Elimination of a hydroxyl group in FTY720 dramatically improves the phosphorylation rate

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Running Title: Deoxy FTY720 Derivative is Much More Rapidly Phosphorylated

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Abbreviations

dhSph, dihydrosphingosine
G3PDH, glyceraldehyde 3-phosphate dehydrogenase
PI, propidium iodide
SphK1, sphingosine kinase 1
SphK2, sphingosine kinase 2
TLC, thin layer chromatography
ABSTRACT
The new immunosuppressant FTY720 (Fingolimod), an analogue 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 S1P1 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, whilst 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, as phosphorylation of FTY720 is much less efficient in humans compared to rodents. Our results suggest that AAL(R) is a better tool than FTY720 for in vivo studies with S1P analogues, and would probably be a more effective immunosuppressant than FTY720.
INTRODUCTION

FTY720 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 amongst pharmacological immunosuppressants, and has made FTY720 the subject of intense interest, both from 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 S1P1 receptor is responsible for sequestration of T-cells in the peripheral lymphoid organs, demonstrated with the observations that a range of S1P1-selective agonists can induce lymphopenia, that this is reversible with an S1P1 antagonist, and that S1P1-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 S1P1 agonists induce lymphopenia (Brinkmann, 2007; Rosen et al., 2008): in one model activation of S1P1 receptors on endothelial cells exposed 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 towards the higher S1P concentration of the blood or lymph, requiring stimulation of their S1P1 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 super-agonist of S1P1, 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 like FTY-P have found application in a wide variety of experimental settings, which include immunosuppression during organ transplant (Brinkmann, 2007; Pan et al., 2006), treatment of autoimmune conditions (Fujino et al., 2003; Maki et al., 2005), recovery after ischaemia/reperfusion injury (Hofmann et al., 2009), and as a means of increasing endothelial barrier function (Sanna et al., 2006). In the clinic, FTY720 has been trialed both in kidney transplant and multiple sclerosis patients (Brinkmann, 2007). The trials in transplant patients 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 analogue 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 (Brinkmann et al., 2002; Don et al., 2007; Kiuchi et al., 2000). As FTY720 and AAL(R) are very similar compounds, they have been used interchangeably. In this research paper, we show 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 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 Chemicon, whilst AAL(R) was a gift from Professor Hugh Rosen, The Scripps Research Institute. AAL-P to use as a standard for mass spectrometry was prepared by chemical phosphorylation: The amino group of AAL was protected (Boc2O, NaHCO3, 56%), then reaction with N,N-diisopropyl phosphoramidite dichloride and 5-ethylthio-1H-tetrazole, followed by oxidation with hydrogen peroxide, gave protected AAL-P in 19% yield. The compound was deprotected with trifluoroacetic acid. DhSph was purchased from Avanti Polar Lipids. 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 RPMI1640 medium supplemented with 10% foetal 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 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 NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, for 5 min on ice. Isolated splenocytes were resuspended at a density of 1.5 x 10⁶ viable cells/mL in complete RPMI medium, cultured in the presence of AAL(R), AAL(S) or FTY720 for 20 h, then stained with PI for flow cytometry. For MTT assays, cells
were cultured in 96-well plates, using 0.1 mL medium per well. Ten μL of 0.5% (w/v) MTT reagent (Sigma) in PBS was added to each well, and cells were returned to the incubator for 2 h. MTT was solubilised by adding 0.1 mL 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 20 mM Hapes, pH 7.4, 150 mM NaCl, and 2.5 mM CaCl₂, and incubated for 15 min on ice with 2 μL Annexin V-APC and 1 μg/mL PI, then subjected to flow cytometry.

**siRNA treatment of HeLa cells and Real-time quantitative PCR**

Cells were transfected in 6-well plates, in 2 mL OptiMEM I medium, using siRNA molecules purchased from Qiagen at a final concentration of 100 nM. siRNA’s were pre-mixed in 200 μL OptiMEM with 4 μL lipofectamine 2000 (Invitrogen) for 30 min, then added to the cells for 6 hours, after which the medium was replaced with standard growth medium. On the day following transfection, the cells were detached and re-seeded into a 96-well plate at a density of 10⁴ 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 post-transfection. Real-time PCR was used to measure transcript levels, using the following primers taken from PrimerBank (Spandidos et al): SphK1 fwd: AGGCTGAAATCTCCTTCACGC; SphK1 rev: GTCTCCAGACATGACCACCAG; SphK2 fwd: GCTGCTGCGCCTTTTCTTG; SphK2 rev: CCTGTAGCGGCCCATACTC; G3PDH fwd: TGTTGCCATCAATGACCCCTT; G3PDH rev: CTCCACGACGTACTCAGCG.

