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Phosphorylation of a constrained azacyclic FTY720 analog enhances anti-leukemic activity without inducing S1P receptor activation

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Abstract

The frequency of poor outcomes in relapsed leukemia patients underscores the need for novel therapeutic approaches. The FDA-approved immunosuppressant FTY720 limits leukemia progression by activating protein phosphatase 2A and restricting nutrient access. Unfortunately, FTY720 cannot be re-purposed for use in cancer patients due to on-target toxicity associated with S1P receptor activation at the elevated, anti-neoplastic dose. Here we show that the constrained azacyclic FTY720 analog SH-RF-177 lacks S1P receptor activity but maintains anti-leukemic activity *in vitro* and *in vivo*. SH-RF-177 was not only more potent than FTY720, but killed via a distinct mechanism. Phosphorylation is dispensable for FTY720's anti-leukemic actions. However,

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Conflict of Interest

The authors declare no conflict of interest.

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chemical biology and genetic approaches demonstrated that the sphingosine kinase 2- (SPHK2) mediated phosphorylation of SH-RF-177 led to engagement of a pro-apoptotic target and increased potency. The cytotoxicity of membrane-permeant FTY720 phosphonate esters suggests that the enhanced potency of SH-RF-177 stems from its more efficient phosphorylation. The tight inverse correlation between SH-RF-177 IC₅₀ and *SPHK2* mRNA expression suggests a useful biomarker for SH-RF-177 sensitivity. In summary, these studies indicate that FTY720 analogs that are efficiently phosphorylated but fail to activate S1P receptors may be superior anti-leukemic agents compared to compounds that avoid cardiotoxicity by eliminating phosphorylation.

Introduction

Although acute lymphoblastic leukemia (ALL) patient outcomes have improved dramatically over the last 50 years, relapse is still common, occurring in 15–20% of pediatric and 30–70% of adult patients^{1, 2}. Only 40% (pediatric) and 10% (adult) of relapsed patients achieve a complete response upon treatment, demonstrating the need for novel therapeutic approaches^{2, 3}. The FDA-approved immunosuppressant FTY720 is effective against many hematologic malignancies, including ALL, both *in vitro* and in animal models^{4–8}. While production of reactive oxygen species^{9, 10} or activation of pro-apoptotic Bim and Bid¹¹ have been proposed as alternate mechanisms of action, PP2A activation is thought to be critical for FTY720's anti-leukemic activity^{5, 7, 12–14}. FTY720 triggers amino acid and glucose transporter internalization downstream of PP2A, thereby restricting access to extracellular nutrients⁴. The therapeutic index for FTY720 stems in part from the fact that oncogenic mutations increase metabolic demand and prevent adaptive metabolic changes under nutrient stress, enhancing sensitivity to nutrient limitation^{4, 15}. Despite its proven efficacy and selectivity for transformed cells, FTY720 cannot be re-purposed as an anti-leukemic agent because it produces profound bradycardia through sphingosine-1-phosphate receptor (S1PR) activation at the elevated anti-cancer dose^{16, 17}.

FTY720 is a pro-drug; intracellular phosphorylation by sphingosine kinase 2 (SPHK2) and export through transporters including spinster 2 (SPNS2) are required for S1PR engagement and immunosuppressive lymphocyte sequestration downstream of S1P₁ activation^{18–20} (Figure 1a). In human patients, S1P₁ activation is sufficient to induce bradycardia, while in mice FTY720 slows heart rate by activating S1P₃ (ref. 21, 22). The FTY720 analog AAL-149(S) is not phosphorylated and does not affect S1PRs but kills cancer cells with similar potency to FTY720, indicating that bradycardia and anti-cancer activity are separable⁴. Because S1PR effects prevent FTY720's use in cancer patients, anti-leukemic FTY720 analogs are generally selected for their lack of phosphorylation^{13, 20, 23}. We have reported that reducing the flexibility of the aminodiol portion of FTY720 is sufficient to prevent S1P_{1/3} activation²⁴. One such constrained azacyclic FTY720 analog, SH-RF-177 (Figure 1b), is particularly active against leukemia cell lines²⁵. We now demonstrate that while SH-RF-177 fails to activate S1P_{1/3}, its ability to selectively kill patient-derived leukemia samples and reduce leukemic burden in animals is enhanced by its efficient phosphorylation. These results suggest an improved strategy for generating safe and effective anti-leukemic FTY720 analogs.

Materials and Methods

See Supplementary Methods for details and additional methods.

Reagents

SH-RF-177, SH-RF-39, and their derivatives and FTY720-P'E were synthesized; FTY720 and FTY720-phosphate were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

NADH Fluorescence Lifetime Imaging Microscopy (FLIM)

Lifetime images were acquired using a Zeiss 780 microscope coupled to a Ti:Sapphire laser system (Spectra-Physics Mai Tai, Santa Clara, CA, USA). Data was processed with SimFCS.

Leukemia studies

Female 14–18 week old NOD *scid* gamma (NSG) mice were injected retro-orbitally with 2 500 000 eGFP-expressing SupB15 cells. Mice were treated with SH-RF-177 (10 mg/kg) or vehicle (0.9% NaCl solution) i.p. daily from day 9 to 30. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Mass spectrometry

Samples were analyzed by UPLC-MS/MS (Waters Micromass Quattro Premier XE) equipped with a C18 reversed-phase column (Waters, Milford, MA, USA) in positive ion mode using an acetonitrile/acetic acid gradient elution.

Sphingosine kinase activity

Phosphorylation by SPHK1 and SPHK2 was determined using the ADP-Glo™ Kinase Assay (Promega, Madison, WI, USA) with kinase-specific buffers.

Statistical methods

n = 3 except where noted; see Supplementary Methods for exact values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$).

