1. Title Page

Sphingosine 1-phosphate Receptor Modulator ONO-4641 Regulates Trafficking of T Lymphocytes and Hematopoietic Stem Cells and Alleviates Immune-Mediated Aplastic Anemia in a Mouse Model

Takaki Komiya, Masashi Gohda, Hiroki Shioya, Seishi Katsumata

Discovery Research Laboratories, Ono Pharmaceutical Co., Ltd., Mishima-gun, Osaka, Japan
2. Running Title

ONO-4641 Alleviates Mouse Immune-Mediated Aplastic Anemia

Takaki Komiya

Discovery Research Laboratories, Ono Pharmaceutical Co., Ltd.

Address: 3-1-1 Sakurai, Shimamoto-cho, Mishima-gun, Osaka, 618-8585, Japan

TEL: 81-75-961-1151, FAX: 81-75-961-1171

E-mail: t.komiya@ono.co.jp

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A list of nonstandard abbreviations used in the paper (in alphabetical order).

BM, bone marrow; HSPC, hematopoietic stem and progenitor cell; LN, lymph node; MS, multiple sclerosis; ONO-4641, 1-{6-[(2-methoxy-4-propylbenzyl)oxy]-1-methyl-3,4-dihydropyridin-2-yl}methyl)azetidine-3-carboxylic acid; PLTs, platelets; RBCs, red blood cells; S1P, Sphingosine 1-phosphate; S1PR, S1P Receptor; TBI, total body irradiation; WBCs, white blood cells; 0.5%MC, 0.5w/v% Methyl Cellulose 400 cP Solution
A recommended section assignment to guide the listing in the table of contents:

Immunopharmacology
3. Abstract

ONO-4641 is a second-generation sphingosine 1-phosphate (S1P) receptor modulator that exhibits selectivity for S1P receptors 1 and 5. Treatment with ONO-4641 leads to a reduction in magnetic resonance imaging disease measures in patients with relapsing-remitting multiple sclerosis. The objective of this study was to explore the potential impact of ONO-4641 treatment based on its immunomodulatory effects. Severe aplastic anemia is a bone marrow (BM) failure disease, typically caused by aberrant immune destruction of blood progenitors. Although the T helper type-1-mediated pathology is well described for aplastic anemia, the molecular mechanisms driving disease progression remain undefined. We evaluated the efficacy of ONO-4641 in a mouse model of aplastic anemia. ONO-4641 reduced the severity of BM failure in a dose-dependent manner, resulting in higher blood and BM cell counts. By evaluating the mode of action, we found that ONO-4641 inhibited the infiltration of donor-derived T lymphocytes to the BM. ONO-4641 also induced the accumulation of hematopoietic stem cells in the BM of model mice. These observations indicate, for the first time, that S1P receptor modulators demonstrate efficacy in the mouse model of aplastic anemia and suggest that treatment with ONO-4641 might delay the progression of aplastic anemia.
4. Significance Statement

ONO-4641 is a second-generation sphingosine 1-phosphate (S1P) receptor modulator selective for S1P receptors 1 and 5. In this study, we demonstrated that ONO-4641 regulates the trafficking of T lymphocytes along with hematopoietic stem and progenitor cells leading to alleviation of pancytopenia and destruction of bone marrow in a bone marrow failure-induced mouse model mimicking human aplastic anemia.
5. Introduction

Sphingolipid 1-phosphate (S1P), a product of membrane sphingolipid metabolism, is a bioactive lipid involved in the regulation of various cellular processes including cell proliferation, cell migration, actin cytoskeletal reorganization, and cell adhesion (Hla, 2005). There are five known G protein-coupled S1P receptors (S1PR1-S1PR5) that respond to extracellular S1P (Cartier and Hla, 2019). Fingolimod (FTY720) is a first-in-class S1PR-targeted drug approved for the prevention of relapsing forms of multiple sclerosis (MS) (Cohen et al., 2010; Kappos et al., 2010), and has led to the expansion of research and drug development efforts targeting S1PRs. FTY720 is a non-selective S1PR modulator acting against four S1PRs (S1PR1, S1PR3, S1PR4, and S1PR5) (Brinkmann et al., 2002). Recently, second-generation S1PR modulators, namely, siponimod (Kappos et al., 2018) and ozanimod (Cohen et al., 2019) that exhibit a higher degree of selectivity for S1PR1 and S1PR5 have been approved for the treatment of MS. The therapeutic effects of these drugs are considered to be mediated by S1PR1 activation, leading to sustained internalization and desensitization and, in turn, functionally suppression of S1PR1 signaling (Oo et al., 2007). In contrast, cardiovascular, hepatic, and respiratory side-effects were also observed upon treatment with FTY720 (Meissner and Limmroth, 2016).

