SPANDIDOS PUBLICATIONS

A novel MEK1/2 inhibitor induces G_1/S cell cycle arrest in human fibrosarcoma cells

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Abstract. Blockade of the ERK pathway has antitumor effects against malignant tumor cells. In this study, we investigated the antitumor activity of JTP-70902, a novel specific MEK inhibitor, against human fibrosarcoma cells in which the ERK pathway is constitutively activated. JTP-70902 was synthesized at Japan Tabacco. Human fibrosarcoma HT1080 cells were cultured. JTP-70902 was added at various concentrations. The number of viable cells was counted employing a trypan blue dye exclusion test. Unsynchronized cells were exposed to JTP-70902 for 24 h. The nuclei were stained with propidium iodide. The DNA content was measured using a FACSCalibur flow cytometer. Protein extraction and Western blot analysis were performed. (1) A dose-dependent inhibition of cell growth was observed at concentrations of 10 nM or more. Forty-eight hours after the treatment, the growth of HT1080 cells was completely inhibited by 200 nM JTP-70902. (2) FACS analysis revealed that a 24-h exposure to JTP-70902 increased the population of G_1/S phase cells in a dose-dependent manner. (3) The phosphorylation of ERK was inhibited by JTP-70902. Furthermore, after the treatment with JTP-70902, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ protein expression increased and the phosphorylation of RB was redued. Our results showed that JTP-70902 inhibits cell growth and induces cell cycle arrest in human Ras mutant fibrosarcoma cells. These results indicate that JTP-70902 might be an attractive compound for molecular-targeting chemotherapy for malignant soft tissue tumors with the activation of the Ras-MEK-ERK pathway.

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Abbreviations: JTP, JTP-70902; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: fibrosarcoma, MEK inhibitor, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, cell cycle arrest

Introduction

Fibrosarcoma is one of the high-grade malignant soft tissue sarcomas that commonly occur in middle-aged and elderly adults. It accounts for approximately 2.6% of soft tissue sarcomas, which, themselves, have an incidence of about 2-4/100000 (1). Although the prognosis of these patients has improved due to the development of surgical treatment and various adjuvant chemotherapies, these therapies are not fully effective and, as a result, their 5-year survival is still 39-54% (2,3). One of the most serious causes of therapeutic failure is the resistance of the tumor cells to chemotherapeutic agents. To overcome this drug resistance, new antitumor agents or chemicals and new antitumor therapeutic approaches need to be developed.

The 41-/43-kDa mitogen-activated protein kinase (MAPK) pathway, also called the extracellular signal-regulated kinase (ERK) pathway, is activated in a variety of malignant tumors. Activation of the ERK pathway involves the activation of Ras at the plasma membrane, and the sequential activation of a series of protein kinases. Ras interacts with and activates Raf-1 and MAP kinase/ERK kinase (MEK)-1 and -2. MEK-1/2 then catalyze the phosphorylation of 41- and 43-kDa MAP kinases (ERK1/2), and these activated ERK can phosphorylate cytoplasmic and nuclear targets. The ERK pathway participates in a wide range of cellular programs including proliferation, differentiation and movement (4,5). Constitutively active mutants of Ras (6) and Raf-1 (7) have been observed in several human tumors. The constitutive activation of MEK and ERK is associated with a large number of tumors. For example, tumor cells derived from tissues of the pancreas, colon, lung, ovary, prostate and kidney showed particularly high frequencies (30-50%) and a high degree of kinase activation (8-10). Thus, specific inhibitors might be developed against these protein kinases for malignant tumors, especially for the treatment of tumors showing constitutive activation of the ERK pathway.

In the present study, we examined the effect of blockade of the ERK pathway on the proliferation of human fibrosarcoma HT1080 cells in which the ERK pathway is constitutively activated. We utilized a novel small-molecule inhibitor of this pathway, JTP-70902, which specifically inhibits MEK activity. Our results demonstrated that JTP induces a marked G_1/S cell cycle arrest through the induction

