

## The Immune Modulator, FTY720, Targets Sphingosine 1-Phosphate Receptors

Volker Brinkmann<sup>1</sup>, Michael D. Davis<sup>5</sup>, Christopher E. Heise<sup>6</sup>, Rainer Albert<sup>1</sup>, Sylvain Cottens<sup>1</sup>, Robert Hof<sup>1</sup>, Christian Bruns<sup>1</sup>, Eva Prieschl<sup>4</sup>, Thomas Baumruker<sup>4</sup>, Peter Hiestand<sup>3</sup>, Carolyn A. Foster<sup>4</sup>, Markus Zollinger<sup>3</sup>, Kevin R. Lynch<sup>5,6</sup>

Departments of <sup>1</sup>Transplantation, <sup>2</sup>Arthritis & Bone Metabolism, <sup>3</sup>Preclinical Safety, Novartis Pharma AG, Basel, Switzerland, <sup>4</sup>Department of Dermatology & Immunopathology, Novartis Research Institute, Vienna, Austria, and Departments of <sup>5</sup>Biochemistry & Molecular Genetics and <sup>6</sup>Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia, USA.

address correspondence to:

Kevin R. Lynch  
Department of Pharmacology, Box 800735  
University of Virginia Health System  
1300 Jefferson Park Avenue  
Charlottesville, VA 22908-0735 USA  
Tele: 434-924-2840, fax: 434-982-3878  
email: krl2z@virginia.edu

**Running Title:** FTY720 and sphingosine 1-phosphate signaling

## Summary

Immunosuppressant drugs such as cyclosporin have allowed widespread organ transplantation but their utility remains limited by toxicities and they are ineffective in chronic management of autoimmune diseases such as multiple sclerosis. In contrast, the immune modulating drug, FTY720, is efficacious in a variety of transplant and autoimmune models without inducing a generalized immunosuppressed state, and is effective in human kidney transplantation. FTY720 elicits a lymphopenia resulting from a reversible re-distribution of lymphocytes from circulation to secondary lymphoid tissues by unknown mechanisms. Using FTY720 and several analogs, we show now that FTY720 is phosphorylated by sphingosine kinase; the phosphorylated compound is a potent agonist at four sphingosine 1-phosphate receptors and represents the therapeutic principle in a rodent model of multiple sclerosis. Our results suggest that FTY720, after phosphorylation, acts through sphingosine 1-phosphate signaling pathways to modulate chemotactic responses and lymphocyte trafficking.

## Introduction

FTY720 was derived from ISP-1 (myriocin), a fungal metabolite that was an eternal youth nostrum in traditional Chinese herbal medicine (1). The compound (2-amino-2-[2-(4-octylphenyl) ethyl] propane-1,3-diol) is a novel, high potency immune modulating agent that is remarkably effective in a variety of autoimmune and transplant models including islet transplantation (2), and has recently proven to be effective in renal transplantation in man (3). Unlike the currently used immunosuppressive agents (e.g. the calcineurin inhibitors cyclosporin and tacrolimus), FTY720 does not inhibit T cell activation and proliferation, and in rodent models does not impair immunity to systemic viral infection (4). If confirmed in man, the latter property provides a striking advantage over current immunosuppressive therapies. FTY720 apparently sequesters lymphocytes from circulation to secondary lymph tissue compartments (5) with concomitant reduction of specific effector T cells re-circulating from the lymph nodes to inflamed peripheral tissues (4) and graft sites (6). FTY720 does not act via the lymphocyte homing chemokine

receptor, CCR-7, because FTY720 is active both in CCR-7 deficient mice and *plt* (paucity of lymph node T-cells) mice, which lack CCR-7 ligands (CCL-19 and CCL-21) (7).

FTY720-induced lymphocyte homing is sensitive to suppression by pertussis toxin (PTX)<sup>1</sup> (6-8), which suggests that the molecular target of the drug is a G protein-coupled receptor (GPCR) interacting with heterotrimeric G proteins of the  $\alpha_{i/o}$ -type. The affected GPCR(s) is on the lymphocyte, since fluorescently-labeled lymphocytes treated with PTX *ex vivo* and transferred to mice are not depleted by FTY720 *in vivo* (8). The structural similarity of FTY720 and sphingosine has prompted speculation that the drug might act via the sphingosine 1-phosphate (S1P) receptor, S1P<sub>4</sub> (formerly Edg-6), that is known to be expressed by lymphocytes (9).

S1P is a pleiotropic lysophospholipid mediator; the prominent cellular responses to applied S1P are transient calcium mobilization, inhibition of adenylyl cyclase, escape from apoptosis (10), increased cell migration (11,12) and mitogenesis (13). The physiologic role of S1P remains undefined although cell culture experiments and the phenotype of a mouse with the S1P<sub>1</sub> receptor gene ablated suggest a role for S1P in vascular maturation (14,15). Responses to S1P are mediated through a set of five, cell surface GPCRs (S1P<sub>1-5</sub>) and the various effects of S1P have been attributed to interactions with one or more of these receptors (16). S1P is formed by the action of sphingosine kinase on sphingosine (17). The activity of this enzyme is increased in response to external stimuli (18,19), and enforced expression of sphingosine kinase increased both cell proliferation and survival (20). Sphingosine is converted rapidly to S1P when added to cells (21) while the route of S1P degradation to sphingosine might proceed via an ectophosphatase (22). To learn whether FTY720 might participate in the sphingosine – S1P signaling cascade, we performed the studies described herein.

## Experimental Procedures

**Sphingosine kinase assay:** The assays were performed as described (23) using mouse recombinant sphingosine kinase 1a expressed in *Escherichia coli*. The reaction buffer contained 50 mM HEPES, pH 7.4, 15 mM MgCl<sub>2</sub>, 10 % glycerol, and 0.05 % Triton X100. Substrates (sphingosine, FTY720 or AAL) were incubated at various concentrations with 10 nM sphingosine kinase, 10  $\mu$ M ATP / 0.5  $\mu$ l [ $\gamma$ -<sup>32</sup>P]ATP

(3000 Ci/mmol, Amersham Pharmacia Biotech, Uppsala, Sweden) at 30 °C for 1 h. Lipids were extracted with two volumes  $\text{CHCl}_3$ /methanol (1:2), the organic extraction product dried and the pellet redissolved. The lipids were separated on a thin layer chromatography plate using a n-butanol/acetic acid/water (6:2:2) solvent system, after which the plate was exposed to X-ray film to detect phosphorus-32 labeled lipids.

