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Elimination of a Hydroxyl Group in FTY720 Dramatically Improves the Phosphorylation Rate

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ABSTRACT

The new immunosuppressant FTY720 (fingolimod), an analog of the endogenous lipid sphingosine, induces transient lymphopenia through the sequestration of lymphocytes in secondary lymphoid organs. Phosphorylation of FTY720 by sphingosine kinase 2 (SphK2) yields the active metabolite FTY720-phosphate (FTY-P), which induces lymphopenia through agonism of the sphingosine 1-phosphate receptor $S1P_1$ on endothelial cells and lymphocytes. Dephosphorylation of circulating FTY-P creates an equilibrium between FTY720 and its phosphate, and results with human patients indicate that phosphorylation of FTY720 could be rate limiting for efficacy. We report that the FTY720 derivative 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol [AAL(R)] is phosphorylated much more rapidly than FTY720 in cultured human cells and whole blood. The K_{cat} for AAL(R) with recombinant SphK2 is 8-fold higher than for FTY720, whereas the K_m for the two substrates is very similar, indicating that the increased rate of phosphorylation results from faster turnover by SphK2 rather than a higher binding affinity. Consequently, treating cells with AAL(R), but not FTY720, triggers an apoptotic pathway that is dependent on excessive intracellular accumulation of long-chain base phosphates. In agreement with the in vitro results, phosphorylation of AAL(R) is more complete than that of FTY720 in vivo (mice), and AAL(R) is a more potent inducer of lymphopenia. These differences may be magnified in humans, because phosphorylation of FTY720 is much less efficient in humans compared with rodents. Our results suggest that AAL(R) is a better tool than FTY720 for in vivo studies with S1P analogs and would probably be a more effective immunosuppressant than FTY720.

Introduction

FTY720 (fingolimod) is a new type of immunosuppressant that induces a transient, reversible lymphopenia by trapping lymphocytes in the secondary lymphoid organs and thereby keeping them out of the circulation. This mode of immunosuppression is unique among pharmacological immunosuppressants and has made FTY720 the subject of intense interest, from both a therapeutic and a mechanistic/physiological perspective. Lymphopenia induced by FTY720 is dependent on the phosphorylation of the compound by sphingosine kinase 2 (SphK2) (Billich et al., 2003; Zemann et al., 2006). The phosphorylated compound acts as an agonist at four of the five sphingosine 1-phosphate (S1P) receptors, a family of G-protein-coupled receptors that respond to extracellular S1P (Brinkmann et al., 2002; Mandala et al., 2002). Activation (agonism) of the $S1P_1$ receptor is responsible for sequestration of T cells in the peripheral lymphoid organs, demonstrated with the observations that a range of $S1P_1$-selective agonists can induce lymphopenia, that this is reversible with an $S1P_1$ antagonist, and that $S1P_1$-deficient lymphocytes are resistant to the effects of FTY720 (Matloubian et al., 2004; Pan et al., 2006; Sanna et al., 2006). Two models have been put forward to explain exactly how $S1P_1$ agonists induce lymphopenia (Brinkmann, 2007; Rosen et al., 2008): in one model activation of $S1P_1$ receptors on endothelial cells ex-

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ABBREVIATIONS: FTY720, fingolimod; SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; FTY-P, FTY720-phosphate; AAL(R), (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol; AAL(S), (*S*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol; AAL-P, phosphorylated (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol; dhSph, dihydrosphingosine; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI, propidium iodide; PCR, polymerase chain reaction; siRNA, small interfering RNA; LC, liquid chromatography; TLC, thin-layer chromatography; HMEC, human microvascular endothelial cell.

posed to the blood or lymph results in the closure of endothelial gates through which lymphocytes exit the lymph nodes; the other model invokes lymphocyte migration from the low S1P environment of the lymph nodes toward the higher S1P concentration of the blood or lymph, requiring stimulation of their $S1P_1$ receptors. In a simple interpretation of this model, the presence of FTY720-phosphate (FTY-P) in lymph nodes disrupts this gradient. However, another interpretation is derived from the observation that FTY-P acts as a superagonist of $S1P_1$, promoting internalization and degradation of the receptor. This impairs the ability of lymphocytes to respond to the proposed S1P gradient (Gonzalez-Cabrera et al., 2007; Oo et al., 2007).

S1P receptor modulating compounds such as FTY-P have found application in a wide variety of experimental settings, which include immunosuppression during organ transplant (Pan et al., 2006; Brinkmann, 2007), treatment of autoimmune conditions (Fujino et al., 2003; Maki et al., 2005), recovery after ischemia/reperfusion injury (Hofmann et al., 2009), and as a means of increasing endothelial barrier function (Sanna et al., 2006). In the clinic, there have been trials of FTY720 in patients undergoing kidney transplant and patients with multiple sclerosis (Brinkmann, 2007). The trials in patients undergoing transplant failed to show any improvement in efficacy over the current standard of care, but the compound has showed great promise in phase III trials in patients with relapsing-remitting multiple sclerosis (Cohen et al., 2010; Kappos et al., 2010). Fewer relapses were reported with FTY720 than with the current treatment, intramuscular interferon β (Cohen et al., 2010).

