

Anti-migration and anti-invasion effects of LY-290181 on breast cancer cell lines through the inhibition of Twist1

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Breast cancer has become the most common cancer among women worldwide. Among breast cancers, metastatic breast cancer is associated with the highest mortality rate. Twist1, one of the epithelial-mesenchymal transition-regulating transcription factors, is known to promote the intravasation of breast cancer cells into metastatic sites. Therefore, targeting Twist1 to develop anti-cancer drugs might be a valuable strategy. In this study, LY-290181 dose-dependently inhibited migration, invasion, and multicellular tumor spheroid invasion in breast cancer cell lines. These anti-cancer effects of LY-290181 were mediated through the down-regulation of Twist1 protein levels. LY-290181 inhibited extracellular signal-regulated kinase and c-Jun N-terminal kinase signaling pathways. Therefore, our findings suggest that LY-290181 may serve as a basis for future research and development of an anti-cancer agent targeting metastatic cancers. [BMB Reports 2023; 56(7): 410-415]

INTRODUCTION

Breast cancer has become the leading cause of death from cancer in women, overtaking lung cancer, which was the most diagnosed cancer in the past two decades (1). Breast cancer usually metastasizes to the lungs, bones, liver, and brain (2). Systemic therapy is the main treatment option for metastatic breast cancer and is associated with significant improvements in survival rate (3). Chemotherapy shrinks cancerous tumors and prolongs the lifespan of patients but has several side effects, such as premature menopause and hair thinning (2). Therefore, it is necessary to develop novel drugs to achieve effective inhibition of breast cancer metastasis.

Metastasis is promoted by epithelial-mesenchymal transition (EMT), a process through which epithelial cells transform into mesenchymal cells with metastatic and invasive abilities (4).

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EMT has been extensively studied in relation to angiogenesis, organogenesis, and metastasis (5). EMT-regulating transcription factors (EMT-TFs), the regulators of the EMT process, include Snail1, ZEB1, and Twist1 (6). These EMT-TFs down-regulate the expression of epithelial markers such as E-cadherin and occludin while up-regulating the expression of mesenchymal markers such as N-cadherin and vimentin (7). High expression levels of EMT-TFs have been reported to be associated with multiple types of aggressive cancer such as breast, liver, gastric, and pancreatic cancers (5). EMT-TFs are regulated by a variety of intracellular signaling pathways, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (8). These signaling pathways play a role in the onset, progression, and poor prognosis of breast cancer and are therefore considered important targets for cancer therapy (9, 10).

Twist1, a basic helix-loop-helix domain-containing transcription factor, is a key regulator of the mesenchymal phenotype (11). The most important role of Twist1 is to promote EMT to induce distant metastasis of cancer cells endowed with highly invasive and aggressive properties (12). Twist1, which is activated in several aggressive carcinomas, exhibits an inverse correlation with E-cadherin expression to promote tumor metastasis (11). Several studies on the correlation between Twist1 and breast cancer metastasis have been reported (13). For instance, a study by Watson *et al.* has demonstrated that increased Twist1 expression in the bone marrow of breast cancer patients is associated with poor prognosis (14). Therefore, it is important to discover novel inhibitors targeting Twist1 and to elucidate their mechanisms of action to facilitate metastatic cancer treatment.

LY-290181 (2-amino-4-[3-pyridyl]-4H-naphtho[1,2-b]pyran-3-carbonitrile) was first reported in 1996 as a compound that inhibits the proliferation of smooth muscle cell (15). Since then, LY-290181 has been studied as a cytostatic agent that induces cell cycle arrest at the G₂/M phase by inhibiting microtubule formation (16, 17). LY-290181 and its analogues have been suggested as anti-cancer agents, which induce apoptosis in cancer cells and inhibit tumor growth by functioning as a tumor vasoconstrictor (18). LY-290181 has been proposed as an anti-cancer agent that inhibits cancer growth by arresting cell cycle at the G₂/M phase, but the molecular mechanisms underlying its anti-cancer effects on breast cancer cells remain to be elucidated (16, 17).

