

The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors¹

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SPECIFIC AIMS

FTY720 is a unique immunosuppressant that acts by sequestering circulating lymphocytes in secondary lymphoid organs and thereby limiting their effector responses. At micromolar concentrations, FTY720 is phosphorylated by cells and the resultant FTY-P is a putative agonist for G-protein-coupled receptors (GPCRs) specific for the lymphocyte migration factor sphingosine 1-phosphate (S1P). The discovery that nonphosphorylated FTY720, at nanomolar concentrations regularly attained in therapeutic use, is a noncompetitive inhibitor of S1P effects on lymphocyte migration led to studies of FTY720 actions on S1P GPCRs.

PRINCIPAL FINDINGS

1. FTY720 inhibits signaling by S1P₁, S1P₂, and S1P₅ GPCRs

FTY720 was shown to suppress S1P-evoked chemotaxis of lymphocytes with nanomolar potency. Now we demonstrate the action of FTY720 on individual S1P GPCRs in S1P-R null cell-transductants. FTY720 inhibited S1P₁- and S1P₅-R signaling with nanomolar potency and S1P₂-R signaling with lower potency, without affecting S1P₃- and S1P₄-R signaling. The nanomolar S1P₁- and S1P₅-R specific inhibitory effects of FTY720 were documented in [³²P]S1P binding studies, chemotaxis assays (Fig. 1a–e), measurements of [Ca²⁺]_i levels, and determinations of cytoskeleton rearrangements.

2. FTY720 inhibitory effects are based on elicitation of intracellular translocation of susceptible S1P GPCRs

a. Requirement for prolonged action of FTY720

FTY720 has no immediate agonistic or antagonistic effect on S1P-R ligand binding and signaling by studies of [³²P]S1P binding and S1P-evoked changes in [Ca²⁺]_i. However, after incubation of S1P₁-, S1P₂-, and S1P₅-R transductants in S1P-R null rat hepatoma HTC₄ cells with low nanomolar concentrations of FTY720 at 37°C, S1P-mediated [Ca²⁺]_i-signals were greatly inhibited, and [³²P]S1P binding was impaired. S1P₁-R signal-

ing of increases in [Ca²⁺]_i was suppressed with a linear FTY720 concentration dependence. Maximal inhibition of [Ca²⁺]_i signals was more pronounced and was attained earlier at higher FTY720 concentrations.

b. Brief contact is sufficient for FTY720 inhibitory effects

The prolonged incubation time required for FTY720 inhibitory effects on S1P-R signaling was divisible into a period of contact with FTY720 as brief as 5 s and a subsequent prolonged response time of 16 h or longer after washing to remove fluid-phase FTY720. Both incubations required a physiological temperature of 37°C, as incubation at 4°C for either of these distinct periods eliminated any effect of FTY720 on S1P-R signaling.

c. FTY720 induces prolonged receptor internalization of S1P₁, but not S1P₄ GPCRs

By analyzing hemagglutinin epitope-tagged S1P₁- and S1P₄-R transfected rat hepatoma HTC₄ cells with confocal microscopy and flow cytometry, FTY720 was shown to induce internalization and subsequent partial degradation of S1P₁, but not S1P₄ (Fig. 2a–d). Recovery of S1P₁-R surface expression after complete removal of FTY720 from the fluid phase took days to weeks depending on the initial FTY720 concentration. The times of onset, peak, and recovery of S1P₁-R internalization followed the same pattern as for FTY720 inhibitory effects on S1P-mediated increases in [Ca²⁺]_i. Thus FTY720 inhibits S1P_{1,2,5}-R signaling by inducing their prolonged internalization and partial degradation.

