Published in final edited form as:

Pediatr Blood Cancer. 2013 September ; 60(9): . doi:10.1002/pbc.24564.

FTY720 Inhibits Tumor Growth and Enhances the Tumor-Suppressive Effect of Topotecan in Neuroblastoma by Interfering With the Sphingolipid Signaling Pathway

Mei-Hong Li, PhD¹, Timothy Hla, PhD², and Fernando Ferrer, MD^{1,3,*}

¹University of Connecticut Health Center, Center for Vascular Biology, Farmington, Connecticut

²Department of Pathology and Laboratory Medicine, Center for Vascular Biology, Weill Medical College of Cornell University, New York, New York

³Department of Urology and Surgery, Connecticut Children's Medical Center, Hartford, Connecticut

Abstract

Background—Neuroblastoma (NB) is the most common extra-cranial solid tumor in childhood. Poor outcomes for children with advanced disease underscore the need for novel therapeutic strategies. FTY720, an immunomodulating drug approved for multiple sclerosis, has been investigated in oncology with promising preclinical activities. To date, its effect in NB has not been explored. Herein we describe our preclinical experience with FTY720, alone or in combination with topotecan, and its putative mechanism of action in NB.

Procedure—MTT assay was performed to assess the effect of FTY720 on cell viability. A NB xenograft model was employed to assess the efficacy of FTY720 on tumor growth. Quantitative real-time PCR and Western blot were employed to determine changes of mRNA and protein expression, respectively. Liquid chromatography/tandem mass spectrometry was used to measure sphingolipid levels.

Results—FTY720, but not FTY720-P induced NB cell death. FTY720 inhibited the growth of NB xenografts and enhanced the tumor-suppressive effect of topotecan both *in vitro* and *in vivo*. FTY720 significantly inhibited sphingosine kinase 2 (SphK2) mRNA and protein expression in NB cells. Pro-apoptotic sphingosine levels were increased in NB cells and NB xenografts treated with FTY720. FTY720-induced cell death was caspase-independent and involved the dephosphorylation of Akt and BAD at Ser136.

Conclusions—Our data demonstrate that FTY720 has potent preclinical anti-cancer activity in NB. Its unique death signaling mechanism, interference with the sphingolipid pathway, acts cooperatively with that of topotecan, suggesting that FTY720 related molecules may be useful in NB treatment.

Keywords

apoptosis; FTY720; neuroblastoma; sphingosine; sphingosine kinase 2

INTRODUCTION

Neuroblastoma (NB) is the most common extra-cranial solid tumor in childhood and the most frequently diagnosed solid neoplasm during infancy. It accounts for 8–10% of all childhood cancers and 15% of all cancer-related deaths in children. It is an extremely heterogeneous disease and can be stratified into three risk categories: low, intermediate, and high. Although we have seen substantial improvements in outcomes of patients with low- and intermediate-risk NB as a result of multimodal therapy, the outcome for children with high-risk NB has improved only modestly, with long-term survival remaining less than 40% [1,2]. Moreover, these therapies are associated with significant late adverse effects such as multi-organ malfunction, sensorineural hearing loss and secondary malignancies, to name but a few [3]. Thus, there is a tremendous need for novel therapeutic strategies for NB.

The sphingosine analogue FTY720 is an immunosuppressive drug which was FDA approved for multiple sclerosis (MS) in 2010 [4]. It has been investigated in pre-clinical models as a therapy for various types of cancers including prostate cancer, hepatocellular carcinoma, multiple myeloma, breast cancer, ovarian cancer, leukemia, and recently glioblastoma [5–11]. Available evidence suggests that FTY720 has potent anti-cancer activity in these cancer models, while the mechanisms responsible for FTY720-induced cancer cell death have been reported to vary based on the type of cancer. Specific potential sites of action have included protein kinase C [7], protein phosphatase 2a [8] and SphK1 [9,12]. To date, its effect or mechanism of action in NB has not been explored.

Topotecan is a water-soluble analogue of the natural product alkaloid camptothecin. It is a potent topoisomerase I inhibitor with a mechanism of action aimed at interrupting DNA replication in cancer cells, resulting in cell death [13,14]. Currently, it has been explored as a combination therapy agent for NB in the Children's Oncology Group clinical trials and has shown efficacy in recurrent disease [15].

