Studies on the anemia-improving effect of prolyl hydroxylase inhibitors

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Abbreviations of common words

ANOVA	analysis of variance
BNx	bilaterally nephrectomized
BUN	blood urea nitrogen
CKD	chronic kidney disease
DMOG	dimethyloxaloylglycine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESA	erythropoiesis stimulating agent
FITC	fluorescein isothiocyanate
*K*i	inhibition constant
*K*m	Michaelis constant
MC	methyl cellulose
mRNA	messenger ribonucleic acid
Nx	nephrectomized
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PK/PD	pharmacokinetics-pharmacodynamics
RNA	ribonucleic acid
RT	room temperature
SD	Sprague-Dawley
S.D.	standard deviation
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
TBS	tris-buffered saline
TP0463518	2-[[1-[[6-(4-Chlorophenoxy)pyridin-3-yl]methyl]-4-hydroxy-6-oxo-2,3-dihydropyridine-5-carbonyl]amino]acetic acid
V_{max}	maximum rate of enzymatic reaction

Abbreviations of protein names

β-Actin	actin beta
dCYTb	duodenal cytochrome b
DMT1	divalent metal transporter 1
EPO	erythropoietin
HIF-1α	hypoxia inducible factor 1 alpha
HIF-2α	hypoxia inducible factor 2 alpha
HPRT	hypoxanthine guanine phosphoribosyltransferase
IL-1β	interleukin 1 beta
IL-6	interleukin 6
KIM-1	kidney injury marker 1
MCP-1	monocyte chemotactic protein 1
NAG	*N*-acetyl-β-D-glucosaminidase
PHD	prolyl hydroxylase
TGFβ1	transforming growth factor beta 1
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau protein

General introduction

Kidney

Kidney is a pair of bean-shaped organs located in the retroperitoneal space below the liver in human (posterior in animals). Blood flows into the hilum of the kidney from the inferior aorta via the renal artery. The blood enter the arcuate artery, which locates between the renal cortex and medulla, and passes through the interlobular artery in the cortex, and enter the glomerulus via the afferent artery. Blood that flows out of the efferent artery enters the veins either directly or via the arteriae rectae. Venous blood returns to the inferior vena cava via the interlobular vein, arcuate vein and the renal vein, in this order. Ureter emerges from the hilum and runs vertically downward in human (horizontally backward in animals), connecting the kidney and urinary bladder.

The main function of the kidney is the excretion of waste products from the blood (e.g. uremic toxin, potassium, etc.), and it is the renal glomerulus that plays the role. The vessel wall of the glomerulus is composed from 3 layers: endothelial cells, basement membrane and

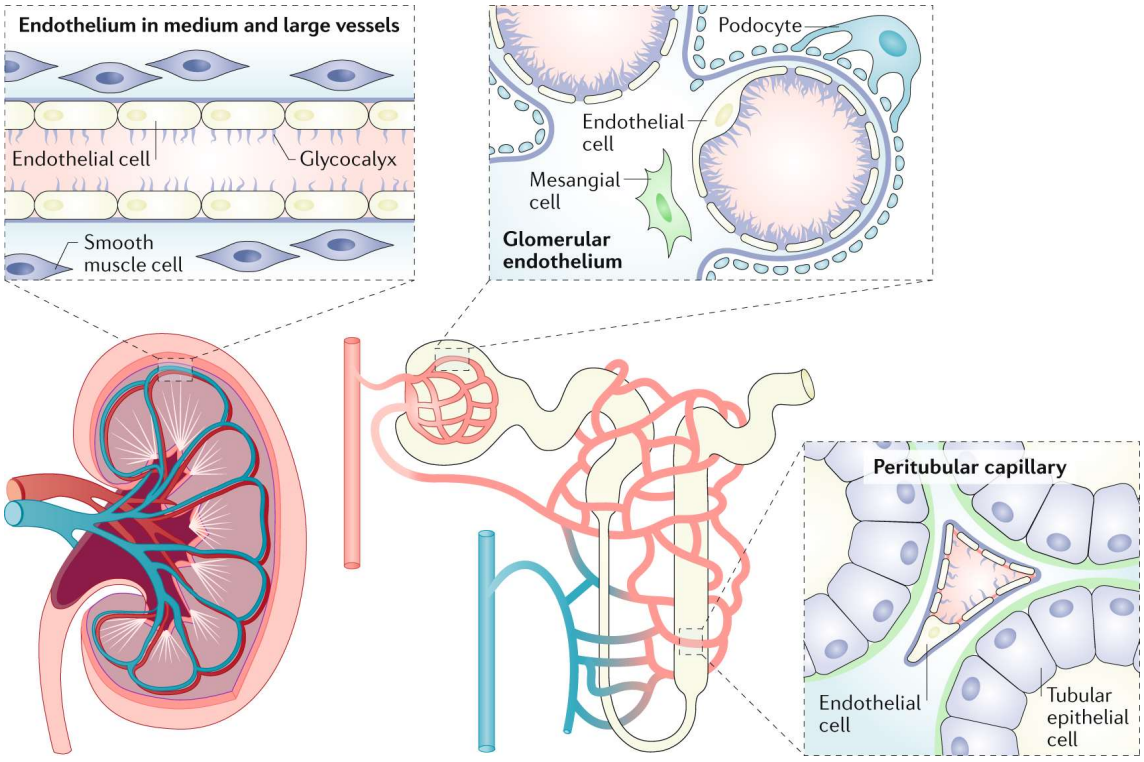


Fig. 1. The anatomy of kidney.

This figure was quoted from Jourde-Chiche et al., 2019.

podocytes. The surface of endothelial cells is negatively charged by proteoglycans and acts as a charge barrier against negatively-charged substances (e.g. albumin). In addition, the unique foot process of podocytes forms tight junction between podocyte-to-podocyte, and acts as a size barrier against large molecules (e.g. proteins). The plasma component of the blood is filtered from the glomerulus into the Bowman's capsule as primary urine. The primary urine undergoes reabsorption or tubular secretion as it passes through the proximal tubules and is further concentrated at the loop of Henle. After passing through the macula densa, the primary urine receives further reabsorption or secretion at the distal tubule and is excreted into the ureter. Functional units consisting of the glomerulus, Bowman's capsule, and tubules (proximal and distal) are called nephron. The number of nephrons in healthy adult is about one million in each kidney.

