# Antitumor Effects of OSU-2S, a Nonimmunosuppressive Analogue of FTY720, in Hepatocellular Carcinoma

Hany A. Omar,[1,2] Chih-Chien Chou,[1] Lisa D. Berman-Booty,[1] Yihui Ma,[1] Jui-Hsiang Hung,[3] Dasheng Wang,[1] Takayuki Kogure,[4] Tushar Patel,[4] Luigi Terracciano,[5] Natarajan Muthusamy,[6] John C. Byrd,[6] Samuel K. Kulp,[1] and Ching-Shih Chen[1]

**Accumulating evidence suggests the therapeutic potential of the immunosuppressive agent FTY720 (fingolimod) in hepatocellular carcinoma (HCC). Based on our previous finding that FTY720 mediates apoptosis in HCC cells by activating reactive oxygen species (ROS)–protein kinase C*δ* (PKC*δ*) signaling independent of effects on sphingosine-1-phosphate (S1P) receptors, we embarked on the pharmacological exploitation of FTY720 to develop a nonimmunosuppressive analogue with antitumor activity. This effort led to the development of OSU-2S, which exhibits higher potency than FTY720 in suppressing HCC cell growth through PKC*δ* activation. In contrast to FTY720, OSU-2S was not phosphorylated by sphingosine kinase 2 (SphK2) *in vitro*, and did not cause S1P1 receptor internalization in HCC cells or T lymphocyte homing in immunocompetent mice. Although devoid of S1P1 receptor activity, OSU-2S exhibited higher *in vitro* antiproliferative efficacy relative to FTY720 against HCC cells without cytotoxicity in normal hepatocytes. Several lines of pharmacological and molecular genetic evidence indicate that ROS–PKC*δ*–caspase-3 signaling underlies OSU-2S–mediated antitumor effects, and that differences in the antitumor activity between FTY720 and OSU-2S were attributable to SphK2-mediated phosphorylation of FTY720, which represents a metabolic inactivation of its antitumor activity. Finally, OSU-2S exhibited high *in vivo* potency in suppressing xenograft tumor growth in both ectopic and orthotopic models without overt toxicity. *Conclusion*: Using the molecular platform of FTY720, we developed OSU-2S, a novel PKC*δ*-targeted antitumor agent, which is devoid of S1P1 receptor activity and is highly effective in suppressing HCC tumor growth *in vivo*. These findings suggest that OSU-2S has clinical value in therapeutic strategies for HCC and warrants continued investigation in this regard. (HEPATOLOGY 2011;53:1943-1958)**

*Abbreviations: CA-Akt, constitutively active Akt; DAPI, 4,6-diamidino-2-phenylindole; DCFDA (5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate); DMEM, Dulbecco's modified Eagle medium; DMS,* N,N*-dimethylsphingosine; DPI, diphenyleneiodonium; FBS, fetal bovine serum; GST-π, glutathione S-transferase-π; HA, hemagglutinin; HCC, hepatocellular carcinoma; Hep3B-luc, luciferase-expressing Hep3B; i.p., intraperitoneal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PARP, poly (ADP-ribose) polymerase; p-FTY720, phosphorylated FTY720; PKCδ, protein kinase Cδ; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; shRNA, short hairpin RNA; SphK2, sphingosine kinase 2; TLC, thin-layer chromatography; TMA, tissue microarray.*

*From the [1]Division of Medicinal Chemistry, College of Pharmacy, and Divisions of [4]Gastroenterology, Hepatology, and Nutrition, and [6]Hematology and Oncology, Department of Internal Medicine, The Ohio State University, Columbus, OH; [2]Department of Pharmacology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt; [3]Department of Biotechnology, Chia Nan University of Pharmacy and Science, Tainan, Taiwan; and [5]Molecular Pathology Division, Institute of Pathology, University Hospital, Basel, Switzerland.*

*Received September 21, 2010; accepted March 1, 2011.*

*Supported by Public Health Service Grants R21 CA133710 and R01 CA112250 from the National Cancer Institute of the National Institutes of Health (to C.S.C.), by the Lucius A. Wing Chair Fund of The Ohio State University Medical Center (to C.S.C.), and a Specialized Center of Research grant from the Leukemia and Lymphoma Society (to C.S.C., N.M., and J.C.B.).*

*Address reprint requests to: Ching-Shih Chen, Ph.D., Division of Medicinal Chemistry, College of Pharmacy, L. M. Parks Hall, Ohio State University, 500 West 12th Avenue, Columbus, OH 43210. E-mail: chen.844@osu.edu; fax: 614-688-8556.*



*View this article online at wileyonlinelibrary.com.*

*DOI 10.1002/hep.24293*

*Potential conflict of interest: Nothing to report.*

*Additional Supporting Information may be found in the online version of this article.*

Hepatocellular carcinoma (HCC) is a leading cause of cancer death worldwide, and is expected to become more prevalent in the United States over the next decade due to the dramatic rise in the incidence of hepatitis C virus infection. A major challenge in the systemic treatment of HCC is cellular resistance to conventional cytotoxic agents, which may be attributed to the heterogeneity of genetic abnormalities acquired during the course of hepatocarcinogenesis.[1] Consequently, targeting molecular defects that allow HCC cells to evade apoptosis represents a viable strategy to improve patient outcome. Accordingly, a number of therapeutic agents targeting aberrant cellular growth and survival signaling pathways have been investigated for the treatment of HCC.[2] Recently, sorafenib, a multikinase inhibitor, was approved for the treatment of advanced HCC.[3] This therapy, however, only works in a subset of patients and is not curative, which underlies the urgency in identifying new therapies.

FTY720, a synthetic sphingosine immunosuppressant, was recently approved for the treatment of relapsing multiple sclerosis.[4] Its immunosuppressive effect is attributed to the ability of its phosphorylated metabolite (p-FTY720) to induce T lymphocyte homing by targeting sphingosine-1-phosphate (S1P) receptors.[5] Recent studies indicate that FTY720 induces apoptosis and inhibits angiogenesis and metastasis in HCC,[6,7] suggesting its translational potential as a cancer therapeutic agent. However, in addition to side effects common to immunomodulatory therapy, FTY720 was reported to cause cardiovascular complications, macular edema, and brain inflammation,[4] presumably the result of interactions with more than one S1P-receptor subtype.[8] Previously, we demonstrated that FTY720 induces apoptosis in HCC cells through the reactive oxygen species (ROS)-dependent activation of protein kinase C (PKC)$\delta$.[7] Dissociation of the apoptosis-inducing activity of FTY720 from its S1P receptor agonist activity provides a basis for its pharmacological exploitation to develop a novel class of antitumor agents. Here, we report the development of a nonimmunosuppressive FTY720 analogue, OSU-2S [(*S*)-2-amino-2-(4-[(6-methylheptyl)-oxy]phenethyl)pentan-1-ol], which exhibits higher *in vitro* and *in vivo* potency than FTY720 in suppressing HCC cell growth through PKC$\delta$ signaling.

## Materials and Methods

Details about reagents, their commercial sources, and experimental procedures are provided in the Supporting Information.

***Cell Lines, Culture, and Reagents.*** The HCC cell lines, Hep3B, PLC5 and Huh7, and primary nonmalignant human hepatocytes were used in this study. FTY720 was synthesized as described,[9] and p-FTY720 was purchased from Cayman Chemical (Ann Arbor, MI). Synthesis of OSU-2S and phosphorylated OSU-2S (p-OSU-2S) will be described elsewhere. Various polyclonal and monoclonal antibodies were used for western blotting, immunocytochemical, and flow cytometric analyses.

***Cell Viability Assay.*** Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assays as previously reported.[10]

***Flow Cytometry.*** For assessment of apoptosis, treated cells were stained with Annexin V-Alexa Fluor 488 and propidium iodide according to the vendor's protocols. For caspase-3 activity, cells were incubated with the fluorogenic caspase-3 substrate (Ac-DMQD)2-Rh110 for 20 minutes. ROS production was detected using the fluorescence probe 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) as described.[7] Data were analyzed by ModFitLT V3.0 software program.

***Immunoblotting.*** Immunoblotting of biomarkers in cell lysates and tumor tissue homogenates was performed as reported.[10]

***Small Interfering RNA- or Short Hairpin RNA–Mediated Knockdown.*** PLC5, Huh7, and Hep3B cells were transfected with siRNA duplexes for knockdown of glutathione S-transferase (GST)-$\pi$ sphingosine kinase (SphK)2, and gp91$^{phox}$, or shRNA plasmid for knockdown of PKC$\delta$ according to the manufacturer's protocol. Stable clones were isolated from Huh7 cells transfected with shRNA plasmids using geneticin. Knockdowns were confirmed by iummunoblotting.

***Overexpression of S1P1 Receptors, GST-π or Constitutively Active Akt (CA-Akt).*** Huh7 or Hep3B cells were transfected with plasmids encoding S1PR1, Flag-tagged GST-$\pi$ or hemagglutinin (HA)-tagged CA-Akt. The corresponding empty vectors served as controls. Stable clones were selected using geneticin, and the expression of cloned proteins was confirmed by immunoblotting.

***Phosphorylation of FTY720 and OSU-2S by SphK2.*** Analysis of SphK2-mediated phosphorylation was performed as reported[11] with modifications. Five $\mu$M FTY720 or OSU-2S was incubated with 0.75 $\mu$g/mL human recombinant SphK2, 5 $\mu$Ci [$\gamma$-$^{32}$P]-ATP, and 0.5 mM cold ATP (37°C, 60 minutes). The reaction products were separated by silica-gel thin layer chromatography (TLC) and visualized by autoradiography.

***Immunocytochemistry.*** Immunocytochemical analysis of S1P1 internalization and PKC$\delta$ nuclear translocation

were performed as described[12] with modifications. After treatment, fixation and permeabilization, cells were incubated with rabbit anti-S1P1 or rabbit anti-PKC$\delta$ antibodies (1:200 dilution, 4°C, 24 hours), followed by Alexa Fluor 488–conjugated goat anti-rabbit IgG (room temperature, 1 hour).

**In Vivo *Evaluation of T-Lymphocyte Homing.*** CD2F1 mice were treated via intraperitoneal (i.p.) injection with FTY720 or OSU-2S, at 1, 2.5, or 5 mg/kg, or vehicle. Six hours later, animals were sacrificed, and peripheral blood mononuclear cells were prepared as described.[13] Cells were stained with FITC-labeled rat anti-mouse CD3 molecular complex and PE-labeled rat anti-mouse CD45RA (4°C, in darkness, 30 minutes), and analyzed by flow cytometry.