In this study, we described our preclinical experience with FTY720, alone or in combination with topotecan and suggest a putative mechanism of action in NB. Utilizing cell systems *in vitro* and a NB xenograft model *in vivo*, our data demonstrate that FTY720 has a potent preclinical anti-cancer activity in NB. Its unique death signaling through inhibition of sphingosine kinase 2 (SphK2) expression and increase of pro-apoptotic sphingosine levels, acts cooperatively with that of topotecan, suggesting that FTY720 related molecules may be useful in NB treatment.

MATERIALS AND METHODS

Reagents

FTY720 and FTY720-P were kindly provided by Novartis (Basel, Switzerland). Topotecan was purchased from Sigma (Saint Louis, MO). Primary antibodies for caspase 8 (#9746), caspase 3 (#9662), p-Akt (#9271), Akt (#9272), p-BAD (#4366), BAD (#9292) were from Cell Signaling Technology (Beverly, MA) while Bax (sc-493), Bcl-2 (sc-7382), and -Actin (sc-8432) were from Santa Cruz Biotechnology (Santa Cruz, CA). SphK1 and SphK2 antibodies were developed and characterized as described previously [16].

Cell Culture

SK-N-AS, SK-N-FI cell lines were obtained from the American Type Culture Collection (ATCC). SK-N-BE(2), originally from ATCC, and CHP-134, originally for Children's Oncology Group Biorepository, were kindly provided by Dr. Nehal Parikh (Connecticut Children's Medical Center). They were cultured as previously described [17].

MTT Assay

The Methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma) assay was performed to evaluate the effect of tested compounds on cell viability as previously described [18]. Briefly, NB cells were seeded in 96-well plates at a density of 2×10^4 cells/well. The next day cells were treated with different compounds at indicated concentrations for different times followed by adding 10 μ l of MTT (5 mg/ml dissolved in PBS) to each well. After incubation at 37°C for 2 hours, the culture medium containing MTT was removed and 100 μ l of DMSO (Sigma) was added to each well. Viable cells were detected by measuring absorbance at 595 nm using Bio-Rad Microplate Reader (Model 680) and results were presented as percentage of growth inhibition compared to the non-drug-treated controls.

Subcutaneous NB Tumor Model

Animal experiments were conducted according to our institution's and the National Research Council's guide for the care and use of laboratory animals. Six-week-old male athymic NCr-nu/nu nude mice (National Cancer Institute, Frederick, MD) were used in this study. Briefly, each mouse received a subcutaneous flank injection containing 1×10^7 SK-N-AS cells in 0.1 ml PBS. Five days later, the mice were randomized into four groups: vehicle control, FTY720 (5 mg/kg in saline, daily, i.p.) alone, topotecan (1 mg/kg in saline, daily, gavage) alone, and FTY720 plus topotecan. Treatments started on the day of randomization. Tumors were measured every other day with a caliper and tumor volumes were calculated using the following formula: $TV = \text{length} \times \text{width}^2 \times 0.52$. Two weeks later, the mice were euthanized; tumor masses were collected and weighted on the OHAUS Adventurer Pro AV114 balance.

Quantitative Real-Time PCR

Total RNA was isolated from treated SK-N-AS cells using Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was generated using Moloney murine leukemia virus reverse transcriptase (Invitrogen) followed by SYBR Green-based quantitative real-time PCR on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) as previously described [19]. Primers were designed using Primer Express 2.0 (Applied Biosystems) and sequences were as follows: 5'-CATCCAGAAGCCCCTGTGTAG-3' (forward) and 5'-CATCCAGAAGCCCCTGTGTAG-3' (reverse) for the SphK1 gene and 5'-CAACCTCATCCAGACAGAACGA-3' (forward) and 5'-TTCACAGCTTCCTCCCAGTCA-3' (reverse) for the SphK2 gene. Results were expressed relative to the internal control gene GAPDH.