The FTY720 analog AAL(R) has been used in a number of studies, because its chiral enantiomer AAL(S) is not a substrate for SphK2 and therefore acts as a useful control for effects of the compound that are not attributed to its phosphorylation (Kiuchi et al., 2000; Brinkmann et al., 2002; Don et al., 2007). Because FTY720 and AAL(R) are very similar compounds, they have been used interchangeably. In this study, we showed that AAL(R) is a much better substrate for SphK2 than FTY720, which translates into a faster rate of phosphorylation by cultured cells and in whole blood and almost complete phosphorylation in living mice. Phosphorylation of FTY720 occurs much more rapidly in rodent than in human blood, suggesting that AAL(R) would prove significantly more effective than FTY720 as a sphingosine 1-phosphate receptor agonist in humans.

Materials and Methods

Materials. FTY720 was purchased from Millipore Bioscience Research Reagents (Temecula, CA), whereas AAL(R) was a gift from Professor Hugh Rosen (The Scripps Research Institute, La Jolla, CA). AAL-P to use as a standard for mass spectrometry was prepared by chemical phosphorylation: The amino group of AAL was protected (Boc_2O, $NaHCO_3$, 56%), then reaction with *N*,*N*-diisopropyl phosphoramidite dichloride and 5-ethylthio-1*H*-tetrazole, followed by oxidation with hydrogen peroxide, gave protected AAL-P in 19% yield. The compound was deprotected with trifluoroacetic acid. Dihydrosphingosine (dhSph) was purchased from Avanti Polar Lipids (Alabaster, AL). Synthesis of 3-deoxy-dhSph has been reported previously (Lim et al., 2004).

Cell Culture and Viability Assays. Jurkat cells and primary splenocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin/streptomycin solution. The human microvascular endothelial cell line HMEC-1 (Ades et al., 1992) was cultured in MCDB131 medium (Invitrogen) supplemented with 10% FBS, glutamine, and antibiotics. Rat splenocytes were isolated by crushing the spleen between frosted glass slides and filtering through a 70 μM filter, followed by two rounds of red cell lysis in 0.17 M NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA for 5 min on ice. Isolated splenocytes were resuspended at a density of 1.5×10^6 viable cells/ml in complete RPMI medium, cultured in the presence of AAL(R), AAL(S), or FTY720 for 20 h, then stained with propidium iodide (PI) for flow cytometry. For MTT assays, cells were cultured in 96-well plates, using 0.1 ml of medium per well. Ten microliters of 0.5% (w/v) MTT reagent (Sigma, St. Louis, MO) in PBS was added to each well, and cells were returned to the incubator for 2 h. MTT was solubilized by adding 0.1 ml of 10% SDS/10 mM HCl to each well and shaking overnight, and absorbance was read at 650 nM. Alternatively, viability was assessed by flow cytometry: cells were resuspended in 100 μl of 20 mM HEPES, pH 7.4, 150 mM NaCl, and 2.5 mM $CaCl_2$ and incubated for 15 min on ice with 2 μl of Annexin V-allophycocyanin and 1 μg/ml PI, then subjected to flow cytometry.

siRNA Treatment of HeLa Cells and Real-Time Quantitative PCR. Cells were transfected in six-well plates, in 2 ml of OptiMEM I medium, using siRNA molecules purchased from QIAGEN at a final concentration of 100 nM. siRNAs were premixed in 200 μl of OptiMEM with 4 μl of lipofectamine 2000 (Invitrogen) for 30 min, then added to the cells for 6 h, after which the medium was replaced with standard growth medium. On the day after transfection, the cells were detached and reseeded into a 96-well plate at a density of 10^4 cells/well for MTT assay. Cells were treated with FTY720 or AAL(R) at 48 h after transfection, and viability was assayed with MTT reagent at 72 h after transfection. Real-time PCR was used to measure transcript levels, using the following primers taken from PrimerBank (Spandidos et al., 2010): SphK1: fwd, AGGCTGAAATCTCCTTCACGC; rev, GTCTCCAGACATGACCACCAG; SphK2: fwd: GCTGCTGCGCCTTTTCTTG; rev, CCTGTAGCGGCCCATACTC; and glyceraldehyde 3-phosphate dehydrogenase: fwd, TGTTGCCATCAATGACCCCTT; rev, CTCCACGACGTACTCAGCG. RNA was prepared with an RNEasy Mini Kit (QIAGEN); cDNA was prepared with Moloney murine leukemia virus reverse transcriptase (Invitrogen); and a SYBR Green with ROX Kit (Invitrogen) was used for quantitative PCR, on an Mx3000 cycler (Stratagene, La Jolla, CA).

Assays of Compound Phosphorylation In Vitro. Jurkat cells were cultured for 2 h in medium containing 5 μM FTY720 or AAL(R), in triplicate, at a density of 4×10^5 cells/ml. The cells were then pelleted and resuspended at the same density in fresh medium. Samples (0.4 ml) were removed from the culture at the times indicated in Fig. 3, snap-frozen, and stored at −80°C. Samples were extracted with ethyl acetate/isopropanol (Bielawski et al., 2006). In total, the culture medium was extracted four times with ethyl acetate/isopropanol, twice under acidic conditions. The four organic extracts were combined, dried under vacuum, and resuspended in 100 μl of 80% methanol/20% water (mobile phase for LC). Lipids were quantified by LC-tandem mass spectrometry, using a C8 column coupled to a Thermo Quantum TSQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) operating in positive ion multiple reaction monitoring mode. The compounds were separated with a gradient of 80% methanol/20% water increasing to 85.5% methanol over 5 min. Precursor and product ion *m/z* values were as follows: FTY720, 308.3 and 255.1; FTY720-P, 388.0 and 255.1; AAL(R), 394.0 and 161.1; AAL-P, 374.1 and 161.1.

