Pharmacologic Restoration of PP2A Activity by FTY720 as a Novel Therapy for Polycythemia Vera

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Abstract

Polycythemia Vera (PV) is an incurable rare myeloproliferative disorder driven by Jak2 V617F with few viable treatment options and no curative therapy available. With the intention of developing a curative treatment modality, we examined the interplay between the known tumor suppressor PP2A and the driver of PV, Jak2 V617F. We found PP2A to be inactivated by Jak2 V617F activity and that forced PP2A activity is produces a loss of Jak2 V617F activity and function. Of particular interest we found that the clinically relevant PP2A activator/immunosuppressant FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride, fingolimod, Gilenya) to activate PP2A in PV model cell lines and primary patient samples. Treatment with FTY720 produced a reduction in Jak2 protein coupled with reduced cellular proliferation in cells expressing Jak2 V617F. The anti-leukemic effects of FTY720 were found to be independent of immunosuppressive activity. To conclude, we found that PP2A activation in cells expressing Jak2 V617F by FTY720 reduced Jak2 levels and proliferation and did so in a mechanism independent of immunosuppression.

Introduction

Polycythemia Vera (PV) is rare myeloproliferative disorder driven by a mutation in the Jak2 kinase (Jak2 V617F) that causes patients to over-produce mature erythrocytes leading to viscous/sticky blood. Patients with PV often die from heart attack or stroke due to the increased blood viscosity. We examined the interactions between Jak2 V617F and the tumor suppressor

PP2A to determine if PP2A is a viable target for the treatment of PV. PP2A was inactive in PV model cell lines and in primary patient samples. Conversely, forced activation of PP2A through PP2A overexpression or treatment with the clinically relevant PP2A activator FTY720 produced reduced Jak2 expression and activity and inhibited cellular proliferation. In particular, FTY720 was found to mimic the endogenous PP2A activator ceramide by binding directly with the PP2A inhibitor SET.

Results/Discussion

Jak2 V617F has is resistant to most endogenous regulators therefore we focused on the tumor suppressor protein phosphatase 2A (PP2A). PP2A is a serine/threonine phosphatase that regulates cell cycle and cell division by targeting a variety of kinases and phosphatases.

Notably, PP2A binds in a complex with Jak2 (1-3).

Results

PP2A is a target of Jak2 V617F.

A key attribute of PV is the escape of Jak2 V617F from control by negative feedback mechanisms. While regulators such as SOCS proteins and loss of stimulatory cytokines are ineffective at inhibiting Jak2 V617F less consideration has be given to regulatory phosphatases. To establish the Jak2/PP2A interplay we transfected the IL-3-dependent pro-B lymphoid Ba/F3 cell line with the MiGR1 Jak2 V617F vector. Using GFP as a marker we established separate low and high expressing populations and measured PP2A activity. Interestingly, all cells expressing Jak2 V617F has reduced PP2A activity while the highest expressing cells produced the most robust inhibition of PP2A (Figure 1 A and B).

Likewise, treatment of Jak2 V617F expressing Ba/F3 cells with either of two Jak2 inhibitors (AG490 25μM, 10 hours or Jak Inhibitor I 1μM, 10 hours) restored PP2A to 68% and 100% or normal controls, respectively (Figure 1C).

We previously found a role for the PP2A inhibitor SET in leukemia (4), we therefore sought to determine it's role in PV. To do this we expressed SET shRNA in the Ba/F3 Jak2 V617F cell line as well as CD34+ PV primary patient samples (Figure 2A). This restored PP2A activity to 80% of normal controls in the Ba/F3 cells (Figure 2B) and produced a 2-fold increase in PP2A activity in the PV patient sample (Figure 2C). Likewise the clonogenic potential of Ba/F3 Jak2 V617F+ cells by 91% (Figure 2D). Together, these data suggest Jak2 V617F requires SET expression to inhibit PP2A. Additionally, knock-down of SET reduced the clonogenic potential of Ba/F3 Jak2 V617F cells by 91% (Figure 2D), a reduction likely caused by restoration of PP2A activity.

To examine the Jak2 V617F/PP2A relationship further we overexpressed PP2A in Ba/F3 Jak2 V617F cells. This overexpression produced a restoration of PP2A activity (Figure 3A) but, more importantly, caused a reduction in Jak2 protein levels and a stark reduction in Jak2 activity levels as measure by Jak2 pY1007/1008 (Figure 3B), consequently these cells also had an 82% reduction in colony forming ability when compared to Ba/F3 Jak2 V617F cells (Figure 3C).

