis induced by H$_2$O$_2$, providing new directions in the search for novel drugs to prevent and treat cataracts.

ORCID IDs

Haijun Ran https://orcid.org/0000-0002-6710-2685
Han Liu https://orcid.org/0000-0002-6039-9362
Ping Wu https://orcid.org/0000-0002-8297-6140

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