Sphingosine 1-phosphate (S1P) is a lysophospholipid mediator that evokes a variety of cell and tissue responses via a set of cell surface receptors. The recent development of S1P receptor agonists, led by the immunomodulatory pro-drug FTY720, has revealed that S1P signaling is an important regulator of lymphocyte trafficking. With the twin goals of understanding structure-activity relationships of S1P ligands and developing tool compounds to explore S1P biology, we synthesized and tested numerous S1P analogs. We report herein that a subset of our aryl amide-containing compounds are antagonists at the S1P1 and S1P3 receptors. The lead compound in the series, VPC23019, was found in broken cell and whole cell assays to behave as a competitive antagonist at the S1P1 and S1P3 receptors. The structure-activity relationship of this series is steep; for example, a slight modification of the lead compound resulted in S1P analogs with the twin goals of expanding the structure-activity relationships associated with S1P receptor interactions and identifying receptor-specific compounds. Our studies have led to the identification of a series of S1P analogs that behave as antagonists at two of the five S1P receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals for syntheses were purchased from Aldrich, Sigma, Advanced ChemTech Chemical Company (Louisville, KY), and/or NovaBiochem and were used without further purification. [γ-32P]ATP and [γ-35S]GTP were purchased from Amersham Biosciences. CyQuant cell proliferation assay kit and Fluo-4 AM calcium indicator were purchased from Molecular Probes (Eugene, OR). Chinese hamster ovary and T24 cells were purchased from the American Type Culture Collection (Manassas, VA). HEK293T cells were a gift from Dr. Judy White (Department of Cell Biology, University of Virginia). Tissue culture media and normal fetal bovine serum (FBS) were obtained from Invitrogen. Charcoal/dextran-stripped FBS was obtained from Gemini Bio-Products (Woodland, CA). G-protein α, β, and γ DNAs were a gift from Dr. Doug Bayliss (Dept. of Pharmacology, University of Virginia). Sphingosine 1-phosphate was purchased from Avanti Polar Lipids (Alabaster, AL).

**Syntheses of** VPC23019, VPC23031, VPC25239, and VPC23089—The synthetic route to the meta-substituted compounds VPC23031, VPC25239, and VPC23089 was initiated with a Sonogashira coupling (6) of 3-iodo-1-nitrobenzene with the appropriate terminal alkyne. The resulting adducts were then subjected to simultaneous hydrogenation of the nitro group and the triple bond to generate the meta-substituted anilines. The anilines were next coupled to a protected serine, and the ensuing amides underwent hydrogenolysis to afford the free alcohols. The alcohol was subsequently phosphorylated, oxidized with hydrogen peroxide, and then subjected to acid-catalyzed global deprotection to provide the final products, VPC23031, VPC25239, and VPC23089.

**Synthesis of the ortho-substituted compound, VPC23089**—commenced with the union of 2-iodoaniline and 1-octyne via a Sonogashira coupling. The ensuing aniline was then coupled to a protected serine utilizing the benzotriazol-1-yloxypyrrolidino-phosphonohexafluorophosphate reagent. The resulting amide was then subjected to a hydrogenation/hydrogenolysis step to remove the benzyl ether-protecting group and simultaneously reduce the aryl triple bond. The liberated alcohol was then phosphorylated, oxidized with hydrogen peroxide, and then sub-

---

* This work was supported by National Institutes of Health Grant R01 GM067958 (to K. R. L.) and National Institutes of Health Predoctoral Fellowship F31 GM064101 (to M. D. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pharmacology, Box 800735, University of Virginia School of Medicine, 1300 Jefferson Park Ave., Charlottesville, VA 22908-0735. Tel.: 434-924-2840; Fax: 434-982-3878; E-mail: klynch@virginia.edu.

‡ The abbreviations used are: S1P, sphingosine 1-phosphate; HCK, human embryonic kidney; FBS, fetal bovine serum; BSA, bovine serum albumin; LPA, lysophosphatidic acid; RFU, relative fluorescence unit.