In this study, we found that LY-290181 exerts anti-invasive and anti-migration effects by reducing Twist1 protein levels in breast cancer cell lines. LY-290181 inhibits the activities of ERK and JNK and results in the suppression of EMT through the regulation of epithelial and mesenchymal markers. Our findings suggest that LY-290181 acts as an anti-metastatic factor by inhibiting Twist1, suggesting that it may constitute a novel therapeutic agent for metastatic cancers.

RESULTS

LY-290181 inhibits the invasive and migratory activities of breast cancer cells

LY-290181 (Fig. 1A) has been proposed as an anti-cancer agent that inhibits the growth of cancer cells by inducing cell cycle arrest at the G₂/M (18), but its effects on metastasis remain largely unknown. To determine the optimal concentration of LY-290181 for subsequent experiments, the cytotoxicity of LY-290181 against breast cancer cell lines was investigated. MCF10A cells (normal breast cells) and breast cancer cells MDA-MB-231, MCF7, and SK-BR-3 were treated with LY-290181 (50 nM or 100 nM) and cell viability was measured (Fig. 1B and Supplementary Fig. 1A). Tested compounds that show cell viability levels at or above 80% have been considered non-cytotoxic in several studies (19, 20). LY-290181 treatment (100 nM) resulted in cell viability levels of 80% or above, indicating that it did not significantly cause cytotoxicity. Accordingly, LY-290181 was utilized in subsequent experiments at concentrations up to 100 nM. The ability of LY-290181 to inhibit the migration of MDA-MB-231 and MCF7 cells was investigated by wound-healing and transwell migration assays (Fig. 1C, D). Wound recovery in MDA-MB-231 and MCF7 cells were significantly reduced upon LY-290181 treatment (48 h) in a concentration-dependent manner. Similarly, cell migration in the transwell assay was significantly reduced by LY-290181 treatment in a concentration-dependent manner. This effect induced by LY-290181 treatment (100 nM) was comparable to the inhibitory effect observed with a much higher dose of emodin (40 μ M), a well-studied anti-cancer compound in various cancer types, suggesting that LY-290181 has a stronger effect compared with emodin (19). To investigate the anti-invasion effect of LY-290181, an invasion assay using Matrigel was performed (Fig. 1E). LY-290181 treatment significantly inhibited invasion in a concentration-dependent manner, and this inhibition was achieved with a much lower dose of LY-290181 (100 nM) compared with emodin (40 μ M). Multicellular tumor spheroid (MTS) invasion assay is used in cancer research and anti-cancer drug screening because it provides a better simulation of *in vivo* tumor environment compared with the two-dimensional (2D) cell culture system (21). In our study, MTS invasion was evaluated using a three-dimensional (3D) culture system to confirm the anti-invasive effect of LY-290181 in conditions similar to the microenvironment of cancer cells (Fig. 1F). As a result, LY-290181 treatment inhibited MTS inva-

