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Effect of 15-deoxyspergualin, a microbial angiogenesis inhibitor, on the biological activities of bovine vascular endothelial cells

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We found recently that 15-deoxyspergualin, an analog of spergualin, which is an antibiotic and includes a spermidine moiety in its structure, exhibits anti-angiogenic activity. We have now carried out *in vitro* experiments with bovine vascular endothelial cells to determine which events occurring during angiogenesis are affected by this microbial angiogenesis inhibitor. 15-Deoxyspergualin did not inhibit the production of urokinase-type plasminogen activator (u-PA) or type IV collagenase by vascular endothelial cells. The direct inhibition of u-PA activity by 15-deoxyspergualin was not observed either. The angiostatic antibiotic neither affected the migration of vascular endothelial cells nor inhibited the endothelial cell proliferation in a two-dimensional culture system. We also examined the effect of 15-deoxyspergualin on the proliferation of endothelial cells in a three-dimensional culture system involving collagen gel, in which cell growth resembles more closely the endothelial cell proliferation during *in vivo* angiogenesis than that in a two-dimensional culture system without collagen gel. The antibiotic inhibited cell proliferation in a dose-dependent manner, indicating that the three-dimensional culture system is useful for finding a new angiogenesis inhibitor with a different mode of action from those of angiogenesis inhibitors found by using a two-dimensional assay system; however, no cause–effect relationship has yet been established. Taken together, these results suggest the possible involvement of the inhibition of vascular endothelial cell growth by 15-deoxyspergualin in its angiogenesis-inhibitory effect. 15-Deoxyspergualin appears to be a promising candidate as an angiogenesis inhibitor for controlling aberrant angiogenic responses occurring in different states, including tumor development. Studies on this angiostatic antibiotic will undoubtedly improve our understanding of the mechanism of angiogenesis.

Key words: Angiostatic antibiotic, angiogenesis inhibitor, 15-deoxyspergualin, vascular endothelial cell growth inhibition.

Introduction

Angiogenesis has become important in studies on cancer therapy, because of the reported finding that various angiogenesis inhibitors also affect the progressive growth of solid tumors. The hitherto identified angiogenesis inhibitors include cartilage-derived factor(s),[1,2] angiostatic steroids,[3–5] angiostatic vitamins,[6,7] microbial products[8–13] and others.

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It was previously reported that angiostatic steroids exhibited angiogenesis-inhibitory activity only with the co-administration of heparin; in the absence of heparin these steroids failed to show anti-angiogenic activity.[3,4] In contrast, we have demonstrated that medroxyprogesterone acetate (MPA) alone effectively inhibits the angiogenic activity triggered by autochthonous rat mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA), probably resulting in the growth inhibition of these mammary tumors.[5] Similarly, other research groups showed that, like MPA, most angiostatic steroids exhibit anti-angiogenic activity in the absence of heparin, although their anti-angiogenic effects are sometimes potentiated by heparin.[9,14] Based on our results obtained with MPA, we attempted to find a new angiogenesis inhibitor.

On the basis of the state of differentiation or dedifferentiation of vascular endothelial cells in the angiogenesis process, we have proposed that a cell differentiation-modifying agent could affect angiogenesis.[6,7] To verify this hypothesis, we examined the anti-angiogenic activities of retinoids and vitamin D_3 analogs, which are both known to be differentiation modifiers, and found that these two groups of vitamins (or hormones) exert angiogenesis-inhibitory effects. We demonstrated recently that 22-oxa-1α,25-dihydroxyvitamin D_3 (22-oxa-1,25$(OH)_2D_3$), the most potent angiostatic vitamin among vitamin D_3 analogs, inhibits the growth of DMBA-induced autochthonous rat mammary tumors in a dose-dependent manner.[15]