Michael D. Davis, Jeremy J. Clemens, Timothy L. Macdonald, and Kevin R. Lynch
From the Departments of §Biochemistry and Molecular Genetics, ¶Pharmacology, and ¶Chemistry, University of Virginia, Charlottesville, Virginia 22908

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org
all cases, each concentration of every compound was tested at least in triplicate. For determination of the reversibility of the antagonism associated with VPC23019 (0.010 mM), the compound was added in triplicate with loading buffer to cell monolayers incubated at 37 °C for 30 min. The cells were washed with phosphate-buffered saline and exposed to compounds immediately, as described above.

**Determination of the Binding Constant for VPC23019 at the S1P1 and S1P3 Receptors**—The binding constant (Kd) for VPC23019 at the S1P1 and S1P3 receptors was determined by Schild analyses from curves that were fitted using the nonlinear regression method discussed by Lew and Angus (9). Briefly, nonlinear analysis of the best fit line generated by plotting the negative log of the EC50 values obtained from agonist dose-response curves, in the absence and presence of varying concentrations of antagonist, was plotted against the concentration of antagonist to give the Kd value. A F-test analysis was also performed to establish whether the antagonist did or did not meet the criteria of a simple competitive interaction.

**SIP Radiolabeling**—[35S]P1 was prepared by incubating sphingosine-1-PATP with cell lysate from HEK293T cells transfected transiently with human sphingosine kinase type 2 DNA. The 0.2-mL reaction contained 0.025 mM sphingosine, 1 μCi of [35S]PATP (7000 Ci/mmol), and kinase buffer (10 mM MgCl2, pH 7.5, 10 mM NaF, and 2 mM semicarbazide). The reaction was initiated by the addition of 0.02 mg of cell lysate protein and incubated at 37 °C for 30 min. The [35S]PATP was detected with P400 and 2.0 x 10^4 cpm trichloroacetic acid, and chloroform to the reaction mixture. The mixture was then vortexed and centrifuged at 1000 x g for 5–10 min. The organic layer was isolated, and the extraction procedure was repeated two additional times with the remaining aqueous fraction. The combined organic fractions were dried under a stream of nitrogen gas and resuspended in aqueous 0.1% fatty acid-free BSA. The specific activity of the product, [35S]PATP, is estimated to be that of the radiolabeled substrate, [35S]PATP, i.e. 7000 Ci/mmol.

**Statistical Analysis**—The EC50 and IC50 values for all dose response curves were determined by nonlinear regression analysis of all data using the Graphpad Prism program. The error associated with the data collected is reported as the standard error of the mean (S.E.).

**RESULTS**

VPC23019 Is Devoid of Agonism at the S1P1 and S1P3 Receptors—In the course of our examinations of SIP analog structure-activity relationship, we discovered that the aryl-amide compound VPC23019 (Fig. 1) lacked agonist activity at the S1P1 (Fig. 2A) and S1P3 (Fig. 2B) receptors in a broken cell assay (10). In applying this equation, the concentration of radioligand (L) is 0.05 mM and the Kd value used was that reported for the S1P1 receptor interaction, i.e. 8.1 nm (10).

