Pectenotoxin-2 induces G1 arrest of the cell cycle in synovial fibroblasts of patients with rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA), a chronic inflammatory disease, is characterized by hyperplasia of the synovial fibroblasts, due in part to increased cell growth. This study investigated the mechanisms underlying the anti-proliferative action of pectenotoxin-2 (PTX-2), isolated from marine sponges, in synovial fibroblasts obtained from RA patients. PTX-2 concentration-dependently inhibited the growth of synovial fibroblasts, arresting them in the G1 phase of their cell cycle. The G1 arrest was correlated with down-regulation of cyclin D1 and cyclin-dependent kinase (Cdk) 6, with a concomitant up-regulation of the tumor suppressor, p53, and the Cdk inhibitor, p21 (WAF1/CIP1). Following PTX-2 treatment of synovial fibroblasts, an increased binding of p21 with Cdk2 and Cdk6 was paralleled by a significant decrease in retinoblastoma protein (pRB) phosphorylation and in the protein levels of E2F transcription factors. Thus, PTX-2 might help identify new therapeutic agents against RA-mediated hyperplasia of synovial fibroblasts.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive joint destruction, deformity, and disability. The initial histological features of RA are characterized by synovial lining hyperplasia, excessive angiogenesis, and the accumulation of mononuclear cells in the synovium (1,2). Notably, both the macrophage-like synovial fibroblasts, in the synovial lining, and the fibroblast-like synovial fibroblasts in the synovial sublining layer expand in RA patients (3). The affected synovial tissues are then infiltrated by T and B lymphocytes and macrophages, promoting the release of high concentrations of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1. In response to these cytokines, the synovial fibroblasts proliferate vigorously and form pannus tissues, which then destroy the cartilage and bone of the joints (4,5). Although the reason for the hyperplasia of the synovial lining in RA is not completely understood, an impaired senescence has been proposed as a mechanism to explain the increased numbers of fibroblasts. Therefore, strategies that lead to inhibition of synovial fibroblast proliferation might be useful in treating RA.

Pectenotoxins are a group of natural toxins present in marine sponges and some shellfish that are toxic to humans (6,7). The most toxic compound in this group is pectenotoxin-2 (PTX-2), which is isolated from *Dinophysis* spp. (8). Previous *in vitro* and *in vivo* studies have shown that PTX-2, through its binding site with actin, can modify the actin cytoskeleton by promoting actin depolymerization (9,10). In addition, this compound also displays selective and potent cytotoxicity against human cancer cells (11,12), as well as inducing Bim/Bax-mediated apoptosis in p53-deficient tumors (13). However, effects of PTX-2 on the cell growth of human synovial fibroblasts have not yet been characterized.

The present study was designed to investigate the effects of PTX-2 on proliferation of human synovial fibroblasts obtained from RA patients. The observed decrease in viability of RA synovial fibroblasts exposed to PTX-2 was mediated by the induction of G1 arrest, in response to inhibition of cyclin D1 and cyclin-dependent kinase (Cdk) 6, and the phosphorylation of retinoblastoma proteins (pRB). Up-regulation of the expression of the Cdk inhibitor, p21, by PTX-2 treatment was also associated with increased binding to Cdk2 and Cdk6.

Materials and methods

Cell culture, PTX-2 treatment and growth study. Synovial fibroblasts were prepared as previously described (14) and cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, gentamicin and L-glutamine

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in a humidified atmosphere containing 5% CO_2. PTX-2 was prepared as previously described (11) and dissolved in DMSO (vehicle control) as a stock solution at 1 mg/ml concentration. Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to MTT-formazan by the mitochondria. For the morphological study, the synovial fibroblasts were treated with curcumin for 24 h and photographed directly with an inverted microscope (Carl Zeiss, Germany).

Nuclear staining with DAPI. After treating synovial fibroblasts with PTX-2, the cells were harvested, washed in ice-cold phosphate-buffered saline (PBS) and fixed with 3.7% para-formaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with a 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The nuclear morphology of the cells was examined by a fluorescence microscope (Carl Zeiss).

Flow cytometry analysis. The cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol and stored at 4˚C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 ml of cold propidium iodide (PI, Sigma) solution containing 100 μg/ml RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACScalibur, Becton-Dickinson, San Jose, CA) and CellQuest software was used to determine the relative DNA content based on the presence of red fluorescence.