Results

Constrained FTY720 analog SH-RF-177 retains anti-leukemic activity but does not activate S1P receptors

Bradycardia downstream of S1P receptor activation precludes the use of FTY720 in cancer patients^{16, 17}. We have previously demonstrated that S1P receptor effects are dispensable for the anti-neoplastic activity of FTY720 and that constrained azacyclic analogs lack activity at S1P_{1/3} (ref. 4, 24). More recently, we generated more synthetically accessible constrained analogs, SH-RF-177 and SH-RF-39 (Figure 1b), and demonstrated that SH-RF-177 is more active than FTY720 or its enantiomer in a subset of leukemia cell lines⁽²⁵⁾ and Supplementary Figure 1A). SH-RF-177 retains FTY720's selectivity for leukemia over non-

transformed cells^{4, 5} as expression of the BCR-ABL oncogene reduced the SH-RF-177 IC₅₀ 10-fold relative to non-transformed cells (Figure 1c). Co-culture with stromal cells reduces leukemia cell sensitivity to multiple chemotherapeutic agents. SH-RF-177 was more active than FTY720 and/or SH-RF-39 against four different patient-derived samples in stroma co-culture assays, which should more stringently evaluate drug activity than continuous cell lines²⁶ (Figures 1d). As expected, the absence of stroma decreased the IC₅₀ without altering the compounds' relative potency (Supplementary Figure 1B). Moreover, normal human peripheral blood mononuclear cells were resistant to concentrations of SH-RF-177 that killed parallel cultures of SFO2 leukemia cells (Figure 1e). These experiments indicate that SH-RF-177 is active in patient samples and suggest that it could have an acceptable therapeutic index.

While SH-RF-177 is derived from molecules that are inactive at S1P_{1/3} (ref. 24), the effect of SH-RF-177 on S1PRs was not previously evaluated. Given the conflicting results in mice and humans regarding the contribution of S1P₁ and S1P₃ to bradycardia^{21, 22}, activity at both receptors was assessed. Consistent with published results²¹, FTY720 decreased heart rate by 50% at the commonly employed anti-cancer dose of 10 mg/kg (Figure 1f and Supplementary Figure 1C). FTY720 must be phosphorylated intracellularly and exported before it can activate S1PRs (Figure 1a). Administration of FTY720-phosphate (FTY720-P) bypassed this phosphorylation step and decreased heart rate more rapidly (Supplementary Figure 1D). In contrast, neither SH-RF-177 (10 or 30 mg/kg) nor SH-RF-177-phosphate (177-P, 10 mg/kg) altered heart rate, indicating that SH-RF-177 does not activate S1P₃ *in vivo*, even when phosphorylated (Figure 1f and Supplementary Figure 1D and E). To evaluate the activity of SH-RF-177 at S1P₁, we utilized MEFs derived from reporter mice that express GFP upon S1P₁ activation²⁷. While S1P, FTY720, and FTY720-P induced GFP expression, SH-RF-177 and 177-P failed to activate S1P₁ (Figure 1g). SH-RF-177 also fails to activate S1P₁ *in vivo*. FTY720 induces immunosuppression by blocking S1P₁-dependent lymphocyte egress from secondary lymphoid organs at doses as low as 0.03 mg/kg in mice and 0.25 mg in humans^{20, 28-30}. Accordingly, both FTY720 and FTY720-P dramatically reduced the number of circulating lymphocytes (Figure 1h). In contrast, neither SH-RF-177 nor 177-P had an effect. Together, these results demonstrate that SH-RF-177 lacks S1P receptor activities that prevent the use of FTY720 in cancer patients.

To determine whether SH-RF-177 could limit leukemia progression, NSG mice were injected with SupB15 human leukemia cells and treated with 10 mg/kg SH-RF-177 i.p. daily beginning on day 9. Three weeks of treatment reduced the average leukemic burden in the bone marrow and spleen by 90% and 45%, respectively, with minimal effect in the blood (Figure 1i). We previously identified nutrient transporter loss as a key mechanism through which FTY720 kills leukemia cells⁴. CD98 (4F2hc) is an amino acid transporter-associated protein that is down-regulated by FTY720, reducing amino acid uptake. Interestingly, SH-RF-177 produced statistically significant CD98 down-regulation in the spleen but not in the bone marrow or blood (Figure 1j). The lack of correlation between anti-leukemic activity and CD98 down-regulation suggested that SH-RF-177 and FTY720 kill leukemia cells through distinct mechanisms.

SH-RF-177 triggers apoptosis independent of its nutrient transporters effects

Because SH-RF-177's anti-leukemic activity did not correlate well with CD98 down-regulation *in vivo*, the relationship between nutrient transporter loss and cell death was more fully evaluated *in vitro*. While FTY720, SH-RF-177 and SH-RF-39 reduce surface CD98 equally well at 10 μ M (²⁵ and Supplementary Figure 2A), SH-RF-177 was less effective when equally cytotoxic doses were compared (Figure 2a). The metabolic effects of FTY720 and SH-RF-177 were next compared to known metabolic inhibitors using NADH fluorescence-lifetime imaging microscopy (FLIM). Restricting nutrient import shifts cellular metabolism towards oxidative phosphorylation to more efficiently generate ATP³¹ and these changes can be tracked using NADH FLIM^{32, 33}. As expected, inhibition of oxidative phosphorylation with oligomycin or rotenone and antimycin A decreased the fraction of bound NADH, while inhibiting glycolysis with 2-deoxy-glucose (2-DG) had the opposite effect (Figure 2b). At its IC₅₀, FTY720 increased the bound NADH fraction similar to 2-DG treatment in keeping with its ability to down-regulate transporters (Figure 2a) and limit nutrient access⁴. In contrast, SH-RF-177 did not shift the metabolic profile of cells at its IC₅₀ (Figure 2b), consistent with its limited effects on CD98 surface expression (Figure 2a). These results suggest that SH-RF-177's increased potency stems from a novel mechanism of action unrelated to nutrient transporter loss.