**Measurement of Intracellular Calcium Mobilization**—A Flexstation™ fluorimeter was used to measure intracellular calcium in native T24 cells and T24 cells stably transfected with either human S1P1 or human S1P3 receptor DNA. Cells were plated (~50,000 cells/cm2) in 96-well, clear bottom black microplates (Corning Costar Corp.) and left overnight at 37 °C. The cells were dyes-loaded with 0.004 mM Fluo-4AM ester in a loading buffer (Hanks' balanced salt solution, pH 6.4, containing 20 mM HEPES, 0.1% fatty acid-free BSA, and 2.5 mM probenecid) for 30 min at 37 °C. After washing cell monolayers three times with phosphate-buffered saline, loading buffer was added, and the cells were exposed to sets of compounds for 3 min at 25 °C in the Flexstation™.
gate individual S1P receptors introduced by transfection. Numerous studies have demonstrated that S1P can promote cell migration; however, it was shown recently that S1P can also inhibit migration, possibly through stimulation of the S1P2 receptor (11). To circumvent this inhibitory effect, we used VPC22277 (Fig. 1), an S1P analog that is an agonist at the S1P1 and S1P3 receptors, but not the S1P2 receptor (Table I). (All of the compounds in the aryl amide series (12) are devoid of detectable activity at the S1P2 receptor.) We found that migration of T24-S1P1 cells could be induced by VPC22277, whereas no migration was evoked in response to VPC23019 (Fig. 2C). Similarly, in whole cell calcium mobilization studies using T24-S1P3 cells, dose-dependent calcium mobilization was observed with S1P (Fig. 2D). Thus, VPC23019 is devoid of agonist activity at the S1P1 and S1P3 receptors using both broken cell and whole cell assays.

VPC23019 Blocks Agonist Activity at the S1P1 and S1P3 Receptors—The finding that VPC23019 exhibited possible inverse agonist activity at the S1P1 or S1P3 receptors prompted us to investigate whether this compound blocked agonist activity. Using the γ[35]S-GTP binding assay, we found that S1P concentration effect curves generated in the presence of VPC23019 at either the S1P1 (Fig. 3A) or S1P3 (Fig. 3B) receptors produced a concentration-dependent, parallel rightward shift in the curves. This shift in agonist-mediated responses was also observed in two whole cell assays, cell migration (Fig. 3C) and calcium mobilization (data not shown). VPC23019 neither exhibited agonist activity at the LPA1–3 endothelial cell differentiation gene family receptors at concentrations up to 0.03 mM nor blocked the action at these sites (data not shown).

VPC23019 S1P Receptor Affinity—Schild analyses of the antagonist activity associated with VPC23019 in the γ[35]S-GTP binding assay gave pKb values at the S1P1 (Fig. 3A) and S1P3 (Fig. 3B) receptors of 7.49 ± 0.15 and 5.98 ± 0.08, respectively. Additionally, the nonlinear regression method of Lew and Angus (9), which predicts whether a compound behaves as a competitive antagonist, suggested that VPC23019 behaves as a competitive antagonist at both receptors. Schild analysis of calcium mobilization in T24-S1P3 cells gave a Kb value for VPC23019 that was ~10-fold less than that observed in the γ[35]S-GTP binding assay. However, the nonlinear regression analysis indicated that VPC23019 did not behave as a competitive antagonist in the calcium mobilization assay with T24-S1P3 cells. Importantly, both whole cell S1P receptor assays (Ca2+ mobilization (Fig. 3D, S1P3) and cell migration (S1P1, not shown)) recovered fully after washing out the antagonist.

Fig. 1. Structures of sphingosine 1-phosphate, VPC22277, VPC23019, VPC23031, VPC23089, and VPC25239.
FIG. 2. **VPC23019 lacks agonist activity at the S1P₁ and S1P₃ receptors.** HEK293T cells were transfected transiently with equal amounts of human S1P₁ or S1P₃ receptor and G_α, G_βγ, and G_γ plasmid DNAs. Membranes were collected after 60 h. Receptor activation was determined using a broken cell binding assay measuring the binding of γ-[^35]S-GTP to the membrane as a function of lipid concentration. Concentration-dependent stimulation of S1P₁ (A) and S1P₃ (B) receptors was observed with S1P (filled circles) but not VPC23019 (open circles). When receptor plasmid DNA was excluded, no significant binding of γ-[^35]S-GTP was observed with 0.010 mM S1P (A, inset), which demonstrates that the activity is a function of receptor expression. Binding of γ-[^35]S-GTP was observed with 0.010 mM S1P in HEK393T cells transfected transiently with only receptor and G_α plasmid DNA; however, the response was at least 3-fold less than that of cells where both receptor and all three G-protein plasmid DNAs were added (A, inset). Data points are in triplicate and are representative of two independent experiments. The percent activation is based on normalization of disintegrations/min values obtained from the minimum and maximum S1P concentration. Typical values for 0 and 100% binding were 300 and 3000 dpm/well, respectively, for both the human S1P₁ and S1P₃ receptors.