Western Blot Analysis

Treated SK-N-AS cells were washed with ice-cold PBS and scraped in RIPA buffer. Cellular homogenates were centrifuged at 14,000g for 20 minutes at 4°C, and protein supernatants were determined by BCA protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein to SDS-PAGE followed by Western blot analysis as described [20].

Sphingolipid Measurements

SK-N-AS cells (2×10^6) were seeded into 60 mm dishes and treated with FTY720 the next day for another 48 hours. Then they were washed with cold PBS, scraped and spin down to yield the cell pellets. The measurements of sphingolipids in those pellets and the tumor tissues from NB xenografts treated with FTY720 (100 mg each) were performed by the liquid chromatography-tandem mass spectrometry (LC/MS/MS) core facility at the Medical University of South Carolina (<http://hcc.musc.edu/research/resources/lipidomics/analysis.htm>) [21].

Detection of Cytochrome c Release

SK-N-AS cells (6×10^6) were seeded into 100 mm dishes and treated with the indicated concentrations of FTY720 for 48 hours. The cytosolic and mitochondrial fractions were then collected using cytochrome c releasing apoptosis assay kit (Enzo Life Sciences, Plymouth Meeting, PA) followed by Western blot analysis to detect the cytochrome *c* in those fractions.

Statistical Analysis

All experiments on cells were performed at least twice on separate occasions. Data were presented as means $\pm$ SE. Statistical significance of differences between two groups was determined by two-tailed homoscedastic Student's *t*-test using Microsoft Excel software.

RESULTS

FTY720, but Not FTY720-P, Induced NB Cell Death *In Vitro*

Previous studies have shown that FTY720 has anti-cancer activity in various preclinical models of adult cancers [5–11]. To determine whether FTY720 has activity against NB, MTT assay was performed to assess the effect of FTY720 on cell viability. The SK-N-AS cell line was used since it has an S1P receptor (S1PR) expression profile consistent with human NB specimens, as we have previously demonstrated [17]. FTY720 strongly induced cell death in this cell line, showing a typical S-shaped dose-response curve with the narrow linear range between 2 and 15 μ M (Fig. 1A). Our previous findings have shown that NB predominantly expresses SphK2 [17]. Aware that FTY720 can be avidly phosphorylated by SphK2 into FTY720-P, an extracellular agonist for all S1PRs except S1P₂ [22], we sought to determine the effect of FTY720-P on cell survival in SK-N-AS cells. Compared to that of FTY720, FTY720-P had minimal inhibitory effect (Fig. 1B) suggesting that FTY720-mediated NB cell death is not related to S1PR activation. This phenomenon was also observed in other NB cell lines including SK-N-FI, SK-N-BE(2), and CHP-134 (Fig. 1). Collectively, the above findings suggest that FTY720 has a broad-spectrum cytotoxic effect in various NB cell lines and that S1PR interaction is not central to this effect.

FTY720 Inhibited the Growth of NB xenografts and Enhanced the Tumor-Suppressive Effect of Topotecan *In Vitro* and *In Vivo*

To further determine FTY720's anti-cancer activity *in vivo*, a NB xenograft model was utilized [17]. Interestingly, FTY720 (5 mg/kg) alone was able to significantly inhibit the growth of NB xenografts from Day 12 onward compared to the control group, with a potency similar to that of the topoisomerase I inhibitor topotecan (1 mg/kg), a commonly used chemotherapy drug in NB [2] (Fig. 2A). Combination therapy with these two further inhibited tumor growth and the significant effect was seen as early as 6-day treatment compared to the control group (Fig. 2A). This finding was consistent with the results on tumor weight, where combination therapy decreased tumor weight beyond that seen with either compound used as a stand-alone agent (Fig. 2B). Moreover, this additive combined effect was also demonstrated in SK-N-AS cells using *in vitro* cell viability assay, in which sub-cytotoxic doses of FTY720 and topotecan were utilized (Fig. 2C). Taken together, the above data demonstrate that FTY720 has significant anti-cancer activity in NB and suggested that its effect may occur through a unique signaling pathway that acts cooperatively with topotecan [23].