RNA extraction and reverse transcription-PCR. The total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA) and primed with random hexamers to synthesize the complementary DNA using AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. A polymerase chain reaction (PCR) was carried out using a Mastercycler (Eppendorf, Hamburg, Germany) with the primers shown in Table I. The following conditions were used for the PCR reactions: one cycle at 94˚C for 3 min; 35 cycles at 94˚C for 45 sec; 58˚C for 45 sec; and 72˚C for 1 min and one cycle at 72˚C for 10 min. The resulting amplification products were separated electrophoretically on 1% agarose gel and visualized by ethidium bromide (EtBr, Sigma) staining.

Immunoprecipitation, gel electrophoresis and Western blot analysis. The cells were harvested, lysed, and protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), following the procedure described by the manufacturer. For immunoprecipitation, cell extracts were incubated with an immunoprecipitating antibody in extraction buffer for 1 h at 4˚C. The immuno-complex was collected on protein G/A-Sepharose beads (Sigma). Western blot analysis was performed as previously described (15). Briefly, the immunoprecipitate or an equal amount of total protein was subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electroblotting. Blots were probed with the desired antibodies for 1 h, incubated with diluted enzyme-linked secondary antibody and then visualized by the enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp.). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulins were purchased from Amersham.

Table I. Gene-specific primers for RT-PCR.

Name	Primer sequences
Cyclin D1	
Sense	5'-TGGATGCTGGAGGTCTGCGAGGAA-3'
Antisense	5'-GGCTTCGATCTGCTCCTGGCAGGC-3'
Cyclin E	
Sense	5'-AGTTCTCGGCTCGCTCCAGGAAGA-3'
Antisense	5'-TCTTGTGTCGCCATATACCGGTCA-3'
Cdk2	
Sense	5'-GCTTTCTGCCATTCTCATCG-3'
Antisense	5'-GTCCCCAGAGTCCGAAAGAT-3'
Cdk4	
Sense	5'-ACGGGTGTAAGTGCCATCTG-3'
Antisense	5'-TGGTGTCGGTGCCTATGGGA-3'
Cdk6	
Sense	5'-CGAATGCGTGGCGGAGATC-3'
Antisense	5'-CCACTGAGGTTAGAGCCATC-3'
p53	
Sense	5'-GCTCTGACTGTACCACCATCC-3'
Antisense	5'-CTCTCGGAACATCTCGAAGCG-3'
p16	
Sense	5'-CGGAAGGTCCCTCAGACATC-3'
Antisense	5'-TCATGAAGTCGACAGCTTCCG-3'
p21	
Sense	5'-CTCAGAGGAGGCGCCATG-3'
Antisense	5'-GGGCGGATTAGGGCTTCC-3'
p27	
Sense	5'-AAGCACTGCCGGGATATGGA-3'
Antisense	5'-AACCCAGCCTGATTGTCTGAC-3'
GAPDH	
Sense	5'-CGGAGTCAACGGATTTGGTCGTAT-3'
Antisense	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

Statistical analysis. Data are presented as the mean ± SD of three separate experiments. Comparisons between groups were analyzed using the Student's t-test. P-values of <0.05 were considered statistically significant.

Results

Growth inhibition by PTX-2 in synovial fibroblasts. To determine if PTX-2 influenced the cell viability of RA synovial fibroblasts, the cells were cultured in the presence of increasing

concentrations of PTX-2 ranging from 5-60 ng/ml. After 48 h, the cell viability was determined by the MTT assay. PTX-2 concentration-dependently led to a reduced viability of synovial fibroblasts (Fig. 1A). Direct observation by inverted microscopy showed numerous morphological changes in synovial fibroblasts treated with PTX-2 compared to the control cells (Fig. 1B). Concentration-dependent cell shrinkage, cytoplasm condensation, and the formation of cytoplasmic filaments appeared after PTX-2 treatment. Further experiments were carried out to determine if the inhibitory effect of PTX on cell viability was the result of apoptotic cell death. As shown in Fig. 1C, very few nuclei with chromatin condensation were observed in the control cells or in the PTX-2-treated cells, indicating that PTX-2 did not induce apoptotic cell death.

Induction of G1 arrest by PTX-2 in synovial fibroblasts. To further characterize the inhibitory effect of PTX-2 on synovial fibroblasts, the cell cycle patterns of the cells were examined to determine whether PTX-2 treatment of cells resulted in alteration of cell cycle progression. Analysis of the cell cycle distribution of cells after exposure to PTX-2 showed that most cells were in the G1 phase of the cell cycle, with fewer in the S and G2/M phase when compared with the untreated control cells (Fig. 2). However, we failed to detect significant evidence of any apoptotic sub-G1 population in the present experimental conditions. Taken together, these results suggest that the growth inhibitory effect of PTX-2 in synovial fibroblasts was the result of a block during this G1 phase, without apoptosis.