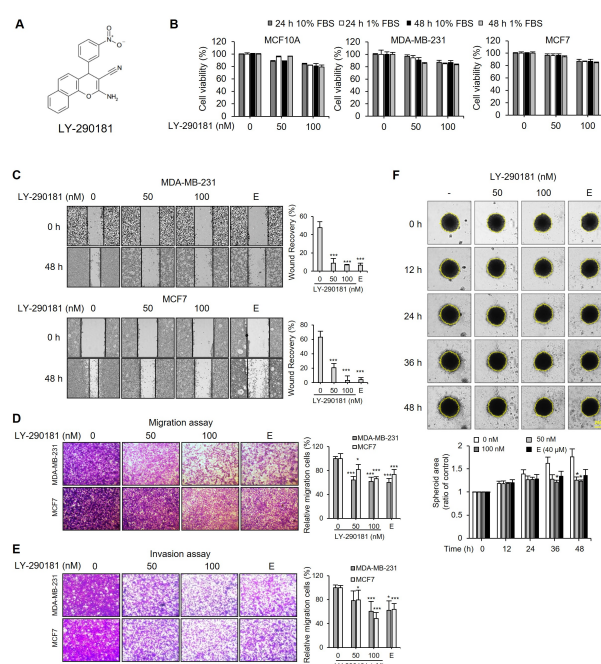


Fig. 1. Inhibitory effects of LY-290181 on migration and invasion ability of breast cancer cells. (A) Chemical structure of LY-290181. (B) MCF10A, MDA-MB-231, and MCF7 cells were seeded in 96-well plates, treated with LY-290181 for 24 h or 48 h, and EZ-Cytox solution was used to evaluate cell viability. (C) The wounds in MDA-MB-231 and MCF7 cells were created using scratcher and then treated with LY-290181 or emodin (E; 40 μ M; positive control) for indicated time periods. Images were captured at 0 h and 48 h. Wound recovery values were quantified by measuring the percentage of wound size compared to the 0 h point for each sample. Quantitative graphs are shown on the right. (D) MDA-MB-231 and MCF7 cells were treated with LY-290181 or emodin (E; 40 μ M; positive control). After incubation for 8 h in transwell plate, cells invading the lower surface of the chambers were fixed and stained. Quantitative graphs are shown on the right. (E) Matrigel invasion assay went through the same procedure as migration assay except that the incubation time was 18 h after coating the inner chamber with Matrigel. Quantitative graphs are shown on the right. (F) The spheroids of MDA-MB-231 cells were incubated with LY-290181 or emodin (E; 40 μ M; positive control) for 48 h. Microscopic images were captured by a JuLi stage real-time imaging system at each time point. Yellow lines indicate the outline of the 3D spheroid on each experiment group at 0 h. The invaded areas or lengths of the spheroids were measured using Image J. Quantitative graphs are shown below. Data are representative of three experiments and expressed as the means $\pm$ SD. * P < 0.05 and *** P < 0.001 relative to the LY-290181 untreated control.

sion compared to the untreated group as well as to the emodin-treated group. Comparing the yellow outline of MTS observed at initial time point with the 3D spheroids at later time points, MTS invasion was significantly suppressed by treatment with LY-290181 (100 nM). These data suggest that LY-290181 is a potent compound capable of inhibiting cancer cell-associated activities such as the migration and invasion in breast cancer cells.

LY-290181 down-regulates Twist1 protein levels

Twist1 and Snail1 are the major EMT-TFs that induce cancer metastasis and promote cancer progression (12). Therefore, immunoblotting analysis was performed to investigate the change of Twist1 and Snail1 by LY-290181 (Fig. 2A). Protein levels of Twist1 decreased upon LY-290181 treatment in MDA-MB-231 and MCF7 cells. However, the protein levels of Snail1 were not altered by LY-290181. When SK-BR-3 cells were treated with LY-290181, Twist1 protein expression levels were also (Supplementary Fig. 1B). As shown in Fig. 2B and Supplementary Fig. 1C, the mRNA levels of *Twist1* were not altered by LY-290181 in breast cancer cells, implying that LY-290181 regulates Twist1 expression at the protein level. To further confirm that Twist1 is regulated at the protein levels by LY-290181, immunoblotting analysis was performed after co-treatment with MG132, a proteasome inhibitor (Fig. 2C). LY-290181-mediated reduction in Twist1 levels in MDA-MB-231 and MCF7 cells was abolished upon MG132 treatment. Taken together, these results indicate that reduction of Twist1 by LY-290181 is regulated by proteolytic degradation rather than changes in gene expression.

Twist1 target gene expression is regulated by LY-290181

Since LY-290181 down-regulated Twist1, it was further investigated whether LY-290181 regulates Twist1 target genes, E-cadherin and N-cadherin. Decreases in E-cadherin and increases

in N-cadherin levels in various malignant tumors are associated with cancer invasion and metastasis, resulting in a poor prognosis (22). Treatment of MDA-MB-231 and MCF7 cells with LY-290181 resulted in the up-regulation of both E-cadherin protein and mRNA levels and down-regulation of both N-cadherin protein and mRNA levels, as shown in Fig. 3A, B. In addition, LY-290181 exhibited a dose-dependent regulation of the protein levels of occludin, an epithelial marker, and vimentin, a mesenchymal marker (Supplementary Fig. 2). When the E-cadherin promoter activity was measured using reporter assays, LY-290181 treatment significantly increased E-cadherin promoter activity (Fig. 3C). These findings suggest that LY-290181 regulates the expression of EMT-related target genes by decreasing Twist1 levels.

