FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation

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Our aim was to study the anticancer effect of the novel immunomodulator FTY720 in vitro and in vivo by investigation of cell cycle entry, cell cycle regulation, cell survival and apoptosis pathways. Three hepatoma cell lines with different p53 statuses (HepG2, Huh-7 and Hep3B) and one non-tumorigenic immortalized liver cell line (MIHA) were used for an in vitro study. The in vivo effects of FTY720 were evaluated in a nude mouse tumor model. Cell cycle distribution and cell cycle regulator proteins p27Kip1 and cyclin D1, together with the PI3-K/Akt pathway, mitogen-activated protein kinases and cleaved caspase-3 and caspase-9, were evaluated. FTY720 selectively induced cell apoptosis in hepatoma cell lines with overexpression of cleaved caspase-3 and caspase-9, but the same phenomena were not found in MIHA cells. FTY720 induced Akt dephosphorylation at Ser473 mediated by phosphoinositide 3-kinase (PI3-K) inhibition. Dephosphorylation led to down-regulation of p42/p44 and dephosphorylation of Forkhead transcription factor and GSK-3β and, subsequently, up-regulation of p27kip1 and down-regulation of cyclin D1. In our in vitro model FTY720 induced apoptosis of tumor cells by down-regulation of the Akt pathway. FTY720 suppressed tumor growth without notable side-effects in normal liver. In conclusion, FTY720 is a novel anticancer agent that induces apoptosis of hepatoma cell lines both in vitro and in vivo through PI3-K-mediated Akt dephosphorylation in a p53-independent manner.

Introduction

Hepatocellular carcinoma (HCC), which is particularly prevalent in Asia and Africa, is a major malignancy worldwide and is one of the most common causes of cancer mortality (1). Although surgical resection is the first treatment of choice for HCC, most cases present at an advanced stage when surgery can no longer cure the disease. Chemotherapy is commonly applied for inoperable HCC, but its results are often discouraging because of chemoresistance conferred by p53 mutation and toxicity to normal cells. Therefore, a novel pharmaceutical therapy needs to be developed. Recently, FTY720, a novel immunomodulator, has been reported to have a strong anti-tumor effect for breast cancer (2), bladder cancer (3) and leukemia (4). So far, the feasibility of using this drug in HCC treatment has not been studied. The precise cellular mechanisms of FTY720 on cancer cells are not completely understood. Therefore, in this study we aimed to investigate the in vitro and in vivo anticancer potential of FTY720 and ascertain its exact mechanism of inducing apoptosis in HCC cells.

The PI3-K and Akt pathway is very important in governing cell survival and prevention of apoptosis in tumors. In this study we have investigated the effect of FTY720 on the Akt cell survival pathway and found that Akt dephosphorylation plays a central role in cell growth arrest and apoptosis upon administration of FTY720 to hepatoma cell lines. Dephosphorylation of AktSer473 but not AktThr308 might lead to dephosphorylation of Forkhead transcription factor (FKHR), GSK-3β and p42/p44, resulting in p53-independent G1 arrest, possibly by up-regulation of p27kip1 and down-regulation of cyclin D1. It might also activate the apoptotic pathway by up-regulation of caspase-3 and caspase-9, both in vitro and in vivo.

Materials and methods

Cell lines and reagents

The human HCC cell line Huh-7 (15) (a gift from Dr H.Nakabayashi, Hokkaido University School of Medicine, Japan), human HCC cell line Hep3B (American Type Culture Collection, HB-8064), and the human
hepatoblastoma cell line HepG2 (American Type Culture Collection, HB-8065) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 50 U/ml penicillin G and 50 µg/ml streptomycin (Gibco BRL) at 37 °C in a humidified atmosphere containing 5% CO₂. The human non-tumorigenic, immortalized liver cell line MIHA (16) (kindly provided by Dr J.R. Chowdhury, Albert Einstein College of Medicine, New York) was maintained in Cheam’s medium (Gibco BRL) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mmol/l l-glutamine, 50 mmol/l dexamethasone and 20 µg/ml recombinant insulin (Boehringer Mannheim, Indianapolis, IN). FTY720 was kindly provided as a powder by Novartis Pharmaceuticals UK Ltd (Basel, Switzerland). It was added to the culture medium at different concentrations after dissolution in normal saline. The constitutively active PI3-K plasmid (CD2-pi10) was a gift from Dr D.Cantrell (University of Dundee, Dundee, UK).