To assay phosphorylation of compounds in whole blood, human or rat blood was collected into heparin-coated tubes, then mixed 1:1 with RPMI 1640 medium. One nanomole of FTY720 or AAL(R) was added directly to 250 μl of blood/RPMI 1640 mix and incubated at 35°C for the indicated times. Reactions were stopped with the addition of 1 ml of ice-cold methanol, and the mixture was cleared by centrifuging at 21,000*g* for 15 min. The insoluble pellets were re-

extracted by sonicating in 1 ml of methanol. The supernatants from both steps were combined in 4 ml of glass tubes, dried down in a SpeedVac SC210 (Thermo Fisher Scientific), and the extracts were resuspended in 200 μl of 80% methanol/20% water (LC mobile phase). Extraction efficiency was determined by spiking blood with compounds, then immediately extracting.

Sphingosine Kinase Assays. The radioactive kinase assays were based on published methods (Olivera et al., 2000; Siow and Wattenberg, 2007). Kinase assays were set up in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.1% fatty acid-free BSA, and 5 μCi/reaction radiolabeled [^{32}P]ATP (PerkinElmer Life and Analytical Sciences). Reactions (0.1 ml) were started with the addition of recombinant human SphK2, produced in insect cells (BIOMOL Research Laboratories, Plymouth Meeting, PA). The final enzyme concentrations were 0.2 μg/ml for dhSph and 3-deoxy-dhSph and 1 μg/ml for AAL(R) and FTY720. Reactions were run for 30 min at 35°C for dhSph, 3-deoxy-dhSph, and AAL(R) and for 150 min for FTY720. Note that the different enzyme concentrations and times were used to ensure that enzyme, and not available substrate, was rate limiting. Reactions were stopped with the addition of 350 μl of methanol/HCl (150:1), followed by 250 μl of 2M KCl, and 350 μl of chloroform. Tubes were vortexed, then spun in a refrigerated Microfuge (Beckman Coulter, Fullerton, CA) at 14,000 rpm to resolve the phases. The upper aqueous phase was discarded, and 4 μl of the (lower) organic phase was spotted onto Silica Gel 60 TLC plates (Fluka, Buchs, Switzerland). TLC plates were resolved in butanol/acetic acid/water (3:1:1), then exposed to Fuji Imaging Plates and imaged by filmless autoradiographic analysis with a Fuji FLA7000 (Fujifilm, Tokyo, Japan). The concentration of product in each spot was derived from a standard curve constructed with the [^{32}P]ATP reaction mix.

Lipid Phosphatase Assay. To prepare radiolabeled FTY-P and AAL-P, solutions of 50 μM FTY720 or AAL(R) were phosphorylated in kinase assay buffer containing 10 μCi/400 μl of reaction [^{32}P]ATP, for 4 h at 35°C, using 3.75 μg/ml (for FTY720) or 0.75 μg/ml [for AAL(R)] recombinant SphK2. Reactions were stopped and extracted with addition of 400 μl of methanol, 40 μl of 3 M NaOH, and 400 μl of chloroform. Tubes were vortexed, phases were separated by centrifugation, and the upper aqueous phase, containing the radiolabeled phosphates, was transferred to a new tube. This aqueous extract was re-extracted by adding 80 μl of concentrated HCl and 400 μl of chloroform, this time discarding the aqueous phase and retaining the lower organic phase. This method effectively separates the sphingoid bases from their phosphates (Maceyka et al., 2007). The organic extract was dried down and resuspended in 400 μl of 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% fatty acid free BSA, and the concentration of the radiolabeled phosphate was measured by resolving the resuspended compound on TLC and quantification of FTY-P or AAL-P spots by filmless autoradiographic analysis.

To assay dephosphorylation, HMEC-1 cells were seeded in a 24-well plate at a density of 2×10^5 cells/well. On the following day, the medium was replaced with 0.3 ml of fresh growth medium containing 100 nM radiolabeled FTY-P or AAL-P. Samples (2.5 μl) were removed at indicated times and spotted onto a TLC plate, then resolved and imaged as described above.

In Vivo Measurement of Compounds and Circulating Lymphocytes. AAL(R) or FTY720 were administered by intraperitoneal injection, in 0.1 ml of sterile water, to groups of four C57BL6 mice (per treatment). Mice were euthanized, and blood was drawn by cardiac puncture 18 h after dosing. A 0.1-ml aliquot of blood from each mouse receiving 0.3 mg/kg AAL(R) or FTY720 was immediately mixed with 0.4 ml of ice-cold methanol, and the samples were processed for mass spectrometry as described above. To assay the proportion of T cells in the blood, 0.3-ml blood samples were first subjected to three rounds of red cell lysis (each 5 min at room temperature) in 0.17 M NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA. The resulting leukocytes were then incubated for 30 min with a 1:100 dilution of both anti-mouse CD4-PE and anti-mouse CD8-eFluor450 (eBioscience, San Diego, CA) in PBS/2% FBS. Cells were washed, then fixed for 10 min at room temperature with 1% paraformaldehyde in PBS, washed once more, and analyzed the following day using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (TreeStar Inc., Ashland, OR). Total T cells shown are the sum of CD4- and CD8-positive cells. These experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales.

