

FTY720 and cisplatin synergistically induce the death of cisplatin-resistant melanoma cells through the downregulation of the PI3K pathway and the decrease in epidermal growth factor receptor expression

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Abstract. Sphingosine kinase (SK), a key enzyme in sphingosine-1-phosphate (S1P) synthesis, is known to be overexpressed in various types of cancer cells. The effects of anticancer agents on SK1/S1P signaling have not yet been fully assessed in melanoma cells. In the present study, we investigated the effects of the combination of FTY720, an S1P receptor antagonist, and cisplatin, a DNA-damaging agent, on the induction of the death of human melanoma cells, as well as the molecular mechanisms involved. The viability of various human melanoma cell lines was examined following treatment with anticancer drugs. The cisplatin-resistant SK-Mel-28 and cisplatin-sensitive A375 cell lines were selected for this analysis. Protein expression and apoptotic rates were evaluated by western blot analysis following treatment with cisplatin and/or FTY720. Following treatment with a combination of FTY720 and cisplatin, cell viability significantly decreased and the expression of apoptosis-associated cleaved poly(ADP-ribose) polymerase (PARP) was significantly higher in comparison to treatment with cisplatin alone in the SK-Mel-28 cells. In addition, the combination of FTY720 and cisplatin reduced the protein expression of SK1 and the phosphorylation levels of phosphoinositide 3-kinase (PI3K), Akt and mTOR in the SK-Mel-28 cells; the expression of epidermal growth factor receptor (EGFR) was also markedly reduced. These findings suggest that FTY720 and cisplatin synergistically induce cell death through the downregulation of the PI3K/Akt/mTOR pathway and the decrease in EGFR expression in SK-Mel-28 cells. Thus, the combination of FTY720 and cisplatin may have therapeutic potential for chemotherapy-resistant melanoma,

and the effects are likely exerted through the downregulation of S1P signaling.

Introduction

Sphingolipids are the main components of lipid rafts and have critical functions as signaling molecules. Sphingosine 1-phosphate (S1P) and ceramide regulate proliferation and apoptosis (1-4). In response to various stimuli, ceramide mediates cell death and apoptosis, whereas S1P abrogates apoptosis and mediates cell proliferation and migration (5). Sphingosine kinase 1 (SK1) is the key enzyme responsible for converting sphingosine to S1P. The signaling pathway via the S1P receptor contributes to cancer cell survival and proliferation (6), apoptosis reduction (7) and oncogenic transformation (8). Various types of cancer cells show high levels of SK1 expression/activity, increasing their resistance to anticancer agents, such as anthracyclines, doxorubicin and camptothecin (9,10). Thus, SK1 may play an important role in the development and proliferation of cancers, such as melanoma (11-13). FTY720 (fingolimod) is phosphorylated by SK1 and SK2 (14), and acts as an S1P receptor antagonist (14,15). FTY720 induces apoptosis in prostate (15), liver (7), bladder cancer (12) and melanoma (16,17) by the direct inhibition of SK1 (18). In the present study, we investigated the combined effects of FTY720 and cisplatin, a DNA damaging agent, on cell death, SK1 expression, S1P signaling and epidermal growth factor receptor (EGFR) expression in anticancer drug-resistant melanoma cells.

Materials and methods

Chemical reagents. The S1P receptor antagonist, FTY720, was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Cisplatin, carboplatin, paclitaxel, dacarbazine, bovine serum albumin, Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimal essential medium (EMEM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). RPMI-1640, non-essential amino acids (NEAA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies against

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Key words: cisplatin, epidermal growth factor receptor, FTY720, melanoma, sphingosine kinase 1, sphingosine 1-phosphate