**C.** Migration of T24 cells transfected stably with human S1P₁ receptor was observed with the S1P₁ agonist VPC22277 (10 nM) but not VPC23019 (1–1000 nM). Data points are in duplicate and are representative of two independent experiments. The percent migrating cells is based on normalization of relative fluorescence unit (RFU) values obtained from the RFU values when migration was observed with 0.1% BSA carrier (minimum) and VPC22277 (maximum). Typical values for 0 and 100% migration were 30,000 and 100,000 RFU/well, respectively.

**D.** Concentration-dependent calcium mobilization of untransfected T24 cells.
The Discovery of Antagonists for the S1P₁ and S1P₃ Receptors

Thus, the antagonist activity exhibited by VPC23019 is reversible as well as fully surmountable, which are essential criteria for a competitive antagonist.

To measure the affinity of VPC23019 for the S1P₁ and S1P₃ receptors directly, we examined the ligand-receptor interaction associated with the S1P₁ and S1P₃ receptors via a receptor binding assay using [³²P]SIP in competition with SIP and VPC23019. Analysis of SIP in the radioligand binding assay (Fig. 4A) yielded pKᵦ values of 8.96 ± 0.14 and 8.12 ± 0.06 at the S1P₁ and S1P₃ receptors, respectively. These values are in agreement with the published pKᵦ values for radiolabeled SIP binding to these receptors (10, 13, 14). The radioligand binding assay also revealed an excellent correlation between the pKᵦ and the pKᵦ for VPC23019 generated from the Schild analysis at both the S1P₁ and S1P₃ receptors, i.e., pKᵦ values of 7.86 ± 0.16 and 5.93 ± 0.19, respectively (Fig. 4B and Table II). Finally, VPC23019 was also found to be devoid of agonist activity at the S1P₂ receptor, and radioligand binding studies with the S1P₂ receptor revealed that VPC23019 did not influence the binding of [³²P]SIP to the S1P₂ receptor at concentrations up to 0.010 mM (data not shown).

Changes in Analog Structure Alter Activity at the S1P₁ and S1P₂ Receptors—Previous studies with para-substituted aryl amide compounds (VPC23019 is meta-substituted, Fig. 1) revealed that they are agonists at all of the S1P receptors, except at the S1P₂ receptor, where they are inactive (12). Furthermore, different potencies and efficacies were observed with changes in either the length of the longest alkyl chain or the spatial configuration about the amino carbon (12). It is reasonable, therefore, to expect that such changes would also influence antagonist activity. Thus, we synthesized several structural analogs of VPC23019 to test the effect of potency at either the S1P₁ or S1P₃ receptor.

If the primary amine of VPC23019 is placed in the opposite spatial configuration (VPC25027), the compound is at least 1 log order less potent than VPC23019 as an antagonist at the S1P₂ receptor (data not shown). Furthermore, these analogs blocked agonist-mediated migration (Fig. 5A) and calcium mobilization (Fig. 5B) in T24 cells stably expressing either the S1P₁ or S1P₃ receptor, respectively. None of these synthetic maneuvers resulted in an antagonist with improved potency except for VPC25239 (Table II), which was equal in potency to the lead compound at the S1P₂ receptor but about 1 log order more potent at the S1P₃ receptor (Table II). Thus, VPC25239 is an equipotent S1P₁/S1P₃ receptor antagonist, whereas the other antagonists in the series are more potent at the S1P₃ receptor. The affinity of VPC23031 observed at the S1P₃ receptor (Table II) does not appear to correlate with the ability of the compound to effectively shift the agonist-mediated activation of the receptor (Fig. 5B). The reason for this disconnect is unclear to us presently.

