TP0463518 (TS-143) Ameliorates Peptidoglycan-Polysaccharide Induced Anemia of Inflammation in Rats

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TP0463518 (TS-143) is a competitive prolyl hydroxylase 1/2/3 pan-inhibitor, and has been shown to specifically stabilize hypoxia-inducible factor-2 alpha in the liver to increase erythropoietin production. While TP0463518 has been shown to improve renal anemia, its effect on anemia of inflammation is still unknown. In this study, we created a rat model of anemia of inflammation by administering peptidoglycan-polysaccharide (PG-PS) to Lewis rats; the PG-PS-treated rats developed anemia within 2 weeks after the PG-PS challenge. The hematopoietic effects of oral TP0463518 administration at 10 mg/kg once daily for 6 weeks were examined in this rat model. The hematocrit values in the TP0463518-treated group increased significantly from 32.8 ± 0.8 to 44.5 ± 2.1% after the treatment, which was comparable to that in the healthy control group. The change of the mean corpuscular volume following TP0463518 treatment was similar to that in the healthy control group up to week 4, and significantly higher than that in the vehicle-treated group. TP0463518 increased divalent metal transporter 1 and duodenal cytochrome b expressions in the intestine. Conversely, TP0465318 did not exert any effects on the expressions of genes involved in iron metabolism in the liver, even though TP0463518 dramatically increased erythropoietin expression. Furthermore, TP0463518 had no effect on the expressions of inflammation markers in the liver. These results suggest that TP0463518 increased iron absorption and improved anemia of inflammation without exacerbating liver inflammation. TP0463518 appears to have an acceptable safety profile and could become a useful new therapeutic option for anemia of inflammation.

Key words TP0463518; TS-143; prolyl hydroxylase inhibitor; inflammatory anemia; iron metabolism

INTRODUCTION

Iron deficiency anemia can occur as a result of absolute iron deficiency or functional iron deficiency.1 In absolute iron deficiency, the total amount of iron in the body is decreased as a result of bleeding, malabsorption of iron, etc. Absolute iron deficiency is treated by intravenous or oral iron supplementation. In contrast, the total amount of iron in the body is sufficient in functional iron deficiency, but the iron is not appropriately distributed. Functional iron deficiency is closely associated with chronic inflammation, because interleukin-1β (IL-1β) and interleukin-6 (IL-6) induce hepcidin, a master regulator of iron metabolism.2 Hepcidin internalizes ferroportin,3,4 which exports intracellular iron to the outside, reducing iron uptake by the intestine and inhibiting transfer of iron from the liver into the blood. As a result, chronic inflammation is associated with low levels of iron in the blood and excess iron stores in the liver. As the erythropoietin (EPO)-producing capacity of the kidney is intact, conventional erythropoiesis-stimulating agents are ineffective for treating anemia of inflammation, and iron supplementation is associated with the risk of further iron accumulation in the liver; therefore, new therapeutic agents are needed for the treatment of functional iron deficiency.

Hypoxia-inducible factor-2 alpha (HIF-2α) regulates iron metabolism. The expression level of HIF-2α is tightly controlled by prolyl hydroxylase (PHD). Under normoxic conditions, the proline residues in HIF-2α are hydroxylated by PHD, and hydroxylated HIF-2α is degraded through the ubiquitin-proteasome pathway.5,6 When PHD is inhibited by the depletion of oxygen, HIF-2α escapes hydroxylation and subsequent degradation, and stabilized HIF-2α binds to the hypoxia response element to directly upregulate the genes involved in iron uptake by the intestine (e.g., divalent metal transporter 1 (DMT-1) and duodenal cytochrome b (dCYTb)).7,8 HIF-2α also indirectly downregulates hepcidin expression via induction of cyclin and EPO expressions.9-12 The role of HIF-2α in maintaining iron homeostasis is supported by the results of studies of PHD-knockout animals and clinical trials of PHD inhibitors, which have demonstrated a decrease in hepcidin levels, increase of gene expressions involved in iron uptake, and an improvement of the serum ferritin levels.13-19 Therefore, PHD inhibitors are among the therapeutic candidates for the treatment of functional iron deficiency.