The kidneys not only excrete waste products, but also act as an endocrine tissue. For example, the kidneys secrete renin from juxtaglomerular cells when they sense a decrease in circulating blood volume (e.g. acutely caused by bleeding or chronically caused by a low-salt diet). Renin converts angiotensinogen, which is secreted from the liver, into angiotensin I. Angiotensin I is further converted to angiotensin II (AngII) by angiotensin-converting enzyme, and then AngII binds to angiotensin receptors in the adrenal gland to promote the synthesis and secretion of aldosterone. Aldosterone acts on the aldosterone receptors in the tubules to increase sodium reabsorption and thereby increase circulating blood volume. In addition, AngII strongly constricts the peripheral blood vessels, thus rescuing the drop in blood pressure associated with a decrease in circulating blood volume. The kidneys convert calcidiols (e.g. calcifediol, ercalcidiol, and secalciferol) to their active form calcitriols (1, 25-$(OH)_2D_3$, ercalcitriol, andcalcitetrol, respectively) by 25-Hydroxyvitamin D 1-alpha-hydroxylase and secrete them. Calcitriol acts on the intestine, bone and kidneys via vitamin D receptor to increase the calcium concentration in the blood. In addition, the renal tubulointerstitial cells sense oxygen concentration in the blood and secrete EPO (see Erythropoietin and renal anemia section for details).

Nephropathy

Usually, the filtration function of the kidney is measured as GFR, which is the plasma volume filtrated out of the glomerulus per body surface area per time. GFR is measured as inulin clearance, as inulin is filtered from plasma to primary urine at the same concentration by glomeruli and does

not receive secretion nor reabsorption at the tubules. However in the real world, the measurement of inulin clearance is time-consuming and labor-intensive, so that renal function is often measured as estimated GFR (eGFR) using creatinine or cystatin C clearance. GFR declines due to aging or nephrotoxic stimuli, as these stimuli reduce the number of glomeruli and/or the filtration capacity of each glomerulus. A GFR approximately 60 mL/min/1.73m^2 or more is classified as normal or mildly impaired, and as the GFR decreases, the risk of death, end-stage renal failure (dialysis), and cardiovascular events increases (Table 1, Japanese Society of Nephrology, 2018). If one completely loses his/her filtering function of the kidneys, his body is unable to excrete sodium, potassium and uremic toxin, etc. and he/she would die within ten days (Ohira, 2008). Therefore, people whose filtration function has been abolished need to undergo dialysis to remove these waste products.

cause		classification of proteinuria		A1	A2	A3
diabetes		urinary albumin excretion (mg/day) urinary albumin Cre ratio (mg/gCr)		normal	micro-albuminuria	macro-albuminuria
				<30	30-299	≧300
hypertension nephritis polycystic kidney disease transplanted kidney unknown others		urinary protein excretion (g/day) urinary protein Cre ratio (g/gCr)		normal	mild proteinuria	severe proteinuria
				<0.15	0.15-0.49	≧0.50
classification of GFR (mL/min/1.73 m^2)	G1	normal or high	≧90			
	G2	normal or mildly decreased	60-89			
	G3a	mildly to moderately decreased	45-59			
	G3b	moderately to severely decreased	30-44			
	G4	severely decreased	15-29			
	G5	kidney failure	<15			

Table 1. Classification of CKD severity.

This table was quoted and translated from Japanese Society of Nephrology. The color bar indicates the risk of the events. Green square is control. Yellow, orange and red squares indicate low, middle and high risk, respectively.

According to the guidelines, dialysis should be considered at GFR < 15 mL/min/1.73m^2 and hopefully started at least by GFR < 2 mL/min/1.73m^2 (Japanese society for dialysis therapy clinical guideline for "hemodialysis initiation for maintenance hemodialysis", Watanabe et al., 2015). The number of dialysis patients in Japan exceeds 340,000, and the prevalence rate is 2,731 per million (1 patient in 366 Japanese), ranking second in the world after Taiwan (Nitta et al., 2020). Chronic kidney disease is a preliminary stage of dialysis. There are 13 million patients with chronic kidney disease in Japan (Evidence-based Clinical Practice Guideline for CKD 2018, Japanese Society of Nephrology, 2018), and the number of dialysis patients is expected to keep increasing.

In the first half of the 1980s, glomerulonephritis, which was largely caused by infectious diseases, accounted for 60% of dialysis patients. However, with the improvement of sanitary environment and the increase of obesity and diabetes due to the improvement of nutritional conditions, glomerulonephritis has decreased and instead diabetic nephropathy has been increasing. Since early 1990s, the number of dialysis patients with nephrosclerosis, which is caused by persistent hypertension, has also increased. Considering that Japanese people traditionally consume more salt than people in other countries (Nutrition Policies of Foreign Countries surveyed by The National Institute of Health and Nutrition), and that there is a close relationship between salt intake and blood pressure, food is thought to have a significant impact on nephrosclerosis.

Erythropoietin and renal anemia

EPO is a hormone that acts on erythroblast in the bone marrow to promote hematopoiesis. When hemoglobin levels drop due to hemorrhage or hematopoietic disorders, blood EPO levels increase. EPO acts on its receptor to activate the Janus Activating Kinase 2 signaling cascade to stimulate the differentiation of erythroid progenitor cells into erythrocytes (Koury and Haase, 2015; Kuhrt and Wojchowski, 2015). EPO is secreted by neural crest cell-derived fibroblasts (renal EPO producing cells: REP cells) located in the tubular interstitium (Obara et al., 2008; Asada et al., 2011). REP cells transform into myofibroblasts when the proximal tubule receives nephrotoxic stimuli. Since myofibroblasts lose their capacity to produce EPO, the amount of EPO is insufficient when the kidneys are damaged, resulting in anemia (renal anemia, Takaori et al., 2016). Since there is no compensate EPO secretion in CKD patients, the absence of an increase

in EPO concentration is a typical symptom of renal anemia (Macdougall, 2015). Takaori et al. pointed that proximal tubule injuries not only lead to transformation of REP cells, but also cause glomerulosclerosis and atubular glomeruli. Therefore, the decrease in GFR, as a result of glomerular damage, and the decrease in EPO production (i.e., anemia), as a result of tubulointerstitial damage, are closely related. For example, in the United States, the prevalence of anemia in patients with GFR >30 mL/min/1.73m^2 is about 20%, while the prevalence jumps to 60% and 75% for GFR 15-29 and <15 mL/min/1.73m^2, respectively (McFarlane et al., 2008). The symptoms of anemia are palpitations, shortness of breath and dizziness, and sometimes pulmonary edema, heart failure, depression and severe impairment of cognitive function (Ludwig and Strasser, 2001). Therefore, renal anemia is treated to improve quality of life and prognosis.

Current trend of anemia treatment and the issues in ESA

Due to differences in body size and physical activity, the diagnostic criteria for anemia differ across Japan, the United States, and Europe (Table 2 and Table 3). The diagnostic criteria for anemia in Japan also vary with age and gender (Table 3). Renal anemia is mainly treated with iron supplements and ESAs. The treatment strategy also differs among countries. For example, iron is often prescribed in the United States, while ESAs are often prescribed in Japan.