3-(4,5-Cimetidiheterazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The hepatoma cells lines and non-tumorigenic, immortalized MIHA cells were seeded on 96-well plates and appropriate concentrations of FTY720 ranging from 0.078 to 200 µM were then added. After 4–24 h 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye, at a concentration of 5 mg/ml (Sigma-Aldrich), was added to the wells and plates were incubated at 37 °C in a moist chamber at 37 °C. Optical density was determined by eluting the dye with dimethyl sulfoxide (Sigma-Aldrich) and the absorbance was measured at 570 nm. At least three independent experiments were performed.

Cell cycle analysis

After FTY720 treatment, the DNA content and cell cycle distribution of hepatoma cells grown in 6-well plates were determined by flow cytometry. The cells were plated at a low density (5 × 10⁵ cells/well) and were harvested at 0, 4, 8, 16 and 24 h. Another set of controls was employed without FTY720 treatment. They were trypsinized and washed once in phosphate-buffered saline (PBS). They were then fixed in cold 70% ethanol and stored at 4 °C before testing. The ethanol was removed and the cells were resuspended in PBS. The fixed cells were then washed with PBS and treated with RNase (1 µg/ml) and stained with propidium iodide (50 µg/ml) for 30 min at 37 °C. Cell cycle analysis was performed in an EPICS profile analyzer, using ModFit LT2.0 software (Coulter Electronics, Hialeah, FL).

Detection of apoptotic cells by 4,6-diamino-2-phenylindole (DAPI) staining and TUNEL assay

Hep3B cells were plated onto 12 mm coverslips in DMEM medium at ~70% confluence for 24 h. The cells were then treated with FTY720 at the IC₁₀ and IC₅₀ doses for 0, 4, 8, 16 and 24 h. They were fixed in ice-cold acetone and methanol (1:1), washed with PBS and then stained with DAPI for 5 min. The stained cells were examined under a fluorescence microscope and cells were considered to undergo apoptosis based on the appearance of nuclear fragmentation. A total of 300 cells were counted in 5 fields/sample. The percentage of apoptotic cells was calculated as (number of apoptotic cells ÷ total number of cells counted) × 100. For the TUNEL assay the procedure was the same as for DAPI staining except that the cells were stained in situ with a POD cell death detection kit (Boehringer Mannheim, Manheim, Germany) and counterstained with propidium iodide.

Assay for PI3-K activity

Cultured Hep3B cells were treated with FTY720 at the IC₁₀ and IC₅₀ doses for 4 h and then stimulated with 100 nM insulin for 10 min at 37 °C. The cells were then lysed in 1 ml of NP-40 containing lysis buffer with protease inhibitors. Equal amounts of protein lysate (Protein Assay Kit, Bio-Rad, Hercules, CA) were then incubated with protein A–agarose beads for 1 h at 4 °C. Immunoprecipitates were washed and evaluated for PI3-K activity by competitive ELISA (Echelon Biosciences, Salt Lake City, UT) as described (17). Briefly, PI3-K bound to the beads was incubated for 2 h with 10 µM diaciglycerol phosphatidylinositol 4,5-diphosphate (diC₂(diP(4,5)P₂)) substrate at room temperature in 50 µl of buffer containing 5 mM HEPES (pH 7), 25 µM ATP and 1.5 mM MgCl₂. The beads removed by centrifugation and the supernatant, or known concentrations of phosphatidylinositol 3,4,5-trisphosphate (Pi[3,4,5]P₃), were incubated for 1 h with 50 µl of PI[3,4,5]P₃-binding reagent and then transferred to a detection plate coated with PI[3,4,5]P₃. Plate-binding binding reagent was quantitated using a secondary detection reagent, peroxidase, and peroxidase substrate, with the reaction product measured by absorbance at 450 nm.

In vivo effect of FTY720 in the nude mouse tumor model

We studied the in vivo effect of FTY720 on the cell survival and apoptosis pathway using a nude mouse tumor model. The mice were housed in microisolator cages under positive air pressure and maintained at a constant temperature (22 °C) and humidity. Surgical operations and drug administration were performed in a laminar flow cabinet. Four-week-old male BALB/c nu/nu mice were used to establish the tumor model by s.c. implantation of 1–2 mm³ tumor tissue originating from the Hep3B strain. FTY720 was given at 10 mg/kg by i.p. injection when the tumor size reached 32 mm³ (L × W²/2) at 10 mg/kg for 10 or 20 days. Tumor size and liver were sampled for detection of protein expression and morphological examination. Tumor growth was also compared between the rats with and without FTY720 treatment.