TP0463518 (also known as TS-143) is a competitive PHD 1/2/3 pan-inhibitor, and has been shown, in both rats and humans, to specifically stabilize HIF-2α in the liver, to increase EPO production.20-22 While TP0463518 has been shown to improve renal anemia in 5/6 nephrectomized rats,22 its effect on functional iron deficiency anemia is still unknown. Therefore, in this study, we examined the effect of TP0463518 on functional iron deficiency anemia associated with inflammation in peptidoglycan-polysaccharide (PG-PS)-treated rats. Our findings showed that TP0463518 ameliorated the anemia through upregulation of the genes involved in iron uptake in the intestine, without aggravating liver inflammation.
findings suggest that TP0463518 can potentially ameliorate anemia of chronic inflammation.

MATERIALS AND METHODS

Reagents 2-[[1-[[6-(4-Chlorophenoxy)pyridin-3-yl]-methyl]-4-hydroxy-6-oxo-2,3-dihydropyridine-5-carbonyl]-amino]acetic acid (TP0463518, Fig. 1) was synthesized at Taisho Pharmaceutical Co., Ltd., according to a previously described method.23) PG-PS was purchased from Becton, Dickinson and Company.

Animal Experiment Protocols All animal experiment protocols were approved by the Animal Committee of Taisho Pharmaceutical Co., Ltd., and all the animal experiments were conducted with the approval of the committee. The animals were reared in cages maintained at a room temperature and humidity level of 23 ± 3°C and 50 ± 20%, respectively, under a light–dark cycle of 12–12 h. Food and water were made freely available to the animals.

Eight-week-old female Lewis rats (Charles River Laboratories Japan) were divided randomly into two groups: the healthy control group and the anemia group. The anemia group received an intraperitoneal injection of PG-PS at the dose of 15 mg/kg to cause anemia, and the healthy control group received the same volume of saline. When the rats became 10 weeks old, the anemic rats were randomly assigned to two groups: the vehicle-treated group and the TP0463518-treated group, while ensuring that the variance and mean hemoglobin levels remained balanced between the groups. The healthy control group and vehicle-treated group received oral administration of 0.5% methylcellulose, and the TP0463518-treated group, while ensuring that the variance and mean hemoglobin levels remained balanced between the groups.

Western Blotting The duodenal tissue samples were homogenized in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail, and the homogenates were sonicated for 20 s and left to stand on ice for 30 min. The homogenates were then centrifuged (14500 g, 15 min, 4°C) and the supernatants collected. The protein concentrations in the supernatants were measured using the bicinchoninic acid method. The supernatants were mixed with sample buffer and incubated for 5 min at 95°C. Then, 40 µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted on to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk and incubated with the primary antibodies (anti-DMT1 antibody from Cell Signaling Technology, Danvers, MA, U.S.A., and anti-dCYTb and anti-vinculin antibodies from Abcam, Cambridge, U.K.) overnight at 4°C, followed by incubation with the secondary antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific). The primer sequences are listed in Table 1. The PCR conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The expression level of each target gene was normalized by that of an internal standard gene (hypoxanthine-guanine phosphoribosyltransferase (HPRT)). The relative expression levels of the relevant mRNAs in the liver and duodenum were calculated as the ratios to those in the healthy control group.

