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Antagonic Effects of Dexamethasone on FK506-induced Antitumor Effects in Hep3B Cells

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Abstract : FK506 is a widespread immunosuppressive drug after liver transplantion in patients with advanced-stage hepatocellular carcinoma. Dexamethasone is frequently used as co-treatment in cytotoxic cancer therapy, e.g. to prevent nausea, to protect normal tissue or for other reasons. Our aim was to investigate antitumor effects of FK506 in Hep3B cells, one of differentiated human hepatocellular carcinoma cell lines and inhibitory effects of dexamethsone on FK506-induced antitumor effects. Cell injury was evaluated by biochemical assays as cell viability, lactate dehydrogenase (LDH) and reactive oxygen species (ROS) in Hep3B cells. Intracellular calcium concentration ($[Ca^{2+}]i$) and the level of activation of the c-Jun-N-terminal kinase (JNK) and the Bax protein in cultured Hep3B cells was measured. Exposure of 0.1 μM FK506 to Hep3B cells led to cell death accompanied by a decrease in cell viability and an increase in LDH, ROS and $[Ca^{2+}]i$. FK506 induced an increase in activity of Bax and JNK protein but inhibited the activity of Bcl-2 protein. Treatment of dexamethsone, *per se*, had no effects on cell viability, LDH and ROS. However, co-treatment of FK506 and dexamethasone diminished the FK506-induced LDH release, ROS generation and JNK activation. These results demonstrate that FK506 has antitumor effect in Hep3B cells but the combination of FK506 and dexamethasone antagonizes the FK506-induced antitumor effects.

***Key words* :** hepatocellular carcinoma cell line (Hep3B), FK506, dexamethasone.

Introduction

Hepatocellular carcinoma is fifth most common cancer and the third most significant cause of cancer-related mortality worldwide in human (10). In dogs, the commonest form of the liver cancer is the metastatic disease and the primary liver cancer is quite rare, and accounts for lower than 2% of all cancers observed in the particular species. When such forms of cancer occurs the most prevalent liver cancer in dogs is found to be hepatocellular carcinoma (4). Liver transplantation is accepted as a primary treatment for prolonging survival times in patients with advanced-stage hepatocellular carcinoma (2). FK506 is one of immunosuppressive drugs after liver transplantion (9). Glucocorticoids, an immunosuppressive drug, were used in combination with cytotoxic drugs to treat all haematological tumours, and to manage treatment- and disease-associated symptoms in solid cancer (13). Glucocorticoids were used on the basis of their recent well-understood pro-death effects in lymphoid cells, and on their effectiveness in treating tumor or treatment-related edema, inflammation, pain, electrolyte imbalance, to stimulate appetite, to prevent nausea and emesis, or toxic reactions caused by cytotoxic treatment in cancer therapy (12). Glucocorticoids are commonly used as co-medication in cancer therapy (12,13).

Tumor recurrence is the primary reason for poor long-term survival times after liver transplantation in hepatocellular carcinoma patients (16). Long-term immunosuppression could facilitate the growth and spread of malignant cells (16) and glucocorticoids could play an important role in tumor recurrence after liver transplantation for hepatoma (8). Dexamethasone administration may intensify hepatocellular cancer cell (HepG2, Huh7 and Hep3B) resistance to cytotoxic therapies with 5-FU, cisplatin, gemcitabine or γ-irradiation (17). FK506 inhibits the proliferation of differentiated human hepatocellular carcinoma cell lines, HLE and HuH-7 (14).

In this study, we tried to investigate antitumor effects of FK506 in Hep3B cells, one of differentiated human hepatocellular carcinoma cell lines and inhibitory effects of dexamethsone on FK506-induced antitumor effects.

Materials and Methods

Cell culture and reagent

Hep3B cell line (ATCC; American Type Culture Collection, Manassas, VA) was grown on 50 mL tissue culture flasks

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(10 Cellstar, greiner bio-one, Frickenhausen, Germany) in the DMEM-F-12 HAM medium mixture. This was supplemented with 10% FCS, 5 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified 5% CO2-95% air environment at 37°C. Confluent cells were washed three times with phosphate-buffered saline (PBS) that contained 5 mM ethylene glycol-bis(B-aminoenylyl ether)N,N,N', N'-tetraacetic acid (EGTA) to conduct the fluorescent studies. Cells were then trypsinized and seeded onto glass coverslips. Aliquots of the harvested cells were allowed to settle onto sterile glass coverslips in 100 mm-diameter Corning tissue culture dishes, and cells were grown to sub-confluence over 1-2 days in supplemented media.

