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Insulin receptor binding motif tagged with IgG4 Fc (Yiminsu) works as an insulin sensitizer to activate Akt signaling in hepatocytes

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ABSTRACT. Insulin resistance is a key feature of obesity and type 2 diabetes mellitus (T2DM). Interaction of insulin with the insulin receptor (IR) leads to both its auto-phosphorylation and phosphorylation of tyrosine residues on the IR substrate (IRS) proteins, initiating the activation of intracellular signaling cascades. The metabolic effects of IRS are known to be mediated through pathways involving phosphatidyl-

inositol 3-kinase (PI-3K), which result in the activation of Akt signaling. The C-terminal region of the IR ectodomain is required to facilitate the conformational changes that are required for high-affinity binding to insulin. Furthermore, the CH2 and CH3 domains in the Fc fragments of immunoglobulins are responsible for their binding to the Fc receptor, which triggers transcytosis. In this study, we created a fusion peptide of the C-terminal end of the human IR ectodomain with the IgG4 Fc fragment, including an intervening polyG fragment to ensure enough space for insulin binding. We named this new peptide "Yiminsu", meaning an insulin sensitizer. The results of our analyses show that Yiminsu significantly facilitates insulin signaling via the activation of Akt in hepatocytes in a dose- and time-dependent manner. Further studies are required to determine whether Yiminsu can act as an insulin sensitizer.

Key words: Yiminsu; Insulin sensitizer; Type 2 diabetes; IgG4 Fc

INTRODUCTION

Insulin resistance is a key feature of obesity and type 2 diabetes mellitus (T2DM). Insulin signaling is a complex process; the interaction of insulin with the insulin receptor (IR) leads to both its auto-phosphorylation and the phosphorylation of tyrosine residues on IR substrate (IRS) proteins, initiating intracellular signaling cascades (Tamemoto et al., 1994). The metabolic effects of IRS have been shown to be mediated through the phosphatidyl-inositol 3-kinase (PI-3K) pathway, which results in the activation of protein kinase B (Akt) signaling. Insulin signaling might also activate the mitogen activated protein kinase-ERK1/2 pathway. In patients with T2DM and insulin resistance, PI-3K activation pathways were found to be blocked (Cusi et al., 2000). Thus, the re-activation of Akt signaling would help to increase the sensitivity to insulin in patients with insulin resistance.

The wild-type IR is a heterotetramer with a molecular weight of approximately 350 kDa, composed of two α and two β subunits. The entire α subunit and 194 amino acids of the β subunit comprise the ectodomain, while the tyrosine kinase domain is located on the intracellular portion of the β subunit (De Meyts, 2004). IRs contain two distinct binding regions (sites 1 and 2), which allow the binding of one insulin molecule with low-affinity (e.g., to site 1), and the binding of a second with high-affinity. It has been demonstrated that transmembrane domain dimerization is a key step for receptor activation (Menghini et al., 2013), suggesting that the ectodomain contributes to the formation of a high-affinity binding site. Secreted IR truncated at the ectodomain/transmembrane junction form dimers with only a low affinity for insulin, in contrast to the full-length receptor (Chung et al., 2011). Truncation at the C-terminal end of the transmembrane region has been shown to be sufficient for the formation of high-affinity binding and negative cooperativity (Tseng et al., 2013), indicating that the C-terminal region of the ectodomain is required to facilitate the conformational changes required for high-affinity. A study reported that wild-type receptor binding characteristics were obtained with soluble insulin receptor ectodomain fusion proteins, in which the self-associating, constant domains from either immunoglobulin Fc domain were placed at the C-terminus (Bass et al., 1996).

In this study, we placed an IgG4 Fc fragment at the C-terminal end of the human IR (hIR) ectodomain, and inserted a polyG fragment between them, which ensured the presence

of enough space for the binding of insulin to the IR. This enabled the production of a soluble form of the hIR ectodomain dimer that exhibited high-affinity insulin binding. The CH2 and CH3 domains in the Fc fragment of immunoglobulins are responsible for their binding to the Fc receptor, and thus triggering transcytosis (Martin et al., 2001), which would be helpful for promoting insulin sensitivity and reducing the serum insulin in patients with insulin resistance.

