## Original Article
# The HMG-CoA reductase inhibitor rosuvastatin promotes complement factor H expression in apolipoprotein E-knockout mice

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**Abstract:** The aim of this study was to investigate the relationship between plaque severity and the expression of complement factor H (CFH) in an apo E-knockout mouse model of atherosclerosis. An additional objective was to determine the effect of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) on this relationship. Eight-week-old apo E-knockout mice were divided randomly into 2 groups: group A (atherosclerotic model group, n=14), and group B (rosuvastatin-treated group, n=8). Ten age- and genetic background-matched healthy C57BL/6 mice were used as a control group. After 20 weeks of continuous intragastric rosuvastatin administration, aorta samples were taken for H&E and immunohistochemical staining. Quantitative real-time PCR was performed to determine the level of CFH transcription, and western blot analysis was performed to measure CFH protein expression. Immunohistochemical staining showed that CFH was expressed in group A, and this was accompanied by increased mRNA and protein levels and the severity of atherosclerosis. However, none of these changes were observed in the control group. Rosuvastatin upregulated CFH mRNA expression and increased CFH protein levels. The plasma level of cholesterol was higher in group A than that in the control group (P<0.05). Furthermore, serum high-sensitive C-reactive protein and C3a levels decreased significantly in group B compared with those in group A (P<0.05). This study verified expression of CFH in atherosclerotic plaques in apo E-knockout mice and shows that rosuvastatin attenuated lesions and stabilized aortic plaques, possibly by increasing the expression of CFH and suppressing inflammation.

**Keywords:** Atherosclerosis, complement factor H (CFH), apolipoprotein E-knockout mice, rosuvastatin, inflammation

## Introduction

Atherosclerosis is the most common pathological process in various cardiovascular diseases (CVDs) that leads to occlusive lesions in the large- and medium-sized arteries. These lesions are characterized by the formation of atherosclerotic plaques consisting of necrotic cores, accumulated modified lipids, inflamed smooth muscle cells (SMCs), endothelial cells (ECs), leukocytes, and foam cells [1]. Because atherosclerosis is a complex disease, many components of the vascular, metabolic, and immune systems are involved in this process. Although low-density lipoprotein cholesterol (LDL-C) remains the most important risk factor for atherosclerosis, immune and inflammatory mechanisms of atherosclerosis have garnered tremendous interest in the past 20 years [1-3].

Complement is the most important component of humoral autoimmunity, and it has been proposed that antibody-independent mechanisms activate complement, with cholesterol and oxysterols activating the alternative pathway and C-reactive protein (CRP) activating the classical pathway [4, 5]. Full activation of the complement cascade in atherosclerotic plaques has been shown, with the generation of the membrane attack complex (MAC) resulting from activation of the classical pathway, alternative pathway, or both pathways [5-7].

Complement factor H (CFH) is a key regulator of the complement system of innate immunity that maintains optimum circulating levels of C3 [8]. CFH influences the risk of age-related macular degeneration by modulating oxidative stress, inflammation, and abnormal angiogenesis [9]. Furthermore, the association of CFH polymorphisms with CVDs is dependent on other cardiovascular risk factors [10]. The use of inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (statins) has become standard treatment in patients with atherosclerotic disease. In addition to their cholesterol-lowering effects [11-13], statins have been shown to retard the progression of atherosclerosis [13], improve endothelial function [14, 15], reduce systemic inflammatory markers [16], and decrease cardiovascular morbidity and morbidity [17]. Recently, the clinical regression of coronary artery atherosclerosis was reported with the use of rosuvastatin, a third-generation, very high-intensity statin [18].

With the exploration of new therapeutic strategies to treat atherosclerotic CVD, it is increasingly important to demonstrate the role of anti-atherosclerotic mechanisms in addition to the cholesterol-lowering effect of statins as well as their potential pleiotropic effects. In line with this approach, atherosclerotic animal models responding to currently available statin therapy are useful for translational research [19]. In the present study, we aimed to explore the effects of the HMG-CoA inhibitor, rosuvastatin, on the progression of lesions and CFH expression in apolipoprotein E-knockout (ApoE-KO) mice.