As observed previously with VPC23019, all of the compounds in this series behaved as agonists at the S1P₂ and S1P₃ receptors, and no agonist activity was observed with the S1P₁ receptor (Table I). Finally, modification of the phosphate head group of VPC23019 (e.g., phosphate) resulted in compounds with an agonist and antagonist activity profile similar to that observed with VPC23019.3

Table I

| Compound | Longest alkyl chain | Ring substitutions | Enantiomer | pECᵦ₀ values |
|----------|---------------------|--------------------|------------|--------------|
|          |                     |                    |            | SIP₁ | SIP₂ | SIP₃ | SIP₄ | SIP₅ |
| SIP      | 18                  | S                  |            | 8.39 ± 0.16 | 8.62 ± 0.10 | 8.65 ± 0.11 | 8.61 ± 0.14 | 8.63 ± 0.06 |
| VPC22277 | 10                  | para S             |            | 8.80 ± 0.06 | <5 | 7.13 ± 0.11 (PA) | 7.05 ± 0.22 (PA) | 7.96 ± 0.10 |
| VPC23019 | 8                   | meta R             |            | NA | <5 | NA | 6.58 ± 0.08 | 7.07 ± 0.12 (PA) |
| VPC25239 | 7                   | meta R             |            | NA | <5 | NA | 6.78 ± 0.09 | 7.94 ± 0.09 (PA) |
| VPC23031 | 6                   | meta R             |            | NA | <5 | NA | 5.96 ± 0.06 | 6.87 ± 0.16 (WPA) |
| VPC23089 | 8                   | ortho R            |            | NA | <5 | NA | 6.07 ± 0.21 | <5 |
| VPC23079 | 9                   | meta R             |            | 6.17 ± 0.24 | <5 | NA | 5.93 ± 0.08 | NA |
| VPC23069 | 10                  | meta R             |            | 7.09 ± 0.16 | <5 | 5.72 ± 0.28 (WPA) | 6.07 ± 0.07 | <5 |
| VPC25027 | 8                   | meta S             |            | 8.65 ± 0.16 (PA) | NA | NA | 6.05 ± 0.04 | 7.20 ± 0.08 (PA) |

DISCUSSION

Understanding of the physiological role of the S1P receptors has been greatly enhanced by using sphingosine- and S1P-related analogs. An example of this is FTY720, a sphingosine-like lipid that has been shown to prolong allograft survival (15–22) and is efficacious in autoimmune disease models such as experimental autoimmune encephalomyelitis (1, 23) and the non-obese diabetic mouse (24, 25). Recent reports demonstrate that FTY720 is a pro-drug; the immunomodulatory effect associated with its administration requires phosphorylation (1, 2, 26). The resultant S1P analog generated (FTY720-phosphate) was found to be a potent agonist at all of the S1P receptors except the S1P₂ receptor (1, 2). Furthermore, studies performed with the S1P₃ receptor-selective agonist SEW2871 (5-(4-phenyl-5-trifluoromethyl-thiophen-2-yl)-3-(3-trifluoromethyl-phenyl)-(1,2,4)oxadiazole) suggest that lymphopenia, which is an index of FTY720 action, is a result of activation of the S1P₁ receptor (4). Furthermore, lymphocyte egress from thymus and peripheral lymphatic tissues is dependent on the S1P₁ receptor (3). Conversely, the toxicity associated with S1P agonist administration in rodents (27, 9837)