**Organ culture:** Mouse organs (as indicated) were prepared and kept for 24 h in medium containing tritiated FTY720. Single cells were prepared from those organs by cell strainer, cells were lysed and a lipid extraction was performed from  $10^6$  cells of each organ or from the corresponding culture supernatant. Extraction and analysis by thin layer chromatography was done as described above.

**GTP[ $\gamma$ - $^{35}\text{S}$ ] binding assays:** Membranes were prepared from either insect Sf9 cells that were infected with recombinant baculoviruses encoding receptor and G proteins (for the  $\text{S1P}_1$  receptor) or HEK293T cells transfected with DNAs encoding  $\text{S1P}$  receptors as well as G proteins ( $\text{S1P}_4$  and  $\text{S1P}_5$  receptors) or rat hepatoma RH7777 cells that were transfected with receptor DNA alone ( $\text{S1P}_3$  receptor). After 48 hours, cells were collected and crude microsomal membranes prepared. Ligand stimulation of GTP[ $\gamma$ - $^{35}\text{S}$ ] binding was performed as described previously (24). In membranes from all three cell types, agonist stimulation of GTP[ $\gamma$ - $^{35}\text{S}$ ] binding was entirely dependent on exogenous receptor.

**Measurement of circulating lymphocytes:** FTY720 and AAL were dissolved in water and administered by gavage to Lewis rats at various doses. FTY720-P and AFD were dissolved in water:DMSO (5:1 v/v) and injected intraperitoneally into C3H mice (1 mg/kg). Blood was collected from the tail vein of mice or the sublingual vein of rats six hours after drug administration and subjected to hematology using an automated Tecnicon H1-E analyzer (Bayer Diagnostics, Zürich, Switzerland)

**Apoptosis assay:** Human  $\text{CD4}^+$  T cells were negatively selected from Ficoll-isolated peripheral blood mononuclear cells by magnetic cell sorting (MACS) according to standard procedures, using anti- $\text{CD8}$ , anti- $\text{CD20}$ , and anti- $\text{CD14}$  coated Miltenyi Biotec (Gladbach, Germany) magnetic beads.  $\text{CD4}^+$  T cells ( $10^6/\text{ml}$ ) were incubated with increasing concentrations of compounds for 4 hours. Cells were stained for

expression of phosphatidylserine in the outer leaflet of the membranes, using the Annexin-V-FLUOS staining kit (Roche) and positive cells were detected by FACS.

**Experimental autoimmune encephalomyelitis (EAE) model:** Wistar rats were immunized with an emulsion of bovine spinal cord in complete Freund's adjuvant as described (25). Two week oral treatment with FTY720 (aqueous solution) or the enantiomers (dissolved in water:DMSO, 10:1 v/v) was started on day 0, using a dose of 0.3 mg/kg/day. Positive controls received vehicle alone. Animals (10 per group) were monitored daily and graded according to disease symptoms: 1, flaccid tail; 2, hind limb weakness or ataxia; 3, full paralysis of hind limbs.

## Results

We used a set of FTY720-like compounds to determine sphingosine kinase and S1P receptor activity and correlate these with assays of lymphocyte function. In addition to FTY720, we tested both enantiomers (AAL) of an analog described by Kiuchi *et al.* (26) wherein a hydroxymethylene substituent of FTY720 was replaced by a methyl group (Fig. 1). These enantiomers have very different activities; the ID<sub>50</sub> values for decreasing circulating T lymphocytes in rats were reported to be 0.009 mg/kg and >1 mg/kg for the *R* and *S* enantiomers, respectively, while the ID<sub>50</sub> value for FTY720 in the same system was 0.024 mg/kg (26).

We asked first whether these compounds were substrates for sphingosine kinase. Recombinant mouse sphingosine kinase 1a catalyzed the phosphorylation of FTY720 and AAL(*R*), but not AAL(*S*) (Fig. 2a,b). Moreover, lymphoid tissue including Peyer's patches, spleen and lymph nodes effectively phosphorylated FTY720 while heart, liver and kidney contained little of the phosphorylated drug (Fig. 2c). This pattern of active tissues best matches the RNA localization of sphingosine kinase type 1 (27). The concept that phosphorylated FTY720 might be the active principle is intriguing and suggests that an alcohol/phosphate cycling of FTY720/FTY720-P takes place *in vivo* as occurs with sphingosine/S1P. Indeed, FTY720 was converted extensively to FTY720-P *in vivo*, resulting in up to 4-fold higher blood levels of FTY720-P compared to parent FTY720 (Table 1). To learn whether FTY720-P is dephosphorylated *in vivo*, we administered single doses of FTY720-P to mice and assayed blood levels of

FTY720 after 24 hours. FTY720 could be detected after the lowest dose (0.1 mg/kg) of FTY720-P and increased in a dose-dependant fashion (Table 1). We next determined whether synthetic phosphate derivatives, namely FTY720-P, AFD(*R*) and AFD(*S*), which resemble S1P (Fig. 1), were agonists at S1P receptors. To interrogate the individual S1P receptors, we used a membrane-based GTP[ $\gamma$ -<sup>35</sup>S] binding assay that allows direct comparison of the rank order potencies (pEC<sub>50</sub>) and relative efficacies (E<sub>max</sub>) of agonist ligands at isolated receptors (24). All of our compounds were agonists at the S1P<sub>4</sub> receptor, although FTY720-P and AFD(*R*) were far more potent than their non-phosphorylated congeners (Fig. 3). Both FTY720-P and AFD(*R*) were high potency agonists also at the S1P<sub>1</sub>, S1P<sub>3</sub> and S1P<sub>5</sub> receptors (Table 2), but the corresponding alcohols (FTY720 and AAL) were not efficacious at these three receptors (data not shown). Although FTY720-P behaved as a partial agonist in the GTP[ $\gamma$ -<sup>35</sup>S] binding assay (Table 2), this compound was a full agonist in whole cell assays of inhibition of cAMP accumulation, where there exists more amplification of signal (data not shown). None of our compounds were active at the S1P<sub>2</sub> receptor in our assays at concentrations up to 10  $\mu$ M. The receptor activation data are consistent with ligand binding measurements, which demonstrate a high affinity interaction between FTY720-P and AFD(*R*), but not AFD(*S*), and the S1P receptors (data not shown). Finally, the compounds were not active at the three receptors for a structurally-related lysophospholipid mediator, lysophosphatidic acid (data not shown).

