

Statin Pretreatment in Experimental Acute Pancreatitis

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ABSTRACT

Context Some authors have found beneficial effect of statins in certain inflammatory conditions, but the effect of statins on acute pancreatitis is not yet defined.

Objective The aim of this study was to evaluate the effect of simvastatin on an experimental model of mild and severe acute pancreatitis.

Animals One hundred and one Wistar rats with cerulein or taurocholate-induced acute pancreatitis were used in this study.

Design The rats were divided into two groups: Group I (n=51) received two previously i.p. injections (18±2 and 3±1 hours) of simvastatin (200 µg/kg) and Group II (n=50) received two previously i.p. injections of saline. Both groups were subdivided into two subgroups: mild pancreatitis (cerulein-induced; IA, n=10; IIA, n=10) and severe pancreatitis (taurocholate-induced; IB, n=41; IIB, n=40).

Main outcome measures The parameters evaluated were: pancreatic vascular permeability, tissue water content, histologic lesion, amylase serum levels in rats with mild pancreatitis (subgroups A); mortality rate, serum levels of IL-6, IL-10, amylase, pulmonary myeloperoxidase activity and ascitic levels of TNF-alpha in rats with severe pancreatitis (subgroups B).

Results Serum levels of IL-10 were significantly lower in the simvastatin-treated group as well as the myeloperoxidase activity. There was no significant difference in any of other studied parameters.

Conclusion Simvastatin appears to reduce inflammatory cytokines and pulmonary neutrophilic activation in the severe acute pancreatitis model, but there is no significant effect on survival curve, in spite of a clear trend towards a better survival in the simvastatin group.

INTRODUCTION

Acute pancreatitis is an inflammatory condition of the pancreas, with varying involvement of other tissues and organs. The disease includes a spectrum of pancreatic lesions, varying from parenchymatous edema to severe hemorrhagic pancreatitis, with necrosis, infection and organ destruction [1]. Therefore, clinical presentation is also variable, including mild abdominal discomfort or metabolic disturbances, hypotension and shock [2].

During the past years, several substances have been tested, with varying degrees of success, in experimental models of acute pancreatitis as an attempt to modify the natural history of the disease by either blocking or neutralizing one or more inflammatory mediators which have a known role in the pathophysiology of

the disease [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14].

Statins, which are 3-hydroxy-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors, have been clinically used for treatment of dyslipidemia. The HMG-CoA reductase is the enzyme that catalyses the conversion of HMG-CoA in mevalonate, the limiting step in the cholesterol synthesis. Several evidences suggest that statins might alter the inflammatory response independently of its antilipemic effect [15, 16, 17, 18, 19]. They also can modulate the production of acute-phase proteins, endothelial leukocyte adherence, NO and macrophage activation [15], besides their immunomodulatory role [20].

The successful *in vitro* inhibition of pancreatic stellate cells activation by lovastatin [21] raises the possibility of a potential beneficial effect of statins also in chronic inflammatory process, such as, chronic pancreatitis.

Merx *et al.*, in a mice experimental model of sepsis, showed that simvastatin pretreatment (200 µg/kg) improved hemodynamics parameters and increased animals survival [22]. In this context, the class of drugs known as statins may be a new hope in the treatment or prevention of acute pancreatitis, considering its anti-inflammatory role in several steps of inflammation cascade [23].

The aim of this study is to evaluate the effect of simvastatin pretreatment in two different models of experimental acute pancreatitis: the cerulein (mild acute pancreatitis) and the taurocholate (severe acute pancreatitis) models.

MATERIAL AND METHODS

Animals

One hundred one male Wistar rats with a body mass varying from 240 to 260 g were used for this experiment. Experimental acute pancreatitis was induced either by cerulein or taurocholate models.

Study Groups

The rats were divided into two treatment groups.

Group I (n=51). Rats received previous treatment with i.p. simvastatin (200 µg/kg).

Group II (n=50). Rats received previous i.p. saline solution and were used as controls.

Study Subgroups

Each group was subdivided according to the model of acute pancreatitis induction.

Subgroups IA (n=10) and IIA (n=10). Rats with cerulein-induced acute pancreatitis (mild acute pancreatitis).