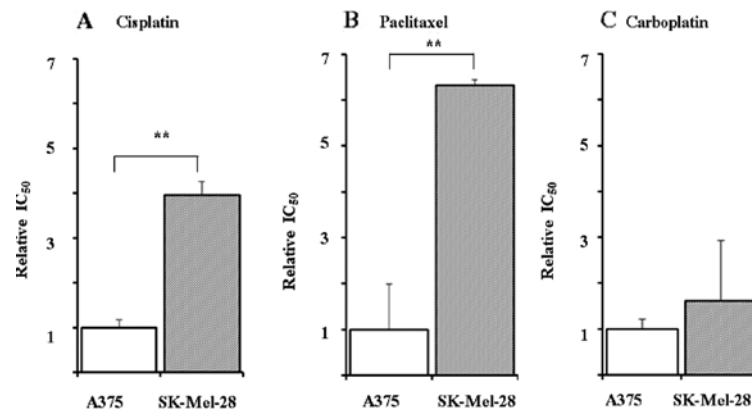


Figure 1. In the SK-Mel-28 cells and A375 cells, the 50% inhibitory concentrations (IC₅₀) for various anticancer drugs were examined by MTT assay. These cell lines (5x10³ cells/well) were cultured in 96-well plates for 24 h, and were then treated with various concentrations of (A) cisplatin, (B) paclitaxel, or (C) carboplatin for 48 h. Each condition was measured in triplicate, and the ratio of the IC₅₀ value in the SK-Mel-28 cells to that of the A375 was calculated. Data are expressed as the means ± SD of 3 independent experiments. **P<0.01.

SK1, p53, cleaved poly(ADP-ribose) polymerase (PARP), phosphoinositide 3-kinase (PI3K), phosphorylated (p)-PI3K, Akt, p-Akt, mammalian target of rapamycin (mTOR), p-mTOR, EGFR and p-EGFR were obtained from Cell Signaling Technology (Beverly, MA, USA) and rabbit polyclonal antibody against SK2 was obtained from Abcam (Cambridge, UK).

Cell lines and measurement of cell viability. The human melanoma SK-Mel-28 cells were obtained from the JCRB Cell Bank (Osaka, Japan), the human melanoma cell lines (A375, A2058 and WM115) and the mouse melanoma B16 cells were purchased from the European Collection of Cell Cultures (ECACC). The SK-Mel-28 and WM115 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) and 0.1% tyrosine. The A375 and B16 cells were cultured in DMEM containing 10% FBS. The A2058 cells were grown in EMEM containing 1% of NEAA and 10% FBS. To assess viability, melanoma cells were plated at 1x10⁴ cells/well and 5x10³ cells/well in 24- and 96-well plates, respectively. The anticancer drugs were dissolved in DMSO. When the determination of IC₅₀ was performed in 96-well plates, cisplatin, paclitaxel, carboplatin and dacarbazine were added at doses of up to 50, 10, 350 and 750 μM, respectively. In 24-well plates, the cells were treated with up to 150 μM of cisplatin. The cells treated with DMSO only were used as controls. Viability was analyzed following 48 h of treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) using a standard assay, and 50% inhibitory concentrations (IC₅₀) were calculated.

Western blot analysis. The cells were lysed by sonication in lysis buffer (1% Nonidet P-40, 0.5% sodium cholate, 1% SDS, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM HEPES, 3 mM MgCl₂, protease inhibitors, 0.5 mM 4-deoxyribose, 20 mM β-glycerophosphate, 1 mM sodium fluoride and 1 mM sodium orthovanadate, pH 7.4). Total cell lysates (20 μg protein) were separated by electrophoresis on 10-7.5% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Danvers, MA, USA). The membranes were blocked with 5% bovine serum albumin. The expression of SK1, SK2, cleaved PARP, total PI3K, p-PI3K, total Akt, p-Akt, total mTOR, p-mTOR, total EGFR and p-EGFR was measured by

western blot analysis. Anti-β-actin antibody was used as the loading control. Following repeated washes, bound antibodies were detected using the ECL Western Blotting Detection system (GE Healthcare Ltd., Buckinghamshire, UK). Protein band density was determined using a densitometer (Multi Gauge, version 3.1; Fuji Film, Tokyo, Japan).

Statistical analysis. Data are expressed as the means ± SD and compared using the Student's t-test. P-values <0.05 were considered to indicate statistically significant differences.