3 M. D. Davis, F. W. Foss, T. L. Macdonald, and K. R. Lynch, unpublished data.
may be because of stimulation of the cardiac S1P3 receptors (29). These studies highlight the potential associated with the further characterization of related molecular entities. We have characterized the activity of a series of S1P analogs that are antagonists at two of the five S1P receptors. Our results show that two of these analogs, VPC23019 and VPC25239, are reasonably potent at the S1P1 receptor (i.e. Kᵢ ≈ 50 nM), and VPC25239 is also potent (Kᵢ < 100 nM) at the S1P3 receptor. By comparison, the Kᵢ value for the S1P-S1P1 receptor interaction is reported to be 8.1 nM (10). Our previous results with para-substituted aryl amide analogs demonstrate that these are uniform S1P receptor agonists (12). The antagonist activity observed with the compounds presented here resulted from minor changes in the aryl amide structures. Given our earlier observations, it was counterintuitive that antagonists would be realized by 1) positioning of the primary amine in a configuration opposite that of naturally occurring sphingosine and S1P, 2) placing the phenyl substituents in a 1,3 (meta) configuration, and 3) limiting the alkyl chain to no more than eight carbon atoms. In view of our present findings, it is possible that one of the enantiomers of the meta-substituted analog of FTY720 described by Kiuchi et al. (15) is an antagonist (following phosphorylation) for one or more S1P receptors; however, the whole animal assays used to evaluate their compounds would not have allowed for this determination. The availability of an S1P receptor antagonist should provide a useful tool for studies of S1P biology. Two predictions made using S1P agonists and genetic models are prominent. First, the history of S1P and the S1P1 receptor in endothelial biology, which is reinforced by the defect in vascular maturation observed in S1P1 receptor gene ablated mice (3), has led to the suggestion that S1P1 receptor antagonists might prove

**FIG. 3. Antagonism at the S1P₁ and S1P₃ receptors by VPC23019.** A and B, HEK293T cells were transfected transiently with equal amounts of human S1P₁ or S1P₃ receptor and Gₛα, Gβ₁, and Gγ₂ plasmid DNAs. Membranes were collected after 60 h. Receptor activation was determined using a broken cell binding assay measuring the binding of γ-³²P-gTP to the membrane as a function of agonist (S1P) stimulation. Blockade of S1P stimulation at S1P₁ and S1P₃ in the γ-³²P-gTP broken cell binding assay was performed in the presence of 10,000 nM (open circles), 1000 nM (open squares), 100 nM (filled squares), or 0 nM (filled circles) of VPC23019. The binding constant (pKᵢ) is reported as pKᵢ ± S.E. Data points are in hexuplicate and are representative of two independent experiments for each receptor. The percent activation is based on normalization of disintegrations/min values obtained from the minimum and maximum S1P concentration. Typical values for 0 and 100% binding were ~300 and 3000 dpm/well, respectively, for both the human S1P₁ and S1P₃ receptors. C, blockade of the migration of T24 cells transfected stably with human S1P₁ receptor obtained with the S1P₁ agonist VPC22277 (10 nM) was observed with 10, 100, and 1000 nM concentrations of VPC23019. Data points are in duplicate and are representative of two independent experiments. The percent migrating cells is based on normalization of RFU values obtained from the RFU values when the migration was observed with BSA (minimum) and VPC22277 (maximum). Typical values for 0 and 100% migration were ~30,000 and 100,000 RFU/well, respectively. D, Ca²⁺ mobilization observed with T24 cells transfected stably with human S1P₃ receptor (solid line) was not altered by pretreatment with VPC23019 (10,000 nM, dashed line) followed by washout. Data points are in triplicate and are representative of two independent experiments. The percent activation is based on normalization of RFU values obtained from the RFU values when the migration was observed with BSA (minimum) and VPC22277 (maximum). Typical values for 0 and 100% Ca²⁺ mobilization were ~400 and 4000 RFU/well, respectively.
useful as anti-angiogenic agents. Second, the suggestion that phospho-FTY720 exerts its effect on lymphocyte trafficking by desensitizing T-lymphocyte S1P1 receptors (and thereby functionally antagonizing S1P signaling in T-cells (3, 30)), suggests that a S1P<sub>1</sub> receptor antagonist should also evoke the lymphopenia that is characteristic of S1P<sub>1</sub> receptor agonists, such as phospho-FTY720 or SEW2871 (4). The presence of the phosphate monoester in our current set of antagonist compounds, however, might result in their rapid hydrolysis by cell surface lipid phosphatases and thus make their use in vivo problematic. Thus, the testing of these ideas may require the synthesis of similar compounds containing hydrolysis-resistant phosphate analogs.

