JS-K enhances chemosensitivity of prostate cancer cells to Taxol via reactive oxygen species activation

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Abstract. The aim of the present study was to investigate the influence of the nitric oxide donor prodrug JS-K (C13H20N2O4) on Taxol-induced apoptosis in prostate cancer cells, and to investigate a potential reactive oxygen species (ROS)-associated mechanism. The effect of JS-K on the anticancer activity of Taxol was assessed in prostate cancer cells; cell viability, colony formation, apoptosis, ROS generation and expression levels of apoptosis-associated proteins were investigated. The function of ROS accumulation in the combined effects of JS-K and Taxol was determined using the antioxidant N-acetylcysteine (NAC) and the pro-oxidant oxidized glutathione (GSSG). The results of the present study demonstrated that JS-K was able to increase Taxol-induced suppression of prostate cancer cell proliferation, apoptosis, ROS accumulation and upregulation of apoptosis-associated proteins. Furthermore, NAC reversed the effect of JS-K on Taxol-induced apoptosis and conversely, the pro-oxidant GSSG exacerbated the effect of JS-K on Taxol-induced apoptosis in prostate cancer cells. In conclusion, JS-K enhances the chemosensitivity of prostate cancer cells to Taxol, via the upregulation of intracellular ROS.

Introduction

Taxol, which is a type of microtubule-stabilizing antitumor drug, has a broad antineoplastic spectrum and induces cell apoptosis in various types of human cancer (1). Taxol exerts its anticancer effects via several mechanisms: Inhibition of microtubule assembly and protein isoprenylation, cell cycle arrest at the G2/M phase, and stimulation of cell apoptosis and DNA fragmentation (2,3). Taxol has been demonstrated to be efficacious for castration-resistant prostate cancer in previous studies (4,5). However, the majority of patients with prostate cancer gradually acquired drug resistance to Taxol following a series of repeated treatments, which may seriously affect their prognosis (1,6). Thus, there is an increasing requirement to develop novel agents which could enhance chemosensitivity to Taxol in patients with prostate cancer.

As an important signaling molecule, antioxidant and toxicant, nitric oxide (NO) is involved in multiple pathological and physiological processes. JS-K (C13H20N2O4; Chemical Abstracts Service no. 205432-12-8), a glutathione transferase-activated nitric oxide-donor prodrug, is reported to promote high intracellular levels of NO and result in cytotoxicity to human prostate cancer cells (7-10). Our previous study demonstrated that JS-K was able to increase the anticancer effects of doxorubicin, which is a type of anthracycline and potent antitumor drug, in human renal carcinoma cells (11). In the present study, the effect of JS-K on the chemosensitivity of human prostate cancer cells to Taxol was investigated. The results of the present study also demonstrated the function of the accumulation of reactive oxygen species (ROS) in apoptosis in prostate cancer cells induced by the combination of JS-K and Taxol. These results revealed the essential functions and mechanisms of JS-K on Taxol-induced apoptosis in prostate cancer cells.

Materials and methods

Drugs and reagents. Taxol and the nitric oxide prodrug JS-K were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and dissolved in 100% dimethylsulfoxide (DMSO) as stock solutions (Taxol, 10 mM; JS-K, 5 mM). The final concentration of DMSO did not exceed 0.1% in any experiment. N-acetylcysteine (NAC) and oxidized glutathione (GSSG) were obtained from Beyotime Institute of Biotechnology (Haimen, China) and dissolved in PBS to concentrations of 100 mM (NAC) and 5 mM (GSSG). Antibodies against BRI1-associated receptor kinase 1 (Bak, cat no. 3814), Bcl-2-associated X protein (Bax, cat no. 2772), B-cell lymphoma (Bcl-2, cat no. 2876), cleaved caspase-3 (cat no. 9661), caspase-7 (cat no. 9494) and caspase-9 (cat no. 9502) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and an antibody against GAPDH...

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was purchased from Abcam (Cambridge, MA, USA) to be used as a loading control. Horseradish peroxidase (HRP) goat anti-rabbit and anti-mouse IgG secondary antibodies were purchased from EarthOx Life Sciences (Millbrae, CA, USA).

**Cell lines and cell culture.** Human prostate cancer 22RV1 and PC-3 cell lines were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). 22RV1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and PC-3 cells were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂. All culture media were supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin.