FTY720 Inhibited SphK2 Expression and Increased Pro-Apoptotic Sphingosine in NB

FTY720 has been reported to be an inhibitor of SphK in cancer models [9,12]. Based on our previous finding, that NB is unique among solid tumors we have studied, in that it

predominantly expresses SphK2 [17], we speculated that FTY720 interference with SphK2 might be responsible for FTY720's antitumor effect in NB. To address this question, we began by utilizing quantitative real-time PCR and Western blot analysis to detect SphK2 mRNA and protein expression. Treatment of SK-N-AS cells with FTY720 resulted in a significant decrease in SphK2 mRNA and protein expression. However, SphK1 mRNA was significantly increased while its protein barely changed in response to FTY720 treatment for 48 hours (Fig. 3A and B), suggesting that SphK2, not SphK1, is the intracellular target of FTY720 in NB. The principle role of SphKs is to convert pro-apoptotic sphingosine into pro-survival S1P. Therefore, LC/MS/MS analyses were conducted to measure sphingosine levels. Interestingly, pro-apoptotic sphingosine levels were dramatically increased in NB cells after treatment with FTY720 (Fig. 3C). This finding was confirmed in FTY720-treated NB xenografts, in which pro-apoptotic sphingosine levels were also increased (Fig. 3D). Pro-apoptotic ceramide has been reported as another SphK effector [24]. Therefore, we also determined its levels in NB cells and NB xenografts treated with FTY720. Of note, the total ceramide levels were increased in both cases (Fig. 3C and D). Taken together, all the above data demonstrate that FTY720 inhibited SphK2 gene expression and increased pro-apoptotic sphingosine and ceramide levels in NB.

Dephosphorylation of Akt and BAD at Ser136 Were Involved in FTY720-Induced NB Cell Death

Multiple sphingosine-induced mechanisms have been shown to lead to apoptosis. Given the apparent enhanced activity seen when combining topotecan and FTY720, understanding the effector mechanisms for FTY720-induced cell death might explain how the two agents cooperate. The effector molecules downstream of sphingosine include caspases, pro-apoptotic proteins BAD/BAX, anti-apoptotic protein Bcl-2, and the protein kinase Akt which is critical for cell survival [25]. Western blot analysis in SK-N-AS cells failed to show cleaved forms of caspase-3 and caspase-8 at 6 hours treatment of FTY720 (Fig. 4A) as well as other time points (16, 24, and 48 hours), suggesting that FTY720-induced cell death in NB was caspase-independent. Interestingly, after treatment of NB cells with FTY720 for 48 hours, we found the phosphorylated forms of Akt and its downstream target BAD at Ser136 [26,27], were strongly inhibited while pro-apoptotic Bax protein was unaffected and anti-apoptotic Bcl-2 protein was greatly increased in these cells (Fig. 4B). FTY720 treatment ultimately led to the release of cytochrome c, a biomarker of apoptosis, from mitochondria into the cytosol (Fig. 4C). Taken together, all the above data indicate that FTY720-induced cell death in NB was caspase-independent while dephosphorylation of Akt and BAD appear to be involved. This may to some extent explain the cooperation exhibited when combined with topotecan, which is known to activate caspase related cell death pathways.

DISCUSSION

The bioactive lipid S1P has been recognized as an important regulator of many pathological diseases including cancer. Two isoforms of SphK have been identified to produce S1P: SphK1 and SphK2. SphK1 is overexpressed in various types of cancers. Its upregulation has been correlated with disease progression and reduced patient survival [28]. However, it was not until recently, that the functional role of SphK2 in cancer has begun to be appreciated. Different approaches, such as SphK2 deficient tumor xenografts, selective SphK2 inhibitors, as well as, siRNA knockdown strategies have been utilized to demonstrate that SphK2 acts as a tumor promoter [29–31]. Interestingly, we have previously shown that unlike many other cancers, NB predominantly expresses SphK2 [17]. Our unpublished data further showed that overexpression of SphK2 in NB cells promoted cell proliferation, suggesting that SphK2 provides a positive growth signal in NB. These findings prompted us to consider

SphK2 as a therapeutic target in NB. While FTY720 is currently approved for clinical use in MS, it remains an important tool and prototype drug for exploring biologic functions of S1P signaling pathways. FTY720 is avidly and specifically phosphorylated by SphK2. This caused us to speculate that it might have a potential beneficial effect in NB. We hypothesized that FTY720 may interfere with the function of SphK2, preventing conversion of pro-apoptotic sphingosine into pro-survival S1P, and thereby providing an anti-cancer effect.