MATERIAL AND METHODS

Reagents

The restriction enzymes *Eco*RI, *Age*I, *Bam*HI, *Kpn*I, and T4-DNA ligase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All plasmids were constructed in our laboratory. Human anti-insulin receptor antibody was obtained from Abcam (Hong Kong, China).

cDNA cloning and plasmid construction

The cDNA of the human insulin receptor Yiminsu were amplified by polymerase chain reaction (PCR), using DNA polymerase (Thermo Fisher Scientific) and primers containing the IgG secretion signal sequence. The primers were as follows: INSR-hIgG4 Fc forward primer: 5'-GCG***AAGCTT***ATGCCACTCTGGGTGTTCTTCTT T-3'; INSR-hIgG4 Fc reverse primer: 5'-CGC***TCTAGA***CTATCATTTACCCAGAGA CAGGGAGAGG-3' (bold sequences indicate the restriction sites). The PCR product was subcloned into the p-CMVIE vector (Cat No. Biovector958235-3, Biovector NTCC Inc., Beijing, China). The resulting clones were then verified by sequencing. The Yiminsu fragment products were gel extracted to release the fragment by using QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), and the resulting solution was sent out for sequencing by Sangon (Shanghai, China). Bidirectional sequencing was used because of the length of the constructed gene fragment (4877 bp). The molecular weight of the recombinant protein was 33 kDa.

Cell culture and treatment

Chinese hamster ovary (CHO)-K1 cells were cultured in American Type Culture Collection (ATCC)-formulated Eagle's minimum essential medium (ATCC, Manassas, VA, USA) with the addition of fetal bovine serum (Life Technology, Waltham, MA, USA) to a final concentration of 10%. When confluency reached 80-90% on 100-mm plates, the cells were transfected with cDNA of the human insulin receptor Yiminsu (2 μg per sample) by using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instruction. After 48-h transfection, selection was effected using the neomycin resistance (*neo*^{r}) gene present in the plasmid by using G418 (600 μg/mL). Surviving clones were identified and expended for analysis.

Heap-1c1c7 cells were obtained from the ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were treated with 0.1 nM insulin alone or co-treated with 0.1 nM insulin and 0.1 nM Yiminsu for 5 min. Cells were collected for further analysis.

Human normal hepatic LO2 cells and CCL13 cells were purchased from the Chinese Academy of Science, Shanghai Cell Library (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. In order to determine the best concentration, the cells were treated with vehicle, 0.01, 0.1, 1, 5, or 10 nM Yiminsu for 5 min. The cells

treated with 5 nM insulin for 5 min were used as a positive control. In order to determine the optimal action time, the cells were treated with 5 nM Yiminsu for 1, 5, 10, 20, and 30 min. Cells treated with 5 nM insulin for 5 min were used as positive control. At the indicated time point, cells were collected for further analysis.

Purification of Yiminsu

Protein A affinity chromatography was used to purify the fusion peptide, Yiminsu. Briefly, protein A resin was equilibrated with phosphate-buffered saline (PBS; pH 7.4), and after sample loading, the column was washed with PBS and the protein eluted with 0.1 M glycine, pH 2.8. The eluted protein was neutralized with 1 M Tris-HCl, pH 8.0, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue.

Western blot analysis of Yiminsu

Prior to protein isolation, transfected cells were harvested and washed three times with PBS. The radioimmunoprecipitation assay (RIPA) lysis buffer (100 μL, cat No. AR0102, Boster, Wuhan, China) was added to the wells for 30-min incubation on ice. After centrifugation at 12,000 rpm for 15 min, the supernatant was extracted and the protein concentration detected using a BCA Protein Assay Kit (Thermo Fisher Scientific). After purification with protein A affinity chromatography as described above, 200, 100, 50, 25, or 12.5 ng reduced protein was separated using 15% SDS-PAGE. The separated proteins were transferred to PVDF membranes, and the membranes were blocked in 5% non-fat milk for 1 h at room temperature. The membranes were washed and incubated with primary antibodies (human insulin receptor antibody; dilution, 1:100; Abcam, United Kingdom) at 4°C overnight, and with secondary antibodies (HRP-anti-human IgG; dilution, 1:2000; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h. After incubations, the proteins were detected by enhanced chemiluminescence (Millipore, Billerica, MA, USA) and photographed. Data were analyzed by densitometry using the Image-Pro plus software 6.0 (Media Cybernetics, Rockville, MD, USA) .

