

A Monoselective Sphingosine-1-Phosphate Receptor-1 Agonist Prevents Allograft Rejection in a Stringent Rat Heart Transplantation Model

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Summary

FTY720 is an immunomodulator with demonstrated efficacy in a phase II trial of relapsing multiple sclerosis. FTY720-phosphate, the active metabolite generated upon phosphorylation *in vivo*, acts as a potent agonist on four of the five known sphingosine-1-phosphate (S1P₁) receptors. AU954, an aminocarboxylate analog of FTY720, is a low nanomolar, monoselective agonist of the S1P₁ receptor. Due to its selectivity and pharmacokinetic profile, AU954 is an excellent pharmacological probe of S1P₁-dependent phenomena. Oral administration of AU954 induces a profound and reversible reduction of circulating lymphocytes and, in combination with RAD001 (Certican/Everolimus, an mTOR inhibitor), is capable of prolonging the survival of cardiac allografts in a stringent rat transplantation model. This demonstrates that a selective agonist of the S1P₁ receptor is sufficient to achieve efficacy in an animal model of transplantation.

Introduction

Sphingosine-1-phosphate (S1P) is an evolutionarily conserved bioactive sphingolipid. S1P is generated as a metabolite of ceramide and secreted into serum by mast cells, platelets, and monocytes. It has been implicated as a second messenger in cellular proliferation and survival and in protection against ceramide-mediated apoptosis. In addition, S1P regulates diverse physiological processes such as cell migration, angiogenesis, vascular maturation [1, 2], and immunity [3–6] by serving as a ligand to cell-surface G-protein-coupled S1P receptors [7, 8].

FTY720 is a synthetic analog of the natural product myriocin that shares structural homology with the endogenous sphingolipid sphingosine. As a novel immunomodulatory agent, FTY720 exhibited promising activity in animal models of organ transplantation and autoimmunity [9, 10]. In combination with cyclosporin (CsA), FTY720 has proven to be efficacious in preventing kidney rejection in humans [11] and entered phase III clinical trials for transplant rejection [12]. However, further development for this indication was discontinued since FTY720 did not provide benefits over standard of care. FTY720 is now being developed for the treatment of multiple sclerosis and is entering a phase III trial after having reduced clinical relapse in a phase II study by greater than 50% relative to placebo in 6 months [13].

Upon phosphorylation *in vivo* primarily by sphingosine kinase 2 (SK2) [14], FTY720 acquires the ability to mimic sphingosine-1-phosphate (S1P) and acts as a potent agonist at four of the five known sphingosine-1-phosphate receptors, namely S1P₁, S1P₃, S1P₄, and S1P₅ (Figure 1) [3, 15]. It has been convincingly demonstrated that FTY720-phosphate-mediated S1P receptor agonism causes inhibition of lymphocyte egress from thymus and lymph nodes, which is—at least in part—responsible for the immunomodulatory activity of FTY720 [16–18]. Recent reports demonstrate the requirement of the S1P₁ receptor for the desired pharmacodynamic response *in vivo* [19]. In addition, a transient and asymptomatic bradycardia has been reported as the primary clinical adverse effect of FTY720 [20], and S1P₃ has been demonstrated to regulate heart rate in rodents [21], apparently by activating G-protein-coupled inward-rectifying potassium (GIRK) channels in atrial myocytes [22]. We therefore sought to determine the minimal receptor profile required to provide full protection of organ transplants *in vivo*. To accomplish this, we prepared a series of novel amino carboxylate analogs and profiled their selectivity against the S1P receptors by using functional GTP γ S binding assays. Here, we demonstrate that a compound with monoselectivity for the sphingosine-1-phosphate receptor-1 (S1P₁) is capable of potentially reducing circulating lymphocytes and protecting rat heart allografts *in vivo* in combination therapy with a subtherapeutic dose of RAD001.

Results and Discussion

Design and Synthesis of AU954: A S1P₁ Monoselective Agonist

FTY720 acts as a prodrug and is converted to an active aminophosphate metabolite through sphingosine-kinase-2-mediated phosphorylation *in vivo*. Due to potential complexities associated with prodrugs such as species differences between the kinase and phosphatase responsible for controlling drug levels, we and others [23] sought to prepare non-prodrug aminocarboxylate bioisoteres. Recently, a series of orally bioavailable tricyclic carboxylate analogs have also been reported as S1P₁ selective agonists [23]. These and earlier compound series developed by the Merck group [24]

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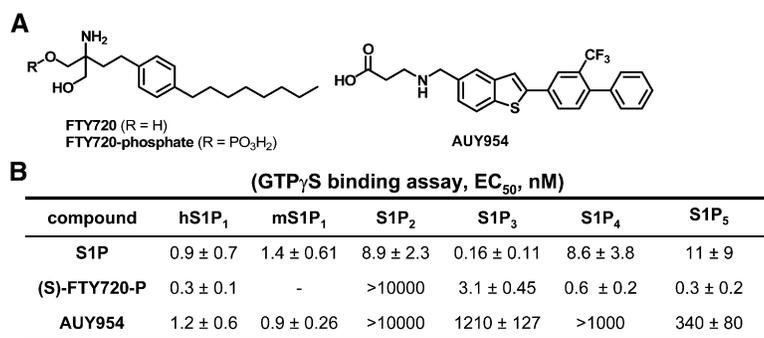


Figure 1. Chemical Structures and Functional Activity of FTY720, FTY720-Phosphate, and AUY954 on S1P Receptors

(A) Chemical structures of FTY720, FTY720-phosphate, and AUY954.