***Measurement of NADPH Oxidase Activity.*** Superoxide production was measured in the membrane fraction of drug- versus vehicle-treated Hep3B cells by using lucigenin-derived chemiluminescence according to a reported procedure.[14]

***Xenograft Tumor Models.*** Ectopic tumors were established in athymic nude mice by subcutaneous injection of Hep3B cells. Mice with established tumors were randomized to five groups (n = 8) receiving daily i.p. injections of OSU-2S or FTY720 at 5 or 10 mg/kg, or vehicle. Tumor burdens were determined weekly using calipers. Body weights were measured weekly. At the study endpoint, tumors were harvested, snap-frozen and stored at −80°C for biomarker analysis. A panel of 22 tissues was collected for toxicopathological evaluation. For further assessment of potential toxicities, additional mice were treated as described above for 21 days, after which blood was collected for determinations of complete blood counts and serum chemistry. To assess effects on intratumoral NADPH oxidase expression, ectopic Hep3B tumor-bearing mice were treated for 7 days as described above, after which $gp91^{phox}$ expression in tumor homogenates was evaluated by western blotting.

Orthotopic tumors were established by direct intrahepatic injection of luciferase-expressing Hep3B (Hep3B-luc) cells and their growth was monitored by bioluminescent imaging (IVIS, Xenogen, Alameda, CA) as described.[10] Two weeks after tumor cell injection, animals with established tumors were randomized to three groups (n = 3) that received daily i.p. injections of FTY720 or OSU-2S at 5 mg/kg, or vehicle for 42 days and were imaged weekly.

All animal use was done in accordance with protocols approved by The Ohio State University Institutional Animal Care and Use Committee.

***Expression of PKCδ in HCC and Non-Neoplastic Human Liver Tissues.*** A tissue microarray (TMA) containing both HCC and non-neoplastic liver tissues was constructed from archival paraffin-embedded tissue samples as described.[15] The TMA was immunostained for PKC$\delta$ and expression was evaluated in 163 HCC and 71 non-neoplastic liver samples using a semiquantitative scoring system (0 = negative; 1 = weak; 2 = moderate; 3 = strong).

***Statistical Analysis.*** Differences among group means were analyzed for statistical significance using one-way analysis of variance followed by the Neuman-Keuls test for multiple comparisons. Differences in the proportions of PKC$\delta$-positive HCC and non-neoplastic liver samples were analyzed by Fisher's exact test. Differences were considered significant at $P < 0.05$. Analysis was performed using GraphPad InStat for Windows (GraphPad Software, San Diego, CA).

## Results

***OSU-2S, a Nonimmunosuppressive FTY720 Analogue With Higher Antiproliferative Potency.*** Based on our finding that the antitumor effect of FTY720 in HCC cells was mediated through PKC$\delta$ activation, we hypothesized that these two pharmacological activities, i.e., immunosuppression and anti-proliferation, could be dissociated via structural modifications. Thus, FTY720 was used as scaffold to establish a small focused compound library for lead identification. Among more than 20 derivatives examined, OSU-2S (Fig. 1A) lacked immunomodulatory activity, yet exhibited higher potency than FTY720 in inducing apoptotic death in HCC cells, thereby providing a proof-of-concept of our hypothesis.

FTY720 acts as a prodrug that undergoes SphK2-catalyzed phosphorylation to mediate its immunomodulatory function.[16] Radiometric analysis, followed by TLC, revealed that, in contrast to FTY720, OSU-2S was not phosphorylated by recombinant SphK2 (Fig. 1B). Although not a SphK2 substrate, OSU-2S's phosphate derivative, p-OSU-2S, was synthesized to test its ability vis-à-vis p-FTY720, FTY720, and OSU-2S to facilitate the internalization of S1P receptors, a key mechanism underlying FTY720-mediated immunomodulation,[17] in S1P1 receptor-overexpressing Huh7 cells. Immunocytochemical analysis indicated a profound effect of FTY720 and p-FTY720 on receptor internalization and clustering in the cytoplasm, whereas OSU-2S or p-OSU-2S failed to show any appreciable effect (Fig. 1C). To confirm these results, the effects of these agents on T-lymphocyte homing *in vivo* were evaluated in CD2F1 mice. Treatment with FTY720 for 6 hours, even at 1 mg/kg, caused a

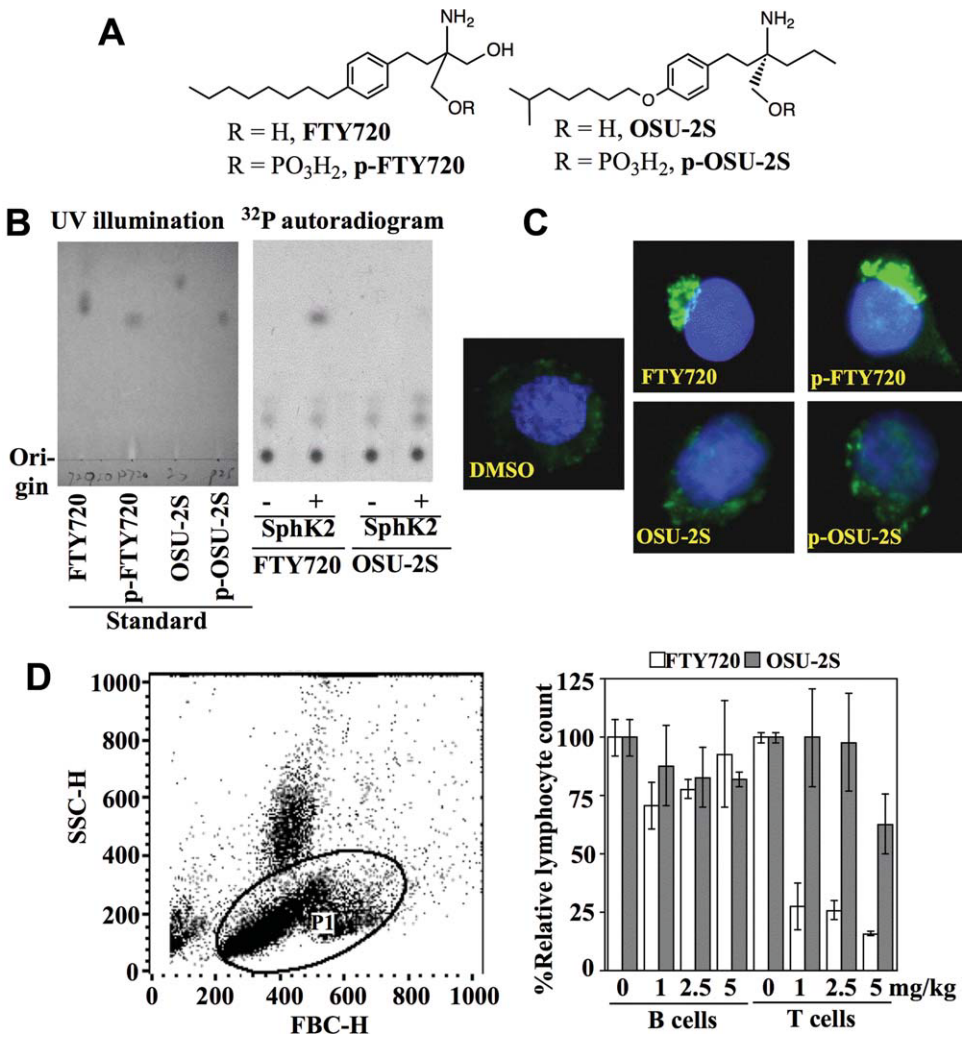


Fig. 1. Evidence that OSU-2S is nonimmunosuppressive. (A) Chemical structures of FTY720 and OSU-2S. (B) OSU-2S is not a substrate of sphingosine kinase 2 (SphK2). Left panel, thin-layer chromatogram of the standard compounds FTY270, p-FTY720, OSU-2S, and p-OSU-2S, visualized by UV illumination. Right panel, autoradiogram of the reaction products from the incubation of FTY720 or OSU-2S with [$\gamma$-$^{32}$P]ATP in the presence (+) or absence (−) of recombinant SphK2 after separation by thin-layer chromatography. (C) Immunocytochemical analysis of sphingosine-1-phosphate (S1P) receptor localization in S1P1 receptor-overexpressing Huh7 cells treated with DMSO vehicle, FTY720, p-FTY720, OSU-2S, or p-OSU-2S, each at 2.5 $\mu$M, in 5% FBS-supplemented DMEM medium for 6 hours. Green and blue colors denote S1P receptors and DAPI-stained nuclei, respectively. (D) Flow cytometric analysis of numbers of lymphocytes in the peripheral blood collected from male CD2F1 mice 6 hours after treatment with FTY720 or OSU-2S, at 1, 2.5, or 5 mg/kg, or vehicle via i.p. injection. Left panel, dot-plot shows the gating of the lymphocyte population as P1 based on forward and side-scatter profiles. Right panel, effect of FTY720 and OSU-2S relative to vehicle control on the numbers of circulating B and T-lymphocytes in the peripheral blood of immunocompetent CD2F1 mice at 6 hours posttreatment. Columns, mean; bars, ± SD (n = 3).

precipitous drop (≥75%) in the number of T lymphocytes in peripheral blood, whereas OSU-2S exerted no appreciable effect at 1 and 2 mg/kg and a modest decrease at 5 mg/kg (Fig. 1D). Because OSU-2S and p-OSU-2S had no effect on S1P1 receptor internalization, the reason for this modest suppression in circulating T lymphocytes is unclear; however, it appears to be a transient change, because total lymphocyte counts were unchanged in mice treated with this dose of OSU-2S for 21 days (Supporting Table 1). In contrast, no comparable changes in circulating B lymphocytes were noted with either agent.

The antitumor effects of OSU-2S vis-à-vis FTY720 were examined in three different HCC cell lines, Huh7, Hep3B, and PLC5, and in normal human hepatocytes by MTT assays. OSU-2S exhibited nearly twofold higher potency than FTY720 in suppressing the viability of HCC cells (Fig. 2A). The $IC_{50}$ values in Huh7, Hep3B, and PLC5 cells after 24 hours of treatment were: OSU-2S: 2.4 $\mu$M, 2.4 $\mu$M, and 3.5 $\mu$M, respectively; FTY720: 4.8 $\mu$M, 4.2 $\mu$M, and 6.2 $\mu$M, respectively. Relative to malignant cells, normal human hepatocytes were resistant to both compounds.