Consistent with its metabolic mechanism, FTY720 induces necrosis in apoptosis-resistant cells⁴. MEFs lacking the pro-apoptotic proteins Bax and Bak were as sensitive as wild type cells to FTY720 and SH-RF-39 (Figure 2c and Supplementary Figure 2B). In striking contrast, *Bax*^{-/-}/*Bak*^{-/-} cells were resistant to SH-RF-177-induced death (Figure 2c and Supplementary Figure 2C), strongly suggesting that SH-RF-177 kills cells by inducing apoptosis. Indeed, SH-RF-177 increased the percent of cells with a sub-G1 DNA content (Figures 2d and e and Supplementary Figure 2D) and triggered caspase 3 cleavage (Figure 2f), whereas an equimolar dose of FTY720 did not. The pan-caspase inhibitor QVD-OPh prevented death, confirming that SH-RF-177-induced cytotoxicity is caspase-dependent (Figure 2g). Furthermore, the Bcl-2 inhibitor ABT-263 (Navitoclax) sensitized patient-derived SFO2 and ICN1 cells to a cytostatic dose of SH-RF-177 (Figure 2h). These data indicate that at its IC₅₀ SH-RF-177 kills cells by triggering apoptosis, although higher doses produce transporter loss and bioenergetic stress similarly to FTY720 (Supplementary Figures 2A and C).

Enhanced activity of SH-RF-177 requires its phosphorylation

While S1P has intracellular anti-apoptotic actions that might be opposed by SH-RF-177 or its phosphate³⁴, both S1P and FTY720-P have been found to inhibit HDACs, which can trigger apoptosis^{35, 36}. Consistent with a potential pro-apoptotic role for 177-P, SH-RF-177 was phosphorylated much more efficiently than FTY270 in MEFs and SupB15 leukemia cells (Figures 3a and b). *In vitro* kinase assays confirmed that SH-RF-177 is a better substrate than FTY720 for both SPHK1 and SPHK2 (Figure 3c). *In vivo*, only 177-P was detected in the plasma (Figure 3d), indicating that SH-RF-177 is very efficiently phosphorylated in the blood. Both SH-RF-177 and 177-P were detected in the spleen and bone marrow (Figures 3e and f). Interestingly, the degree of transporter loss in leukemic animals correlated with the concentration of unphosphorylated SH-RF-177 present in the

tissue (Figure 1j, Figures 3d–f). Transporter down-regulation results from PP2A activation^{4, 37}. Consistent with distinct mechanisms of action, SH-RF-177 but not 177-P activated PP2A (Fig. 3g). Taken together, these results suggest that 177-P is responsible for the enhanced anti-leukemic, pro-apoptotic activity of SH-RF-177, while the unphosphorylated form is linked to transporter loss.

To test whether conversion to 177-P accounts for the enhanced cytotoxicity of SH-RF-177, we evaluated MEFs isolated from Sphk1- or Sphk2-deficient animals. SPHK2 is the predominant FTY720 kinase³⁸ and *Sphk2*^{-/-} MEFs failed to phosphorylate both FTY720 and SH-RF-177 (Figure 3a). As expected if FTY720-induced cytotoxicity is independent of phosphorylation^{4, 9}, absence of Sphk1 or Sphk2 did not affect sensitivity to FTY720 (Figure 3h). In contrast, *Sphk2*^{-/-} MEFs were resistant to SH-RF-177-induced death (Figure 3i). Moreover, *Sphk2*^{-/-} MEFs failed to exhibit apoptotic features observed in wild type MEFs (Figures 2d and f). These studies indicate that SPHK2-dependent phosphorylation is required for the pro-apoptotic effects that increase the cytotoxicity of SH-RF-177.

To complement this genetic strategy, we synthesized SH-RF-177 analogs that eliminated or mimicked phosphorylation. A non-phosphorylatable version of SH-RF-177 (177-nonP) was generated by replacing the hydroxymethyl group with a methyl or methoxy group (²⁵ and Supplementary Figure 3). Consistent with results in *Sphk2*^{-/-} MEFs (Figure 3i), 177-nonP was less potent than SH-RF-177 but as active as FTY720 in SupB15 cells and MEFs (Figure 3j). 177-nonP also activated PP2A similarly to SH-RF-177 and FTY720 (Figure 3g). However, in contrast to SH-RF-177, 177-nonP down-regulated nutrient transporters with similar efficiency to FTY720 and *Bax*^{-/-}/*Bak*^{-/-} cells were not resistant to 177-nonP (Figures 2a and c). As rapid conversion of 177-P to SH-RF-177 by phosphatases made it difficult to parse the roles of the two compounds (data not shown), the phosphonic acid (177-P'A) was synthesized to mimic the structural features of the “locked” phosphate (Supplementary Figure 3). Because 177-P'A was likely too polar to cross the plasma membrane, the phosphonate ester (177-P'E) was also generated. Esterification neutralizes the charge present in the free acid, increasing membrane permeability; cytosolic esterases will produce the phosphonic acid intracellularly (Figure 4a). As previously reported for FTY720-P^{4, 20, 39}, 177-P'A was not cytotoxic (Figure 4b). 177-P'E was cytotoxic, although slightly less active than SH-RF-177. As expected, *Sphk2* deletion did not confer resistance to 177-P'E (Figure 4c). These results indicate that extracellular S1P receptors are not the pro-apoptotic target of 177-P. Furthermore, increasing intracellular 177-P 1.5-fold by eliminating Spns2 (Supplementary Figure 4A) increased sensitivity to SH-RF-177 1.5 times (Supplementary Figure 4B). If the 177-P target is intracellular, the reduced cytotoxicity of 177-P'E relative to SH-RF-177 (Figure 4b) may stem from the fact that, once de-esterified, the compound is exported by Spns2 and is then too polar to re-enter the cell (Figure 4a). Taken together, these results are consistent with a model where 177-P and SH-RF-177 engage distinct intracellular targets, both of which contribute to cytotoxicity.

It was unclear whether 177-P engaged a pro-apoptotic target not accessible to FTY720-P or whether the enhanced phosphorylation of SH-RF-177 (Figures 3a and b) simply generated more ligand to engage a common target. To distinguish between these possibilities, a racemic mixture of FTY720 phosphonate ester (FTY720-P'E) was generated

(Supplementary Figure 3). FTY720-P'E was as active as 177-P'E in cytotoxicity assays (Figure 4b), suggesting that enhanced phosphorylation rather than a differential affinity for the pro-apoptotic target accounts for SH-RF-177's increased potency. Similar results were obtained with the less potent SH-RF-177 stereoisomer, SH-RF-39. SH-RF-39 was a poor sphingosine kinase substrate (Supplementary Figures 5A-C), but the phosphonate ester of SH-RF-39 (39-P'E) was as cytotoxic as 177-P'E (Supplementary Figures 3 and 5D). In summary, the equivalent activities of the phosphonate esters of FTY720, SH-RF-177, and SH-RF-39 demonstrate that the enhanced cytotoxicity of SH-RF-177 stems from the increased intracellular accumulation of the phosphorylated form rather than structural features that allow 177-P to engage a target not accessible to FTY720-P.