Western blot analysis of pAkt and Akt

Total protein was extracted from indicated cells by cold RIPA lysis buffer. Protein concentration was determined by a BCA Protein Assay Kit. The protein was then separated with 10% SDS-PAGE and transferred to a nitrocellulose membrane. After being blocked in 8% nonfat dried milk in PBS for 4 h, the membrane was incubated with the indicated primary antibody (pAkt and Akt, mouse, 1:2000, Cell Signaling Technology, Danvers, MA, USA; β-actin, mouse, 1:3000, Boster, Wuhan, China) overnight at 4°C. The membrane was then washed and incubated with the secondary antibody for 1h at 37°C. Signals were detected as described above, and normalized to internal control (β-actin) expression.

Statistical analysis

Data were analyzed using the GraphPad Prism5 software (LaJolla, CA, USA). Student *t*-tests or one-way ANOVA were used depending on the experimental conditions. Data shown in the figures are reported as means ± standard deviation. Statistical significance was

represented by P values of <0.05.

RESULTS

Yiminsu sequence

The construction scheme of the Yiminsu fusion gene is shown in Figure 1. The target genes were constructed within the P-CMVIE vector, containing an eomycin resistance gene. In order to identify the sequence of the Yiminsu gene, the Yiminsu fragment products were sent to Sangon for sequencing (data not shown). Furthermore, CLUSTAL 2.1 multiple sequence alignment was used to examine whether the Yiminsu sequence matched with the original *Homo sapiens* insulin receptor (INSR) (range from 146238 to 146271) and immunoglobulin heavy constant gamma 4 (G4m marker) (range from 87400952 to 87401277) sequence. As expected, the sequence of the reconstructed plasmid sequence exactly matched with the original sequence (data not shown).

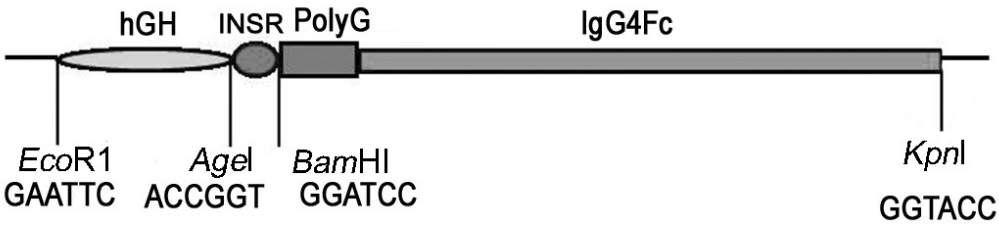

Figure 1. Construction scheme of the novel Yiminsu fusion-gene plasmid. Restriction enzyme cleavage sites are shown, including *Eco*RI, *Age*I, *Bam*HI, and *Kpn*I. hGH, human growth hormone; INSR, C-terminal end of the human insulin receptor ectodomain; IgG4Fc, Fc fragment of human immunoglobulin.

Expression and purification of Yiminsu fusion proteins

In order to obtain cells expressing high levels of Yiminsu, after G418 selection, five clones were picked and analyzed. As shown in Figure 2A, we found that overexpression of the Yiminsu fusion protein was observed in clones No.1 and 4, with highest expression seen in clone No. 4. Recombinant Yiminsu fusion proteins transiently expressed in CHO-K1 cells were secreted into the medium, suggesting that the proteins were folded properly. To further validate the fusion protein, the fusion proteins were purified by protein A affinity chromatography. The fusion proteins appeared on an SDS-PAGE gel as a 33-kDa monomer under reducing conditions by Coomassie brilliant blue staining (Figure 2B). This result was also validated by western blotting analysis (Figure 2C).