LY-290181 suppresses ERK and JNK signaling pathways

Since LY-290181 treatment down-regulates Twist1 protein levels in breast cancer cells, it was hypothesized that LY-290181 might regulate signal transduction that controls the stability of the Twist1 protein. Previous studies have shown that MAPK signaling pathway phosphorylates serine 68 (S68) of Twist1 and PI3K/AKT signaling pathway phosphorylates serine 42 of Twist1 to increase its stability (23). Therefore, MAPK and PI3K/AKT signaling pathways were investigated to determine the mechanism by which LY-290181 regulates Twist1. Total and phosphorylation levels of ERK, JNK, p38, and AKT were measured by immunoblotting analysis after treating MDA-MB-231 cells with various concentrations of LY-290181 for 48 h (Fig. 4A). LY-290181 treatment significantly suppressed phosphory-

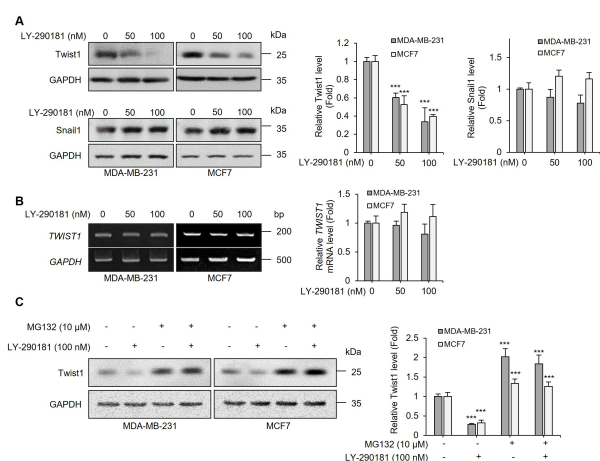


Fig. 2. Effect of reducing Twist1 protein stability by LY-290181. (A) MDA-MB-231 and MCF7 cells were treated with LY-290181 for 48 h. Subsequently, whole cell lysates were harvested and analyzed by immunoblotting. (B) RNA was obtained from MDA-MB-231 and MCF7 cells after treatment with LY-290181 for 48 h. Transcriptional levels of *Twist1* and *GAPDH* were measured by RT-PCR. (C) The cells were treated with each LY-290181 or MG132 alone, or with a combination of both for 3 h. The expression levels of Twist1 and *GAPDH* in the cell lysates were analyzed by immunoblotting. Quantitative graphs are shown on the right. Data are representative of three experiments and expressed as the means $\pm$ SD. *** P < 0.001 relative to the LY-290181 untreated control.

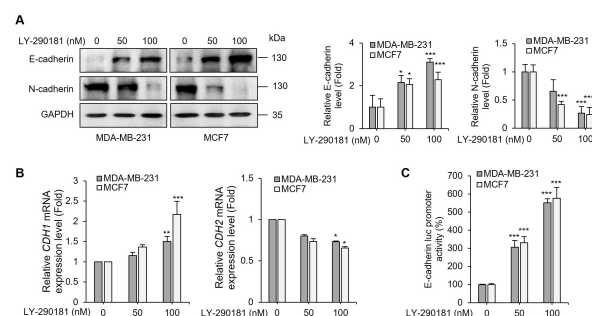


Fig. 3. Expression levels of target genes of Twist1. (A) MDA-MB-231 and MCF7 cells were treated with LY-290181 for 48 h. Subsequently, whole cell lysates were harvested and analyzed by immunoblotting. Quantitative graphs are shown on the right. (B) RNA was obtained from MDA-MB-231 and MCF7 cells after treatment with LY-290181 for 48 h. Transcriptional levels of *CDH1*, *CDH2*, and *GAPDH* were measured by RT-qPCR and then normalized against the *GAPDH* levels. (C) MDA-MB-231 and MCF7 cells were co-transfected with the E-cadherin-Luc reporter and gWIZ-GFP. The cells were treated with the indicated concentrations of LY-290181 for 48 h. The luciferase activities were normalized against the GFP levels. Data are representative of three experiments and expressed as the means $\pm$ SD. * P < 0.05, ** P < 0.01, and *** P < 0.001 relative to the LY-290181 untreated control.