**Cell viability assay.** The viability of prostate cancer cells was detected using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. Briefly, 22RV1 and PC-3 cells were seeded in 96-well plates (NEST Biotechnology Co., Ltd, Jiangsu, China) at a density of 5x10⁴ cells/well and then cultured for 24 h. Cells were pretreated with or without JS-K (1 µM) for 24 h and then exposed to Taxol (2 µM) for another 24 h, the culture medium was removed and replaced with 100 µl medium containing CCK-8 reagent and then incubated at 37°C for 2 h. Absorbance was recorded at a wavelength of 450 nm in a 96-well plate reader (EnSpire 2300 Multilabel Reader; PerkinElmer, Inc., Waltham, MA, USA). Half-maximal inhibitory concentrations (IC₅₀) were calculated by probit method using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA).

**Colony formation assay.** Cells were pretreated with or without JS-K (1 µM) for 6 h and then exposed to Taxol (2 µM) for another 6 h. Viable cells were harvested and seeded into 6-well plates (NEST Biotechnology Co., Ltd.) at a density of 1.5x10⁴ cells/well. Cells were cultured for 2 weeks for assessment of colony formation. Colony formation was analyzed by staining the cells with a Crystal Violet Staining Solution (Beyotime Institute of Biotechnology). Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were stained with crystal violet staining solution (10 mg/ml) for 30 min at room temperature and then washed with ultrapure water. The macroscopic clones (>1 mm) were photographed with digital camera.

**Apoptosis assay.** Apoptotic cells were quantified using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Briefly, following pretreatment with or without JS-K (1 µM) for 24 h and then exposure to Taxol (2 µM) for another 24 h, the prostate cancer cells were collected, washed with PBS and resuspended in binding buffer. Cells were incubated with annexin V-FITC and propidium iodide for 15 min at room temperature in the dark, prior to flow cytometric analysis. The stained cells were analyzed by flow cytometry within 1 h.

**Measurement of intracellular ROS.** To determine the accumulation of intracellular ROS in prostate cancer cells, a ROS assay kit (Beyotime Institute of Biotechnology) was used. Briefly, following pretreatment with or without JS-K (1 µM) for 24 h and then exposure to Taxol (2 µM) for another additional 24 h, cells were collected and resuspended with serum-free medium containing dihydrofluorescein diacetate (10 µM). Following incubation at 37°C for 20 min in the dark, the cells were harvested and analyzed using flow cytometry with excitation at 488 nm and emission at 525 nm.

**Measurement of mitochondrial membrane potential.** 22RV1 and PC-3 cells were seeded into 6-well plates at a density of 3x10⁵ cells/well and cultured for 24 h, then were pretreated with or without JS-K (1 µM) for 6 h and then exposed to Taxol (2 µM) for an additional 6 h. The JC-1 Mitochondrial Membrane Potential assay kit (Beyotime Institute of Biotechnology) was used according to the manufacturer's protocol. The cells were analyzed using flow cytometry.

**Glutathione assay.** Cells were plated in 6-well plates and pretreated with or without JS-K (1 µM) for 6 h and then exposed to Taxol (2 µM) for an additional 6 h. The cells were then harvested and lysed by two successive rounds of freezing and thawing. The supernatant was collected by centrifuging at 10,000 x g at 4°C for 10 min and analyzed for reduced glutathione (GSH) and GSSG levels using a GSH and GSSG assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol.

**Measurement of adenosine triphosphate (ATP) levels.** Intracellular ATP levels were measured using an ATP assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, prostate cancer cells were pretreated with or without JS-K (1 µM) for 6 h and then exposed to Taxol (2 µM) for another 6 h and incubated in 200 µl lysis buffer at 4°C for 5 min with gentle shaking. The supernatant of lysis buffer was then collected by centrifugation at 12,000 x g at 4°C for 10 min. Firefly luciferase activity was detected using a Sirius L luminometer (TiterTek-Berthold, Pforzheim, Germany) Preferably.

**Caspase-Glo 3/7 and Caspase-Glo 9 assays.** To examine cell apoptosis following treatment with JS-K and Taxol, caspase-3/7 and caspase-9 activities were analyzed using Caspase-Glo 3/7 and Caspase-Glo 9 assays (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates (NEST Biotechnology Co., Ltd.) and pretreated with or without JS-K (1 µM) for 24 h and then exposed to Taxol (2 µM) for another 24 h and incubated in 200 µl lysis buffer at 4°C for 5 min with gentle shaking. The supernatant of lysis buffer was then collected by centrifugation at 12,000 x g at 4°C for 10 min. Firefly luciferase activity was detected using a Sirius L luminometer (TiterTek-Berthold, Bad Wildbad, Germany).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from 22RV1 and PC-3 cells using an E.Z.N.A. Total RNA Kit I (Omega Bio-tek, Inc. Norcross, GA) and the mRNAs were reversely transcribed into...
stable cDNA with a PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Inc., Dalian, China). RT-qPCR analysis was performed by using the SYBR Premix Ex Taq II kit (TaKaRa). The thermocycling conditions included: 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 5 sec and 60°C for 30 sec. The levels of mRNAs were expressed as \(2^{-\Delta\Delta CT}\) (12). The sequences of forward and reverse primers for each gene are listed in Table I.