Western blotting

The cells were lysed and protein extraction was performed. The samples were separated in a 10% SDS-acrylamide gel and electrophoretically transferred to PVDF membrane (Amersham, Little Chalfont, UK). The membrane was blocked with 10% non-fat milk, washed and then probed with PI3-K (1:1000) (Upstate Biotechnology), p53 (1:1000) (Dako, Carpinteria, CA), p21WAF1 (1:500) (Dako), p73 (1:2000) (Transduction Laboratories, Lexington, KY), phospho-p42/p44 and p42/p44 (Cell Signaling Technology, Beverly, MA), phospho-Akt and Apoptosis Pathway Sampler Kits (Cell Signaling Technology) and actin (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Amersham) and then visualized by enhanced chemiluminescence plus according to the manufacturer’s protocol.

Morphological examination

TUNEL staining was performed in situ using a POD cell death detection kit (Boehringer Mannheim) following the manufacturer’s protocol. Apoptotic cells were determined with careful observation of TUNEL-positive nuclei. For hematoxylin and eosin (H&E) staining the samples were stained with H&E. The images were captured using an image analysis system (Eclipse E600; Nikon, Japan).

Results

FTY720 selectively induces significant hepatoma cell death in vitro

To determine the cytotoxic effect of FTY720 on hepatoma cell lines, the MTT assay was performed to determine the IC₁₀ and IC₅₀ doses. The MTT assay demonstrated that FTY720 treatment induced dramatic cell death in hepatoma cell lines in a time- and dose-dependent manner (Figure 1). As shown in Table I, there was no significant difference in drug sensitivity (IC₅₀ range 12–14 µM) among the three hepatoma cell lines (HepG2, Huh-7 and Hep3B) with different p53 statuses. To examine its toxicity, the effect of FTY720 on MIHA cells was also investigated. The results showed that FTY720 conferred greater chemoresistance to MIHA, with an IC₅₀ up to 60 µM (Table I).

FTY720 induces p53-independent G₁ arrest in hepatoma cell lines

FTY720 was previously reported to induce G₁ arrest in prostate and breast cancers (2,11). To evaluate its possible effects on the cell cycle of cells with different p53 statuses, cell cycle changes were analyzed by flow cytometry in HepG2, Huh-7 and Hep3B cells. The effect of FTY720 on G₁ arrest was examined by excluding cells of small size in the sub-G₁ phase. When FTY720 was administered at the IC₁₀ and IC₅₀ doses, all three hepatoma cell lines showed increased G₁ arrest in a dose- and time-dependent manner. The cell cycle distribution of Hep3B cells upon FTY720 treatment is shown in Figure 2. The percentage of sub-G₁ phase cells was analyzed by DAPI staining and TUNEL assay. The DAPI result showed that the number of apoptotic nuclei increased significantly after FTY720 administration compared with the control starting at 8 h at the IC₅₀ dose and at 24 h at the IC₁₀ dose (Figure 3). The percentages of apoptotic nuclei as determined by the TUNEL assay were 1.2 ± 1.0, 4.3 ± 0.3, 7.9 ± 0.9 and 10.5 ± 1.5 at 4, 8, 16 and 24 h, respectively, at the IC₁₀ dose.
and 8 ± 0.2, 17.5 ± 2, 35 ± 1.1 and 48 ± 2.5 at 4, 8, 16 and 24 h, respectively, at the IC50 dose.

**FTY720 induces G1 arrest and apoptosis through Akt dephosphorylation**

We then examined the effect of FTY720 on Akt and MAPK. The PI3-K/Akt/MEK/MAPK pathway is essential for cell growth, migration and survival (18). The Akt pathway has been reported, at least in part, to activate the MAPK pathway (19). In the current study we found that the phosphorylation level of AktSer473, but not AktThr308, decreased in a time- and dose-dependent manner upon treatment with FTY720 in Hep3B cells (Figure 4). The Akt protein level remained unaffected (Figure 4). The phosphorylation level of p42/p44 was also found to be down-regulated in response to FTY720 treatment, while the p42/p44 protein level remained unchanged in the course of FTY720 treatment (Figure 4). Moreover, the phosphorylation level of FKHR and GSK-3β decreased, accompanied by up-regulation of p27kip1 and down-regulation of cyclin D1 (Figure 5). Therefore, down-regulation of both phospho-GSK-3β and phospho-FKHR might contribute to the G1 arrest of hepatoma cells. For FTY720-induced apoptosis, dephosphorylation of AktSer473 led to increased cleaved forms of both caspase-9 and caspase-3 (Figure 5).