## Material and methods

### Creation of a murine atherosclerotic model

Homozygous apoE-deficient (C57BL/6 background) male mice were purchased from Beking University. Male mice were used in the subsequent experiments and kept in a temperature-controlled facility on a 14- and 10-hour light-dark cycle with free access to food and water.

After weaning at 4 weeks of age, the mice were fed a normal-chow diet (Oriental Yeast) until 8 weeks of age, and then they were switched to a high-fat diet containing 21% fat and 0.15% cholesterol as previously described [20]. In addition, 10 C57BL/6 mice were fed a normal-chow diet to serve as a control group. All mice were inspected each day. The experimental protocols were approved by the Ethics Committee for Animal Experiments of Zhongda Hospital, Southeast University.

### Treatment protocol

At 18 weeks of age, 13 mice were treated by intragastric administration of rosuvastatin (0.8 mg·kg$^{-1}$·day$^{-1}$, AstraZeneca R&D, Sweden) for 10 weeks accompanied by a high-fat diet. The therapeutic dose of rosuvastatin for mice was calculated according to the conversion coefficient (10.00) between humans and mice.

### Tissue processing

Mice were killed by exsanguination after puncturing the left ventricle. The aorta was removed immediately, and the thoracic aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning. Four sections of 6-μm thickness were placed on each slide, and 8 slides were prepared from each mouse. Pathological changes were observed using H&E staining.

### Lipid and inflammatory parameter measurement

Serum was separated by centrifugation and stored at -80°C. Total cholesterol levels were analyzed continuously during the study using commercial reagents (Cat. No. 12016630, Roche Diagnostics, Mannheim, Germany). The levels of the acute inflammatory marker, high-sensitive CRP (hsCRP), were analyzed at the time of death using a murine serum CRP immunoassay kit (Cat. No ab80205, Abcam).

### Immunohistochemistry

Anti-CFH (1:100, Abcam Biotechnology, Abcam Hong Kong Limited) antibodies were applied to acetone-fixed cryosections of the thoracic aorta. After washing, the sections were then exposed to the second antibody (horseradish peroxidase-conjugated antibody), and antibody binding was visualized with diaminobenzidine. Sections were counterstained with methyl green or Mayer's hematoxylin. Sections that were positive for CFH staining were observed in several fields at 100× magnification.

### RNA Isolation and cDNA synthesis

RNA was isolated from all the collected tissues using TRIzol reagent, according to the manufacturer's protocol. To remove genomic DNA contamination from isolated RNA, total RNA was

**Table 1.** Lipid and inflammatory parameters over time in control and rosuvastatin-treated apolipoprotein E-knockout mice

| Group | N | BW (g) | TC (mmol/L) | TG (mmol/L) | HDL-C (mmol/L) | LDL-C (mmol/L) | hsCRP (ng/mL) | C3a (ng/mL) |
|---|---|---|---|---|---|---|---|---|
| Control | 5 | 26.60±2.01 | 2.7±0.23 | 0.90±0.24 | 0.45±0.06 | 1.12±0.18 | 512.10±2.81 | 486.95±16.72 |
| AS model | 13 | 31.00±1.73[a],* | 26.05±2.02[a],* | 1.30±0.27[a],* | 2.03±0.39[a],* | 11.81±1.54[a],* | 3242.19±853.47[a],* | 2977.09±104.75[a],* |
| Rosuvastatin-treated model | 8 | 30.89±1.69 | 24.83±2.18 | 1.11±0.15 | 2.14±0.20 | 10.89±0.31 | 1697.16±120.56[b],* | 1012.18±10.44[b],* |

BW, Body weight; TC, Total cholesterol; TG, Triglyceride; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; hsCRP, High-sensitive C-reactive protein; AS, Atherosclerosis. [a]Compared with the control group; [b]Compared with the AS model group; *P<0.01.

treated with Rnasefree DNase (Promega, Madison, WI) and was purified with an Rneasy minikit (Qiagen, Valencia, CA). First-strand cDNA synthesis was carried out (SuperScript II First-Strand Synthesis System; Invitrogen) for RT-PCR. First-strand cDNA was used for RT-PCR and qRT-PCR.

