FTY720 Induces Apoptosis in Hepatocellular Carcinoma Cells through Activation of Protein Kinase C \( \delta \) Signaling

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Abstract

This study was aimed at elucidating the mechanism by which FTY720, a synthetic sphingosine immunosuppressant, mediated antitumor effects in hepatocellular carcinoma (HCC) cells. The three HCC cell lines examined, Hep3B, Huh7, and PLC5, exhibited differential susceptibility to FTY720-mediated suppression of cell viability, with IC\(50\) values of 4.5, 6.3, and 11 \(\mu\)mol/L, respectively. Although FTY720 altered the phosphorylation state of protein kinase B and p38, our data refuted the role of these two signaling kinases in FTY720-mediated apoptosis. Evidence indicates that the antitumor effect of FTY720 was attributable to its ability to stimulate reactive oxygen species (ROS) production, which culminated in pro-apoptosis. FTY720 was synthesized according to a published procedure (25).

Introduction

FTY720 is a synthetic sphingosine immunosuppressant, which is currently undergoing clinical trials for the prevention of kidney graft rejection (1) and the treatment of relapsing multiple sclerosis (2). Previous studies indicate that the effect of FTY720 on prolonging the survival of allografts is attributable to the ability of its phosphorylated metabolite to inhibit T-lymphocyte infiltration by targeting several of the sphingosine-1-phosphate (S1P) receptors (3, 4). In addition to immunosuppression, FTY720 has also been shown to induce apoptosis in several human cancer cell lines, including Jurkat T cells (5), multiple myeloma cells (6), and those of liver (7, 8), prostate (9–12), breast (13), kidney (14), and bladder (15). This antitumor effect is noteworthy because of the involvement of S1P receptor-independent mechanisms (16). To date, a number of signaling pathways have been proposed to account for the ability of FTY720 to facilitate apoptosis in different cancer cell lines, including those mediated by protein kinase B (Akt) (5, 6, 8), mitogen-activated protein (MAP) kinases (6, 10), focal adhesion kinase (10), Rho-GTPase (12), signal transducers and activators of transcription 3, I\(\kappa\)B, nuclear factor-\(\kappa\)B, and Bcl-xL (6). Mechanistically, the targeting of this wide spectrum of signaling elements underscores the effectiveness of FTY720 in suppressing cell growth in a broad range of cancer cells that exhibit distinct mechanisms in governing cell cycle progression and apoptosis.

Hepatocellular cancer occurs both sporadically and is also related to chronic viral infection, environmental exposure, and alternative causes of hepatic cirrhosis. The US Surveillance, Epidemiology, and End Results database estimates that 19,160 men and women will be diagnosed with and 16,780 men and women will die of cancer of the liver and intrahepatic bile duct in 2007 within the United States (17). The incidence of hepatocellular carcinoma is even higher in the Asian cultures due to the higher frequency of chronic active viral hepatitis. Until only recently, effective treatment of hepatocellular cancer has been essentially absent (18). Recently, the RAF inhibitor sorafenib has been shown to be beneficial for the treatment of metastatic hepatocellular carcinoma and was approved for marketing by the Food and Drug Administration in this indication (19). However, this therapy only works in a subset of patients and is not curative. This emphasizes both the potential for multitargeted kinase inhibitors in hepatocellular carcinoma and the need to identify new therapies.

In this study, we describe a novel mechanism by which FTY720 induces apoptosis in hepatocellular carcinoma cells (HCC). We obtain evidence that FTY720 facilitates reactive oxygen species (ROS)-dependent activation of protein kinase C \(\delta\) (PKC\(\delta\)), resulting in caspase-3–mediated apoptotic death in HCC cells. PKC\(\delta\), a member of the novel PKC subfamily, has been shown to play a pivotal role in mediating apoptosis induced by oxidative stress (20, 21), Fas ligands (22), and various genotoxic agents including DNA damaging agents (23) and paclitaxel (24), thereby representing an important target for cancer therapy. Consequently, this mechanistic finding provides a molecular basis to pharmacologically exploit FTY720 to develop potent PKC\(\delta\)-targeted antitumor agents.