3. FTY720 inhibition of S1P-R signaling is the same in lymphocytes as in transductants

Since the primary target for FTY720-mediated immunosuppression in vivo are lymphocytes that undergo a different migration pattern upon FTY720-treatment, we examined the main elements of FTY720-induced inhi-

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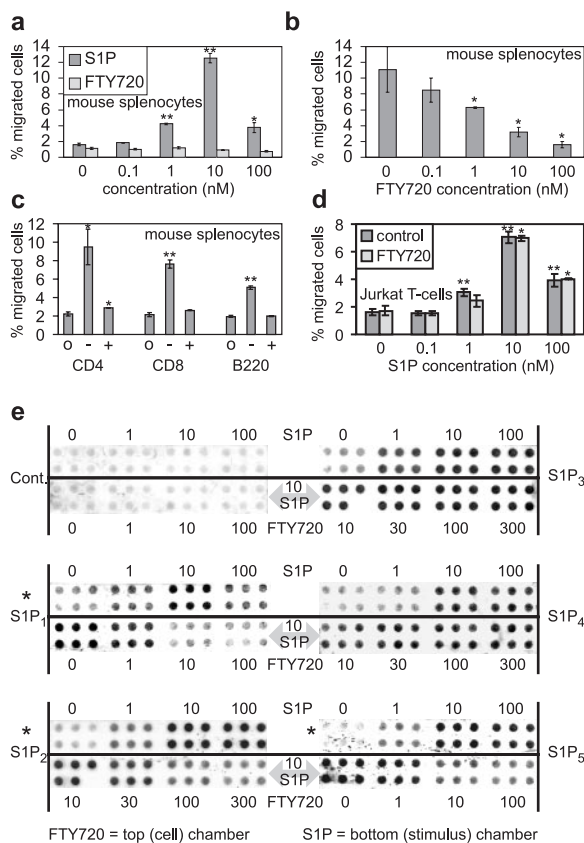


Figure 1. Transwell and modified Boyden-chamber chemotaxis assays with lymphocytes and single S1P-R expressing HTC₄ cell lines. *a–d*) Transwell chemotaxis assay with *a*) mouse primary naive splenocytes responding to S1P, but not FTY720; *b*) mouse primary naive splenocytes responding to 10 nM S1P in the presence of different amounts of FTY720 in the cell chamber; *c*) mouse magnetic bead-sorted primary naive splenocyte subsets to medium alone (o) or to 10 nM S1P in the presence (+) or absence (–) of 100 nM FTY720 in the upper chamber; *d*) Jurkat cells in the presence (FTY720) or absence (control) of 100 nM FTY720 in the cell chamber. (*a–d*) Means of duplicates of one representative experiment out of two ± SD. Statistical significance of the difference between each mean value and the value for medium alone (*a, c*) or the value in the absence of FTY720 (*b*) was determined by a two-sample *t* test and depicted as **P* < 0.05 and ***P* < 0.01. *e*) Modified Boyden-chamber chemotaxis assays with HTC₄ S1P-R null control cells (Cont.) and single human S1P-R expressing HTC₄ cells. Cells migrated toward the S1P-concentration in the upper row and to 10 nM S1P in the presence of a range of concentrations of FTY720 in the upper chamber in the lower row in sets of six replicates. One representative micropore membrane out of three similar experiments is shown for each cell line. One missing dot in the lower row of both SIP₂- and SIP₃-HTC₄ cells were created for orientation purposes. Receptors showing FTY720-elicited inhibition of their migratory response to 10 nM S1P are marked with asterisks.

bition of S1P signaling in primary lymphocytes. Chemotaxis was chosen as the most prominent S1P-mediated cellular effect on freshly isolated mouse splenocytes. A substantial inhibitory effect of low nanomolar FTY720 concentrations was shown on chemotaxis of CD4 and CD8 T cells, and B cells to S1P, but not to other chemotactic stimuli (Fig. 1*a–c*). Preincubation

of lymphocytes with FTY720 for only 5 s at 37°C significantly inhibited S1P-mediated chemotaxis, whereas no inhibition was achieved at 4°C.

4. Immunosuppression by FTY720 is mediated by S1P₁ on lymphocytes

To find out which S1P-R is targeted by FTY720 on primary lymphocytes we analyzed the mRNA expression pattern of all S1P-Rs in human peripheral blood lymphocytes and primary mouse splenocytes. These realtime-PCR studies revealed that S1P₁ and S1P₄ are the only S1P-Rs that are highly expressed on naive lymphocytes. S1P₃ is also expressed on marginal zone B cells, and S1P₅ is expressed selectively on naive CD8 T cells. Since S1P₄ is not a target for FTY720-induced inhibitory effects, S1P₁ is the most likely S1P-R on all naive lymphocytes mediating lymphocytopenia after administration of FTY720.