To learn whether this pattern of activity is recapitulated *in vivo*, we determined the potency of our compounds in reducing numbers of circulating T-lymphocytes. FTY720 and AAL(*R*) potently reduced circulating T-lymphocyte levels in rats in a dose dependent manner, whereas AAL(*S*) and sphingosine were completely inactive at doses up to 1 mg/kg (Fig. 4a). We obtained the analogous result with the respective phosphorylated compounds; a 1 mg/kg bolus injection of FTY720-P or AFD(*R*) reduced circulating T cells by about 70%, whereas AFD(*S*) and S1P were inactive with this dosing regimen (Fig. 4b). The lymphopenic activity of FTY720 and AAL(*R*) *in vivo* (Fig. 4 and reference 25) thus can be explained by their metabolism to the phosphorylated forms (FTY720-P and AFD(*R*)), which are potent

agonists at multiple S1P receptors. The lack of efficacy of AAL(*S*) in decreasing circulating lymphocytes cannot be credited only to its failure as a substrate for sphingosine kinase for even when phosphorylated (synthetically) to form AFD(*S*), it lacks affinity for S1P receptors (Table 2).

FTY720 evokes apoptosis in lymphocytes at micromolar concentrations, prompting the idea that the drug acts by killing lymphocytes (28). We consider this mechanism highly unlikely in view of the low nanomolar levels ( $C_{\max} < 50$  nM) of FTY720 realized in the blood of rats treated with the high dose of 1 mg/kg (29). Nevertheless, we determined whether the apoptotic potential of our compounds correlated with activities *in vitro* or *in vivo*. We observed apoptotic responses only in T cells treated with micromolar concentrations of non-phosphorylated compounds (Table 3). This pattern is reminiscent of reports of the activity of sphingosine and S1P where sphingosine is associated with apoptosis and S1P with protection from the same (10,30). The apoptotic responses elicited by the non-phosphorylated compounds were neither stereoselective nor inhibited by prior pertussis toxin treatment (data not shown) – both properties contrast the behavior of these compounds in mice or rats regarding the depletion of circulating lymphocytes (7,26).

The unique mechanism that apparently underlies the immune modulating effects of FTY720, i.e. increased homing of T cells towards the lymphatic system and away from inflammatory tissues (5), provides an opportunity for therapy of autoimmune disorders that does not exist with current immunosuppressive agents. Therefore, we tested FTY720 and both enantiomers of AAL in experimental autoimmune encephalomyelitis (EAE), which is a primary model of human multiple sclerosis (31). Treatment of Wistar rats with FTY720 or AAL(*R*) (0.3 mg/kg/day) completely prevented the development of EAE whereas the AAL(*S*) was entirely inactive (Fig. 5). Thus the prophylactic activities of the compounds in this model are entirely consistent with their activity on S1P receptors and their potential to reduce circulating lymphocytes.

## Discussion

Administration of FTY720 somehow re-sets a rheostat that apportions lymphocytes between the circulatory and secondary lymphoid tissue compartments. We have now documented that FTY720 and an

analog (AAL(*R*)), after phosphorylation to FTY720-P and AFD(*R*), are high affinity agonists at four (of five) S1P receptors. The correlation of substrate activity (Fig. 2), agonism at S1P receptors (Fig. 3 and Table 2), induction of lymphopenia (Fig. 4) and activity in the EAE model (Fig. 5) suggests strongly that FTY720 and an analog (i.e. AAL(*R*)) function ultimately as S1P mimetics that increase the lymphocyte homing response.

The failure of sphingosine and S1P to evoke lymphopenia when administered to rats (Fig. 4b) might relate to an approximately one log order higher potency of FTY720-P and AFD(*R*) compared to S1P at the S1P<sub>1</sub> and S1P<sub>4</sub> receptors (Table 1), but could also be due to a different metabolic rate for the naturally occurring compound. For example, the rate of de-phosphorylation of S1P, FTY720-P and AFD(*R*) – presumably proceeding either through the non-selective lipid phosphate phosphohydrolases (22) or the S1P phosphatase (30) – might be different with resultant differences in accumulation. Alternately, FTY720 and AAL(*R*) might be more effective substrates of the sphingosine kinase *in vivo*.

Our discovery that FTY720 can be phosphorylated to yield a potent S1P mimetic has substantial implications for S1P biology. This pleiotropic lipid mediator is most often characterized as promoting angiogenesis, cell proliferation and escape from apoptosis. To this list must now be added immune system modulation via changes in lymphocyte trafficking. In addition to increasing knowledge of lysophospholipid medicinal chemistry, our results reinforce the notion that sphingosine participates in a cycle of phosphorylation/de-phosphorylation that governs the levels of the alcohol (sphingosine) and phosphate (S1P) forms and thus establishes the S1P tone of tissues. The ability of FTY720 to participate in this cycle to establish an exaggerated S1P tone probably underlies the high potency and efficiency of this drug. The existence of this cycle and its effect on lymphocyte trafficking might confound development of drugs that are sphingosine kinase inhibitors or S1P receptor antagonists.