RNA was prepared with an RNEasy Mini Kit (Qiagen); cDNA was prepared with MMLV reverse transcriptase (Invitrogen); and a SYBR Green with ROX Kit (Invitrogen) was used for qPCR, on a Stratagene Mx3000 cycler.
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 x 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 indicated times, 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 80% methanol/20% water (mobile phase for LC). Lipids were quantified by LC-MS/MS, using a C8 column coupled to a Thermo Quantum TSQ mass spectrometer 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 RPMI1640 medium. One nmole FTY720 or AAL(R) was added directly to 250 μL blood/RPMI mix, and incubated at 35 °C for the indicated times. Reactions were stopped with the addition of 1 mL 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 methanol. The supernatants from both steps were combined in 4 mL glass tubes, dried down in a Speedivac, and the extracts were resuspended in 200 μL 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 MgCl₂, 1 mM DTT, 2 mM ATP, 0.1% fatty acid free BSA, and 5 μCi/reaction radioactively labeled ³²P-ATP (Perkin Elmer). Reactions (0.1 mL) were started with the addition of recombinant human SphK2, produced in insect cells (Biomol). The final enzyme concentration was 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 150 min in the case of 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 methanol/HCl (150:1), followed by 250 μL 2M KCl, and 350 μL chloroform. Tubes were vortexed, then microfuged at 14,000 rpm to resolve the phases. The upper aqueous phase was discarded, and 4 μL of the (lower) organic phase was spotted on to Silica Gel 60 TLC plates (Fluka). TLC plates were resolved in butanol/acetic acid/water (3:1:1), then exposed to Fuji Imaging Plates and imaged with a Fuji FLA7000 phosphorimager. The concentration of product in each spot was derived from a standard curve constructed with the ³²P-ATP reaction mix.

**Lipid phosphatase assay**

To prepare radiolabelled FTY-P and AAL-P, solutions of 50 μM FTY720 or AAL(R) were phosphorylated in kinase assay buffer containing 10 μCi/400 μL reaction ³²P-
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 methanol, 40 μL 3M NaOH, and 400 μL chloroform. Tubes were vortexed, phases were separated by centrifugation, and the upper aqueous phase, containing the radiolabelled phosphates, was transferred to a new tube. This aqueous extract was re-extracted by adding 80 μL concentrated HCl and 400 μL 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 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% fatty acid free BSA, and the concentration of the radiolabelled phosphate was measured by resolving the resuspended compound on TLC and quantification of FTY-P or AAL-P spots with a phosphorimager.

To assay dephosphorylation, HMEC-1 cells were seeded at a density of 2 x 10⁵ cells/well, in a 24-well plate. On the following day, the medium was replaced with 0.3 mL fresh growth medium containing 100 nM radiolabelled 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 intra peritoneal injection, in 0.1 mL sterile water, to groups of 4 (per treatment) C57BL6 mice. Mice were euthanased, 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 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 in 0.17 M NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, each for 5 min at RT. 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) in PBS/2% FBS. Cells were washed, then fixed for 10 min at RT with 1% paraformaldehyde in PBS, washed once more, and analysed the following day using a BD FACS Canto II flow cytometer (BD Bioscience) and FlowJo software (TreeStar). 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 previously shown 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: firstly AAL(R) was much more efficient than its non-phosphorylatable enantiomer, AAL(S), at inducing loss of viability; secondly, 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(R)-phosphate (AAL-P). To our surprise, we have found that FTY720 is much less potent than AAL(R), and equipotent with AAL(S), at inducing loss of viability in cultured mouse splenocytes (Figure 1A). FTY720 was also less efficient than AAL(R) at inducing apoptosis in the Jurkat T-lymphoblast cell line, at concentrations below 10 µM (Figure 1B 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 (Figure 1C 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 pre-treated with siRNA to SphK2, then treated for 24 h with AAL(R) or FTY720 (Figure 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 liquid chromatography mass spectrometry (LC-MS/MS) (Figure 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 (Figure 3B and Table 1). Both compounds were phosphorylated much more rapidly in rat blood, when compared to 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 to 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 to 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 (Figure 4A). The enzyme turnover rate was 7.9 times higher with AAL(R) as the substrate, whilst 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 HEK293 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, whilst the $K_m$ was similar (7.4 μM for AAL(R); 13.2 μM for FTY720). These results indicate that whilst there appears to be no difference in the affinity of SphK2 for the two substrates, the active site is better able to turnover AAL(R) than FTY720.