SPHK2 expression predicts SH-RF-177 sensitivity

Patient selection based on established biomarkers is a key component of successful clinical trials. The data presented here suggest that SPHK2 plays a critical role in determining cellular sensitivity to SH-RF-177. When the IC₅₀ values of six different NCI60 cancer cell lines were plotted against their relative *SPHK2* mRNA expression, a tight inverse correlation was observed (Pearson's $r = -0.91$, P value = .01) (Figure 5a, left). Sensitivity to SH-RF-177 was not correlated with the expression of other sphingolipid metabolism genes or the PP2A protein inhibitor *SET/I2PP2A*, which is reported to play a critical role in sensitivity to FTY720^{7, 12, 40} (Supplementary Figure 6). Consistent with the less efficient phosphorylation of FTY720 (Figures 3a and b), the correlation between FTY720 IC₅₀ and *SPHK2* expression was weaker (Pearson's $r = -0.62$, P value = .19) (Figure 5a, right). To further test the hypothesis that *SPHK2* expression was sufficient to predict sensitivity to SH-RF-177, *SPHK2* was over-expressed in a resistant cell line with low endogenous levels of *SPHK2*, DU145 (Figure 5a, red arrow). Over-expression of *SPHK2* increased production of 177-P and dramatically sensitized DU145 human prostate cancer cells to SH-RF-177, decreasing the IC₅₀ by a factor of 10 (Figures 5b and c, left). As would be predicted, *SPHK2* over-expression did not sensitize DU145 cells to 177-nonP (Figure 5c, right). These results suggest that *SPHK2* expression level would provide a useful biomarker to identify leukemias and other tumors sensitive to SH-RF-177.

Discussion

FTY720 displays cancer-selective activity against acute and chronic lymphoblastic leukemias, acute and chronic myeloid leukemias, and multiple myeloma⁵⁻⁸. However, bradycardia downstream of S1P receptor activation precludes its re-purposing as an anti-leukemic therapy^{16, 17}. FTY720 derivatives that lack S1P receptor activity such as AAL-149(S), OSU-2S, and MP07-66 retain anti-leukemic activity and might be used in cancer patients^{4, 13, 20, 23, 41, 42}. However, all three compounds are rendered safe for use at the anti-leukemic dose by elimination of SPHK2-mediated phosphorylation. SH-RF-177 is the first anti-leukemic FTY720 analog that is efficiently phosphorylated yet fails to activate S1P receptors or sequester lymphocytes (Figures 1f-h). By generating non-phosphorylatable and phosphomimetic versions of SH-RF-177 and testing them in *Sphk2*^{-/-}, *Spns2*^{-/-}, and *Bak*^{-/-}*Bax*^{-/-} MEFs, we clearly demonstrate that phosphorylation enhances the anti-leukemic activity of SH-RF-177 by allowing it to engage an intracellular, pro-apoptotic

target. Interestingly, the FTY720 analog AAL-149(*R*) also triggers SPHK2-dependent apoptosis⁴¹. However, because AAL-149(*R*)-phosphate potently activates S1P₁ and S1P₃, this compound is unsuitable for use in leukemia patients²¹. Whether apoptosis induction was a general feature of FTY720-P analogs or specific to AAL-149(*R*)-phosphate was not evaluated in this earlier study. The equivalent activity of the phosphomimetic phosphonate esters of SH-RF-177, SH-RF-39, and FTY720 (Figure 4b and Supplementary Figure 5D) suggests that the pro-apoptotic target is accessible to multiple FTY720 analogs upon their phosphorylation. Our demonstration that FTY720-P'E is toxic suggests that FTY720-P failed to induce apoptosis in previous studies because it was too polar to enter cells^{4, 20, 39}. Although *Sphk2*^{-/-} MEFs were not resistant to FTY720, FTY720-P may contribute to FTY720's anti-cancer activity as sensitivity is somewhat correlated to SPHK2 expression (Figure 5a, right). The efficient phosphorylation of SH-RF-177 *in vitro* (Figure 3c), in cells (Figures 3a and b), and in mice (Figures 3d–f) is consistent with previous reports that (*R*) stereoisomers of FTY720 analogs are better SPHK2 substrates²⁰ and with observations that removing the non-phosphorylated hydroxyl group from the aminodiol portion of FTY720 enhances phosphorylation⁴³. In sum, our findings suggest that SH-RF-177 represents a novel class of anti-leukemic FTY720 analogs with increased therapeutic potential: compounds that are efficiently phosphorylated but fail to activate S1PRs.

While phosphorylation of SH-RF-177 leads to apoptotic death, a key conclusion from our studies is that phosphorylation is dispensable for killing through FTY720's bioenergetic mechanism (Figures 2a and c). Moreover, while IC₅₀ concentrations of SH-RF-177 cannot trigger sufficient nutrient transporter loss to induce metabolic changes consistent with starvation (Figures 2a and b), it down-regulates nutrient transporter proteins as efficiently as FTY720 at equimolar doses (Supplementary Figure 2A). Thus, SH-RF-177 has not lost the anti-leukemic metabolic actions of FTY720 but rather gained additional pro-apoptotic actions through its efficient phosphorylation. The identification of two distinct mechanisms by which FTY720 induces cell death helps to explain conflicting reports regarding the role of phosphorylation in FTY720-induced cytotoxicity and its ability to kill through necrosis or apoptosis^{4, 5, 20, 39, 41}. While the phosphates of other FTY720 analogs may engage both the apoptotic and metabolic anti-leukemic targets, 177-P may be unique in its lack of S1P_{1/3} activity (Figures 1f–h), a key requirement for a therapeutic compound.