Subgroups IB (n=41) and IIB (n=40). Rats with taurocholate-induced acute pancreatitis (severe acute pancreatitis).

Acute Pancreatitis Induction

The cerulein model of experimental acute pancreatitis was developed in our laboratory and is described elsewhere [24]. The taurocholate experimental model was performed according to the technique previously reported by Lankisch *et al.* [25], using a 5.0% concentration of sodium taurocholate.

Statin Pretreatment

Simvastatin was used in a dosage of 200 µg/kg. The drug was administered i.p. twice (18±2 h and 3±1 h) before acute pancreatitis induction. The substance was dissolved in ethyl alcohol to a concentration of 10 mg/mL [22]. In order to obtain a dose of 200 µg/kg, solution was diluted in sterile saline solution (NaCl 0.9%) until reach the concentration of 10 µg/mL. Final volume injected into the rat was 0.02 mL/g of corporal mass.

Sacrifice

Two hours after the end of the cerulein or taurocholate acute pancreatitis induction period, rats underwent a median laparotomy (mild acute pancreatitis model; subgroups A) or a thoracotomy (severe acute pancreatitis model; subgroups B) and cardiac puncture for blood samples.

The portal vein was clamped, pancreas was exposed and removed in two parts: proximal and distal. The organ was dissected and set free of all nodes and adjacent fat tissue. The lungs were washed with 50 mL of a PBS

solution using a pump infusion (model 975, Harvard Apparatus, Holliston, MA, USA) through a catheter introduced into the pulmonary artery. The lungs were then extracted and the animals sacrificed by exsanguinations.

Main Outcome Measures

Vascular permeability, water tissue content, histopathology and serum levels of amylase were evaluated in subgroups A (mild acute pancreatitis). Mortality rate after 72 hours (evaluated in 30 rats), serum levels of interleukin-6, interleukin-10, amylase, pulmonary myeloperoxidase (MPO) activity and ascitic levels of tumor necrosis factor-alpha were evaluated in subgroups B (severe acute pancreatitis).

Vascular Permeability Evaluated by Evans Blue Dye Extravasation

Pancreata was put on a test tube with 3 mL of formamide, in a dose of 4 µg/mg of tissue for 24 hours, at room temperature for dye extraction.

The concentration of Evans blue in formamide was assessed by 620 nm spectrophotometer. The results were compared with standard dye curve (from 0.5 to 20 µg/mL) and were expressed as concentration of Evans blue in the dry tissue (µg/g).

Pancreas Water Content

Pancreata was weighed on an analytic balance (fresh weight) and dehydrated by heating at 56°C during 48 hours (dry weight). Water content was calculated as percentage of dry weight according to the formula:

$$((\text{fresh weight} - \text{dry weight}) / \text{dry weight}) \times 100$$

Histologic Evaluation

Representative tissue samples collected from the proximal and distal parts of the pancreas were fixed in 10% formalin for 24 h and embedded in paraffin. Five-µm-thick sections were stained with hematoxylin and eosin, and the degree of tissue damage was graded in a blinded fashion by a single pathologist, using an established scoring system [30].

Serum Levels of Amylase and Interleukins and Ascitic Levels of Tumor Necrosis Factor (TNF)-alpha

Serum amylase levels were assessed by the colorimetric method described by Bernfeld *et al.* [26] and modified by Jamieson *et al.* [27]. Serum levels of interleukins-6 and 10, as well as the ascitic levels of TNF-alpha, were determined by ELISA using commercial available kits (Cytoscreen, Biosource International, Camarillo, CA, USA).

Pulmonary Myeloperoxidase (MPO) Activity

Lung MPO activity were determined as previously described [28, 29] and were expressed as optic density (OD).

ETHICS

All animals experiments were previously approved by the Ethic Committee of the University of São Paulo Medical School and received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences [31].