Dulbecco's Modified Eagle's Medium (DMEM) nutrient mixture and F-12 HAM medium was purchased from ATCC. Fetal calf serum (FCS) was obtained from Gibco (Grand Island, NY), and Fura-2/AM and 2,7'-dichlorofluorescin diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR). Dexametasone was purchased from Sigma-Aldrich Ltd (St. Louis, MO, USA) and FK506 was purchased from Fujisawa Ireland Ltd (Kerry, Ireland).

Measurement of lactate dehydrogenase (LDH) activity

Hep3B cells were grown in wells of 6 well plates (1×10^5 cells/well) and cultured in the DMEM-F-12 HAM medium containing 10% FBS for 48 h. After 48 h incubation, the Hep3B cells were either left untreated or treated with 0.1 μM FK506 or 1 μM dexamethasone for 24 h. After 24 h, culture medium was collected and LDH activity was determined through the measurement of pyruvic acid levels with a SpectraMax fluorometer with a SoftMax Program (Molecular Probes) at an absorbance of 490 nm.

MTT-based viability assay

After 24 h, control or treated cells were washed twice with PBS. 50 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL in PBS) was added to each well and incubated for an additional 4 h. The MTT solution was removed and MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to dissolved the formed formazan crystals. One hundred fifty microliters of the purple-blue colored MTT solubilization solution containing the dissolved formazan was collected from each sample and added to a well in a 96-well plate. Absorbance was read at 570 nm using a microplate reader (SpectraMax M5, Molecular Devices, USA). MTT solubilization solution without cells was used as a blank contol.

Measurement of reactive oxygen species (ROS) generation

DCFH-DA is a non-fluorescent probe that becomes highly fluorescent DCFH upon reaction with ROS, and so can be used to monitor ROS production. Briefly, after application of FK506 or dexamethason, medium was collected in wells of a 96-well microplate and diluted DCFH-DA was added to each well at a concentration of 10 μM. Plates were incubated for 60 min at 37°C and DCFH fluorescence was determined with the aforementioned fluorescence plate reader (at an excitation and emission wavelength of 488 nm and 515 nm, respectively).

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$)

Coverslips were mounted into perfusion chambers, and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) was determined with Ca^{2+}-sensitive fluorescent dye (Fura-2; Molecular Probes). The cell-permeant aceoxymethyl ester (AM) of the dye was dissolved in dimethyl sulfoxide (DMSO) to make a 5 mM stock concentration. This stock concentration was further diluted to 5 mM Fura-2AM in medium for 30 min at 37°C. Cells were washed three times with buffered salt solution containing 145 mM NaCl, 4.0 mM KCl, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1.0 mM $CaCl_2$, 5.0 mM glucose, and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)/ Tris (hydroxymethyl) aminomethane (Tris) at pH 7.4. The Hep3B cells were incubated for an additional 30 min to allow for complete deesterification. Cells were washed once with this buffer solution prior to measurement of fluorescence. Epifluorescence microscopy was used to monitor the Fura-2 changes within single Hep3B cells. The chamber was mounted on an inverted Nikon Diaphot-TMD microscope equipped with a FluorX100 objective, and fluorescence was monitored within a single cell under oil immersion over the course of the study. Fluorescence was recorded at 1 s intervals using a Delta-scan dual-excitation wavelength spectrophotometer (Photon Technologies, Princeton, NJ) with excitation wavelengths for Fura-2 of 340 and 380 nm (the chopper speed was set at 100 Hz), and an emission wavelength of 510 nm. All the experiments were performed at 23°C with continuous replacement of the bathing solution (1 mL/min). Medium changes were done without interrupting the recording.