CONCLUSIONS AND SIGNIFICANCE

The present findings prove that FTY720 is a noncompetitive inhibitor of S1P₁- and S1P₅-Rs with high potency and of S1P₂-Rs with moderate potency (Fig. 1*e*) at 37°C, but not at 4°C. FTY720 rapidly elicited long-lasting internalization of the susceptible S1P-Rs (Fig. 2*a, c*). This profile of S1P-R selectivity is different from the preferential targeting of S1P_{1,3,4,5} by phosphorylated FTY720. Our results are not contradictory to the previously described phosphorylation of FTY720 and its consequent action as an agonist in its phosphorylated state. Instead, they offer a new possibility to explain both therapeutic potency and selective activity of this immunosuppressive compound in the persistent presence of blood and lymph S1P (Fig. 3). S1P₁ is the only S1P-R that is both highly expressed on human and mouse T- and B-lymphocytes and inhibited by FTY720 with high potency (Fig. 1*e, 2a, c*). Considering its dominant role in inducing and modulating lymphocyte migration in vitro, S1P₁ is the most likely lymphocyte receptor to mediate FTY720-induced lymphopenia in vivo. S1P₄ was proposed in earlier studies to be the most likely mediator of FTY720-induced lymphopenia because of its selective expression on immune cells, but S1P₄ is not affected by FTY720 concentrations up to 300 nM (Fig. 1*e, 2b, d*). S1P₃ is not a target for FTY720 inhibition (Fig. 1*e*). In contrast to primary naive T cells, Jurkat cells mainly express S1P₃ at high levels. S1P₃ mediates a migratory response to S1P in transductants (Fig. 1*e*). Consistent with these data, Jurkat cells show a chemotactic response to S1P, but their chemotaxis is not inhibited by FTY720 (Fig. 1*d*). Although it has been suggested that S1P-Rs on endothelial cells and other elements of SLOs may be responsible for the induction of lymphopenia, *aly/aly* mice which lack LNs and PPs still develop lymphopenia upon FTY720 treatment, which strongly indicates that lymphocyte S1P₁ GPCRs are targeted by the drug.

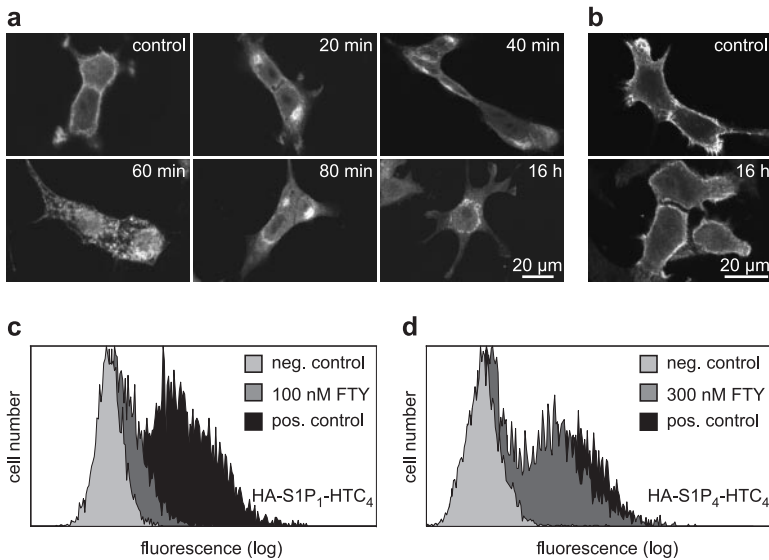


Figure 2. Effects of FTY720 on surface expression of HA-SIP₁ and HA-SIP₄ of transfected HTC₄ cells. *a)* Confocal microscopy of HA-SIP₁ and *b)* HA-SIP₄ transfected HTC₄ cells in the presence or absence (control) of FTY720. *c–d)* Flow cytometry of HA-SIP₁ (*c*), HA-SIP₄ (*d*), or untagged SIP₁ (negative control) transfected HTC₄ cells in the presence or absence (controls) of FTY720.