Table 1. Primer Sequences

| Gene     | Sequence (5’ to 3’)     |
|----------|-------------------------|
| HPRT     | TTGGTGGATATGCGCCTTGACT  |
|          | CGGCTGCTTTTGGGCTTTC    |
| dCYTb    | TCTTGACCTCCTCTTCTTGG   |
|          | RCTGGTGGAAGAATGGAGT    |
| DMT1     | GCTGACGAAGATACCGAGG   |
|          | RGTTGCAACGGCACACTTG    |
| ferroportin | TTGGAGGAGTGCATTTGCTGA |
|          | TGGAGTCTGGACACATCGG    |
| hepcidin | GAAGCGAAGATGCGCCTAACG |
|          | RCTGGTGACATTTGGGAGATG |
| MCP-1    | AGCATCACCAGTGCTGCTC   |
|          | RGTACATTCGCTGAAATGAG  |
| IL-1β    | TGTTAGTAAAGACGCGACAC  |
|          | RCTCCTCTTTGGGATGGTTTGG |
| TNFa     | TCAGTTGCAATGCAGCAG     |
|          | RGTGTTGGTATGATTGGG     |
| TGFβ     | GCTGAAACAAAGGACGGCAAATA |
|          | RACCCTGGTTTGAGGACCTG  |
| EPO      | ACCAGGAGGATCATTGCTCA   |
|          | RAGGGCGACATAATTCCCTC   |

Fig. 1. Chemical Structure of TP0463518

Measurement of mRNA The pieces of liver and duodenum were homogenized using Tissue Lyser (Qiagen, Venlo, Netherlands) and mRNAs were extracted using the RNeasy Plus Mini Kit (Qiagen), in accordance with the instructions in the manufacturer’s manual. The samples were reverse-transcribed using the High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The mRNA levels were determined using Fast SYBR Green PCR Master Mix and the ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The primer sequences are listed in Table 1. The PCR conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The expression level of each target gene was normalized by that of an internal standard gene (hypoxanthine-guanine phosphoribosyltransferase (HPRT)). The relative expression levels of the relevant mRNAs in the liver and duodenum were calculated as the ratios to those in the healthy control group.

Determination of the Plasma Iron and Transferrin Saturation (TSAT) The plasma iron levels and unsaturated iron binding capacity (UIBC) were measured in a HITACHI 7180 automatic biochemical analyzer. The total iron binding capacity (TIBC) was calculated as the sum of the plasma iron concentration and the UIBC. TSAT was calculated as the ratio of the plasma iron concentration to the TIBC.
oxidase (Abcam) for 1 h at room temperature. The membranes were visualized and quantified using an enhanced-chemiluminescence (Cytiva, Marlborough, MA, U.S.A.) and FUSION imaging system (Vilber-Lourmat, Marne-la-Vallée, France).

Enzyme-Linked Immunosorbent Assay (ELISA) The tumor necrosis factor α (TNFα) and IL-6 levels in the liver were measured according to a previously reported method, with slight modification.20 The liver tissue specimens were homogenized in 5 volumes of Tris-buffered saline containing 5 mM EDTA, 1% Triton X100, and a protease inhibitor cock-
tail. The homogenates were centrifuged at 11000 × g for 5 min at 4 °C, and the TNFα and IL-6 levels in the supernatants were measured using ELISA kits (R&D Systems, Minneapolis, MN, U.S.A.).

Plasma ferritin levels were measured using the Rat ferritin ELISA kit (Kamiya Biomedical Company, Seattle, WA, U.S.A.), and the serum EPO and hepcidin levels were measured using the rat EPO and hepcidin ELISA kits (BioLegend, San Diego, CA, U.S.A. and Novus Biologicals, Centennial, CO, U.S.A.), respectively, in accordance with the instructions in the respective manufacturers’ manuals.

Statistics Data shown are the means ± standard errors of the mean (S.E.M.). As the mRNA expression levels increased exponentially, the means and S.E.M. were calculated after logarithmic transformation of the mRNA expression levels. The statistical significances of differences were determined by two-way repeated measures ANOVA for time-course studies or Student’s t-test for comparisons between two groups, using SAS 9.2. Statistical significance was defined as p < 0.05.