$[Ca^{2+}]i$ was calculated from the ratio of the fluorescence at the two excitation wavelengths using a dissociation constant (Kd) of 1.4 mM for the Fura-2 Ca^{2+} complex. The Rmax for Fura-2 was determined by the addition of 50 mM $MgCl_2$ in the absence of Ca^{2+}, and the Rmin was obtained by Ca^{2+} removal and the addition of 100 mM EDTA at pH 7.2. The change in $[Ca^{2+}]i$ with time ($d([Ca^{2+}]i)/dt$) was determined by linear regression analysis of fluorescence tracing over the initial 500 s.

Western blot analysis

The medium was aspirated after incubation and cells were washed three times with 100 μL of ice-cold PBS sodium dodecyl sulfate (SDS) sample buffer (2.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue, pH 6.8). The cells were immediately homogenized and the extract was transferred to a 1.5 mL microfuge tube on ice. The sample was lsonicated for 5 pulses at a 40% duty cycle using a Sonics & Materials Ultrasonic Processor and was microcentrifuged at 14000 rpm for 30 min. Supernatants (30 μL each) were loaded onto a 7.5% SDS-polyacrylamide gel electrophoresis gel. Resolved proteins were electro-

transferred after electrophoresis to a Hybond-ECL nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (20 mM Tris and 140 mM NaCl, pH 7.6) containing 0.1% Tween20 (TBST) and 5% milk at room temperature for 2 h. The membrane was incubated overnight with the primary monoclonal antibody at a 1:1000 dilution in TBST with 5% milk at 4°C. The blot was washed three times for 10 min in TBST and the bands were detected using enhanced chemiluminenscence with exposure to X-OMAT AR film. The blots were quantified by laser scanning densitometry (Bio-Rad, Hercules, CA).

Statistical analysis

The results were expressed as the means ± standard error of the mean (SEM). The data was analyzed via analysis of paired student's *t*-test, repeated measures of the analyses of variance (ANOVA), and the Bonferroni test using Prism 5.03 (GraphPad Software Inc, San Diego, CA). A p value of < 0.05 was considered to significant.

Results

Effect of FK506 and dexamethasone on viability and LDH activity of Hep3B cells

As shown in Fig. 1A, treatment with 0.1 μM FK506 to Hep3B cells attenuated cell growth compared to controls. This effect was corroborated by results from the MTT test (Fig. 1B). FK506 induced an increase in cellular LDH activity by 18 ± 0.5%, but combination with FK506 and 1 μM dexamethasone significantly diminished the FK506-induced increase in LDH activity (Fig. 1C)

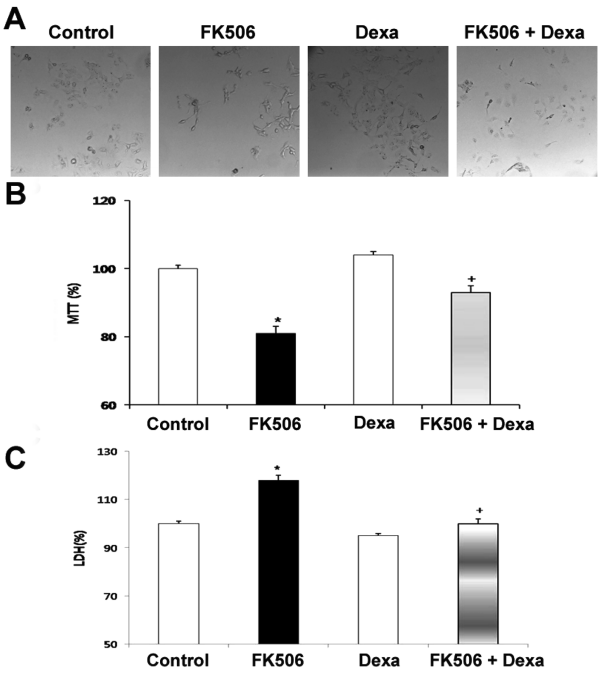

Fig. 1. Effects of FK506 and dexamethasone on cell viability and LDH activity in Hep3B cells. Hep3B cells plated in wells of 6-well plates were treated for 24 h with 0.1 μM FK506 and 1 μM dexametasone (Dexa). (A) Cell morphology was assessed by microscopy (× 200). Cell viability was determined using the MTT test (B) and by LDH activity (C). The viability was expressed relative to the control (100%). The experiments were conducted at least in triplicate. *$P < 0.05$ vs. control, +$P < 0.05$ vs. 0.1 μM FK506.

