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# Optimization of a Potent, Orally Active $S1P_1$ Agonist Containing a Quinolinone Core

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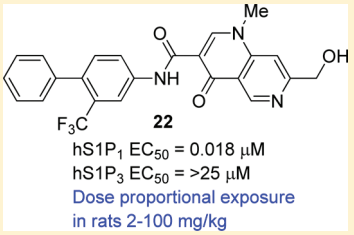
Supporting Information

**ABSTRACT:** The optimization of a series of $S1P_1$ agonists with limited activity against $S1P_3$ is reported. A polar headgroup was used to improve the physicochemical and pharmacokinetic parameters of lead quinolinone **6**. When dosed orally at 1 and 3 mg/kg, the azahydroxymethyl analogue **22** achieved statistically significant lowering of circulating blood lymphocytes 24 h postdose. In rats, a dose-proportional increase in exposure was measured when **22** was dosed orally at 2 and 100 mg/kg.




**KEYWORDS:** *Sphingosine-1-phosphate receptor, $S1P_1$, agonist, multiple sclerosis*

The lysophospholipid sphingosine-1-phosphate **1** has been implicated in an array of cellular signaling pathways such as apoptosis, proliferation, migration, and differentiation (Figure 1).[1] S1P signaling is mediated by five G-protein coupled receptor (GPCR) subtypes known as $S1P_{1-5}$.[2] The role of $S1P_1$ in lymphocyte trafficking in the lymph system, where it is highly expressed, has been the focus of intense research.[3] These efforts were aided by the disclosure that **2** (FTY720P), which is derived from **3** (FTY720) in vivo by selective phosphorylation by sphingosine kinase, is a non-selective $S1P_1$ agonist.[4,5] In addition, FTY720P is also an agonist of $S1P_{3-5}$. Agonists[6,7] such as **2**, **4** (AMG 369),[8] and **5**[9] bind to the $S1P_1$ receptor, resulting in receptor internalization, which prevents lymphocyte egress from lymphoid organs.[10,11] The sequestration of lymphocytes in the lymphoid organ and resulting decrease in circulating lymphocytes has been postulated to explain the efficacy of $S1P_1$ agonists in preclinical models of autoimmune diseases including multiple sclerosis (MS),[12,13] where these cells participate in the pathogenesis of the disease.[14]

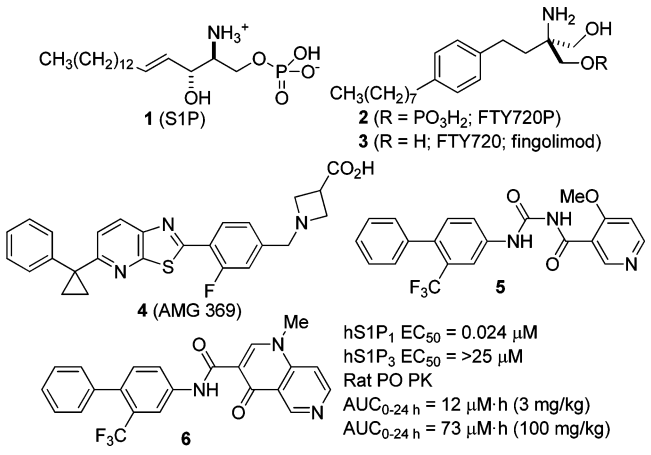



**Figure 1.** Agonists of $S1P_1$.

Until recently, agonism of $S1P_3$ by **2** was believed to be the exclusive cause of the transient dose-dependent bradycardia that was observed in clinical trials of FTY720.[15] This conclusion was supported by rodent data wherein dosing of **2** to normal mice resulted in bradycardia, whereas administration of **2** to $S1P_3$ knockout mice caused no change in heart rate.[16,17] More recent literature[18,19] suggests that $S1P_1$ rather than $S1P_3$ is responsible for the observed heart rate changes in humans. Despite this side effect, FTY720 was approved by the United States Food and Drug Administration for the treatment of relapsing forms of MS.[20,21] Herein, we report the discovery of a $S1P_1$ agonist with minimal $S1P_3$ activity and excellent pharmacokinetic parameters.