The finding that the membrane-impermeant 177-P'A does not reduce cell viability or proliferation while 177-P'E is cytotoxic (Figure 4b) establishes that the anti-leukemic target of 177-P is intracellular. Previous studies with FTY720-P and S1P suggest several pro-apoptotic intracellular targets for 177-P. For example, it has recently been reported that FTY720-P produced by nuclear SPHK2 inhibits class I HDACs³⁵. Intriguingly, HDAC inhibitors such as the FDA-approved drug vorinostat can induce apoptosis in leukemias and other cancers³⁶. However, neither FTY720, SH-RF-177, nor their phosphates reproducibly increased histone acetylation in whole cell lysates or isolated nuclei from MEFs or ALL cell lines despite robust increases in histone acetylation in vorinostat-treated controls. FTY720-P and 177-P also failed to inhibit the enzymatic activity of purified HDAC1 despite strong inhibition with vorinostat (data not shown). This may indicate either that culture conditions or cell type affect the ability of FTY720 to function as an HDAC inhibitor or that this assay is very sensitive to small experimental differences. Moreover, while our independent

modeling confirms that FTY720-P fits into the binding pocket of HDAC2³⁵, the nitrogen atom in the pyrrolidine ring of 177-P assumes a spatial orientation incompatible with interaction with the Zn²⁺ ion (Supplementary Figure 7). It is therefore unlikely that 177-P triggers apoptosis by inhibiting HDACs.

Other proposed mechanisms for apoptosis include SPHK1 inhibition or PP2A activation. FTY720 can inhibit SPHK1, and several molecules structurally related to SH-RF-177 have been suggested to kill cancer cells through this mechanism^{44, 45}. However, independent studies from Pfizer and Amgen have demonstrated that dramatically reducing S1P levels by inhibiting SPHK1 and/or 2 does not limit cancer cell proliferation or viability or inhibit tumor growth^{46, 47}. Our observation that eliminating SPHK1 affords no protection from either FTY720 or SH-RF-177 (Figures 3h and i) also suggests that SPHK1 inhibition is not a major contributor to the anti-neoplastic activity of SH-RF-177. Endogenous sphingolipids and FTY720 activate PP2A^{4, 5, 7, 12–14, 40, 48, 49}. We have demonstrated that nutrient transporter loss is PP2A-dependent, and others have reported that FTY720 stops proliferation of Ph⁺ ALL or CML cells and induces apoptosis of CLL cells by reactivating PP2A, possibly by disrupting the interaction with the inhibitor SET^{5, 7, 12, 13, 40}. While SH-RF-177 activates PP2A, 177-P does not (Figure 3g). In our hands, chemical PP2A inhibitors kill cells within 6 h at concentrations sufficient to correct PP2A-induced dephosphorylations, while the less toxic approach of over-expressing the protein inhibitors SET or SV40 small t was insufficient to block cell death (data not shown). Thus the extent to which SH-RF-177-induced death depends on PP2A activation remains unclear. Ongoing efforts to identify the specific PP2A heterotrimers affected by FTY720 analogs and to isolate the binding partners of SH-RF-177 and 177-P should facilitate future experiments addressing this point.

In summary, we propose a new paradigm for generating the most potent and effective anti-leukemic sphingolipids: retaining phosphorylation but ablating S1P receptor activity. Previous studies indicating that FTY720-P was not cytotoxic are readily explained by our demonstration that its pro-apoptotic target is intracellular. SH-RF-177 is currently unique among anti-neoplastic FTY720 analogs in retaining phosphorylation without activating S1P receptors. However, the equivalent potency of phosphonate esters of FTY720, SH-RF-177, and SH-RF-39 suggests that other phosphorylated analogs may also bind this target. A critical goal of future work will be to isolate the target(s) of SH-RF-177 and 177-P. This knowledge would clarify which cancer subtypes are likely to be most sensitive and how cancer cells might acquire resistance, aid in the design of rational drug combinations to further improve the therapeutic index, and facilitate medicinal chemistry optimization for enhanced target engagement. Optimization of SH-RF-177 dosing strategies and mode of administration will also be important in the continued preclinical evaluation of this analog.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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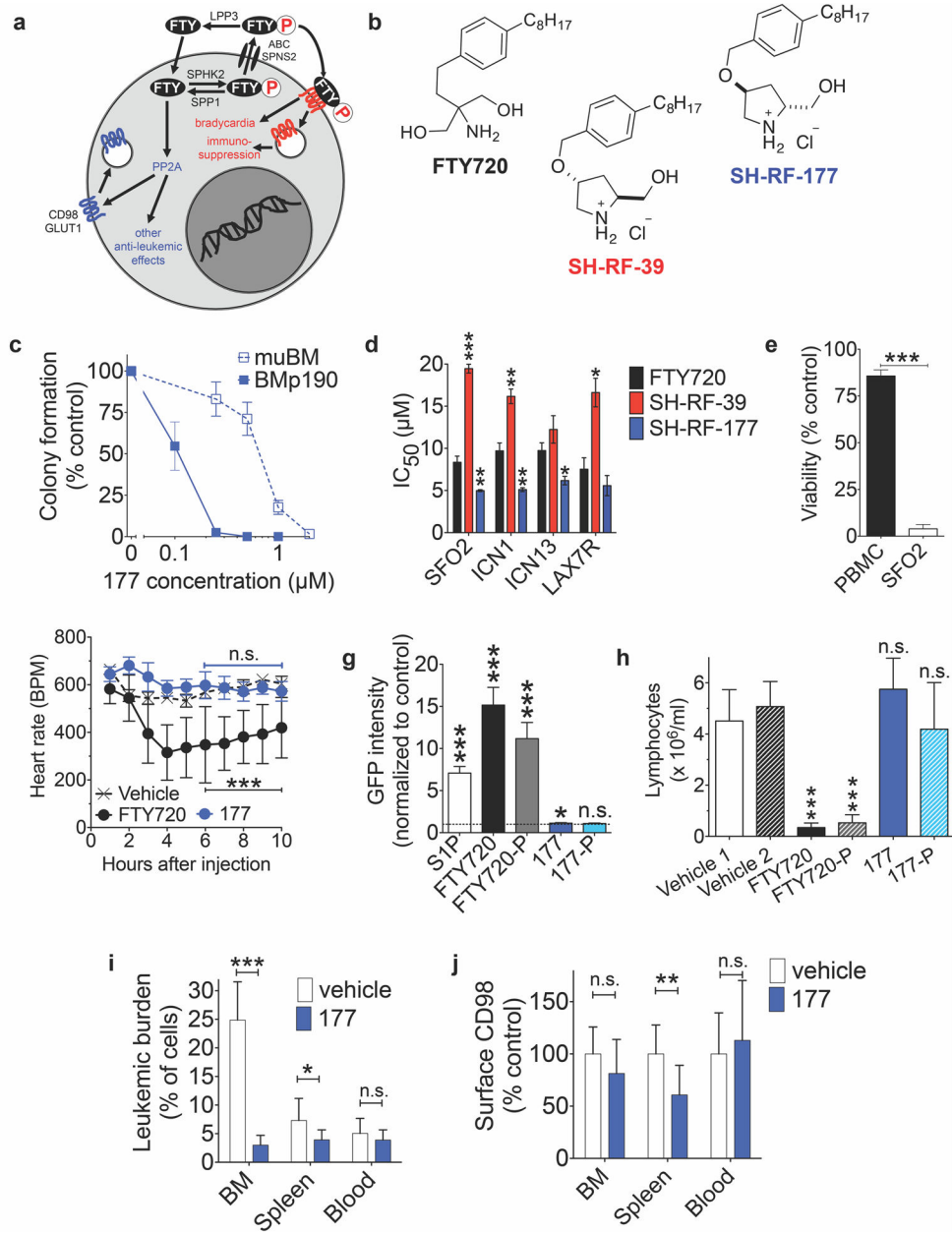