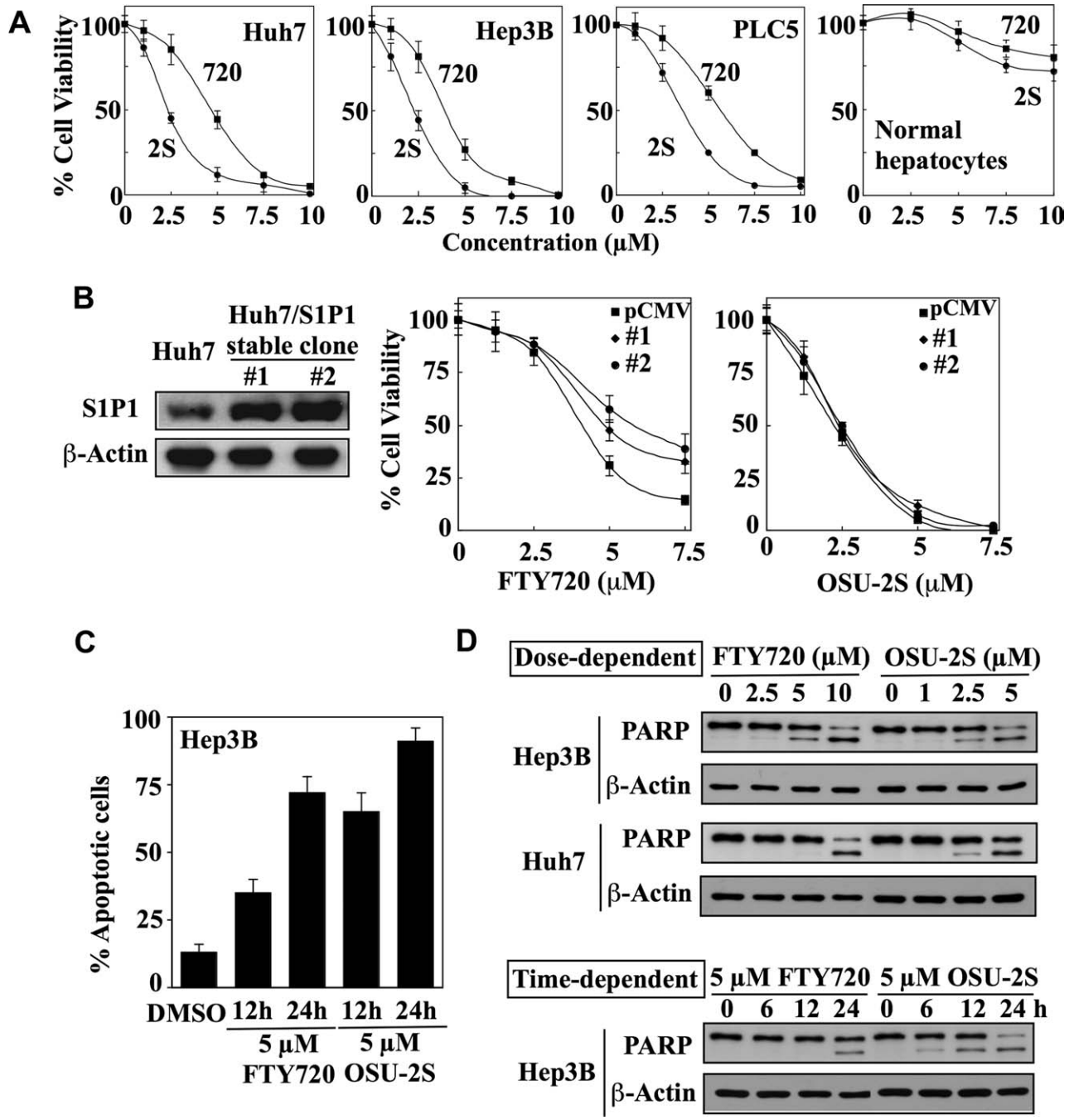


Fig. 2. OSU-2S exhibits a higher potency than FTY720 in inducing apoptotic death in HCC cells independent of sphingosine-1-phosphate 1 (S1P1) receptor agonist activity. (A) Dose-dependent suppressive effects of OSU-2S (2S) and FTY720 (720) on the viability of Huh7, Hep3B, and PLC5 cells relative to normal human hepatocytes after 24 hours of treatment. Points, mean; bars, ± SD (n = 6). (B) Effect of ectopic S1P1 receptor expression on the sensitivity of Huh7 cells to FTY720- versus OSU-2S-mediated inhibition of cell viability. Left, western blot analysis of the differential expression levels of S1P1 receptors in untransfected Huh7 cells versus two stable S1P1 receptor transfectants. Right, effect of FTY720 and OSU-2S on the viabilities of untransfected Huh7 cells and stable S1P1 receptor-overexpressing clones (#1 and #2). Points, mean; bars, ± SD (n = 6). (C) Flow cytometric analysis of apoptosis in Hep3B cells treated with 5 $\mu$M of FTY720 or OSU-2S for 12 and 24 hours. The cells were stained with fluorescein-conjugated Annexin V and propidium iodide (PI). Apoptotic cells included both Annexin V+/PI− (early apoptotic) and Annexin+/PI+ (late apoptotic) cells. Columns, mean; bars, SD (n = 3). (D) Western blot analysis of the dose- and time-dependent effects of FTY720 and OSU-2S on poly (ADP-ribose) polymerase (PARP) cleavage in Hep3B and Huh7 cells.

As mentioned, OSU-2S exhibits higher antitumor activity than FTY720 but lacks immunosuppressive activity. To demonstrate that its antitumor effect was independent of S1P1 receptors, we evaluated the effect of ectopic S1P1 receptor expression on the antiproliferative activities of OSU-2S vis-à-vis FTY720 in

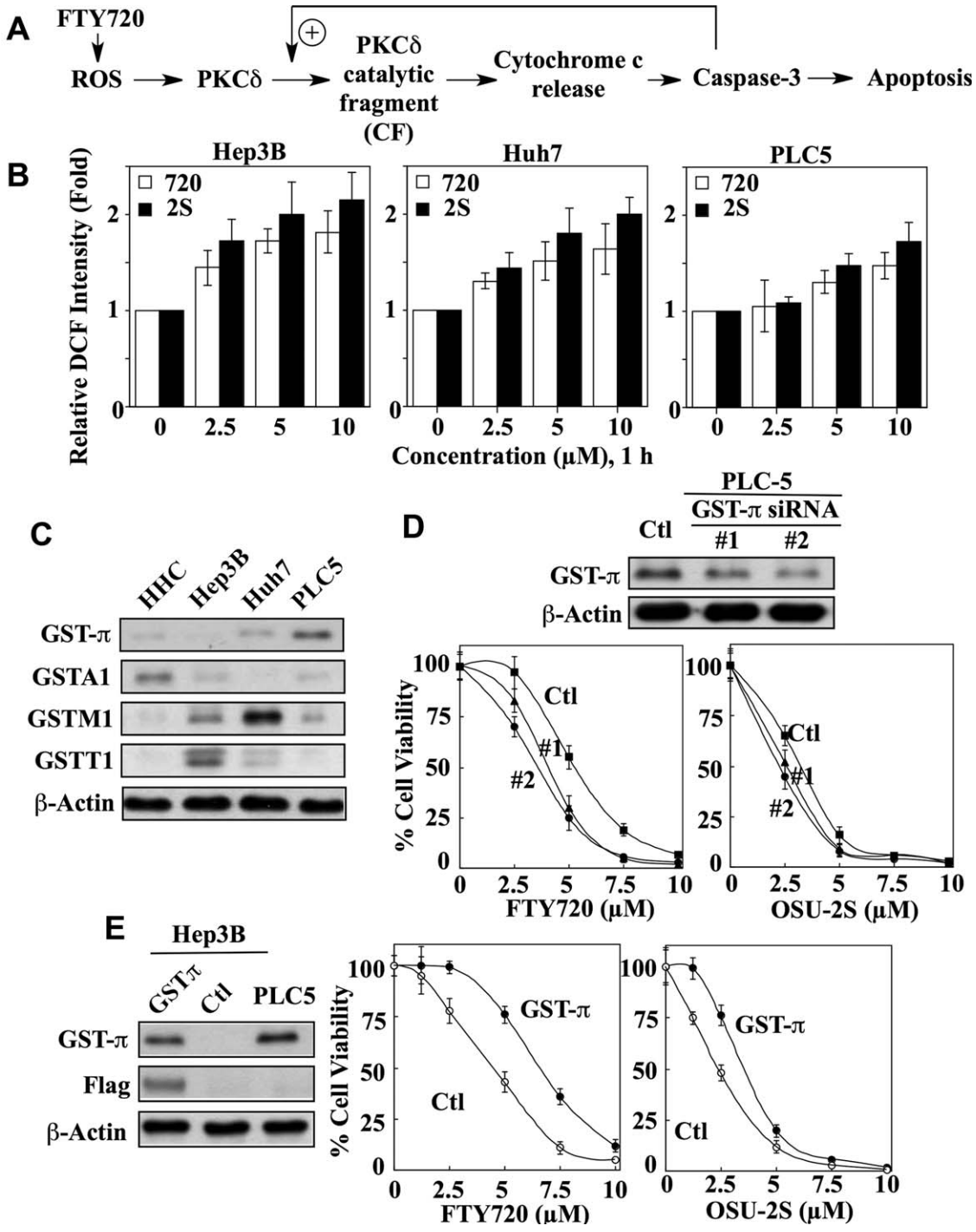


Fig. 3. The reactive oxygen species (ROS)-PKC$\delta$ signaling pathway is involved in OSU-2S–mediated apoptosis in HCC cells. (A) Schematic diagram depicting the mode of action of FTY720 in eliciting PKC$\delta$-dependent apoptosis. (B) Dose-dependent effect of OSU-2S (2S) versus FTY720 (720) on ROS production in Hep3B, Huh7, and PLC5 cells after 1 hour of treatment. DCF, dichlorofluorescein. Columns, mean; bars, $\pm$ SD (n $=$ 3). (C) Western blot analysis of the expression of the four GST isozymes (GST-$\pi$, GSTA1, GSTM1, and GSTT1) in normal human hepatocytes (HHC), Hep3B, Huh7, and PLC5 cells. (D) Effect of siRNA-mediated GST-$\pi$ knockdown on the sensitivity of PLC5 cells to FTY720 and OSU-2S. Upper, differential expression of GST-$\pi$ in PLC5 cells transiently transfected with scrambled (Ctl) or GST-$\pi$ siRNA (#1 and #2). Lower, the antiproliferative activities of FTY720 and OSU-2S in the two GST-$\pi$ siRNA transfectants (#1 and #2) versus control (Ctl) cells. Points, mean; bars, $\pm$ SD (n $=$ 6). (E) Effect of ectopic expression of GST-$\pi$ on the sensitivity of Hep3B cells to FTY720 and OSU-2S. Left, expression levels of GST-$\pi$ in Hep3B cells transiently transfected with a Flag-GST-$\pi$ plasmid versus untransfected Hep3B (Ctl) and PLC5 cells. Middle and right panels, the antiproliferative activities of FTY720 and OSU-2S in GST-$\pi$–overexpressing Hep3B cells versus control. Points, mean; bars, $\pm$ SD (n $=$ 6).