FTY720-P and its active analog, AFD(*R*), are potent agonists at four S1P receptors, three of which (S1P<sub>1</sub>, S1P<sub>4</sub> and S1P<sub>5</sub>) are expressed by lymphocytes. Although pertussis toxin treatment of lymphocytes *ex vivo* demonstrates that a G protein signaling pathway in the lymphocyte is essential for the FTY720-

promoted homing response (7,8), S1P receptors on endothelial cells (including S1P<sub>1</sub> and S1P<sub>3</sub>) might participate in the process also. Thus only the S1P<sub>2</sub> receptor, at which FTY720-P and AFD(R) are inactive, is eliminated from contention. Likewise, we cannot know from present data what sphingosine kinase isoform or what phosphatase isoform are relevant to the metabolism of FTY720. Additional chemical entities and genetically-modified mice lacking one or more S1P receptor genes or sphingosine kinase genes are needed to define the FTY720 target(s) more precisely.

An interesting finding reported recently by Hla and colleagues (21) is that sphingosine kinase 1a can be released from cultured cells. Although their experimental system was necessarily artificial and its prediction requires confirmation, taken at face value it suggests the intriguing notion that the phosphorylation of sphingosine might be extracellular. Since the lipid phosphate phosphohydrolases are clearly ectophosphatases (22), the entire cycle might proceed in the extracellular compartment. Such a system would provide a route whereby lysophospholipid receptors could be accessed using orally available lipid alcohol compounds as exemplified by FTY720.

FTY720 is the first in a class of new immune system modulators that may allow both better management of allograft recipients and more effective treatment of patients with autoimmune disorders, which is a substantially unmet medical need. The drug is apparently less toxic than existing regimens and, in striking contrast to classical immunosuppressants, FTY720 did not impair immunity to systemic viral infection (4), suggesting that treatment with FTY720 could reduce the incidence of opportunistic infections in transplant patients. The drug may even be used to treat inflammatory processes associated with chronic viral infection, since in a model of viral myocarditis, FTY720 (but not cyclosporin), reduced inflammatory processes and pathology without accelerating virus replication (32). Very encouraging is the activity of FTY720 in models of autoimmune disorders such as EAE (Fig. 5), a model of human multiple sclerosis. Calcineurin inhibitors (cyclosporin, tacrolimus), which block T cell activation, are of limited use in the chronic management of such diseases. Perhaps the effects of FTY720 in EAE may relate to a direct effect on neuronal cells and/or oligodendrocytes expressing S1P receptors (33).

Activation of S1P receptors can antagonize apoptotic processes (21), which are associated with early stages of progressive neurodegenerative and demyelinating diseases (34,35).

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### Footnotes

1. abbreviations used: PTX pertussis toxin, GPCR G protein-coupled receptor, S1P sphingosine 1-phosphate, EAE experimental autoimmune encephalomyelitis
2. The International Union of Pharmacology subcommittee on lysophospholipid receptor nomenclature has recommended that the colloquial 'Edg' nomenclature be replaced with S1P (or LPA) subscript number, where the number indicates chronology of molecular cloning. Thus for S1P receptors, Edg-1 becomes S1P<sub>1</sub>, Edg-5 S1P<sub>2</sub>, Edg-3 S1P<sub>3</sub>, Edg-6 S1P<sub>4</sub>, and Edg-8 S1P<sub>5</sub>.
3. Work at the University of Virginia was supported by NIH grants (R01 GM52722, R01 CA88994) and a Research Contract Grant from Novartis Pharma AG to K.R.L as well as a NIH predoctoral fellowship (F31 GM64101) to M.D.D. We thank C. Wilt, C. Kristofic, C. Pally, C. Simeon, H. Wiegand, and J.-P. Baldeck of Novartis and R. Jarosz of the University of Virginia for excellent technical assistance.

4. While this paper was in review, a report (Mandala et al. (2002) *Science* online publication, 10.1121/science.1070238) appeared describing the metabolism of FTY720 in rodents and the agonist activity of FTY720-P at S1P receptors.

### Figure Legends

**Figure 1:** Structures – not shown are the *S* enantiomers of AAL and AFD.

**Figure 2:** A,B) *In vitro* kinase assay with recombinant mouse sphingosine kinase 1a using FTY720, AAL(*R*), AAL(*S*) and sphingosine as substrates. The phosphorylated compounds, which were labeled with phosphorus-32, were detected by autoradiography. The substrate alcohols were detected by spraying the t.l.c. plate with Fluram® (a fluorescent dye that binds to compounds with a primary amine) followed by photography with UV illumination. Thus the image shown in A or B is a composite of two images. The compound used as a substrate as well as the concentration (in  $\mu\text{M}$ ) is indicated underneath the figure, 10  $\mu\text{M}$  sphingosine was used as a reference point. No phosphorylated form(s) was detected in this *in vitro* assay when omitting either the enzyme or the substrates. N gives a normalization control for equal loading / extraction after “staining” the thin layer chromatography plate with Fluram®. Conversion rates of sphingosine to S1P ranged – depending on the enzyme preparation and the assay conditions – between 0.5 % and 10 % in this assay.. The autoradiogram in B represents an approximately 50-fold longer exposure time than that presented in A. C) The fate of [ $^3\text{H}$ ]-FTY720 in various mouse tissues (normalized on a cell basis of  $10^6$  cells). Shown are autoradiograms of thin layer chromatography plates whereby FTY720 and its phosphorylated form were resolved; the positions of FTY720 and FTY720-P are indicated at the right. Further details are provided in Methods.