We next introduced the clinically relevant PP2A activator FTY720. Using this compound we restored PP2A activity to 78% of normal controls when treating Ba/F3 Jak2 V617F cells (data not shown) and produced a 2-fold increase in PP2A activity in CD34+ PV primary patient samples (Figure 4A). Similarly, treatment with FTY720 reduced Jak2 levels (Figure 4B) and colony forming of PV patient sample CD34+ cells by 62% Figure 4C).

In sum, these data indicate that pharmacologic activation of PP2A by FTY720 neutralizes Jak2 V617F-driven proliferation PV cells through reduction of Jak2 activity.

Reportedly, for FTY720 to be immunosuppressive necessitates phosphorylation by sphingosine kinase (SPHK) 2 which permits the drug to bind to the sphingosine-1-phosphate receptor 1 (S1PR1) which induces lymphocyte sequestration (5). To determine whether phosphorylation of FTY720 into FTY720-P is required for its anti-leukemic activity in Jak2 V617F-expressing cells, we co-treated Ba/F3 Jak2 V617F cells with the SPHK inhibitor dimethylsphingosine (DMS, 2.5μM, 6h) which had no effect of the ability of FTY720 to activate PP2A. Likewise direct treatment with immunosuppressive FTY720-P was unable to activate PP2A in Ba/F3 Jak2 V617F cells (Figure 5A). Likewise, treatment with FTY720 or FTY720 + DMS reduces Jak2 levels while FTY720-P does not (Figure 5B). Additionally, loss of the S1PR1 through shRNA-mediated knock down had no affect on the PP2A activating effects of FTY720 signifying the receptor is not required for FTY720 to modulate PP2A (Figure 5C and D). These data indicate that FTY720 activates PP2A using a mechanism independent of its immunosuppressive properties.

As FTY720 is a sphingolipid analog and a sphingolipid (ceramide) is known to activate PP2A through binding of SET (6) we sought to determine if FTY720 may bind to SET in a manner similar to that of ceramide. FTY720/SET binding was measured in three independent experiments. In the first experiment normal Ba/F3 cells were retrovirally transduced with either wild-type SET or SET with a mutated ceramide binding site (SET K209D). Overexpression of either SET reduced PP2A activity, but only the wild-type SET responded to FTY720 treatment with an increase in PP2A activity indicating FTY720 likely binds SET on the ceramide-binding region (Figure 6A). Likewise, an avidin pull-down of a biotin-labeled FTY720 analog followed

by western blotting for SET showed a direct FTY720/SET interaction (Figure 6B). Finally, an avidin pull-down from lysates treated with biotin labeled ceramide showed the expected ceramide/SET interaction while adding FTY720 to the lysate disrupted the ceramide/SET binding again suggesting that FTY720 binds to SET on the same region as ceramide (Figure 6C).

Finally, as we demonstrated that the anti-leukemic and immunosuppressive properties of are independent, work as begun on new FTY720 analogs that lack immunosuppressive properties yet retain the PP2A-activating effects of FTY720. One of these analogs, QC-FTYSM, increased PP2A activity in Ba/F3 Jak2 V617F cells, mimicking FTY720. Unlike the immunosuppressive FTY720-P, however, treatment with QC-FTYSM did not induce internalization of a GFP-tagged S1PR1 suggesting the drug and receptor do not bind. Likewise while FTY720 decreased circulating CD19+/B220+ lymphocytes from 7.76% to 1.21%, QC-FTYSM produced no reduction (CD19+/B220+ remained at 7.37%) again showing this compound is not immunosuppressive.

Conclusions

Polycythemia Vera is a rare incurable disease caused by the constitutively-active Jak2 V617F. Our data indicate that Jak2 V617F produces a functional inhibition of the tumor suppressor PP2A. However, forced reactivation of PP2A, either through over expression or pharmacologic activation by FTY720, causes a loss of Jak2 V617F protein and activity with a corresponding decrease in cellular proliferation. Interestingly we found that FTY720 activates PP2A through a novel mechanism of binding to the PP2A inhibitor SET. As FTY720 functions through SET to activate PP2A it does not require phosphorylation or expression of the S1PR1 which are required for immunosuppression. Based on this divorcing of immunosuppression and

PP2A activation we are currently working novel FTY720 analogs which can serve as affective treatment of PV without producing the immunosuppression found with FTY720 itself.