Materials and Methods

Reagents. FTY720 was synthesized according to a published procedure (25). The identity and purity were verified by nuclear magnetic resonance,
absorbance at 570 nm.

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death. In brief, Huh7 cells (5 × 10⁴) were incubated with the Cell Death Detection ELISA kit, which is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death. In brief, Huh7 cells (5 × 10⁴) were seeded and incubated in 96-well plates containing DMEM with 10% FBS for 16 h and were then subjected to various drug treatments for 24 h. After incubation, the plates were centrifuged at 200 × g for 10 min and incubated with 200 μL of lysis buffer at room temperature for 30 min. Twenty microliters of supernatant from each sample were used in the ELISA by following the manufacturer's instruction.

Detection of ROS. DCFDA is an indicator for ROS that is nonfluorescent until its acetate groups are removed by intracellular esterases and oxidation occurs in the cell. The ROS production in Huh7 cells after FTY720 treatment was detected using 5 μM/L DCFDA. Huh7 cells (7 × 10⁴) in 10-cm dishes were treated with FTY720 for 1 h, washed with PBS twice, and then exposed to 5 μM/L DCFDA for 30 min at 37°C. Cells were collected and analyzed by flow cytometry.

Detection of hydrogen peroxide. Quantitative determination of hydrogen peroxide in the culture medium was performed by using a colorimetric hydrogen peroxide kit (Assay Designs, Inc.) according to the vendor’s instruction. In brief, Huh7 cells (7 × 10⁴) were seeded and incubated in 10-cm dishes containing 10% FBS-supplemented DMEM for 16 h. Because the culture medium would interfere with the detection of hydrogen peroxide, it was replaced with an equal volume of Hanks’ balanced Salt Solution (HBSS) containing 10 μM/L FTY720. After 3-h exposure, 50 μL of the collected HBSS solution was incubated with 100 μL of the colorimetric solution provided in the kit in 96-well plates, and incubated at room temperature for 30 min. Absorbance at 570 nm was measured.

ShRNA-mediated knockdown of PKCo, PKCe, and PKCc. Huh7 cells (1 × 10⁵) were treated with 1.8 μg of individual shRNA plasmids (pKD-PKCo-v1, pKD-PKCe-v2, and pKD-PKCc-v5) and 0.2 μg of pCDNA 3.1 (+) plasmids using Invitrogen Lipofectamine 2000 reagent according to the manufacturer’s protocol. Cells were then selected by G418 for 2 weeks. The stable clones were established and confirmed by Western blotting using individual antibodies against PKCo, PKCe, and PKCc.

Ekctopic expression of constitutively active Akt. The pCDNA 3.1 (+)/CA-Akt-HA plasmid that encodes AktT308D/S473D, a constitutively active form of Akt, was provided by Dr. Matthew D. Ringel (The Ohio State University, Columbus, OH). Huh7 cells were transfected via nucleofection by using program T-022 of the AMAXA Nucleofector system according to the manufacturer’s instructions. Expression of constitutively active Akt was confirmed by Western blotting using antibodies against Akt and hemagglutinin tag.

Subcellular fractionation. Subcellular fractionation was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit according to the manufacturer’s instruction. In brief, Huh7 (7 × 10⁵) cells in 10-cm dishes were exposed to 10 μM/L FTY720 in 10% FBS–containing DMEM for different time intervals. After treatment, the cells were washed with cold PBS, scraped, and harvested by centrifugation. The cell pellets were suspended in 200 μL of Cytoplasmic Extraction Reagent I solution and incubated on ice for 10 min, followed by adding 11 μL of Cytoplasmic Extraction Reagent II solution and incubation on ice for 1 min. The cell suspensions were centrifuged at 16,000 × g for 5 min to collect supernatant as the cytoplasmic fraction. The pellets were resuspended with 100 μL of Nuclear Extraction Reagent on ice for 40 min. The cell suspension was centrifuged at 16,000 × g for 10 min at 4°C to collect supernatant as the nuclear fraction.