*Real-time quantitative RT-PCR*

Forward and reverse primers for qRT-PCR were designed from the mouse CFH coding regions in such a way that they expanded over at least 1 intron to nullify the chance of amplification from residual genomic DNA contamination. Primer pairs were used for qRT-PCR (forward, 5'-GCCTCCTCCACCTATTGACA-3'; reverse, 5'-G-TACGAAATGGCGGTGAATC-3'). Primers for mouse CFH were designed from exons 14 and 15, which should amplify the major transcript of CFH rather than the truncated isoform. Control primers derived from mouse β-actin were used to normalize and validate CFH expression [21]. Quantitative PCR (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA) and melt-curve analysis (iCycler; Bio-Rad) were performed. The relative expression of CFH in the samples was calculated using the comparative CT (threshold cycle)-value method [22, 23]. Expression data were calculated from 3 independent qRT-PCR reactions carried out for each sample, and the mean values (± SD) are presented.

*Protein isolation and western blotting*

Total protein was extracted from frozen thoracic aorta using a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100 and 0.1% (wt/vol) SDS with protease inhibitor cocktail (Sigma, St. Louis, MO). Isolated protein was quantitated using BCA reagent (Pierce Biotechnology, Inc., Rockford, IL) that was prepared in Laemmli sample buffer. Equal amounts of protein from each sample were resolved on reducing 10% SDS-PAGE. Western blot analysis was carried

out using goat polyclonal anti-CFH antibodies (1:100, Abcam Biotechnology, Abcam Hong Kong Limited). The signal was detected using an enhanced chemiluminescence kit (ECL Kit; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

*Statistical analysis*

All analyses were performed using SPSS software (version 11.5). Values are expressed as the mean ± SD. Differences in body weight, main lipid levels, hsCRP, and C3a between interventional groups and controls over time were tested using 2-way repeated measures ANOVA. Comparisons between each time point were performed using the Student *t* test only when the 2-way ANOVA showed significance, and the *p*-values are corrected for multiple comparisons using the Bonferroni correction factor whenever necessary. A P<0.05 was considered statistically significant.