RESULTS

Effects of TP0463518 on Anemia of Inflammation To examine whether TP0463518 ameliorates anemia of inflammation, the hematopoietic effects of TP0463518 were examined, as illustrated in Fig. 2A. There were no significant differences in the body weight measured after TP0463518 treatment among the groups (Fig. 2B). The hematocrit values, hemoglobin levels and red blood cell counts in the PG-PS-treated group at 2 weeks after PG-PS administration were significantly lower than those in the healthy control group, indicating that the PG-PS-treated rats had developed anemia (Figs. 2D–F). Then, the PG-PS-treated rats were divided into vehicle-treated and TP0463518-treated groups. The reticulocyte count in the vehicle-treated group increased transiently, to reach the peak at week 2, and then returned to a level similar to that in the healthy control group by week 6. The reticulocyte count in the TP0463518-treated group, on the other hand, increased significantly as compared to that in the vehicle-treated group (Fig. 2C). The hematocrit value in the TP0463518-treated group increased significantly from 32.8 ± 0.8% prior to TP063518 treatment to 44.5 ± 2.1% after 6 weeks of treatment, which was comparable to the hematocrit value in the healthy control group (Fig. 2D). The hemoglobin levels and red blood cell counts in the TP0463518-treated group were higher than those in the vehicle-treated group throughout the experimental period, although the differences were not statistically significant (Figs. 2E, F). At the end of the study, there was no statistically significant difference in the serum EPO concentration between the healthy control group and the vehicle-treated group (10.8 ± 2.1 vs. 33.8 ± 15.1 pg/mL). However, TP0463518 treatment significantly increased the serum EPO concentration (117.9 ± 14.4 pg/mL, p < 0.01).

As the baseline MCV was not balanced among the groups, the baseline MCV in each rat was subtracted from the MCV at every time point and the values were analyzed as ΔMCV. The ΔMCV was significantly lower in the vehicle-treated group as compared to the healthy control group. On the other hand, the ΔMCV in the TP0463518-treated group was similar to that in the healthy control group up to week 4 and significantly higher than that in the vehicle-treated group (Fig. 2G). Change in mean corpuscular hemoglobin from the baseline (ΔMCH) was also significantly lower in the vehicle-treated group as compared to the healthy control group. The ΔMCH in the TP0463518-treated group was higher than that in the vehicle-treated group up to week 4, but the difference was not statistically significant (data not shown).

Measurement of Iron-Related Parameters As the ΔMCV in the TP0463518-treated group was higher than that in the vehicle-treated group, we also analyzed the changes in the iron-related parameters at the end of the study. PG-PS treatment reduced the plasma iron levels and TSAT (Figs. 3A, B), and increased the serum ferritin levels (Fig. 3C). However, TP0463518 treatment of the PG-PS-treated rats for 6 weeks had no significant effects on the plasma iron levels, TSAT, or plasma ferritin levels.

Effects of TP0463518 on the Expressions of Genes Involved in Iron Metabolism To investigate the effects of TP0463518 on iron metabolism, we analyzed the expression levels of iron metabolism-related genes. The mRNA and protein levels of dCYTb in the duodenum were lower in the vehicle-treated group than in the healthy control group (Figs. 4A, D). On the other hand, the dCYTb mRNA/protein expression levels were significantly increased in the TP0463518-treated group. The mRNA expression level of DMT1 in the duodenum was lower in the healthy control group as compared to the TP0463518-treated group than in the healthy control group (Figs. 4A, D). On the other hand, the dCYTb mRNA/protein expression levels were significantly increased in the TP0463518-treated group.
Fig. 4. Effects of TP0463518 on the Expression Levels of Genes Involved in Iron Metabolism in the Duodenum and the Liver

The mRNA expression levels of (A) dCYTb, (B) DMT1, and (C) ferroportin, and the protein expression levels of (D) dCYTb and (E) DMT1 were analyzed in the duodenum. (F) Serum hepcidin concentration. The mRNA expression levels of (G) DMT1, (H) ferroportin, and (I) hepcidin were analyzed in the liver. Data are presented as the mean values ± S.E.M. n = 5–6. Student’s t-test was performed. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the corresponding values in the healthy control rats. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the corresponding values in the vehicle-treated PG-PS rats.
DMT1 protein level (Fig. 4E). The mRNA expression levels of ferroportin in the duodenum were significantly lower in the vehicle-treated group than in the healthy control group, however, the expression levels of ferroportin were not increased in the TP0463518-treated group either (Fig. 4C).