Analysis of caspase-3 activity. Caspase-3 activity was determined using (Ac-DMQD)2-Rh110 as the fluorogenic substrate for active caspase-3. Briefly, Huh7 (7 × 10⁵) cells in 10-cm dishes were exposed to 10 μM/L FTY720 in 10% FBS–containing DMEM for different time intervals. After treatment, the cells were washed with cold PBS, and fluorescence signals generated by caspase-3–cleaved substrate were analyzed by flow cytometry.

Cytochrome c release. Drug-treated Huh7 cells were collected and triritated with 100 μL of chilled hypotonic lysis solution [220 mmol/L mannitol, 68 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L EDTA, 2 mmol/L MgCl₂, and 1 mmol/L DTT in 50 mmol/L PIPES-KOH (pH 7.4)] for 45 min. The solution was centrifuged at 600 × g for 10 min to collect the supernatant. The supernatant was further centrifuged at 14,000 rpm for 30 min, and equal amounts of proteins (50 μg) from the supernatant were

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resolved in 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-cytochrome c antibodies.

**Statistical analysis.** The JMP5.0.1 software package was used to perform all analyses. Data were analyzed by the Student’s t test. Differences were considered significant at a P value of <0.05.

**Results**

**Differential susceptibility of HCC cell lines to FTY720-induced apoptotic death.** The in vitro antitumor efficacy of FTY720 was evaluated in three human HCC cell lines, Huh7, Hep3B, and PLC5, which are resistant to cytotoxic drugs due to loss of p53 function and/or overexpression of Bcl-xL (26). As these three cell lines harbor different cellular and genetic abnormalities, they showed differential susceptibility to the antiproliferative effect of FTY720. The IC50 values for Hep3B, Huh7, and PLC5 cells were 4.5, 6.3, and 11 μmol/L, respectively (Fig. 1A). The antiproliferative activity of FTY720 was, at least in part, attributable to apoptosis, as evidenced by poly(ADP)ribose polymerase (PARP) cleavage in each of these three cell lines and Annexin V/propidium iodide staining in Huh7 cells in a dose- and/or time-dependent manner (Fig. 1B and C).

**Involvement of PKCδ in FTY720-mediated apoptotic death.** To date, various signaling pathways have been reported to account for FTY720-induced apoptosis in different cancer cell systems, which might be reconciled by differences in the molecular abnormalities associated with the oncogenesis of individual cancer types. Of these proposed mechanisms, the ability of FTY720 to affect the activation status of p38 MAP kinase and Akt in prostate cancer cells (10) and HCC cells (8), respectively, was especially noteworthy.

To discern the role of Akt and p38 in FTY720-mediated apoptosis in HCC cells, we investigated the effect of FTY720 on the phosphorylation of these two signaling kinases in Huh7, Hep3B, and PLC5 cells. As these HCC cell lines contained functional PTEN, they exhibited low levels of Akt phosphorylation at Ser173 compared with the PTEN-defective PC-3 cancer cells (Fig. 2A). In contrast, this PTEN functional status had no direct correlation with the Thr308 phosphorylation, as all these three HCC cell lines exhibited high levels of p-Thr308-Akt relative to PC-3 (Fig. 2B). This site-specific phenomenon is consistent with the finding that levels of PTEN modulate Akt phosphorylation on Ser173, but not on Thr308, in rhabdomyosarcomas cells (27).