Results

AAL(R) but Not FTY720 Treatment Triggers SphK2-Dependent Cell Death. We have shown previously that phosphorylation of AAL(R) by SphK2 is required for this compound to induce a loss of viability in cultured murine splenocytes, based on two observations: first, AAL(R) was much more efficient than its nonphosphorylatable enantiomer, AAL(S), at inducing loss of viability; second, splenocytes derived from SphK2 knockout mice were resistant to AAL(R) (Don et al., 2007). These findings led us to propose that a specific apoptotic response is triggered by excessive intracellular accumulation of AAL-P. To our surprise, we have found

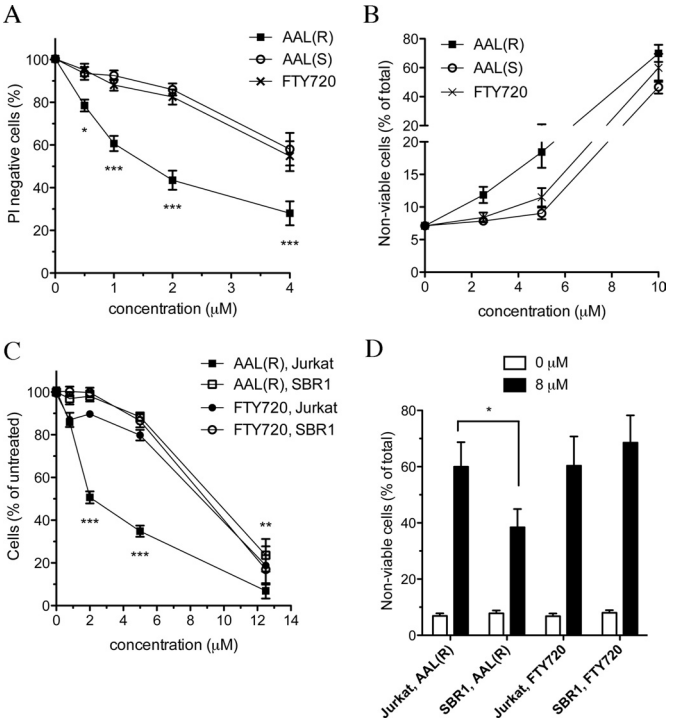

Fig. 1. AAL(R) but not FTY720 triggers a SphK2-dependent apoptosis pathway in lymphocytes. A, viability of mouse splenocytes incubated for 24 h with FTY720 (×), AAL(R) (■), or AAL(S) (○) was assessed by PI exclusion. Proportion of viable cells was normalized relative to vehicle-treated. B, viability of Jurkat cells treated for 24 h with FTY720 (×), AAL(R) (■), or AAL(S) (○) was assessed by annexin V/PI staining. Non-viable cells are those that were positive for annexin V, PI, or both. C, MTT assay was used to asses viability of Jurkat cells (closed symbols) or the SphK2-deficient Jurkat derivative cell line SBR1 (open symbols) (Don et al., 2007), after a 20-h treatment with FTY720 (circles) or AAL(R) (squares). D, Jurkat or SBR1 cells were treated for 24 h with 0 or 8 μM AAL(R) or FTY720. Viability was assessed by annexin V/PI staining. All results shown are the combined results of three separate experiments, each consisting of triplicate treatments (i.e., $n = 9$ per data point). Two-way analysis of variance with Bonferroni post test was used to determine the statistical significance of differences between AAL(R) and both FTY720 and AAL(S) (in A and B) and between AAL(R)-treated Jurkat and SBR1 cells (in C and D); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

that FTY720 is much less potent than AAL(R), and equipotent with AAL(S), at inducing loss of viability in cultured mouse splenocytes (Fig. 1A). FTY720 was also less efficient than AAL(R) at inducing apoptosis in the Jurkat T-lymphoblast cell line, at concentrations below 10 μM (Fig. 1, B and C). As observed previously (Don et al., 2007), the SphK2-deficient Jurkat cell line SBR1 was resistant to apoptosis induced with AAL(R). However, these cells were not resistant to FTY720 (Fig. 1, C and D). These results indicated that a SphK2-dependent apoptotic pathway is triggered by treating cells with AAL(R), but not FTY720. At higher concentrations, apoptosis induced with AAL(R) becomes SphK2-independent, suggesting activation of a second apoptotic pathway, which is the same as that triggered by treating cells with FTY720 or AAL(S). Similar results were seen in HeLa cells pretreated with siRNA to SphK2, then treated for 24 h with AAL(R) or FTY720 (Fig. 2). As a potential explanation for why AAL(R) but not FTY720 triggers a SphK2-dependent apoptotic response, we investigated whether AAL-P accumulates inside cells to a greater extent than FTY-P.