In prior work,[22] we reported a series of conformationally restricted $S1P_1$ agonists derived from **5**.[9] Quinolinone **6** demonstrated excellent potency in an assay that measured receptor internalization (RI) of $hS1P_1$-GFP fusion protein in U2OS cells, in addition to limited $hS1P_3$ activity as determined

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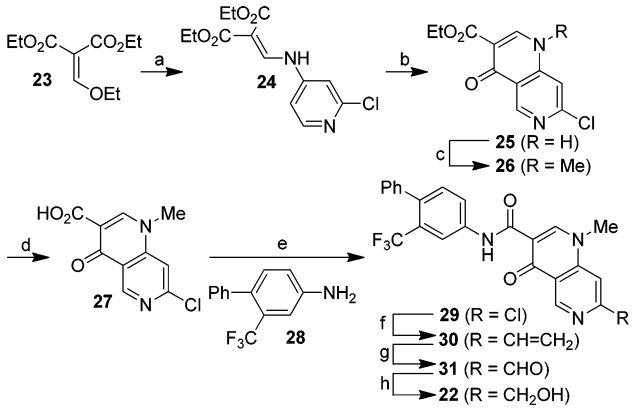
by $Ca^{2+}$ mobilization in $hS1P_3/G_{q/i5}$-transfected Chinese hamster ovary (CHO)-K1 cells. In vivo, **6** lowered circulating lymphocytes in rats after a single oral dose of 1 mg/kg when measured at 24 h (vide infra). However, when dosed orally in rats at 100 mg/kg, the exposure of **6** failed to increase proportionally relative to the 3 mg/kg dose despite efforts to optimize the formulation (Figure 1). The lack of dose-proportional exposure limited further development of **6**. Furthermore, **6** suffered from poor solubility (5 $\mu$g/mL) in simulated intestinal fluid (SIF) and relatively high lipophilicity (cLog $P$ = 3.5).[23] Efforts aimed at introducing additional nitrogen atoms to the carbon skeleton were only moderately successful. Thus, more significant structural modifications of **6** were directed at decreasing the cLog $P$ and improving the aqueous solubility while maintaining or improving the pharmacological profile.

Many of the $S1P_1$ agonists that have been disclosed contain a polar headgroup (Figure 1).[6,7] This group is usually comprised of an amino acid, amino alcohol, or a diol that is attached to a lipophilic carbon skeleton. Quinolinone **6** lacked a polar headgroup, which could provide a handle to modulate the physicochemical properties without detrimentally affecting the pharmacological profile. To investigate whether attachment of a polar moiety to **6** would result in improved properties, a hydroxymethyl group was attached at several positions about a fused bicyclic core of the scaffold. Compounds derived from the carbon analogue **7** were initially targeted to simplify the challenge of synthesizing azaquinolinones containing a polar headgroup. Gratifyingly, as shown in Table 1, alcohols **8** and **9** demonstrated that a hydroxymethyl group could be incorporated into the carbon skeleton at either the 8- or the 7-position, respectively, without compromising potency (**8**, $EC_{50}$ = 0.039 $\mu$M; **9**, $EC_{50}$ = 0.018 $\mu$M vs **7**, $EC_{50}$ = 0.070 $\mu$M). However, the 6-substituted analogue **10** was substantially less potent than **7**. Additional efforts aimed at improving $S1P_1$ potency by incorporating different groups (**11**, **12**, and **13**) at the 8-position resulted in diminished activity relative to **8**. Furthermore, the significant activity of **12** and **13** in our $S1P_3$ assay suggested that maintaining minimal activity against $S1P_3$ may be a challenge with 8-substituted analogues. Therefore, we focused on optimizing the polar headgroup at the 7-position.

The chain length of the primary alcohol was extended by two (**14**, $EC_{50}$ = 0.008 $\mu$M) and three (**15**, $EC_{50}$ = 0.015 $\mu$M) carbon atoms without significantly impacting $S1P_1$ potency or $S1P_3$ activity. Carboxylic acids were also examined at the 7-position. The propionic acid analogue **18** provided excellent $S1P_1$ potency ($EC_{50}$ = 0.004 $\mu$M) and limited activity against $S1P_3$ ($EC_{50}$ = 0.94 $\mu$M). Two amino acids (**19** and **20**) were also examined. Although **19** had only moderate $S1P_1$ activity ($EC_{50}$ = 0.15 $\mu$M), incorporating the azetidine carboxylic acid found in AMG 369 led to an analogue, **20** ($EC_{50}$ = 0.025 $\mu$M), with little difference in potency relative to **7** ($EC_{50}$ = 0.070 $\mu$M). With several polar head groups identified, the corresponding 6-aza analogs of two analogues (**18** and **9**) that provided an acceptable combination of potency, limited activity against $S1P_3$, solubility, and structural diversity were prepared with the expectation that the combination would provide improved physicochemical properties. Analogue **22** was equipotent ($EC_{50}$ = 0.018 $\mu$M) with **9**, while maintaining limited activity against $S1P_3$ ($EC_{50}$ >25 $\mu$M). However, **21** had diminished potency relative to the parent compound **18** in addition to increased potency against $S1P_3$ ($EC_{50}$ = 1.3 $\mu$M). This result was somewhat unexpected since the 6-aza analogue **6** ($EC_{50}$ = 0.024 $\mu$M) was slightly more potent than the parent **7** ($EC_{50}$ = 0.070 $\mu$M) and **22** was equipotent with **9**.