Effects of FK506 on intracellular calcium concentration ($[Ca^{2+}]i$)

The mean $[Ca^{2+}]i$ at 1 mM extracellular Ca^{2+} concentration was 104.2 ± 2.9 nM in Hep3B cells (n = 10). The addition of FK506 provoked a dose-dependant increase in $[Ca^{2+}]i$. Panel A of Fig. 2 showed a typical recording of $[Ca^{2+}]i$ changes after exposure to FK506 (0.03 and 0.1 μM). The value of $[Ca^{2+}]i$ after exposure to 0.03 μM and 0.1 μM FK506 were 130.3 ± 3.5 nM and 284.6 ± 7.8 nM, respectively.

Effects of FK506 and dexamethasone on reactive oxygen species (ROS) generation

The treatment with 0.1 μM FK506 produced an increase in ROS production by 1.5-fold after 24 h (Fig. 3) compared to control. However, combination with FK506 and 1 μM dexamethasone significantly diminished the FK506-induced increase in ROS generation (Fig. 1C).

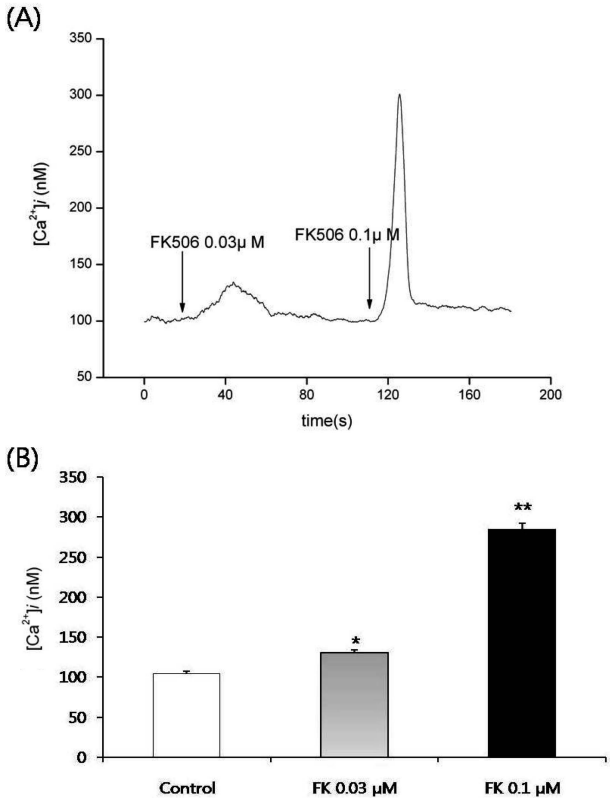

Fig. 2. Effects of FK506 and dexamethasone on $[Ca^{2+}]i$ in Hep3B cells. A typical recording (A) and data summary (B) of $[Ca^{2+}]i$ induced by 0.03 and 0.1 μM FK506 in Hep3B cells. The data are reported as the mean ± SEM of eight different preparations. *$P <$ 0.05 vs. control, **$P < 0.005$ vs. control.

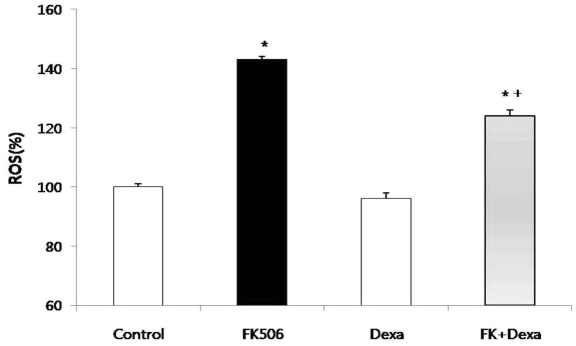

Fig. 3. Effect of FK506 and dexamethasone on ROS generation. Hep3B cells plated in wells of 6-well plates were treated for 24 h with 0.1 μM FK506 and 1 μM dexametasone (Dexa). The cells were incubated with 10 mM DCFH-DA as described in Materials and Methods section. *$P < 0.05$ vs. control, +$P < 0.05$ vs. 0.1 μM FK506.