**FTY720 induces apoptosis through inhibition of PI3-K activity**

To examine whether dephosphorylation of AktSer473 was mediated by PI3-K, PI3-K activity in Hep3B cells was evaluated by competitive ELISA. FTY720 inhibited the PI3-K activity in a dose-dependent manner 4 h after treatment. There was 39.4 ± 2.9 and 79.2 ± 3.4% PI3-K inhibition at the IC10 and IC50 doses, respectively. To further determine that inhibition of PI3-K was critical to the apoptosis-inducing activity of FTY720, a constitutively active PI3-K plasmid (CD2-p110) was transiently transfected into Hep3B cells. Flow cytometry analysis showed that the transfection efficiency of PI3-K cDNA was 59.3% (data not shown). PI3-K-transfected Hep3B cells showed decreased apoptosis by the TUNEL assay, up-regulation of AktSer473 and down-regulation of cleaved caspase-3, caspase-9, p27kip1 and cyclin D1 protein levels (Figure 6). However, there was no significant change in AktThr308 (Figure 6) and no obvious change in the percentage of cells in G1 phase (from 54.84 to 58.98%) compared with parental Hep3B cells (from 54.84 to 67.73%) (Figure 6).

**FTY720 suppresses tumor growth and selectively induces tumor cell apoptosis in vivo**

In the nude mouse tumor model tumor growth was suppressed by FTY720 treatment (Figure 7). Consistent with our in vitro results, we also observed down-regulation of AktSer473 but not AktThr308 in Hep3B-bearing nude mice, together with an increased amount of cleaved caspase-9 and caspase-3 in a time-dependent manner upon FTY720 treatment (Figure 8).
There was a remarkable difference in the number of apoptotic nuclei in tumor tissues treated with FTY720 on day 10 and patchy necrosis on day 20 (Figure 9B) when compared with the untreated samples (Figure 9A) by TUNEL assay and H&E staining. However, the architecture of the liver of the nude mice remained intact after FTY720 treatment (Figure 10). There was no difference in the number of apoptotic nuclei of the liver between mice with and without FTY720 treatment (Figure 10).
Discussion

Chemotherapy is the current therapy for inoperable HCC. For single agent therapy doxorubicin is widely adopted clinically. However, HCC is considered to be resistant to chemotherapy (20). The response rates for single agent chemotherapy, usually doxorubicin, are ~15–20% (21–23). Combination chemotherapy appears to give a higher response rate (20–30%), although in both cases remissions are usually short and a survival advantage has not been convincingly demonstrated (24). Many reports have linked chemoresistance to p53 mutation and Mdr1 overexpression (25). Therefore, there is an urgent need to develop a novel drug that specifically targets cancer cells but is not harmful to normal liver cells. Recently, several reports have demonstrated that FTY720 selectively kills hepatoma cells and inhibits in vivo tumor growth in the nude mouse model without notable side-effects. The above data are promising and make FTY720 a new candidate for HCC therapy.