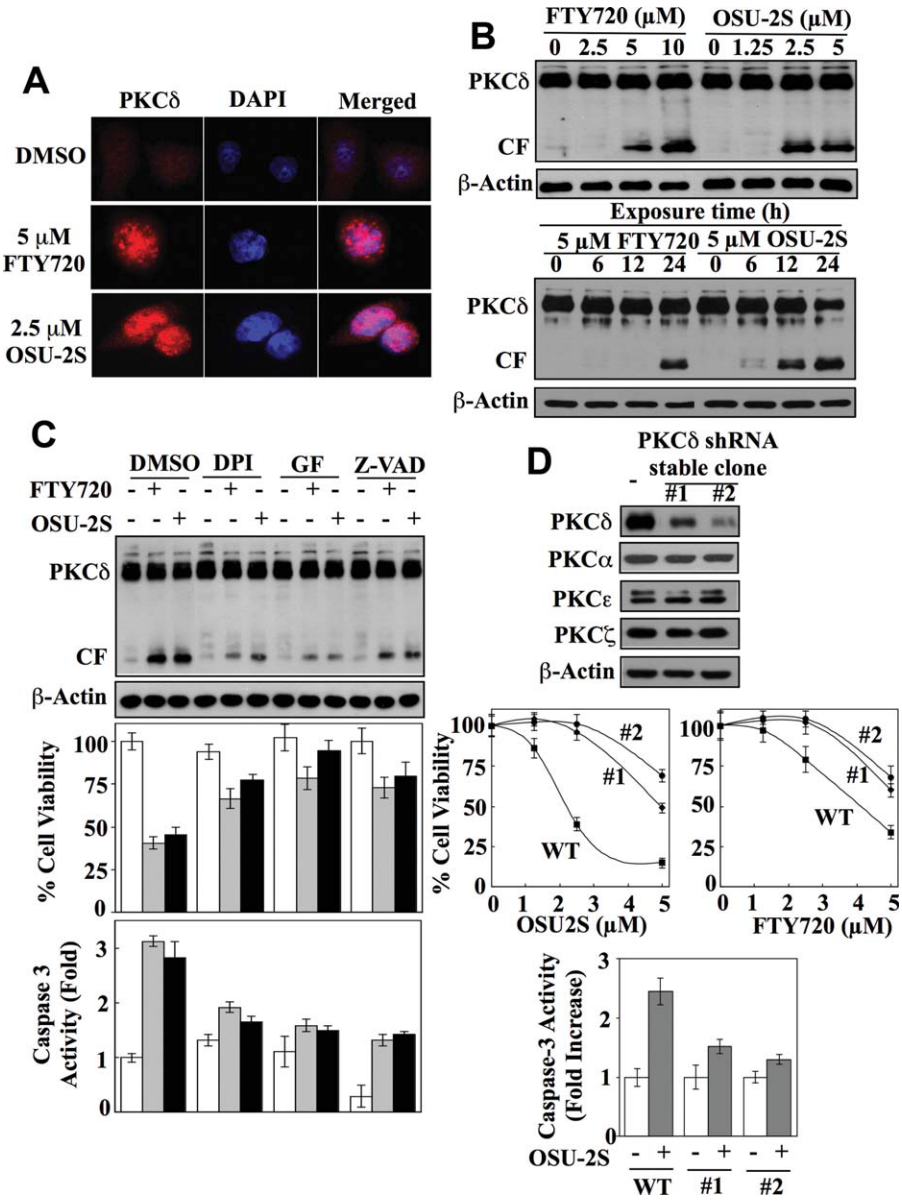


Fig. 4. Role of PKC$\delta$–caspase-3 signaling in mediating the antiproliferative effect of OSU-2S. (A) Immunocytochemical analysis of the effect of FTY720 and OSU-2S, each at 5 $\mu$M, on the nuclear localization of PKC$\delta$ (red) in Huh7 cells after 24 hours of treatment. Cells were counterstained with DAPI (blue) to visualize the nuclei. (B) Dose- (upper panel) and time-dependent (lower panel) effects of FTY720 and OSU-2S on the activation of PKC$\delta$ by proteolytic cleavage in Huh7 cells as indicated by the appearance of the catalytic fragment (CF). (C) Effects of the pharmacological inhibitors of NADPH oxidase (diphenyleneiodonium [DPI], 10 $\mu$M), PKC$\delta$ (GF-109293X [GF], 5 $\mu$M), and caspases (Z-VAD-FMK [Z-VAD], 20 $\mu$M) on the abilities of FTY720 (5 $\mu$M) and OSU-2S (2.5 $\mu$M) to induce PKC$\delta$ activation (top), to suppress cell viability (middle), and to enhance caspase-3 activity (lower) in Huh7 cells. CF, catalytic fragment. Columns, mean; bars, $\pm$SD (n = 6). (D) Knockdown of PKC$\delta$ expression diminishes the ability of FTY720 and OSU-2S to mediate cell death and caspase-3 activation in Huh7 cells. Upper panel, western blot analysis of shRNA-mediated knockdown of PKC$\delta$ in Huh7 cells showing the expression levels of PKC$\delta$, PKC$\alpha$, PKC$\epsilon$, and PKC$\zeta$ in two stable clones (#1, #2) relative to the untransfected control. Central panel, knockdown of PKC$\delta$ protects Huh7 cells from FTY720- and OSU-2S-mediated suppression of cell viability. WT, wild-type. Points, mean; bars, $\pm$ SD (n = 6). Lower panel, knockdown of PKC$\delta$ protected Huh7 cells from OSU-2S–induced activation of caspase-3 activity. Columns, mean; bars, $\pm$ SD (n = 6).

Huh7 cells. Two stable clones exhibiting different levels of ectopic S1P1 receptor expression and wild-type Huh7 cells were treated with different concentrations of FTY720 or OSU-2S. Although S1P1 receptor overexpression partially protected Huh7 cells against FTY720 in an expression level-dependent manner, no protective effect was noted in OSU-2S-treated cells (Fig. 2B). Annexin V/PI staining and PARP cleavage indicated that OSU-2S mediated cell death primarily through apoptosis in a manner similar to FTY720 with relative potency paralleling that determined by MTT assays (Fig. 2C,D).

***OSU-2S Induces Caspase-Dependent Apoptosis Through ROS-Dependent PKCδ Activation.*** Previously, we demonstrated that FTY720 facilitates ROS-dependent PKCδ activation, leading to increased caspase-3 activity, which, in turn, activates PKCδ via proteolytic cleavage in HCC cells (Fig. 3A).[7] The

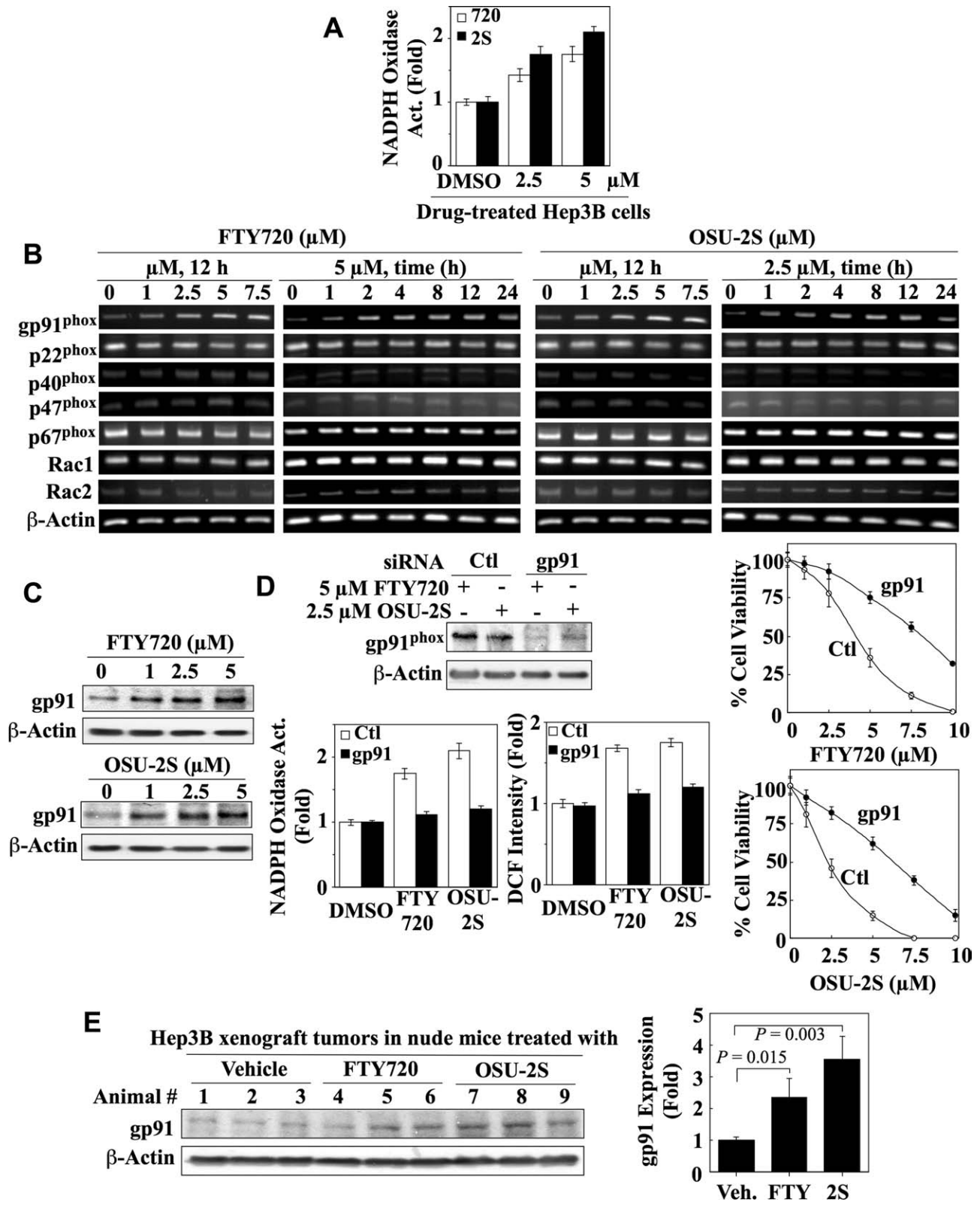


Fig. 5.

following evidence indicates that this signaling axis also underlies OSU-2S–mediated apoptosis.