**Figure 3:** Concentration response curves of compounds at the recombinant human S1P<sub>4</sub> receptor. A membrane-based GTP[ $\gamma$ - $^{35}\text{S}$ ] binding assay was used to determine the relative potencies and efficacies of the compounds. Each point represents triplicate measurements and these data are representative of those used to generate the values presented in Table 2. Symbols: ○ AFD(*R*), △ FTY720-P, ■ S1P, ▲ FTY720, ◆ AAL(*R*), ▼ AAL(*S*), □ AFD(*S*)

**Figure 4:** Dose-dependent depletion of circulating peripheral blood lymphocytes in rats. Phosphorylated compounds (panel b) were administered at 1 mg/kg. In all cases, blood was drawn six hours after drug administration. Symbols: ◆ FTY720, ▲ AAL(R), ● AAL(S), ○ sphingosine

**Figure 5:** Prophylactic effect of FTY720, AAL(R) and AAL(S) in EAE in rats. Compounds were administered orally to rats at 0.3 mg/kg/day Disease symptom grade: 1, flaccid tail; 2, hind limb weakness or ataxia; 3, full paralysis of hind limbs. Symbols: ○ vehicle, ● FTY720, ■ AAL(R), ▼ AAL(S)

**Table 1:** Metabolism of FTY720 and FTY720-P in rodents.

Compound administered	Dose (mg/kg)	Compound detected (ng/ml) <sup>a</sup>							
		FTY720				FTY720-P			
		3h	8h	24h	72h	3h	8h	24h	72h
FTY720 <sup>b</sup>	7.5	85	133	73	24	179	383	437	109
FTY720 <sup>c</sup>	0.1	Nd	Nd	1.15	Nd	Nd	Nd	Nd	Nd
“	0.3	Nd	Nd	6.20	Nd	Nd	Nd	Nd	Nd
FTY720-P <sup>c</sup>	0.1	Nd	Nd	0.15	Nd	Nd	Nd	Nd	Nd
“	0.3	Nd	Nd	1.06	Nd	Nd	Nd	Nd	Nd
“	1.0	Nd	Nd	4.91	Nd	Nd	Nd	Nd	Nd

<sup>a</sup> Compound was detected by after h.p.l.c. separation after extraction from blood pools from 3 animals; <sup>b</sup> FTY720 administered to Wistar rats (p.o.) or to <sup>c</sup> C3H mice (i.p.); Nd, not determined

**Table 2:** Potency (pEC<sub>50</sub>) and efficacy (E<sub>max</sub>) values of phosphorylated compounds at human S1P receptors.

Drug:		S1P	FTY720-P	AFD (R)	AFD (S)
Receptor:	Parameter:				
S1P <sub>1</sub>	pEC <sub>50</sub> <sup>a</sup>	7.1 (7.0-7.3) <sup>b</sup>	8.2 (8.1-8.2)	8.6 (8.4-8.8)	<5
	E <sub>max</sub>	1.00	0.92 (0.84-1.03)	0.92 (0.84-1.03)	—
S1P <sub>3</sub>	pEC <sub>50</sub>	8.7 (8.4-8.9)	8.4 (7.8-9.4)	8.4 (7.8-9.4)	<5
	E <sub>max</sub>	1.00	0.34 (0.27-0.38)	0.34 (0.27-0.38)	—
S1P <sub>4</sub>	pEC <sub>50</sub>	6.1 (5.9-6.1)	7.2 (6.6-7.9)	8.4 (8.3-8.4)	<5
	E <sub>max</sub>	1.00	0.70 (0.62-0.75)	1.01 (0.92-1.09)	—
S1P <sub>5</sub>	pEC <sub>50</sub>	7.7 (7.4-8.0)	8.2 (7.2-9.1)	8.9 (8.0-9.7)	<5
	E <sub>max</sub>	1.00	0.61 (0.56-0.66)	0.64 (0.57-0.68)	—

<sup>a</sup> pEC<sub>50</sub>, -log molar concentration of compound resulting in 50% of maximal GTPγS binding; E<sub>max</sub>, maximal GTP[γ-<sup>35</sup>S] binding as a fraction of S1P signal (set at 100%=1.00); <sup>b</sup> average value of 3-6 experiments (range of values)

**Table 3:** Induction of apoptosis in human CD4<sup>+</sup> T cells

Compound:	Apoptosis (%) in the presence of compound				
concentration:	30 μM <sup>a</sup>	10 μM	3 μM	1 μM	0.3 μM
FTY720	24.0	12.7	0.9	1.4	0
FTY720-P	0.4	0.8	0.7	0.7	1.8

AAL( <i>R</i> )	33.7	10.6	2.6	0.6	0.5
AFD( <i>R</i> ), phos <sup>b</sup> .	2.6	1.0	0	1.2	1.3
AAL( <i>S</i> )	34.9	13.1	2.9	0.4	2.4
AFD( <i>S</i> ), phos.	1.3	0.9	1.6	0.6	0.2

<sup>a</sup>Data are representative of three experiments, <sup>b</sup>phos. = phosphorylated compound