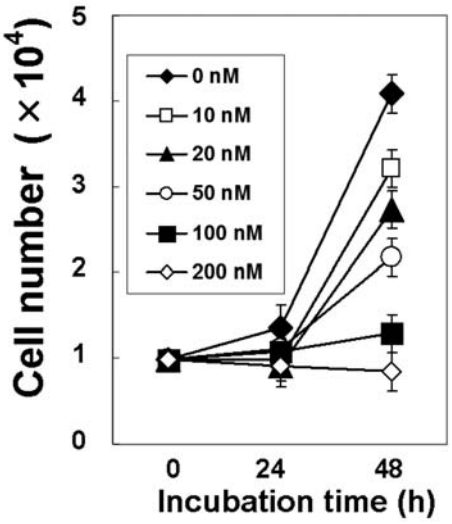


Figure 1. Effect of JTP on the growth of HT1080. Twenty-four hours after the seeding of HT1080 cells, JTP at 10 (□), 20 (▲), 50 (○), 100 (■) or 200 (◇) nM was added, and the cell number was compared with control culture with equivalent DMSO (♦) by counting the cells employing the trypan blue dye exclusion test. The values shown are means (bars, SD) (n=3).

of the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. This result raises the possibility that JTP is a promising candidate for molecular-targeting chemotherapy against fibrosarcoma.

Materials and methods

Reagents. JTP-70902 (N-{3-[5-(4-bromo-2-fluorophenyl-amino)-3-cyclopropyl-8-metyl-2,4,7-trioxo-3,4,7,8-tetrahydro-*2H*-pyrido(2,3-*d*)pyrimidin-1-yl]-phenyl}-methanesulfonamide) was synthesized at Japan Tabacco (Osaka, Japan) (11). JTP was dissolved in DMSO. The maximum volume (%) of DMSO in the assays was 0.1%.

Cell culture. Human fibrosarcoma HT1080 cells were cultured in RPMI-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) containing 10% fetal bovine serum and incubated at 37˚C in a humidified atmosphere of 5% CO_2. The cells were seeded at a density of $1x10^5$ cells in 6-well plates. Twenty-four hours after seeding, the confluency of cells at the time of treatment was 30-40%.

Cell growth study. For the cell growth study, HT1080 cells were seeded at a density of $1x10^4$ cells in 12-well plates. Twenty-four hours after seeding, JTP was added at various concentrations. From 24 to 48 h after treatment, the number of viable cells was counted employing a trypan blue dye exclusion test. The data are presented as the means ± SD of at least three independent experiments.

Analysis of cell cycle progression. Unsynchronized cells were exposed to JTP for 24 h and harvested from culture dishes. After washing with PBS, the cells were suspended in PBS containing 0.1% Triton X-100, treated with RNase A, and the nuclei were stained with propidium iodide (PI). The DNA content was measured using a FACSCalibur flow cytometer with Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ). For all assays, 10,000 events were counted. The ModFit LT V2.0 software package (Verity Software, Topsham, ME) was used to analyze the data.

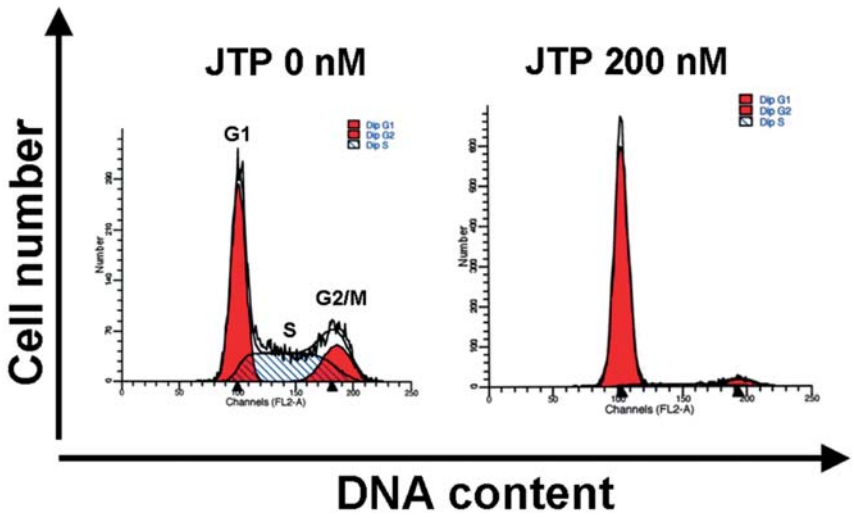
Western blot analysis. Cells were lysed in lysis buffer [50 mmol/l Tris-HCl (pH 7.5), 1% SDS]. The protein extract was then boiled for 5 min and loaded onto a 12% (for $p21^{WAF1/CIP1}$, $p27^{KIP1}$, cyclin D1 and GAPDH), 10% [for phosphor-p42/44 MAPK (p-ERK1/2), p42/44 MAPK (ERK1/2)] or 7% (for RB detection) polyacrylamide gel, subjected to electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were used as the primary antibody: rabbit polyclonal anti-$p21^{WAF1/CIP1}$ antibody (Santa Cruz Biotechnology), anti-$p27^{KIP1}$ antibody (Santa Cruz Biotechnology), mouse monoclonal anti-pRB antibody (Pharmingen), mouse monoclonal anti-phospho-pRB (ser780) antibody (Cell Signaling Technology, Inc.), rabbit polyclonal anti-phospho-p42/44 MAPK antibody (Cell Signaling Technology, Inc.), rabbit polyclonal anti-p42/44 MAPK antibody (Cell Signaling Technology, Inc.) and mouse monoclonal anti-GAPDH anti-body (Immunotech, Marseille, France). Enhanced chemiluminescence (GE Science, Piscataway) was used for detection.