Results

Changes in cell viability induced by anticancer drugs. We examined the sensitivity of 5 melanoma cell lines (i.e., SK-Mel-28, A375, WM115, A2058 and B16) to 4 anticancer drugs (cisplatin, paclitaxel, carboplatin and dacarbazine). The IC₅₀ value for cisplatin was the highest in the SK-Mel-28 cells and the lowest in the A375 cells (data not shown). The relative IC₅₀ values for cisplatin, paclitaxel and carboplatin in the SK-Mel-28 cells vs. the A375 cells were 3.97-, 6.33- and 1.62-fold greater, respectively (Fig. 1). The IC₅₀ value for dacarbazine was >20-fold greater than that of cisplatin in the SK-Mel-28 cells (data not shown). Therefore, for further analysis, the SK-Mel-28 and A375 cells were used as cisplatin-resistant and cisplatin-sensitive cell lines, respectively.

Effects of FTY720 on viability of cisplatin-resistant cells. We investigated the effects of FTY720 on the viability of the anticancer drug-resistant cell line, SK-Mel-28. In the SK-Mel-28 cells treated with cisplatin, the IC₅₀ value was reduced from 85.9 to 54.2 μM in the presence of 3 μM FTY720 (an approximate 37% reduction) (Fig. 2). The IC₅₀ value for cisplatin in the A375 cells (1.3 μM) was lower than that in the SK-Mel-28 cells and the combination with FTY720 had no effects on the IC₅₀ value for cisplatin in the A375 cells (data not shown). Treatment with FTY720 alone at 3 μM had no significant effect on cell viability, although treatment with 7 μM FTY720 reduced viability to 10% of the control (data not shown). These results suggest that the combination of FTY720 and cisplatin significantly reduces viability in comparison to treatment with cisplatin alone in SK-Mel-28 cells.

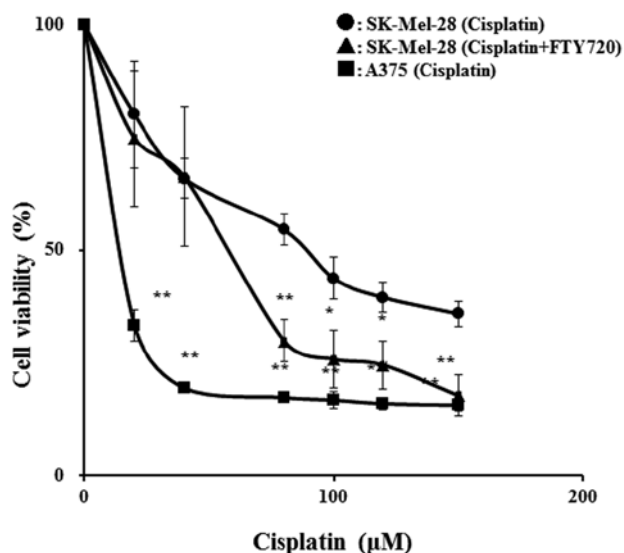


Figure 2. Effects of FTY720 on the viability of SK-Mel-28 cells. After the SK-Mel-28 or A375 cells (1×10^4 cells/well) were incubated with various concentrations of cisplatin in the presence or absence of $3 \mu\text{M}$ FTY720 for 48 h, MTT assays were performed. Each condition was measured in triplicate, and data are expressed as the means \pm SD of 3 independent experiments. Viability was reduced by the addition of FTY720 in the SK-Mel-28 cells. * $P < 0.05$, ** $P < 0.01$.