Our data for the first time demonstrate that FTY720 has potent anti-cancer activity in NB both *in vitro* and *in vivo*, alone or in combination with topotecan, a commonly used chemotherapeutic drug in NB (Figs. 1 and 2). It is noted that the therapeutic dose that we and others have used in oncology [6,8,32–36] is much higher than that used in MS [37]. Two facts likely contribute to this inconsistency: (1) the lipophilic nature of FTY720 allows it to cross the blood–brain barrier so that FTY720 could accumulate in the central nervous system [38]; and (2) FTY720 exerts its inhibition on lymphocyte migration in MS through interacting with S1P₁ on the cell surface [37], while its anti-cancer effect in NB is not mediated via S1PR activation (Fig. 1B), consistent with prior findings [9,39]. This suggests that FTY720-induced apoptosis was probably mediated through intracellular targets in NB. As a consequence a higher dose of FTY720 might be needed to produce its anti-cancer effects. FTY720's adverse effects have been noted to include head cold, sinusitis, mild headaches, fatigue, gastrointestinal dysfunction, cancer, and hemorrhaging focal encephalitis [37]. These side effects are potentially related to its immunosuppressive actions and will require careful consideration, as immunologic “off-target” effects could be detrimental in the cancer setting. Ideally, FTY720 derivatives with diminished S1P₁ binding affinity would resolve this issue (a current focus of our work). Other side effects of FTY720 due to “off-target” S1P signaling in other organ system such as bradycardia and macular edema have also been observed, the underlying mechanism of these effects remain elusive [37]. But it must be remembered that current anticancer agents such as topotecan and others also have substantial side effects. In-depth analysis of the mechanism of FTY720 action is required and will provide the basis for development of more specific and efficient FTY720 derivatives for MS and cancer treatment.

It has been suggested that FTY720 is a SphK1 inhibitor, acting through inhibition of its enzymatic activity or by promoting its proteasomal degradation in prostate cancer cells [9,12]. This has led some to believe its anti-cancer effects are related to SphK1 inhibition. Interestingly, in our NB model system, we demonstrate that FTY720 potently inhibited the mRNA and protein expression of SphK2, the predominant isoform of SphKs in NB while it did not have inhibitory effect on SphK1 (Fig. 3A and B), suggesting that SphK2 might be the potential intracellular target of FTY720 in NB. Further functional assay by LC/MS/MS analysis revealed that treatment of NB cells or NB xenografts with FTY720 increased pro-apoptotic sphingosine and ceramide levels (Fig. 3C), suggesting that FTY720, by acting as a SphK2 inhibitor, disrupted the ceramide-sphingosine-S1P balance which has been proposed by the others as a factor in cell death signaling pathways [28].

The mechanisms responsible for FTY720-induced cancer cell death are poorly understood. Explanations vary based on the type of cancer and have included caspase-dependent [9,12] or caspase-independent mechanisms [11,33]. Further exploration in NB revealed that FTY720-induced cell death was caspase-independent (Fig. 4A). Assuming that FTY720 exerts its apoptotic effect in NB through an increase in sphingosine levels, we examined other effector molecules downstream of sphingosine and found that FTY720 caused dephosphorylation of the survival factor Akt and its downstream effector BAD at 136 as well as subsequent release of cytochrome *c* (Fig. 4B and C), which strongly indicated that 14-3-3 protein might be involved in this process [40]. However, we are also aware that other

intracellular mechanisms of sphingosine function, such as ANP32a which binds sphingosine and activates the phosphatases PP2A may be involved [41]. Further studies are warranted to gain a better understanding of the mechanisms involved in FTY720's anti-cancer effect in NB.