STATISTICS

Means, standard deviations (SD), and 95% confidence intervals were used as descriptive statistics, while interquartile values and ranges were used to show data in the figures. Results were tested with unpaired t-Student test (continuous variables) or the Mann-Whitney U test (histologic score). Mortality rates were estimated by the Kaplan-Meier method and the comparisons were made by the log-rank test. Significant difference was accepted when the two-tailed P value was less than 0.05. GraphPad Prism 4.0 Software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Taurocholate-Induced Acute Pancreatitis (Severe Pancreatitis. Subgroups B)

Significantly lower levels of IL-10 (P=0.046; Figure 1) and pulmonary MPO activity (P=0.018; Figure 2) were found in the simvastatin treated group (IL-10: 8.2±5.5

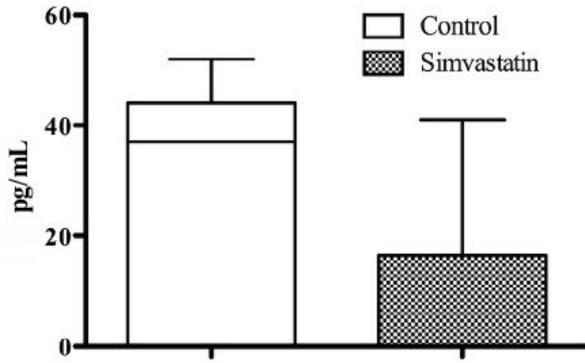


Figure 1. Box and whiskers plot of serum levels of IL-10 in rats with taurocholate-induced severe acute pancreatitis treated with simvastatin (subgroup IB, No. 41) and in controls (subgroup IIB, No. 40). Boxes represent the interquartile ranges and whiskers extend to the maximum values. The median values are shown within the boxes.

pg/mL, 95% CI: 0.4 to 38.7 pg/mL; MPO: 0.085 ± 0.011 OD, 95% CI: 0.011 to 0.105 OD) than in the control group (IL-10: 27.8 ± 7.2 pg/mL, 95% CI: 11.2 to 44.4 pg/mL; MPO: 0.143 ± 0.019 OD, 95% CI: 0.098 to 0.188 OD). There were no significant differences in serum levels of IL-6 (simvastatin: 137 ± 52 pg/mL, 95% CI: 18 to 256 pg/mL; control: 170 ± 99 pg/mL, 95% CI: 57.6 to 398.5 pg/mL; $P=0.765$; Figure 3) and in ascitic levels of TNF-alpha (simvastatin: 74.6 ± 12.3 pg/mL; 95%CI: 46.2 to 120.9 pg/mL; control: 80.6 ± 17.3 pg/mL, 95% CI:

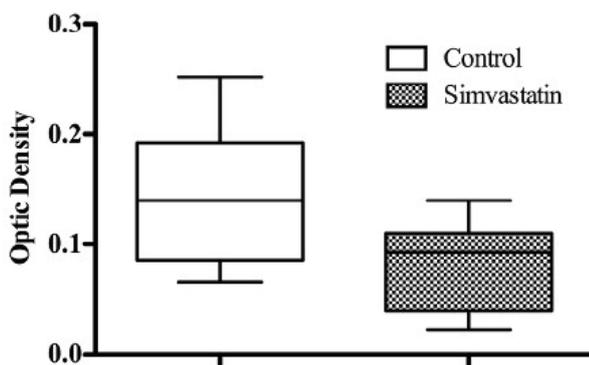


Figure 2. Box and whiskers plot of pulmonary myeloperoxidase activity in rats with taurocholate-induced severe acute pancreatitis treated with simvastatin (subgroup IB, No. 41) and in controls (subgroup IIB, No. 40). Boxes represent the interquartile ranges and whiskers extend to the minimum and maximum values. The median values are shown within the boxes.

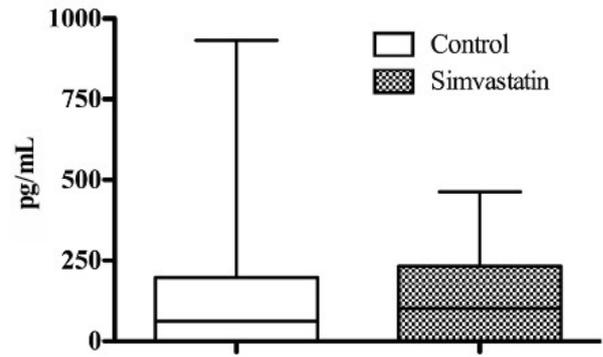


Figure 3. Box and whiskers plot of serum levels of IL-6 in rats with taurocholate-induced severe acute pancreatitis treated with simvastatin (subgroup IB, No. 41) and in controls (subgroup IIB, No. 40). Boxes represent the interquartile ranges and whiskers extend to the maximum values. The median values are shown within the boxes.