Flow cytometry analysis using the ROS-sensitive probe DCFDA showed that OSU-2S stimulated ROS production to a greater extent than FTY720 in all three HCC cell lines examined (Fig. 3B). Moreover, the degree to which these two agents induced ROS levels paralleled their relative antiproliferative potencies in these cell lines, i.e., Hep3B → Huh7 → PLC-5. We rationalized that the differential induction of ROS production resulted from differences in the enzyme antioxidant capacity among these cell lines. Of the four representative GST isozymes examined (GST-$\pi$, GSTA1, GSTM1, GSTT1), the expression levels of GST-$\pi$ in Hep3B, Huh7, and PLC5 cells was inversely related to their respective sensitivities to FTY720- and OSU-2S–induced cell death (Fig. 3C versus Fig. 2A). For example, PLC5 cells, which exhibited the highest GST-$\pi$ expression, were least sensitive among these three cell lines. These findings are consistent with the notion that GST-$\pi$ represents a marker of drug resistance in HCC.[18] In contrast, normal human hepatocytes expressed low GTS-$\pi$ levels, suggesting a GST-independent basis for their resistance to these compounds.

To validate the relationship between GST-$\pi$ and drug resistance, we evaluated the effects of altering GST-$\pi$ expression on drug sensitivity. First, siRNA-mediated knockdown of GST-$\pi$ in PLC5 cells shifted the dose-response curves of OSU-2S and FTY720 to the left in two transient transfectants exhibiting different levels of target suppression relative to the scrambled siRNA control (Fig. 3D). Second, ectopic expression of GST-$\pi$ in Hep3B cells via transient transfection with a Flag-tagged GST-$\pi$ plasmid (Fig. 3E, left) conferred protection against the suppressive effect of FTY720 and OSU-2S on cell viability (right).

The stimulation of ROS generation by FTY720 and OSU-2S was accompanied by PKC$\delta$ activation in drug-treated Huh7 cells with parallel potency, as manifested by nuclear translocation (Fig. 4A) and dose- and time-dependent accumulation of the catalytic fragment (Fig. 4B). Translocation of PKC$\delta$ to the nucleus and subsequent proteolytic cleavage are necessary and sufficient to induce apoptosis in cancer cells.[19] The role of the ROS-PKC$\delta$-caspase-3 signaling axis in mediating OSU-2S's antiproliferative effect was further corroborated by the use of pharmacological inhibitors of pertinent cellular responses, i.e., the NADPH oxidase inhibitor diphenyleneiodonium (DPI), the PKC$\delta$ inhibitor GF-109293X, and the pancaspase inhibitor Z-VAD-FMK. These inhibitors blocked the abilities of OSU-2S (2.5 $\mu$M) and FTY720 (5 $\mu$M) to induce the proteolytic cleavage of PKC$\delta$ (Fig. 4C, upper), to suppress cell viability (middle), and to stimulate caspase-3 activity (lower).

To confirm the intermediary role of PKC$\delta$ in OSU-2S's antiproliferative effect, we assessed the effect of shRNA-mediated PKC$\delta$ knockdown on the viability and caspase-3 activity of drug-treated Hep3B cells. Transfection with plasmids encoding shRNA against PKC$\delta$ followed by clonal selection yielded two stable clones expressing different residual levels of PKC$\delta$ without cross-silencing of the other PKC isozymes examined ($\alpha$, $\epsilon$, and $\zeta$) (Fig. 4D, upper). These two stable clones displayed differential protection against the antiproliferative effects of OSU-2S and FTY720 (middle). Moreover, this silencing of PKC$\delta$ expression suppressed the ability of OSU-2S to enhance caspase-3 activity (lower).

***OSU-2S Activates NADPH Oxidase by Up-Regulating gp91$^{phox}$ Expression.*** Our finding that DPI inhibited PKC$\delta$ activation by FTY720 and OSU-2S suggests the involvement of NADPH oxidase in drug-induced ROS production. This mechanistic link was supported by concentration-dependent increases in NADPH oxidase activity in the membrane fractions of FTY720- and OSU-2S–treated Hep3B cells (Fig. 5A). This activation, however, was not attributable to a direct effect on the enzyme as neither agent stimulated

Fig. 5. Evidence that OSU-2S and FTY720 activate NADPH oxidase by up-regulating the gp91$^{phox}$ expression in Hep3B cells. (A) Dose-dependent increase in NADPH oxidase activity in the membrane fraction isolated from Hep3B cells treated with OSU-2S (2S) or FTY720 (720) at the indicated concentration for 24 hours. Columns, mean; bars, $\pm$ SD (n = 3). (B) RT-PCR analysis of the dose- and time-dependent effects of FTY720 and OSU-2S on the mRNA expression of NADPH oxidase subunits in Hep3B cells. (C) Western blot analysis of the dose-dependent effects of FTY720 and OSU-2S on gp91$^{phox}$ expression in Hep3B cells after 24 hours of treatment. (D) Left panels, siRNA-mediated knockdown of gp91$^{phox}$ protects Hep3B cells from the stimulatory effects of FTY720 (5 $\mu$M) and OSU-2S (2.5 $\mu$M) on membrane-associated NADPH oxidase activity (lower left) and ROS production (lower right). Hep3B cells were treated with scrambled (Ctl) or gp91$^{phox}$ siRNA for 24 hours, washed, and exposed to either agent for 12 hours. Columns, mean; bars, $\pm$ SD (n = 3). Right panels, effect of siRNA-mediated knockdown of gp91$^{phox}$ on FTY720- and OSU-2S–mediated suppression of Hep3B cell viability. Hep3B cells were treated with scrambled (Ctl) or gp91$^{phox}$ siRNA for 24 hours, washed, and exposed to either agent for 24 hours. Points, mean; bars, $\pm$ SD (n = 6). (E) *In vivo* effect of FTY720 and OSU-2S (5 mg/kg daily, i.p.) on the expression of gp91$^{phox}$ in Hep3B xenograft tumors after 7 days of treatment as determined by western blotting. Left, Immunoblot analysis of gp91 expression in Hep3B tumor homogenates. Right, Relative expression levels of gp91. Amounts of immunoblotted proteins were quantitated by densitometry and normalized to that of $\beta$-actin. Columns, mean; bars, $\pm$ SD (n = 3).

