



Published in final edited form as:

*J Pharmacol Sci.* 2017 March ; 133(3): 187–189. doi:10.1016/j.jphs.2017.02.006.

## FTY720-derivatives do not induce FTY720-like lymphopenia

Ismael Segura-Ulate, Troy K. Belcher, Guadalupe Vidal-Martinez, Javier Vargas-Medrano, Ruth G. Perez\*

Department of Biomedical Sciences, Graduate School of Biomedical Sciences, Center of Emphasis in Neurosciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center El Paso, El Paso, TX, United States

### Abstract

FTY720 is an immunosuppressive multiple sclerosis (MS) drug that stimulates the expression of neuroprotective brain-derived-neurotrophic-factor (BDNF). In vivo preclinical data suggest that FTY720 could be beneficial for treating Parkinson's patients, though its immunosuppressive effects might limit its efficacy. Two novel FTY720-derivatives, FTY720-C2 and FTY720-Mitoxo, also stimulate BDNF expression and enter brain like FTY720 but are not phosphorylated, suggesting they will not produce FTY720-like immunosuppression. Using FTY720 as a positive control, we measured low and high dose FTY720-derivatives, which did not stimulate FTY720-like lymphopenia or immunosuppressive signaling. These findings support the further preclinical assessment of the derivatives as potential novel Parkinson's therapies.

### Keywords

Anti-inflammatory; Fingolimod; Neuroprotection

Parkinson's disease (PD) is a neurodegenerative disorder in which motor symptoms develop after the loss of substantia nigra dopaminergic neurons (1). The substantia nigra in PD brain also exhibits loss of brain-derived neurotrophic factors (BDNF); while increasing BDNF in PD models protects nigral neurons (2). Furthermore, PD duration correlates with low serum BDNF levels, and exercise can stimulate BDNF expression which may help slow PD progression (3). New data reveal that treating A53T parkinsonian mice with FTY720 (fingolimod) increases gut BDNF protein and mRNA levels while reducing constipation (4), a common premotor PD symptom. In that same report, blocking BDNF signaling in mice accelerates constipation and  $\alpha$ -synuclein aggregation that is counteracted by FTY720 (4). Moreover, FTY720 significantly improves motor behavior and reduces dopaminergic neurotoxicity in cell and mouse PD models (5,6). Thus, therapies that increase endogenous BDNF levels hold promise for treating both non-motor and motor symptoms of PD. Such therapies may also slow disease progression; though there are currently no BDNF-

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\*Corresponding author. Fax: +1 915 783 1230. ruth.g.perez@ttuhsc.edu (R.G. Perez).

Conflict of interest statement

The corresponding author has filed a patent, "Compositions and Methods for the Treatment of Parkinson's Disease", US 20150290145, CA 2888634, which does not alter adherence to Journal of Pharmacological Sciences policies.

stimulatory drugs in the clinic. Many laboratories have shown that FTY720 improves endogenous BDNF expression, promotes neuroprotection, and enhances neurogenesis in animal models (4,5,7–10). Furthermore, FTY720 increases BDNF in dopaminergic cells and protects them from neurotoxicity (5,11). Additionally, upregulating BDNF with FTY720 in a Rett syndrome mouse model (10), encouraged fast-tracking of FTY720 to treat Rett syndrome children ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02061137) identifier [NCT02061137](https://clinicaltrials.gov/ct2/show/study/NCT02061137)), raising the possibility that the neurotrophin stimulating activity of FTY720 could benefit patients with PD.