In summary, the results presented here clearly illustrate the discrete chemical space associated with the interaction between these ligands and the S1P receptors. As demonstrated amply by FTY720, such chemical tools can provide unique insights into the physiological roles of S1P and expand our understanding of the S1P receptor family.

**Table II**

| Compound     | Longest alkyl chain | Ring substitutions | pK<sub>i</sub> S1P<sub>1</sub> | pK<sub>i</sub> S1P<sub>3</sub> | pK<sub>b</sub> S1P<sub>1</sub> | pK<sub>b</sub> S1P<sub>3</sub> |
|--------------|---------------------|---------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| VPC23019     | 8                   | meta                | 7.86 ± 0.16                 | 7.49 ± 0.16                 | 5.98 ± 0.08                 | 5.98 ± 0.08                 |
| VPC25239     | 7                   | meta                | 7.87 ± 0.04                 | 7.01 ± 0.14                 | 6.25 ± 0.23                 | 5.85 ± 0.10                 |
| VPC23031     | 6                   | meta                | 7.21 ± 0.07                 | 2.56 ± 13.4                 | 6.87 ± 0.15                 | 4.98 ± 0.62                 |
| VPC23089     | 8                   | ortho               | 6.65 ± 0.16                 | 5.80 ± 0.16                 | 6.31 ± 0.23                 | 6.36 ± 0.67                 |

* Based on Schild analysis, the antagonism observed is not competitive.
understanding of S1P receptor signaling, a significant undertaking given their potential roles as therapeutic targets.

Acknowledgment—We thank Dr. Mark Alexandrow for helpful discussions.

REFERENCES

1. Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C. A., Zollinger, M., and Lynch, K. R. (2002) J. Biol. Chem. 277, 21453–21457

2. Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., and Rosen, H. (2002) Science 296, 346–349

3. Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L., and Cyster, J. G. (2004) Nature 427, 355–360

4. Sanna, M. G., Luo, Y., Wu, E., Alfonso, C., Aho, M. Y., Peterson, M. S., Webb, B., Lefebvre, S., Chun, J., Gray, N., and Rosen, H. (2002) J. Biol. Chem. 277, 13839–13848

5. Kimura, T., Sato, K., Malchinkhuu, E., Tomura, H., Tamama, K., Kuwabara, A., Murakami, M., and Okajima, F. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 1283–1288

6. Jones, L., Schummi, J. S., and Tour, J. M. (1997) J. Org. Chem. 62, 1588–1410

7. Zhang, T., Nanney, L. B., Luongo, C., Lamps, L., Heppner, K. J., Dubois, R. N., and Beaudette, R. D. (1997) Cancer Res. 57, 169–175

8. Im, D. S., Heise, C. E., Ancellin, N., O'Dowd, B. F., Shei, G. J., Heavens, R. P., Rigby, M. R., Hla, T., Mandala, S., McAllister, G., George, S. R., and Lynch, K. R. (2000) J. Biol. Chem. 275, 14281–14286

9. Lew, M. J., and Angus, J. A. (1995) Trends Pharmacol. Sci. 16, 328–337

10. Lee, M. J., Van Brocklyn, J. R., Huang, S., Liu, C. H., Hand, A. R., Menzelleev, R., Spiegel, S., and Hla, T. (1998) Science 279, 1552–1555

11. Clair, T., Aoki, J., Koh, E., Bandle, R. W., Nam, S. W., Ptaszynska, M. M., Mills, G. B., Schiffman, E., Liotta, L. A., and Stracke, M. L. (2003) Cancer Res. 63, 5446–5453

12. Clemens, J. J., Davis, M. D., Lynch, K. R., and Macdonald, T. L. (2003) Bioorg. Med. Chem. Lett. 13, 3401–3404

13. Van Brocklyn, J. R., Tu, Z., Eadsall, L. C., Schmidt, R. R., and Spiegel, S. (1999) J. Biol. Chem. 274, 4626–4632

14. Kon, J., Sato, K., Watanabe, T., Tomura, H., Kuwabara, A., Kimura, T., Tamama, K., Ishizuka, T., Murata, N., Kanda, T., Kobayashi, I., Ohtsora, H., Ui, M., and Okajima, F. (1999) J. Biol. Chem. 274, 23940–23947