Effects of FK506 and dexamethasone on JNK, Bcl-2 and Bax

As shown in Fig. 4, treatment with 0.1 μM FK506 caused an increase in total and phosphorylated JNK and Bax protein, but a decrease total and phosphorylated in Bcl-2 protein. The percentage variations of the FK506 treated groups (n = 4) compared to control groups (100%) were 291 ± 6% (phosphorylated JNK), 54 ± 2% (phosphorylated Bcl-2 protein) and 143 ± 1% (Bax protein) as determined by densitometry. However, combination with FK506 and 1 μM dexamethasone significantly attenuated the FK506-induced changes in JNK, Bcl-2 and Bax.

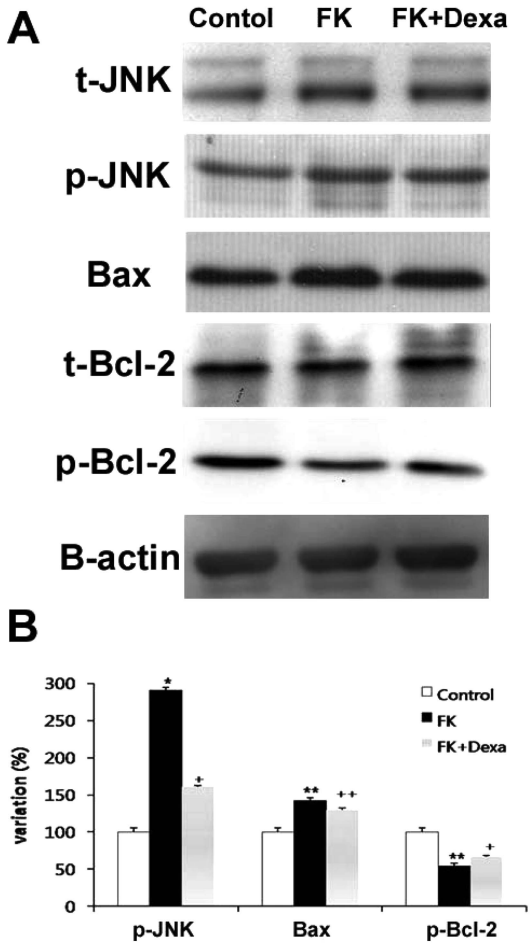

Fig. 4. Effects of FK506 and dexamethasone on JNK, Bcl-2 and Bax in cultured Hep3B cells. The amount of JNK, Bcl-2 and Bax of Hep3B cells was measured by Western blot analysis. Hep3B cells were treated for 24 h with no treatment (control), with 0.1 μM FK506 treatment (FK), or with FK506 plus 1 μM dexametasone treatment (FK + Dexa). A typical changes in JNK, Bcl-2 and Bax (A). The blots were quantified by scanning densitometry. The data are reported as a mean ± SEM (n = 4) for each group (B). *$P < 0.05$ and **$P < 0.005$ vs. control, +$P < 0.05$ and ++$P < 0.005$ vs. 0.1 μM FK506.