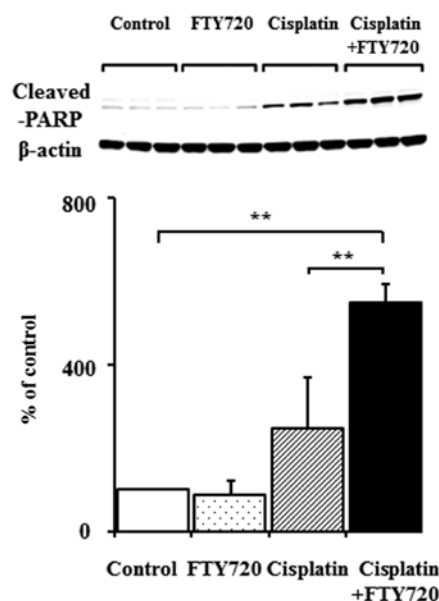


Figure 3. Effects of FTY720 on the apoptosis of SK-Mel-28 and A375 cells. SK-Mel-28 and A375 cells were treated with $3 \mu\text{M}$ FTY720 and $30 \mu\text{M}$ cisplatin, alone or in combination for 48 h. Cell lysates were subjected to western blot analysis with antibody against cleaved PARP, and the band intensity was measured. Results are expressed as a percentage increase relative to the untreated controls. Data are expressed as the means \pm SD. ** $P < 0.01$.

Effects of FTY720 on apoptosis. We investigated the effects of FTY720 on the apoptosis of SK-Mel-28 and A375 cells. Cleaved PARP, a marker of apoptosis, was examined by western blot analysis. In the A375 cells, the expression of cleaved PARP significantly increased following treatment with cisplatin, but not following treatment with FTY720 alone (Fig. 3). Although PARP degradation increased in the SK-Mel-28 cells treated with cisplatin alone, combined treatment with FTY720 induced a synergistic increase in PARP degradation (Fig. 3). These results demonstrated that the combination of FTY720 and cisplatin enhanced apoptosis in comparison to treatment with cisplatin alone in the SK-Mel-28 cells, but not in the A375 cells.

Changes in SK1 and SK2 expression induced by FTY720. We investigated the changes in SK expression levels in the SK-Mel-28 cells following treatment with FTY720, cisplatin or a combination of FTY720 and cisplatin. Treatment with FTY720 or cisplatin alone produced no change in SK1 protein expression (Fig. 4A). Treatment with a combination of FTY720 and cisplatin for 24 and 48 h caused a synergistic decrease in SK1 expression, while SK2 expression remained unaltered (Fig. 4B). Thus, the combination of FTY720 and cisplatin mainly reduced SK1 expression in the SK-Mel-28 cells within 48 h.

Downregulation of SIP signaling and the decrease in EGFR expression following treatment with a combination of FTY720 and cisplatin. To investigate the effects of FTY720 on signal transduction via the SIP receptor, the expression levels of total and phosphorylated forms of PI3K, Akt and mTOR were examined by western blot analysis in the SK-Mel-28 cells following treatment with FTY720, cisplatin alone, or a combination of FTY720 and cisplatin. The combination of FTY720 and cisplatin reduced the expression of the phosphorylated forms of PI3K,

Akt and mTOR, but not the total protein expression (Fig. 5A-C). Unexpectedly, the expression of total and p-ERK remained unaltered following combination treatment (data not shown). Cross-talk between SK1/S1P and EGFR-dependent pathways has been previously reported (19-21). Subsequently, we examined the effects of FTY720 on EGFR expression in the SK-Mel-28 cells. As shown in Fig. 5D, total EGFR expression was decreased following treatment with a combination of FTY720 and cisplatin, whereas total EGFR expression remained unaltered following treatment with cisplatin and FTY720 alone.

Discussion

Combination chemotherapy is an important strategy for improving therapeutic outcomes and reducing the side-effects of anticancer drugs. Various studies have indicated the possible utility of combining anticancer drugs with agents associated with the SK1/S1P pathway for cancer therapy (22-24). The combination of SK inhibitors, including siRNA, with anticancer drugs has been shown to be effective against drug-resistant colon cancer (22,23) and pancreatic cancer (24). The combination of doxorubicin and SK1-locked acid-antisense oligonucleotides has also been shown to be effective against gastric cancer (25). Recent studies have indicated that FTY720, which is phosphorylated by SK and acts as an antagonist of S1P, may enhance the efficacy of anticancer therapies. This compound has been shown to induce dose-dependent cell death in several human tumor cell lines, such as prostate and bladder cancer, as well as hepatocellular carcinoma (7,12,15). Moreover, FTY720 has been shown to reduce tumor growth and metastasis by increasing radiotherapeutic sensitivity in prostate cancer (26). In murine melanoma, FTY720 reduces the development of metastatic melanoma *in vivo* and apoptosis in tumor cells (16). Furthermore, FTY720