Figure 1. Constrained FTY720 analog SH-RF-177 retains anti-leukemic activity and selectivity for transformed cells without affecting S1P_{1/3}

(a) FTY720 diffuses into the cell where it is phosphorylated by SPHK2. FTY720-P is membrane-impermeant and must be exported by SPNS2 (or possibly ABC transporters) before it can bind to surface S1P receptors. FTY720-P is dephosphorylated by SPP1 and LPP3. (b) Structures of FTY720, SH-RF-39, and SH-RF-177. (c) Colony formation assays with primary murine bone marrow cells (muBM) or bone marrow cells expressing the BCR-ABL p190 fusion (Bmp190) in SH-RF-177 at 7 d ($P = 0.001$). (d) FTY720, SH-RF-177 and SH-RF-39 IC₅₀ values in patient-derived ALL samples at 96 h. (e) Peripheral blood mononuclear cells (PBMCs) from healthy donors or patient-derived SFO2 ALL cells were

treated with 5 μ M SH-RF-177 in liquid culture at 3 d. **(f)** Heart rate was monitored continuously for 10 h after a single dose of the indicated compound. **(g)** S1P₁ reporter MEFs were treated with 1 μ M of the indicated compounds. Fluorescence was quantified by flow cytometry at 24 h. **(h)** Peripheral blood was collected and the number of CD4⁺, CD8⁺, or B220⁺ lymphocytes per ml quantified 12 h after administration of the indicated compounds. **(i)** Frequency of GFP⁺ SupB15 cells in bone marrow (BM), spleen, or blood of NSG mice. **(j)** Surface CD98 on leukemia cells in **(i)**. Compounds were given to C57BL/6 **(f–h)** or NSG **(i and j)** mice at 10 mg/kg by i.p. injection. FTY720 and SH-RF-177 were delivered in saline (vehicle 1) and FTY720-P and 177-P were delivered in acidified DMSO in saline (vehicle 2). Means shown \pm SEM for *in vitro* experiments **(c–e)** and \pm SD for *in vivo* experiments **(f–j)**. In **(d)**, statistics indicate comparison to FTY720 IC₅₀.

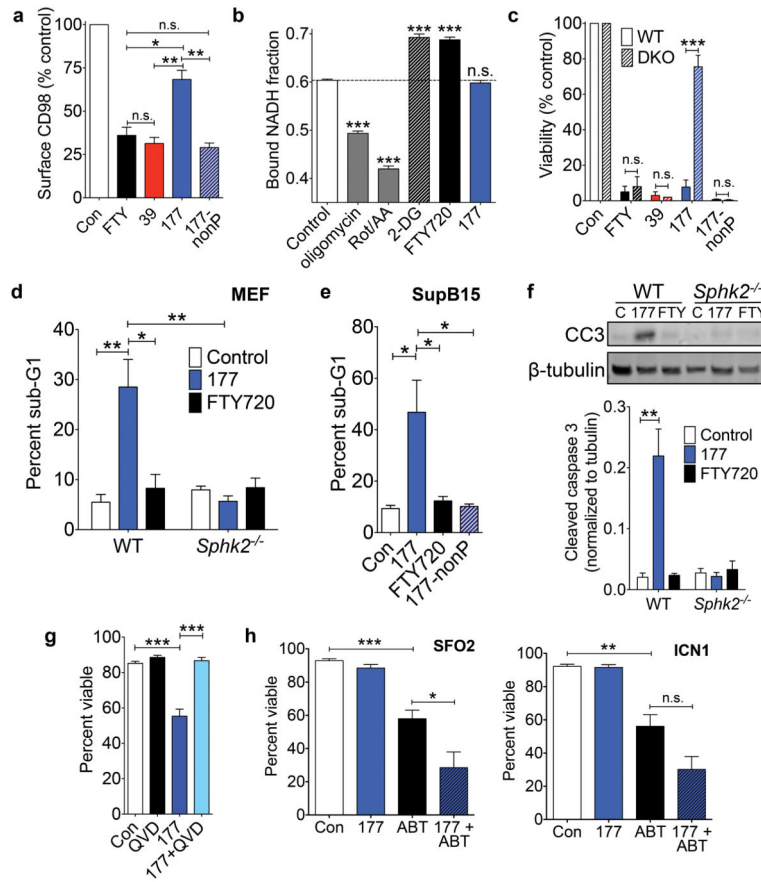


Figure 2. SH-RF-177 has a pro-apoptotic activity independent of its effects on nutrient transporters