Unlike the case in the duodenum, the DMT1 mRNA expression levels in the liver were higher in the vehicle-treated group than in the healthy control group, and the TP0463518-treated group showed no change of the DMT1 mRNA expression level in the liver either (Fig. 4G). Neither PG-PS treatment nor TP0463518 exerted any effects on the mRNA expression levels of hepcidin and ferroportin (Figs. 4H, I). The serum hepcidin concentration also remained unchanged in all the groups (Fig. 4F).

**Effects of TP0463518 on the Expression Levels of Genes Involved in Inflammation** As PG-PS induces inflammation in the liver, we investigated the expression levels of genes involved in inflammation at the end of the study. The mRNA expression levels of monocyte chemoattractant protein-1 (MCP-1), IL-1β, TNFα, and transforming growth factor β (TGFβ) in the liver were higher in the vehicle-treated group than in the healthy control group (Figs. 5A–D). No changes in the mRNA expression levels of MCP-1, IL-1β, TNFα and TGFβ in the liver were observed in the TP0463518-treated group. The protein levels of TNFα and IL-6 were also increased in the liver of the vehicle-treated group (Figs. 5E, F). TP0463518 treatment had no effect on the expression levels of TNFα and IL-6.

**Effect of TP0463518 on the Expression Level of EPO**
TP0463518 treatment had no effects on the expression levels of either genes involved in iron metabolism or those involved in inflammation in the liver in this study, whereas in a previous study, we showed that TP0463518 induced EPO expression in the liver. 22) Therefore, to examine the effect of TP0463518 treatment on the expression of EPO in the liver, we examined the EPO mRNA expression level in the liver. PG-PS treatment significantly increased the EPO mRNA expression in the liver as compared to the level in the healthy control rats (Fig. 6). TP0463518 treatment further increased the EPO mRNA expression in the liver.

**DISCUSSION**

We previously reported that TP0463518, a competitive PHD 1/2/3 pan-inhibitor, induced EPO expression specifically in the liver and improved anemia in 5/6 nephrectomized rats. 22) In this study, we investigated whether TP0463518 improved the anemia of inflammation induced by PG-PS in Lewis rats. Two weeks after the PG-PS challenge, the rats developed severe anemia, with an increase of the reticulocyte count, consistent with previous reports. 25,26) Administration of TP0463518 further increased the reticulocyte count, possibly via inducing EPO production, and also improved the hematocrit values, which became comparable to those in the healthy control group. The ΔMCV was markedly reduced by the PG-PS treatment, whereas the ΔMCV in the TP0463518-treated group was higher than that in the vehicle-treated group throughout the experimental period, indicating that more iron was utilized for erythropoiesis in this group. These results
were consistent with a previous report that PHD inhibitors improved PG-PS-induced anemia.13,27) We further investigated the effects of TP0463518 on the iron metabolism in the liver and intestine. While PG-PS treatment reduced the plasma iron levels and TSAT values, TP0463518 treatment had no effect on these parameters. PG-PS treatment increased the plasma ferritin concentration, possibly via inducing DMT1 expression in the liver. TP0463518 treatment had no effects on the serum ferritin levels or expressions levels of genes involved in iron metabolism in the liver, even though TP0463518 dramatically increased the EPO expression in the liver. On the other hand, in the intestine, TP0463518 treatment increased the expression levels of dCYTb and DMT1. The promoter region of DMT1 and dCYTb contain hypoxia response elements to which HIF-2α, but not HIF-1α, binds directly.28) Intestine-specific deletion of HIF-2α decreased the DMT1 and dCYTb expressions.29) In contrast, intestine-specific von Hippel-Lindau-knockout mice showed protection against HIF-2α degradation and increased DMT1 and dCYTb gene expressions.28) These previous studies clearly demonstrate that HIF-2α directly regulates the gene expressions of DMT1 and dCYTb. Intestine-specific DMT1-knockout mice have been reported to show severe iron deficiency.29) dCYTb is a ferric reductase expressed only in the duodenal mucosa. Deletion of dCYTb decreased the reticulocyte hemoglobin level.30) These results indicate that DMT1 and dCYTb are key players in the absorption of iron. Therefore, since TP0463518 is a PHD inhibitor, TP0463518 may increase DMT1 and dCYTb expressions via stabilizing HIF-2α, and consequently, increase iron uptake by the intestine.