Table 2. European and American criteria for anemia

	EBPG (g/dL)	KDOQI (g/dL)
Adult male	Hb <13.5	Hb <13.5
Adult female	Hb <11.5	Hb <12.0
Male ≧ 70 years	Hb <12.5	-

EBPG: European Best Practice Guidelines (European guidelines)

KDOQI: Kidney Disease Outcomes Quality Initiatives (American guidelines)

Table 3. Japanese criteria for anemia

	<60 years (g/dL)	60-69 years (g/dL)	≧70 years (g/dL)
Male	Hb <13.5	Hb <12.0	Hb <11.0
Female	Hb <11.5	Hb <10.5	Hb <10.5

Guidelines for renal anemia in chronic kidney disease from Japanese Society for Dialysis Therapy (Yamamoto et al., 2017)

Epoetin, a thrice-weekly ESA, was launched in 1990 in Japan, and darbepoetin, in which the sugar chains of EPO have been modified to allow administration at two-week intervals, was launched in 2007. In addition, Mircera, a PEGylated EPO with a longer dosing interval, was launched in 2011. These drugs increase blood hemoglobin levels in patients with renal anemia and are known to improve quality of life (Johansen et al., 2012). On the other hand, ESAs are expensive and must be administered by injection (Engelberg et al., 2009). In addition, once antibodies to exogenous ESAs are produced after chronic administration of ESAs, the remaining endogenous EPO is also eliminated by the antibodies, resulting in more severe anemia (pure red cell aplasia, Means, 2016). More importantly, treatment with ESAs has been reported to promote the development of hypertension, possibly by increasing the viscosity of the blood (Letcher et al., 1981; Raine, 1988; Steffen et al., 1989). Several studies have explored the effects of ESAs on renal function in a variety of CKD models in normotensive strains of rats and mice (Lee et al., 2005b; Katavetin et al., 2007; Toba et al., 2009; Cañadillas et al., 2010; Rjiba-Touati et al., 2012). In general, these studies indicate that ESAs reduced renal inflammation and fibrosis regardless of its hematopoietic effect. However, subsequent three pivotal clinical trials have raised concerns about the safety of high dose of ESAs for the treatment of anemia. The CREATE and TREAT trials showed that high target hemoglobin levels are associated with an increased risk of hypertension, dialysis, and stroke (Drüeke et al., 2006; Pfeffer et al., 2009). The CHOIR trial showed that high doses of ESAs, which greatly exceeded normal physiologic ranges of EPO, may have increased the risk of cardiovascular events independent of target hemoglobin levels or elevated blood pressure (Szczech et al., 2008; Inrig et al., 2012). Since hypertension and diabetes are the primary risk factors for the development of CKD; it is possible that these patients may be more susceptible to potential adverse effects of ESAs on blood pressure and hypertension-induced renal injury. For these reasons, a new type of anemia treatment is needed to replace ESAs.

Physiological Hypoxic Response

A healthy human body has a mechanism to respond to hypoxia. The PHD-HIF system is the mechanism. There are three known isoforms of PHD (PHD1, PHD2 and PHD3), which were cloned in 2001 (Bruick and McKnight, 2001). All PHD isoforms hydroxylate HIF-α with iron, oxygen and 2-oxoglutarate (Dao et al., 2009). The consensus mechanism of hydroxylation by PHD is as follows (see Fig. 2). First, 2-oxoglutarate coordinates to the divalent iron coordinated

to the PHD (Fe^{2+}—). Then, oxygen (O^2) is oxidatively bound to the iron, and the oxygen becomes negatively charged ($(O\text{-}O)^-$—Fe^{3+}—). The electrons of the oxygen nucleophilically attack the carbonyl carbon in the ketone of 2-oxoglutarate, the carboxylic acid of 2-oxoglutarate is cut off as carbon dioxide (2-oxoglutarate → succinate), and the oxygen and iron form a double bond (O=Fe^{4+}—). Finally, the oxygen removes hydrogen from the proline of HIF-α (HO—Fe^{3+}—) and the hydroxyl group returns to the proline (Fe^{2+}—). The succinate dissociates from the iron, and the enzyme-iron complex returns to its original state.

HIF has three isoforms: HIF-1α, HIF-2α, and HIF-3α. HIF-1/2α have two, and HIF-3α has one, proline residues that are hydroxylated by PHD in each of their central parts. Hydroxylated HIF-α is recognized by VHL, undergoes ubiquitination by ubiquitin ligase, and finally degraded in the proteasomal pathway (Maxwell et al., 1999; Ivan et al., 2001; Jaakkola et al., 2001). For this reason, these proline-containing areas are termed as oxygen-dependent degradation (ODD)

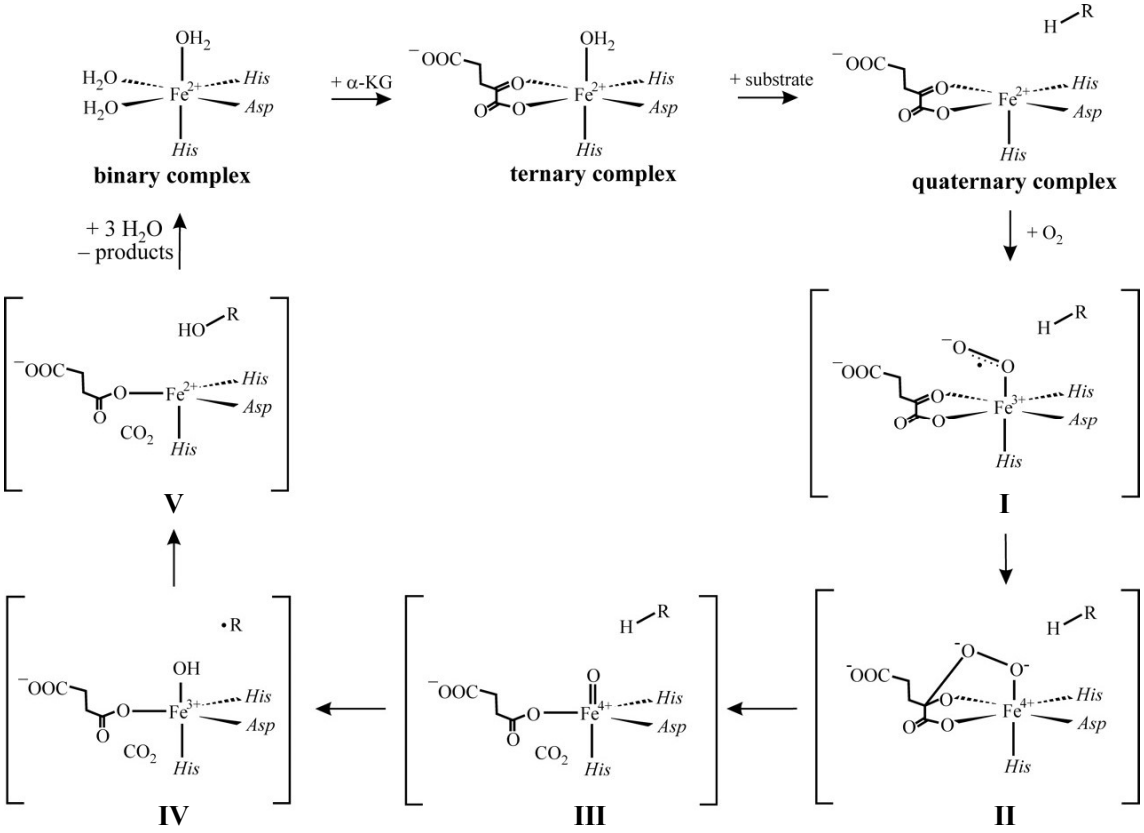


Fig. 2. The consensus mechanism of hydroxylation by PHD.
This figure was quoted and edited from Hoffart et al., 2006.