Figure 1

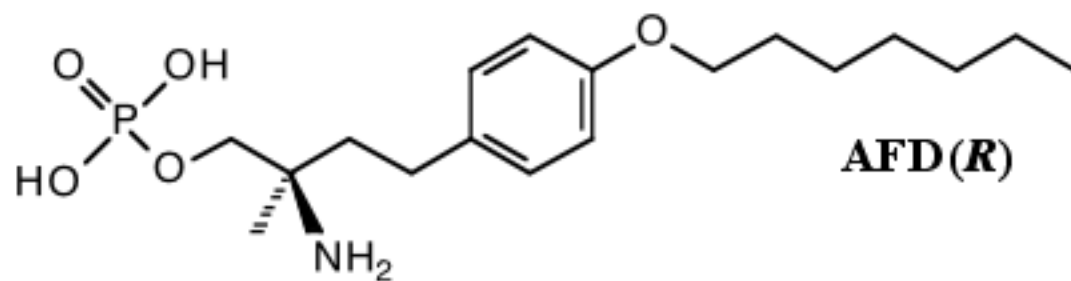
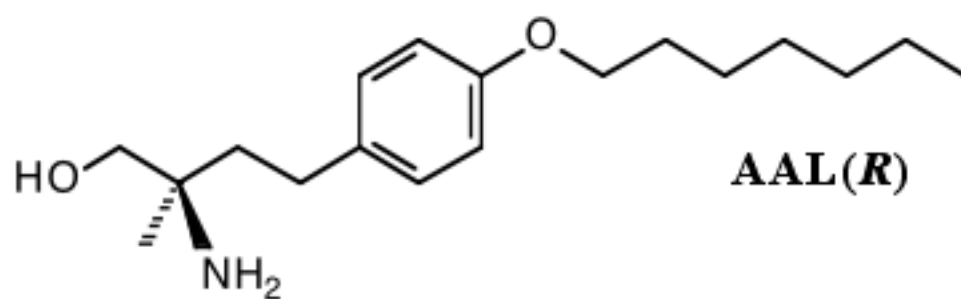
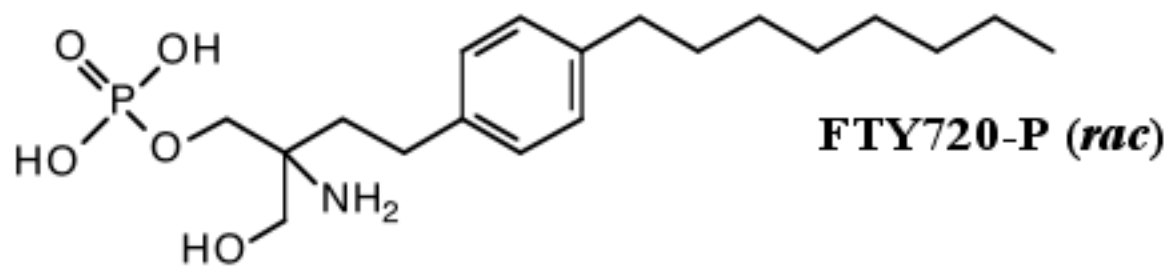
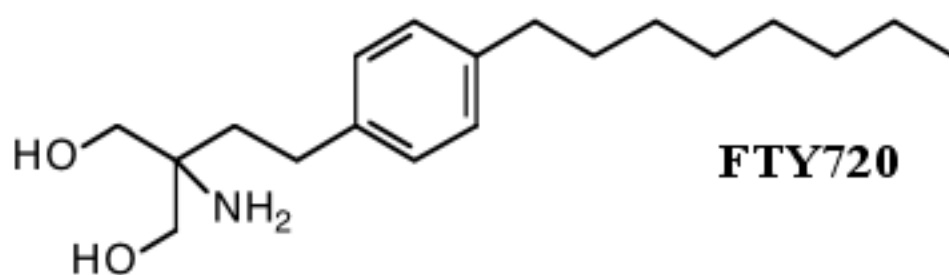