AAL(R) Is More Rapidly Phosphorylated than FTY720. We found that AAL(R) is phosphorylated much more rapidly than FTY720 by cultured Jurkat cells, using LC-tandem mass spectrometry (Fig. 3A). We therefore compared the phosphorylation rate for these compounds in whole blood, which is rich in sphingosine kinase 2 activity (Billich et al., 2003). The rate of phosphorylation in human blood was 8.9-fold faster with AAL(R) than with FTY720 as substrate (Fig. 3B and Table 1). Both compounds were phosphorylated much more rapidly in rat blood, compared with human blood: the rate of phosphorylation was 35-fold higher for FTY720 and 27-fold higher for AAL(R), in rat versus human blood. The more rapid conversion of FTY720 by mouse or rat blood, compared with human blood, has been reported previously, although without quantification of the difference in rate (Billich et al., 2003). The difference was attributed to the higher SphK2 activity of rodent blood compared with human blood, rather than any difference in the rate of FTY720 phosphorylation by rodent versus human SphK2.

We next investigated whether AAL(R) is a better substrate for SphK2 than FTY720, using an in vitro reaction with recombinant human SphK2 (Fig. 4A). The enzyme turnover rate was 7.9 times higher with AAL(R) as the substrate, whereas the ability of the enzyme to bind the substrate (measured as K_m) was very similar (Table 2). This difference in phosphorylation rate is very similar to that observed with whole human blood [8.9-fold higher with AAL(R) as substrate]. Similar results were seen when lysates of human embryonic kidney 293 cells overexpressing human SphK2 were used as the source of SphK2 activity: the turnover rate was 14 times higher with AAL(R) than with FTY720 as the substrate, whereas the K_m was similar [7.4 μM for AAL(R); 13.2 μM for FTY720]. These results indicate that although there seems to be no difference in the affinity of SphK2 for the two substrates, the active site is better able to turn over AAL(R) than FTY720.

The key structural difference between FTY720 and AAL(R) is a hydroxymethyl to methyl substitution on the quaternary

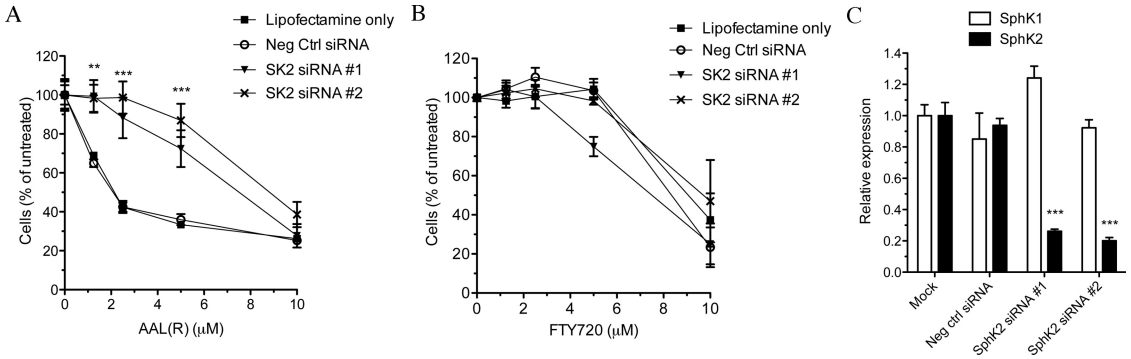

Fig. 2. AAL(R) but not FTY720 triggers a SphK2-dependent apoptosis pathway in HeLa cells. HeLa cells were pretreated for 48 h with two different siRNA molecules targeting SphK2 (▼ or ×, universal negative control siRNA (○), or lipofectamine only (■), then incubated for 24 h in the presence of AAL(R) (A) or FTY720 (B). Viability was determined by MTT assay and normalized to vehicle control-treated cells. Results shown are the combined results of two separate experiments, each consisting of triplicate treatments (i.e., $n = 6$ per data point). C, expression of SphK2 (closed bars) and, as a control, SphK1 (open bars) was measured 48 h after siRNA treatment, using real-time PCR. Expression was normalized relative to glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and is expressed proportional to the lipofectamine only control (Mock). Results shown are mean and S.E. of four data points, derived from two separate experiments for each siRNA. Two-way analysis of variance with Bonferroni post test was used to determine the statistical significance of differences between negative control and both SK2-specific siRNAs (A and B) or all siRNAs compared with mock transfected (C); ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.0001$.

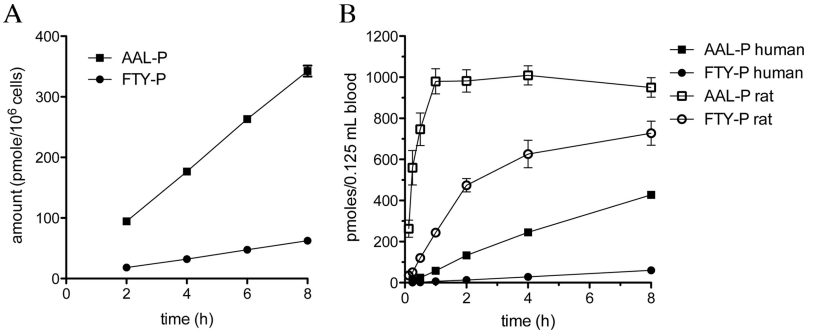

Fig. 3. AAL(R) is more rapidly phosphorylated than FTY720. A, phosphorylation of AAL(R) or FTY720 by cultured Jurkat cells was measured over time, by quantifying the amount of AAL-P (■) or FTY-P (●) in both cells and culture medium. Results are mean and S.E. derived from triplicate cell treatments and representative of two independent experiments. B, formation of AAL-P (squares) or FTY-P (circles) in human (solid symbols) or rat (open symbols) blood was measured as a function of time after addition of 1 nmol of AAL(R) or FTY720 to 125 μl of whole blood, as described under *Materials and Methods*. Results shown are combined data from two separate experiments ($n = 5$, rat blood; $n = 6$, human blood).