Effects of PTX-2 on the levels of G1 phase cell cycle regulators. Because PTX-2 arrested the synovial fibroblasts in the G1 phase of the cell cycle, we determined the expression levels of the cell cycle regulating factors at the G1 boundary, such as cyclin D1, cyclin E, Cdk2, Cdk4 and Cdk6, by RT-PCR and Western blotting. As shown in Fig. 3, the protein, but not the mRNA levels, of cyclin D1 and Cdk6 were markedly inhibited after exposure to PTX-2; however, the levels of cyclin E, Cdk2 and Cdk6 were not.

PTX-2 induces the association of p21 with Cdk2 and Cdk4. We next examined the possible up-regulation of the tumor suppressor, p53, and Cdk inhibitors in synovial fibroblasts treated with PTX-2. In untreated control cells, the protein and mRNA levels of p53 and p21 were undetectable by Western blotting and RT-PCR analysis (Fig. 4A and B). However, incubation of synovial fibroblasts with PTX-2 resulted in concentration-dependent increases in p53 and p21 at both the transcriptional and translational levels, whereas PTX-2 treatment did not significantly affect the expression levels of other Cdk inhibitors, including p16 and p27. In addition, co-immunoprecipitation analysis indicated that treatment of cells with PTX-2 resulted in a significant increase in the binding of Cdk2 and Cdk4 with p21 (Fig. 4C).

Down-regulation of pRB phosphorylation and inhibition of E2Fs by PTX-2. Since the RB gene product, pRB, is an important checkpoint protein in the G1 phase of the cell cycle, we next determined the kinetics between the phosphorylation of pRB and the transcription factors, E2F-1 and E2F-4. As shown in Fig. 5, total protein levels of E2F-1 and E2F-4 were down-regulated in PTX-2-treated cells, and pRB expression showed a pronounced alteration from the hyperphosphorylated to the hypophosphorylated form after PTX-2 treatment. This effect of PTX-2 was dose-dependent.

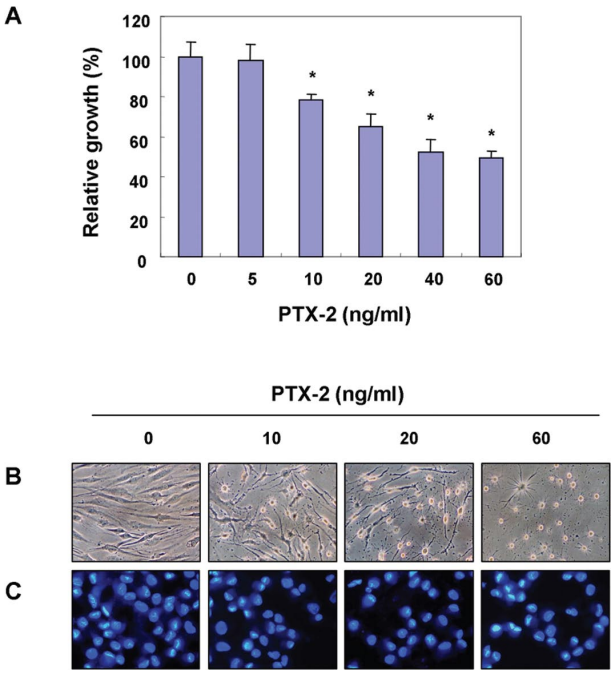
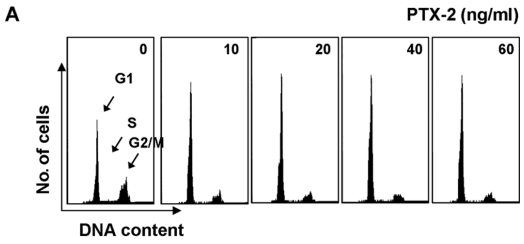


Figure 1. Growth inhibition and morphological changes in response to PTX-2 treatment of synovial fibroblasts. (A) Cells plated at $1x10^5$ cells per 35-mm dish were treated with different concentrations of PTX-2 for 48 h, and the level of growth inhibition was measured using a metabolic-dye-based MTT assay. The data are reported as the mean ± SD of three independent experiments. The significance was determined by a Student's t-test (*P<0.05, compared to control). (B) After incubation with PTX-2 for 48 h, the cells were examined by an inverted microscope. These are representative examples of duplicate tests. Magnification x200. (C) The cells were sampled, fixed and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope using a blue filter. Magnification x400.