While much recent work has focused on BDNF modulation by FTY720, the drug was originally approved as an immunomodulator that reduces ~80% of circulating blood lymphocytes (12,13), producing a condition called *lymphopenia*. FTY720-induced lymphopenia results after the prodrug is phosphorylated by ubiquitous sphingosine kinases to form FTY720-P, a sphingosine-1 phosphate analog (14). FTY720-P can bind sphingosine-1 phosphate receptor 1 (S1PR<sub>1</sub>) on T cells to prevent their egress from lymphoid tissues into circulating blood (13). This has long-been considered the main therapeutic effect of FTY720 for treating autoimmune-related neurological disorders like MS (12) or chronic-inflammatory-demyelinating-polyneuropathy ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01625182) identifier [NCT01625182](https://clinicaltrials.gov/ct2/show/study/NCT01625182)). And though more than 130,000 MS patients worldwide have safely used FTY720; the immunosuppressive effects of the drug can lead to reactivation of dormant infections or a more serious condition, progressive multifocal leukoencephalopathy. This activity contraindicates FTY720 use in certain individuals (12). It also raises concern about using FTY720 to stimulate BDNF expression in patients with non-autoimmune conditions, like PD. This makes it important to identify new compounds with FTY720-like neuroprotective benefits that will not induce lymphopenia.

We have created two FTY720-derivatives with modifications adjacent to the site that is phosphorylated on FTY720 (11), and both retain many positive characteristics of FTY720. For example, all three FTY720s stimulate BDNF expression in dopaminergic cells and protect against neurotoxicity (11). Like FTY720, both new derivatives also cross the brain blood barrier, yet unlike FTY720, neither is phosphorylated in human or rat liver hepatocytes (15). As mentioned above, phosphorylation of FTY720 leads to lymphopenia (13). Here we measured the effects of low dose and high dose FTY720-C2 and FTY720-Mitoxo on lymphopenia. Briefly, 6–12 week old C57BL/6 and Swiss Webster male and female mice were given single equivalent low molar doses by i.p. injection (1.25 mg/kg FTY720, 1.25 mg/kg FTY720-C2 or 2.5 mg/kg of FTY720-Mitoxo; 3 mice per drug, N = 9) modeling Morris et al. (13); or 10× subcutaneous high doses (12.5 mg/kg FTY720-C2 or 25 mg/kg of FTY720-Mitoxo; to 3 mice per drug, N = 6 total) to eliminate hepatic first pass effects. Blood was collected by tail-vein puncture into 4 heparinized micro-hematocrit tubes (VWR Micro Capillary Tubes, Radnor, PA, USA, 15401–560) per mouse per time point. Blood was collected just before drug (0 h) and at 24 h (Fig. 1A). Blood smears were prepared on glass microscope slides, air dried, and Wright-stained (Thermo Fisher, USA, Cat. 3121TS) (Fig. 1B). Lymphocytes and neutrophils were manually counted by bright field analysis of blood smear monolayers. For low dose experiments we counted 3 independent 2 mm<sup>2</sup> fields on 3–5 slides per mouse per time point (0 h and 24 h). For high dose experiments we counted 100 total lymphocytes and neutrophils on 3–5 slides per mouse per time point (0 h and 24 h). Each set of data was evaluated by two different investigators, one of whom was

blinded to treatment conditions. Data were analyzed using Student's t-tests and normalized to % control  $\pm$  SD for all experiments (GraphPad Prism 6, San Diego, CA, USA).

As a positive control we used a dose of FTY720 that causes ~80% reduction in circulating lymphocytes at 24 h but has little effect on neutrophils (Fig. 1C, right data set), closely replicating the work of Morris et al. (13). Equivalent low molar doses of FTY720-C2 (Fig. 1C, left data set) or FTY720-Mitoxy (Fig. 1C, middle data set) also had little effect on lymphocytes or neutrophils. To eliminate hepatic first-pass metabolic effects related to i.p.-delivered drugs, we used 10 $\times$  higher doses of FTY720-C2 and FTY720-Mitoxy, delivered subcutaneously. Overall, the high dose data (Fig. 1D) were similar to low dose data for both novel FTY720-derivatives (Fig. 1C), except that high dose FTY720-Mitoxy (Fig. 1D, right data set) reduced lymphocyte numbers by ~20%, which though significant, is within the normal range and far below the 80% loss induced by FTY720. High doses of FTY720-C2 and FTY720-Mitoxy were also well tolerated in mice.