In summary, our data demonstrate that SphK2 may play an important role in human NB. Inhibition of SphK2 by FTY720 resulted in increased levels of pro-apoptotic sphingolipid species. FTY720 acts cooperatively with topotecan, suggesting that FTY720 derivatives may hold promise for NB treatment.

Acknowledgments

Grant sponsor: NIH; Grant number: R01CA168903; Grant sponsor: Seraph Foundation

REFERENCES

1. Maris JM, Hogarty MD, Bagatell R, et al. Neuroblastoma. *Lancet*. 2007; 369:2106–2120. [PubMed: 17586306]
2. Park JR, Eggert A, Caron H. Neuroblastoma: Biology, prognosis, and treatment. *Pediatr Clin North Am*. 2008; 55:97–120. x. [PubMed: 18242317]
3. Perwein T, Lackner H, Sovinz P, et al. Survival and late effects in children with stage 4 neuroblastoma. *Pediatr Blood Cancer*. 2011; 57:629–635. [PubMed: 21319289]
4. Chun J, Brinkmann V. A mechanistically novel, first oral therapy for multiple sclerosis: The development of fingolimod (FTY720, Gilenya). *Discov Med*. 2011; 12:213–228. [PubMed: 21955849]
5. Azuma H, Takahara S, Ichimaru N, et al. Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. *Cancer Res*. 2002; 62:1410–1419. [PubMed: 11888913]
6. Estrada-Bernal A, Palanichamy K, Ray Chaudhury A, et al. Induction of brain tumor stem cell apoptosis by FTY720: A potential therapeutic agent for glioblastoma. *Neuro Oncol*. 2012; 14:405–415. [PubMed: 22351749]
7. Hung JH, Lu YS, Wang YC, et al. FTY720 induces apoptosis in hepatocellular carcinoma cells through activation of protein kinase C delta signaling. *Cancer Res*. 2008; 68:1204–1212. [PubMed: 18281497]
8. Neviani P, Santhanam R, Oaks JJ, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest*. 2007; 117:2408–2421. [PubMed: 17717597]
9. Pchejetski D, Bohler T, Brizuela L, et al. FTY720 (fingolimod) sensitizes prostate cancer cells to radiotherapy by inhibition of sphingosine kinase-1. *Cancer Res*. 2010; 70:8651–8661. [PubMed: 20959468]
10. Yasui H, Hideshima T, Raje N, et al. FTY720 induces apoptosis in multiple myeloma cells and overcomes drug resistance. *Cancer Res*. 2005; 65:7478–7484. [PubMed: 16103102]
11. Zhang N, Qi Y, Wadham C, et al. FTY720 induces necrotic cell death and autophagy in ovarian cancer cells: A protective role of autophagy. *Autophagy*. 2010; 6:1157–1167. [PubMed: 20935520]
12. Tonelli F, Lim KG, Loveridge C, et al. FTY720 and (S)-FTY720 vinylphosphonate inhibit sphingosine kinase 1 and promote its proteasomal degradation in human pulmonary artery smooth muscle, breast cancer and androgen-independent prostate cancer cells. *Cell Signal*. 2010; 22:1536–1542. [PubMed: 20570726]
13. Arun B, Frenkel EP. Topoisomerase I inhibition with topotecan: Pharmacologic and clinical issues. *Expert Opin Pharmacother*. 2001; 2:491–505. [PubMed: 11336601]
14. Kingsbury WD, Boehm JC, Jakas DR, et al. Synthesis of water-soluble (aminoalkyl)camptothecin analogues: Inhibition of topoisomerase I and antitumor activity. *J Med Chem*. 1991; 34:98–107. [PubMed: 1846923]