(a) Surface CD98 levels in SupB15 cells treated with the indicated compounds at twice their IC₅₀ for 3 h. (b) MEFs were treated with 1 μM oligomycin, 1 μM rotenone and antimycin A (Rot/AA), 1 mM 2-deoxy glucose (2-DG), or FTY720 or SH-RF-177 at their IC₅₀ for 16 h and the bound NADH fraction determined (n = 2 biological replicates, 50 cells for each). (c) Wild type (WT) and *Bax*^{-/-}/*Bak*^{-/-} MEFs (DKO) were treated with the indicated compounds at twice their IC₅₀ in WT MEFs for 48 h. (d-e) WT and *Sphk2*^{-/-} MEFs (d) or SupB15 cells (e) were treated with SH-RF-177 at twice their IC₅₀ in WT cells and percent of cells with sub-G1 DNA content determined at 17 (d) or 24 h (e). (f) WT and *Sphk2*^{-/-} cells were treated as in (d) and cleaved caspase 3 measured by western blot. A representative blot and quantification are shown. (g) SupB15 cells were treated with SH-RF-177 at twice the IC₅₀ ± 20 μM QVD-Oph for 24 h. (h) Patient-derived SFO2 (left) and ICN1 (right) ALL cells were cultured with stroma and treated with 1.25 μM SH-RF-177 ± 100 nM ABT-263 for 8 d. In all panels, means ± SEM shown.

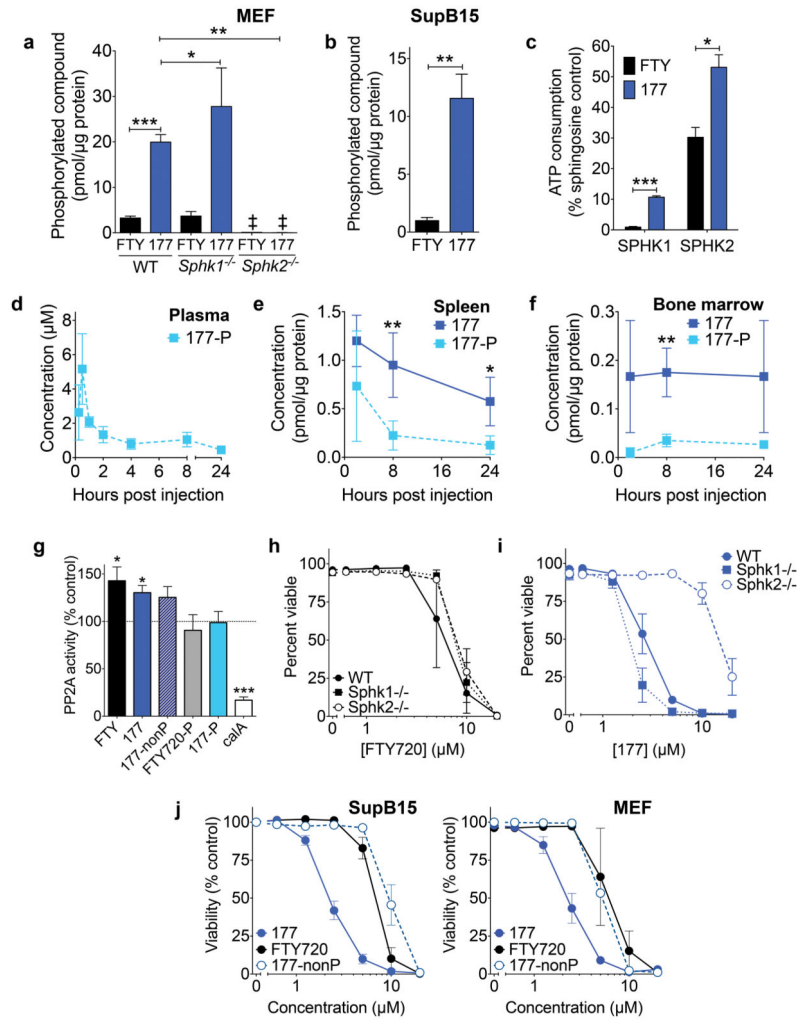


Figure 3. FTY720 and SH-RF-177 are differentially phosphorylated and phosphorylation leads to enhanced potency

(a–b) FTY720-P and 177-P were quantified by UPLC-MS/MS in WT, *Sphk1*^{-/-}, and *Sphk2*^{-/-} MEFs (a) or SupB15 cells (b) treated with FTY720 or SH-RF-177 at 10 μM for 6 h (a) or 5 μM for 24 h (b). ‡Below limit of detection. (c) Phosphorylation of FTY720 and SH-RF-177 by recombinant SPHK1 or SPHK2 as measured by ATP consumption. (d–f) Concentrations of SH-RF-177 and 177-P in plasma (d), spleen (e), and bone marrow (f) after a single 10 mg/kg i.p. dose of SH-RF-177. (g) Activity of immunoprecipitated PP2A in the presence of 5 μM of the indicated compounds. The PP2A inhibitor calyculin A (calA, 5nM) is included as a control. (h–i) WT, *Sphk1*^{-/-}, and *Sphk2*^{-/-} MEFs were treated with FTY720 (h) or SH-RF-177 (i) for 48 h. IC₅₀ values for FTY720 are not significantly different; IC₅₀ for SH-RF-177 is significantly different between WT and *Sphk2*^{-/-} (*P* = 0.001) but not WT and *Sphk1*^{-/-}. (j) SupB15 cells (left) or MEFs (right) were treated with 177-nonP for 72 h (left) or 48 h (right). For SupB15 cells, IC₅₀ value for 177-nonP is significantly different from values for both FTY720 (*P* = 0.01) and SH-RF-177 (*P* = 0.001). For MEFs, IC₅₀ value for 177-nonP is significantly different from the value for SH-RF-177 (*P* = 0.01) but not

FTY720. Means are shown \pm SEM in *in vitro* experiments (**a–c** and **g–j**) and \pm SD in *in vivo* experiments (**d–f**).