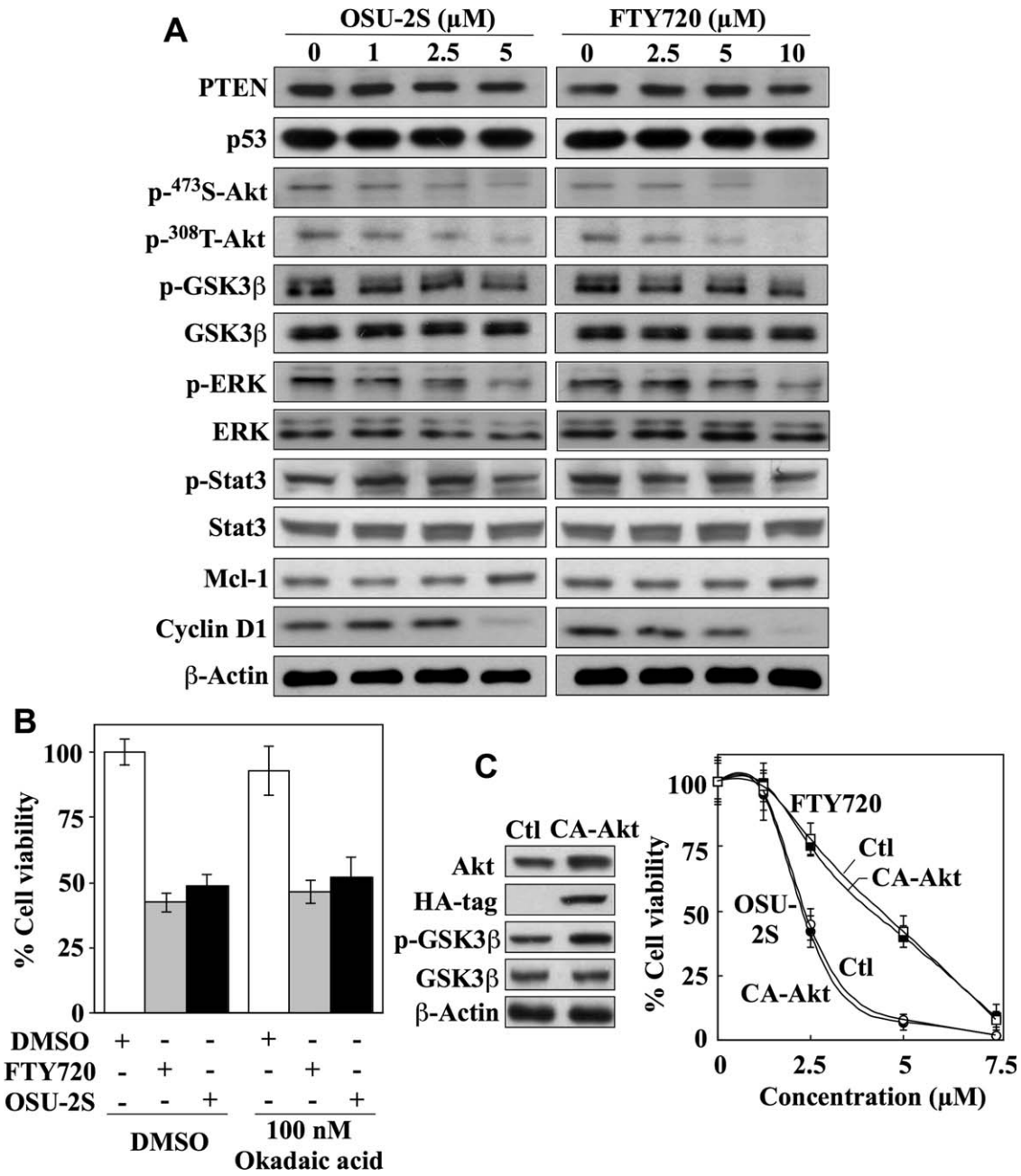


Fig. 6. Evidence that refutes the involvement of protein phosphatase 2A, PTEN, and Akt as targets for OSU-2S. (A) Western blot analysis of the dose-dependent effects of OSU-2S and FTY720 on the phosphorylation/expression levels of PTEN, p53, Akt, GSK3$\beta$, ERK, Stat3, Mcl-1, and cyclin D1 in Huh7 cells after 24 hours of treatment. (B) Effect of okadaic acid (100 nM) on the antiproliferative activities of 5 $\mu$M FTY720 and 2.5 $\mu$M OSU-2S in Huh7 cells after 24 hours of treatment. Columns, mean; bars, $\pm$ SD (n = 6). (C) Effect of ectopic expression of constitutively active Akt (CA-Akt) on the sensitivity of Huh7 cells to FTY-720- and OSU-2S–induced suppression of cell viability. Left, western blot analysis of the expression level of Akt, HA-tag, p-GSK3$\beta$, and GSK3$\beta$ in a stable CA-Akt-overexpressing clone versus untransfected Huh7 cells. Right, effect of FTY720 and OSU-2S on the viabilities of untransfected Huh7 cells (Ctl) and stable CA-Akt–overexpressing clones. Points, mean; bars, $\pm$ SD (n = 6).

activity in cell membranes isolated from untreated cells (data not shown).

NADPH oxidase is a complex consisting of six subunits (gp91phox, p22phox, p40phox, p47phox, p67phox, Rac1/Rac2).[20] Several lines of evidence indicate that FTY720 and OSU-2S activated NADPH oxidase by up-regulating gp91$^{phox}$ subunit expression in Hep3B cells. First, treatment with either agent caused concentration-dependent increases in gp91$^{phox}$ mRNA and protein expression (Fig. 5B,C), which were highly specific because the mRNA levels of the other five subunits remained unaltered (Fig. 5B). Second, siRNA-

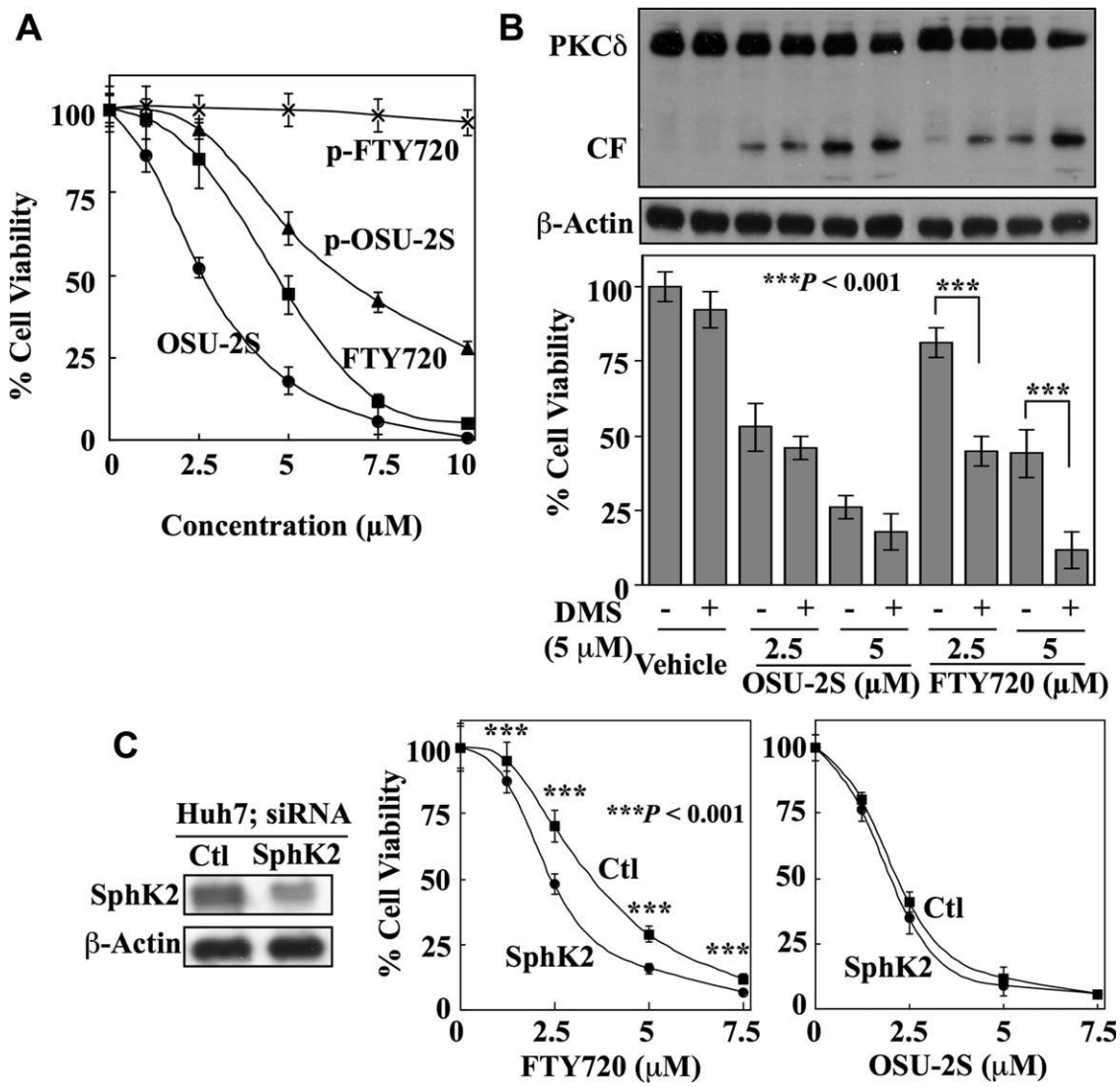


Fig. 7. Evidence that SphK2-mediated phosphorylation of FTY720 may underlie its lower antiproliferative potency relative to OSU-2S. (A) MTT assays of the dose-dependent suppressive effects of OSU-2S, phospho-OSU-2S (p-OSU-2S), FTY720, and p-FTY720 on the viability of Huh7 cells after 24 hours of treatment. Points, mean; bar, + SD (n = 6). (B) Effect of *N*,*N*-dimethylsphingosine (DMS, 5 $\mu$M), a pharmacological inhibitor of sphingosine kinase-2 (SphK2), on the dose-dependent activation of PKC$\delta$ (upper) and suppression of cell viability (lower) by OSU-2S versus FTY720 in Hep3B cells. Columns, mean; bars, ± SD (n = 6). (C) Effect of siRNA-mediated knockdown of SphK2 expression on the sensitivity of Huh7 cells to the antiproliferative effects of FTY720 and OSU-2S. Left, expression levels of SphK2 in cells transfected with SphK2 siRNA or scrambled siRNA (Ctl). Right, knockdown of SphK2 expression enhances the effect of FTY720, but not OSU-2S, on cell viability. Points, mean; bars, ± SD (n = 6).

mediated knockdown of gp91$^{phox}$ (Fig. 5D, upper left) blocked FTY720- and OSU-2S–stimulated NADPH oxidase activity and ROS production (lower left), thereby protecting against the dose-dependent suppression of Hep3B cell viability by these agents (right). Third, in an *in vivo* study, we confirmed that tumor-suppressive doses of FTY720 and OSU-2S (5 mg/kg daily, i.p.) could up-regulate gp91$^{phox}$ expression in Hep3B xenograft tumors (Fig. 5E). Western blot analysis indicates that FTY720 and OSU-2S increased intratumoral gp91$^{phox}$ expression by 2.4 and 3.6-fold, respectively (n = 3).

***Evaluation of Other Potential Mechanisms.*** Besides PKC$\delta$, several distinct mechanisms have been proposed for the proapoptotic effects of FTY720 in different cancer cell systems, including induction of PTEN and p53 expression,[21] activation of protein phosphatase (PP)2A and down-regulation of Mcl-1,[22] and down-regulation of p-Akt and cyclin D1.[23] To discern these possible mechanisms, we examined the expression/functional status of various markers pertinent to these pathways in drug-treated Huh7 cells by western blot analysis. Neither OSU-2S nor FTY720 had an appreciable effect on the expression levels of

PTEN, p53, or Mcl-1 (Fig. 6A). Moreover, the involvement of PP2A was refuted by the inability of okadaic acid to protect cells from OSU-2S- or FTY720-induced cell death (Fig. 6B), even though OSU-2S and FTY720 modestly decreased the phosphorylation of various PP2A target proteins, including