15. Park JR, Scott JR, Stewart CF, et al. Pilot induction regimen incorporating pharmacokinetically guided topotecan for treatment of newly diagnosed high-risk neuroblastoma: A Children's Oncology Group study. *J Clin Oncol*. 2011; 29:4351–4357. [PubMed: 22010014]
16. Venkataraman K, Thangada S, Michaud J, et al. Extracellular export of sphingosine kinase-1a contributes to the vascular SIP gradient. *Biochem J*. 2006; 397:461–471. [PubMed: 16623665]
17. Li MH, Hla T, Ferrer F. Sphingolipid modulation of angiogenic factor expression in neuroblastoma. *Cancer Prev Res (Phila)*. 2011; 4:1325–1332. [PubMed: 21576349]
18. Li MH, Sanchez T, Pappalardo A, et al. Induction of antiproliferative connective tissue growth factor expression in Wilms' tumor cells by sphingosine-1-phosphate receptor 2. *Mol Cancer Res*. 2008; 6:1649–1656. [PubMed: 18922980]
19. Li MH, Sanchez T, Milne GL, et al. SIP/SIP2 signaling induces cyclooxygenase-2 expression in Wilms tumor. *J Urol*. 2009; 181:1347–1352. [PubMed: 19157443]
20. Li MH, Sanchez T, Yamase H, et al. SIP/SIP1 signaling stimulates cell migration and invasion in Wilms tumor. *Cancer Lett*. 2009; 276:171–179. [PubMed: 19131156]
21. Bielawski J, Pierce JS, Snider J, et al. Comprehensive quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Methods Mol Biol*. 2009; 579:443–467. [PubMed: 19763489]
22. Brinkmann V, Davis MD, Heise CE, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem*. 2002; 277:21453–21457. [PubMed: 11967257]
23. Staker BL, Hjerrild K, Feese MD, et al. The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc Natl Acad Sci U S A*. 2002; 99:15387–15392. [PubMed: 12426403]
24. Baran Y, Salas A, Senkal CE, et al. Alterations of ceramide/sphingosine 1-phosphate rheostat involved in the regulation of resistance to imatinib-induced apoptosis in K562 human chronic myeloid leukemia cells. *J Biol Chem*. 2007; 282:10922–10934. [PubMed: 17303574]
25. Suzuki E, Handa K, Toledo MS, et al. Sphingosine-dependent apoptosis: A unified concept based on multiple mechanisms operating in concert. *Proc Natl Acad Sci U S A*. 2004; 101:14788–14793. [PubMed: 15466700]
26. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. 1997; 91:231–241. [PubMed: 9346240]
27. del Peso L, Gonzalez-Garcia M, Page C, et al. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science*. 1997; 278:687–689. [PubMed: 9381178]
28. Pyne NJ, Pyne S. Sphingosine 1-phosphate and cancer. *Nat Rev Cancer*. 2010; 10:489–503. [PubMed: 20555359]
29. French KJ, Zhuang Y, Maines LW, et al. Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. *J Pharmacol Exp Ther*. 2010; 333:129–139. [PubMed: 20061445]
30. Gao P, Smith CD. Ablation of sphingosine kinase-2 inhibits tumor cell proliferation and migration. *Mol Cancer Res*. 2011; 9:1509–1519. [PubMed: 21896638]
31. Weigert A, Schiffmann S, Sekar D, et al. Sphingosine kinase 2 deficient tumor xenografts show impaired growth and fail to polarize macrophages towards an anti-inflammatory phenotype. *Int J Cancer*. 2009; 125:2114–2121. [PubMed: 19618460]
32. Lee TK, Man K, Ho JW, et al. FTY720: A promising agent for treatment of metastatic hepatocellular carcinoma. *Clin Cancer Res*. 2005; 11:8458–8466. [PubMed: 16322309]
33. Liu Q, Alinari L, Chen CS, 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. [PubMed: 20460491]
34. Chua CW, Lee DT, Ling MT, et al. FTY720, a fungus metabolite, inhibits in vivo growth of androgen-independent prostate cancer. *Int J Cancer*. 2005; 117:1039–1048. [PubMed: 15986440]
35. Zheng T, Meng X, Wang J, 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. [PubMed: 20506484]
36. Ubai T, Azuma H, Kotake Y, et al. FTY720 induced Bcl-associated and Fas-independent apoptosis in human renal cancer cells in vitro and significantly reduced in vivo tumor growth in mouse xenograft. *Anticancer Res*. 2007; 27:75–88. [PubMed: 17352219]