ONO-4641 (ceralifimod) is also a second-generation S1PR modulator selective for S1PR1 and S1PR5. S1PR modulators such as FTY720 and ONO-4641 cause the S1PR1 down-regulation on lymphocytes, which renders lymphocytes unresponsive to the S1P gradient in secondary lymphoid tissues. As a consequence, S1PR modulators decrease peripheral blood lymphocyte counts by inhibiting egress of lymphocytes from secondary lymphoid tissues.
(Matloubian et al., 2004; Komiya et al., 2013; Kurata et al., 2017). The phase II clinical trial DreaMS study (Drug Research Evaluation for MS; NCT01081782) demonstrated that ONO-4641 (0.05, 0.1, and 0.15 mg once daily) significantly reduced magnetic resonance imaging (MRI) disease measures across all doses in patients with relapsing-remitting MS and reduced the annualized relapse rate by 6 months in MS patients treated with the 0.1 mg dose (Zipp et al., 2013; Vollmer et al., 2013). The study further demonstrated a dose-dependent lymphocyte count reduction from baseline established at week 2: 40%, 60%, and 65% with the administration of 0.05, 0.1, and 0.15 mg/day, respectively. The efficacy of ONO-4641 was demonstrated in a phase II clinical trial for the treatment of MS. As ONO-4641 demonstrated the reduction of lymphocyte infiltration in clinical study in MS, ONO-4641 is considered to be also effective in other lymphocyte-mediated diseases.

Severe aplastic anemia is an acquired BM failure syndrome (Young, 2018). Evidence in the majority of cases suggests that a breakdown in self-tolerance leads to the infiltration of destructive T helper type-1 (Th1) lymphocytes into the BM, where they target hematopoietic stem cells and compromise stromal cells via bystander effects (Chen et al., 2005; Young et al., 2008). As a result, the population of hematopoietic stem and progenitor cells (HSPCs) in the BM is destroyed. Without the ability to replenish platelets and red and white blood cells, patients with aplastic anemia are at an increased risk of bleeding episodes, hypoxia, and infection. If left untreated, aplastic anemia is uniformly fatal (Dezern and Brodsky, 2011).

Although the pathology of aplastic anemia is well defined, the molecular mechanisms that drive disease progression remain to be elucidated. Unlike other autoimmune diseases such
as MS, an inciting self-antigen has not been identified for aplastic anemia. However, mouse models of immune-mediated BM failure have been developed successfully by transferring parental splenocytes or lymph node (LN) cells into minor histocompatibility- or major histocompatibility-mismatched recipients, followed by robust immune responses that target BM cells and HSPCs (Bloom et al., 2004; Chen, 2005; Chen et al., 2007). The utility of such aplastic anemia mouse models is well established. Mice exhibit many of the clinical features of aplastic anemia and provide an excellent system for studying the underlying mechanisms of the disease as well as testing the efficacy of potential therapeutics (Bloom et al., 2004; Chen et al., 2007). In such a model, massive lymphocyte infiltration in the BM led to the rapid destruction of HSPCs. It has been demonstrated that S1P acts as a chemoattractant for HSPCs. Their egress from extramedullary tissues depends on S1PR1 (Massberg et al., 2007).

In this study, we evaluated the efficacy of ONO-4641 in a mouse model of aplastic anemia generated by infusing parental LN cells into the irradiated recipient mice. The efficacy of ONO-4641 when combined with cyclosporine, an immunosuppressive drug was also investigated. ONO-4641 regulates the trafficking of T lymphocytes and HSPCs and alleviates pancytopenia and the destruction of BM in this model. Our results confirm the therapeutic benefits of ONO-4641, implying its potential as a novel therapeutic agent for aplastic anemia.
6. Materials and Methods

**Chemicals** ONO-4641 (more than 95% purity as determined by chiral high-performance liquid chromatography) was provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Cyclosporine was obtained from Novartis (Switzerland) as Neoral Oral Solution 100 mg/ml. Plerixafor was obtained from Sigma-Aldrich (MO, USA).

**Peripheral Blood Lymphocyte Counts in Mice** CBF1 mice were administered a single oral dose of 0.01, 0.03, 0.1, or 0.3 mg/kg of ONO-4641 or 0.5w/v% Methyl Cellulose 400 cP Solution (0.5%MC; WAKO, Osaka, Japan) (vehicle). At 24 h after the administration, blood was collected from the abdominal vena cava of the mice for the measurement of peripheral blood lymphocyte count with an automated hematology analyzer (SF-3000, Sysmex Corporation, Hyogo, Japan).

**Experimental Aplastic Anemia Model** Inbred C57BL/6NCr (B6) and hybrid (B6 and BALB/c) F1 (CBF1) mice were obtained from Japan SLC Inc. (Shizuoka, Japan). Male mice of 8 to 10 weeks of age were used in each experiment. Based on the national regulations and guidelines, animal experiments were reviewed by the Institutional Animal Care and Use Committee and finally approved by the director of the research institution. The animal experiments were performed in accordance with the regulations for animal experiments of Ono Pharmaceutical Co., Ltd.

CBF1 recipient mice were subjected to 4 Gy total body irradiation (TBI) from an X-ray irradiation device (model MBR-1520R-3, Hitachi Power Solutions, Ibaraki, Japan). BM
failure was induced 5 to 8 h later, by administering 5 x 10^6 LN cells with an intravenous injection. The LN cells were harvested from B6 donor mice. Inguinal, brachial, axillary, and mesenteric LNs were obtained for the experiments. In each experiment, mice that received 4 Gy TBI only and TBI plus LN cell infusion with 0.5%MC administration were used as controls. Mice were bled at 14 days after TBI plus LN cell infusion for peripheral blood lymphocyte counts performed with an automated hematology analyzer and for the measurement of BM cells in femurs with FACSCalibur flow cytometer (BD Biosciences, CA, USA).