Alcohol **22** was prepared as described in Scheme 1. Condensation of **23** with 2-chloropyridin-4-amine provided

**Scheme 1. Synthesis of 22**[a]




[a]Reagents and conditions: (a) 2-Chloropyridin-4-amine, 120 °C. (b) $Ph_2O$, 220 °C. (c) $K_2CO_3$, MeI, 7% yield (3 steps). (d) KOH, 89% yield. (e) Compound **28**, HBTU, $Et_3N$, 92% yield. (f) Tributyl(vinyl)stannane, Pd[P(*tert*-Bu)$_3$]$_2$, CsF, 80 °C, 76% yield. (g) $OsO_4$, $NaIO_4$, 97% yield. (h) LiAlH(O*tert*-Bu)$_3$, 68% yield.

**24**, which was cyclized to **25**. Methylation of **25** gave **26** in 7% yield over three steps from **23**. Ester **26** was hydrolyzed to **27** and coupled with **28**[9] to provide **29**. The hydroxymethyl was introduced using a three step sequence.

With several polar head groups identified, the most potent and $S1P_3$ sparing analogues were further profiled. As anticipated, the addition of the polar headgroup increased the solubility in SIF of most of the analogs shown in Table 2 relative to **6** and **7**. None of the compounds distinguished themselves in terms of cell permeability, and only one, **16**, was an efflux substrate in the parental porcine proximal tubule cell line (LLC-PK1). All of the compounds in Table 2 exhibited excellent stability in rat liver microsomes ($CL_{int}$ < 14 $\mu$L/min/mg). The rat plasma protein binding was similar (91.7−92.7% bound) for the analogues containing a polar headgroup derived from an alcohol (**9**, **14**, and **22**), whereas acids **16** (98.0% bound) and **18** (97.8% bound) were more extensively protein-bound. All five analogues that contained a polar headgroup had acceptable characteristics for advancement into an in vivo assay that measures circulating lymphocytes.

Compounds were dosed orally at 1 mg/kg in female Lewis rats, and after 24 h, blood lymphocyte counts and plasma concentration of total drug were measured. The 24 h time point was chosen to minimize the impact that dosing has on lymphocyte counts. Both **16** and **18** had low plasma concentrations 24 h postdose and did not lower blood lymphocytes at 24 h. However, **9**, **14**, and **22** achieved statistically significant lowering of blood lymphocytes. Of the compounds in Table 2, **22** appeared the most promising with high lymphocyte reduction and excellent SIF solubility. Therefore, **22** was investigated in a dose−response experiment. When dosed orally, **22** achieved statistically significant ($P$ < 0.05) reduction of circulating blood lymphocytes after 24 h when dosed at 1 and 3 mg/kg (Figure 2). The 3 mg/kg dose corresponds to a 88% reduction in lymphocytes relative to vehicle at 24 h.


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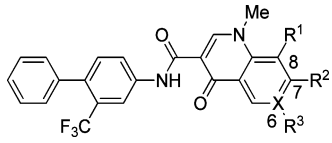
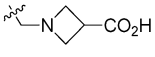
**Table 1. SAR of Analogues Containing a Polar Head Group**[a]