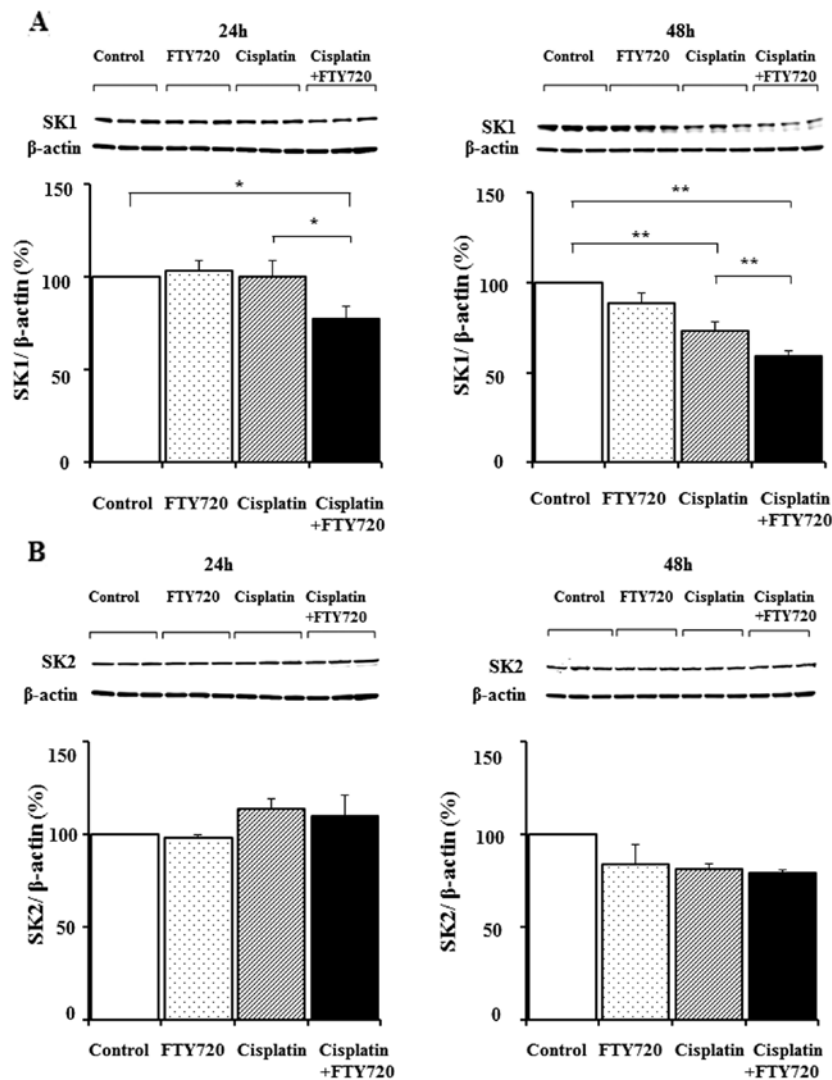


Figure 4. Effects of FTY720 and cisplatin on the expression of (A) sphingosine kinase (SK)1 and (B) SK2 in SK-Mel-28 cells. SK-Mel-28 cells were treated with 3 μ M FTY720 and 30 μ M cisplatin, alone or in combination for 24 and 48 h. Cell lysates were subjected to western blot analysis with antibodies against SK1 and SK2, with β -actin as the loading control. Each condition was examined in triplicate, and data are expressed as the means \pm SD. * P <0.05.