ONO-4641 at 0.03, 0.1, or 0.3 mg/kg was orally administered once a day for 13 days starting from next day, corresponding to day 1 after TBI plus LN cell infusion. In the therapeutic regimen administered after the reduced platelet counts in mice, which corresponds to day 4 after TBI plus LN cell infusion, ONO-4641 at 0.3 mg/kg was treated daily for 10 days. Cyclosporine at 6 or 30 mg/kg was administered orally twice a day for 13 days. For the two-drug combination experiment, ONO-4641 0.1 mg/kg was administered either alone or in combination with cyclosporine 15 mg/kg for 13 days starting from day 1 after disease induction. Control mice received equivalent volumes of 0.5%MC.

**Flow cytometry and intracellular cytokine staining** BM cells were prepared by flushing the femurs with 6 mL PBS. The cells were lysed with ACK lysis buffer (Lonza, Basel, Switzerland) to remove red blood cells, followed by washing and suspending in stain buffer (BD Biosciences, CA, USA). The cells were then incubated with anti-CD16/CD32 (BD Biosciences) to block IgG Fc receptors, followed by surface staining with PE-conjugated
anti-H-2Kd, PerCP-conjugated anti-CD3, FITC-conjugated anti-CD4, and APC-conjugated anti-CD8a (all reagents from BD Biosciences) for staining of donor-derived T lymphocyte subsets. To detect cytokines, the BM cells were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of brefeldin A (5 mg/mL) for 4 h at 37°C. The PerCP-conjugated anti-CD4, and APC-conjugated anti-CD8a (both reagents from BD Biosciences) were used for surface antigens. For the detection of intracellular cytokines, the cells were fixed with 2% PFA and permeabilized with 0.5% saponin (Sigma-Aldrich, MO, USA), followed by staining with Alexa fluor 647-conjugated anti-IFN-γ and PE-conjugated IL-17A (both reagents from BD Biosciences). For the detection of HSPCs, the cells were first stained with a premixed antibody cocktail of biotinylated anti-mouse antibodies to CD3ε, CD11b, CD45R, Ly-6G/Ly6C, TER-119, and then with PerCP-conjugated streptavidin, FITC-conjugated anti-CD34, PE-conjugated anti-Sca-1(Ly-6A/E), and APC-conjugated anti-c-Kit (all reagents from BD Biosciences). Cells were recovered by centrifugation at 400 g at 4°C after each staining/washing step. All samples were analyzed on FACSCalibur flow cytometer.

**Histology** On day 14 following induction, femurs were harvested, fixed in 10% neutral buffered formalin for 48 h, decalcified, and preserved in 70% ethanol at 4°C until further use. The femurs were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination.
Colony Forming Assay  Femoral BM cells were harvested from mice on day 14 after disease induction for evaluating the number of colonies forming units (CFU). BM cells were suspended in methylcellulose medium (MethoCult M03434; Stem Cell Technologies, Vancouver, Canada) containing recombinant IL-3, SCF, and IL-6 but not G-CSF and GM-CSF. BM cells harvested from mice that received TBI only were plated at $2 \times 10^4$ cells/mL for the determination of granulocyte/macrophage colony-forming unit (CFU-GM). BM cells from mice that received TBI plus LN cell infusion were prepared with the same dilution method as the TBI only mice group. Morphological analysis of colony formation was performed after 12 days of incubation at 37°C using an inverted microscope.

HSPC Mobilization in Mice  Male B6 mice were orally administered with 0.3 mg/kg ONO-4641 or injected subcutaneously with 3 mg/kg plerixafor daily for 8 days. After 3 h following the last dosing, mice were bled for the measurement of HSPCs and BM cells in femurs and of HSPCs in blood with FACSCalibur flow cytometer.

Statistical Analysis  Statistical analysis was performed using SAS System Release 9.2 TS2M3 (SAS Institute Japan Inc., Tokyo, Japan) and its cooperative system EXSUS Version. 7.7.1 (CAC Croit Corporation, Tokyo, Japan). Statistical significance was determined with Wilcoxon signed-rank sum test in experiments comparing 2 groups and by Steel's multiple comparison test in experiments comparing the treatment groups with a control group. A p-value of less than 5% indicated statistical significance.
7. Results

Lymphocyte-reducing effect of ONO-4641 in CBF1 mice ONO-4641 is a selective dual modulator that acts on S1PR1 and S1PR5, and decreases the peripheral blood lymphocyte count in animals by inhibiting lymphocyte egress from secondary lymphoid tissues (Komiya et al., 2013). The lymphocyte-reducing effect by ONO-4641 was observed in patients with multiple sclerosis (Krösser et al., 2015). First, we evaluated the lymphocyte-reducing effect of ONO-4641 in CBF1 mice. After 24 h following the administration of a single ONO-4641 oral dose of 0.01, 0.03, 0.1, or 0.3 mg/kg, the peripheral blood lymphocyte count was decreased by 6%, 34%, 62%, and 86% in a dose-dependent manner, respectively (Fig. 1).