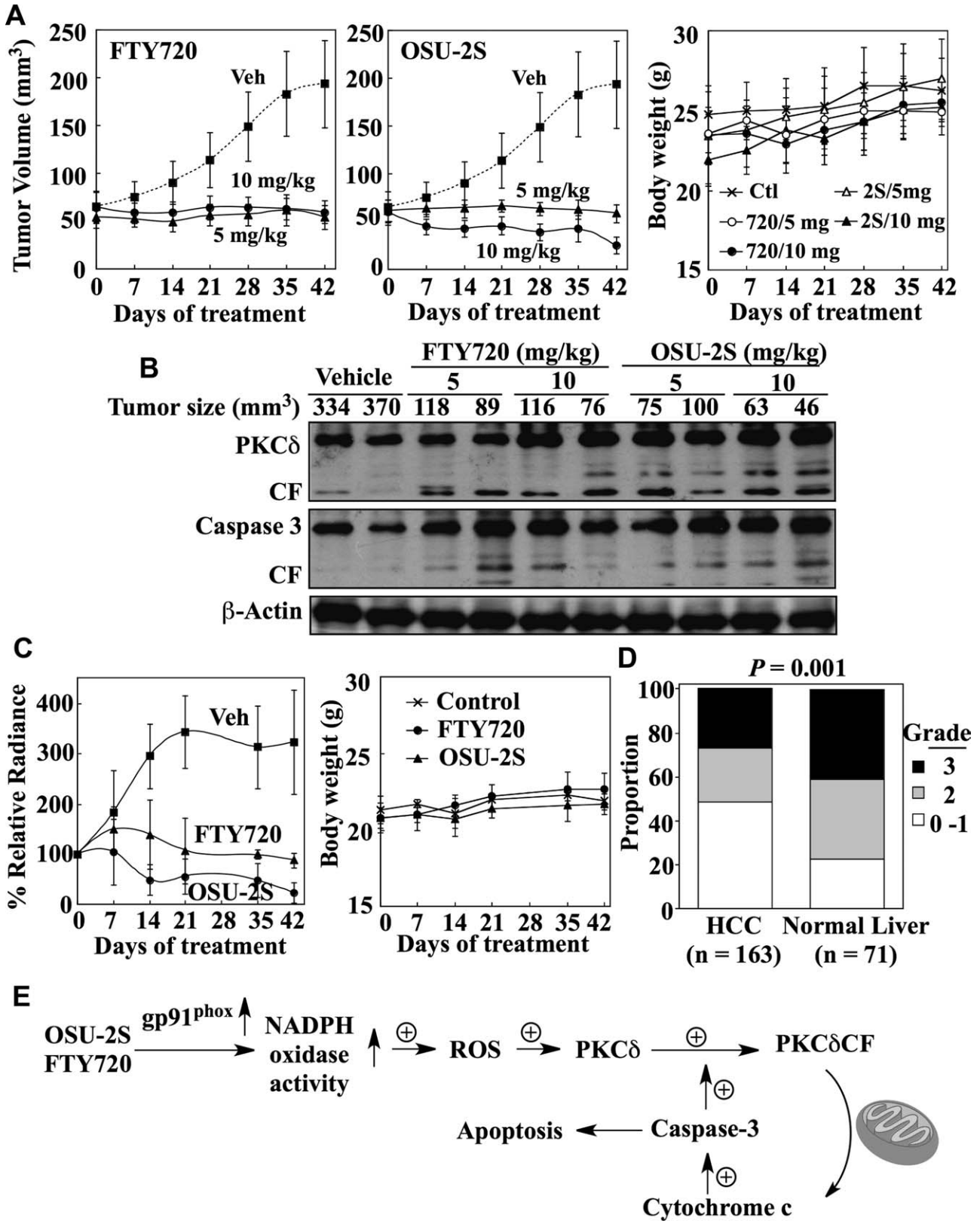


Fig. 8.

Thr-308- and Ser-473-Akt, ERK, and, to a lesser extent, Stat3 (Fig. 6A). As Huh7 cells expressed low p-Akt levels due to functional PTEN,[7] this drug-induced Akt dephosphorylation was confirmed by parallel decreases in p-GSK3$\beta$ levels accompanied by reduced cyclin D1 expression. To assess the role of Akt, the effect of ectopic expression of CA-Akt ($Akt^{T308D/S473D}$) on OSU-2S-induced cell death was examined. The overexpression of CA-Akt, as evidenced by HA tag expression and increased GSK3$\beta$ phosphorylation, did not protect against FTY720- or OSU-2S-induced cell death (Fig. 6C).

***The Lower Antiproliferative Potency of FTY720 Relative to OSU-2S Is, in Part, Attributable to SphK2-Mediated Metabolic Inactivation.*** Although SphK2-mediated phosphorylation of FTY720 is required for its effect on S1P receptors and consequent immune modulation, evidence suggests that this phosphorylation represents a metabolic inactivation of its ability to elicit intracellular apoptosis signaling as p-FTY720 lacks *in vitro* efficacy in suppressing HCC cell viability (Fig. 7A). We have shown above (Fig. 1B) that OSU-2S is not a substrate of SphK2; thus, it is not subject to phosphorylating inactivation. Consistent with this premise, synthetic p-OSU-2S retained partial antitumor activity, in contrast to p-FTY720 (Fig. 7A).

Consequently, we hypothesize that SphK2-mediated phosphorylation underlies the lower antiproliferative activity of FTY720 and that inhibition of SphK2 activity would enhance its anticancer activity in HCC cells. This premise was supported by the potentiation of FTY720-induced PKC$\delta$ activation and inhibition of Hep3B cell viability by the SphK2 kinase inhibitor *N*,*N*-dimethylsphingosine (DMS) (5 $\mu$M) (Fig. 7B). DMS alone had no appreciable activity on either marker, but when combined with FTY720, achieved effects on PKC$\delta$ activation and cell viability equivalent to those of OSU-2S as a single agent at the same concentration. Consistent with our finding that OSU-2S is not a SphK2 substrate, this enhanced effect was absent in cells cotreated with DMS and OSU-2S.

Similar to the effect of pharmacological inhibition, siRNA-mediated silencing of SphK2 expression in Huh7 cells significantly increased the antiproliferative activity of FTY720 ($P < 0.001$) to a level comparable to that of OSU-2S. This sensitization, however, was not observed with OSU-2S (Fig. 7C). These results suggest that the different antitumor potencies of OSU-2S and FTY720 are attributable to differences in their susceptibility to SphK2-mediated phosphorylation.

***OSU-2S Exhibits Potent* In Vivo *Efficacy in Suppressing HCC Tumor Growth in Two Different Murine Models.*** The *in vivo* antitumor efficacy of OSU-2S was evaluated vis-à-vis FTY720 in both ectopic and orthotopic Hep3B tumor xenograft models. Athymic nude mice bearing established subcutaneous Hep3B tumors were treated by i.p. injection once daily with OSU-2S or FTY720 at 5 and 10 mg/kg, or with vehicle. Both agents, at 5 mg/kg, completely suppressed Hep3B tumor growth relative to the vehicle control ($P < 0.001$) (Fig. 8A). Although no dose-dependency in the response to FTY720 was noted, OSU-2S at 10 mg/kg reduced tumor volume by more than 50% by the end of treatment. Examination of intratumoral biomarkers of drug activity showed that PKC$\delta$ and caspase-3 were activated in the tumors from FTY720- and OSU-2S–treated mice (Fig. 8B), confirming the *in vitro* mechanistic findings.

The daily administration of both drugs was well-tolerated as no overt signs of toxicity and no loss of body

Fig. 8. *In vivo* efficacies of OSU-2S versus FTY720 in ectopic and orthotopic xenograft models of HCC tumor growth. (A) Effects of FTY720 (left) and OSU-2S (center) on the growth of subcutaneous Hep3B tumors in athymic nude mice. Mice bearing established Hep3B tumors were treated with FTY720 or OSU-2S at 5 or 10 mg/kg via daily i.p. injection for 42 days. Veh, vehicle. Points, mean; bars, $\pm$ SD (n = 8). Right panel, treatments had no effect on the body weights of ectopic tumor-bearing mice. Ctl, vehicle-treated control; 720, FTY720; 2S, OSU-2S. Points, mean; bars, + SD (n = 8). (B) Western blot analysis of intratumoral biomarkers of drug activity in the homogenates of two representative subcutaneous Hep3B tumors from each treatment group. CF, catalytic fragment. (C) Left panel, effects of FTY720 and OSU-2S on the growth of established orthotopic luciferase-expressing Hep3B tumors in athymic nude mice. Tumor burden was determined by weekly measurements of bioluminescence. The mice were treated with FTY720 or OSU-2S at 5 mg/kg or with the vehicle via daily i.p. injection for 42 days. Veh, vehicle. Points, mean; bars, $\pm$ S.D. (n = 3). Right panel, treatments had no effect on the body weights of mice bearing orthotopic tumors. Points, mean; bar, + SD (n = 3). (D) Immunohistochemical analysis of PKC$\delta$ expression levels in HCC (n = 163) and normal human liver tissues (n = 71) on a tissue microarray. Intensities of immunohistochemical staining were semiquantitatively scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong. Fisher's exact test shows a significant difference ($P = 0.001$) between the HCC and normal liver tissues with respect to the proportions of tissues that are PKC$\delta$-positive. (E) Schematic diagram depicting the mechanism by which OSU-2S and FTY720 mediate apoptosis in HCC cells through the ROS–PKC$\delta$–caspase-3 signaling pathway. We demonstrated that OSU-2S and FTY720 stimulate NADPH oxidase activity by up-regulating the expression of the $gp91^{phox}$ subunit, leading to increased ROS production. This study provides evidence that PKC$\delta$ represents a major downstream effector of drug-induced ROS production to facilitate caspase-3–dependent apoptosis through a positive feedback loop between the proteolytic cleavage of PKC$\delta$ and caspase-3 activation.[6,7]

weight were observed (Fig. 8A, right). Moreover, no histologic lesions consistent with toxic injury were seen in any of the organs examined microscopically, with the exception of mesentery and mesenteric blood vessels. Specifically, of the six mice per group examined histologically, three that received 5 mg/kg FTY720 and all that received 10 mg/kg FTY720 and either dose of OSU-2S had evidence of abdominal adhesions with varying amounts of peritonitis. In some of these, the inflammation exhibited varying degrees of angiocentricity with vasculitis in some cases. Significant hematological findings after 21 days of treatment included a pronounced reduction in lymphocytes in FTY720-treated mice and, to a lesser extent, in mice treated with 10, but not 5 mg/kg OSU-2S (Supporting Table 1), which was reflected in corresponding decreases in leukocytes, despite a marked eosinophilia in FTY720-treated mice. Although liver enzymes were not significantly elevated, alanine aminotransferase was moderately reduced in FTY720-treated and the high dose OSU-2S-treated mice (Supporting Table 2). Alkaline phosphatase was also mildly reduced in the high dose OSU-2S-treated group. Nonetheless, levels of the affected parameters were within the normal ranges for mice. In the absence of corresponding histologic lesions, the clinical significance of these changes is unclear.

To confirm that these *in vivo* tumor-suppressive activities could also occur in the context of a relevant tumor microenvironment, *in vivo* efficacy was assessed in an orthotopic xenograft model. Orthotopic tumors were established by intrahepatic injection of Hep3B-luc cells and monitored by bioluminescent imaging. Mice were treated with the agents at 5 mg/kg daily or with vehicle for 42 days. Figure 8C (left) shows that orthotopic tumors in vehicle-treated mice grew during the first 21 days of treatment, after which a plateau was reached. Tumors in FTY720-treated mice showed a gradual rise in bioluminescence over the first week of treatment, but, by 3 weeks, mean tumor burden was suppressed to the original level. OSU-2S exhibited a higher tumor-suppressive potency than FTY720 in this model, achieving 80% reduction in bioluminescence at the end of treatment. Both treatments were well tolerated as indicated by stable body weights (Fig. 8C, right).