Western blot analysis. Cells were lysed with radiolabeled sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (Beyotime Institute of Biotechnology) supplemented with 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) and proteins were extracted by centrifugation at 10,000 x g at 4°C for 15 min. Total protein was determined by a BCA Protein assay kit (Beyotime Institute of Biotechnology). Proteins (40 μg/lane) were loaded onto SDS-PAGE gels (12%) then transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany), which were blocked with 5% non-fat milk in Tris-buffered saline and 1% Tween 20 (TBS-T) and incubated with the aforementioned primary antibodies (1:1,000) in diluent (5% BSA in TBS-T) overnight at 4°C. Following six washes with TBS-T for 5 min each, the membranes were probed with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (#E030120, #E030110; EarthOx Life Sciences, Millbrae, CA, USA; dilution 1:10,000 in TBS-T containing 5% BSA) for 2 h at room temperature.

Statistical analysis. All experiments were performed in triplicate. The results are presented as the mean ± standard deviation. All data were analyzed using one-way analysis of variance using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). Differences between treatments were assessed using a Fisher’s least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

JS-K promotes Taxol-induced suppression of proliferation and apoptosis of prostate cells. Prostate cancer cells were exposed to Taxol, JS-K or their combination for 24 h. A CCK-8 assay (Fig. 1A) and a colony formation assay (Fig. 1B and C) were performed to evaluate the effects of Taxol and JS-K on prostate cancer cells, and the data indicated that the number of viable cells was significantly decreased by treatment with Taxol and JS-K together compared with Taxol alone. The half-maximal inhibitory concentrations of Taxol were significantly decreased when cells were pretreated with JS-K compared with Taxol alone.

JS-K increases Taxol-induced ROS production and decreases the Taxol-induced decrease in the GSH/GSSG ratio in prostate cancer cells. The effects of JS-K on Taxol-induced ROS production were assessed; it was revealed that ROS production was significantly increased in prostate cancer cells that were treated with Taxol and JS-K together (Fig. 2A). The levels of GSH and GSSG were investigated; it was revealed that the GSH/GSSG ratio was significantly decreased in cells treated with Taxol and JS-K together compared with Taxol alone (Fig. 2B).

Table I. Sequences for target gene primers.

| Gene      | Primer sequence (5'-3') | \(T_m\), °C |
|-----------|-------------------------|-----------|
| GAPDH     | TGCACCACCAACTGTTAG (F)  | 60.07     |
|           | AGTGAAGGCGGGATGTGTC (R) | 59.72     |
| Bak       | CCAACCCATTCACTACAGG (F) | 59.80     |
|           | CCACTTAGAACCCCTCCAGA (R) | 59.80     |
| Bcl-2     | CTATTGAGTCGGTGGTGTC (F) | 59.80     |
|           | GGCCGTACAGTTCCACAAA (R) | 59.80     |
| Bax       | AAGCTAGCCAGTGTCTCAAG (F) | 60.00     |
|           | CAAAGTGAAGGCGACAAAC (R) | 58.20     |

\(T_m\), melting temperature; Bak, BR1-associated receptor kinase 1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; F, forward; R, reverse.

JS-K exacerbates the Taxol-induced decrease in mitochondrial membrane potential and ATP levels in prostate cancer cells. The Taxol and JS-K-induced increase in total ROS and decrease in GSH/GSSG ratio (Fig. 2A and B) may contribute to increased mitochondrial dysfunction and lead to mitochondria-mediated apoptosis. To investigate the dysfunction in mitochondrion, the mitochondrial membrane potential and intracellular levels of ATP in prostate cancer cells treated with JS-K and Taxol was determined. It was revealed that the mitochondrial membrane potential (Fig. 2C) and intracellular levels of ATP (Fig. 2D) was significantly decreased in cells treated with Taxol and JS-K together compared with Taxol alone.