domain. All HIF-α have a DNA-binding basic helix-loop-helix (bHLH) at the N-terminus and a Per-Arnt-Sim (PAS) domain that binds to HIF-1β, a constitutively expressing transcriptional cofactor, next to the bHLH. However, the C-terminus differs between HIF-1/2α and HIF-3α. HIF-1/2α have a C-terminal acidic domain (CTAD) at the C-terminus that binds to coactivator CBP/p300, and the CTAD is functionally regulated by factor inhibiting HIF (FIH) (not quantitatively by PHD, Mahon et al., 2001). HIF-3α, on the other hand, lacks CTAD and thus does not have the transcriptional activity like HIF-1/2α. Although some literatures reported that HIF-3α repressed the function of HIF-1/2α (Hara et al., 2001; Makino et al., 2001; Maynard et al., 2005), there were also reports that HIF-3α increased the mRNA expression of *EPO* (Heikkilä et al., 2011), hence there is still no established theory for HIF-3α.

The *K*m values of PHD2 for oxygen vary widely in the literature, from 67 to 1746 μM (Hirsilä et al., 2003; Ehrismann et al., 2007; Dao et al., 2009), but all the values are higher than the intracellular oxygen concentration (30-60 μM, Ehrismann et al., 2007). Since the kidney is the organ with the lowest partial oxygen pressure in the body (Safran et al., 2006), the fluctuations in oxygen concentration in the kidney directly regulate the activity of PHD. When PHD activity

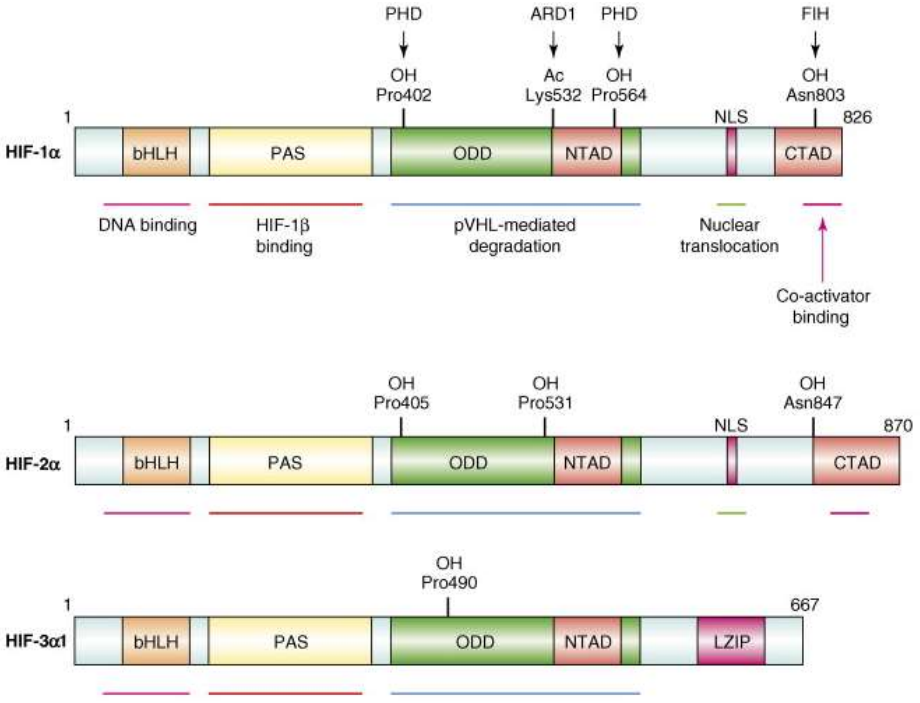


Fig. 3. The structure of HIF-1α, HIF-2α, and HIF-3α.
This figure was quoted from Rocha, 2007.

is inhibited by hypoxia, HIF-α escapes hydroxylation and enters the nucleus, where it binds to the hypoxia response element together with HIFβ and CBP/p300, and regulates the transcriptional activity of target genes (Semenza et al., 1991; Wang et al., 1995). HIF-1α and HIF-2α induce different groups of genes. For example, HIF-1α induces genes involved in angiogenesis and anaerobic respiration, such as *VEGF* and *GLUT1* (Warnecke et al., 2004), while HIF-2α induces genes involved in hematopoiesis, such as *EPO* and *DMT1* (Mastrogiannaki et al., 2009; Kapitsinou et al., 2010; Anderson et al., 2011). Thus, hypoxia itself promotes adaptation to hypoxia through the inhibition of PHD and the following activation of HIF-α. For these advancements in research of hypoxic response, the 2019 Nobel Prize in Physiology or Medicine has been awarded to Gregg L. Semenza, Peter J. Ratcliffe and William Kaelin Jr.