Results

*Effects of rosuvastatin on organ weights and plasma lipid levels*

Body weight (BW) was not significantly different between the rosuvastatin-treated and AS model groups (**Table 1**). Total cholesterol levels were not different between the rosuvastatin-treated and control groups in the ApoE-KO mice before the high-fat diet. After the start of the high-fat diet, plasma cholesterol levels were markedly increased in the AS model group. After 10 weeks of treatment and until euthanasia at 20 weeks of treatment, total cholesterol levels were reduced in the rosuvastatin-treated group (P<0.05, **Table 1**). At the end of the study, the amount of hepatic cholesterol ester was significantly decreased in the rosuvastatin-treated group (24.83±2.18 mmol/L) compared with the AS model group (26.05±2.02 mmol/L, P<0.01), along with lowered triglyceride content and LDL-C levels (the
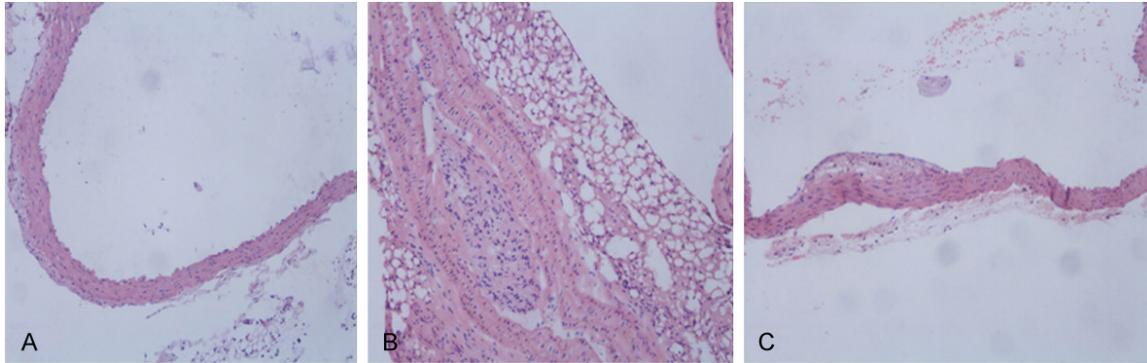
**Figure 1.** Effects of rosuvastatin on atherosclerotic lesions. The AS model group developed extensive lesions in the thoracic aorta (A, B). In the rosuvastatin-treated group, the fractional area of the lesions was reduced compared with that in the AS model group (C). (A) Control group (10×10). (B) AS model group (10×10). (C) Rosuvastatin-treated group (10×10). AS: Atherosclerosis.
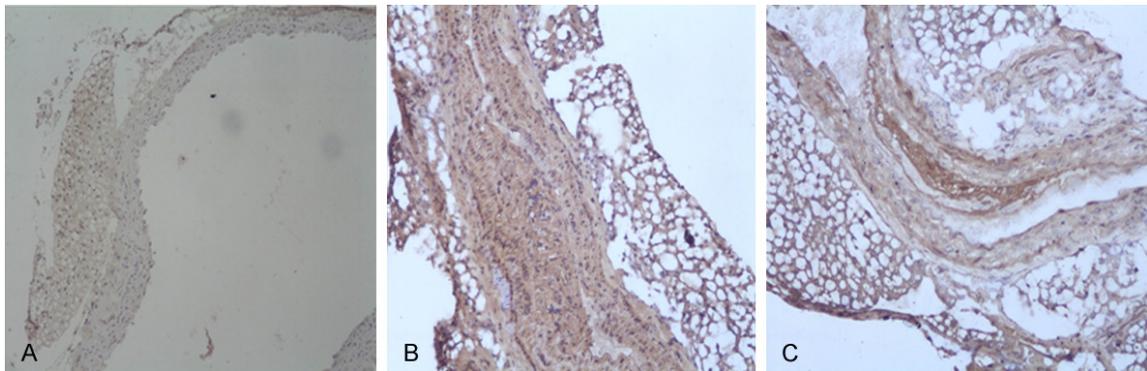


**Figure 2.** Results of immunohistochemical staining. There was no CFH expression in the control group (A), whereas there was over CFH expression in the AS model group (B), and the expression was more obvious in the rosuvastatin-treated group than in the AS model group (C). (A) Control group (10×10). (B) AS model group (10×10). (C) Rosuvastatin-treated group (10×10). AS: Atherosclerosis.

decreases were not significant). Plasma hs-CRP and C3a increased with the progression of AS plaque, and decreased significantly after the administration of rosuvastatin.

*Effects of rosuvastatin on atherosclerotic lesions*

ApoE-deficient mice were kept on a cholesterol-rich diet for 20 weeks to induce AS plaque formation. The surface area covered by lesions was quantified by H&E staining, and specimens from the control group were compared with those from the other 2 groups. The AS model group developed extensive lesions in the thoracic aorta (**Figures 1A**, **2B**). Histopathology showed that the thickness of the vessel wall was uneven, the endomembranes were not smooth, the plaques were thick, the fibrous caps were thin, and the lipid cores were large.

Foam cells and cholesterol crystals were found in the atheromatous plaques, which were not firmly attached to the vessel wall. In mice treated with rosuvastatin, the fractional area of the lesions was reduced compared with that in the AS model group (**Figure 1C**). These mice showed a small quantity of foam cells and few cholesterol crystals, and the lipid plaques were firmly adhered to the vessel wall.