***PKCδ Expression in Human HCC Versus Non-Neoplastic Human Liver Tissues.*** Although down-regulation of PKC$\delta$ expression has been reported in many cancer types, including squamous cell carcinoma,[24] urinary bladder carcinoma,[25] and endometrial cancer,[26] information regarding the expression of this proapoptotic kinase in HCC is lacking. Thus, we used a TMA to evaluate PKC$\delta$ expression in 163 human HCC and 71 non-neoplastic liver tissue samples. Our data show a lower expression level of PKC$\delta$ in HCC relative to non-neoplastic liver ($P = 0.001$) (Fig. 8D).

## Discussion

Considering the translational potential of FTY720 as a therapeutic agent for HCC, it is desirable to dissociate its S1P receptor agonist activity from its antitumor effects to avoid untoward side effects associated with immunomodulatory therapies. OSU-2S represents a proof-of-concept that these two pharmacological activities could be separated via structural modifications to develop novel antitumor agents with a unique mode of action. In contrast to FTY720, OSU-2S lacks significant effects on S1P1 receptor internalization in Huh7 cells and T lymphocyte homing in immunocompetent mice. Though devoid of immunosuppressive activity, OSU-2S exhibits twofold higher *in vitro* antiproliferative efficacy relative to FTY720 against HCC cells. Moreover, like FTY720, this antitumor activity is mediated, in part, through the activation of PKC$\delta$ signaling. PKC$\delta$ plays an intriguing role in regulating apoptosis, eliciting either proapoptotic or antiapoptotic effects in a cell type–specific and context-specific manner.[27] In this study, we demonstrated that OSU-2S shared the ability of FTY720 to mediate PKC$\delta$-dependent apoptosis through NADPH-dependent ROS production, and that caspase-3 not only represents a downstream effector of PKC$\delta$, but also provides positive feedback by facilitating PKC$\delta$ activation via proteolytic cleavage (Fig. 8E).

This unique mechanism might underlie the high potency of OSU-2S and FTY720 in mediating apoptotic death in HCC cells as somatic *GST-π* gene silencing is a frequent feature of HCC leading to low antioxidant capacity.[28] This premise was corroborated by the ability of siRNA-induced repression of GST-$\pi$ to sensitize PLC5 cells, which exhibit high levels of endogenous GST-$\pi$, to OSU-2S- and FTY720-mediated growth inhibition.

In contrast to the gain of S1P receptor agonist activity by FTY720 after SphK2-mediated phosphorylation, metabolic transformation of FTY720 to its phosphate derivative results in the loss of its antitumor activity. Because FTY720 is gradually phosphorylated and secreted, this inactivation/sequestration may explain the lower antiproliferative potency of FTY720 relative to OSU-2S, which is not phosphorylated by SphK2.

Indeed, our data show that the suppression of SphK2 activity by pharmacological inhibition or knockdown of gene expression enhanced the antitumor activity of FTY720 to the same level as that of OSU-2S.

As a single agent *in vivo*, OSU-2S exhibited high tumor-suppressive activity against both subcutaneous and intrahepatic HCC xenograft tumors through the activation of PKC$\delta$ and caspase-dependent apoptosis without overt toxicity. The abdominal adhesions and peritonitis observed in drug-treated mice were likely a response to the chronic irritation associated with repeated i.p. injections of the agents. The angiocentric inflammation noted in the mesenteric vasculature of some mice may represent a localized hypersensitivity reaction to the compounds or a localized vascular toxicity, the significance of which is unclear. The mechanism for the lymphocyte reduction seen after prolonged treatment with 10 mg/kg OSU-2S is unknown, but is apparently independent of effects on S1P1 receptors as OSU-2S is devoid of S1P1 receptor-targeted activity. Moreover, this effect occurred at a dose that exceeds the 5 mg/kg dose needed to completely suppress tumor growth.

Evaluation of PKC$\delta$ expression in a human TMA revealed lower PKC$\delta$ expression levels in HCC than in nonmalignant liver tissues, suggesting that the down-regulated expression of this proapoptotic kinase may provide survival advantages. Our finding that shRNA-mediated knockdown of PKC$\delta$ reduced the sensitivity of Huh7 cells to the antiproliferative effects of OSU-2S supports this premise. From a clinical perspective, the expression levels of PKC$\delta$ and GST-$\pi$ represent two key determinants for cellular sensitivity to OSU-2S, and thus may be useful as criteria for selecting patients for OSU-2S therapy; i.e., OSU-2S would benefit HCC patients with moderate to high PKC$\delta$ and low GST-$\pi$ expression.

In summary, we report the development of OSU-2S, a nonimmunosuppressive analogue of FTY720. Unlike FTY720, OSU-2S is not subject to SphK2-mediated phosphorylation and thus exhibits higher antitumor potency than FTY720. These findings, along with the potent *in vivo* tumor-suppressive activity, support the translational potential of OSU-2S as a component of therapeutic strategies for advanced HCC, for which systemic therapies have been largely unsuccessful. In support of the translation of these promising preclinical findings to clinical use of OSU-2S, investigations of combining OSU-2S with chemotherapy or other targeted agents, and the development of an analytical method to support pharmacokinetic analysis of OSU-2S are underway.

## References


1. Moradpour D, Blum HE. Pathogenesis of hepatocellular carcinoma. Eur J Gastroenterol Hepatol 2005;17:477-483.
2. Thomas MB, Abbruzzese JL. Opportunities for targeted therapies in hepatocellular carcinoma. J Clin Oncol 2005;23:8093-8108.
3. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359:378-390.
4. Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med 2010;362:402-415.
5. Takabe K, Paugh SW, Milstien S, Spiegel S. "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. Pharmacol Rev 2008;60: 181-195.
6. Ho JW, Man K, Sun CK, Lee TK, Poon RT, Fan ST. Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma. Mol Cancer Ther 2005;4:1430-1438.
7. Hung JH, Lu YS, Wang YC, Ma YH, Wang DS, Kulp SK, et al. FTY720 induces apoptosis in hepatocellular carcinoma cells through activation of protein kinase C delta signaling. Cancer Res 2008;68: 1204-1212.
8. Martin R. Multiple sclerosis: closing in on an oral treatment. Nature 2010;464:360-362.
9. Kiuchi M, Adachi K, Kohara T, Minoguchi M, Hanano T, Aoki Y, et al. Synthesis and immunosuppressive activity of 2-substituted 2-aminopropane-1,3-diols and 2-aminoethanols. J Med Chem 2000;43: 2946-2961.
10. Omar HA, Sargeant AM, Weng JR, Wang D, Kulp SK, Patel T, et al. Targeting of the Akt-nuclear factor-kappa B signaling network by [1-(4-chloro-3-nitrobenzenesulfonyl)-1H-indol-3-yl]-methanol (OSU-A9), a novel indole-3-carbinol derivative, in a mouse model of hepatocellular carcinoma. Mol Pharmacol 2009;76:957-968.
11. Prieschl EE, Csonga R, Novotny V, Kikuchi GE, Baumruker T. The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fc epsilon receptor I triggering. J Exp Med 1999;190:1-8.
12. Tseng PH, Wang YC, Weng SC, Weng JR, Chen CS, Brueggemeier RW, et al. Overcoming trastuzumab resistance in HER2-overexpressing breast cancer cells by using a novel celecoxib-derived phosphoinositide-dependent kinase-1 inhibitor. Mol Pharmacol 2006;70: 1534-1541.
13. Dragun D, Bohler T, Nieminen-Kelha M, Waiser J, Schneider W, Haller H, et al. FTY720-induced lymphocyte homing modulates post-transplant preservation/reperfusion injury. Kidney Int 2004;65: 1076-1083.
14. Vaquero EC, Edderkaoui M, Pandol SJ, Gukovsky I, Gukovskaya AS. Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. J Biol Chem 2004;279:34643-34654.
15. Baumhoer D, Tornillo L, Stadlmann S, Roncalli M, Diamantis EK, Terracciano LM. Glypican 3 expression in human nonneoplastic, preneoplastic, and neoplastic tissues: a tissue microarray analysis of 4,387 tissue samples. Am J Clin Pathol 2008;129:899-906.
16. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem 2002;277:21453-21457.
17. Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, Seuwen K. Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. Nat Chem Biol 2009;5:428-434.
18. Yusof YA, Yan KL, Hussain SN. Immunohistochemical expression of pi class glutathione S-transferase and alpha-fetoprotein in hepatocellular carcinoma and chronic liver disease. Anal Quant Cytol Histol 2003;25: 332-338.
19. Reyland ME. Protein kinase Cdelta and apoptosis. Biochem Soc Trans 2007;35:1001-1004.
20. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004;4:181-189.

21. Zheng T, Meng X, Wang J, Chen X, Yin D, Liang Y, et al. PTEN- and p53-mediated apoptosis and cell cycle arrest by FTY720 in gastric cancer cells and nude mice. J Cell Biochem 2010;111:218-228.
22. Roberts KG, Smith AM, McDougall F, Carpenter H, Horan M, Neviani P, et al. Essential requirement for PP2A inhibition by the oncogenic receptor c-KIT suggests PP2A reactivation as a strategy to treat c-KIT+ cancers. Cancer Res 2010;70:5438-5447.
23. Liu Q, Alinari L, Chen CS, Yan F, Dalton JT, Lapalombella R, et al. FTY720 shows promising in vitro and in vivo preclinical activity by downmodulating Cyclin D1 and phospho-Akt in mantle cell lymphoma. Clin Cancer Res 2010;16:3182-3192.
24. D'Costa AM, Robinson JK, Maududi T, Chaturvedi V, Nickoloff BJ, Denning MF. The proapoptotic tumor suppressor protein kinase C-delta is lost in human squamous cell carcinomas. Oncogene 2006;25: 378-386.
25. Varga A, Czifra G, Tallai B, Nemeth T, Kovacs I, Kovacs L, et al. Tumor grade-dependent alterations in the protein kinase C isoform pattern in urinary bladder carcinomas. Eur Urol 2004;46:462-465.
26. Reno EM, Haughian JM, Dimitrova IK, Jackson TA, Shroyer KR, Bradford AP. Analysis of protein kinase C delta (PKC delta) expression in endometrial tumors. Hum Pathol 2008;39:21-29.
27. Jackson DN, Foster DA. The enigmatic protein kinase Cdelta: complex roles in cell proliferation and survival. FASEB J 2004;18:627-636.
28. Tchou JC, Lin X, Freije D, Isaacs WB, Brooks JD, Rashid A, et al. GSTP1 CpG island DNA hypermethylation in hepatocellular carcinomas. Int J Oncol 2000;16:663-676.