The key structural difference between FTY720 and AAL(R) is a hydroxymethyl to methyl substitution on the quaternary (second) carbon of the headgroup (Figure 4C), suggesting that the presence of this second hydroxyl group interferes with catalysis or release of the product. In the natural substrates sphingosine and dihydrosphingosine (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 (Figure 4D) by SphK2 (Figure 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. As FTY-P is membrane impermeable, 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 not likely to contribute significantly to the compound’s phosphorylation (Anada et al., 2007). FTY-P is a membrane impermeable 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). In order to determine whether there are any differences in the ability of lipid phosphatases to dephosphorylate the two compounds, we tested the ecto-phosphatase activity of cultured human endothelial cells towards both FTY-P and AAL-P (Figure 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 (Figure 6). For AAL(R), 3.7 ± 1.3% of the compound remained unphosphorylated (i.e. 96% phosphorylated), whilst 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 whilst the total amount of compound in blood declined over time, the proportion of phosphorylated compound remained steady.

DISCUSSION

Induction of lymphopenia with FTY720 is dependent on stimulation of the S1P1 receptor by FTY-P. The potency (EC50) and efficacy (E_{max}) for AAL-P, FTY-P and S1P on the human S1P1 receptor are essentially identical (Brinkmann et al., 2002). Despite this, two publications have reported that the EC50 for induction of lymphopenia in rats is 3 times lower with AAL(R) than with FTY720 (Hogenauer et al., 2008; Kiuchi et al., 2000). Our own measurements of blood T-cells in mice confirm the greater potency of AAL(R) as an inducer of lymphopenia: the EC50 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 (Figure 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, whilst FTY720 does not. The apoptotic response appears 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 depolarisation (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 around 10 μM. Apoptosis induced with FTY720 forms the basis for its anti-cancer properties, and is believed to occur through activation of the broad spectrum serine/threonine phosphatase PP2A, at least in leukaemia cells (Liu et al., 2008; Matsuoka et al., 2003; Neviani et al., 2007). Our results are in agreement with those of others, who have shown that FTY720 does not need to be phosphorylated in order to induce apoptosis in leukaemia cells (Liu et al., 2008; Neviani et al., 2007). Neither the SphK2-dependent nor the SphK2-independent apoptotic pathway are relevant to immunosuppression, since these pathways are activated at concentrations of the drug that are one to two 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 (Figure 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 (Figure 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 20-30% of the compound in the non-phosphorylated 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 favour of the phosphate (Figure 6), and this gives rise to an increase in potency. For this reason, AAL(R) is probably superior, compared 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, non-phosphorylatable 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 appears 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) (Figure 3B). Results with human patients indicate that the equilibrium between FTY720 and its phosphate rests more heavily in favour of dephosphorylation: the plasma concentration of FTY-P drops below that of FTY720 12 – 24 h after a single 5 mg dose of FTY720, and thereafter FTY-P declines as a proportion of the FTY720 concentration (Kovarik et al., 2009; Kovarik et al., 2008). 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) (Brinkmann, 2007; Kahan et al., 2003). At this concentration FTY720 may have effects that are not dependent on its phosphorylation, such as inhibition of cytosolic phospholipase A2, with consequently inhibition of prostaglandin and prostacyclin synthesis (Payne et al., 2007), or inhibition of Protein Kinase C isoforms (Sensken and Graler, 2010).

In summary, in this paper we report that the R enantiomer of the FTY720 derivative 2-amino-1,3-propanediol is a 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, since the efficacy of FTY720 as an immunosuppressant is dependent on the phosphate rather than the parent compound. Firstly, effective immunosuppression could be achieved with lower doses of AAL(R) than are needed with FTY720. Secondly, use of AAL(R) would reduce the amount of non-phosphorylated compound in circulation, thus obviating any effects associated with the non-phosphorylated compound.
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FOOTNOTES:

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FIGURE LEGENDS

Figure 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 (X), AAL(R) (■), or AAL(S) (○), was assessed by propidium iodide (PI) exclusion. Proportion of viable cells was normalized relative to vehicle-treated. (B) Viability of Jurkat cells treated for 24 h with FTY720 (X), AAL(R) (■), or AAL(S) (○), was assessed by annexin V/PI staining. Non-viable cells are those that were positive for either annexin V or PI, or both. (C) MTT assay was used to assess viability of Jurkat cells (closed symbols), or the SphK2-deficient Jurkat derivative cell line SBR1 (open symbols) (Don et al., 2007), following 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 ANOVA 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.