As shown in Fig. 2B, exposure to FTY720 for 24 h led to a dose-dependent decrease in p-Thr308-Akt levels in these three HCC cell lines (Fig. 2B), which is consistent with that previously reported (8). Moreover, the relative potency in Akt dephosphorylation correlated with the respective susceptibility to FTY720-induced cell death. In contrast, the effect of FTY720 on modulating p38 phosphorylation was cell line specific among the three cell lines examined, i.e., only PLC5 cells exhibited a dose-dependent increase in p-p38 levels after drug treatment.
To further examine whether Akt inhibition represented a major underlying antitumor mechanism for FTY720, we assessed the effect of the ectopic expression of a constitutively active form of Akt (AktT308D/S473D) on FTY720-induced cell death by transiently transfecting Huh7 cells with HA-CA-Akt plasmids (Fig. 2C). The constitutively activated status of Akt was manifested by the multifold increase in the phosphorylation level of GSK3β. However, this ectopic AktT308D/S473D expression did not provide a significant protection against the suppression of cell viability by 10 μmol/L FTY720. Together, these findings argued against the involvement of Akt and p38 in FTY720-induced apoptosis at least in these two sensitive cell lines.

To delineate the underlying mechanism, we further assessed the effect of a panel of pharmacologic inhibitors, including those of various signaling enzymes on FTY720-mediated cell death in Huh7 cells (left). Huh7 cells were exposed to 5 μmol/L FTY720 in the presence of one of the following inhibitors, okadaic acid (100 nmol/L), PD98059 (10 μmol/L), SB203580 (10 μmol/L), Ro6976 (10 μmol/L), rottlerin (10 μmol/L), and U3122 (10 μmol/L), for 24 h in 10% FBS–supplemented DMEM for 24 h. Cell viability was assessed by MTT assays. Right, dose-dependent effect of rottlerin on protecting Huh7 cells against FTY720-mediated cell death. Huh7 cells were exposed to DMSO vehicle or the indicated dose of rottlerin in the absence or presence of 10 μmol/L FTY720 for 24 h, and cell viability was analyzed by MTT assay. Columns, mean; bars, ±SD (n = 3).

E. rottlerin (10 μmol/L) inhibits the effect of FTY720 (10 μmol/L) on cytochrome c release into the cytoplasm and PARP cleavage (right) in Huh7 cells. Huh7 cells were exposed to 10 μmol/L FTY720 in the presence of 10 μmol/L rottlerin or DMSO vehiele in 10% FBS–containing DMEM for the indicated time intervals. Mitochondria-free lysates and total lysates were prepared as described in Materials and Methods for the Western blot analysis of cytochrome c release and PARP cleavage, respectively.

FTY720 Mediates Apoptosis through PKCα Activation
FTY720-mediated apoptotic death. It is noteworthy that PKCδ was differentially expressed among the three HCC cell lines (Fig. 3A). Relative to Huh 7 and Hep3B cells, PLC5 cells exhibited low levels of PKCδ expression, which might, in part, attribute to their relative insensitivity to FTY720-induced apoptosis.

**Role of PKCδ in FTY720-induced apoptosis.** To validate this premise, we examined the effect of shRNA-mediated knockdown of PKCδ vis-à-vis two other PKC isoforms (PKCα and PKCζ, representing conventional and novel PKC isoforms, respectively) on rescuing FTY720-mediated apoptotic death in Huh7 cells. Huh7 cells were transfected with plasmids encoding shRNA against individual PKC isoforms, followed by clonal selection. This selection led to three stable clones with substantially reduced PKCδ expression, and one stable clone each for PKCα and PKCζ (Fig. 3B-D). This shRNA knockdown was highly specific because no cross silencing of other PKC isoforms examined (α, ε, and η) was noted. As shown, only the knockdown of PKCδ rendered Huh7 cells resistant to FTY720-mediated apoptotic death (Fig. 3B), whereas that against PKCα or PKCζ exhibited no appreciable effect (Fig. 3C and D).

**Effect of FTY720 on PKCδ activation.** We showed that treatment of FTY720 led to PKCδ activation in Huh7 cells via two distinct mechanisms, i.e., phosphorylation and proteolytic cleavage. Of the four potential phosphorylation sites examined, FTY720 caused a rapid increase in the phosphorylation at Tyr<sup>311</sup>, Thr<sup>307</sup>, and Ser<sup>464</sup>, without affecting that of Tyr<sup>155</sup> (Fig. 4A, left). Moreover, this FTY720-facilitated phosphorylation at Thr<sup>307</sup> and Ser<sup>464</sup> could be blocked by 1 μmol/L rottlerin (right), suggesting that PKCδ mediated autophosphorylation on these two sites.