(B) EC₅₀ in nanomolar (mean ± SEM) for the compounds against S1PRs in a γ -GTPS-binding assay.

demonstrated that the aminophosphate “head group” can be functionally mimicked by an amino carboxylate and is sufficient to achieve potent agonist activity. The discovery of the S1P₁ selective agonist SEW02871 [21] demonstrated that modifications made to the parts of the inhibitor that interact with the hydrophobic channel of the S1P receptor allow S1P₁ subtype selectivity to be achieved. SEW02871 achieves significant agonist activity (EC₅₀ = 14 ± 8 nM) despite being incapable of forming ionic interactions with the receptor [25]. It is postulated that the trifluoromethyl groups contributes significantly to the binding affinity. AUY954 (Figure 1) was inspired by combining structural features derived from screening leads related to SEW02871 that were obtained from a high-throughput screen of the S1P₁ receptor. Although the trifluoromethyl biphenyl substituent of AUY954 was identified by a systematic structure activity relationship (SAR) analysis, it can be retrospectively viewed as an isosteric replacement of the trifluoromethyl-thiophene-phenyl fragment of SEW02871. The

potential for molecular mimicry between AUY954 and SEW02871 [25] can be visualized when these compounds are docked to a homology model of S1P₁ (Figure 2). Interestingly, an energetically preferred binding mode has the two compounds closely overlapped, especially in the trifluoromethyl region. AUY954 was finally obtained after installing different amino carboxylate “head groups” to a diverse assortment of benzo-fused heterocycles that possessed the trifluoromethyl-biphenyl pharmacophore (unpublished data) [26]. AUY954 exhibited monoselectivity for S1P₁ over all other S1P receptors (S1P₂₋₅) as assessed by GTP γ S-binding assays (see section below). The synthesis of AUY954 was completed in seven steps (Figure 3). Thus, the boronic acid 2 was obtained from benzothiophene compound 1 upon treatment of lithium diisopropylamide and trimethyl borate. Its coupling counterpart (5) was prepared in two steps starting with Suzuki coupling of commercially available 3-trifluoromethyl-4-bromoaniline (3) with phenylboronic acid followed by a diazotization-bromination

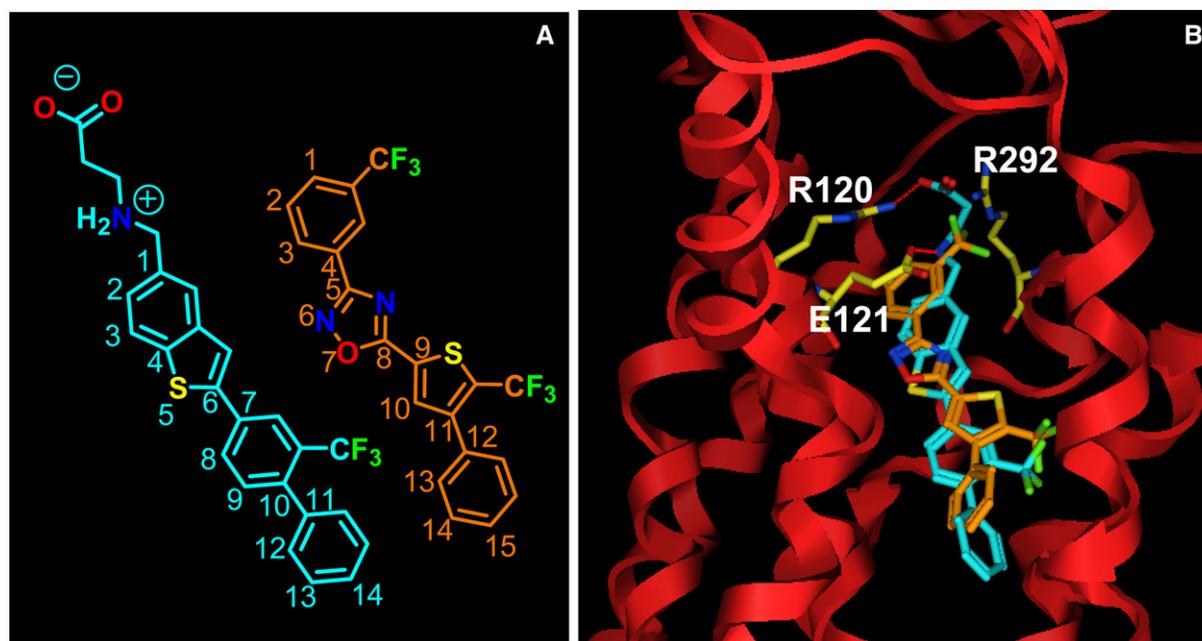


Figure 2. Comparative Binding Mode of AUY954 and SEW02871 Bound to Homology Model of S1P₁

AUY954 and SEW02871 are shown in cyan and brown, respectively (heteroatoms are colored red for oxygen, blue for nitrogen, green for fluorine, and yellow for sulfur), and S1P₁ in red ribbons. Possible ionic interactions are shown as dotted lines to Glu121 to amine and Arg120 to acid of AUY954.