Yiminsu fusion protein activates the Akt signaling pathway

We further examine the effect of Yiminsu on insulin signaling in the condition of insulin resistance. Akt signaling plays an important role in insulin resistance. Considering the short half-life of insulin ($t_{1/2}$<10 min), Hepa-1c1c7 cells were treated with 0.1 nM insulin alone or co-treated with 0.1 nM insulin and 0.1 nM Yiminsu for 5 min, followed by analysis of the action of Akt in these hepatic cells. As shown in Figure 3, the cells treated with insulin alone presented a slight increase in expression of pAkt. However, pAkt was significantly activated by co-treatment

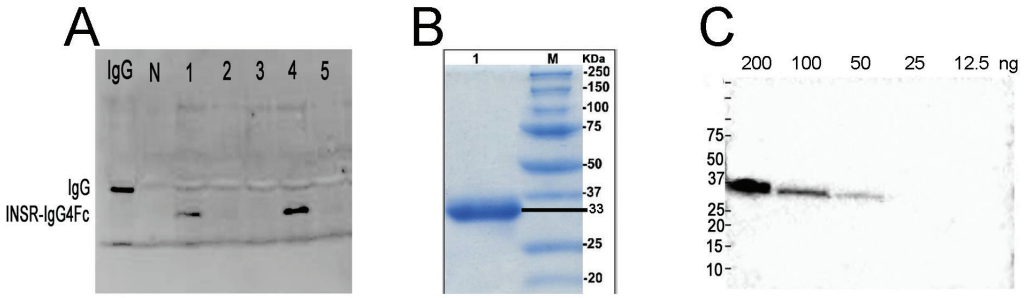

Figure 2. Expression of INSR-hIgG4Fc fusion protein. **A.** Electropherogram of PCR products detected the fusion protein mRNA expression: *lane N* = negative; *lanes 1-5* = clone numbers. **B.** Purified fusion protein (20 µg) was analyzed by SDS-PAGE under reducing conditions, and visualized by Coomassie brilliant blue R250 staining. *Lane 1* = INSR-hIgG4Fc fusion protein; *lane M* = marker. **C.** Western blotting analysis of the INSR-hIgG4Fc fusion protein. Reduced proteins were loaded on a gradient at 200, 100, 50, 25, and 12.5 ng per lane and were incubated with horseradish peroxidase-anti-human IgG secondary antibody.

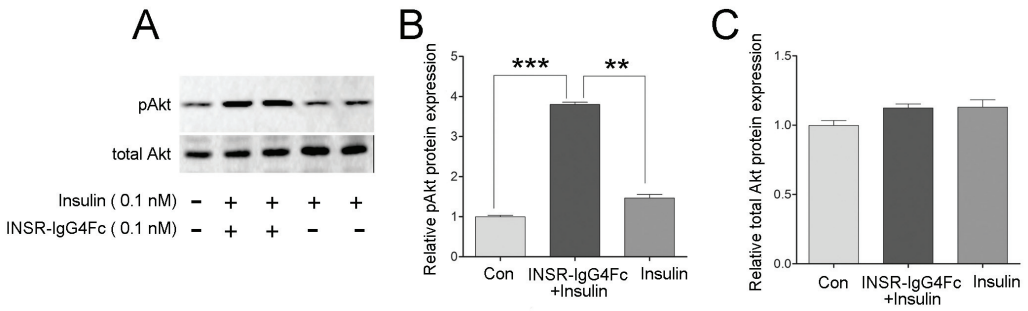

Figure 3. Yiminsu activates the Akt signaling pathway. **A.** pAkt and total Akt were measured by western blotting after treatment with the Yiminsu peptide combined with insulin, or with insulin alone. **B. C.** Quantification of pAkt and total Akt protein. Data are reported as means ± standard deviation. **P < 0.01; ***P < 0.001 *vs* control.

with insulin and Yiminsu, indicating that Yiminsu can increase the activation of Akt signaling.