Figure 2. AAL(R) but not FTY720 triggers a SphK2-dependent apoptosis pathway in HeLa cells. HeLa cells were pre-treated for 48 h with two different siRNA molecules targeting SphK2 (▼ or X), universal negative control siRNA (○), or lipofectamine only (■), then incubated for 24 h in the presence of (A) AAL(R) or (B) FTY720. 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 G3PDH, and is expressed proportional to the lipofectamine only control (Mock). Results shown are mean and standard error of four data points, derived from two separate experiments for each siRNA. Two-way ANOVA with Bonferroni post-test was used to determine the statistical significance of differences between negative control and both SK2-specific siRNAs in (A) and (B), or all siRNAs compared to mock transfected (C); * P < 0.05, ** P < 0.01, *** P < 0.0001.

Figure 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 standard error 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 following addition of 1 nmole AAL(R) or FTY720 to 125 μL whole blood, as described in methods. Results shown are combined data from two separate experiments (n = 5, rat blood; n = 6, human blood).

Figure 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 GraphPad PRISM.
software, 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.

**Figure 5. Dephosphorylation of FTY-P and AAL-P.** Radiolabelled AAL-P (■) or FTY-P (○) was added at 100 nM to the medium of cultured Human Microvascular Endothelial (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 phosphorimaging. Image from the 48 h incubation, plus no-cell control, is shown on the right, and graph shows loss of radiolabelled substrate over time. Results shown are mean of triplicate incubations, and representative of three independent experiments. Data points were fitted to a one phase decay model using GraphPad PRISM.

**Figure 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 *intra peritoneal* 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, whilst base phosphate (clear bars) refers to AAL-P and FTY-P.
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 Figure 3B. Units are nmoles product/h/mL blood (±
standard error).

<table>
<thead>
<tr>
<th></th>
<th>FTY720</th>
<th>AAL(R)</th>
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<tbody>
<tr>
<td>Human blood</td>
<td>0.056 ± 0.001</td>
<td>0.496 ± 0.014</td>
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<tr>
<td>Rat blood</td>
<td>1.93 ± 0.040</td>
<td>13.3 ± 1.13</td>
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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, using GraphPad PRISM. Values shown are best fit values ± standard error. $V_{\text{max}}$ is expressed as pmoles product/min/μg enzyme.

<table>
<thead>
<tr>
<th></th>
<th>$K_{m}$</th>
<th>$V_{\text{max}}$</th>
<th>$K_{\text{cat}}$</th>
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<tbody>
<tr>
<td>dhSph</td>
<td>28.8 ± 7.2</td>
<td>1281 ± 131</td>
<td>89.6</td>
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<tr>
<td>3-deoxy-dhSph</td>
<td>38.9 ± 6.8</td>
<td>781 ± 62.8</td>
<td>54.6</td>
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<tr>
<td>AAL(R)</td>
<td>15.6 ± 2.7</td>
<td>86.8 ± 5.0</td>
<td>6.07</td>
</tr>
<tr>
<td>FTY720</td>
<td>13.1 ± 2.3</td>
<td>11.0 ± 0.61</td>
<td>0.77</td>
</tr>
</tbody>
</table>
Figure 2

A

- Lipofectamine only
- Neg Ctrl siRNA
- SK2 siRNA #1
- SK2 siRNA #2

B

- Lipofectamine only
- Neg Ctrl siRNA
- SK2 siRNA #1
- SK2 siRNA #2

C

<table>
<thead>
<tr>
<th></th>
<th>SphK1</th>
<th>SphK2</th>
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<tbody>
<tr>
<td>Mock</td>
<td></td>
<td></td>
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<tr>
<td>Neg ctrl siRNA</td>
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<tr>
<td>SphK2-siRNA #1</td>
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<tr>
<td>SphK2-siRNA #2</td>
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</tbody>
</table>
Figure 3

A

B

amount (pmole/10^6 cells)

pmoles/0.125 mL blood

time (h)

AAL-P

FTY-P

AAL-P human

FTY-P human

AAL-P rat

FTY-P rat
Figure 4

A

Molecular Pharmacology Fast Forward. Published on July 7, 2010 as DOI: 10.1124/mol.110.064873

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Figure 6

The graph shows the comparison of base phosphate and free base compounds (p moles compound/mL blood) for AAL(R) and FTY720. The error bars indicate the variability of the measurements.