Herbimycin A was found to inhibit tyrosine kinase activity,[16] although it was originally identified as an antibiotic. The receptor for fibroblast growth factor, one of the putative angiogenesis factors, was reported to be associated with tyrosine kinase activity and subseqently the basic fibroblast growth factor receptor was shown to include a tyrosine kinase domain.[17,18] These findings led us to examine the anti-angiogenic activity of herbimycin A. This antibiotic exhibits a dose-dependent angiogenesis-inhibitory effect, as assessed in an *in vivo* assay system involving chorioallantoic membrane (CAM) of growing chick embryo.[8] Based on this fact and the previous finding that microorganisms provide a variety of useful products, we hypothesized that microorganisms produce different angiogenesis inhibitors.[8] 15-Deoxyspergualin is an analog of an antibiotic, spergualin, which was discovered by Umezawa and his co-workers,[19] and which shows the most potent inhibitory activity against several experimental tumors among its analogs.[20] However, the mechanism of the antitumor action of the antibiotic has not been fully elucidated. 15-Deoxyspergualin contains both spermidine and guanidine moieties in its chemical structures. Spermidine is one of the polyamine family, which is known to play key roles in cell proliferation and cell differentiation.[21,22] In addition, previous studies showed that derivative(s) of an enzyme substrate sometimes inhibit its enzymatic activity. Considering these findings, we proposed that 15-deoxyspergualin could have the ability to inhibit angiogenesis. To verify this, we examined the angiogenesis-inhibitory effect of 15-deoxyspergualin and found that it exerts a dose-dependent anti-angiogenic action.[11] The angiostatic antibiotic has the distinctive property that it exhibits anti-angiogenic activity in a relatively wide dose range. Interestingly, 15-deoxyspergualin was found to exhibit its antitumor activity in a wide dose range as well, supporting the possible involvement of the anti-angiogenic activity of 15-deoxyspergualin in its antitumor effect.

The present study was performed to determine which event(s) occurring during angiogenesis was affected by 15-deoxyspergualin. This angiostatic antibiotic did not influence the production of plasminogen activator or type IV collagenase by vascular endothelial cells, nor was the inhibition of cell migration or cell proliferation observed in a two-dimensional culture system. 15-Deoxyspergualin inhibited the proliferation of vascular endothelial cells in a three-dimensional culture system involving collagen gel, which seems to be closer to *in vivo* angiogenesis than a two-dimensional culture system. These results indicate that the anti-angiogenic activity of 15-deoxyspergualin is at least in part due to its inhibitory action toward vascular endothelial cell proliferation.

Materials and methods

Materials

15-Deoxyspergualin was isolated as described in the previous paper.[11] Its structure is shown in Figure 1. It was dissolved in saline at a concentration of 2 mg/ml, sterilized by passage through a Millipore

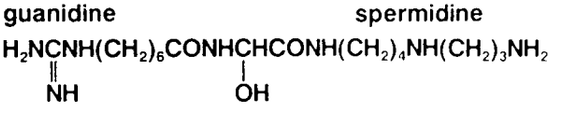

Figure 1. Structure of 15-deoxyspergualin.

filter with a pore size of 0.2 μm and stored at −20°C until use. Eagle's minimum essential medium (MEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum was a product of Biocell Laboratories (Carson, CA, USA). Vitrogen was obtained from Collagen Corp. (Palo Alto, CA, USA).

Vascular endothelial cells

Vascular endothelial cells from bovine capillary or carotid artery were used. Their characteristics were described in previous papers.[13,23] The capillary and arterial endothelial cells used in these experiments were passages 10–13 and 9–15, respectively.

Plasminogen activator and type IV collagenase secreted by endothelial cells into the conditioned medium

Endothelial cells (2×10^5 cells/well) were plated onto the wells of 24-multiwell dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ, USA) containing 1 ml of MEM supplemented with 10% FBS. After 16 h incubation, the medium was aspirated off, and the cells were washed carefully twice with serum-free MEM and then cultured in 1 ml of serum-free medium containing various doses of 15-deoxyspergualin for 24 h. After the culture, the serum-free conditioned medium was collected and centrifuged. The resulting supernatant was subjected to assaying for both plasminogen activator and type IV collagenase.

Plasminogen activator activity was assayed using plasminogen and the synthetic substrate, S-2251, according to the method of Shimada *et al.*[24] The activity was expressed in urokinase units.