Alleviation of disease phenotype in mouse BM failure model Mouse model of immune aplastic anemia was made by the infusion of major histocompatibility complex-mismatched LN cells from B6 mice into irradiated CBF1 mice (Chen et al, 2005) and therapeutic effect of ONO-4641 was investigated. The recipient CBF1 mice demonstrated a loss of red blood cells (RBCs), platelets (PLTs), white blood cells (WBCs), and peripheral blood neutrophils on day 14 after 4 Gy TBI and infusion of $5 \times 10^6$ LN cells compared to those in the mice that were subjected to TBI only. Since ONO-4641 significantly decreased the peripheral blood lymphocytes from 0.03 mg/kg in normal CBF1 mice (Fig. 1), 0.03, 0.1, and 0.3 mg/kg were employed in this BM failure model. ONO-4641 or 0.5%MC (vehicle) was administered with daily oral dosing for 13 days starting from next day, corresponding to day 1 after disease induction (Fig. 2A). We observed that ONO-4641 significantly
increased the RBC, PLT, and neutrophil counts in a dose-dependent manner on day 14. In contrast, ONO-4641 did not increase the WBC counts, which may be attributed to its lymphocyte-reducing effect (Fig. 2B). We also tested ONO-4641 at 0.3 mg/kg in therapeutic regimes, in which treatment started from day 4 after TBI plus LN cell infusion when reduced PLT counts were observed. The delayed treatment with ONO-4641 demonstrated significantly higher levels of PLTs, but did not increase the levels of RBCs and neutrophils compared to those in the vehicle (Fig. 2C).

Next, we tested the immunosuppressive effect of cyclosporine, a drug widely used in the treatment of acquired aplastic anemia in human and myelodysplastic syndromes. Cyclosporine at 6 or 30 mg/kg was administered orally twice a day for 13 days after disease induction. Mice that received TBI plus LN cell infusion along with cyclosporine demonstrated significantly higher levels of PLTs and neutrophils on day 14. The levels of RBCs in the cyclosporine treatment groups were not different or lower than those in the vehicle group (Supplemental Fig. 1). In addition, mice belonging to the cyclosporine 30 mg/kg group lost body weight by up to 18% during the evaluation period, which might be a result of the treatment toxicity. We examined the combinatorial effect of ONO-4641 and cyclosporine by using sub-therapeutic doses for both substances. The decrease of RBCs by cyclosporine 15 mg/kg was recovered by the co-treatment with ONO-4641 0.1 mg/kg. In addition, ONO-4641 0.1 mg/kg combined with cyclosporine 15 mg/kg demonstrated higher levels of PLTs and neutrophils compared to those treated with vehicle on day 14 (Fig. 2D).
Reduction of BM hypopcellularity by inhibiting infiltration of donor-derived T lymphocytes to BM

The severity of pancytopenia induced by TBI plus LN cell infusion implicated an immune response that attacked and destroyed BM cells. The femurs demonstrated a moderate loss of BM cellularity on day 6 in mice that were subjected to TBI only. However, BM cellularity was recovered spontaneously afterwards. In contrast, mice that received TBI plus LN cells harvested from B6 mice showed a progressive loss of BM cellularity with numerous empty spots in marrow cavities on day 14 (Fig. 3A and B). We observed that ONO-4641 treatment increased BM cellularity in mice that received TBI plus LN cell infusion (Fig. 3B and C).

ONO-4641 has been previously shown to inhibit lymphocyte infiltration into disease lesions by regulating lymphocyte recirculation (Komiya et al., 2013). To understand the mechanism underlying the effect of ONO-4641 in the BM failure model, we attempted to clarify the contribution of different T lymphocyte populations and activation by analyzing T lymphocyte markers in the BM. The LN cells from B6 mice (H\(^2b/b\)) were injected into CBF1 mice (H\(^2b/d\)), utilizing the difference in the H-2Kd haplotype between the donor and recipient mice to identify the transferred T lymphocytes by flow cytometric analysis. Marked expansion of both donor-derived CD4\(^+\) and CD8\(^+\) T lymphocytes was observed in the BM of LN cell-infused mice on day 14. ONO-4641-treated mice demonstrated significantly diminished infiltration of donor-derived CD4\(^+\) and CD8\(^+\) T lymphocytes (Fig. 3D). Next, we analyzed the intracellular IFN-\(\gamma\) and IL-17A expressions in BM CD4\(^+\) T lymphocytes as representative markers for the Th1 and Th17 immune responses. The number of donor-derived CD4\(^+\) IFN-\(\gamma^+\) (Th1), CD4\(^+\) IL-17A\(^+\) (Th17) lymphocytes, and...
CD8$^+$ T lymphocytes increased in the BM in the vehicle group (Fig. 3D). We observed a decrease of Th1 and Th17 lymphocytes as well as other CD8$^+$ T lymphocyte subsets in mice that received ONO-4641 treatment (Fig. 3D and E). This inhibition of donor-derived T lymphocyte infiltration may be associated with peripheral blood lymphocyte-reducing effects by ONO-4641. Taken together, these results suggest that ONO-4641 reduces the destruction of BM cellularity by inhibiting the infiltration of donor-derived CD4$^+$ and CD8$^+$ T lymphocytes into the BM.