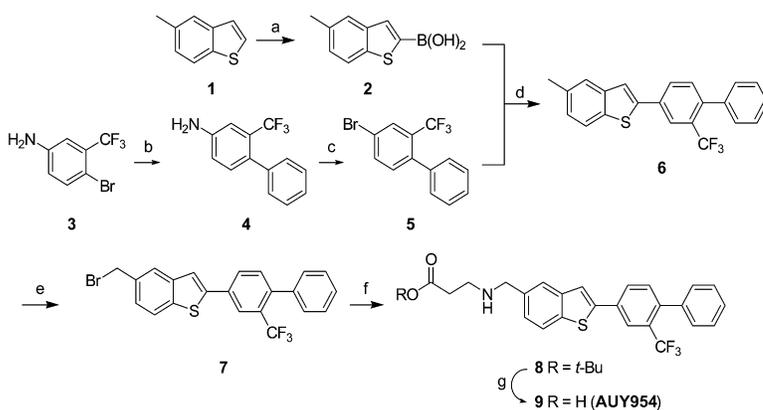


Figure 3. Synthetic Scheme for AUY954
Reagents: (a) i. LDA, B(OMe)₃, THF, -78°C to RT, overnight; ii. HCl, 88%; (b) PhB(OH)₂, Pd(OAc)₂, phosphine ligand, KF, THF, reflux, overnight, 70%; (c) CuBr₂, t-BuONO, CH₃CN, 0°C to RT, overnight, 65%; (d) Pd(PPh₃)₄, Na₂CO₃, PhCH₃-EtOH-H₂O, 80°C, 6 hr, 74%; (e) NBS, AIBN, CCl₄, reflux, 10 hr; (f) NaH, β-alanine t-butyl ester, DMF, RT, overnight, 62% for two steps; (g) TFA, CH₂Cl₂, 66%.

reaction. Bromide 7 was obtained by a Suzuki coupling reaction between 2 and 5 followed by bromination with *N*-bromosuccinimide. AUY954 (9) was obtained by amination with β-alanine *t*-butyl ester, followed by removal of the *t*-butyl protecting group with trifluoroacetic acid.

AUY954 Is Selective for S1P₁

AUY954 was tested for its ability to stimulate the binding of GTPγS to S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ receptor G-protein complexes expressed in recombinant human CHO cell membranes (Figure 1). The EC₅₀ of AUY954 for S1P₁ receptor activation was 1.2 nM, which is comparable to the measured and reported [15] EC₅₀ for FTY720-phosphate. AUY954 exhibited at least 280-fold greater selectivity against the other four receptor subtypes. In addition, AUY954 also showed comparable activity

on the murine S1P₁ receptor (Figure 1). AUY954 was also tested at a concentration of 10 μM for selectivity against a diverse panel of receptors and ion channels, and no appreciable binding affinity (IC₅₀ > 1 μM) was observed.

AUY954 Induces ERK and AKT Phosphorylation

In order to ascertain whether AUY954 induces activation of kinase cascades downstream of receptor activation as has been reported for S1P₁ and SEW02871, we measured the levels of phosphorylation of Erk and Akt following treatment of S1P₁ expressing CHO cells with AUY954. AUY954 increased Erk (tyrosine residue 204) and Akt phosphorylation (serine residue 473) levels with an EC₅₀ of approximately 0.1 and 1 nM, respectively (Figure 4). This EC₅₀ is in close agreement with the EC₅₀

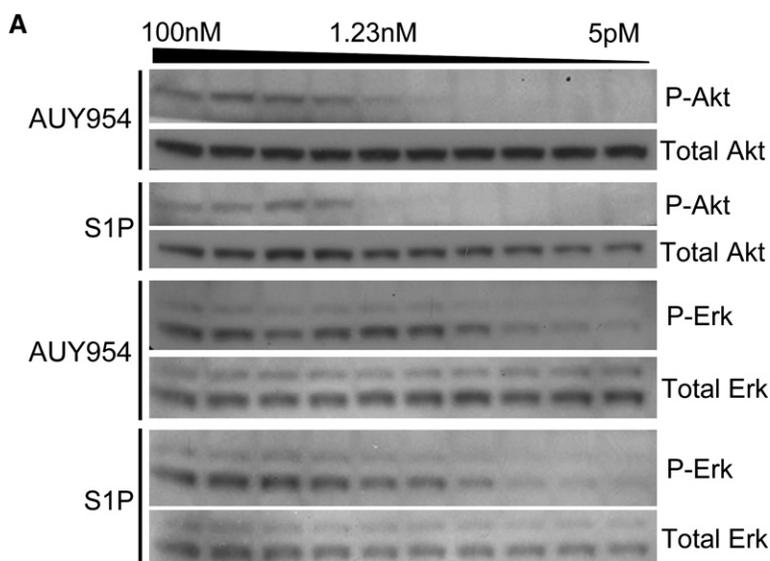


Figure 4. AUY954 Induces Activation of Erk and Akt Kinases in CHO Expressing S1P₁
(A) Western blot analysis of phospho-Akt, total Akt, phospho-Erk, and total Erk, following 5 min stimulation of CHO-S1P₁ cells with varying concentrations (3-fold dilution series starting at 100 nM) of AUY954 or S1P.
(B) EC₅₀ (mean ± SEM) of S1P and AUY954 for phospho-Akt and phospho-Erk as calculated from western blot analysis.