B

PTX-2	% of cell		
(ng/ml)	G1	S	G2/M
0	56.21	2.27	41.52
10	87.26	0.65	12.97
20	87.77	0.63	11.70
40	90.33	0.33	9.34
60	91.01	0.56	8.43

Figure 2. Induction of G1 arrest of synovial fibroblasts by PTX-2 treatment. (A) The cells were grown under the same conditions as Fig. 1 and were collected, fixed, and stained with propidium iodide (PI) for flow cytometry analysis. (B) The percentages of cells in the each phase are presented. The data represent the average of two independent experiments.

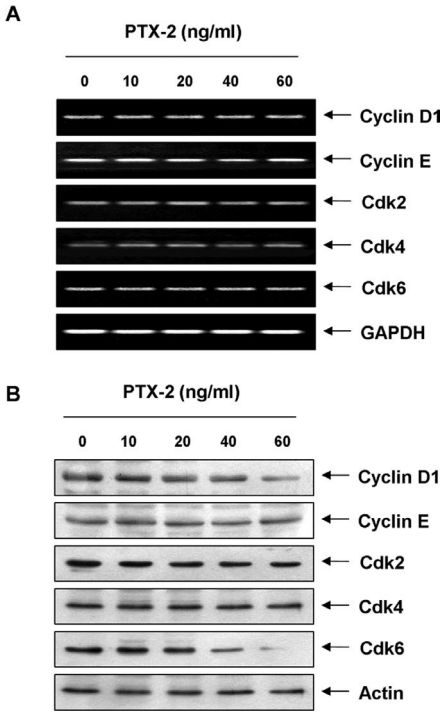


Figure 3. Effects of PTX-2 on the expression of G1 phase-associated cyclins and Cdks in synovial fibroblasts. (A) The cells were treated with different concentrations of PTX-2 for 48 h. Total RNAs were then isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis on a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) Cells grown under the same conditions as (A) were lysed and the cellular proteins were then separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Next, the membranes were probed with the indicated antibodies and the proteins were visualized using an ECL detection system. Actin was used as an internal control.

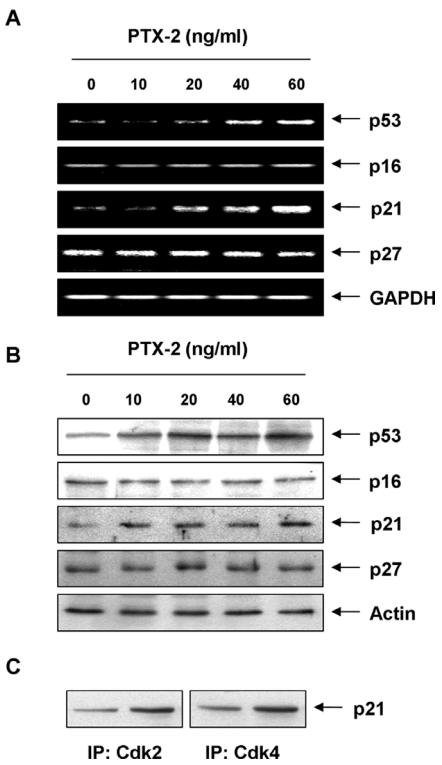


Figure 4. Effects of PTX-2 on the expression of p53 and Cdk inhibitors in synovial fibroblasts. (A) The cells were treated with different concentrations of PTX-2 for 48 h. Total RNAs were then isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis on a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) Cells grown under the same conditions as (A) were lysed and the cellular proteins were then separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Next, the membranes were probed with the indicated antibodies and the proteins were visualized using an ECL detection system. Actin was used as an internal control. (C) Whole cell lysates (0.5 mg of protein) from control cells and cells treated with 60 ng/ml of PTX-2 for 48 h were immunoprecipitated with anti-Cdk2 or anti-Cdk4 antibody. Immuno-complexes were separated on 12% SDS-polyacrylamide gels, and then transferred to nitrocellulose. p21 levels were detected with an anti-p21 antibody and ECL detection.

Discussion

Recent studies have reported that PTX-2, isolated from marine sponges, displays selective and potent cytotoxicity against various types of cancer cells (11). This compound has been reported to display significant cytotoxicity to p53-deficient cancer cell lines (12,13,16). In the present study, we evaluated the ability of PTX-2 to inhibit the growth of synovial fibroblasts obtained from patients with RA. We found that treatment of cells with PTX-2 resulted in a concentration-dependent inhibition of cell viability, without apoptosis induction. The inhibition was associated with a cell-cycle block at the G1 to S phase transition, suggesting that PTX-2 interferes with the proliferation of synovial fibroblasts, possibly through a mechanism that involves arrest at G1, induced by modulation of cell cycle-regulators.