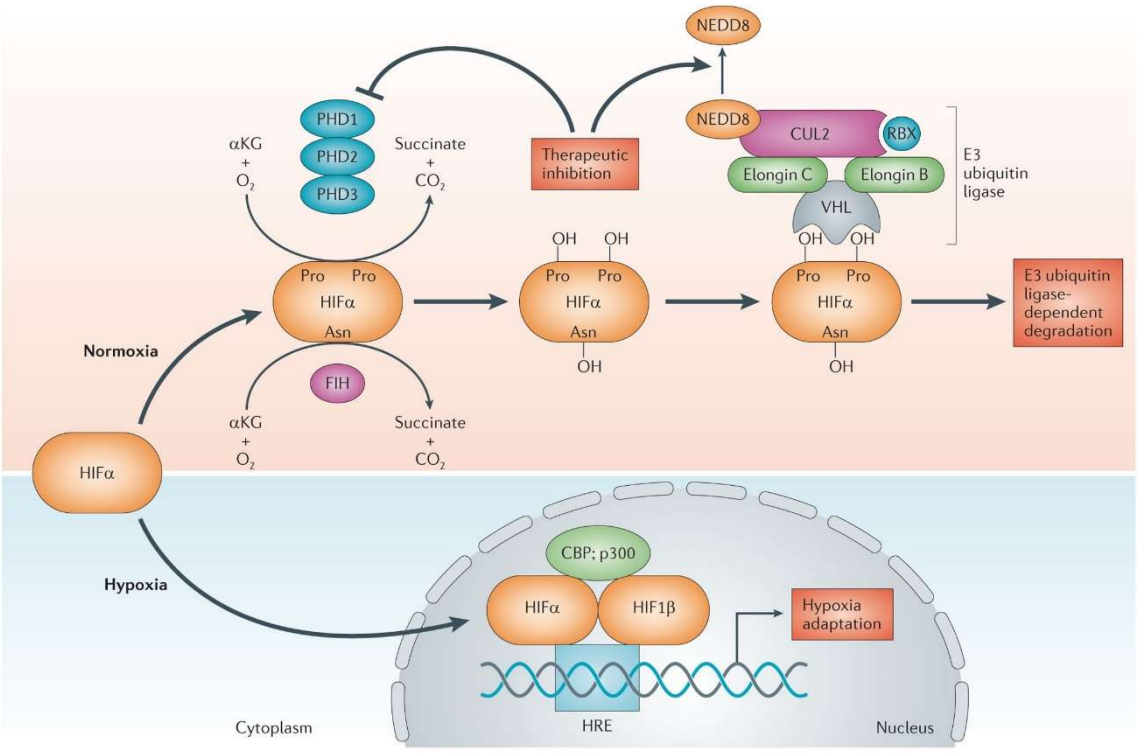


Fig. 4. Molecular mechanism of oxygen sensing and hypoxic response.
This figure was quoted from Eltzschig et al., 2014.

PHD regulation of EPO

It has long been known from the studies using bilaterally nephrectomized rats that the kidney is the major organ for EPO production in the adult and that non-renal EPO production is also observed at hypoxia (Jacobson et al., 1957; Mirand and Prentice, 1957). It was also suggested that the liver was the major organ for EPO production in fetal or neonatal (Peschle et al., 1975). After the cloning of *EPO* (Lee-Huang, 1984; McDonald et al., 1986; Shoemaker and Mitsock, 1986), Eckardt examined EPO-producing organs by molecular biology (Eckardt et al., 1992). Eckardt et al. applied hypoxic stimulation to various ages of rats and measured the *Epo* mRNA concentration in the kidney and liver. They also measured organ weights and total mRNA amounts in the tissues, and quantitatively compared the total amount of *Epo* mRNA in the whole kidneys and whole liver. As a result, they found that liver *Epo* was more than 90% of the total EPO up to 14 days of age, but at 28 days of age or later, liver *Epo* was below the detection limit. They also found that hypoxic stimulation (low O_2 or 0.1% CO) increased the liver *Epo* to more than 30% of the total EPO in adult rats.

EPO is regulated by the PHD-HIF system. *Phd2* knockout mice showed a significant increase in *Epo* expression in the kidney and increased hemoglobin concentration, while single/double knockout mice of *Phd1* or/and *Phd3* showed no change in these parameters (Minamishima et al., 2008, 2009; Takeda et al., 2008). Hemoglobin concentration in *Phd2* heterozygous knockout mice was also increased (Li et al., 2010), suggesting that anemia would be improved if PHD2 activity in the kidney is decreased to half. On the other hand, knockout of one of the *Phd1/2/3* alone is not sufficient to increase *Epo* expression in the liver. Knockout of two of these *Phd1/2/3* in combination increased liver *Epo*, and knockout of all three in combination markedly increased the *Epo* expression in the liver (Minamishima and Kaelin, 2010; Tojo et al., 2015). Because *Hif1a* was cloned first and studied well, some of the old literature stated that it is HIF-1α that regulates EPO (*Hif1a* cloned in Wang et al., 1995; *Hif2a* cloned in Tian et al., 1997). However, phenotypic analysis of liver-specific *Hif1a* or *Hif2a* conditional knockout mice showed that it is HIF-2α that regulates *Epo* expression in the liver (Rankin et al., 2007). In the kidney, a detailed investigation of the distribution of HIF-1α, HIF-2α and EPO in the rat renal cortex by immunohistochemistry revealed that EPO is produced by tubulointerstitial cells and that HIF-2α, not HIF-1α, is coexpressed with EPO (Paliege et al., 2010). Furthermore, kidney-specific knockdown of *Hif2a* in mice resulted in decreased EPO production in the kidney

and developed anemia, indicating that HIF-2α induces *Epo* also in the kidney (Kapitsinou et al., 2010).

For a long time, hepatoma (HepG2, Hep3B and H4-II-E-C3) and neuroblastoma (Kelly cells) have been used to study EPO production in cultured cell systems, while cell lines established from kidney have not (Goldberg et al., 1987; Warnecke et al., 2004; Kato et al., 2019). This is because kidney-derived cell line have lost the ability to produce EPO. Recently, a method for differentiating iPS or ES cells into EPO-producing cells has been established (Hitomi et al., 2017). In this method, iPS cells or ES cells are stimulated with activin A for 7 days and then maintained without differentiation step to liver. As a result, these cells differentiate into EPO-producing cells with characteristics similar to those of fetal liver. Transplantation of differentiated EPO-producing cells into mice with renal failure induced by adenine resulted in improvement of anemia in the mice. Furthermore, these cells have been shown to increase EPO production by hypoxic stimulation or PHD inhibitor treatment. These cells are expected to be used as a new tool for hypoxia research.

Regulation of EPO by the PHD-HIF system has been shown not only in rodents but also in human. For example, the mutations in PHD2 (Pro317Arg, Arg371His or His374Arg) reduced the activity of PHD2 by decreasing the affinity to HIF-2α (loss-of-function of PHD2). People with these mutations develop polycythemia due to the lack of hydroxylation of HIF-2α (Percy et al., 2006, 2007; Ladroue et al., 2008). In addition, the mutations in HIF-2α (Gly537Trp, Gly537Arg or Met535Val) reduce its affinity to PHD2, thereby inhibiting hydroxylation and stabilizing HIF-2α itself (gain-of-function of HIF-2α). As a result, people with these mutations also develop polycythemia (Percy et al., 2008a, 2008b; Furlow et al., 2009). There are no reports linking mutations in PHD1 or PHD3 to polycythemia, reflecting the results of knockout mice in which single gene mutations of *Phd1/3* did not increase *Epo* expression. These reports strongly suggest that pharmacological inhibition of PHD in human would result in hematopoiesis.

PHD Inhibitors

PHD inhibitors are expected to treat anemia. Cobalt competes with iron to inhibit PHD. In the 1940s, cobalt was known to have hematopoietic effects (Wintrobe et al., 1947), but the use of cobalt was very limited due to its toxicity. In the early 1990s, specific small molecule inhibitors for PHD were first reported (Cunliffe et al., 1992). In 2007, FG-2216 from FibroGen was shown

to induce EPO in not only cultured cell lines but also non-human primates (Hsieh et al., 2007), but later FG-2216 caused fulminant hepatitis in phase 2 clinical trials. No clinical trials of FG-2216 have been registered on clinicaltrial.gov thereafter. FibroGen instead advanced clinical development of roxadustat (also known as FG-4592), and roxadustat was approved in China for the first time in the world in 2018. Roxadustat, daprodustat, vadadustat, enarodustat, and molidustat were subsequently approved in Japan.