| Compound | $R^1$ | $R^2$ | $R^3$ | X | cLog P | hS1P$_1$ RI $EC_{50}$, μM (% efficacy) | hS1P$_3$ $Ca^{2+}$ $EC_{50}$, μM (% efficacy) |
|---|---|---|---|---|---|---|---|
| **6** | H | H | – | N | 3.5 | 0.024 (103) | >25 |
| **7** | H | H | H | C | 4.8 | 0.070 (105) | 1.5 (92) |
| **8** | $CH_2OH$ | H | H | C | 3.7 | 0.039 (104) | >25 |
| **9** | H | $CH_2OH$ | H | C | 3.7 | 0.018 (125) | >25 |
| **10** | H | H | $CH_2OH$ | C | 3.7 | 0.70 (46) | >25 |
| **11** | $(CH_2)_2OH$ | H | H | C | 4.0 | 0.39 (106) | >25 |
| **12** | $CH_2NH_2$ | H | H | C | 3.7 | 0.081 (118) | 5.6 (48) |
| **13** | $CO_2H$ | H | H | C | 4.6 | 2.0 (103) | 1.1 (11) |
| **14** | H | $(CH_2)_2OH$ | H | C | 4.0 | 0.008 (118) | >25 |
| **15** | H | $(CH_2)_3OH$ | H | C | 4.3 | 0.015 (118) | >25 |
| **16** | H | $CO_2H$ | H | C | 4.6 | 0.021 (98) | >25 |
| **17** | H | $CH_2CO_2H$ | H | C | 4.0 | 0.18 (138) | 1.6 (28) |
| **18** | H | $(CH_2)_2CO_2H$ | H | C | 4.5 | 0.004 (85) | 0.94 (82) |
| **19** | H | $NH(CH_2)_2CO_2H$ | H | C | 4.3 | 0.15 (81) | >25 |
| **20** | H |  | H | C | 3.0 | 0.025 (108) | >25 |
| **21** | H | $(CH_2)_2CO_2H$ | – | N | 3.3 | 0.038 (88) | 1.3 (49) |
| **22** | H | $CH_2OH$ | – | N | 2.9 | 0.018 (106) | >25 |

[a]Data represent an average of at least two determinations. The percent efficacy is reported relative to 1.00 μM 1-(4-(6-benzylbenzofuran-2-yl)-3-fluorobenzyl)azetidine-3-carboxylic acid (see ref 8) and 0.200 μM S1P for hS1P$_1$ RI and hS1P$_3$ $Ca^{2+}$ assays, respectively; > [highest concentration tested] is reported for compounds that do not achieve >10% of control activity.

**Table 2. Solubility, Permeability, Protein Binding, and in Vivo Characterization of Select Compounds**

| compd | SIF solubility (mg/mL) | $P_{app}$ (LLC-PK1) × $10^{-6}$ cm/s[a] | LLC-PK1 parental (ER)[a] | rat plasma protein binding (%) | PLC POC[b] 1 mg/kg, 24 h (%) | $C_{plasma}$ at 24 h (ng/mL) |
|---|---|---|---|---|---|---|
| **6** | 0.005 | 5.7 | 0.8 | 91.7 | −64[c] | 350 |
| **9** | 0.027 | 2.8 | 1.0 | 92.4 | −44[c] | 65 |
| **14** | 0.006 | 2.2 | 1.0 | 92.7 | −48[c] | 27 |
| **16** | 0.048 | 3.0 | 6.7 | 98.0 | +4 | BQL[d] |
| **18** | 0.20 | 1.6 | 1.0 | 97.8 | −25 | 4 |
| **22** | >0.20 | 2.5 | 1.0 | 91.7 | −60[c] | 84 |

[a]Apparent permeability ($P_{app}$) through porcine proximal tubule cells (LLC-PK1 cell line) and efflux ratio (ER). [b]Percent-of-control (POC) reduction vs vehicle in peripheral lymphocyte count (PLC) 24 h after a single oral dose (1 mg/kg; vehicle = 20% captisol, 1% HPMC, and 1% pluronic F68, pH 2.1, with MSA) administered to female Lewis rats ($N$ = 5/group). [c]The measured POC reduction in PLC is statistically significant ($P$ < 0.05 vs vehicle by ANOVA/Dunnett's multiple comparison test). [d]BQL, below the quantifiable limit.

Although **22** achieved dose-proportional exposure over a narrow range of 0.3−3 mg/kg, the exposure at higher doses was critical for advancement of **22**. The exposure of **22** in rats was measured after single oral doses of 2 and 100 mg/kg (Table 3). Its oral absorption was complete, with % $F$ of 132 at 2 mg/kg.[24]

**Table 3. Preclinical Pharmacokinetic Parameters for 22**

| species | IV/oral dose (mg/kg) | CL (L/h/kg) | Vss, iv (L/kg) | MRT, iv (h) | oral $AUC_{0\text{-}inf}$ (μM·h) | $F$ (%) |
|---|---|---|---|---|---|---|
| mouse[a] | 1/3 | 0.31 | 6.0 | 20 | 16 | 75 |
| rat[b] | 1/2 | 0.31 | 5.8 | 19 | 19 | 132 |
| rat[b,c] | −/100 | – | – | – | 1630 | >93 |
| NHP[d] | 4/10 | 0.11 | 4.3 | 40 | 141 | 67 |

[a]Studies were conducted in female C57BL/6 mice; iv doses were administered in 40% hydroxypropyl β-cyclodextrin/60% water; oral doses in 1% HPMC/1% pluronic F68/20% HPBCD/78% water, MSA, pH 2. [b]Studies were conducted in male Sprague−Dawley rats; iv doses were administered in DMSO; oral doses in 20% Captisol/1% Tween 80, pH 2, MSA. [c]Oral data only. [d]PK studies were conducted in male nonhuman primates (NHP); iv doses were administered in 40% hydroxypropyl β-cyclodextrin/60% water, MSA, pH 3; oral doses in 1% HPMC/1% pluronic F68/20% HPBCD/78% water, MSA, pH 2.