	AUY954		S1P	
	P-Akt	P-Erk2	P-Akt	P-Erk2
EC ₅₀ (nM)	1.43	0.09 ± 0.03	2.11	0.12 ± 0.02
% Efficacy	151	134 ± 1	100	100 ± 0

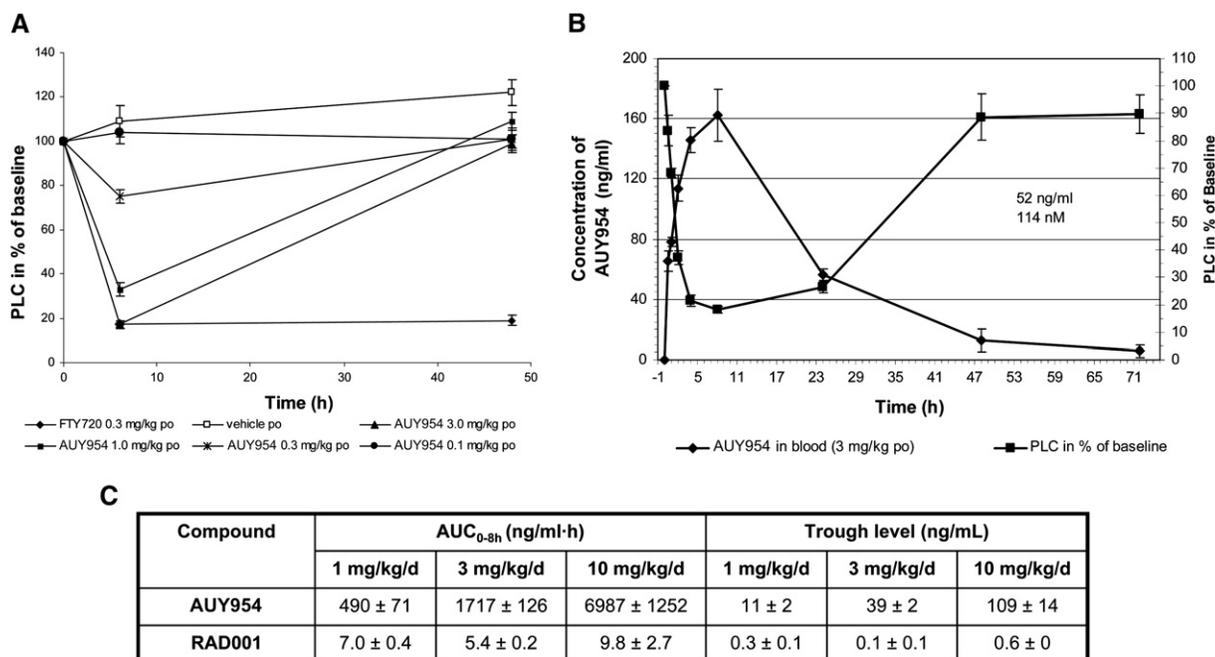


Figure 5. AUY954 Induced Potent and Reversible Peripheral Lymphocyte Depletion in Lewis Rats
(A) AUY954 was administered orally at 3, 1, 0.3, and 0.1 mg/kg to Lewis rats and dose-dependently depleted peripheral lymphocytes. Results are expressed as peripheral lymphocyte counts (PLC) as compared to baseline. The mean \pm SEM of five animals is shown.
(B) Profile of peripheral lymphocyte counts related to plasma concentrations of AUY954 after oral administration at 3 mg/kg.
(C) Comparison of AUC and trough level (mean \pm SEM) of AUY954 and RAD001 for different dosing groups.

for S1P₁ receptor activation measured by GTP γ S binding. No activation of Erk or Akt was observed when wild-type CHO cells were incubated with S1P and AUY954 (data not shown).

AUY954 Causes a Profound, Reversible, and Dose-Dependent Reduction of Circulating Lymphocytes Sustained by Blood Agonist Levels

Oral administration of AUY954 induces a rapid, potent, and dose-dependent decrease in the number of peripheral lymphocytes in Lewis rats (Figure 5). The effective dose required to achieve a 50% reduction (ED₅₀) in circulating lymphocytes is 0.7 mg/kg, which represents the time at which the nadir of circulating lymphocyte reduction is reached. Peripheral lymphocyte counts return to untreated levels 48 hr after compound treatment. The pharmacokinetic profile of AUY954 in Lewis rats and cynomolgus monkeys further reveals good oral bioavailability (33% and 74%, respectively) and short elimination half lives ($t_{1/2}$ of 5.0 and 11.6 hr, respectively) compared to FTY720 and its phosphate ($t_{1/2}$ of 21–28 hr and 33–52 hr, respectively). In order to investigate pharmacodynamics, drug levels and decrease of circulating lymphocytes were simultaneously assessed in Lewis rats following an oral dose of 3 mg/kg of AUY954. There is an inverse relationship between the number of circulating blood lymphocytes and blood concentration of AUY954 over a 72 hr time interval following oral administration (Figure 5B). The rapid onset and maintenance of circulating lymphocyte reduction was associated with a systemic exposure of agonist in the range of 100–300 nM, with a recovery of peripheral lymphocytes concomitant with the decline of blood

levels. Therefore, this pharmacodynamic effect of AUY954 appeared to be a response to S1P₁ agonism.