**Regulation of hematopoietic stem/progenitor cell trafficking after treatment with ONO-4641**

S1PR1 is also expressed on hematopoietic stem cells, and S1P is known to facilitate the egress of hematopoietic stem and progenitor cells (HSPCs) from the BM into the blood (Massberg et al., 2007). Next, we investigated whether ONO-4641 would affect the number of BM HSPCs in this model. Subsequently, we analyzed BM cells for the Lin$^-$ Sca-1$^+$ c-Kit$^+$ marker phenotype and further divided them into CD34$^+$ and CD34$^-$ fractions. It was reported that mouse BM Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells consist mostly of HSPCs, whereas BM Lin$^-$ Sca-1$^+$ c-Kit$^+$ CD34$^-$ cells contained long-term hematopoietic stem cells (Osawa et al., 1996). The number of both cell populations in the BM was decreased in mice that received TBI plus LN cell infusion relative to that in mice that were subjected to TBI only (Fig. 4A). Treatment with ONO-4641 at 0.3 mg/kg significantly increased the number of both cell populations when compared to treatment with vehicle in the TBI plus LN cell and with vehicle in the TBI only groups (Fig. 4A and B). To determine the functional role of these changes, we assessed the hematopoietic activity by quantifying the CFU. We found
reduction in the number of CFU-GM colonies present in the BM on day 14 in the vehicle-treated TBI plus LN cell group relative to that in the TBI only group. A significant increase was observed in the ONO-4641 0.3 mg/kg-treated group compared to that in the vehicle-treated TBI plus LN cell group (Fig. 4C). Collectively, these results indicate that ONO-4641 induces the accumulation of HSPCs in the BM of aplastic anemia model mice.

**Regulation of plerixafor-induced HSPC mobilization after cotreatment with ONO-4641**

Plerixafor (AMD3100) is a rapid mobilizing agent that enhances CXCR4-dependent HSPC recruitment to the blood by antagonizing CXCR4, the receptor of SDF-1, in the bone stromal cells (Broxmeyer et al., 2005). The cross-talk between S1P and SDF-1 modulates BM stromal cells and hematopoietic progenitor cell motility (Golan et al., 2012). To further investigate the involvement of ONO-4641 in the regulation of HSPCs, we used a model of plerixafor-induced progenitor cell mobilization. Normal mice were administered ONO-4641 orally or plerixafor subcutaneously once a day for 8 days to evaluate the changes in HSPCs. Notable changes in HSPCs were not observed in the BM with either ONO-4641 or plerixafor treatment while BM cells were decreased with the plerixafor treatment (Fig. 5A). As previously reported (Golan et al., 2012), an increase in the number of HSPCs after plerixafor treatment was detected in the blood. In contrast, the number of HSPCs in the blood was decreased upon treatment with ONO-4641. When mice were treated with both plerixafor and ONO-4641, the HSPC increase in the blood caused by plerixafor was blocked by ONO-4641 (Fig. 5B), suggesting that ONO-4641 interferes with the HSPC mobilization caused by CXCR4-dependent mechanism.
8. Discussion

In this study, we characterized the preclinical *in vivo* effects of ONO-4641 in a mouse model of aplastic anemia. The roles of S1PR modulators that have been demonstrated with the translational mouse models of human diseases have been ascribed to various mechanisms. The primary mechanism is thought to be the drug-induced S1PR1 downregulation on the cell surface. This S1PR1 downregulation called functional antagonism causes the reduction of lymphocytes in the peripheral blood by preventing the egress of them from secondary lymphoid tissues (Mandala S, et al., 2002; Schwab SR and Cyster JG, 2007). ONO-4641 is a second-generation S1PR modulator selective for S1PR1 and S1PR5. ONO-4641 also induces S1PR1 downregulation on the cell surface (Komiya et al., 2013). ONO-4641 administration decreased the number of peripheral blood lymphocytes in a dose-dependent manner. The S1PR5 is almost exclusively expressed in the central nervous system, predominantly on oligodendrocytes (Jaillard et al., 2005), suggesting that the S1PR5 modulation of ONO-4641 might not contribute to the efficacy observed in the mouse model of aplastic anemia.