Discussion

This study examined the cytotoxic effects of FK506 on Hep3B cells and particularly inhibitory dexamethsone on FK506-induced antitumor effects. The results suggest that decreased cell viability in Hep3B cells induced by FK506 is related to ROS generation and change in $[Ca^{2+}]i$. To our knowledge, this is the first examination of the mechanisms of FK506-induced apoptosis by ROS in Hep3B cells. ROS can induce apoptosis through DNA damage, oxidization of membrane lipids and direct activation of the expression apoptosis genes and proteins (1). ROS and Ca^{2+} are important signal mediators that regulate programmed cell death. The results of several studies have indicated that Ca^{2+} and ROS affect common signals that regulate apoptotic pathways. Previous study shows that an increase in cytosolic Ca^{2+} leads to intracellular ROS accumulation in apoptotic cells (6). The present study clearly showed that intracellular Ca^{2+} could be increased by the addition of FK506. This enhanced Ca^{2+} accumulation might be associated with ROS generation, mitochondrial damage and cell death. We studied JNK and Bcl-2 family protein expression to elucidate the mechanism of apoptosis by FK506. Treatment with FK506 led to JNK phosphorylation and Bax activation, but inhibited Bcl-2 phosphorylation in Hep3B cells. In general, JNK activation is associated with apoptosis induction. The ability of ROS induced apoptosis by regulating phosphorylation and ubiquitination of the Bcl-2 family proteins, resulting in increased pro-apoptotic protein levels and decreased anti-apotic protein expression (5). Bcl-2 and Bcl-xL protect against mitochondrial dysfunction and, therefore, inhibit apoptosis. In contrast, Bid, Bax and Bak promote apoptosis (3). Therefore, the enhancement of intracellular ROS levels could be related to promotion of JNK and Bax activation and Ca^{2+} release, ultimately resulting in apoptosis. FK506 previously was demonstrated to inhibit the growth of liver cancer cells, HLE and HuH-7 at 1 and 10 ng/mL and exhibit cytotoxicity at concentrations over 100 ng/mL (14).

Most chemotherapeutic treatments are mitigated by the

addition of dexamethasone and similar glucocorticoids (13). Glucocorticoids are commonly used in comedication in cancer therapy (8,13,16). A previous study reported that dexamethasone inhibited cell growth in four cell lines (MCF-7, MCF-7/MXR1, MCF-7/TPT300 and HeLa), increased cisplatin cytotoxicity in one cell line (SiHa) and decreased cisplatin cytotoxicity in two cell lines (H460 and Hep3B) (7). Another report demonstrated that dexamethasone protected thyroid cancer cells from tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis induced by TRAIL (11). Dexamethasone acts through glucocorticoid receptor activation and up-regulation of the expression of the anti-apoptotic protein Bcl-xL (11). However, dexamethasone administration inhibited cytotoxicity of anti-tumor drugs in hepatocellular cancer cells (17). Also, glucocorticoids inhibited neutrophil-mediated tumor cell cytostasis (15). In the present study, we showed co-treatment with dexamethasone inhibited the FK506-induced cytotoxic effects.

In view of the above arguments and the new data presented herein, we strongly propose that FK506 has antitumor effect in Hep3B cells but the combination of FK506 and co-treatment of dexamethasone antagonizes the FK506-induced antitumor effects.

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간암세포주(Hep3B cell)에서 FK506의 항암효과에 대한 dexamethasone의 길항효과

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요 약 : FK506은 말기 간암환자의 간이식 후 널리 사용되는 면역억제제이다. Dexamethasone은 세포독성 암 치료에서 오심 방지, 정상세포의 보호와 기타 이유 등의로 빈번하게 병용처치된다. 본 연구의 목적은 간암세포주(Hep3B)에서 FK506의 항암효과와 FK506에 의한 항암효과에 대한 dexamethasone의 억제효과를 알아보기 위함이다. 세포의 손상은 세포 생존성 평가와 LDH 및 세포내 ROS 양의 측정으로 평가 하였다. 세포내 칼슘 농도($[Ca^{2+}]i$)와 JNK, Bax 단백질의 발현 정도도 평가하였다. FK506의 처치는 Hep3B의 세포사를 유도하였으며 세포생존성의 감소와 LDH, ROS 및 $[Ca^{2+}]i$ 를 증가시켰다. FK506은 Bax와 JNK 의 활성을 증가시켰으며 Bcl-2의 활성을 억제하였다. Dexamethasone 처치 그 자체는 세포생존성, LDH와 ROS에 영향을 주지 않았다. 그러나 dexamethasone과 FK506의 병용처치는 FK506에 의한 LDH 방출, ROS 생성 및 JNK의 활성을 감소시켰다. 이 결과는 간암세포주에서 FK506은 항암효과를 가지지만 dexamethasone의 병용처치는 FK506에 의한 항암효과를 길항한다.

주요어 : 간암세포주(Hep3B), FK506, dexamethasone