The area under the plasma concentration−time curve ($AUC_{0\text{-}inf}$) increased from 19 to 1630 μM·h for the 2 and 100 mg/kg doses, respectively, thereby confirming that the exposure was dose porportional in rats up to at least 100 mg/kg. In addition, the clearance (CL = 0.31 L/h/kg) and long mean residence time (19 h) were favorable. In nonhuman primates, **22** displayed excellent pharmacokinetic parameters with low clearance (CL = 0.11 L/h/kg), long mean residence time (40 h), and good oral bioavailability ($F$ = 67%).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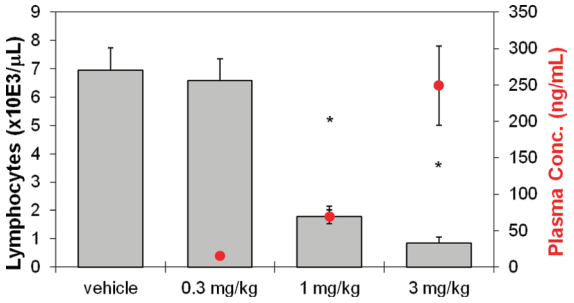


**Figure 2.** After a single oral dose, **22** reduced blood lymphocyte counts in female Lewis rats 24 h postdose ($N$ = 3/group; bars represent average blood lymphocyte counts ± SE; circles represent average plasma concentration ± SE; *$P$ < 0.05 vs vehicle by ANOVA/Dunnett's multiple comparsion test). Compound was administered in 20% captisol/1% HPMC/1% pluronic F68, pH 2, with MSA.

In summary, a polar headgroup was used to improve the physicochemical and pharmacokinetic parameters of a series of $S1P_1$ agonists without detrimentally affecting the pharmacological profile. Three positions of the quinolinone scaffold were examined for appending the polar headgroup. The 7-position was found to be optimal for $S1P_1$ agonist activity with limited $S1P_3$ activity. Analogues that contained a hydroxyl-based polar headgroup improved the solubility in SIF relative to **6** and lowered circulating blood lymphocytes in vivo. Analogue **22** lowered circulating lymphocytes when measured at 24 h after oral doses of 1 and 3 mg/kg. In rats, **22** achieved a dose-proportional increase in exposure when dosed at 2 and 100 mg/kg, thus providing a significant advantage over **6**. The favorable in vitro and in vivo profile of **22** supported selection of this molecule for further preclinical development.

## ■ ASSOCIATED CONTENT

### Supporting Information

Statistical analysis for in vitro data in Table 1, experimental procedures and characterization data for **8**–**22**, PK data for **22**, and experimental details for in vitro and in vivo assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

AUC, area under the plasma concentration–time curve; CHO, Chinese hamster ovary; CL, clearance; $CL_{int}$, intrinsic clearance; cLog $P$, calculated logarithm of octanol/water partition coefficient; $C_{plasma}$, total compound concentration in plasma; DMSO, dimethyl sulfoxide; $EC_{50}$, molar concentration that produces half maximal response; $F$, bioavailability; HBTU, $O$-(benzotriazol-1-yl)-$N$,$N$,$N'$,$N'$-tetramethyluronium hexafluorophosphate; HPBCD, hyroxypropyl $\beta$-cyclodextrin; HPMC, hydroxypropyl methylcellulose; iv, intravenous; LLC-PK1, porcine proximal tubule cell line; MRT, mean residence time; MSA, methanesulfonic acid; $P_{app}$, apparent permeability; Vss, volume of distribution in steady state

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(23) cLog *P* values were calculated using software from Daylight Chemical Information Systems.
(24) The % *F* was not accurately estimated for the 100 mg/kg oral dose due to the long half-life of 57 h with up to 48 h of plasma collection. In contrast, the half-lives after either iv or oral dose (1−2 mg/kg) were only 13−16 h, and thus, the AUC for these two doses was sufficiently characterized by collecting plasma samples up to 48 h. However, the % *F* for the 100 mg/kg oral dose would be at least 93% based on estimation using $AUC_{0-48\ h}$ for the 100 mg/kg oral dose.