(second) carbon of the headgroup (Fig. 4C), suggesting that the presence of this second hydroxyl group interferes with catalysis or release of the product. In the natural substrates sphingosine and dhSph, a second hydroxyl group located on the third carbon of the acyl chain is not accessible for phosphorylation by sphingosine kinases. To gain some insight into whether this 3-OH group influences the phosphorylation rate or substrate affinity, we determined the Michaelis-Menten kinetics for phosphorylation of dhSph and 3-deoxy-dhSph (Fig. 4D) by SphK2 (Fig. 4B and table 2). Removal of the hydroxyl group reduced the enzyme turnover rate and slightly increased the K_m, but the effects were not dramatic, indicating that the 3-OH group plays a minor role in substrate recognition and turnover by SphK2.

FTY720 Is Dephosphorylated Faster than AAL(R). The steady-state level of FTY-P achieved in living organisms is a function not only of phosphorylation but also of dephosphorylation. A likely candidate organ for dephosphorylation of circulating FTY-P is the endothelium, which on the other hand has very little SphK2 activity and is therefore unlikely to contribute significantly to the compound's phosphorylation (Anada et al., 2007). FTY-P is a membrane-impermeant compound, and recent evidence indicates that it may be dephosphorylated extracellularly by endothelial lipid phosphate phosphatases, specifically subtypes 1a and 3 (Mechtcheriakova et al., 2007; Yamanaka et al., 2008). To determine whether there are any differences in the ability of lipid phosphatases to dephosphorylate the two compounds, we tested the ectophosphatase activity of cultured human endothelial cells toward both FTY-P and AAL-P (Fig. 5). In direct contrast to the rate of phosphorylation by SphK2, the rate of dephosphorylation was faster with FTY-P. Using a one-phase exponential decay model to fit the data, the difference in dephosphorylation rate was 1.5, 1.6, and 2.9-fold (faster in the case of FTY-P) in three separate experiments, and was statistically significant ($P < 0.001$, sum-of-squares test).

AAL(R) Is More Fully Phosphorylated In Vivo. Our in vitro results indicated that AAL(R) should be more completely phosphorylated than FTY720, at steady state, in vivo. To test this, we administered a single 0.3 mg/kg dose of AAL(R) or FTY720 to mice, and measured the amount of AAL(R) and AAL-P, or FTY720 and FTY-P, in the blood 18 h later (Fig. 6). For AAL(R), 3.7 ± 1.3% of the compound remained unphosphorylated (i.e., 96% phosphorylated), whereas for FTY720, this was 19.1 ± 3.1% (81% phosphorylated), a statistically significant difference ($P < 0.001$, unpaired t test). Measurements of FTY720 phosphorylation in mice at 2 h (82%) and 6 h (83%) indicated that a steady-state balance was rapidly achieved, and although the total amount of compound in blood declined over time, the proportion of phosphorylated compound remained steady.

TABLE 1

Rate of FTY720 and AAL(R) phosphorylation in whole blood

Phosphorylation rates were calculated using only the linear portion of the phosphorylation curves shown in Fig. 3B. Data are presented as mean ± S.E.

Blood	FTY720	AAL(R)
	nmol product/h/ml of blood	
Human	0.056 ± 0.001	0.496 ± 0.014
Rat	1.93 ± 0.040	13.3 ± 1.13

Discussion

Induction of lymphopenia with FTY720 is dependent on stimulation of the $S1P_1$ receptor by FTY-P. The potency (EC_{50}) and efficacy (E_{max}) for AAL-P, FTY-P, and S1P on the human $S1P_1$ receptor are essentially identical (Brinkmann et al., 2002). Despite this, two publications have reported that the EC_{50} for induction of lymphopenia in rats is three times lower with AAL(R) than with FTY720 (Kiuchi et al., 2000; Högenauer et al., 2008). Our own measurements of blood T cells in mice confirm the greater potency of AAL(R) as an inducer of lymphopenia: the EC_{50} for depletion of blood T cells after 18 h was 27 μg/kg with AAL(R) and 51 μg/kg with FTY720 ($n = 4$, $P = 0.036$, by sum-of-squares F test). These results support the conclusion that the more rapid phosphorylation of AAL(R) in vitro translates into a greater proportion of phosphorylated compound in vivo, and a consequent increase in potency. Although AAL(R) is a better substrate than FTY720 for phosphorylation, dephosphorylation of FTY-P by human endothelial cells was faster than for AAL-P