Evidence suggests that phosphorylation at Tyr<sup>311</sup> and that at Tyr<sup>155</sup> connote different physiologic roles in regulating the function of PKCδ (see Discussion). Moreover, proteolytic activation of PKCδ, which generated an active kinase domain, was also noted after 12 h of exposure to FTY720 (Fig. 4B, left). In addition to the cytoplasm, PKCδ and its processed form were also detected, to a lesser extent, in the nucleus (right), suggesting the potential involvement of a nucleus-dependent pathway in the antitumor effects of FTY720.

As there exists a mechanistic link between caspase-3 activation and the proteolytic cleavage of PKCδ (28), we examined the effect of FTY720 on modulating the activity of caspase-3 in Huh7 cells by using flow cytometric analysis. As shown, exposure to FTY720 led to a dose-dependent stimulation of caspase-3 activity (Fig. 4C). For example, FTY720 caused 3.5-fold and 4.8-fold increases in caspase activity at 5 and 10 μmol/L, respectively. Equally important, cotreatment with the caspase inhibitor Z-VAD-FMK abrogated the effect of FTY720 on facilitating the proteolytic cleavage of PKCδ (Fig. 4D), confirming the involvement of caspase-3 in PKCδ proteolysis. Overall, this suggests that proteolytic cleavage of PKCδ is a secondary event that occurs after activation of caspase family members.

**FTY720 activates PKCδ through a ROS-dependent mechanism.** We next sought to determine the upstream activator of caspases in hepatocellular cancer cell lines that promoted PKCδ activation and cleavage. A variety of reported caspase enzyme–activating stimuli have been shown to trigger PKCδ activation in different cell systems, including ROS (29), ceramide (30), tumor necrosis factor α (31), Fas ligand (31), and radiation (32). The
The present study shows that the effect of FTY720 on PKCδ activation was attributable to its ability to stimulate ROS generation in HCC cells.

Flow cytometric analysis using the ROS-sensitive probe DCFDA indicates that FTY720 at 10 μmol/L stimulated an immediate robust increase in ROS production in Huh7 cells, and that the level of intracellular ROS levels remained high even at 6 h after the treatment (Fig. 5A). The time course of this ROS production paralleled that of PKCδ phosphorylation. Moreover, FTY720 induced different levels of ROS production among the three HCC cell lines, in the order of Hep3B > Huh7 >> PLC5. It is noteworthy that this FTY720-stimulated ROS generation inversely correlated with the expression of GST-π (Fig. 5C), a phase II detoxification enzyme, which has been implicated in protection against apoptosis and drug resistance in cancer cells (33). However, the major ROS produced in response to FTY720 remained unclear because no significant increase in H2O2 generation was detected in FTY720-treated Huh7 cells (data not shown).

The effect of FTY720 on increasing intracellular ROS levels was independent of PKCδ activity. As shown in Fig. 6A, although the NADPH oxidase inhibitor DPI could significantly inhibit FTY720-stimulated production of ROS, rottlerin exhibited no appreciable effect on ROS levels. Moreover, DPI was able to suppress the effect of FTY720 on stimulating the phosphorylation and proteolytic cleavage of PKCδ (Fig. 6B), indicating the causative role of ROS in PKCδ activation. As a consequence, blockade of ROS generation by DPI protected Huh7 cells from FTY720-induced caspase-3 activation and apoptotic death, with potency similar to that of rottlerin (Fig. 6C). In contrast, DPI lacked appreciable effect on rescuing FTY720-facilitated Akt dephosphorylation, suggesting that Akt deactivation was not mechanistically linked to ROS-PKCδ signaling and did not play a crucial role in FTY720-mediated cell death (Fig. 6D). Overall, this suggests that FTY720 production of ROS and secondary activation of PKCδ contributes to the cytotoxicity observed in hepatocellular carcinoma cell lines treated with this agent.