Experimental Procedures

Materials- FTY720-phenoxy-biotin, FTY720, FTY720-P, dimethylsphingosine (DMS), and SEW2871 were from Cayman Chemical (Ann Arbor, Michigan).

PP2A activity

PP2A activity was measured a phosphatase assay kit per manufacturer's instructions (Millipore, Billerica, MA).

Ceramide displacement assay

Lysates from A549 cells (1mg) were suspended in immunoprecipitation buffer with 10μM biotin, biotin-ceramide with or without pretreatment of FTY720 (10μM), and processed through an avidin column as described by the manufacturer. Bound proteins were eluted using 2mM biotin solution; SET was detected via western blot.

Immunoprecipitation

Ba/F3 Jak2 V617F cell lysates (1mg) were prepared in immunoprecipitation buffer and incubated for 1 hour at 4ºC with 10μM FTY720-phenoxy-biotin and passed through an avidin column. SET was detected via western blot.

Sphingosine Kinase Assay

Ba/F3 Jak2 V617F cells (untreated, treated with FTY720 (2.5μM 6 hours), plus or minus okadaic acid (0.25nM, 1.5 hours)) were lysed in assay buffer. Lysates were incubated with 32P and sphingosine (1mM,) at 37ºC for 1 hour. Following lipid extraction, TLC was performed and S1P was visualized via autoradiography

Constructs

MSCV-puro and MigR1-Jak2 V617F

Jak2 V617F was ligated into MigR1 and MSCV-puro using Bgl II and EcoR1 sites of MSCV-puro and the bicistronic GFP-containing MigRI vector.

MigRI-HA-PP2Ac

The HA-tagged PP2Ac cDNA was PCR amplified from pHM6-HA-PP2Ac and subcloned into the HpaI/EcoRI sites of Migr1.

pLL3.7-shSET

The shRNA SET construct was obtained by subcloning the double-stranded 60 mer oligonucleotide containing the SET target sequence (5#-TGAAATAGACAGACTTAAT-3#) into the pLL3.7 vector (Luk Van Parijs (17)).

pCDH-Flag-SET

The human SET cDNA was obtained from K562 mRNA by RT-PCR using an upstream primer containing a HpaI site, the FLAG epitope, and the first 16 nucleotides of SET cDNA, and a downstream primer containing the last 21 nucleotides of SET linked to an EcoRI restriction site. The XbaI/EcoRIdigested PCR product was subcloned into the pCDH-CMV-MCS-EF1-copGFP vector.

pCDH-K209DSET

K209D SET was generated using site-directed mutagenesis and subcloned into pCHD using the same sites as wild-type FLAG-SET.

Figure 1.