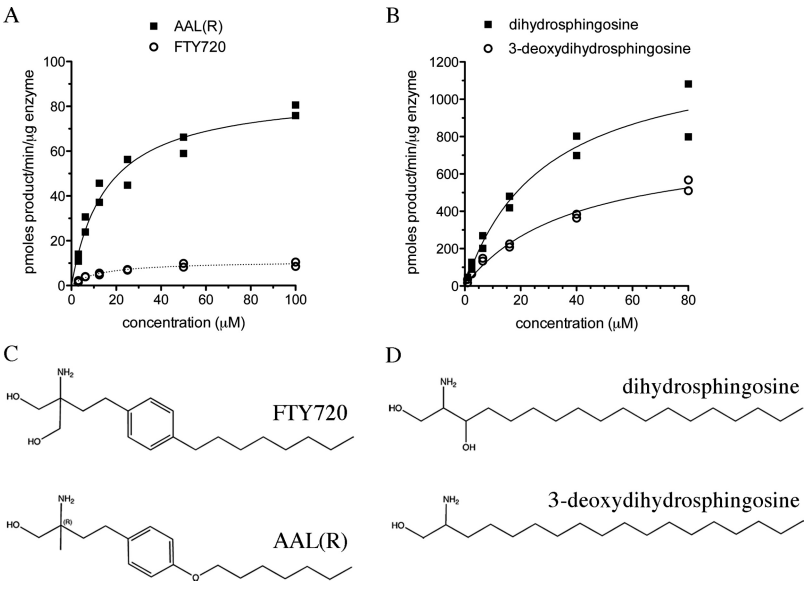

Fig. 4. Turnover rate by SphK2 is higher with AAL(R) than with FTY720. A, phosphorylation of AAL(R) (■) or FTY720 (○) by recombinant human SphK2, as a function of substrate concentration. B, phosphorylation of dhSph (■) or 3-deoxy-dhSph (○) by recombinant human SphK2 as a function of substrate concentration. Michaelis-Menten curves were fitted to 12 data points with Prism (GraphPad Software, San Diego, CA), and V_{max} and K_m values are shown in Table 2. C, structures for FTY720 and AAL(R). D, structures for dhSph and 3-deoxy-dhSph.

(Fig. 5), suggesting that FTY-P is a better substrate for lipid phosphate phosphatases. This would further exacerbate the difference between the two compounds in terms of steady-state phosphorylation.

The more rapid phosphorylation of AAL(R) by SphK2 is supported by our initial observation that AAL(R) induces a SphK2-dependent apoptosis pathway, whereas FTY720 does not. The apoptotic response seems to be triggered by excessive intracellular accumulation of long-chain base phosphates such as AAL-P (Don et al., 2007), *cis*-4-methylsphingosine 1-phosphate (van Echten-Deckert et al., 1997), or, in yeast, phytosphingosine 1-phosphate (Zhang et al., 2001). Further experiments have shown that apoptosis triggered by AAL(R) proceeds through mitochondrial depolarization (not shown), but the precise nature of the intracellular target for AAL-P that triggers apoptosis is currently unknown. AAL(R) and FTY720 both trigger a SphK2-independent apoptotic pathway at concentrations of approximately 10 μM. Apoptosis induced with FTY720 forms the basis for its anticancer properties, and is believed to occur through activation of the broad-spectrum serine/threonine protein phosphatase 2A, at least in leukemia cells (Matsuoka et al., 2003; Neviani et al., 2007; Liu et al., 2008). Our results are in agreement with those of others, who have shown that FTY720 does not need to be phosphorylated to induce apoptosis in leukemia cells (Neviani et al., 2007; Liu et al., 2008). Neither the SphK2-dependent nor the SphK2-independent apoptotic pathway is relevant to immunosuppression, because these pathways are activated at concentrations of the drug that are 1 to 2 orders of magnitude higher than the concentration required to achieve effective immunosuppression in humans or rodents.

There are two structural differences between AAL(R) and FTY720 (Fig. 4C): the introduction of an ether linkage (replacing a carbon) between the lipid tail and the aromatic ring in AAL(R), and elimination of one of the FTY720 hydroxyl headgroups. It has been shown previously (Kiuchi et al., 2000) that introduction of the ether linkage into FTY720 does not improve, or significantly alter, its potency as an inducer of lymphopenia. We therefore conclude that elimination of one of the hydroxyl groups improves catalysis by SphK2. The hydroxyl group on the third carbon of dhSph does not slow down its phosphorylation by SphK2 relative to 3-deoxy-dhSph (Fig. 4B), indicating that it is the position of the second hydroxyl group in FTY720 that interferes with catalysis. It is likely that the presence of two hydroxyl groups in FTY720, both accessible to the SphK2 catalytic site, interferes with release of the product or the transfer of phosphate from ATP.

In rodents, a cycle of phosphorylation and dephosphorylation maintains an equilibrium between FTY720 and FTY-P in the blood, with 20 to 30% of the compound in the nonphosphorylated form (Brinkmann et al., 2002; Mandala et al., 2002). In the current study, we show that when AAL(R) is used, the equilibrium is shifted in favor of the phosphate (Fig. 6), and this gives rise to an increase in potency. For this reason, AAL(R) is probably superior to FTY720 as a research tool for determining the effects the sphingosine 1-phosphate receptor agonists on animal physiology and pathophysiology, especially given the availability of a chemically identical, nonphosphorylatable control compound in the form of the *S*-enantiomer. We note that asymmetric synthesis of AAL(R) or AAL(S) is not difficult and can be achieved by starting with the chiral headgroup and adding the lipophilic portion of the molecule to this. This approach circumvents the need for any chiral separation (Hinterding et al., 2003).