Additionally, we evaluated FTY720-C2 and FTY720-Mitoxy in short term cell culture experiments in which we measured activation of the S1PR<sub>1</sub> downstream kinase, ERK1/2 (10). In dopaminergic MN9D cells we modeled the work of Hait et al., who teased apart the effects of FTY720-P from FTY720 with regard to ERK1/2 (16). Briefly, MN9D cells were seeded  $6 \times 10^5$  onto 6 well plates then treated 16–18 h later with Vehicle, or 1  $\mu$ M FTY720-P, FTY720, FTY720-C2 or FTY720-Mitoxy prepared in the same vehicle solution. At 30 min, cells were lysed and equivalent amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked, then incubated overnight in anti-pERK1/2 (Tyr204) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat. sc-7383) followed by anti-total ERK1/2 (Santa Cruz Biotechnology, Cat. sc-93) to quantify pERK1/2 to total ERK1/2 levels. Blots were imaged using Odyssey (LiCor Biosciences, Lincoln, NE, USA) and quantified using Image-Quant (GE Healthcare, Little Chalfont, UK). ERK1/2 data from 4 independent experiments were analyzed by ANOVA, using Dunnett's multiple comparisons test for post-hoc analysis (Prism 6, GraphPad Inc.). This allowed us to show that while the positive control, FTY720-P, significantly increased pERK1/2 levels; unphosphorylated FTY720 and FTY720-derivatives did not significantly increase pERK1/2 levels (Fig. 2). These results also suggest that FTY720-C2 and FTY720-Mitoxy do not stimulate S1PR<sub>1</sub> signaling in our cells, similarly to what Hait and colleagues saw using this method (16).

We previously demonstrated that all three FTY720s increase BDNF mRNA (11) and our newly published data show that S1PR<sub>1</sub>-signaling through ERK1/2 activation is not necessary for FTY720-mediated increases in BDNF expression (17). This is also supported by our current findings in which only the positive control, FTY720, caused lymphopenia in vivo (Fig. 1C). As FTY720-mediated S1PR<sub>1</sub> activation can also cause first-dose bradycardia in patients, our findings with FTY720-C2 and FTY720-Mitoxy further suggest that neither is likely to produce that effect. Finally, as both FTY720-C2 and FTY720-Mitoxy cross the blood brain barrier (15), they also have the potential to protect brain regions susceptible to neurodegeneration, such as substantia nigra in PD or frontal cortex in patients suffering from neuropathic pain. Taken together, the data encourage further preclinical assessment of

FTY720-C2 and FTY720-Mitoxo for disorders where stimulating BDNF expression may counteract neuropathology and slow disease progression.