AUY954 Treatment, in Combination with Subtherapeutic Dose of RAD001, Significantly Prolongs Graft Survival of Heterotopic Cardiac Transplants

Sequestration of circulating mature lymphocytes into secondary lymphoid organs is thought to be the main mechanism underlying the immunomodulatory action of FTY720. In light of the high efficacy of FTY720 in the prevention of transplant rejection in several animal models and the comparable potency of AUY954 in inducing peripheral lymphocyte depletion in vivo, we conducted a study to investigate the ability of this S1P₁ selective agonist to prolong allograft survival. As previous studies have demonstrated that FTY720 synergizes effectively with inhibitors of T cell activation and proliferation [16, 27, 28], we examined whether treatment with AUY954 in combination with RAD001 would extend rat heart allografts by using the stringent DA to Lewis strain combination. A subtherapeutic dose of RAD001 of 0.3 mg/kg/d was coadministered with AUY954 at 1, 3, and 10 mg/kg/day for 26 days with 3 days of pretreatment before transplantation (Table 1). In placebo-treated rats, all cardiac allografts were rejected within 10 days, with a median graft survival time (MST) of 6 days, while a MST of 7 days was obtained by using RAD001 at 0.3 mg/kg/d alone. In contrast, at an oral dose of 3 and 10 mg/kg/day of AUY954 in combination with RAD001, all grafts reached the termination point of 26 days. However, signs of cellular infiltration were evident in all AUY954/RAD001 combination groups.

Table 1. Efficacy of AU954 in DA-to-Lewis Rat Heart Transplantation in Combination with RAD001

AUY954/Dose (mg/kg/day)	RAD001 Dose (mg/kg/day)	Individual Survival (days)	Survival MST (days)	C _{max} (nM)	Mean Cellular Rejection Score	Lymphocyte Count (units/ μ l)
0	0	6, 6, 6, 6, 6, 7, 10	6	—	4 \pm 0	11,173 \pm 716
0	0.3	7, 7, 7, 7, 11, 19	7	—	3.7 \pm 0.2	8,670 \pm 230
1	0.3	9, 15, 17, >28, >28	17	240 \pm 101	3.6 \pm 0.2	4,810 \pm 330
3	0.3	>26, >26, >26, >26, >26	>26	576 \pm 40	2.6 \pm 0.2	1,860 \pm 190
10	0.3	>26, >26, >26, >26, >26	>26	2,295 \pm 171	2.2 \pm 0.2	1,460 \pm 100

Histology of heart allografts at the termination point of 9–26 days showed moderate to severe acute cellular rejection dependent on the doses of AU954. These results were comparable to FTY720 in the same model (data not shown).

Measurement of the number of peripheral blood lymphocytes 8 hours after the last dosing reveals reduction percentages of 43.0, 16.6, and 13.1 for 1, 3, and 10 mg/kg/day dosing groups, respectively. Analysis of the blood levels of AU954 demonstrates a linear dose-dependent exposure as assessed by C_{max}, C_{last}, and AUC (Figure 5). The blood exposure of RAD001 remained unaltered regardless of the AU954 dose, which suggests that AU954 does not alter the metabolism of RAD001.

In our rat transplantation model, which strongly induces acute rejection within 6 days, AU954 in synergy with RAD001 effectively prolonged the survival of cardiac allografts. These data prove that graft protection can be achieved by specifically targeting the S1P₁ GPCR receptor in vivo and further define an essential requirement for this specific receptor in mediating useful immune suppression in transplantation.

Significance

The functional roles of the S1P receptor subtypes in different tissues remains an area of active research. Growing evidence, especially from the use of the promising immunomodulatory drug FTY720, supports a role for S1P receptors in regulating lymphocyte recirculation and trafficking. Recent reports using selective agonists implicate S1P₁ as the crucial target for agonist-induced peripheral lymphocyte reduction, while they suggest a role for S1P₃ in the regulation of heart rate in rodents [21, 29]. Studies using hematopoietic cells genetically deficient in S1P₁ further confirmed that S1P₁ is required for lymphocytes to egress from thymus and lymphoid organs and suggested that agonist-induced S1P₁ receptor desensitization (functional antagonism) could account for FTY720-induced lymphocyte sequestration [19, 30, 31]. Pharmacological probes with specificity for S1P₁ will be useful in elucidating the function of this receptor in a variety of biological settings. Here, we report the discovery of such a tool, a monoselective S1P₁ agonist (AU954), which has desirable pharmacokinetic properties and demonstrates comparable activity to FTY720 in mediating peripheral lymphocyte depletion. Furthermore, the discovery of AU954 as a monoselective S1P₁ agonist has allowed pharmacological validation of the S1P₁ receptor as a target to prevent allograft rejection in a stringent rat heart transplantation model.