40.8 to 120.3 pg/mL; $P=0.780$; Figure 4). Amylase serum levels also did not show significant difference between the two groups (simvastatin: 18.7 ± 1.2 U/mL; 95%CI: 16.0 to 21.5 U/mL; control: 18.3 ± 0.9 U/mL, 95% CI: 16.2 to 20.4 U/mL; $P=0.787$). The 72-h survival rate of the simvastatin group was 73.3% (22/30) vs. 53.3% of the control group (16/30). The mean survival time was no significantly different between the two groups of animals (simvastatin 18.8 ± 2.1 h, 95% CI: 13.8 to 23.7 h; control 24.4 ± 5.1 h, 95% CI: 13.5 to 35.3 h; $P=0.422$; Figure 5).

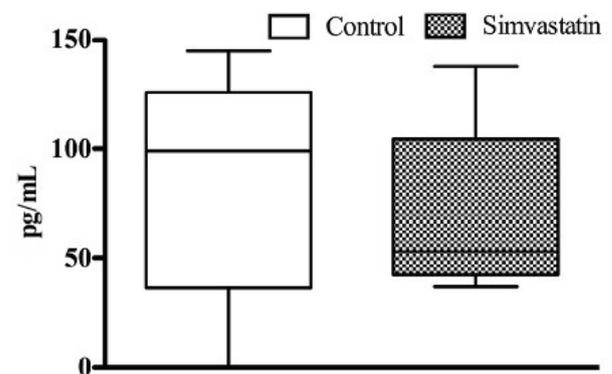


Figure 4. Box and whiskers plot of ascitic levels of TNF-alpha in rats with taurocholate-induced severe acute pancreatitis treated with simvastatin (subgroup IB, No. 41) and in controls (subgroup IIB, No. 40). Boxes represent the interquartile ranges and whiskers extend to the minimum and maximum values. The median values are shown within the boxes.

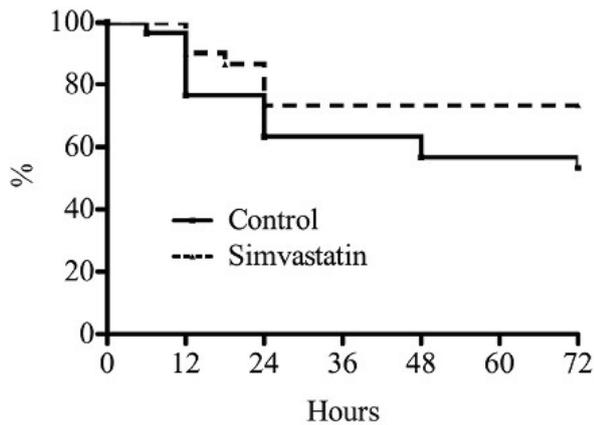


Figure 5. Seventy-two-hour survival curve of rats with taurocholate-induced severe acute pancreatitis treated with simvastatin (subgroup IB, No. 30) and in controls (subgroup IIB, No. 30).

Cerulein-Induced Acute Pancreatitis (Mild Pancreatitis. Subgroups A)