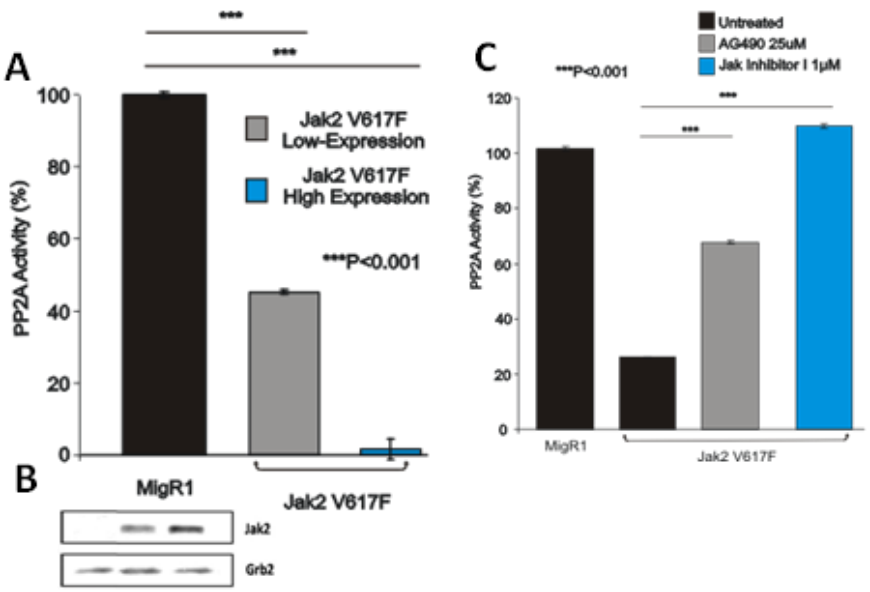

Figure 1. (A and B) Ba/F3 murine hematopoietic progenitor cells were retrovirally transduced with Jak2 V617F-MigR1-GFP and separate high and low expressing populations. Following verification of Jak2 levels via western blot a functional PP2A activity assay was performed. (C) Ba/F3 Jak2 V617F cells were treated with either AG490 (25μM, 10 hours or Jak Inhibitor I (1μM, 10 hours) and PP2A activity was measured.

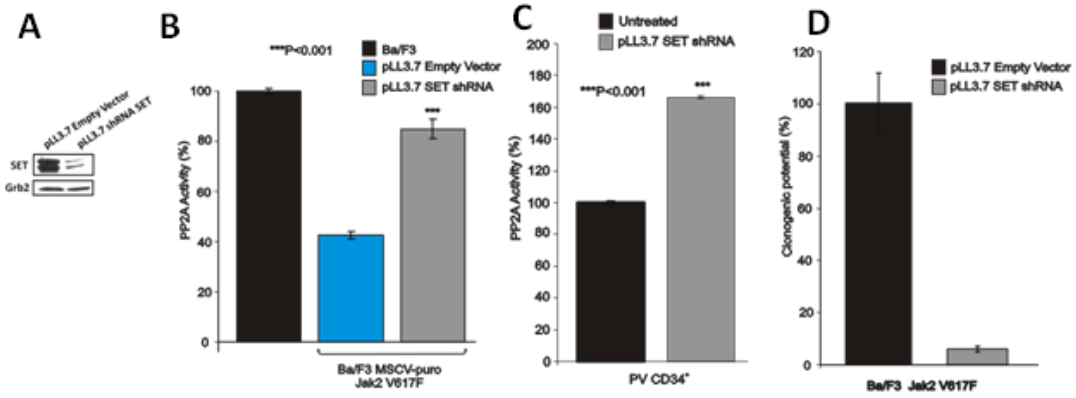

Figure 2. (A) SET shRNA delivered in the pLL3.7 lentiviral vector reduces total SET levels in Ba/F3 Jak2 V617F cells. (B) PP2A activity is restored to 80% of Jak2 V617F-negative controls in Ba/F3 Jak2 V617F-expressing cells. (C) PP2A activity is increased 2-fold in CD34+ PV primary patient samples. (D) Colony forming is reduced by 91% in Ba/F3 Jak2 V617F+ cells.

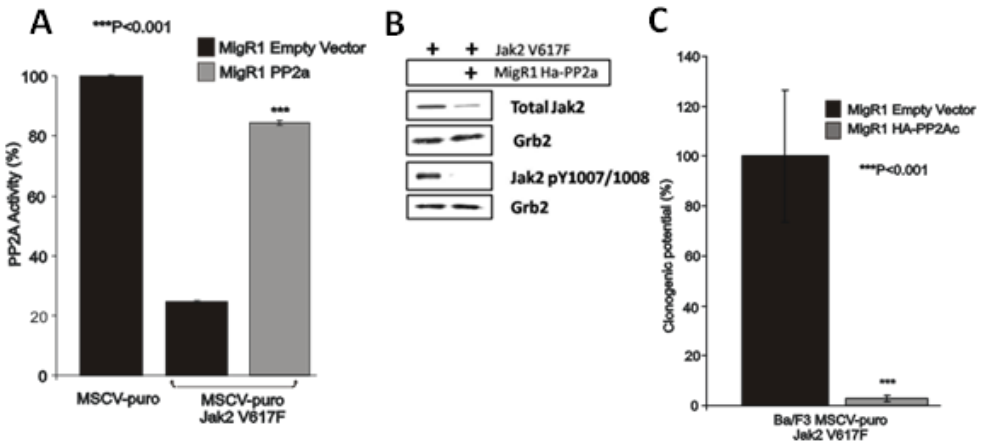

Figure 3. (A) Overexpression of PP2A restores PP2A activity in Ba/F3 Jak2 V617F cells. (B) Increased PP2A activity results in loss of total Jak2 and pY1007/1008 protein in Ba/F3 Jak2 V617F+ cells. (C) Colony forming is reduced by 82% following restoration of PP2A activity.