Type IV collagenase was measured using ^{3}H-labelled human type IV collagen (NEN Research Products, Boston, MA, USA) as a substrate according to the method of Irimura *et al.*[25] with some modifications. The activation of type IV procollagenase (i.e. inactive form) in the vascular endothelial cell conditioned medium to type IV collagenase (i.e. active form) was performed using 0.5 mM 4-aminophenylmercuric acetate (Aldrich Chemical Co., Milwaukee, WI, USA).

Assay for endothelial cell proliferation

Endothelial cells were grown in the presence or absence of various concentrations of 15-deoxyspergualin in a two- or three-dimensional culture system.

The endothelial cell proliferation assay in a two-dimensional culture system was carried out as described previously.[26] In brief, endothelial cells (2×10^4 cells/well of 24-multiwell dishes) were incubated at 37°C for 3 days in a 5% CO_2 incubator in the presence of the indicated concentrations of 15-deoxyspergualin, after which the cell number was determined with a Coulter counter ZBI (Coulter Electronics Inc., Hialeah, FL, USA) after trypsinization.

The endothelial cell proliferation assay in a three-dimensional culture system involving collagen gel was performed as described in a previous paper.[13] The assay procedure is outlined in Figure 2. Briefly, endothelial cells (1×10^5 cells/well of 12-multiwell dishes) were cultured at 37°C under 5% CO_2 in air in the presence of various concentrations of 15-deoxyspergualin. After 3 days of incubation, the cell number was determined with a hemocytometer after treatment with collagenase and then trypsin.

Assay for endothelial cell migration

The endothelial cell migration assay was carried out as described in a previous paper,[13] using a Boyden

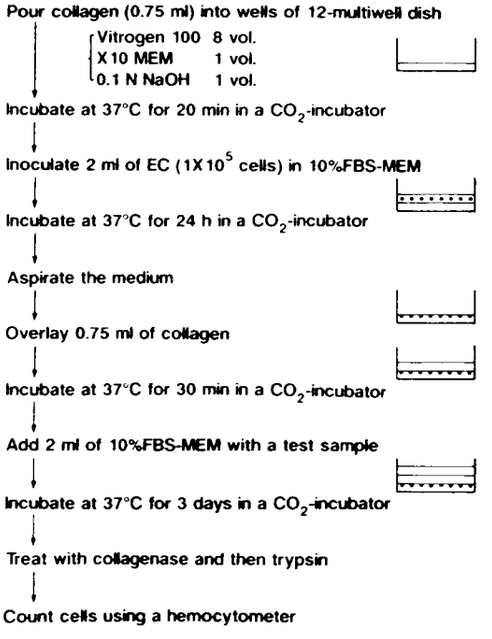

Figure 2. Outline of the assay for endothelial cell proliferation in a three-dimensional collagen culture.

chamber equipped with a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD, USA), each well containing a nucleopore membrane filter of 10 μm thickness and 8 μm pore size. In brief, the lower wells of the microchemotaxis chamber were filled with MEM supplemented with 10% FBS and the upper ones with endothelial cells (2×10^4 cells) suspended in 50 μl of 2% FBS–MEM. 15-Deoxyspergualin was added to the lower well. After the migration assays had been performed at 37°C for 3 h in a 5% CO_2 incubator, the cells which had migrated to the lower surface of membrane filters were counted.

Statistics

Data were analyzed by means of Student's t-test, $p < 0.05$ being taken as the level of significance.

Results

Angiogenesis comprises sequential events including the enhancement of plasminogen activator and type IV collagenase production by vascular endothelial cells, the migration and proliferation of endothelial cells, and subsequent tube formation.[27,28] To facilitate the elucidation of the mechanism of the anti-angiogenic activity elicited by 15-deoxyspergualin, *in vitro* experiments involving cultured vascular endothelial cells were carried out.

Effect of 15-deoxyspergualin on the production of plasminogen activator and type IV collagenase by endothelial cells

First, experiments were performed to determine whether or not 15-deoxyspergualin affected the levels of plasminogen activator and type IV collagenase produced by endothelial cells.

15-Deoxyspergualin, unlike MPA, our angiostatic steroid,[23] influenced the production of plasminogen activator by endothelial cells little or not at all (Figure 3A). The angiostatic antibiotic did not inhibit the production of type IV collagenase by the cells (Figure 3B).