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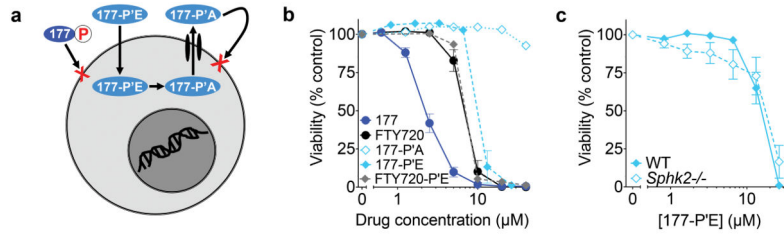


Figure 4. The SH-RF-177 target is intracellular

(a) Model of import/export cycle for SH-RF-177 derivatives. (b) SupB15 cells were treated with the indicated compounds for 72 h. IC₅₀ value for 177-P'E is significantly different from values for FTY720 ($P = 0.001$), SH-RF-177 ($P = 0.001$), and FTY720-P'E ($P = 0.01$). (c) WT and *Sphk2*^{-/-} MEFs were treated with 177-P'E for 48 h. IC₅₀ values for 177-P'E in WT and *Sphk2*^{-/-} cells were not significantly different.

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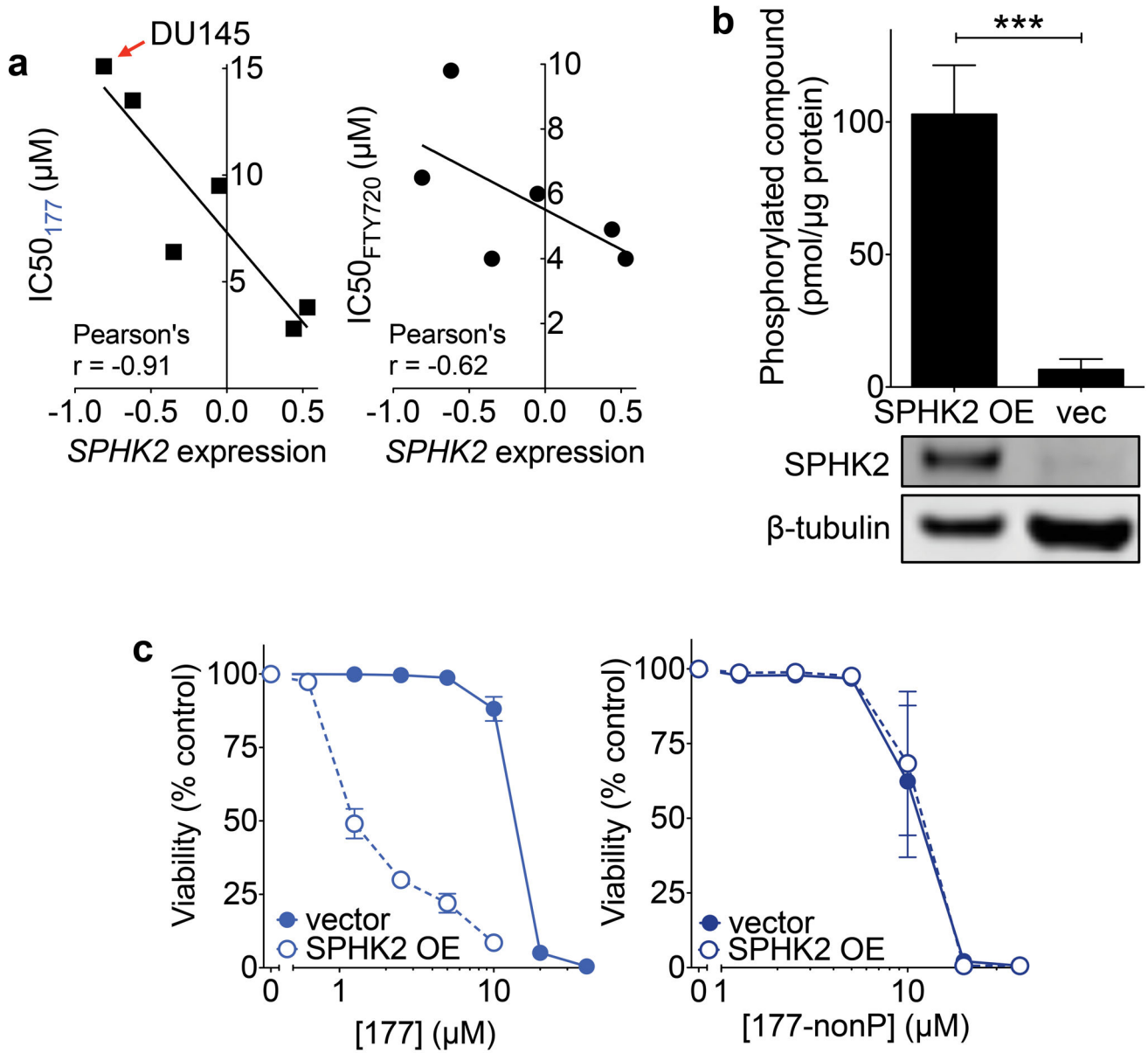


Figure 5. *SPHK2* expression level determines sensitivity to SH-RF-177

(a) Relative expression of *SPHK2* mRNA plotted against the IC₅₀ values for SH-RF-177 (left) or FTY720 (right) for 6 NCI60 cell lines. Pearson's r coefficients were -0.91 ($P=0.01$) for SH-RF-177 and -0.62 ($P=0.19$) for FTY720. (b) Total 177-P was quantified by UPLC-MS/MS in control and *SPHK2* over-expressing DU145 cells treated with 10 μM SH-RF-177 for 6 h. Protein expression in control and *SPHK2* over-expressing (OE) DU145 cells is also shown. (c) Cells as in (b) were treated with SH-RF-177 (left) or 177-nonP (right) for 72 h. The IC₅₀ values for SH-RF-177 in *SPHK2* over-expressing cells and vector control are significantly different ($P=0.001$) while those for 177-nonP are not. Values shown in (b) and (c) are means ± SEM.