has been shown to inhibit SIP- and VEGF-induced angiogenesis and tumor vascularization in a murine model of melanoma cells (17). These studies suggest that functional antagonism between SIP receptors and FTY720 inhibits angiogenesis and provides a novel anticancer drug in chemotherapy. However, the precise mechanisms underlying the induction of cell death have not yet been elucidated. In this study, we demonstrated that the SK-Mel-28 cell line was the most resistant to anticancer drugs, such as cisplatin (Fig. 1), and that the combination of FTY720 and cisplatin significantly reduced viability in this cell line (Fig. 2). The viability of the SK-Mel-28 cells remained unaltered by low concentrations of FTY720 alone, but treatment with over 7 μ M FTY720 induced cell death (data not shown). Several mechanisms have been proposed to explain the anticancer efficacy of FTY720; for instance, it induces apoptosis in various types of cancer (9,12,16,17,25) through the direct inhibition and proteasomal degradation of SK1 at a high concentration (10 μ M) in human prostate cancer cells (18). Other studies have indicated that SK1 is downregulated by genotoxic stress and proteolysis of p53 induced by caspases (27,28). The resistant melanoma

cell line, SK-Mel-28, harbors a p53 mutation, and cell death induced by anticancer drugs is independent of p53 (29). In the present study, the expression of SK1, but not that of SK2, in the SK-Mel-28 cells was reduced following treatment with a combination of FTY720 and cisplatin, but not following treatment with cisplatin or FTY720 alone (Fig. 4). Combination treatment with FTY720 and cisplatin likely induced SK1 degradation by p53-independent caspase activation, resulting in a significant increase in PARP cleavage and the induction of apoptosis. Further studies required to investigate the involvement of SK2 in melanoma, as the roles of SK2 in cancer cells have not yet been elucidated.

Moreover, we investigated the molecular mechanisms underlying the induction of apoptosis following treatment with a combination of FTY720 and cisplatin in the SK-Mel-28 cells. Previous studies have demonstrated that the interaction between FTY720 and the SIP₁ receptor blocks STAT3 signaling in B-cell lymphoma (30) and the FTY-720-induced apoptosis of renal cancer cells is mediated by the inhibition of ERK activation (13). Apoptosis induced by chemotherapeutic agents in SK-Mel-28