15-Deoxyspergualin at concentrations up to 200 μM did not inhibit the enzymatic activity of type IV collagenase from endothelial cells (Figure 4), suggesting that the mechanism underlying its anti-angiogenic activity is different from that in the case of minocycline, an angiostatic antibiotic, which probably exerts its anti-angiogenic effect through the inhibition of the activity of collagenase.[12]

Effect of 15-deoxyspergualin on the proliferation of endothelial cells in a two-dimensional culture system

Some of the hitherto identified angiogenesis inhibitors were found to inhibit endothelial cell proliferation in a two-dimensional culture system,[1,10] which is one of the important events in the

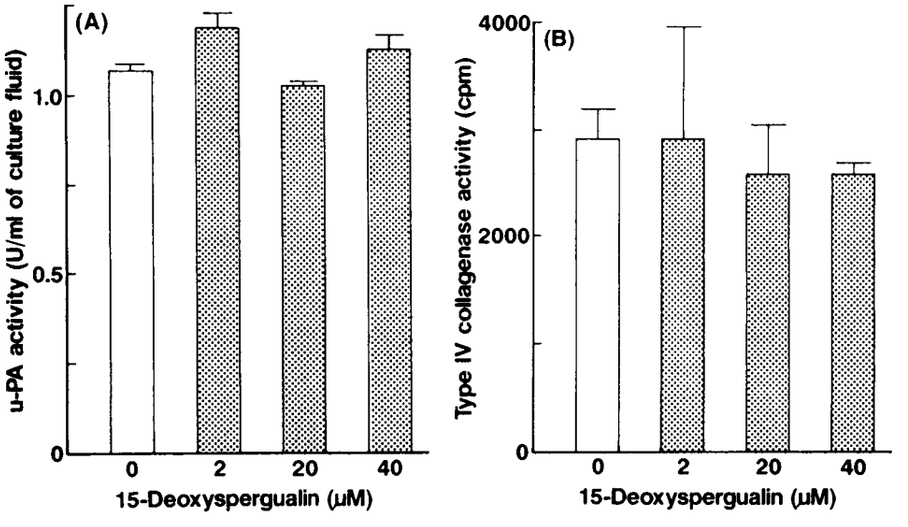

Figure 3. Effect of 15-deoxyspergualin on the production of plasminogen activator (A) or type IV collagenase (B) by vascular endothelial cells. Control endothelial cells secreted 1.07 ± 0.02 urokinase units into the conditioned medium in 24 h. Values are the means ± SD for three determinations.

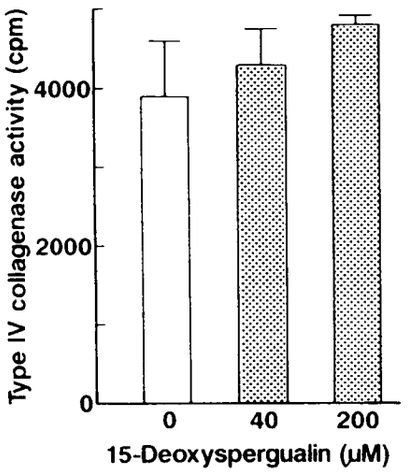

Figure 4. Effect of 15-deoxyspergualin on type IV collagenase activity. Type IV collagenase activity secreted into the serum-free conditioned medium by vascular endothelial cells was assayed in the presence or absence of 15-deoxyspergualin, using ^{3}H-labelled human type IV collagen, after treatment of the conditioned medium with 0.5 mM 4-aminophenylmercuric acetate. Values are the means ± SD for three determinations.

angiogenesis process. Different angiogenesis factors, such as acidic and basic fibroblast growth factors, vascular endothelial growth factor (or vascular permeability factor) and platelet-derived endothelial cell growth factor, exhibit growth stimulating activity toward endothelial cells.[28] Thus experiments were carried out to determine whether or not 15-deoxyspergualin inhibits the proliferation of endothelial cells. 15-Deoxyspergualin at concentrations of 10^{-4} to 10^{-10} M did not inhibit the cell proliferation (Figure 5), indicating that the mode of action in the case of its anti-angiogenic activity is different from those in the cases of several angiogenesis inhibitors identified so far.[1,10]