Chen J et al. developed mouse models for immune-mediated BM failure by infusion of allogeneic LN cells into irradiated recipients (Chen et al., 2005). First, we have demonstrated that the S1PR modulator ONO-4641 alleviated the morbidity of the immune-mediated aplastic anemia mouse model. ONO-4641 treatment starting from the next day after TBI plus LN cell infusion increased the RBC, PLT, and neutrophil counts in a dose-dependent manner. In contrast, ONO-4641 did not increase the WBC counts, most of which compose the peripheral blood lymphocytes in mice. The ratio of lymphocyte to WBC
counts in the vehicle-treated normal mice was 81% and ONO-4641 at 0.3 mg/kg decreased the ratio to 43% by reducing the lymphocytes (Fig. 1). It is assumed that the peripheral blood lymphocyte-reducing effect of ONO-4641 is associated with the minute changes in the WBC counts. Furthermore, the delayed treatment of ONO-4641 from reduced PLT counts, corresponding to day 4 after TBI plus LN cell infusion, was still effective. However, it exhibited a lower potency. The delayed treatment with ONO-4641 increased the levels of PLTs but not RBCs and neutrophils compared to that with vehicle. In this therapeutic regime, however, the number of BM cells was still increased by ONO-4641, suggesting that a longer treatment period may be needed to recover the levels of RBCs and neutrophils. Cyclosporine is widely used for standard therapy of aplastic anemia with anti-thymocyte globulin (Peslak et al., 2017). In this model mice, cyclosporine with a dose of 30 mg/kg decreased the RBC counts, which might resemble clinically observed side effects such as lytic anemia. We evaluated the efficacy of ONO-4641 when combined with cyclosporine. The combination of a suboptimal dose (0.1 mg/kg) of ONO-4641 with a suboptimal dose (15 mg/kg) of cyclosporine resulted in increasing counts of RBC, PLT, and neutrophil compared to those by the treatment with vehicle. This result indicates individual effects that occur when both lymphocyte-trafficking (ONO-4641) and immunological suppressive (cyclosporine) mechanisms are targeted.

We observed that ONO-4641 significantly alleviated the destruction of BM cells by inhibiting the infiltration of the donor-derived T lymphocytes to the BM. We postulate that the peripheral blood lymphocyte-reducing effect by ONO-4641 involves the inhibition of donor-derived lymphocyte infiltration. In addition, ONO-4641 caused a significant
decrease in IFNγ+ CD4+ T (Th1) and IL17+ CD4+ T (Th17) as well as CD8+ T lymphocytes in the BM. Th1 and Th17 immune responses have been reported to play a role in the development of BM failure in aplastic anemia and aplastic anemia mouse models (de Latour et al., 2010), suggesting that the lymphocyte-reducing effect accompanied by the reduction of T lymphocyte infiltration by ONO-4641 may provide a beneficial effect by preserving BM cellularity in the disease.

Interestingly, our data suggest that treatment with ONO-4641 resulted in the accumulation of HSPCs in the BM of aplastic anemia model mice. The egress of the HSPCs from extramedullary tissues depends on S1PR1 and HSPCs migrates into the peripheral blood toward higher S1P concentrations (Massberg et al., 2007). The chemokine SDF-1, which is another potent chemoattractant of HSPCs, and its receptor CXCR4 is a key player in HSPC mobilization. In normal mice, the CXCR4 antagonist plerixafor, a drug used to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation, induced the mobilization of HSPC as reported previously (Golan et al., 2012). In contrast, ONO-4641 decreased the HSPC counts in the blood and blocked the plerixafor-induced HSPC mobilization. The data reveal a cross talk between S1P/S1PR1 and SDF-1/CXCR4 signaling in the HSPC egress and mobilization. ONO-4641 down-regulates the S1PR1 on the cell surface (Komiya et al., 2013), resulting in unresponsiveness to S1P, and thus blocking the egress and the subsequent mobilization of HSPCs. Moreover, in the mouse models, S1P levels in the blood could be lower than that in steady-state conditions as the number of RBC and PLT, both of which produce S1P, was decreased. Accordingly, mice that received TBI plus LN cell infusion treated with ONO-4641
demonstrated a significantly increased number of HSPCs compared to that in the mice that received TBI plus LN cell infusion treated with vehicle and mice that were subjected to TBI only treated with vehicle. It is noteworthy that ONO-4641 induced the accumulation of HSPCs in the BM although its potential contribution to overall therapeutic benefits provided by ONO-4641 remains unclear.

In conclusion, we have demonstrated that ONO-4641 alleviated pancytopenia and the destruction of BM by inhibiting the infiltration of donor-derived T lymphocytes and accumulated hematopoietic stem cells in the BM (Fig.6). The S1PR modulators might be promising targets for strategies aimed at ameliorating the progression of aplastic anemia.

9. Acknowledgments

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10. Authorship Contributions

Participated in research design: Komiya, Gohda, and Katsumata.

Conducted experiments: Komiya, Gohda and Shioya.

Performed data analysis: Komiya and Shioya.

Wrote or contributed to the writing of the manuscript: Komiya and Shioya.

11. References


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12. Footnotes

The authors are employees of Ono Pharmaceutical Co., Ltd. This work received no external funding.

13. Figures legends

**Fig. 1.** ONO-4641 decreases peripheral blood lymphocytes. Effect of ONO-4641 (0.01, 0.03, 0.1 or 0.3 mg/kg) on the number of peripheral blood lymphocytes in CBF1 mice 24 h after the oral administration of a single dose. Data are represented as mean and individuals, n=6 per each group. The p-values were determined by Steel's multiple comparison test; # p<0.05 vs 0.5%MC.