*Immunohistochemistry*

Immunohistochemical staining showed that there was no CFH expression in the control group, whereas there was overt CFH expression in the AS model group, and the expression of CFH in the rosuvastatin-treated group was greater than that in the AS model group (**Figure 2A-C**).
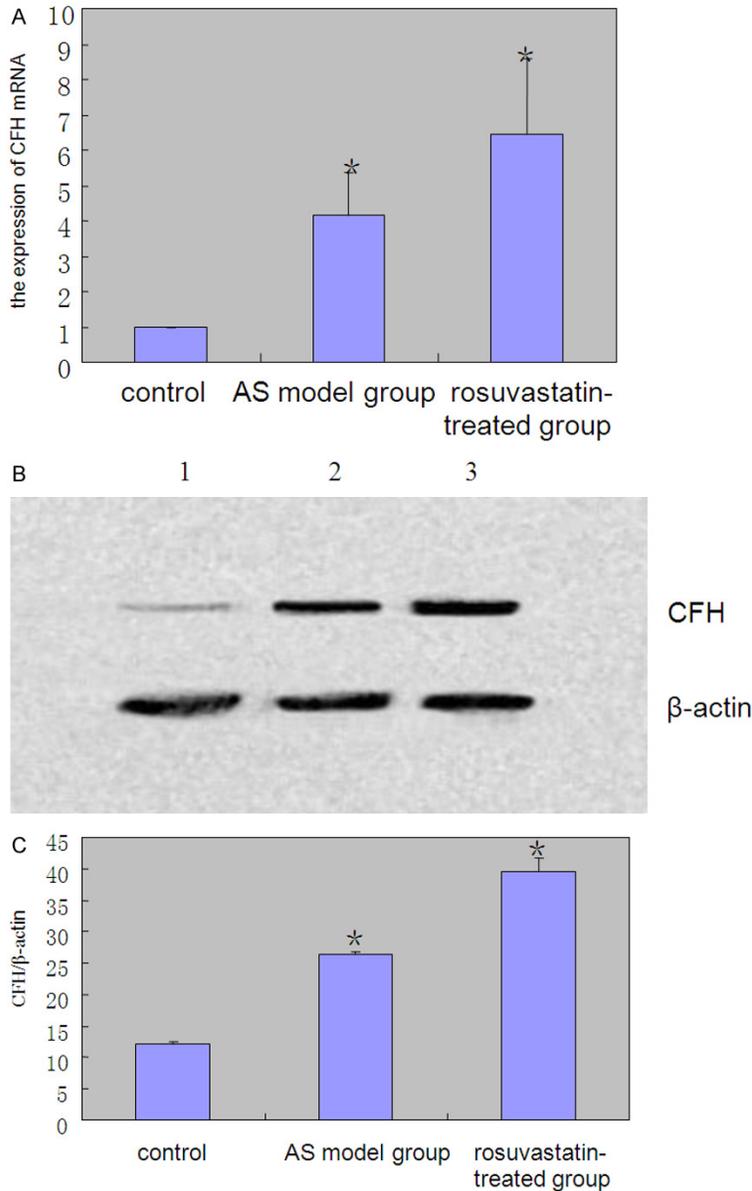
Figure 3. The effect of rosuvastatin on tissue expression of CFH. The effect of rosuvastatin on the level of CFH mRNA detected by qRT-PCR (A). CFH mRNA was higher in the rosuvastatin-treated group, whereas low-level expression was observed in the control group. The effect of rosuvastatin on the level of CFH mRNA detected by western blot analysis (B, C). The results were the same as those of qRT-PCR. (A) Effect of rosuvastatin on the level of CFH mRNA. AS: Atherosclerosis. B1. control; B2. AS model group; B3. rosuvastatin-treated group. (C) CFH/β-actin. AS: Atherosclerosis.

*Effects of rosuvastatin on tissue expression of CFH*

The highest level of CFH mRNA was detected by qRT-PCR in the rosuvastatin-treated group in the thoracic aorta, whereas low-level expression was observed in the control group (**Figure 3A**). The amount of RNA observed in different parts of the aorta was not uniform. Hence, CFH expression in these tissues was studied using qualitative rather than quantitative techniques. A higher level of CFH expression was observed in the rosuvastatin-treated group in the thoracic aorta, and the levels of CFH protein expression (~155 kDa) were higher in the AS plaque and intima (**Figure 3C**).

## Discussion

The important findings of the present study are that CFH plays a vital role in the formation of atherosclerotic lesions, and the lipid-modifying agent rosuvastatin could attenuate and stabilize aortic plaques through increased expression of CFH and an anti-inflammatory effect. The results of the current study prove that rosuvastatin treatment increased CFH expression and suppressed the development of experimental atherosclerosis in apoE-deficient mice. In addition, rosuvastatin had an anti-inflammatory effect, because there was a significant decrease in hsCRP and C3a levels in mice treated with rosuvastatin.