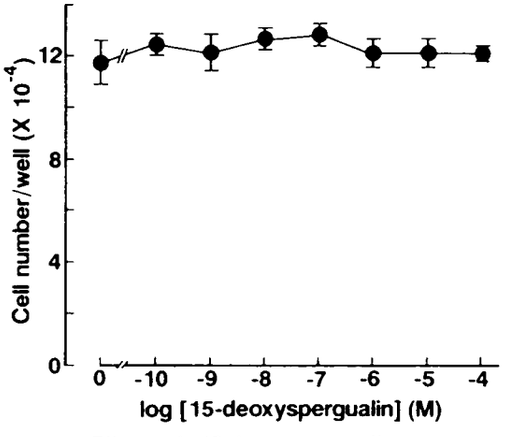

Figure 5. Effect of 15-deoxyspergualin on vascular endothelial cell proliferation in a monolayer culture. Values are the means ± SE for at least two experiments, each assay being performed in triplicate.

Effect of 15-deoxyspergualin on the migration of endothelial cells

To examine the effect of 15-deoxyspergualin on the migration of endothelial cells, cells were allowed to migrate in the Boyden chamber in the presence of various concentrations of the agent for 3 h. 15-Deoxyspergualin at concentrations up to 10^{-5} M was not effective in inhibiting the cell migration (Figure 6), which suggests that the mechanism of its anti-angiogenic activity differs from that of a synthetic peptide of laminin exhibiting angiogenesis inhibitory activity.[29]

Effect of 15-deoxyspergualin on the proliferation of endothelial cells in a three-culture system involving collagen gel

Vascular endothelial cells in the angiogenesis process *in vivo* proliferate in the interstitum, which is mainly composed of collagen. Thus, it is conceivable that endothelial cell growth occurring in a three-dimensional culture system involving collagen gel would reflect more that in *in vivo* angiogenesis than that in a two-dimensional culture system.[13] On the basis of this conception, further experiments were performed to determine whether or not 15-deoxyspergualin influenced the endothelial cell proliferation between collagen gels.

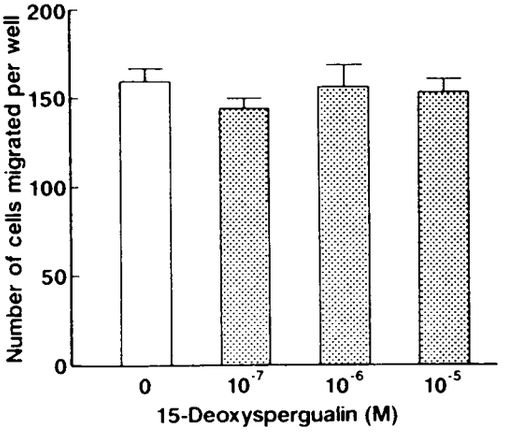

Figure 6. Effect of 15-deoxyspergualin on vascular endothelial cell migration. Endothelial cell migration was assayed in the Boyden chamber. Values are the means ± SE for at least two experiments, each assay being performed in duplicate.

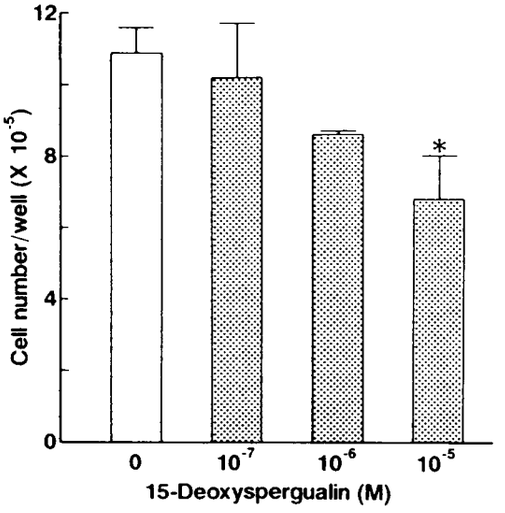

Figure 7. Effect of 15-deoxyspergualin on vascular endothelial cell proliferation in a three-dimensional culture system. Values are the means ± SE for at least two experiments, each assay being performed at least in triplicate. *Significant difference from the control, $p < 0.05$.