**Fig. 2.** ONO-4641 and/or cyclosporine treatment alleviates the induction of BM failure in mice. CBF1 mice were subjected to 4 Gy total body irradiation (TBI), and 5 x 10⁶ LN cells from B6 mice were administered via caudal vein injection to induce aplastic anemia model. On day 14 after disease induction, peripheral blood cell count was determined by the hematology analyzer. (A) Schema of the ONO-4641 and/or cyclosporine treatment experiments. (B) CBF1 mice were treated daily with ONO-4641 (0.03, 0.1 or 0.3 mg/kg) or 0.5%MC (vehicle) by oral gavage from day 1 after disease induction. On day 14 after disease induction, peripheral blood cells were determined by a hematology analyzer. Indicated values are presented as mean and individuals, n=8 per each group except for vehicle, n=7. (C) CBF1 mice were treated daily with ONO-4641 0.3 mg/kg or 0.5%MC (vehicle) by oral gavage from day 4 after disease induction. Indicated values are presented
as mean and individuals, n=8 per each group except for vehicle, n=10. (D) In the two-drug combination experiment, CBF1 mice were treated with either ONO-4641 0.1 mg/kg, cyclosporine 15 mg/kg (Cys), 0.5%MC alone, or ONO-4641 0.1 mg/kg plus cyclosporine 15 mg/kg from day 1 after disease induction. Indicated values are presented as mean and individuals, n=8 per each group except for vehicle, n=7. Steel's multiple comparison test was performed for comparison between the vehicle and the ONO-4641 0.03, 0.1, 0.3 mg/kg groups, with a p-value of less than 5%. #, p<0.05. ##, p<0.01. Wilcoxon signed-rank sum test was performed for comparison between the vehicle and the TBI only, the ONO-4641 0.1, 0.3 mg/kg, the cyclosporine 15 mg/kg, or the ONO-4641 0.1 mg/kg plus cyclosporine 15 mg/kg groups, with a p-value of less than 5%. *, p<0.05. **, p<0.01. ***, p<0.001. Wilcoxon signed-rank sum test was performed for comparison between the ONO-4641 0.1 mg/kg plus cyclosporine 15 mg/kg and the ONO-4641 0.1 mg/kg, or the cyclosporine 15 mg/kg groups, with a p-value of less than 5%. +, p<0.05.

**Fig. 3.** ONO-4641 treatment reduces the destruction of BM cells by inhibiting the infiltration of donor-derived T lymphocytes to BM. (A) The counts of total cells in the BM of mice that received TBI only, TBI plus LN cell infusion, or were untreated (normal mice) at the indicated days after disease induction. Indicated values are presented as mean and individuals, n=5 or 6 per each group. (B) Representative hematoxylin and eosin stained femur section (original magnification x10) in the treatment groups of TBI only with 0.5%MC (vehicle), TBI plus LN cell infusion with vehicle, and TBI plus LN cell infusion with ONO-4641 0.3 mg/kg. (C) Total cell counts in the BM of mice on day 14. Mice were
treated daily with ONO-4641 at 0.03, 0.1, 0.3 mg/kg, or vehicle by oral gavage starting from day 1 after disease induction. Indicated values are presented as mean and individuals, n=8 per each group except for vehicle, n=7. (D) Representative flow cytometric analysis of IFN-γ or IL-17A expressions in donor-derived CD4⁺ and CD8⁺ T lymphocytes in the vehicle or ONO-4641 0.3 mg/kg-treated groups on day 14. (E) Absolute numbers of donor-derived CD4⁺ and CD8⁺ T lymphocyte subsets in the vehicle or ONO-4641 0.3 mg/kg-treated groups on day 14. Indicated values are presented as mean and individuals, n=6 for the vehicle and n=5 for ONO-4641 0.3 mg/kg-treated group. Steel's multiple comparison test was performed for comparison between the vehicle and the ONO-4641 groups, with a p-value of less than 5%. ##, p<0.01. Wilcoxon signed-rank sum test was performed for comparison between the TBI only and the TBI plus LN cell infusion with vehicle, or the ONO-4641 0.3 mg/kg groups, with a p-value of less than 5%. *, p<0.05. **, p<0.01.

Fig. 4. ONO-4641 treatment increases the number of HSPCs in the BM of model mice. Mice received TBI only with vehicle, TBI plus LN cell infusion with 0.5%MC (vehicle), or TBI plus LN cell infusion with ONO-4641 0.3 mg/kg treatment. Mice were treated daily with ONO-4641 0.3 mg/kg or vehicle for 13 days starting from day 1 after disease induction. On day 14, mouse femurs were collected for HSPC analysis. (A) Representative FACS dot plots of HSPCs. Data represent the expressions of Sca-1 and c-Kit on Lin⁻ BM cells (top panels) and the expressions of CD34 on Lin⁻ Sca1⁺ c-Kit⁺ BM cells (bottom panels) of TBI only with vehicle, TBI plus LN cell infusion with vehicle, and TBI plus LN cell infusion with ONO-4641 0.3 mg/kg treatment groups in the femur. (B) The numbers of
HSPCs of TBI only with vehicle, TBI plus LN cell infusion with vehicle, and TBI plus LN cell infusion with ONO-4641 0.3 mg/kg treatment groups in the femur. (C) The number of CFU-GM progenitors was enumerated in an *in vitro* colony-forming assay. Indicated values are presented as mean and individuals, n=8 per each group except for vehicle, n=10. Wilcoxon signed-rank sum test was performed for comparison between the TBI plus LN cell infusion with vehicle and the TBI only with vehicle, or the TBI plus LN cell infusion with ONO-4641 0.3 mg/kg groups, with a p-value of less than 5%. **, p<0.01. ***, p<0.001. Wilcoxon signed-rank sum test was performed for comparison between the TBI only and the TBI plus LN cell infusion with ONO-4641 0.3 mg/kg groups, with a p-value of less than 5%. ++, p<0.01. +++*, p<0.001.