Experimental Procedures

Synthesis of AU954

Boronic Acid 2

To a solution of 5-methylbenzo[*b*]thiophene (6.12 g, 41.3 mmol) in THF (138 ml) at -78°C was added LDA (41.3 ml of a 2 M solution, 82.6 mmol), followed by the addition of trimethyl borate (9.26 ml, 82.6 mmol) in neat. The mixture was allowed to warm up to room temperature overnight. The mixture was cooled to 0°C and treated with 2 N HCl until pH was about 1. After stirring for 1 hr, the mixture was extracted with EtOAc, and the combined organic solution was dried (Na₂SO₄) and concentrated. The crude was washed with hexanes to remove trace amount of unreacted 5-methylbenzo[*b*]thiophene. The crude product, 5-methylbenzo[*b*]thiophenylboric acid (7.01 g, 88%), was used without further purification. ¹H NMR (600 MHz, CD₃OD) δ 7.78 (s, 1 H), 7.75 (d, *J* = 8.2 Hz, 1 H), 7.66 (s, 1 H), 7.21 (d, *J* = 8.0 Hz, 1 H), 2.45 (s, 3 H).

Aniline 4

To a solution of 4-bromo-3-(trifluoromethyl)aniline (91 g, 379 mmol) in THF (750 ml) were added phenylboric acid (49.23 g, 404 mmol), Pd(OAc)₂ (1.7 g, 7.6 mmol), (dicyclohexylphosphino)biphenyl (5.44 g, 15.2 mmol), and KF (66.09 g, 1138 mmol). The resulting mixture was heated at reflux under nitrogen overnight. The mixture was filtered through Celite, which was washed with EtOAc. The filtrate was then concentrated and the residue was purified by column chromatography (EtOAc:hexanes = 1:4) to afford 63 g (70%) of aniline 4 as oil. LC-MS *m/z* 238.1 (M+H⁺).

Bromide 5

To a solution of aniline 4 (63 g, 266 mmol) in CH₃CN (850 ml) at 0°C was added CuBr₂ (62 g, 278 mmol) and then *t*-BuONO (33 ml, 277 mmol) slowly. The resulting mixture was allowed to warm to room temperature and continued to stir until the completion of reaction (by TLC). The mixture was diluted with EtOAc (1.5 liter) and washed with brine (500 ml \times 3). The organic layer was separated, dried over Na₂SO₄, and concentrated. The crude was purified by column chromatography (100% hexanes) to yield 52 g (65%) of bromide 5 as colorless oil. ¹H NMR (600 MHz, CDCl₃) δ 7.88 (d, 1 H), 7.69 (d, 1 H), 7.44–7.38 (m, 3 H), 7.32–7.28 (m, 2 H), 7.21 (d, 1 H); GC-MS *m/z* 302 (M+2), 300 (M).

Benzothiophene 6

Pd(PPh₃)₄ (803 mg, 0.70 mmol) was added to a solution of boric acid 2 (7.01 g, 36.5 mmol) and bromide 5 (10.46 g, 34.76 mmol) in toluene (160 ml) and ethanol (40 ml), followed by addition of a solution of Na₂CO₃ (14.74 g, 139 mmol) in water (160 ml). The mixture was stirred at 80°C for 6 hr until the completion of reaction (by GC-MS). The reaction mixture was cooled to room temperature and the top organic layer was separated. The aqueous phase was extracted with 10% EtOAc/hexane. The combined organic solution was dried over MgSO₄ and filtered through Celite, which was washed with EtOAc. After concentration, the crude product was purified by recrystallization with hexane (\sim 50 ml) to give 9.34 g (73%) of compound 6 as light brown solid. ¹H NMR (600 MHz, CDCl₃) δ 8.06 (s, 1 H), 7.87 (d, 1 H), 7.74 (d, 1 H), 7.61 (s, 1 H), 7.58 (s, 1 H), 7.45–7.35 (m, 6 H), 7.20 (d, 1 H), 2.49 (s, 3 H); GC-MS *m/z* 368 (M).

Benzyl Bromide 7

To a solution of compound 6 (4.88 g, 13.25 mmol) in carbon tetrachloride (130 ml) was added *N*-bromosuccinimide (2.47 g, 13.9 mmol) and 2,2'-azobisisobutyronitrile (AIBN, 435 mg, 2.65 mmol). The mixture was stirred at 95°C for 10 hr and then cooled to room temperature. The solution was passed through a pad of silica gel, which was further rinsed with EtOAc-hexanes (1:9). The

combined organic solution was concentrated to afford 5.7 g (96%, crude) of compound 7, which was used without further purification.

***t*-Butyl Ester 8**

Sodium hydride (1.27 g, 60% dispersion in mineral oil, 31.7 mmol) was added to a solution of β -alanine *t*-butyl ester hydrochloride (3.46 g, 19.1 mmol) in DMF (20 ml) at 0°C. After stirring at room temperature for 10 min, a solution of crude compound 7 (5.7 g, 12.7 mmol) in DMF (25 ml) was added slowly, and the mixture was stirred at room temperature overnight. The reaction was quenched with water (45 ml). The mixture was extracted with EtOAc-hexanes (1:1). The combined organic solution was washed with water, brine, and dried over Na₂SO₄. After concentration, the residue was purified with column chromatography (gradient, 10%–60% EtOAc/hexanes) to yield 4.20 g (62% over 2 steps) of compound 8 as viscous oil. ¹H NMR (400 MHz, CDCl₃) δ 8.05 (s, 1 H), 7.90–7.75 (m, 3 H), 7.61 (s, 1 H), 7.45–7.30 (m, 7 H), 5.08 (br s, 2 H), 3.97 (s, 2 H), 2.94 (t, 2 H), 2.54 (t, 2 H), 1.45 (s, 9 H); LC/MS *m/z* 512.4 (M+H⁺).