Results

JTP inhibits the growth of human fibrosarcoma HT1080 cells. We investigated the effects of JTP on the growth of human fibrosarcoma HT1080 cells. Fig. 1 shows the growth of HT1080 cells in the presence of various concentrations of JTP. A dose-dependent inhibition of cell growth was observed at concentrations of 10 nM or more. Forty-eight hours after treatment, the growth of HT1080 cells was completely inhibited by 200 nM JTP. The growth of cells was inhibited to 78.6, 66.8, 53.3, 31.6 and 20.7% of the control level by 10, 20, 50, 100 and 200 nM JTP, respectively, 48-h after the addition of JTP.

JTP arrests HT1080 cells at the G_1/S phase in cell cycle progression. To elucidate the effect of JTP on the cell cycle progression of HT1080 cells, the DNA content of nuclei of HT1080 cells was measured by flow cytometric analysis. As shown in Fig. 2A, treatment with 200 nM JTP markedly induced G_1/S phase cell cycle arrest. FACS analysis revealed that 24-h exposure to JTP increased the population of G_1/S phase cells in a dose-dependent manner. Cells at the G_1/S phase increased from 46.5% in medium alone to 54.9, 76.1, 85.9 and 89.6% in the presence of 10, 50, 100 and 200 nM JTP, respectively (Fig. 2B).

JTP up-regulates $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression in HT1080 cells. To investigate whether cell cycle regulatory proteins are involved in JTP-induced cell cycle arrest in HT1080 cells, we examined the expression of cycle regulatory proteins after JTP treatment. As shown in Fig. 3A, we found that the phosphorylation of ERK was inhibited by JTP at a concentration of 10 nM or more in HT1080 cells. Furthermore, JTP increased $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ protein expression in a



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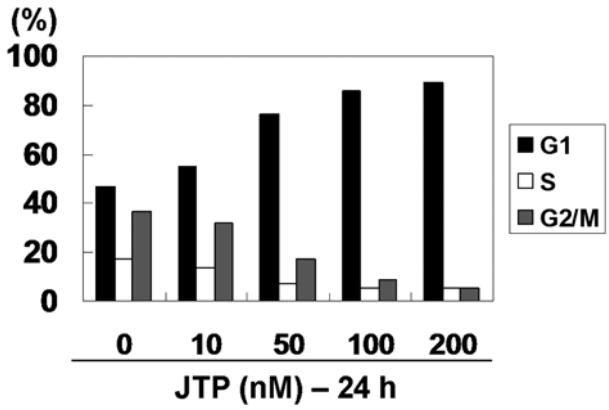

Figure 2. The effect of JTP on cell cycle progression in HT1080 cells. (A) HT1080 cells were treated with JTP at the indicated concentrations for 24 h. The DNA content of propidium iodide-stained nuclei was analyzed by FACSCalibur flow cytometry, as described in Materials and methods. The experiments were repeatedly performed to confirm the results. (B) The percentage of cells in phases G_1, S and G_2/M.

dose-dependent manner (Fig. 3B). The expression of $p15^{INK4B}$ and cyclin D1 was not significantly affected. The proteins $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ are specific inhibitors of cyclin-dependent kinases, and the subsequent dephosphorylation of the RB protein causes G_1/S cell cycle arrest. We therefore examined whether JTP could alter the phosphorylation status of the RB protein in HT1080 cells. A hyperphosphorylated form of the RB protein (ppRB) was converted into a hypo-phosphorylated form (pRB) 24-h after treatment. The phosphorylation of pRB at Ser780 was drastically diminished after 24-h exprosure to JTP (Fig. 3C). These results suggest that JTP induces $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ protein expression, and consequently inhibits the growth of HT1080 cells by arresting the cell cycle at the G_1 phase.