**Fig. 5.** ONO-4641 regulates plerixafor-induced mobilization of HSPC in mice. (A) Mice were administered orally with 0.5%MC (vehicle), ONO-4641 0.3 mg/kg, or injected subcutaneously with PBS, plerixafor 3 mg/kg, or ONO-4641 plus plerixafor (oral administration of ONO-4641 30 min before subcutaneous injection of plerixafor) for 8 days. After 3 h following the last dosing, femurs were collected for BM and HSPC cell analysis. Blood was also collected for HSPC cell analysis. (A) The numbers of Lin− Sca1+ c-Kit+ HSPC and BM cells in the femur. (B) The number of Lin− Sca1+ c-Kit+ HSPC cells in the blood. Indicated values are presented as mean and individuals, n=5 per each group. Wilcoxon signed-rank sum test was performed for comparison between the vehicle and the ONO-4641 0.3 mg/kg, the plerixafor 3 mg/kg, or the ONO-4641 plus plerixafor groups, with a p-value of less than 5%. *, p<0.05. Wilcoxon signed-rank sum test was performed.
for comparison between the ONO-4641 plus plerixafor and the ONO-4641 0.3 mg/kg, or the plerixafor 3 mg/kg groups, with a p-value of less than 5%. +, p<0.05.

**Fig. 6.** Scheme of the proposed mechanistic pathway of ONO-4641 in aplastic anemia model. (A) In the aplastic anemia model, T lymphocytes infiltrate into the BM, where they target and destruct hematopoietic stem cells. (B) ONO-4641 inhibits the infiltration of T lymphocytes into BM and the destruction of hematopoietic stem cells by preventing the egress of T lymphocytes from secondary lymphoid tissues. ONO-4641 also accumulates hematopoietic stem cells in the BM.
Fig. 1
Fig. 2

(A)

ONO-4641 and/or cyclosporine, or 0.5% MC
ONO-4641 or 0.5% MC

Day 0 1 4 13 14

TBI 4 Gy and LN cell infusion into CBF1 mice Analysis

(B)

Bar charts showing changes in RBC, PLT, WBC, and Neutrophil counts for different treatment groups:

- RBC (x10^12/µL)
- PLT (x10^9/µL)
- WBC (x10^9/µL)
- Neutrophil (x10^9/µL)

Different concentrations and treatments are compared, with statistical significance indicated (e.g., **, #, ##).

Legend:
- 0.5% MC
- 0.03 ONO-4641
- 0.1 ONO-4641
- 0.3 ONO-4641

Groups: TBI and TBI + LN cell
Fig. 3

(A) BM cells (x10^6/femur)

- TBI
- TBI + LN cell
- TBI + LN cell
- TBI + LN cell
- TBI + LN cell
- Normal

Day 6
Day 8
Day 11
Day 14

(B) TBI with 0.5%MC
TBI+LN cell with 0.5%MC
TBI+LN cell with ONO-4641 0.3 mg/kg
(C)

![Bar graph showing BM cell counts (x10^6/femur) for different conditions and treatments.](image)

(D)

![Flow cytometry plots showing IL-17A and IFN-γ expression in CD4^+ and CD8^+ cells with vehicle and ONO-4641 treatments.](image)
Fig. 4

(A)

(B)

Hematopoietic cells/femur

Sca1⁺c-Kit⁺Lin⁻

CD34⁻Sca1⁺c-Kit⁺Lin⁻
Fig. 5

(A)

(B)
Fig. 6

(A) Pathology in aplastic anemia model

(B) Treatment with ONO-4641

S1PR1  T lymphocytes  Hematopoietic stem cells
Article Title
Sphingosine 1-phosphate Receptor Modulator ONO-4641 Regulates Trafficking of T Lymphocytes and Hematopoietic Stem Cells and Alleviates Immune-Mediated Aplastic Anemia in a Mouse Model

Authors
Takaki Komiya, Masashi Gohda, Hiroki Shioya, Seishi Katsumata

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Supplemental Fig. 1. Cyclosporine treatment alleviates the induction of BM failure in mice. CBF1 mice were subjected to 4 Gy TBI, and 5 x 10^6 LN cells harvested from B6 mice were injected into pre-irradiated CBF1 mice to induce aplastic anemia model. CBF1 mice were treated twice a day with cyclosporine with a dose of 6, 30 mg/kg or 0.5%MC (vehicle) by oral gavage from day 1 after disease induction. On day 14, peripheral blood cell count was determined. Indicated values are presented as mean and individuals, n=8 per each group except for vehicle, n=7, Steel's multiple comparison test was performed for comparison between the TBI plus LN cell with vehicle and the TBI plus LN cell with cyclosporine groups, with a p-value of less than 5%. ##, p<0.01. Wilcoxon signed-rank sum test was performed for comparison between the TBI only with vehicle and the TBI plus LN cell with vehicle groups, with a p-value of less than 5%. **, p<0.01.
Supplemental Fig. 1