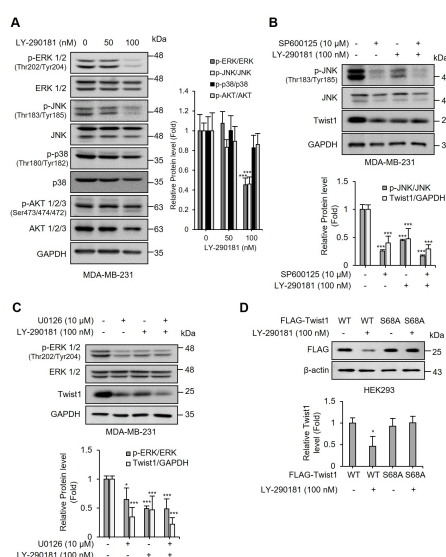


Fig. 4. Inhibitory effects of LY-290181 on signaling pathways regulating invasion and migration. (A) MDA-MB-231 cells were treated with LY-290181 for 48 h. Subsequently, whole cell lysates were harvested and analyzed by immunoblotting. Quantitative graphs are shown on the right. (B) After pre-treatment with SP600125 (10 μ M) for 1 h, LY-290181 was added and incubated for 48 h in MDA-MB-231 cells. Whole cell lysates were harvested and analyzed by immunoblotting. Quantitative graphs are shown below. (C) After pre-treatment with U0126 (10 μ M) for 1 h, LY-290181 was added and incubated for 48 h in MDA-MB-231 cells. Whole cell lysates were harvested and analyzed by immunoblotting. Quantitative graphs are shown below. (D) FLAG-Twist1- or FLAG-Twist1 S68A-transfected HEK293 cells were treated with LY-290181 for 24 h. Whole cell lysates were harvested and analyzed by immunoblotting. Quantitative graphs are shown below. Data are representative of three experiments and expressed as the means $\pm$ SD. * P < 0.05 and *** P < 0.001 relative to the LY-290181 untreated control.

lation of ERK and JNK without changing total protein levels. However, phosphorylation levels of AKT and p38 were not altered by LY-290181. These results suggest that suppressive effects of LY-290181 on Twist1 expression are due to the inhibition of ERK and JNK signaling pathways. In addition, similar effects of LY-290181 on anti-migration and anti-invasion were detected in Hep3B cells, a hepatocellular carcinoma (HCC) cell line (Supplementary Fig. 3A). The phosphorylation levels of ERK and JNK were also down-regulated by LY-290181 in Hep3B cells (Supplementary Fig. 3B). These results suggest that LY-290181 regulates HCC and breast cancer cells by a similar mechanism. Next, MDA-MB-231 cells were treated with the SP600125 (JNK inhibitor) and U0126 (ERK inhibitor) to determine whether the decrease in Twist1 was affected by the reduced phosphorylation of ERK and JNK (Fig. 4B, C). SP600125 treatment inhibited JNK activation and Twist1 expression, showing effects similar to those of LY-290181. U0126 also inhibited ERK activation and Twist1 expression. These re-

sults suggest that LY-290181 inhibited phosphorylation of ERK and JNK. To further confirm that the decrease in Twist1 protein levels by LY-290181 is mediated by inhibiting ERK and JNK, S68 of Twist1, which is the target phosphorylation site by ERK and JNK, was mutated to alanine (A) and then the mutant protein was expressed in HEK293 cells. LY-290181 treatment down-regulated protein levels of Twist1 wild type but had no effect on Twist1 S68A (Fig. 4D). These results imply that LY-290181 induces Twist1 degradation through ERK- and JNK-mediated Twist1 S68 phosphorylation.

DISCUSSION

Cancer growth and metastasis are the main causes of cancer-associated death in humans, and the discovery of powerful anti-cancer drugs to overcome cancer metastasis is essential for cancer treatment (24). Chemotherapy plays an important role in metastatic breast cancer treatment (25). LY-290181 has been studied as a cytostatic anti-cancer agent that inhibits microtubule formation and induces cell cycle arrest (16, 17), but other molecular mechanisms potentially involved in the effects of LY-290181 remained elusive. In this study, we found that LY-290181 acts as a potent Twist1 modulator and evaluated the mechanisms of EMT regulation at molecular and cellular levels.