Discussion

Fibrosarcoma is a rare entity within the heterogeneous group of soft tissue sarcomas. Surgical resection is key in primary treatment, and radiation can improve local control, but once the disease has spread, the remaining treatment options are very limited. Response rates for established chemotherapeutic agents such as doxorubicin and ifosfamide (up to 30% at best) are still disappointing (2). To improve the prognosis of fibrosarcoma, new strategies are necessary.

Mitogen-activated protein kinase (MAPK) kinase (MKK) signal transduction pathways are critical for many aspects of normal cell function, including cell cycle progression and differentiation (12). In addition, activated MAPK or elevated MAPK expression has been detected in a variety of human tumors, and they promote tumor growth and metastasis (13). In soft tissue sarcoma, elevated levels of active MAPK have been detected in cell lines derived from fibrosarcoma (14-16), rhabdomyosarcoma (17,18), and Kaposi's sarcoma (19,20). In addition, Ding *et al* reported that MKK signaling is essential for the growth and vascularization of fibrosarcoma (21). These observations suggest the development of drugs that target the MKK pathways as potential soft tissue tumor therapeutics (22,23).

In tumor cells, the loss of G_1/S transition control often arises from the aberrant activation of cell-proliferative signaling pathways or inactivation of cell cycle-regulating proteins. The Ras-MAPK pathway has been investigated extensively. The MAPK cascade, comprising c-Raf or B-Raf, MEK1/2 and ERK1/2, is constitutively activated in various malignant tumors, often through gain-of-function mutations of Ras and Raf family members (24). Activation of the ERK pathway is essential for cells to pass the G_1 restriction point (25). The MEK-ERK pathway was regarded to promote cellular proliferation and the down-regulation of cyclin-dependent kinase inhibitors, such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ are members of the cyclin-dependent kinase inhibitors, and induce G_1/S- and G_2/M-phase cell cycle arrest (26-31). In addition, $p21^{WAF1/CIP1}$ induces the differentiation of both normal and transformed cells and suppresses the growth of malignant cells *in vitro* and *in vivo* (32,33). In a recent study, Abukhdeir and Park reported that the functional loss of $p21^{WAF1/CIP1}$ or $p27^{KIP1}$ can mediate a drug-resistant phenotype in malignant tumor cells (34). Therefore, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ are attractive molecular targets to suppress cell growth in soft