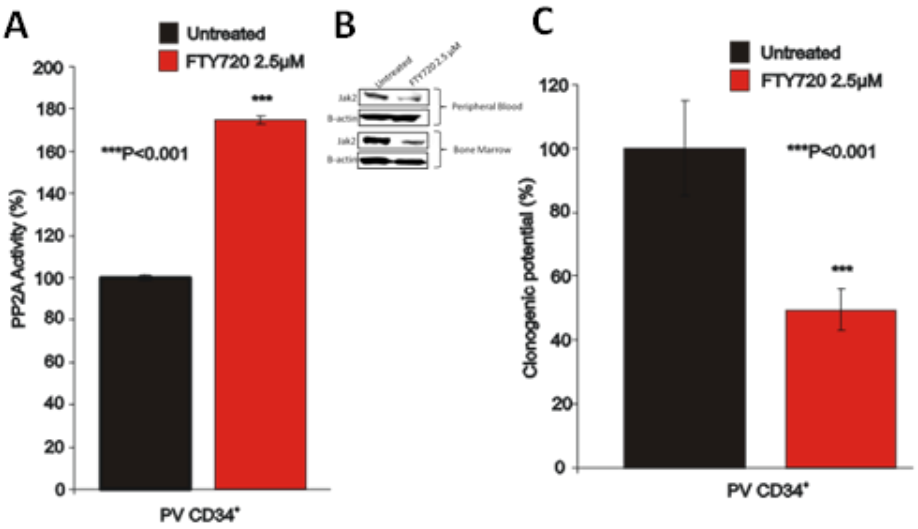

Figure 4. (A) FTY720 (2.5μM, 6 hours) Increases PP2A activity in CD34+ PV primary patient samples. (B) FTY720 (2.5μM, 24 hours) decreases total Jak2 levels in CD34+ patient cells obtained from peripheral blood (top) and bone marrow (bottom). (C) Colony forming is reduced by 62% in PV patient samples by treatment with FTY720 (2.5μM).

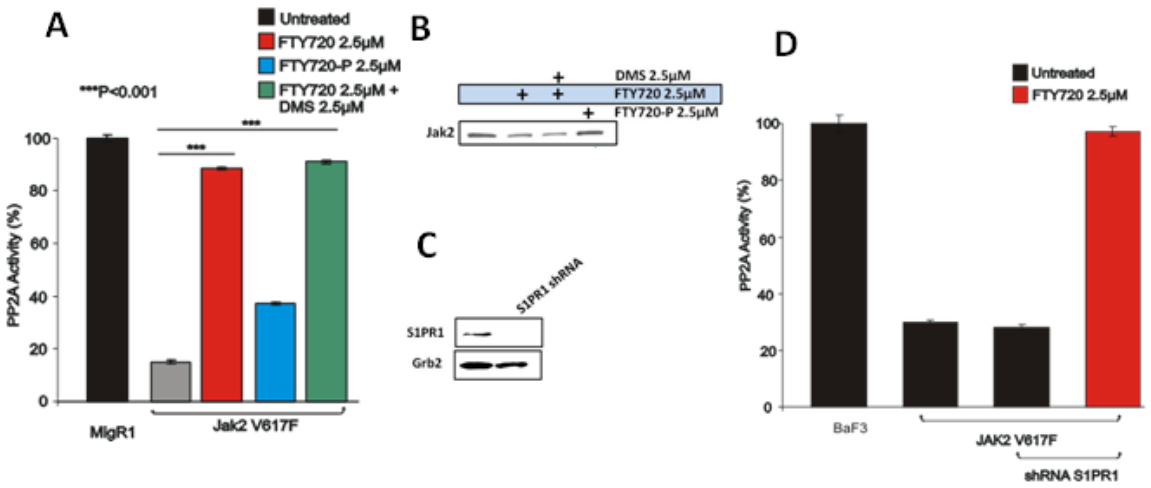

Figure 5. (A) FTY720 (2.5μM, 6 hours) activates PP2A in Ba/F3 V617F, an effect that is unaltered by co-treatment with the sphingosine kinase inhibitor dimethylsphingosine (DMS 2.5μM, 6 hours). Treatment with immunosuppressive FTY720-P (2.5μM, 6 hours) does not activate PP2A in these cells. (B) FTY720 and FTY720 + DMS treatment reduces Jak2 levels while FTY720-P does not. (C and D) Knock-down the S1PR1 does not alter FTY720-mediated PP2A activation