According to the interview form for each drug, thromboembolism has been warned in the Red box warning as a side effect. This is because ESAs increase the risk of thromboembolism due to increased blood viscosity associated with improved anemia; PHD inhibitors may cause thromboembolism due to excessive hematopoiesis; and some of the PHD inhibitors actually increased the incidence of thromboembolism compared to ESAs. In addition, the incidence of hypertension is comparable to that of ESAs and is therefore listed as a common side effect of these PHD inhibitors. Furthermore, due to the possibility of retinal hemorrhage caused by the angiogenesis-promoting effects of these drugs, caution is called for in prescribing these drugs to anemic patients with proliferative diabetic retinopathy, macular edema, age-related macular degeneration and retinal vein occlusion. However, the incidence of retinal hemorrhage in the clinical trials is very low (<1%), and it is unclear whether the incidence of retinal hemorrhage is increased by PHD inhibitors versus placebo or ESAs. In the clinical trials of each drug, about 10,000 patients (P1 to P3 total) were recruited, and these patients were relatively homogeneous populations because they were selected according to inclusion and exclusion criteria. For this reason, side effects of 1 in 10,000 patients or side effects that tend to develop in a specific patient population could be overlooked. On the other hand, it is estimated that up to 100,000 people per year in Japan will be prescribed daprodustat or vadadustat respectively after the market launch (according to information from Central Social Insurance Medical Council). There is a possibility that side effects that were not detected in clinical trials would be discovered after marketing. More information on side effects and also on pharmacology would become available as PHD inhibitors are used in the real world.

Interestingly, 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 (Besarab et al., 2016; Provenzano et al., 2016). In anemia of inflammation, liver inflammation causes accumulation of iron in the liver. As a result, the total

amount of iron in the whole body is sufficient, but the amount of available iron in the blood is insufficient, resulting in functional iron deficiency. More specifically, IL-6 and IL-1β are increased in chronic inflammation of the liver (Lee et al., 2005a). These cytokines increase hepcidin, a master regulator of iron metabolism. Hepcidin internalizes ferroportin, which pumps intracellular iron out of the cells (Nemeth et al., 2004; Ramey et al., 2010). As a result, the small intestine is unable to take iron into the body, and the liver is unable to release liver iron into the bloodstream. As the EPO-producing capacity of the kidney is intact, conventional ESAs are ineffective for treating anemia of inflammation. Furthermore, iron supplementation is associated with the risk of further iron accumulation in the liver; therefore, new therapeutic agents are needed for the treatment of anemia of inflammation. The effect of PHD inhibitors on inflammatory anemia has also been shown in animal studies. Intraperitoneal administration of PG-PS, the cell walls of *Streptococcus pyogenes* Group A D58 strain, in Lewis rats induces inflammation in the liver, resulting in anemia of inflammation. PHD inhibitors have been reported to increase mRNA expression of genes involved in iron metabolism and to improve anemia of inflammation in this model (Barrett et al., 2011, 2015; Flamme et al., 2014). These clinical and non-clinical evidences suggest that PHD inhibitors may also ameliorate inflammatory anemia also in human.

The effects of the PHD inhibitors on the renal EPO production seem to vary depending on the type of PHD inhibitor used and the experimental conditions of nephropathy. When unilateral ureteral obstruction was induced in knockout mice lacking *Phd1/2/3*, myofibroblast-transformed REP cells proliferated in the damaged kidney and higher levels of *Epo* mRNA were found in the damaged kidney than in the healthy kidney (Souma et al., 2016). In contrast, the increase of the renal *Epo* mRNA expression induced by a PHD inhibitor was significantly lower in gentamicin-induced nephropathic rats as compared to healthy control rats, possibly due to the loss of the kidney parenchyma (Flamme et al., 2014). Therefore, drugs that induce renal EPO may have different effects on EPO production in different pathologies or stages of nephropathy. All the PHD inhibitors approved so far inhibit all three PHD isoforms and potentially induce EPO in both the kidney and liver (Table 4). Actually, enarodustat and molidustat were reported to increase *Epo* mRNA in the kidney and liver in normal rats (Flamme et al., 2014, Fukui et al., 2019). Interestingly, hepatic *Epo* expression induced by molidustat was reportedly almost comparable between gentamicin-induced nephropathic rats and healthy control rats (Flamme et al., 2014). Therefore, PHD inhibitors that induce EPO exclusively in the liver are expected to stably increase

blood EPO levels regardless of the pathologies or stages of nephropathy, and could be used in the same regimen among various populations of patients.

Table 4. Inhibition activity of PHD inhibitors

	PHD1	PHD2	PHD3		Ref
roxadustat	100 nM	84 nM	360 nM	*K*i	Inteview form (Evrenzo)
daprodustat	3.5 nM	22 nM	5.5 nM	IC_{50}	Ariazi et al., 2017
vadadustat	15 nM	12 nM	7.6 nM	IC_{50}	Inteview form (Vafseo)
enarodustat	16 nM	61 nM	100 nM	*K*i	Fukui et al., 2019
mooidustat	480 nM	280 nM	450 nM	IC_{50}	Flamme et al., 2014

*The values were rounded to two significant digits from the original values.

Genetic suppression of all the *Phd1/2/3* in the liver has been reported to be associated with severe hepatotoxicity. On the other hand, as hepatotoxicity was not observed in knockout mice of a single or a combination of two *Phd* isoforms, partial inhibition of all the PHD 1/2/3 may not cause liver damage. Therefore, it is important to determine which tissue is the target organs of a given PHD inhibitor and to show how effective the inhibitor is in the liver.

Many of these compounds have a glycinamide structure which mimics 2-oxoglutarate, the substrate of PHD. We also focused on the glycinamide structure and explored PHD inhibitors with the following characteristics: 1) Once-daily oral administration for preferable medication adherence, 2) Low urinary excretion rate so that pharmacokinetics would not change even in patients with reduced GFR.

Purpose and Scope

TP0463518 is a PHD inhibitor discovered in Taisho Pharmaceutical. Co.Ltd. (Fig. 5). The structure of TP0463518 was confirmed by ^{1}H-NMR, infrared ray and mass spectrum analysis (Hamada et al., 2018). The purpose of this study is to examine the pharmacological effects of PHD inhibitors, using TP0463518 and DMOG, which has the same tissue specificity as TP0463518 (see Chapter 2).