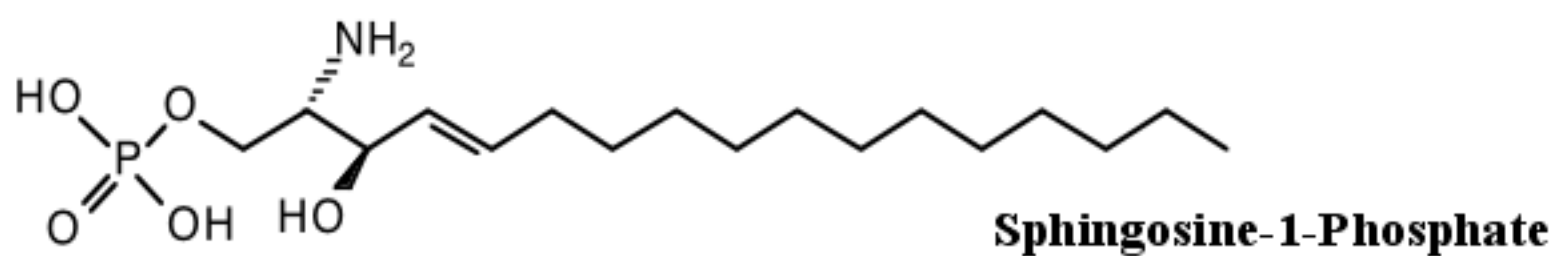
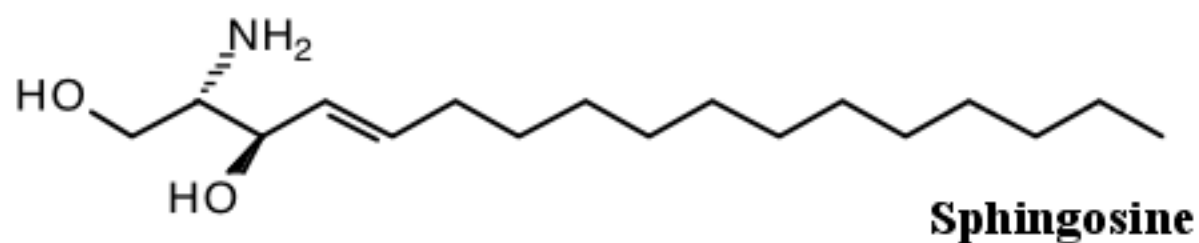
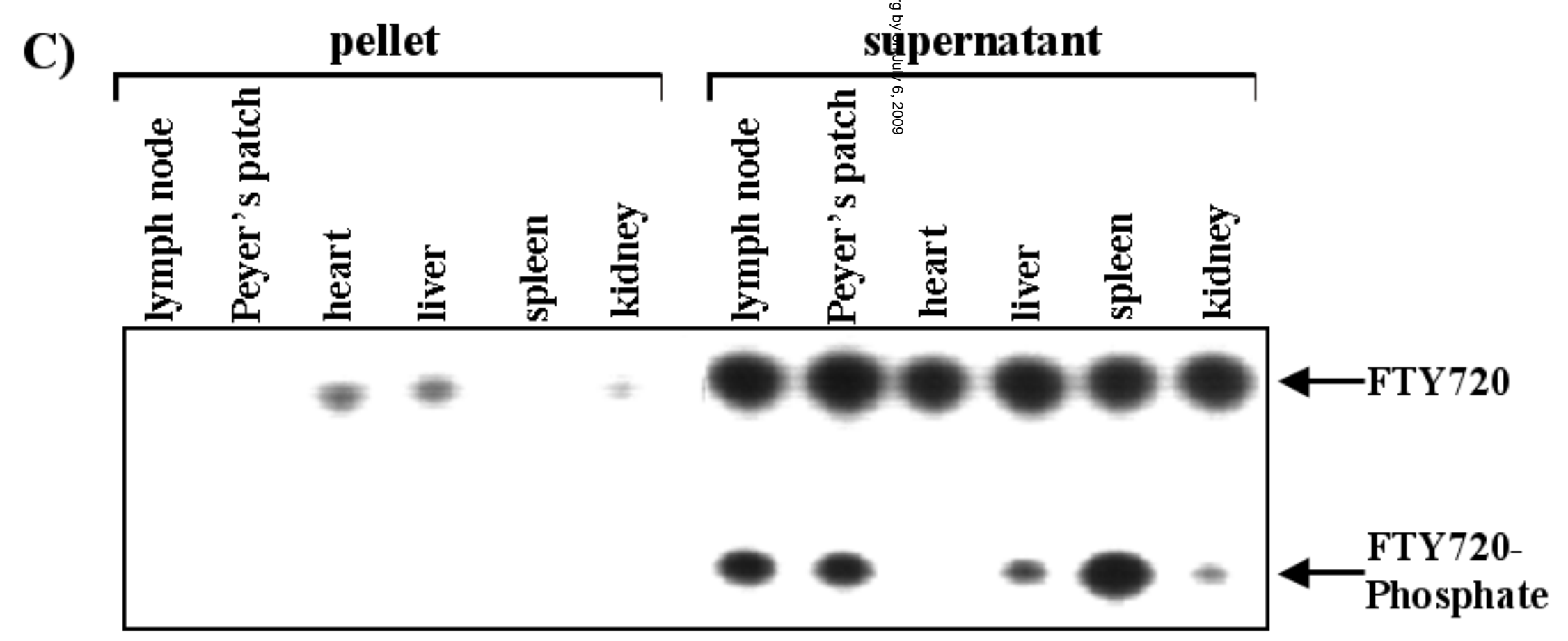
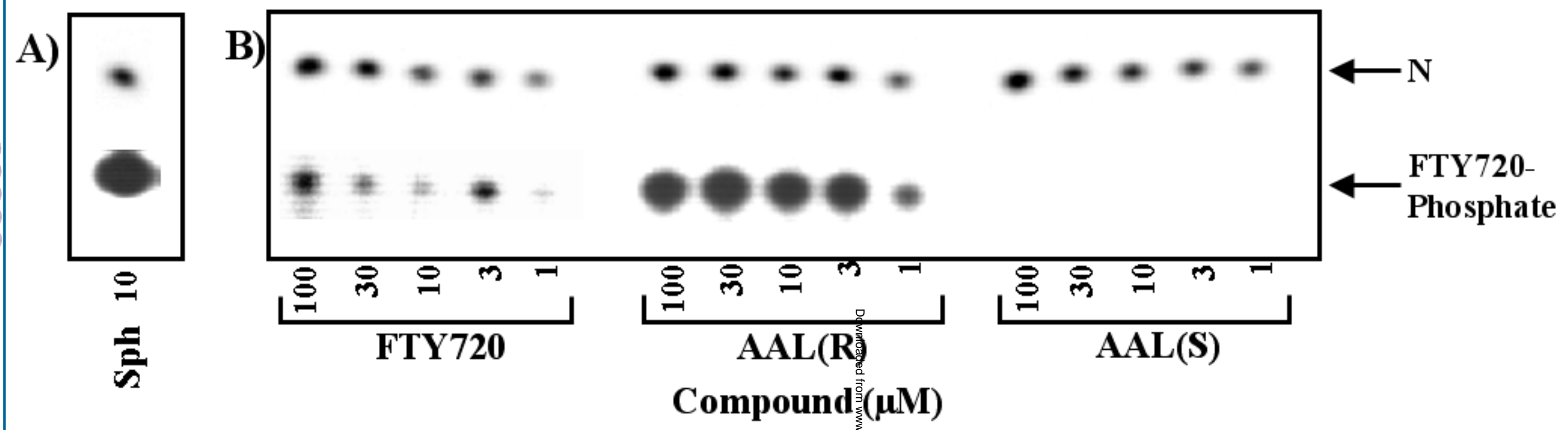
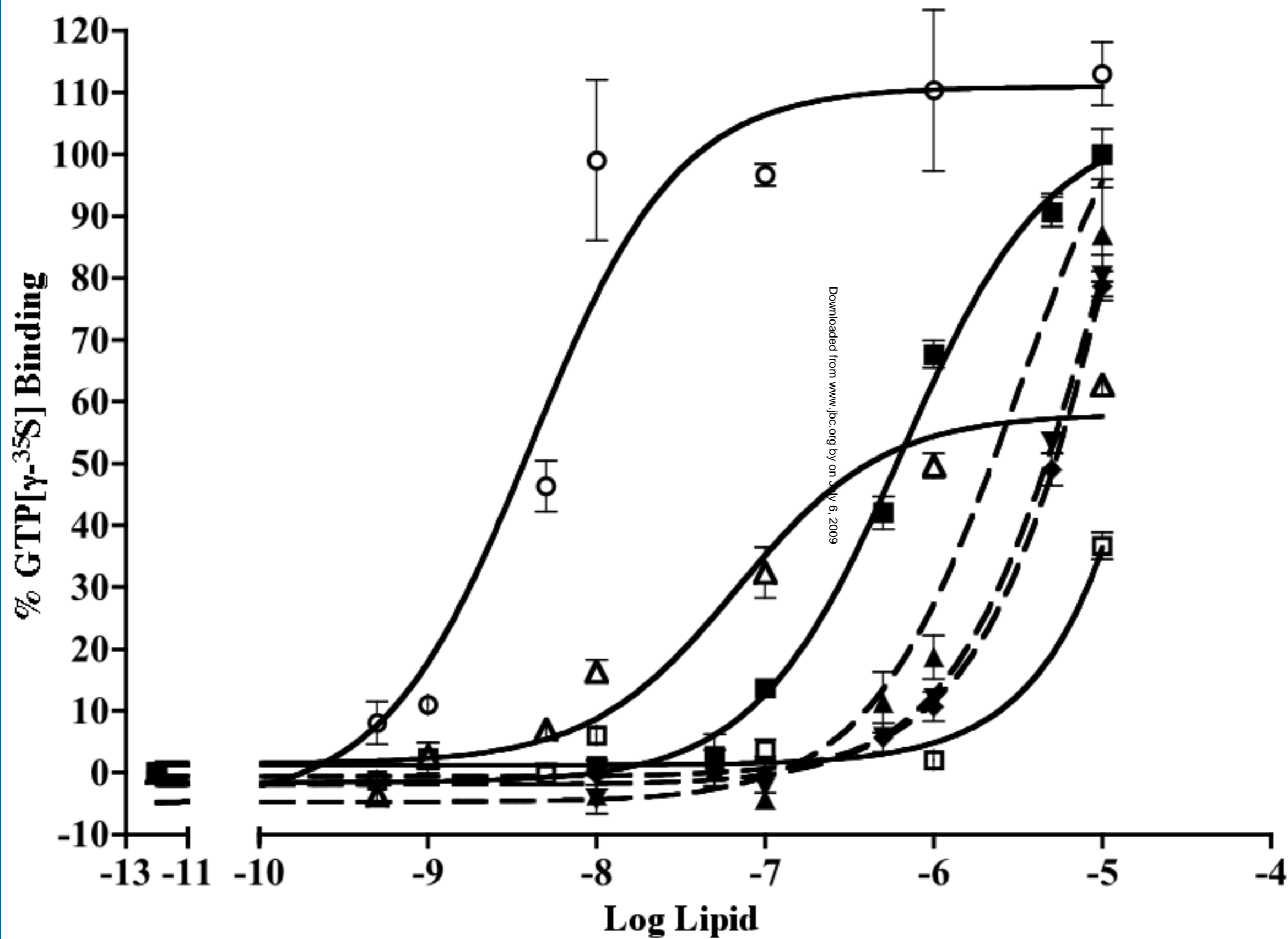