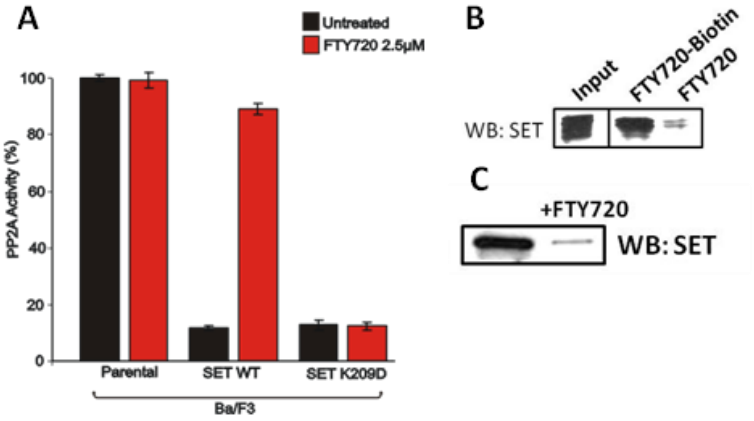

Figure 6. (A) Overexpression of wild-type or K209D SET inhibits PP2A in Ba/F3 cells however only the wild-type SET responds to FTY720 treatment. (B) An avidin pull-down of Ba/F3 Jak2 V617F cell lysates treated with biotin-labeled FTY720 demonstrate direct FTY720/SET binding. (C). Avidin pull-down of Ba/F3 Jak2 V617F cell lysates treated with biotin-labeled ceramide show ceramide/SET binding, an interaction which is displaced by co-treatment of FTY720.

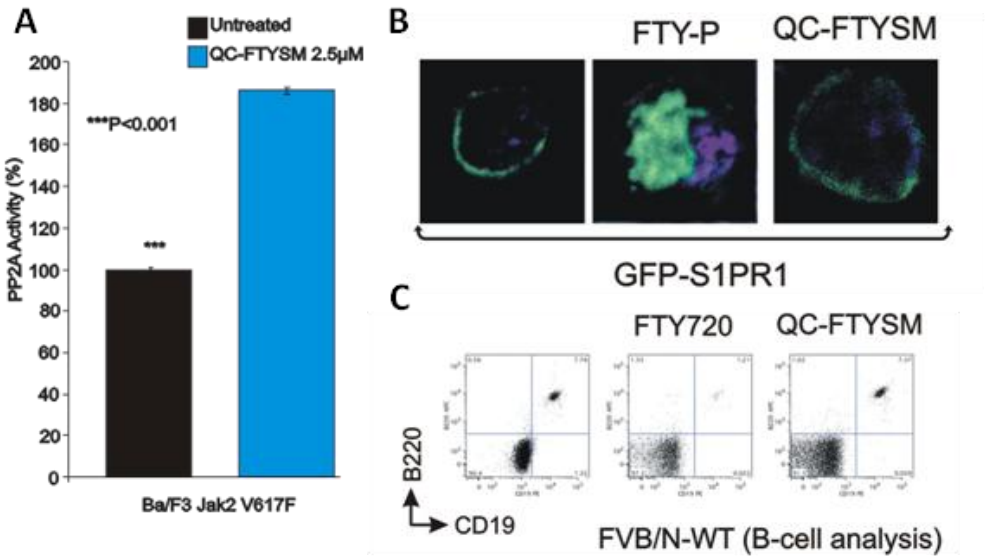

Figure 7. (A) Treatment with FTY720 analog QC-FTYSM (2.5μM 6 hours) increased PP2A activity 2-fold in Ba/F3 Jak2 V617F cells. 2(B) QC-FTYSM does not induce internalization of a GFP-tagged S1PR1 receptor. (C) FTY720 induces lymphopenia in mice treated at 10/mg/kg/d for 24 hours. Treatment using the same dose of QC-FTYSM does not reduce circulating lymphocyte numbers in mice.

References

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