In terms of the regulation of the cell cycle, Cdks play a critical role. The two major mechanisms for Cdk regulation are via binding with cyclin, its catalytic subunit, followed by activation of Cdk/cyclin complexes, and via binding with Cdk inhibitors followed by inactivation of Cdk/cyclin complexes. Progression from the G1 to S phase of the cell cycle is regulated by D-type cyclins and cyclin E, and their cognate kinases, Cdk2, Cdk4 and Cdk6, which act by phosphorylating and inactivating pRB family proteins prior to the restriction point at which cells commit to DNA synthesis. Cyclin E-Cdk2 acts later in the G1 phase (17,18). Therefore, any factor affecting the activity of any of these kinases could abrogate the normal inactivation of pRB and cause cells to arrest in the G1 phase.

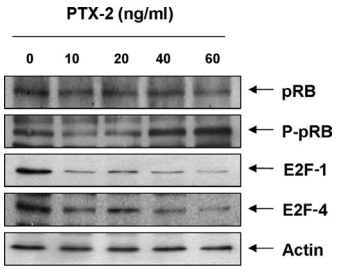


Figure 5. Hypophosphorylation of pRB and reduced protein levels of E2Fs in synovial fibroblasts after exposure to PTX-2. After incubation with PTX-2 for 48 h, the total cell lysates were prepared and separated by electrophoresis on an 8% or 10% SDS-polyacrylamide gel. Western blotting was then performed using anti-pRB, anti-p-pRB, anti-E2F-1 and anti-E2F-4 antibodies. Actin was used as an internal control.

Under normal conditions, pRB binds to the members of the E2F family of transcription factors. However, growth factors induce phosphorylation and dissociation of the pRB from E2F, which triggers G1 cell cycle progression (19). Thus, the complex between cyclin D/E and Cdks is an obvious candidate for control of pRB phosphorylation. If decreased levels of either protein or the association between respective binding partners were to be observed, a concomitant decrease in the degree of pRB phosphorylation would be expected (17,18). Based on these ideas, we investigated the effects of PTX-2 on the expression of G1 phase regulatory factors in synovial fibroblasts. The data generated in the present study demonstrated that PTX-2 treatment did not affect the intracellular mRNA and protein levels of cyclin E, Cdk2 and Cdk4 in the fibroblasts. However, immunoblotting analysis demonstrated that PTX-2 markedly inhibited the levels of cyclin D1 and Cdk6 proteins (Fig. 3), and the PTX-2 selectively induced the expression of the Cdk inhibitor, p21, along with induction of the tumor suppressor, p53, at both mRNA and protein levels (Fig. 4A and B).

In general, p21 is well known to inhibit the activity of Cdks by direct association with various cyclin/Cdk complexes, so that the complex formation of cyclins/Cdks/p21 is increased in cells arrested by DNA damaging agents (20-22). Therefore, we further investigated whether PTX-2 might increase the binding activity between Cdks and p21 proteins. As shown in Fig. 4C, association of p21 with Cdks was almost undetectable by co-immunoprecipitation analysis of the untreated control cells. However, treatment of synovial fibroblasts with PTX-2 resulted in a significant increase in the binding of Cdk2 and Cdk2 with p21. The Cdk inhibitor, p21, has been reported as being potently transactivated by p53, which has further been shown to mediate the induction of cell cycle arrest at the G1 phase by inhibiting Cdk4/Cdk2 activities (20,21). Thus the present results clearly demonstrated that the induction of p21 by PTX-2 might be dependent on p53. Furthermore, PTX-2 also blocks pRB phosphorylation and decreases the levels of E2F-1 and E2F-4 expression (Fig. 5). These results demonstrate that PTX-2 may be inducing G1 phase arrest of synovial fibroblasts through the down-regulation of Cdks kinase activity, via the selective induction of p21 expression and inhibition of pRB phosphorylation.

Although an elucidation of a detailed molecular mechanism for induction of cell cycle arrest by PTX-2 is beyond the scope of this study, the present study demonstrates that i) reduced survival of synovial fibroblasts after exposure to PTX-2 is associated with G1 phase cell cycle arrest, ii) PTX-2 can inhibit cell cycle progression at the G1 phase by decreasing cyclin D1 and Cdk6 expression and pRB phosphorylation, and iii) treatment with PTX-2 results in an increase in a p53-dependent p21 expression that leads to its increased binding with Cdk2 and Cdk6. These novel phenomena have not been previously described for PTX-2, suggesting that PTX-2 and related compounds may have significant potential as targets for RA treatment.

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