Invasion and migration in metastasis is the initial stage of metastasis, in which cancer cells escape from cancer tissues to infiltrate and settle in distant tissues (26). LY-290181 showed anti-migration and anti-invasive effects on breast cancer cells in a 2D cell culture environment. MDA-MB-231 cell line, which is known as triple-negative breast cancer (TNBC) due to its lack of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2, was used in the MTS assay in the current study. Patients with TNBC have the poorest prognosis among breast cancer patients and TNBC frequently metastasizes (27). In this study, LY-290181 significantly inhibited the invasion of MTS in MDA-MB-231 cells. These data demonstrate that LY-290181 effectively inhibits invasion and migration of breast cancer cells in both 2D and 3D environments, suggesting that it may reduce cancer metastasis.

Several studies have revealed a correlation between the MAPK signaling pathway and cancer metastasis (28). In metastatic cancer cells, ERK, one of the MAPKs, shows higher activity compared with non-metastatic cells (29). Increased activation of JNK is associated with cell invasion and motility, and p38 regulates cell migration and invasion through Ras-dependent signaling pathways (30). In our study, LY-290181 inhibited ERK and JNK signaling pathways in MDA-MB-231 cells, suggesting that the suppressive effect of LY-290181 on cancer cell invasion is mediated by these pathways. Activation of ERK and JNK signaling pathways increases EMT-TFs, leading to EMT (31). In this study, LY-290181 was found to down-regulate Twist1 in breast cancer cells, while there was no observed regulation of Snail1. In addition, LY-290181 did not alter *Twist1* mRNA

levels, implying that LY-290181 targets Twist1 at the protein level. Although treatment with U0126, SP600125, and LY-290181 resulted in similar levels of reduction in Twist1 protein levels, LY-290181 treatment effectively reduced Twist1 levels at a lower concentration compared with the MAPK inhibitors. These results suggest that LY-290181 strongly inhibits Twist1 by regulating ERK and JNK signaling pathways.

The MAPK signaling pathway regulates the activities of EMT-TFs in various ways both at the transcriptional level and through phosphorylation (23). As described above, LY-290181 down-regulated Twist1 protein levels in breast cancer cell lines. Activated ERK and JNK phosphorylate S68 of Twist1, leading to stabilization of Twist1 (32). Based on our data, we infer that ERK and JNK-mediated Twist1 down-regulation by LY-290181 led to inhibition of cell motility and regulation of EMT-related markers in breast cancer cell lines. LY-290181 showed similar effects on invasion and migration also in HCC cells through the same mechanism, suggesting that the ability of LY-290181 to control EMT is applicable to a variety of cancer types. Future research is needed to delineate the precise molecular processes of Twist1 regulation by LY-290181 in invasive cancer cell lines other than HCC.

In this study, it was confirmed that LY-290181 inhibits the EMT process by specifically regulating Twist1, not Snail1, although the inactivation of ERK and JNK signaling pathways lead to regulation of both Twist1 and Snail1. To understand these results, we would like to suggest several possibilities. In the case of JNK inactivation by LY-290181, it has been reported that JNK regulates Twist1 through phosphorylation, but the regulation of Snail1 remains elusive (23). On the other hand, p38 and PI3K/AKT signaling pathways that regulate both Snail1 and Twist1 (23) are not affected by LY-290181. It can be suggested that JNK is the major signaling pathway by which LY-290181 reduces Twist1 protein levels. Several studies have revealed that the regulation of Twist1 is not mediated by a single signaling pathway but by multiple signaling pathways (11). Therefore, further studies on additional Twist1-regulating signaling pathways are needed. This discrepancy suggests the possibility that additional signaling pathways may be regulated by LY-290181.

In conclusion, this study demonstrated the anti-migratory and anti-invasive effects of LY-290181 with the underlying molecular mechanisms. Inhibition of Twist1 by LY-290181 leads to the suppression of cancer cell migration and invasion as well as regulation of EMT-related gene expression. Thus, this study proposes that LY-290181 may serve as a basis for developing potential cancer inhibitors to target various invasive cancers.

MATERIALS AND METHODS

Materials and methods are available in the supplemental section.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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