AUY954

To a solution of ester 8 (4.20 g, 8.21 mmol) in methylene chloride (30 ml) was added trifluoroacetic acid (15 ml), and the mixture was stirred at room temperature until the completion of reaction (by LC-MS). The mixture was concentrated, and the resulting solid was fully dissolved in hot EtOAc (~45 ml) followed by addition of hexanes (~3 ml). The solution was cooled to room temperature and solid was formed. After filtration, the solid was redissolved in a hot mixture of CH₃CN (30 ml) and 2 N HCl (15 ml). The solution was concentrated to dryness to afford 2.7 g (66%) of AUY954 as HCl salt. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.20 (br s, 2 H), 8.20 (s, 2 H), 8.13 (s, 1 H), 8.11 (s, 1 H), 8.04 (s, 1 H), 7.59–7.52 (m, 2 H), 7.50–7.43 (m, 3 H), 7.40–7.34 (m, 2 H), 4.31 (s, 2 H), 3.15 (t, 2 H), 2.73 (t, 2 H); ¹⁹F NMR (400 MHz, DMSO-*d*₆) δ -55.48; MS *m/z* 456.1 (M+H⁺).

S1P₁ Homology Model and Docking Studies

The S1P₁ model was developed by homology modeling in the MOE program suite 2003.02 (Chemical Computing Group, Montreal, Canada). The template used in homology modeling was the bovine rhodopsin crystal structure (PDB code 1F88) [32], and the sequence alignment between S1P₁ and Rhodopsin was taken from the papers published by A. Parrill et al. [33, 34]. The extracellular β sheet 4 (β 4) that forms the top of the retinal binding site in the rhodopsin crystal structure was found to block the docking of S1P into the transmembrane region. Therefore, residues on the β 4 sheet from Val173 to His195 of rhodopsin were deleted from the homology template, leaving the corresponding residues Met180 to Leu195 on S1P₁ as a gap and modeled without a template.

Docking was performed with GOLD v1.2 (Cambridge Crystallographic Data Center) [35]. All atom types and charges were assigned in GOLD. The protein was fixed while the ligands were flexible in the docking. All parameters were taken from the standard default settings. One-hundred thousand independent genetic algorithm (GA) runs were performed for each compound. The radius of the search was set to 15 Å.

Receptors and Cell Lines

Stable cell lines expressing human S1P receptors were prepared by transfecting CHO cells with pcDNA3.1 (Invitrogen Corporation) expression vectors encoding N-terminally myc-tag hS1P₁ and S1P₃ (cDNAs were a gift from J.P. Hobson [36]), with pRc/CMV encoding S1P₄ [36] and with pcDNA 3.1 Topo V (Invitrogen Corporation) in which PCR-amplified (sequence verified) mS1P₁, S1P₂, and S1P₅ were cloned. Stable clones were selected in the presence of 0.5 mg/ml G418 (Invitrogen Corporation) and evaluated for high expression of functional receptors.

GTP γ -³⁵S-Binding Assay

The cells expressing S1P receptors were harvested in 20 ml cold 10 mM HEPES (pH 7.5), 0.1% fatty-acid-free bovine serum albumin (BSA), and protease inhibitors cocktail (1/50 complete, Roche Applied Science), centrifuged at 750 \times g for 10 min at 4°C, and resuspended in 10 ml 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.1% BSA, and protease inhibitors cocktail. The cell suspension was homogenized on ice with a Polytron homogenizer at 25,000 rpm, centrifuged at 26,900 \times g for 30 min at 4°C, and resuspended in 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM

EDTA, and 0.1% fat-free BSA at 2–3 mg protein/ml. To characterize the GTP γ -³⁵S binding, membrane proteins were resuspended at 75 μ g/ml in 50 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 20 μ g/ml saponin, and 0.1% fat-free BSA (pH 7.4) mixed with 5 mg/ml with WGA-coated SPA-bead (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), 10 μ M GDP, and different concentrations of agonists and incubated for 10–15 min at RT. The GTP γ -³⁵S-binding reaction was started by the addition of 200 pM GTP γ -³⁵S (Amersham, >1000 Ci/mmol). After 120 min at room temperature, the plates were centrifuged for 10 min at 2000 rpm and counted with a TopCount instrument (Packard Instruments).