The complement system plays a central role in innate immunity and also regulates the adaptive immune response [24]. Complement activation is essential to the host's immune defense, but its uncontrolled or inappropriately targeted activation leads to various diseases, such as glomerulonephritis, rheumatoid arthritis, psoriasis, and CVDs [5, 24]. Modified lipoproteins and apoptotic/necrotic cells have been shown to activate the alternative complement pathways.

The role of the complement system in the advanced stages of atherosclerosis is not known,

but examination of human tissues demonstrates activated complement in human vulnerable plaques prone to rupture [5, 25]. In this study, we confirmed that rosuvastatin promotes tissue CFH expression and CFH mRNA in apolipoprotein E-knockout mice. In addition to a key role of CFH as a complement regulator in the plasma and on particular vascular sites, mutations in CFH have been associated with coronary artery disease (CAD) and myocardial infarction [26-28].

Statins inhibit HMG-CoA reductase, an enzyme crucial to cholesterol synthesis, and randomized controlled trials have demonstrated a reduction in the risk of CVDs. The use of statins for secondary prevention of CAD has been firmly established [11, 12, 29]. In the present study, the fractional area of the lesions was reduced significantly in the rosuvastatin-treated group, which further supports the concept that the clinical benefits of statins are mediated in large part through lipid modulation [11, 12]. However, there is also emerging evidence that supports the existence of other mechanisms for the clinical benefit of rosuvastatin's action.

The beneficial influences may include activation of peroxisome proliferative activated receptors [30] and indirect control of glucose metabolism, inflammation, immune response, thrombosis, and NF-κB-dependent activation of SMCs and monocytes [13, 16, 31]. In the present study, rosuvastatin significantly decreased the levels of hs-CRP and C3a in the experimental group compared with the atherosclerotic group. The level of CRP can be regulated in an LDL-independent manner by statins, but the mechanisms of this influence and the role of CRP in atherogenesis are not clear [32]. This means there are still difficulties in translating all available experimental data into new clinical therapies, and additional studies are in progress [33]. Even so, our results are consistent with data demonstrating that rosuvastatin reduces atherosclerotic plaque and has an anti-inflammatory effect independent of its lipid-lowering effect [34, 35].

The atherosclerotic process is normally initiated at sites where a disturbed flow pattern is present, such as at bifurcations where there is a more pronounced curvature of vessels. The disturbed flow patterns enhance the gene expression of adhesion receptors on the endothelial surface, increasing the recruitment of inflammatory cells from the circulation to the arterial wall [36]. The present study revealed that rosuvastatin reduced atherosclerotic lesion size in the thoracic artery, but not in the aortic arch. In addition, there was a decrease in foam-cell content of the histological sections from the thoracic artery in the rosuvastatin-treated group. Although atherosclerosis could be seen at other sites of the artery, the rosuvastatin-treated mice expressed a more stable phenotype in the thoracic aorta. It is very important to understand how to better balance the function of the immune response in atherosclerosis and the manipulation of this response to develop novel therapeutic strategies to treat CVDs [2].

In conclusion, we observed retarded lesion progression and plaque stabilization in the thoracic artery of ApoE-KO mice following 16 weeks of rosuvastatin treatment, partially through increased expression of CFH and an anti-inflammatory role. Because the pharmacokinetics of rosuvastatin may differ between mice and humans, further studies are needed to confirm our findings and to determine the optimal dose-effect relationship in humans.

### Acknowledgements

### Disclosure of conflict of interest

None.

### Authors' contribution

ZC conceived and designed the study, approved the manuscript submission. QQ and YYY participated in the laboratory tests and finished the data analysis. QQ wrote the manuscript. GSM helped interpret the results. All authors read and approved the final manuscript.

### Abbreviations

ApoE-KO, apolipoprotein E-knockout; CAD, coronary artery disease; CFH, complement factor H; CVDs, cardiovascular diseases; ECs, endothelial cells; H&E, hematoxylin and eosin; hsCRP, high-sensitive C-reactive protein; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; LDL-C, low-density lipoprotein cholesterol; MAC, membrane attack complex; SMCs, smooth muscle cells.

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