In human patients, FTY720 phosphorylation seems to be rate limiting for efficacy. Human blood possesses a much lower intrinsic SphK2 activity than rodent blood (Billich et al., 2003), resulting in a much slower rate of phosphorylation for both FTY720 and AAL(R) (Fig. 3B). Results with human patients indicate that the equilibrium between FTY720 and its phosphate rests more heavily in favor of dephosphorylation: the plasma concentration of FTY-P drops below that of FTY720 12 to 24 h after a single 5-mg dose of FTY720; thereafter, FTY-P declines as a proportion of the FTY720 concentration (Kovarik et al., 2008, 2009). On this basis, one would predict that AAL(R) would achieve effective immunosuppression at a significantly lower dose in humans than FTY720. Lymphopenia in humans is achieved with doses of FTY720 at or above 1 mg/day. At this dose, the steady-state FTY720 concentration in plasma reaches 5.7 ng/ml (18.6 nM). More effective lymphopenia is achieved at 2.5 mg/day (steady state FTY720 concentration of 36.5 nM in plasma) (Kahan et al., 2003; Brinkmann, 2007). At this concentration FTY720 may have effects that are not dependent on its phosphorylation, such as inhibition of cytosolic phospholipase A_2, with consequent inhibition of prostaglandin and prostacyclin synthesis (Payne et al., 2007), or inhibition of protein kinase C isoforms (Sensken and Gräler, 2010).

In summary, in this article we report that the *R*-enantiomer of the FTY720 derivative 2-amino-1,3-propanediol is a

TABLE 2

Michaelis-Menten kinetics for phosphorylation of dhSph, 3-deoxy-dhSph, FTY720, and AAL(R) by purified recombinant human SphK2

Michaelis-Menten curves were fitted to 12 data points for each compound with the use of GraphPad Prism. Values shown are best fit values ± S.E.

	K_m	V_{max}	K_{cat}
	μM	*pmol product / min / μg of enzyme*	
dhSph	28.8 ± 7.2	*1281 ± 131*	89.6
3-Deoxy-dhSph	38.9 ± 6.8	781 ± 62.8	54.6
AAL(R)	15.6 ± 2.7	86.8 ± 5.0	6.07
FTY720	13.1 ± 2.3	11.0 ± 0.61	0.77

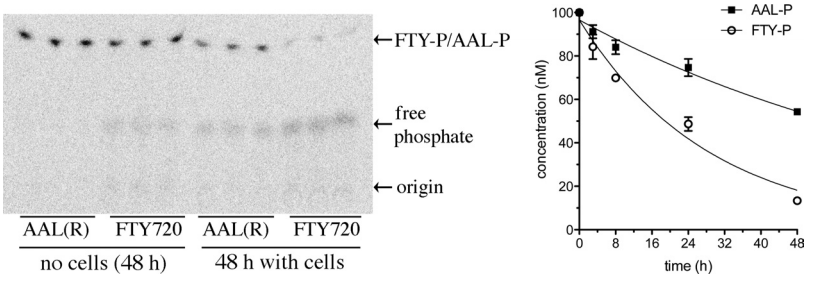

Fig. 5. Dephosphorylation of FTY-P and AAL-P. Radiolabeled AAL-P (■) or FTY-P (○) was added at 100 nM to the medium of cultured HMEC-1 cells. The medium was sampled at indicated times and loss of the phosphorylated substrate from the growth medium was assessed by TLC and filmless autoradiographic analysis. Image from the 48-h incubation, plus no-cell control, is shown on the right, and graph shows loss of radiolabeled substrate over time. Results shown are mean of triplicate incubations and are representative of three independent experiments. Data points were fitted to a one phase decay model using GraphPad Prism.

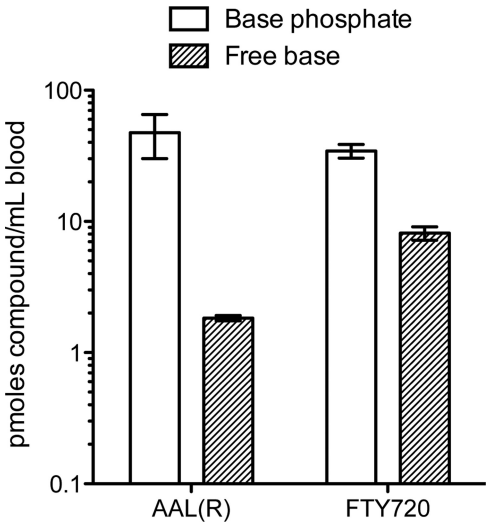

Fig. 6. AAL(R) is more completely phosphorylated in vivo. Amount of AAL(R) and AAL-P, or FTY720 and FTY-P, in the blood, 18 h after intraperitoneal administration of 0.3 mg/kg AAL(R) or FTY720 to C57BL6 mice (n = 4). Free base (shaded bars) refers to AAL(R) and FTY720, whereas base phosphate (clear bars) refers to AAL-P and FTY-P.

much better substrate for SphK2 than FTY720 itself. This results from a faster enzyme turnover rather than higher affinity and leads to a significantly more rapid rate of phosphorylation in human blood. These results have therapeutic importance, because the efficacy of FTY720 as an immunosuppressant is dependent on the phosphate rather than the parent compound. First, effective immunosuppression could be achieved with lower doses of AAL(R) than are needed with FTY720. Second, use of AAL(R) would reduce the amount of nonphosphorylated compound in circulation, thus obviating any effects associated with the nonphosphorylated compound.

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