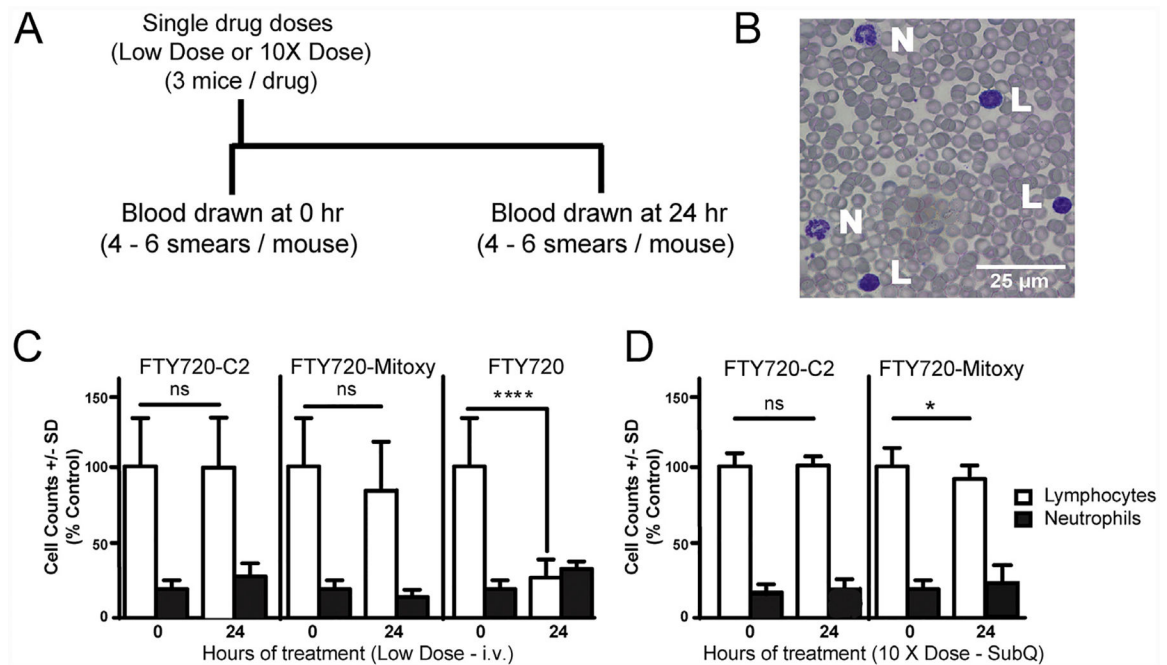
## Acknowledgements

The authors thank Texas Tech University Health Sciences Center El Paso SABR Program (183119) (to TKB), and Hoy Family Research (243337) and Lizanell and Colbert Coldwell Foundation (243036) (RGP) for their generous support. We also thank Tania Fertl of the TTUHSC LARC for help with drug delivery and blood collection. This work is dedicated to D. Byer and M. J. Fox, and in loving memory of S. M. Hoy, J. Cordy, and L. “Rusty” Lanelli.