15. Kusuki, M., Adachi, K., Kohara, T., Minamigu, M., Hanano, T., Aoki, Y., Mishina, T., Arita, M., Nakao, N., Ohtsuki, M., Hoshino, Y., Teshima, K., Chiba, K., Sasaki, S., and Fujita, T. (2000) J. Med. Chem. 43, 2946–2961

16. Yamagawa, Y., Hoshino, Y., and Chiba, K. (2000) Int. J. Immunopharmacol. 22, 597–602

17. Chiba, K., Yamagawa, Y., Masubuchi, Y., Katoaka, H., Kawaguchi, T., Ohtsuki, M., and Hoshino, Y. (1998) J. Biol. Chem. 263, 5037–5044

18. Hoshino, Y., Yamagawa, Y., Ohtsuki, M., Nakayama, S., Hashimoto, T., and...
19. Yanagawa, Y., Hoshino, Y., Kataoka, H., Kawaguchi, T., Obitsu, M., Sugahara, K., and Chiba, K. (1999) Transplant. Proc. 31, 1224–1226
20. Brinkmann, V., Pinschewer, D. D., Feng, L., and Chen, S. (2001) Transplantation 72, 764–769
21. Suzuki, S., Enosawa, S., Kakefuda, T., Li, X. K., Mitsusada, M., Takahara, S., and Amemiya, H. (1996) Transpl. Immunol. 4, 252–255
22. Xie, J. H., Nomura, N., Koprak, S. L., Quackenbush, E. J., Forrest, M. J., and Rosen, H. (2003) J. Immunol. 170, 3662–3670
23. Fujino, M., Funeshima, N., Kitazawa, Y., Kimura, H., Amemiya, H., Suzuki, S., and Li, X. K. (2003) J. Pharmacol. Exp. Ther. 305, 70–77
24. Yang, Z., Chen, M., Fialkow, L. B., Ellett, J. D., Wu, R., Brinkmann, V., Nadler, J. L., and Lynch, K. R. (2003) Clin. Immunol. 107, 30–35
25. Maki, T., Gottschalk, R., and Monaco, A. P. (2002) Transplantation 74, 1684–1686
26. Sanchez, T., Estrada-Hernandez, T., Paik, J. H., Wu, M. T., Venkataraman, K., Brinkmann, V., Claffey, K., and Hla, T. (2003) J. Biol. Chem. 278, 47281–47290
27. Hale, J. J., Doherty, G., Toth, L., Mills, S. G., Hajdu, R., Keohane, C. A., Rosenbach, M., Milligan, J., Shei, G. J., Chebet, G., Bergstrom, J., Card, D., Forrest, M., Sun, S. Y., West, S., Xie, H., Nomura, N., Rosen, H., and Mandala, S. (2004) Bioorg. Med. Chem. Lett. 14, 3501–3505C. A.
28. Hale, J. J., Doherty, G., Toth, L., Li, Z., Mills, S. G., Hajdu, R., Ann Keohane, C., Rosenbach, M., Milligan, J., Shei, G. J., Chebet, G., Bergstrom, J., Card, D., Rosen, H., and Mandala, S. (2004) Bioorg. Med. Chem. Lett. 14, 3495–3499
29. Forrest, M., Sun, S. Y., Hajdu, R., Bergstrom, J., Card, D., Doherty, G., Hale, J., Keohane, C., Meyers, C., Milligan, J., Mills, S., Nomura, N., Rosen, H., Rosenbach, M., Shei, G. J., Singer, II, Tian, M., West, S., White, V., Xie, J., Proia, R. L., and Mandala, S. (2004) J. Pharmacol. Exp. Ther. 309, 758–768
30. Gra¨ler, M. H., and Goetzl, E. J. (2004) FASEB J. 18, 551–553