No differences in the cerulein-induced acute pancreatitis model were found in any of the assessed parameters: vascular permeability of the proximal pancreas (simvastatin 504 ± 94 $\mu\text{g/g}$, 95% CI: 2,874 to 720 $\mu\text{g/g}$; control 450 ± 90 $\mu\text{g/g}$, 95% CI: 237 to 663 $\mu\text{g/g}$; $P=0.686$); vascular permeability of the distal pancreas (simvastatin 653 ± 114 $\mu\text{g/g}$, 95% CI: 391 to 915 $\mu\text{g/g}$; control 620 ± 166 $\mu\text{g/g}$, 95% CI: 227 to 1,013 $\mu\text{g/g}$; $P=0.870$); free water percentage (proximal pancreas) (simvastatin 80.0 ± 1.6 , 95% CI: 76.2 to 83.8; control 77.7 ± 2.1 , 95% CI: 72.8 to 82.6; $P=0.405$); free water percentage (distal pancreas) (simvastatin 82.8 ± 1.5 , 95% CI: 79.4 to 86.2; control 80.9 ± 2.3 , 95% CI: 75.5 to 86.2; $P=0.313$), and amylase serum levels (simvastatin: 19.1 ± 1.2 U/mL; 95%CI: 16.3 to 21.8 U/mL; control: 18.8 ± 0.9 U/mL, 95% CI: 16.8 to 20.9 U/mL; $P=0.872$). Finally, no significant difference ($P=0.739$) was also found in the histologic evaluation between simvastatin (grade 3: 5, 50.0%; grade 4: 5, 50.0%) and control group (grade 3: 6, 60.0%; grade 4: 4, 40.0%).

DISCUSSION

Acute pancreatitis is characterized by local pancreatic inflammation as well as a systemic inflammatory response [1]. According to Choi

et al. [19], there is a therapeutic window between onset of symptoms and the development of distant organ damage in acute pancreatitis, allowing the employment of therapeutic and preventive options that might alter the natural history of the disease. The search for this therapeutic option is still a challenge in pancreatic research. Our group had already experimented the use of cyclooxygenase-2 inhibitors (COX-2) [32], anti-oxidants [6], hypertonic solution [33], antagonists of platelet-activating factor [5] and somatostatin analogs [4], but the results turned out to be disappointing.

In complex situations such as the systemic inflammatory response syndrome that occurs in the acute pancreatitis, multiple cellular activation processes are involved and many humoral cascades are triggered, so that merely blocking a single component may be insufficient to arrest the inflammatory process. Therefore, any intervention affecting this complex syndrome should be able to do so by modifying several arms of the inflammatory cascade [17]. The rationale for using statins to prevent and alter the natural history of acute pancreatitis is their pleiotropic effect, as it is well known that these substances have a wide variety of potent anti-inflammatory properties. According to Almog *et al.* [17], the possible effects of statins are: 1) ligand receptor interaction step in the SIRS cascade. Statins could interfere with this presentation process and its downstream consequences; 2) statins could attenuate the acute-phase response and its immediate consequences; 3) statins could have an important protective effect on the delicate sequence of endothelial activation, dysfunction, and apoptosis; this sequence correlates with worsening severity of illness and organ dysfunction; 4) statins could contribute to a favorable balance between cNOS and iNOS such that vasodilatation is diminished and hemodynamic stability and vasopressor response are restored [17, 18, 34, 35].

All the above are steps of inflammatory cascade and therefore their modulation bring about a theoretical benefit. In addition, many

trials have previously addressed the effects of statins in the primary prevention of atherosclerosis and ischemic heart disease and have invariably demonstrated a strong reduction in all-cause of mortality including noncoronary ones [36, 37].

The numerous non lipid-lowering effects of statins are being elucidated nowadays. Merx *et al.* [22], showed a significant survival rate improvement after statin usage in an experimental model of sepsis, and concluded that its beneficial effect was probably due to preserved cardiac function and hemodynamic stability. In this same study, they also reported a decrease in mononuclear cell adhesiveness in statin-treated mice, which might have contributed to the dramatic improvement in survival. Other reported effects are suppression of T-cell response, reduced expression of class II major histocompatibility complexes on antigen-presenting cells, reduced chemokine synthesis in peripheral blood mononuclear cells and blocking the LFA-1/ICAM-1 interaction. This latter effect might be independent of HMC-CoA reductase pathway [22].

In another study, lovastatin was administered previously to the induction of severe acute pancreatitis by cholecystinin (CCK) infusion in rats [19]. Besides the statin pretreatment, the whole process of statin infusion and acute pancreatitis induction was repeated for five days; rising the possibility of statin effect only take place in a chronic basis. The authors showed that in the statin-pretreated group, the pancreas weight/body ratio was significantly lower, the pancreatic level of heat shock protein 60 (HSP60) was increased and the secretions of IL-1beta, TNF-alpha and IL-6, as well as the lipase levels, were diminished. The aim of the present study was to compare that mortality rates, serum and ascitic cytokines levels, pulmonary neutrophilic activation, pancreatic edema and pancreatic vascular permeability in rats undergoing both mild and severe acute pancreatitis. In the mild model, no difference was observed in the parameters evaluated, whereas in the severe model, there was a tendency towards better survival in the statin

treated group. Serum levels of IL-10 and pulmonary MPO activity were significantly reduced in the statin animal group. These results suggest that statin has no role in local tissue injury but might play a role in the prevention of the pulmonary damage that occurs in severe acute pancreatitis.