Analysis of Erk and Akt Activation

hS1P₁-CHO cells were plated in 6-well dishes at 300,000 cells per well in normal medium. Cells were serum starved overnight before stimulation and then cultivated in fresh serum-free medium. Cells were stimulated at 37°C for 5 min with various dilutions of AUY954 or S1P. Cells were harvested by using CHAPS lysis buffer (50 mM Tris-HCl [pH 8.0], 125 mM NaCl, 20 mM CHAPS, 2 mM DTT, 1 mM EDTA, 2 mM NaVO₄, 10 mM NaF, 1 mM PMSF, 1 \times Complete EDTA-free protease inhibitor cocktail [Roche; 300 ul/well]). Cell lysates were vortexed for 10 s and spun at 15,000 \times g for 5 min at 4°C. Resulting supernatants were then subjected to standard SDS-PAGE and western blotting analysis. The following primary antibodies were used: monoclonal anti-phospho Erk antibody (Santa Cruz Biotechnology, catalog number sc-7383), polyclonal anti-phospho Akt antibody (Cell Signaling catalog number 4051), polyclonal anti-Erk antibody (Santa Cruz Biotechnology), and polyclonal anti-Akt antibody (BD Pharmingen). Anti-mouse IgG HRP-conjugated and anti-rabbit IgG HRP-conjugated (Promega) were used as secondary antibodies. ECL Plus Western Blotting Detection Kit (Amersham) was used for signal detection. The film was exposed between 3 s to 1 min for optimal resolution of the specific bands.

Peripheral Blood Lymphocyte Depletion Assay

Lewis rats were subjected to a single dose of either vehicle (control) or AUY954 via oral administration. Sublingual blood for hematological monitoring was obtained before AUY954 administration (baseline) and 6 and 48 hr after drug application.

Rats were anaesthetized with isoflurane 5% v/v (Forene, Abbott, Baar, CH). Whole blood was sampled from the sublingual vein in EDTA-coated Eppendorf tubes and subjected to hematology analysis. Absolute and differential leukocyte counts, including lymphocyte counts were analyzed with the Technicon H1-E analyzer (Bayer Diagnostics, Zürich, CH).

Pharmacokinetic Analysis

The whole blood sample from Lewis rats treated with compound AUY954 was taken sublingually in EDTA tubes and was stored at -80°C. One hundred microliters of each blood sample was transferred to 2.0 ml Eppendorf tube. Fifty microliters of internal standard solution at a concentration of 0.2 μ g/ml was added to each sample. After mixing with vortex for 10 s, 50 μ l of a boric acid/borax buffer (pH 9.0) and 750 μ l of ethyl acetate were added. After vortexing for 10 s, samples were ultrasonicated for 5 min and then rotated for 10 min on Rotator Drive. The two liquid layers were separated by a short centrifugation step. A 650 μ l aliquot of upper organic layer was transferred into 1.5 ml Eppendorf tube. The organic supernatant was evaporated to dryness. The residue was dissolved in 35 μ l 50% methanol by ultrasonication and vortexing for 5 min. Then 35 μ l of 0.4% formic acid was added. After a further step of ultrasonication and vortexing for 5 min, the residual particles were separated by centrifugation for 5 min at 16,100 \times g at room temperature. Sixty microliters of each sample was transferred to microvial and stored at 12°C prior to analysis. Ten microliters of the above prepared sample was directly injected onto a Zorbax SB-C18 (0.5 mm \times 75 mm, 3.5 μ m) analytical column, and target analyte (AUY954) was eluted by a gradient method with a mobile phase consisting of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The gradient started at 95% A for the first 4 min, changed to 80% A at 5 min, further changed to 5% A at 14 min and kept constant to 16 min, and reverted to 95% A at 16.1 min to re-equilibrate the column for 4 min. The flow rate was 20 μ l/min. The retention time of AUY954 was 11.6 min. The HPLC system was interfaced to

a Micromass Micro triple quadrupole mass spectrometer with an electrospray source. AUY954 was monitored by multiple-reaction monitoring (MRM). The limit of quantitation (LOQ) of AUY954 was 1 ng/ml, and the linear dynamic range was 1–10,000 ng/ml.

Heterotopic Vascular Heart Transplantation in DA to Lewis Rats
Inbred male DA (RT1^a) and Lewis (RT1^b) rats obtained from Harlan, Zeist, Netherlands were used as mismatched donors and recipients, respectively. Donor heart grafts were transplanted onto the recipient abdominal aorta and inferior vena cava by standard microsurgical techniques [37]. Either 0.3 mg/kg/day of RAD001 or different doses (1, 3, or 10 mg/kg/day) of AUY954, in combination with 0.3 mg/kg/day of RAD001 was given to the recipients by gavage. Treatment started 3 days before transplantation and lasted for 26 days after transplantation. Graft survival was monitored daily by palpation for the ventricular contraction. Hearts possessing a complete cessation of ventricular motion were considered rejected. At autopsy, heart allografts were harvested for histopathological evaluation. The degree of acute cardiac rejection was scored with a scale of zero to four: 0, no rejection; 1, slight; 2, moderate; 3, marked; 4, severe.

Five groups were studied as follows: group 1 (n = 7) received pyrogen free water as placebo; group 2 (n = 6) received RAD001 (0.3 mg/kg/d); group 3 (n = 5) received AUY954 (1 mg/kg/d) and RAD001 (0.3 mg/kg/d); group 3 (n = 5) received AUY954 (3 mg/kg/d) and RAD001 (0.3 mg/kg/d); and group 3 (n = 5) received AUY954 (10 mg/kg/d) and RAD001 (0.3 mg/kg/d).

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