JS-K and Taxol synergistically regulates the apoptosis-associated signaling pathway. In the present study, prostate cancer cells were treated with Taxol, JS-K or their combination for 24 h. mRNA (Fig. 3A and B) and protein (Fig. 3C and Table II) levels of Bax and Bak were upregulated in response to the combination of Taxol and JS-K, whereas Bcl-2 levels were downregulated. The levels and activities of caspase-3/7/9 were investigated; increased levels of cleaved caspase-3, cleaved caspase-7 and cleaved caspase-9 (Fig. 3C) and increased activity of caspase-3/7 (Fig. 3D) and caspase-9 (Fig. 3E) in cells treated with Taxol and JS-K together compared with the cells treated with Taxol alone was revealed.

NAC reverses JS-K-induced anticancer activity of Taxol and GSSG exacerbates the effects of JS-K. To investigate the effects of ROS on Taxol and JS-K-induced prostate cancer cell proliferation suppression and apoptosis, prostate cancer cells were treated with Taxol and JS-K in the presence or
Figure 1. JS-K promotes Taxol-induced suppression of prostate cancer cell proliferation and apoptosis. (A) Cell viability following treatment with JS-K and Taxol was determined using a Cell Counting Kit-8 assay. (B) Representative images and (C) cumulative data from colony formation assay following treatment with JS-K and Taxol in 22RV1 and PC-3 cells. (D) Effects of JS-K on IC_{50} of Taxol. (E) Apoptosis induced by JS-K and Taxol was examined using FITC-annexin V/PI staining; representative dot plots (left panel) and cumulative data (right panel). The results are presented as the mean ± SD for three independent experiments. *P<0.05, **P<0.01. IC_{50}, half-maximal inhibitory concentration; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Figure 2. Synergistic effects of JS-K and Taxol on ROS production, GSH/GSSG ratio, mitochondrial membrane potential and ATP levels in prostate cancer cells. Prostate cancer cells were treated with JS-K and Taxol for 6 h and (A) ROS generation, (B) GSH/GSSG ratio, (C) mitochondrial membrane potential and (D) ATP levels were determined. The data are presented as the mean ± standard deviation for three independent experiments. *P<0.05, **P<0.01. GSH, reduced glutathione; GSSG, oxidized glutathione; ATP, adenosine triphosphate; ROS, reactive oxygen species; ΔΨ(m), mitochondrial membrane potential.
Table II. Expression levels of related proteins following treatment with JS-K and Taxol.

| Treatment     | Expression levels of 22RV1 | Expression levels of PC-3 |
|---------------|---------------------------|----------------------------|
|               | Control JS-K Taxol JS-K+Taxol | Control JS-K Taxol JS-K+Taxol |
| Bak           | 1.00 1.06 1.68 3.74 | 1.00 1.23 2.04 3.12 |
| Bax           | 1.00 1.01 1.26 1.82 | 1.00 1.11 4.56 6.44 |
| Bcl-2         | 1.00 0.85 0.62 0.62 | 1.00 0.81 0.65 0.79 |
| Cl-caspase-3  | 1.00 1.10 1.72 2.46 | 1.00 1.37 2.21 3.42 |
| Caspase-7 (35 kDa) | 1.00 1.68 157 1.81 | 1.00 0.92 1.02 1.21 |
| Caspase-7 (30 kDa) | - - - - | 1.00 1.32 2.26 2.06 |
| Caspase-7 (20 kDa) | 1.00 2.56 2.76 2.76 | 1.00 4.35 13.04 21.74 |
| Caspase-9 (47 kDa) | 1.00 0.89 0.70 0.84 | 1.00 1.40 1.57 1.83 |
| Caspase-9 (37 kDa) | 1.00 0.89 1.00 1.53 | 1.00 2.04 2.54 2.71 |

Figure 3. Effects of JS-K and Taxol on the apoptosis-associated signaling pathway. mRNA levels of Bcl-2, Bax and Bak in (A) 22RV1 and (B) PC-3. (C) Expression levels of apoptosis-associated proteins. (D) Activity of caspase-3/7 and (E) activity of caspase-9. The data are presented as the mean ± standard deviation for three independent experiments. *P<0.05, **P<0.01. Bak, BRI1-associated receptor kinase 1; Bax, Bcl-2-associated X protein; Bcl2, B-cell lymphoma 2; ROS, reactive oxygen species; ATP, adenosine triphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione.
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absence of the antioxidant NAC (100 µM) or pro-oxidant GSSG (5 µM) for 24 h. Cells treated with JS-K and Taxol together was demonstrated to decrease the number of viable cells (Fig. 4A) and increase ROS levels (Fig. 4B), caspase-3/7 activity (Fig. 4C) and apoptosis (Fig. 4D and E) in prostate cancer cells; whereas NAC (100 µM) reversed the JS-K-induced increase in chemosensitivity of cells to Taxol (Fig. 4A-D). Furthermore, GSSG (5 µM) exacerbated the JS-K-induced increase in chemosensitivity of cells to Taxol (Fig. 4A-D). These results indicated the functions of ROS in JS-K-sensitized Taxol exerting anticancer activity in human prostate cancer cells.