Proinflammatory cytokines, especially IL-6, are known to upregulate hepcidin expression via the Janus kinase/signal transducer and activator of transcription 3 pathway.31) Increased hepcidin not only suppresses ferroportin mRNA expression, but also facilitates internalization of ferroportin.32) Schwartz et al. reported that intestine-specific ferroportin conditional-knockout mice showed increased iron retention in the enterocytes.33) This mouse model showed decreased expression of HIF-2α and its target genes (e.g., DMT1 and dCYTb) in the intestine via activation of PHD, which requires iron as a substrate. Based on the above, inflammation is considered to cause anemia by downregulating the genes related to iron absorption via the hepatic hepcidin-intestinal ferroportin/HIF-2α axis. In the current study, while hepatic IL-6 expression was increased in the PG-PS-treated rats, the expression of hepcidin was not. Our result is consistent with a previous report of the absence of any significant increase of the hepcidin mRNA levels at eight weeks after PG-PS administration.13) In addition, it has been reported that the serum hepcidin concentration increases up to 2 weeks after PG-PS administration, to start decreasing thereafter.34) Interestingly, Schwartz et al. also found that iron deficiency suppressed liver hepcidin expression.35) Therefore, it is possible that iron deficiency attenuated the induction of hepcidin expression by IL-6 in our eight-week experiment, as in the study reported by Schwartz et al.

Roxadustat, daprodustat, vadadustat, and enarodustat are approved drugs for the treatment of anemia in Japan, and molidustat is now under regulatory review.35) Among the clinical trials, although no studies have yet recruited patients with anemia of inflammation, decrease of hepcidin and ferritin concentrations is widely observed in patients with renal anemia.16–19) Roxadustat reportedly increased the hemoglobin concentration regardless of the serum C-reactive protein levels in the patients, implying that PHD inhibitors are effective in patients with anemia of inflammation.16,36,37) TP0463518 ameliorated anemia of inflammation, associated with an increase in the expressions of the genes involved in iron uptake in the intestine. The effect of TP0463518 in patients with anemia of inflammation is expected to be investigated in the future.

Genetic suppression of all of PHD 1/2/3 in the liver has been reported to be associated with severe hepatotoxicity.38,39) Although TP0463518 did not induce hepatotoxicity in our previous studies performed using healthy and 5/6 nephrectomized rats,20,22) it is possible that rats with liver inflammation may be more susceptible to the potential adverse effects of PHD 1/2/3 pan-inhibitors on the liver. Therefore, we investigated whether the administration of TP0463518 affected the severity of inflammation in the liver. Consistent with previous findings, PG-PS treatment increased the expression levels of all the inflammation marker genes.33,40) However, pharmacological suppression of PHD 1/2/3 by TP0463518 was not associated with any effects on the expression levels of the inflammation marker genes. As shown by Taniguchi et al., hepatotoxicity was not observed in association with suppression of a single or a combination of 2 PHD isoforms.39) TP0463518 at the 10 mg/kg was considered to partially inhibit PHD in the liver, as TP0463518 increased the HIF-2α level and EPO mRNA expression in the liver in a dose-dependent manner from 5 to 40 mg/kg in our previous study.22) In addition, PHD 1/2/3 activity in the liver was considered to be intact at the trough level, as evidenced by the fact that the increased HIF-2α expression recovered within 24 h of the administration of TP0463518.22) Taking these results into account, we would like to suggest that although TP0463518 is a PHD 1/2/3 pan-inhibitor, TP0463518 does not completely inhibit PHD 1/2/3, so that the risk of hepatotoxicity of this compound is very limited.

In summary, TP0463518 increased the expression levels of genes involved in iron uptake in the intestine, and ameliorated anemia of inflammation. TP0463518 did not aggravate inflammation in the liver. These results suggest that TP0463518 has an acceptable safety profile and may be promising as a new therapeutic option for anemia of inflammation.

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