37. Ingwersen J, Aktas O, Kuery P, et al. Fingolimod in multiple sclerosis: Mechanisms of action and clinical efficacy. *Clin Immunol.* 2012; 142:15–24. [PubMed: 21669553]
38. Miron VE, Schubart A, Antel JP. Central nervous system-directed effects of FTY720 (fingolimod). *J Neurol Sci.* 2008; 274:13–17. [PubMed: 18678377]
39. Don AS, Martinez-Lamenca C, Webb WR, et al. Essential requirement for sphingosine kinase 2 in a sphingolipid apoptosis pathway activated by FTY720 analogues. *J Biol Chem.* 2007; 282:15833–15842. [PubMed: 17400555]
40. Woodcock JM, Ma Y, Coolen C, et al. Sphingosine and FTY720 directly bind pro-survival 14-3-3 proteins to regulate their function. *Cell Signal.* 2010; 22:1291–1299. [PubMed: 20403428]
41. Habrukowich C, Han DK, Le A, et al. Sphingosine interaction with acidic leucine-rich nuclear phosphoprotein-32A (ANP32A) regulates PP2A activity and cyclooxygenase (COX)-2 expression in human endothelial cells. *J Biol Chem.* 2010; 285:26825–26831. [PubMed: 20558741]

Fig. 1.

The effect of FTY720 and FTY720-P on cell viability in NB cells. NB cells were plated to 96-wells. The next day they were incubated with different concentrations of FTY720 (**A**) or FTY720-P (**B**) for 72 hours before the MTT assay was conducted. Data are mean $\pm$ SE of triplicates. Representative data from two independent experiments was shown.

Fig. 2.

FTY720 displayed strong anti-cancer activity and enhanced tumor-suppressive effect of topotecan in NB. **A:** NB xenograft model: the tumor growth curves in mice treated with vehicle controls, FTY720 (5 mg/kg, daily, i.p.) alone, topotecan (1 mg/kg, daily, gavage) alone and FTY720 plus topotecan (n=5 per group). * $P < 0.05$, ** $P < 0.01$ versus control. # $P < 0.05$, ## $P < 0.01$ versus FTY720 group. **B:** The tumor weights of four treated groups on NB xenografts. Data are mean ± SE (n=5). * $P < 0.05$, ** $P < 0.01$ versus control. The P values between drug-treated groups were as listed. **C:** SK-N-AS cells were seeded in 96-well plates and treated with FTY720 (8 μM) alone, topotecan (0.5 μM) alone, or FTY720 plus topotecan for 24–48 hours followed by MTT assay. Data are mean ± SE of triplicates from one representative experiment of three independent experiments. # $P < 0.05$, ## $P < 0.01$ versus as indicated.

Fig. 3.

FTY720 inhibited SphK2 expression and increased pro-apoptotic sphingosine in NB. **A:** SK-N-AS cells were treated with different concentrations of FTY720 for 48 hours followed by quantitative real-time PCR. Data are mean±SE from two independent experiments. * $P<0.05$, ** $P<0.01$ versus control. **B:** SK-N-AS cells were treated with FTY720 for 48 hours followed by Western blot analysis. Representative data from two independent experiments was shown. **C:** SK-N-AS cells were treated with or without FTY720 (5 μM) for 48 hours followed by LC/MS/MS analysis. Data are mean±SE from two independent experiments. * $P<0.05$ versus control. **D:** Fresh tumor tissues from NB xenografts treated with FTY720 or control (100 mg each) were collected and followed by LC/MS/MS analysis (n=3).

Fig. 4.

FTY720-induced NB cell death was caspase-independent and involved dephosphorylation of Akt and BAD at Ser136. **A:** SK-N-AS cells were treated with FTY720 for 6 hours followed by Western blot analysis to detect total and cleaved caspase bands. Jurkat cells served as positive controls for cleaved caspase-8 and caspase-3 bands. **B:** SK-N-AS cells were treated with FTY720 for 48 hours followed by Western blot analysis to detect the indicated proteins. **C:** SK-N-AS cells were treated with the indicated concentrations of FTY720 for 48 hours, followed by the preparation of cytosolic and mitochondrial fractions using cytochrome *c* releasing apoptosis assay kit. Western blot analysis was performed to detect the cytochrome *c* in those fractions. Representative data from at least two independent experiments was shown.