Yiminsu fusion proteins activate the Akt signaling pathway in a dose- and time-dependent manner

To further analyze the effects of Yiminsu on Akt signaling, we first determined the optimal concentration of Yiminsu. The dose range of Yiminsu was set from 0.01 up to 10 nM. The cells treated with 5 nM insulin for 5 min were used as a positive control. We found that 5 min after treatment, doses of 0.01 nM Yiminsu did not significantly induce pAkt expression, whereas doses up to 0.1 nM significantly induced pAkt expression in a dose-dependent manner, which reached a peak at 10 nM in LO2 cells and at 5 nM in CCL13 cells (Figure 4A and B). Thus, 5 nM Yiminsu treatment was considered as the optimal concentration and was used in the subsequent experiments. Next, we determined the optimal action time of Yiminsu in hepatic cells. LO2 and CCL13 cells were treated with 5 nM Yiminsu for 1, 5, 10, 20, and 30 min. We found that pAkt expression was induced by Yiminsu in a time dependent manner, which reached the maximum at the 20 min time point in both cell lines (Figure 4C and D). Therefore, our evidence showed that the Yiminsu fusion protein activates Akt signaling in a

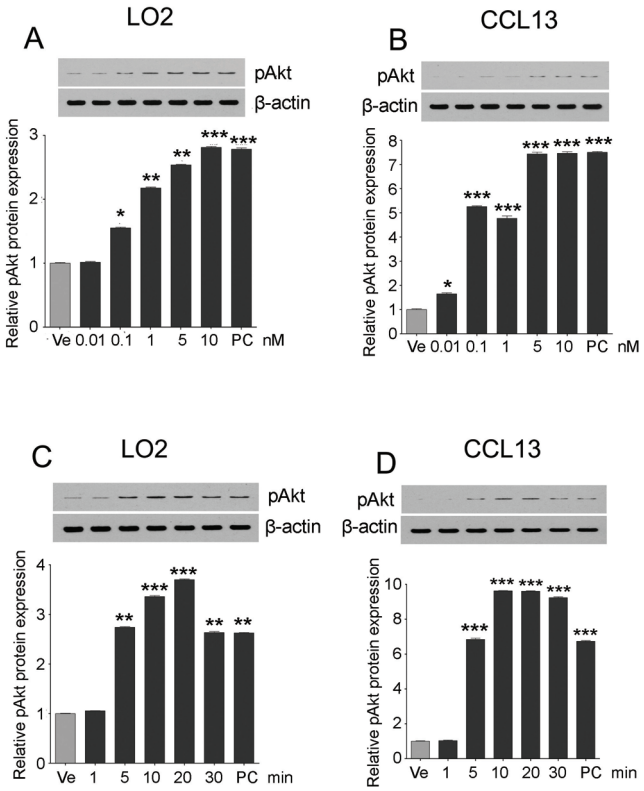

Figure 4. Yiminsu induces pAkt expression in a dose- and time-dependent manner. **A.** Western blotting analysis of pAkt expression in LO2 cells treated with different concentrations of Yiminsu. **B.** Western blotting analysis of pAkt expression in CCL13 cells treated with different concentrations of Yiminsu. **C.** Western blotting analysis of pAkt expression in LO2 cells treated with 5 nM Yiminsu at different time points. **D.** Western blotting analysis of pAkt expression in CCL13 cells treated with 5 nM Yiminsu at different time points. Ve, vehicle control; PC, positive control: the cells were treated with 5 nM insulin for 5 min. Data are reported as means ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001*vs* vehicle control.
dose- and time-dependent manner.

DISCUSSION

T2DM has become a worldwide health problem, with an increasing number of global cases expected to reach 350 million by the year 2030 (Ogden et al., 2006). Insulin resistance, an impaired responsiveness of the body to insulin (Muoio and Newgard, 2008), is usually observed long before the development of diabetes. Additionally, it has been documented that hyperglycemia at prediabetic levels is also an independent risk factor for cardiovascular disease (Haffner et al., 1990). Thus, insulin resistance has become a promising target for therapeutic developments for T2DM and its cardiovascular complications. However, many limitations are associated with pharmacological agents that improve the sensitivity to insulin. For example, thiazolidinediones and biguanides were shown to improve peripheral insulin sensitivity, but also exhibited serious adverse effects (Panagoulias and Doupis, 2014). Therefore, additional

efforts are needed to identify novel agents that are targeted for insulin sensitivity augmentation, thereby potentially overcoming the complications of diabetes.