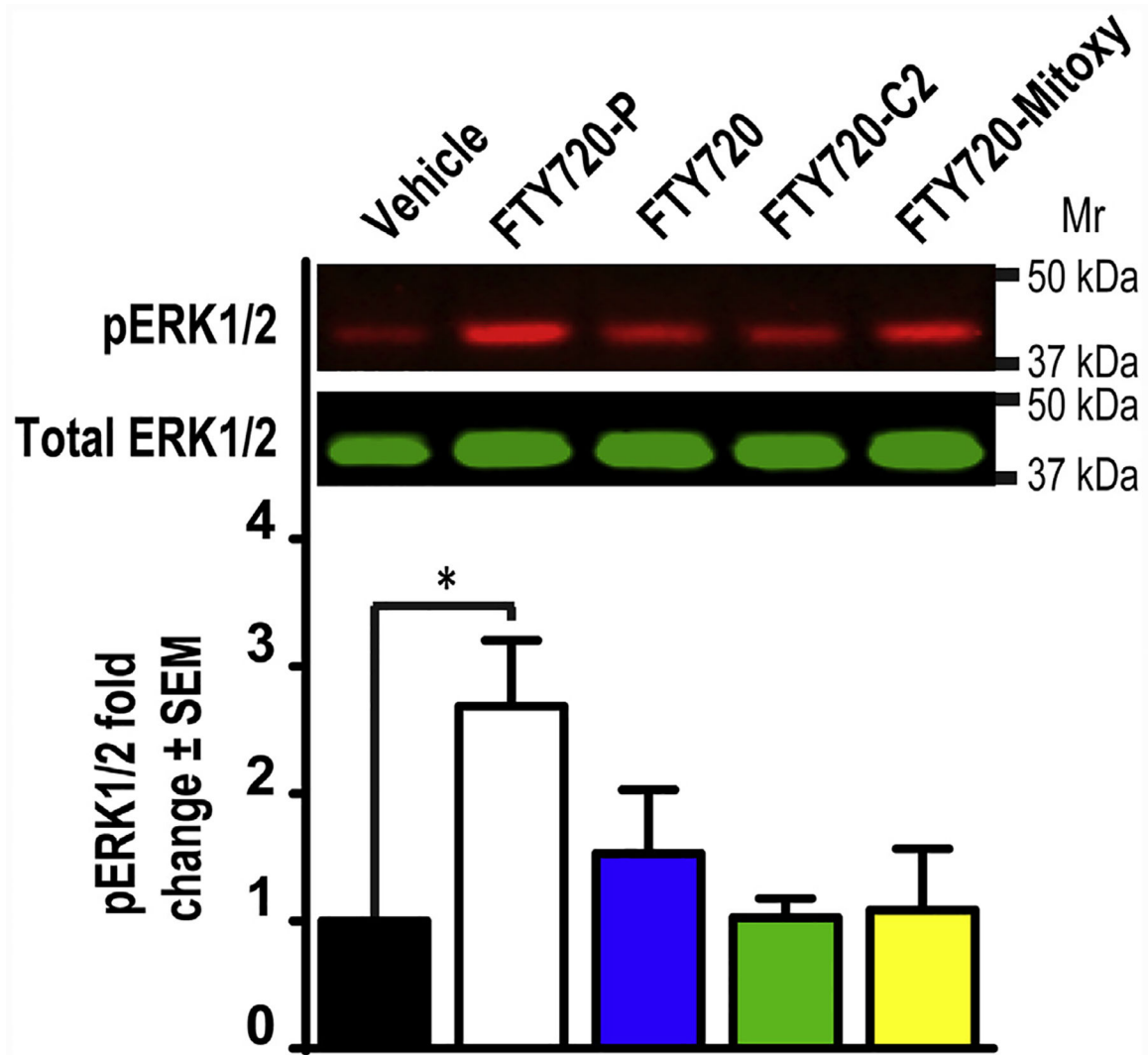
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**Fig. 1. FTY720-C2 or FTY720-Mitoxy treatment does not produce FTY720-like lymphopenia.** **A.** Timeline of drug dosing and blood collection for mice treated with low dose or high dose FTY720 or FTY720-derivatives. **B.** Bright field microscopic image from a representative blood smear in our studies, showing Wright-stained lymphocytes (L) and neutrophils (N) among many red blood cells in a single microscopic field. **C.** Cell counts from mice ( $n = 3$ /condition) treated with i.p. delivery of various FTY720s. FTY720, as a positive control, confirms that it can significantly decrease lymphocyte numbers in the blood within 24 h. No significant change in lymphocytes or neutrophils is noted in mice given low equivalent molar doses of intravenously delivered FTY720-C2 or FTY720-Mitoxy. **D.** Blood cell counts from mice ( $n = 3$ /condition) given high dose FTY720-C2 or FTY720-Mitoxy by subcutaneous delivery to avoid first-pass effects. No significant change in lymphocytes or neutrophils is noted after high dose FTY720-C2, and though lymphocyte numbers were significantly lower after high dose FTY720-Mitoxy, the effect does not replicate FTY720-associated lymphopenia. C and D, white bars = lymphocytes; black bars = neutrophils. SubQ = subcutaneous dosing. Scale bar = 25  $\mu$ m. Student's t-tests. Data represent the mean  $\pm$  SD. \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ ; ns = not significant.



**Fig. 2. Only FTY720-P significantly activates the S1PR1-downstream-kinase, ERK1/2 in 30 min treatments.**

Representative immunoblots of MN9D lysates after 30 min treatment with Vehicle (control), 1  $\mu$ M of FTY720-P, FTY720, FTY720-C2, or FTY720-Mitoxy prepared in the same control solution. ERK1/2 activation was assessed by probing immunoblots for pERK1/2 normalized to total ERK1/2. The histogram shows that only FTY720-P significantly increases pERK1/2 at the 30 min timepoint. Data were analyzed by ANOVA using Dunnett's multiple comparisons test for post-hoc analysis. Data represent the mean  $\pm$  SEM of 4 independent experiments. \* $p < 0.05$ .