Figure 4. FTY720 facilitates phosphorylation and caspase-3–dependent cleavage of PKCδ in Huh7 cells. A, time-dependent effect of FTY720 (10 μmol/L) on the phosphorylation of PKCδ at different sites (left). Right, effect of 1 μmol/L rottlerin on FTY720-facilitated PKCδ phosphorylation at Thr507 and Ser664. B, time-dependent effect of FTY720 (10 μmol/L) on the proteolytic cleavage of PKCδ in whole cell lysates (left) and in the cytosolic versus nuclear fractions (right). C, flow cytometric analysis of dose-dependent effect of FTY720 on increasing caspase-3 activity in Huh7 cells. Left, increased caspase-3 activity was observed in a dose-dependent manner after 24-h exposure to FTY720. Right, relative caspase-3 activities, normalized to DMSO control, at the indicated concentrations of FTY720. Columns, means; bars, ± SD (n = 3). D, the caspase inhibitor Z-VAD-FMK blocks the effect of FTY720 (10 μmol/L) on proteolytic cleavage of PKCδ. Cells were treated with DMSO vehicle or 10 μmol/L FTY720 in the presence of increasing doses of Z-VAD-FMK for 24 h, and cell lysates were subjected to immunoblotting with antibodies against anti-PKCδ and β-actin.
Discussion

In light of the therapeutic potential of FTY720, the mechanism underlying its antitumor effect warrants investigations. In this study, we obtained evidence that FTY720 suppressed the proliferation of HCC cells, at least in part, by stimulating ROS production, leading to PKC\(\gamma\) activation and subsequent caspase-3–dependent apoptosis. From a mechanistic perspective, ROS production represents an early hallmark event in the apoptotic effect of many therapeutic agents, including \(N\)-(4-hydroxyphenyl)-retinamide (34, 35), arsenic trioxide (36), parthenolide (37), and cisplatin (38). Relative to these agents, FTY720 represents a structurally distinct type of small-molecule agent with unique mechanistic features, which could be exploited to foster novel therapeutic strategies for HCC treatment.

It is well-documented that drug-induced ROS stress triggers a cascade of redox-dependent signaling events at difference cellular levels, culminating in mitochondria-dependent apoptosis (39). This study obtains evidence in HCC cells that PKC\(\gamma\) represents a major downstream effector of FTY720-generated ROS to facilitate caspase-3–dependent apoptosis, and that blockade of ROS production by the NADPH oxidase inhibitor DPI would protect caspase-3–dependent apoptosis, and that blockade of ROS major downstream effector of FTY720-generated ROS to facilitate mediated apoptosis. As shown, the expression levels of PKC\(\gamma\) mediated apoptosis. As shown, the expression levels of PKC\(\gamma\) on stimulating different extents of ROS production in Hep3B, Huh7, and PLC5 cells after 1 h of treatment. Columns, means; bars, ± SD (n = 3).

C, differential expression of GST-\(\pi\) protein in the three HCC cell lines.

Figure 5. FTY720 stimulates ROS production in HCC cells. A, time-dependent effect of FTY720 (10 \(\mu\)mol/L) on ROS production. Left, Huh7 cells were exposed to 10 \(\mu\)mol/L FTY720 in 10% FBS–containing DMEM for the indicated time intervals, stained with DCFDA, and subjected to flow cytometric analysis as described in Materials and Methods. Right, relative ROS production in Huh7 cells treated with 10 \(\mu\)mol/L FTY720 for the indicated time intervals. Columns, means; bars, ±SD (n = 3). B, dose-dependent effect of FTY720 on stimulating different extents of ROS production in Hep3B, Huh7, and PLC5 cells after 1 h of treatment. Columns, means; bars, ± SD (n = 3).