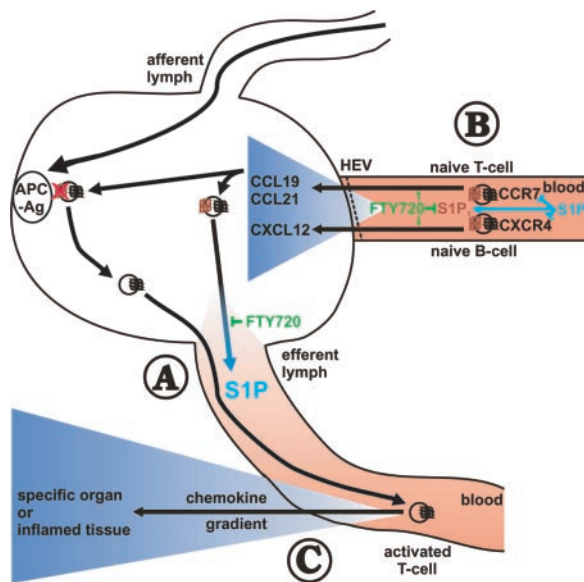


Figure 3. Model of FTY720-induced inhibition of S1P-SIP₁-dependent regulation of lymphocyte circulation. *A)* S1P is a chemoattractant for naive lymphocytes. S1P binding to SIP₁ induces migration of lymphocytes and enables their re-entrance from secondary lymphoid organs (SLOs) into blood. FTY720 induces prolonged internalization and partial degradation of SIP₁ and therefore blocks S1P binding to SIP₁, inhibits lymphocyte migration to S1P, and blocks the re-entrance of lymphocytes from SLOs into blood. *B)* Naive lymphocytes are trapped in blood by S1P-SIP₁ axis-mediated inhibition of their response to chemokines. Only a small number of lymphocytes enter the lymph node via high endothelial venules (HEV) under direction of chemokine gradients. FTY720 inhibits SIP₁ signaling, neutralizes S1P inhibitory effects on chemokine-induced migration, and enhances lymphocyte homing into SLOs. *C)* Antigen-activated T cells down-regulate expression of SIP₁, which enhances their responsiveness to chemokines and permits them to follow a different organ-specific homing pattern and to enter inflamed sites. They are able to leave the circulating blood-stream. The S1P-SIP₁ axis is no longer controlling their fate.

The observation that an optimal *in vitro* and *in vivo* effect of FTY720 requires preincubation is well documented. Our results also indicate that prolonged incubation at 37°C is critical for the inhibitory effects of low nanomolar FTY720 concentrations. SIP_{1,2,5}-Rs are retained intracellularly for several days after FTY720-treatment. No immediate effects of FTY720 have been observed in analyses of S1P binding and S1P-evoked calcium studies, confirming previous results that FTY720 does not show any immediate stimulatory or inhibitory effects.

This striking mechanism provides new insights into GPCR signaling and function. For the first time a small synthetic nonlipid compound is shown to induce internalization of a GPCR for an amphipathic lipid without activating or inducing recycling of the receptor. Activation-induced internalization and recycling of a GPCR are thought to be closely linked processes. However, FTY720 induces internalization of SIP₁ without stimulating the receptor and favors the degradation pathway by blocking receptor recycling. How FTY720 induces receptor internalization and retains the receptor intracellularly requires further investigations.

In contrast to the serendipitous finding of FTY720, drug screening methods could be modified to permit systematic discovery of such noncompetitive inhibitors. Current techniques for identifying receptor agonists and antagonists focus on the induction or inhibition of receptor signaling that is detected by a single immediate biochemical event. Our results may enable us to develop systems that screen for FTY720-like drugs by including a prolonged incubation to permit intracellular relocalization of receptors. The advantages of FTY720-type GPCR inhibitors are 1) high potency by a noncompetitive mechanism; 2) less dependence on stability as only short incubation times are required for prolonged activity; 3) enhanced biopotency and lower toxicity since there is no need to compete with higher concentrations of endogenous ligands; and 4) high specificity by targeting individual site-critical GPCRs within a subset specific for the same ligand. Thus FTY720 may be taken as a lead compound for future pharmaceutical drugs. **FJ**