Discussion

Taxol is recognized as an effective anticancer drug for treating castration-resistant prostate cancer and has been clinically challenged by its acquisition of drug resistance following repeated treatments (1,4,6). In the present study, it was indicated that JS-K increased Taxol-induced proliferation suppression and apoptosis in prostate cancer cells. Taxol was identified to increase ROS generation in cancer cells (13). Our
previous study demonstrated that JS-K promotes apoptosis in prostate cancer cells via induction of intracellular ROS accumulation (10), and the results of the present study were consistent with these results.

Apoptosis is a major process of programmed cell death, which involves two distinct apoptosis pathways including mitochondrial and death receptor pathways (14,15). Abnormal ROS generation is a critical event during mitochondria-associated apoptosis. As vital signaling molecules, ROS regulate multiple intracellular signal transduction pathways that are involved in various cellular processes including cell proliferation, cell cycle progression, invasion, migration and apoptosis in cancer (16,17). Our previous studies have demonstrated that JS-K, an important glutathione transferase-activated NO donor prodrug, was able to stimulate intercellular accumulation of ROS which resulted in cytotoxicity and apoptosis in cancer cells derived from human urogenital tumors (10,18). In the present study, it was demonstrated that JS-K markedly increased intracellular ROS generation in Taxol-treated prostate cancer cells. It was also revealed that administration of the prooxidant GSSG exacerbated the inducing effect of JS-K on Taxol-induced apoptosis. However, the inducing effect of JS-K was inhibited in the presence of the antioxidant NAC. These data suggested that upregulated ROS generation may be a mechanism by which JS-K increases the anticancer activity of Taxol in prostate cancer cells.

GSH, as well as its oxidized form GSSG, are crucial elements involved in mitochondrial dysfunction, redox balance and apoptosis (19,20). The physiological balance of GSH and GSSG is used to indicate the general cellular oxidation/reduction microenvironments and the redox balance (21). The results of the present study have demonstrated that JS-K significantly decreased the GSH/GSSG ratio, which resulted in a redox imbalance. Apoptosis induced by mitochondrial dysfunction is a fundamental apoptotic pathway accompanied by a series of events including decreases in mitochondrial membrane potential and ATP production, and activation of caspase-9 (15,22,23). As an energy source in cellular metabolism, ATP production would be influenced by a decline in mitochondrial membrane potential during cellular apoptosis (24). The results of the present study demonstrated that JS-K significantly promoted Taxol-induced mitochondria-associated apoptosis, and exacerbated the decrease in mitochondrial membrane potential and ATP levels, and exacerbated the activation of caspase-9. In addition, Bcl-2 family members are important regulators of apoptosis, and dysregulation of Bcl-2 family members occurs in a number of diseases and stress-induced apoptosis (25,26). As members of the Bcl-2 family, Bak, Bax and Bcl-2 serves crucial functions in mitochondrial stress-induced cellular apoptosis, and the appropriate balance of Bcl-2 and Bax is vital for cellular survival (27). In the present study, JS-K significantly exacerbated the Taxol-induced increase in expression levels of Bax and Bak proteins, and the decrease in expression of Bcl-2 protein. These results suggested that JS-K promoted Taxol-induced apoptosis through a mitochondria-mediated pathway.

In conclusion, the results of the present study indicated that the addition of JS-K increased the anticancer effect of Taxol on prostate cancer cells. It was revealed that the effects of combined treatment with JS-K and Taxol were reversed by an antioxidant and exacerbated by a pro-oxidant. It was hypothesized that ROS activation, induced by the combination of JS-K and Taxol, induced apoptosis in prostate cancer cells (Fig. 5). Further investigation is required to identify the roles and mechanisms underlying the combination of JS-K and Taxol.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MNQ and JDL designed the experiments. MNQ, SZ, LZK, HCT and XZ performed the experiments. MNQ and JDL were involved in data and statistical analyses. MNQ and JDL wrote the article and prepared figures. JDL provided guidance and the financial support. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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