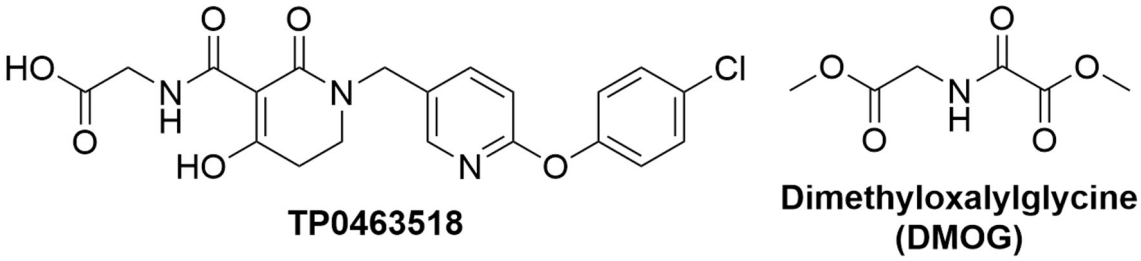


Fig. 5. Structure of TP0463518 and DMOG.

Chapter 1

The purpose of this chapter was to clarify the basic characteristics of TP0463518. The inhibitory activities of TP0463518 against human PHD isoforms and rat and monkey PHD2 were examined in detail. The type of inhibition of TP0463518 was also investigated. Furthermore, to investigate the effect of TP0463518 on EPO production in healthy and anemic animals, TP0463518 was administered to normal mice, rats and monkeys and anemic 5/6 Nx rats.

Chapter 2

The results of Chapter 1 suggested that TP0463518 induce EPO mainly in the liver. Therefore, the purpose of this chapter is to investigate this hypothesis. The sites of action of TP0463518 were investigated by measuring the amount of HIF-2α protein and *Epo* mRNA in the kidney and liver. This study was also conducted for DMOG, a known PHD inhibitor. In addition to mRNA, extra-renal EPO production was evaluated in BNx rats treated with TP0463518. From these results, it was concluded that TP0463518 (and DMOG) induces EPO mainly in the liver. Furthermore, TP0463518 was administered to examine whether liver-derived EPO actually ameliorates anemia.

Chapter 3

Inflammation in the liver reduces the amount of iron available in the blood, resulting in functional iron deficiency anemia (anemia of inflammation). Since roxadustat improved the anemia independent of CRP, we hypothesized that TP0463518 could improve anemia of inflammation. In this chapter, TP0463518 was administered to a rat model of inflammatory anemia induced by PG-PS, and the effects of TP0463518 on hematocrit, iron metabolism, and liver inflammation

were examined.

Chapter 4

Several studies have investigated the effects of recombinant human EPO (rHuEPO) on renal function in several CKD models in normotensive strains of rats and mice (Lee et al., 2005b; Katavetin et al., 2007; Toba et al., 2009; Cañadillas et al., 2010; Rjiba-Touati et al., 2012). However, subsequent clinical trials indicate that the use of rHuEPO increased the risks of hypertension and dialysis in CKD patients (Drüeke et al., 2006). Since hypertension is the primary risk factors for the development of CKD; it is possible that these patients may be more susceptible to potential adverse effects of rHuEPO on blood pressure and hypertension-induced renal injury. To explore this possibility, rHuEPO was repeatedly administered to a high-salt diet-induced hypertensive rat model for 3 weeks to investigate the effects of rHuEPO on blood pressure and renal function. In addition, DMOG was administered to obtain comparable hematopoietic effects to rHuEPO, and the effects of rHuEPO and DMOG on blood pressure and nephropathy were compared.

Chapter 1

TP0463518, a novel inhibitor for hypoxia-inducible factor prolyl hydroxylases, increases erythropoietin in rodents and monkeys with a good pharmacokinetics-pharmacodynamics correlation

Introduction

Anemia frequently develops in patients with kidney disease. The prevalence of anemia is 60% for stage 4 renal failure patients and 75% for stage 5 patients (McFarlane et al., 2008). Anemia caused by chronic renal failure is mainly the result of reduced renal EPO production in response to hypoxia (Nangaku and Eckardt, 2006). EPO is a hematopoietic factor that activates the Janus Activating Kinase 2 signaling cascade in erythroid progenitors through the EPO receptor, leading to erythroid maturation (Kuhrt and Wojchowski, 2015). To treat anemia, recombinant EPO is widely used as an erythropoiesis-stimulating agent. Though EPO ameliorates anemia and improves quality of life (activity levels, health status, etc.) (Evans et al., 1990), it is expensive (Engelberg et al., 2009), it must be injected, and hypertension is a known adverse effect (Raine, 1988; Vaziri, 1999). Consequently, new types of drug are currently being developed (Schmid and Jelkmann, 2016).

One potential alternative to EPO is PHD inhibitor. PHD hydroxylates proline residues of HIFα, a master regulator of the hypoxic response, and hydroxylated HIF is recognized by VHL, leading to the degradation of HIFα through the ubiquitin-proteasome pathway (Jaakkola et al., 2001; Pappalardi et al., 2011). As PHD requires oxygen as a substrate, PHD activity is suppressed under hypoxic conditions and HIF escapes from hydroxylation and subsequent degradation. Once HIFα is stabilized, HIFα binds to the hypoxia response element together with CBP/p300 and constitutively active HIFβ to upregulate target genes, *EPO*, *VEGF*, etc. (Haase, 2006). As a consequence, a hypoxic response is observed, such as an increase in hemoglobin (Percy et al., 2008). PHD has three isoforms: PHD1, PHD2, and PHD3. Genetically, the suppression of only *Phd2* increases the renal EPO and hemoglobin levels (Takeda et al., 2008). On the other hand, the triple knockout of *Phd1/2/3* dramatically increases liver EPO expression (Minamishima and Kaelin, 2010; Tojo et al., 2015). So, PHD2 is thought to be engaged in renal EPO production and all PHDs cooperate to regulate liver EPO expression.

According to the European clinical practice guideline for anemia in chronic kidney disease, it is recommended that the hemoglobin level not be increased to over 13 g/dL, so as to avoid increase in the risk of development of cardiovascular events (KDIGO guideline, 2012). Rapid fluctuations of the hemoglobin level (i.e., increase/decrease by ≥1 g/dL within 2 weeks) and the ESA dose have also been identified as possible risk factors (Bradbury et al., 2008; Lau et al., 2010). Long-term PHD inhibition is associated with the risk of polycythemia-related events. Furthermore, as HIF is involved in the regulation of a variety of genes, unnecessary/excessive stabilization of HIF could be associated with diverse adverse events (Rabinowitz, 2013; Maxwell and Eckardt, 2016). According to the results of clinical trials, roxadustat has a half-life of 13 hours (Groenendaal-van de Meent et al., 2016), and can be administered three times a week to obtain minimum necessary stabilization of HIF and thereby, a reduced likelihood of adverse effects, while securing the appropriate amount of EPO production for

the correction of renal anemia. A once-daily dosing regimen was set for daprodustat (half-life: 2.7 hours) and vadadustat (4.5 hours) (Johnson et al., 2014; Gupta and Wish, 2017). We believe that a once-daily dosage regimen would be the most suitable for an orally administered drug, to ensure adequate compliance, and therefore, we selected a clinical candidate with a predicted half-life in humans of approximately 2 to 5 hours. Therefore, we conducted a metabolic stability test in vitro, a pharmacokinetic study in rats, and an EPO induction test in mice, and successfully found TP0463518. TP0463518 is a novel glycineamide-type PHD inhibitor that is being examined in a clinical trial with a once-daily dose regimen (Shinfuku et al., 2018). Here, we report the inhibitory profiles of TP0463518 using in vitro enzymatic assays and the pharmacological effect of TP0463518 in rodents and monkeys. TP0463518 has the potential to become a new therapeutic option for easily controlling hemoglobin levels in renal anemia patients.