Figure 2



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Figure 3



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Figure 4

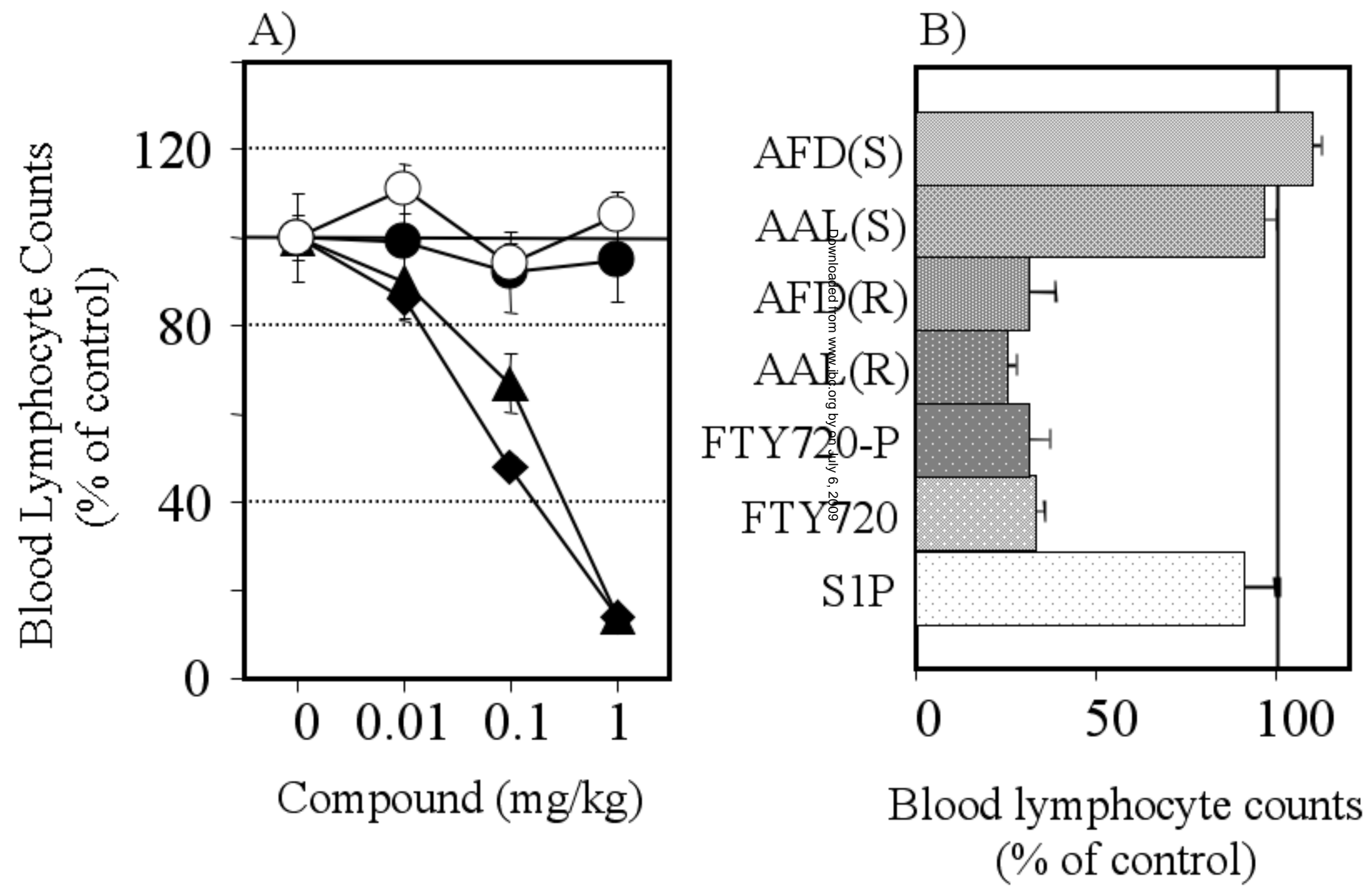


Figure 5