Although the mechanism of FTY720-induced cell death has been studied, most of the in vitro studies focused only on the pro-apoptotic death signaling pathways, such as the c-Jun, NH2-terminal kinase and p38 pathways, and little is known about the anti-apoptotic pathways and the effectiveness of FTY720 in in vivo systems (26). This study aimed to demonstrate for the first time both the in vitro and in vivo effects of FTY720 on HCC by studying the Akt pathway, an important survival pathway. We demonstrated that dephosphorylation of Akt played a central role in FTY720-induced G1 arrest and apoptosis in hepatoma cells. However, interestingly, FTY720 preferentially dephosphorylated Akt at Ser473 but not significantly at Thr308. This result was not only observed in our
in vitro experiment but also in our in vivo nude mouse model. For apoptosis induced by FTY720, dephosphorylation of AktSer473 led to dephosphorylation of phospho-caspase-9, resulting in an increase in the cleaved form of caspase-9 and, consequently, caspase-3. Apoptosis induced by Akt dephosphorylation in hepatoma cell lines was found through inhibition of PI3-K activity and was confirmed by transfection of a constitutively active PI3-K enzyme into Hep3B cells. Transfection of PI3-K into Hep3B cells protected them from apoptosis, with increased AktSer473 and decreased cleaved caspase-3, cleaved caspase-9, p27Kip1 and cyclinD1, while AktThr308 remained unchanged. This result was different from a previous study of FTY720-induced apoptosis in leukemia cell lines, in which both AktSer473 and AktThr308 were dephosphorylated independent of PI3-K inactivation (4), suggesting...
a differential effect of FTY720 on different tumor cell lines. This discrepancy could be attributed to the fact that sphingosine kinase could be found in different forms in different tissues and showed different tissue distributions (27). Therefore, FTY720 may show different kinetic properties and substrate specificities in different tissues. The level of protein p27kip1 is regulated directly by Akt phosphorylation and indirectly by FKHR. FKHR is the downstream substrate of Akt. FKHR plays a major role in cell cycle progression by inhibition of p27kip1 transcription through PI3-K-induced FKHR-L1 phosphorylation (28). In addition to Akt, p42/p44 activation triggers p27kip1 degradation independent of CDK2/cyclin E in NIH 3T3 cells (29). There is cross-talk between Akt and p42/p44. Several reports have demonstrated that expression of p42/p44 is partly mediated by the Akt pathway (19). Akt can also affect cellular proliferation by inactivating GSK-3β, resulting in stabilization of cyclin D1 (30). p27kip1 and cyclin D1 have been shown to play an important role in cell cycle progression from G1 to S phase (31). From our western blot results, up-regulation of p27kip1 and down-regulation of cyclin D1 were observed in a time- and dose-dependent manner after FTY720 administration and the alterations were consistent with

Fig. 9. FTY720 induces remarkable tumor cell apoptosis in vivo. Both H&E staining (a and b) and the TUNEL assay (c and d) of FTY720-treated cells (B) demonstrated significant tumor cell apoptosis on day 10 (a and c) and significant necroses without defined nuclei and the cytoplasm on day 20 (b and d), when compared with untreated controls (A) on days 10 (a and c) and 20 (b and d). In the control group only a few apoptotic nuclei were observed, as indicated by arrows (×200).

Fig. 10. FTY720 has minimal toxicity to normal liver cells. Both H&E staining (a and b) and the TUNEL assay (c and d) showed that normal livers of nude mice were healthy in the control group (A) on days 10 (a and c) and 20 (b and d). After FTY720 administration (B) the liver tissues remained intact without apoptosis and necroses on days 10 (a and c) and 20 (b and d).
induction of $G_1$ arrest. Therefore, up-regulation of $p27^{kip1}$ and down-regulation of cyclin D1 might be correlated with FTY720-induced $G_1$ arrest. This conclusion was further confirmed by transfection of constitutively active PI3-K enzyme into Hep3B cells. There was no apparent change in $p27^{kip1}$ and cyclin D1 protein levels in PI3-K-transfected Hep3B cells, with only an insignificant change in the percentage in $G_1$ phase from 54.84 to 58.98%, in contrast to parental Hep3B cells, which changed from 54.84 to 67.73%, thus suggesting the role of $p27^{kip1}$ and cyclin D1 in FTY720-induced $G_1$ arrest. Therefore, we propose that FTY720-induced apoptosis is mediated by PI3-K Akt dephosphorylation (Figure 11).

The main hurdle to chemotherapy for HCC is chemoresistance and toxicity to normal liver cells (32). In this study we have found that FTY720 induces hepatoma cell death in a p53-independent manner. This result is an advantage in preventing chemoresistance induced by $p53$ mutation in HCC (33). FTY720 selectively induces hepatitis C virus (HCV)-infected human hepatoma cell death, which is not that of immortalized, non-tumorigenic liver cell lines. Consistent with the *in vitro* study, we found that FTY720 selectively induces apoptosis in tumors in a time- and dose-dependent manner, but not in normal liver in the nude mouse tumor model.

In conclusion, FTY720 is a novel anticancer agent that induces apoptosis of hepatoma cell lines in a p53-independent manner through PI3-K-mediated Akt dephosphorylation. FTY720 may be a promising agent for the treatment of HCC patients.

Supplementary material

Supplementary material is available online at [http://www.carcin.oupjournals.org/](http://www.carcin.oupjournals.org/).

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