In the present study, we successfully constructed a novel plasmid that expressed the fusion protein Yiminsu. Positive clones screened using the G418 resistance marker gene of the p-CMVIE vector were sent for sequencing. The sequencing results showed that the selected positive clones were positive recombinants, and that the sequence of the constructed gene matched those of its components. Furthermore, protein sequence prediction showed that Yiminsu contained 301 amino acids and that the molecular weight of the recombinant protein should be 33 kDa, which was consistent with the results of SDS-PAGE analysis of the purified recombinant protein. In addition, we found that Akt signaling was significantly activated in Hepa-1c1c7 cells by co-treatment with the Yiminsu fusion protein and insulin, to a greater extent than that occurred following insulin treatment alone.

Insulin, a pleiotropic hormone with metabolic and mitogenic functions (Magon and Seshiah, 2014) is important for the regulation of blood glucose levels. In skeletal muscles, insulin promotes glucose uptake by stimulating a cascade of signaling processes initiated by the binding of insulin to the extracellular α-subunit of the IR on the cellular membrane (D'Souza et al., 2013). After binding, the intracellular tyrosine kinase domain of the IR becomes activated by IR autophosphorylation of its intracellular β-subunit. Once activated, the IR phosphorylates multiple tyrosine residues downstream, including IRS-1 and 2. This promotes their binding to Src-homology 2 domains, leading to an association between IRS-1 and PI-3K. Finally, these events lead to the phosphorylation of Akt. Activated pAkt in turn phosphorylates multiple downstream effectors, including the Rab-GTPase activating protein, eventually leading to the translocation of glucose transporter-4 (Glut4)-vesicles from the cytoplasm to the cell surface, regulating cellular glucose uptake. Akt also phosphorylates glycogen synthase kinase-3 to stimulate glycogen formation (De Meyts, 2008). Previous data have suggested that Akt represented the key point for augmentation of insulin sensitivity (Mackenzie and Elliott, 2014). Our results showed that the Yiminsu fusion protein efficiently activated Akt signaling in a dose- and time-dependent manner, thereby indicating that it might be useful for management of insulin resistance.

Two major obstacles limited the therapeutic effects of administered proteins (e.g., insulin) in the gastrointestinal tract, namely, inactivation and malabsorption. Inactivation issues can be solved by the use of genetically engineered food-grade microorganisms that secrete therapeutic protein drugs (Yu et al., 2014) or by microencapsulation of the therapeutic protein drug (Shin and Yoo, 2013) to target the therapeutic proteins to the absorption site. However, because of the general large molecular size and hydrophilicity of therapeutic proteins, the issue of malabsorption is difficult to solve.

Malabsorption might be overcome by the induction of receptor-mediated transcytosis (Bahhady et al., 2008). Transcytosis is the process by which cargo proteins internalized at one plasma membrane of a polarized cell are transported via vesicular carriers to the contralateral plasma membrane (Lee et al., 2007). It is well known that the CH2 and CH3 domains in the Fc fragment of immunoglobulins are responsible for their binding to the Fc receptor, triggering transcytosis (Hornby et al., 2014). In addition, adults express the Fc receptor on endothelial cells in their blood vessels (Tanigaki et al., 2013). Furthermore, it has been demonstrated that erythropoietin and follicle-stimulating hormone have been successfully delivered via the pulmonary route into non-human primates by recombinant fusion of the Fc fragment (Low et al., 2005). Taken together, the previous study suggested that recombinant fusion of the Fc

fragment would contribute to the delivery of the fusion protein into the target cells. Thus, we infer that the fusion of Fc fragment and the ectodomain of the IR may facilitate Yiminsu into plasma of target cell when binding with insulin.

In conclusion, the fusion protein Yiminsu was successfully constructed in this study. We confirmed that Akt signaling was activated by Yiminsu in hepatic cell lines in a dose- and time- dependent manner. This protein might be helpful in promoting insulin sensitivity and reducing serum insulin in patients with insulin resistance.

Conflicts of interest

The authors declare no conflict of interest.

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