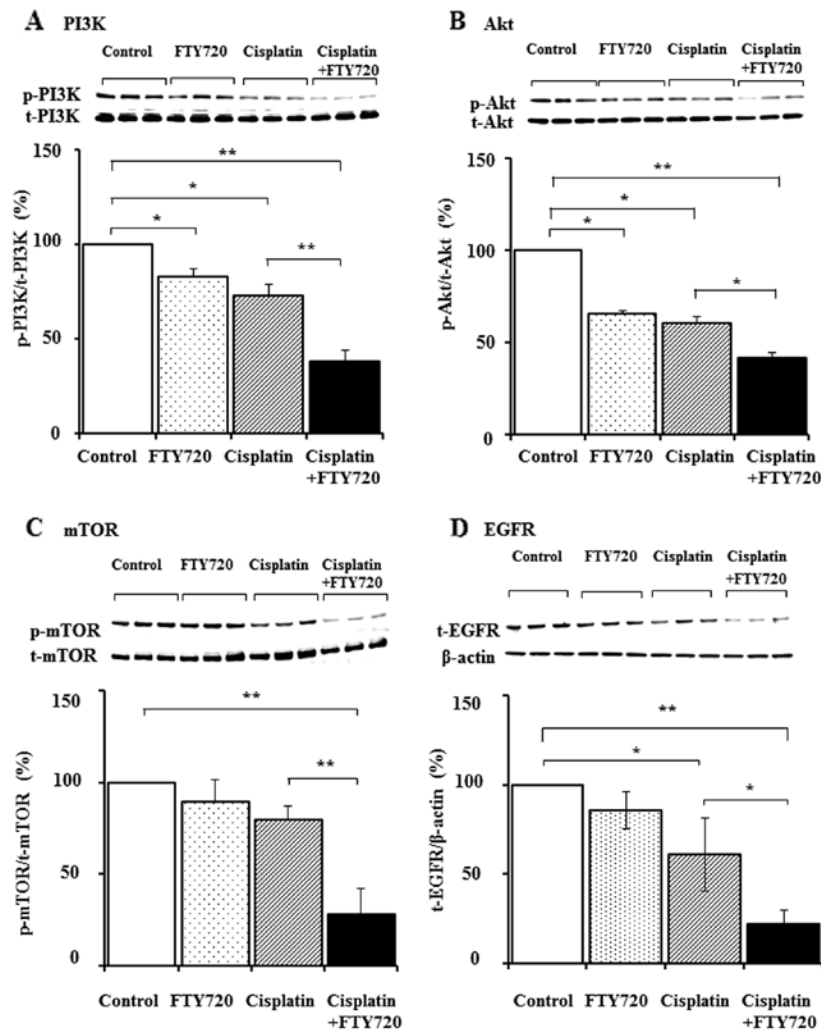


Figure 5. Effects of FTY720 and cisplatin on signaling transduction in SK-Mel-28 cells. SK-Mel-28 cells were treated with $3 \mu\text{M}$ of FTY720 and $30 \mu\text{M}$ cisplatin, alone or in combination; for 48 h, and cell lysates were subjected to western blot analysis with antibodies to total (t) and phosphorylated (p) forms of (A) PI3K, (B) Akt, (C) mTOR and (D) EGFR. The ratios of phosphorylated to total PI3K, Akt, mTOR were calculated. The expression of EGFR is shown as the ratio of total-EGFR to β -actin. Results are expressed as ratios of treated to untreated cells. Each condition was examined in triplicate, and data are expressed as the means \pm SD. * $P < 0.05$, ** $P < 0.01$.

cells is mediated by the activation of stress kinases, such as p38 MAP kinase and SAPK/JNK (29), and by the increased expression of the transactivation factor, E2F-1 (31). Furthermore, accumulating evidence suggests that the PI3K/Akt pathway is related to chemoresistance, mainly through escape from apoptosis, and these signaling pathways promote cell survival and proliferation (32). The MAPK pathway via ras-raf-MEK-ERK and the PI3K-Akt pathway are major pathways in cancer development and progression (33), and the latter is particularly important in cell survival in melanoma (34). Our results revealed that combination treatment with FTY720 and cisplatin reduced the levels of pp-PI3K and p-Akt (Fig. 5). However, this treatment did not reduce ERK activation, suggesting that signal transduction via the PI3K/Akt pathway through the SIP_1 receptor was suppressed by FTY720. Furthermore, mTOR, which belongs to the PI3K family, regulates cell growth and survival via p70S6 kinase activation in cancer cells (35,36). Therefore, studies have noted that the inhibition of mTOR reduces the proliferation of colorectal cancer, prostate cancer and esophageal squamous cell carcinoma (37). In the present study, we demonstrated that

the combination of FTY720 and cisplatin, but not cisplatin alone, suppressed p-mTOR levels (Fig. 5). Thus, FTY720 may inhibit the PI3K/Akt/mTOR pathway in cancer therapy. We also demonstrated that the combination of FTY720 and cisplatin reduced EGFR expression in the SK-Mel-28 cells (Fig. 5). It is well known that estrogen induces SIP release via SK1 activation, and that the subsequent activation of the SIP receptor SIP_3 , leads to receptor transactivation in human breast cancer (19). Such cross-talk between SIP/SK1 and EGFR has also been reported in gastric cancer (20) and human glioblastoma (21). Therefore, our results suggest that the transactivation of EGFR by SIP was also reduced by combination treatment with FTY720 in melanoma cells. EGFR expression is enhanced in various types of cancer cells and anti-EGFR drugs have been developed as anticancer agents (38). Thus, the decrease in EGFR expression may be an important mechanism through which the combination of FTY720 and cisplatin induces apoptosis in SK-Mel-28 cells.

In conclusion, compared to treatment with alone, combination treatment with FTY720 and cisplatin exerted synergistic effects on the induction of apoptosis in drug-resistant melanoma cells

by SK1 degradation, possibly due to the downregulation of the PI3K/Akt/mTOR pathway via the SIP receptor and the reduced EGFR expression. These results indicate that the inhibition of survival signals via the SK/SIP pathway may be a molecular target for cancer therapy, and FTY720 may serve as a useful agent in chemotherapy.

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