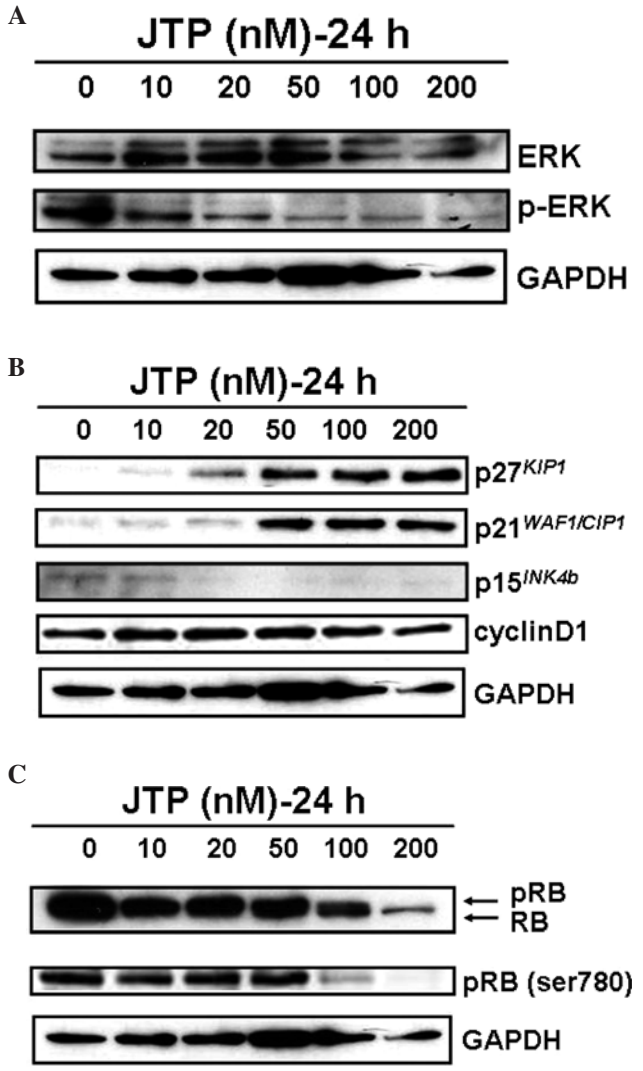

Figure 3. Up-regulation of $p21^{WAF1/CIP1WAF1}$ and $p27^{KIP1}$ expression by JTP in HT1080 cells. (A) The phosphorylation of ERK was inhibited by JTP at a concentration of ≥10 nM. (B) JTP up-regulated $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ protein expression in a dose-dependent manner. (C) The phospho-rylation of RB was inhibited after the treatment with JTP. HT1080 cells were treated with JTP at the indicated concentrations for 24 h. Western blotting was then performed, as described in Materials and methods. GAPDH was used to ensure equal gel loading.

tissue sarcoma cells, and $p21^{WAF1/CIP1}$- and $p27^{KIP1}$-inducing agents might be effective for the chemotherapy of poor-prognostic fibrosarcoma.

In the present study, we examined the effect of a specific blockade of the ERK pathway on the growth of human fibrosarcoma HT1080 cells *in vitro*, using the specific inhibitor JTP. JTP binds to MEK1/2 and inhibits its kinase activity, and the inhibitory activity was much stronger than that of the known MEK inhibitor U0126 (11).

JTP efficiently suppressed ERK activation in HT1080 cells, and suppressed the growth of HT1080 cells. JTP induced striking G_1/S cell cycle arrest. JTP exhibits $p21^{WAF1/CIP1}$- and $p27^{KIP1}$-inducing activity, as previously reported for other known MEK inhibitors (35,36). JTP induces $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ through the inhibition of ERK phosphorylation, resulting in hypophosphorylation of the RB protein. A large number of cancer studies have shown that the RB pathway is the most frequently inactivated in human malignant tumors (37,38). If the RB pathway is inactivated, the activation of CKI will lead to the functional restoration of this pathway. Therefore, anticancer agents activating RB function, such as JTP, may contribute to new strategies for the therapy of malignant soft tissue tumors.

It was reported that JTP down-regulated c-Myc, which is one of the target proteins of the MEK-ERK pathway. Phosphorylation by ERK1/2 enhances the stability of c-Myc protein (39), and the expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ is inhibited by c-Myc (40-42). These findings raise the possibility that ERK1/2 inactivation causes the reduction of c-Myc protein and results in the induction of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in HT1080 cells.

Furthermore, conventional anticancer agents which are cytotoxic in normal cells, cause side effects and reduce the quality of life of the patient. It is necessary to develop anticancer agents exhibiting tumor-selective cytotoxicity. In a chemotherapeutic study, JTP drastically inhibited the growth of xenografts of human colon cancer by oral administration, and no marked signs of toxicity were observed following JPT administration (11). Therefore, JTP might be an attractive compound for molecular-targeting chemotherapy or chemoprevention due to showing little or no toxicity against normal cells.

In conclusion, we demonstrated that JTP induced the selective up-regulation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, resulting in G_1/S phase arrest of the cell cycle progression in HT1080 cells. These results suggest that specific blockade of the ERK pathway induces the marked up-regulation of $p21^{WAF1/CIP11}$ and $p27^{KIP1}$ in tumor cells with a constitutively high level of ERK activation. Although further studies of the chemotherapeutic effect *in vivo* are needed, these results raise the possibility that MEK inhibitors such as JTP might be effective chemotherapeutic agents for the treatment of not only fibrosarcoma but also a broad spectrum of sarcomas, including malignant fibrous histiocytoma, liposarcoma, melanoma and Kaposi's sarcoma.

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