15-Deoxyspergualin inhibited the proliferation of endothelial cells in a concentration-dependent manner (Figure 7). The angiostatic antibiotic at a concentration of 10^{-5} M induced about 40% growth inhibition, as compared with the control.

Discussion

Based on our previous finding that the antitumor activity of MPA against DMBA-induced rat mammary tumors probably includes its anti-angiogenic action,[5] we attempted to find a new angiogenesis inhibitor by employing an *in vivo* assay (i.e. the CAM assay) for the detection of anti-angiogenic activity. Our strategy for finding a useful angiogenesis inhibitor was based on a distinctive idea of ours.[13] Namely, we deduced that microbial products and cell differentiation modifiers are the targets of angiogenesis inhibitors, because a variety of microbial products have been found to be useful in maintaining human life and because cell differentiation or dedifferentiation is one of the crucial events in the angiogenesis process. We have found that both microbial products and cell differentiation modulators exhibit angiogenesis-inhibitory activity.[6–8,11,13] Our microbial angiogenesis inhibitors include herbimycin A, 15-deoxyspergualin and eponemycin.[8,11,13] Angiogenesis inhibitors showing cell differentiation activity include retinoids and vitamin D_3 analogs.[6,7] We have shown the antitumor effect of 22-oxa-$1,25(OH)_2D_3$ in a DMBA-induced autochthonous rat mammary tumor model system, which is a useful model system for studying breast cancer and for developing drugs against this cancer.

In this study we have demonstrated that the anti-angiogenic activity of 15-deoxyspergualin is mediated through the inhibition of endothelial cell proliferation by the angiostatic antibiotic. The important point is that the cell growth inhibition by 15-deoxyspergualin was found only in a three-dimensional culture system involving collagen gel, i.e. not in a two-dimensional culture system, although it remains to be determined what causes the difference in the inhibitory effect on cell proliferation between these two experimental systems. However, the present finding will undoubtedly contribute to the finding of useful and potent angiogenesis inhibitors with unique modes of action. In addition, it might facilitate elucidation of the angiogenesis mechanism. Similarly, Kanayasu *et al.*[30] reported that eicosapentaenoic acid does not inhibit endothelial cell proliferation in the monolayer culture, but exhibits a concentration-dependent inhibitory effect on endothelial cell growth in a three-dimensional collagen culture,[30] although the agent has not yet been examined as to its *in vivo* anti-angiogenic activity. In this regard, we would like to emphasize that most of our inhibitors of endothelial cell growth, which were detected in a monolayer culture assay, did not exhibit anti-angiogenic activity, as assessed by the CAM assay (unpublished data).

Our previous study showed that the ID_{50} value, i.e. anti-angiogenic potency, of 15-deoxyspergualin is 960 pmol (480 ng) per egg.[11] The present study revealed that 15-deoxyspergualin at a concentration of 10^{-5} M, the highest concentration used in this experiment, inhibited the proliferation of endothelial cells by 40%. Taking all the results together it is highly likely that 15-deoxyspergualin exhibits anti-angiogenic activity not only through its inhibition of endothelial cell proliferation but also through an unknown mechanism. With respect to this, it is interesting that the angiostatic antibiotic contains a spermidine moiety, which is a member of the polyamine family which is known to play important roles in cell proliferation and cell differentiation.[21,22] Additionally, we have found that cell differentiation modifiers, including retinoids and vitamin D_3 analogs, are potent angiogenesis inhibitors based on our working hypothesis that a cell differentiation modifier shows anti-angiogenic activity.[6,7] Experiments are currently in progress to determine whether or not 15-deoxyspergualin

exhibits cell differentiation modifying activity and also whether or not the angiostatic antibiotic inhibits an angiogenic response(s) other than embryonic angiogenesis, in particular, tumor angiogenesis.

Conclusion

15-Deoxyspergualin, a new angiostatic antibiotic, inhibited the proliferation of vascular endothelial cells in a three-dimensional culture system involving collagen gel, but not in a two-dimensional culture system. The present data suggest that 15-deoxyspergualin is a promising angiogenesis inhibitor. In addition, these results might facilitate the finding of unique angiogenesis inhibitors and also the understanding of the mechanism underlying angiogenesis.

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