It is well known that interleukin-10 is a major and most potent anti-inflammatory cytokine, and it is able to inhibit the *in vitro* production of proinflammatory cytokines by activated monocytes/macrophages [38, 39]. It is primarily synthesized by monocytes and T and B lymphocytes and modulates Th cell transdifferentiation into the Th2 subset [40, 41]. Administration of recombinant IL-10 before the induction of pancreatitis prevents necrosis and reduces severity in cerulein induced necrotizing pancreatitis in mice and others experimental models of acute pancreatitis, independent of the timing of administration [42, 40, 43, 44, 45]. It potently inhibits the release of proinflammatory cytokines, including TNF-alpha and IL-1-alpha, as well as IL-6, IL-8, colony stimulating factor (CSF) and nitric oxide probably through an inhibitory action on the transcriptional factors [46]. In addition to these activities, IL-10 stimulates the production of naturally produced modulators such as IL-1 receptor antagonist and soluble p75 TNF receptors and also decreases the cellular immune response by suppressing IL-2 and IFN-gamma production [46, 47]. This anti-inflammatory response parallels the release of proinflammatory cytokines.

Among these suggested mechanisms of action, the inhibition of the local release of TNF-alpha by monophages/macrophages is most established [43, 48]. According to Van Laethem *et al.* [49], endogenous IL-10 controls pancreatic, liver, and lung TNF-alpha production and plays a protective role in the development of pancreatic inflammation and necrosis as well as the associated lung injury. Systemic release of IL-10 parallels the rise of TNF-alpha in serum and in the pancreatic and lung parenchyma during the progress of pancreatitis. Another major finding from Van Laethem *et al.* [42] is the increased severity of

pancreatitis after blockade of endogenous IL-10. indicating that endogenous IL-10 plays a strategic and early role in regulating and limiting events promoting inflammation and necrosis.

In turn, IL-6 has already been recognized as an early mediator involved in acute pancreatitis, and thus as an early determinant of the sequence of inflammatory events leading to the injuries observed in the most severe cases [50]. IL-6 has been shown to be associated with distant organ complications and increased IL-6 has been linked to adult respiratory distress syndrome [51]. In isolated peripheral blood monocytes, increased IL-6 release was associated with systemic complications [52]. Considering the results of the present study and the fact that inflammation in acute pancreatitis results of a misbalance between proinflammatory and anti-inflammatory cytokines, we postulate that IL-10 has diminished because there was not enough inflammatory stimuli to its rising. On the other hand, a degree of activation of the systemic inflammatory response syndrome did occurred and simvastatin was unable to reduce serum levels of IL-6 (which is marker of systemic inflammation) [44, 50]. This finding confirms the fact that the activation of IL-10 is paralleled with TNF-alpha and not with IL-6, which might be related with other anti-inflammatory cytokine. In conclusion, there was no beneficial effect on survival or on local pancreatic inflammation in these models of acute pancreatitis, but there is a trend towards a better survival rate in the statin group. In addition, serum levels of interleukin-10, which has a key role in the inflammation cascade and in the neutrophilic activation of target-organs of acute pancreatitis, were diminished.

Besides, new acute pancreatitis experimental studies addressing the effects of statins therapy in a chronic basis, the employment of statins after acute pancreatitis induction, the assessment of the effect of statins on serum levels of other cytokines (such as TNF-alpha and prostaglandin E2) and influence of these

drugs in the activity of transcription factors (such as NF-kappa B) are welcome.

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Keywords Hydroxymethylglutaryl-CoA Reductase Inhibitors; Inflammation; Pancreatitis

Abbreviations HMG-CoA: 3-hydroxymethylglutaryl coenzyme A; MPO: myeloperoxidase

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