\[\text{Relative DCF intensity (Fold Increase)} \]

\[\text{Exposure time (h) 10 \(\mu\)mol/L FTY720} \]

\[\begin{array}{c|c|c|c|c}
\text{FTY720 (\(\mu\)mol/L), 1 h} & \text{Relative DCF intensity} \\
\hline
DMSO & 1 & 2 & 3 \\
5 & 1.5 & 2 & 3 \\
7.5 & 3 & 3.5 & 4 \\
10 & 4 & 4.5 & 5 \\
\end{array} \]

\[\text{B, dose-dependent effect of FTY720 on stimulating different extents of ROS production in Hep3B, Huh7, and PLC5 cells after 1 h of treatment. Columns, means; bars, ± SD (n = 3).} \]

\[\text{C, differential expression of GST-\(\pi\) protein in the three HCC cell lines.} \]

chemotherapy and low patient survival rates (40). For a mechanistic perspective, therapeutic interference of GST-\(\pi\) expression/activity by antisense or small-molecule agents provides a viable strategy to overcome this resistance (41, 42).

PKC\(\gamma\) has been shown to play an intriguing role in regulating apoptosis, either proapoptotic or antiapoptotic, in different cell systems (43, 44). This dichotomous behavior might be, in part, controlled by phosphorylation at different tyrosine residues of PKC\(\gamma\) by different kinases. For example, stress signals such as etoposide and \(H_2O_2\) promoted the phosphorylation of tyrosine residues at 64 and 187 (45), and especially 311 (29), respectively, which presumably committed PKC\(\gamma\) to activating caspases through phosphorylation. On the other hand, phosphorylation on Tyr155 was reported to promote the antiapoptotic effect of PKC\(\gamma\) in Sindbis virus–infected glioma cells (46). Accordingly, the selective phosphorylation of PKC\(\gamma\) at Tyr311 but not Tyr155, in FTY720-treated Huh7 cells underscores the proapoptotic nature of this signaling pathway.

An earlier report indicates that FTY720 induced apoptosis in HCC cells through phosphoinositide 3-kinase–mediated Akt dephosphorylation (8). This premise, however, was disputed by the finding that overexpression of constitutively active Akt could not rescue Huh7 cells from FTY720-mediated cell death. However, it is noteworthy that the mode of antitumor action of FTY720 might vary in different types of tumor cells. For example, in hematologic malignant cells such as those of chronic myelogenous leukemia and chronic lymphocytic leukemia, FTY720 induced caspase-independent apoptosis by activating PP2A signaling (47, 48). This PP2A activation, however, was not noted in HCC or prostate cancer cells.\(^4\) This cell line specificity

\(^4\) Y.S. Lu and C.S. Chen, unpublished data.
underlines the pleiotropic nature of FTY720 in killing cancer cells. In light of the heterogeneity in molecular and cellular abnormalities associated with oncogenesis and tumor progression, the ability of FTY720 to target different clinically relevant signaling mechanisms in different cancer types might have therapeutic relevance in cancer therapy. Consequently, this rationale constitutes the impetus of defining the causative mechanisms for the antitumor effects of FTY720 in different types of cancer cells.

In conclusion, this study shows the involvement of ROS-PKCδ activation in FTY720-mediated apoptosis in HCC cells, of which the therapeutic relevance is multifold. First, as PKCδ is mostly activated by genotoxic signals such as ionizing radiation (32) and etoposide (49), FTY720 represents a novel PKCδ activator without known genotoxic effects. Second, dissociation of the antitumor effect of FTY720 from its S1P activity provides a molecular basis to use it as a scaffold to develop potent antitumor agents devoid of the immunosuppressive activity. Third, FTY720 and its derivatives will be inherently suitable to treat cancers exhibiting increased oxidative stress with high levels of cellular ROS and a low antioxidant capability. Human HCC is prevalent in somatic GST-p gene silencing due to CpG island DNA hypermethylation (50), which provides a mechanistic rationale to include this type of ROS-generating agents in HCC therapy.

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