Materials and Methods

Reagents

TP0463518 was synthesized at Taisho Pharmaceutical Co., Ltd. according to a previously described method (Hamada et al., 2018). FITC-HIF peptides were synthesized by Peptide Institute, Inc. The sequences of the peptides were as follows: FITC-X-DLDLEMLAPYIPMDDDFQL (for human and monkey HIF-1$\alpha_{556\text{-}574}$) and FITC-X-ELDLETLAPYIPMDGEDFQL (for human HIF-2$\alpha_{523\text{-}542}$), where X represents aminocaproic acid.

Preparation of enzymes

cDNA containing human PHD1 (NP_444274.1), human PHD2 (NP_071334.1), and human PHD3 (NP_071356.1) were sub-cloned into pcDNA3.1/hygro (+) (Life Technologies, Grand Island, NY). Monkey *phd2* was cloned from a *Macaca fascicularis* cDNA library (Zyagen), and the obtained clone was subcloned into pcDNA3.1/hygro (+). Each vector was transfected into 293FT cells. Then, the cells were harvested 1-3 days after transfection with lysis buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.1% Triton X-100, and proteinase inhibitor cocktail). The cell lysates were sonicated and centrifuged (4˚C, 100,000 xg, 30 min) to prepare the enzyme solution.

Enzymatic assay

The PHDs inhibition studies were performed using fluorescence polarization. FITC-HIF and 2-oxoglutarate were mixed with enzyme solution in a reaction buffer (32 mM (for human PHD2) or 20 mM (for other PHD isoforms, and rat and monkey PHD2) Tris-HCl [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 10 μM $FeSO_4$, 2 mM ascorbic acid, 1 mM DTT) with or without various concentrations of TP0463518. The concentrations of FITC-HIF and 2-oxoglutarate were twice the *K*m values of each enzyme. The reaction temperature was 30˚C, and the reaction time was optimized to each PHD enzyme to obtain the initial velocity (9 to 20 min). At the end of the reaction, a stop solution containing 20 mM of EDTA and anti-hydroxylated HIF antibody (Cell Signaling Technology, Inc.) was added to the reaction buffer. Then, the fluorescence (ex: 480 nm, em: 535 nm) was measured using EnVision (PerkinElmer Japan Co., Ltd.) to calculate the millipolarization (mP) value as follows:

$$\mathrm{mP} = \frac{(\mathrm{S}-\mathrm{GP})}{(S+GP)} * 1000, \qquad \text{equation 1-1}$$

where S is fluorescence intensity parallel to the excitation plane, P is fluorescence intensity perpendicular to the excitation plane, and G is G-factor. The mP values and the corresponding hydroxylated HIF concentration were proportional, so we used the mP values as the activities. The

IC_{50} values were calculated using SAS version 9.2 (SAS Institute, Tokyo, Japan) using a nonlinear least squares method.

To determine the mode of inhibition, the activity of PHD2 was measured with various concentrations of 2-oxoglutarate (0.025 to 8 μM) and TP0463518 (0 to 40 nM). Then, the apparent V_{max} and *K*m corresponding to each TP0463518 concentration were compared. The mode of inhibition was confirmed using a double reciprocal plot. To determine the *K*i value, the activity of PHD2 was measured with various concentrations of 2-oxoglutarate (0.08 to 0.32 μM) and TP0463518 (0 to 12 nM). Then, the *K*i value was calculated according to the determined mode of inhibition using non-linear least square fitting (SAS 9.2).

Animal protocol

All animal protocols in this chapter were approved by the Animal Committee of Taisho Pharmaceutical Co., Ltd., and all the animal experiments were conducted under the regulation of the committee (approval number: 92011, 92354, 101013, 141007 and 143006). The room temperature and humidity were kept at 23 ± 3˚C and 50% ± 20% with a light-dark cycle of 12/12 h. Food and water were freely available, if not otherwise specified.

Nine-week-old Balb/c mice (Charles River Laboratories Japan, Inc.) were randomly assigned to a vehicle or a 5 to 40 mg/kg dose of TP0463518 group. The mice were orally treated with 0.5% methyl cellulose or a TP0463518 dosing suspension. Blood was collected at 6 h after administration from the orbital plexus under deep anesthesia, and euthanasia was performed without awakening. An aliquot of blood was mixed with EDTA, and the remaining blood sample was left to stand at room temperature for 15 min. The samples were then centrifuged (2130 xg for 10 min at 4°C) to prepare the plasma and serum.

For the healthy rats study, 7-week-old SD rats (Japan SLC, Inc.) were randomly assigned to a vehicle or 1.25 to 160 mg/kg dose of TP0463518 group. For the CKD model study, 5/6 Nx SD rats were prepared at Japan SLC, Inc., as follows. Two-thirds of the left kidney were resected at 4 weeks of age and the right kidney was removed at 5 weeks of age. The rats were then transferred to our facility and kept until 10 weeks of age, at which time they had developed anemia. The rats were assigned to a vehicle or a 2.5 to 80 mg/kg dose of TP0463518 group, while ensuring that there was no imbalance in the variance and mean of their whole-blood hemoglobin levels. SD rats and 5/6 Nx rats were orally treated with 0.5% methyl cellulose or a TP0463518 dosing suspension. Approximately 0.6 mL of blood was collected from the tail vein at 8 h (SD rats) or 4 h (5/6 Nx rats) after administration. The serum samples were prepared using the same method as that used for mice.

Eight monkeys (9 to 12-year-old *Macaca fascicularis*; Hamri Co., Ltd.) were subjected to a fast for 16 h before administration and were re-fed at 8 h post-administration. Blood was collected from the cephalic vein or the femoral vein before (0) and 0.5, 1, 2, 4, 8, 12 and 24 h after administration.

Plasma samples were prepared at all the time points, and serum samples were prepared at 0, 4, 8, 12 and 24 h after administration. The experiments were repeated weekly with increasing doses of TP0463518 from 0 (vehicle) to 20 mg/kg.

Determination of serum EPO

The serum EPO levels in mice, 5/6 Nx rats, and monkeys were measured using a commercially available EPO ELISA kit (mouse: MEP00B, R&D Systems; rat: 442807, BioLegend; monkey: 01630, Stemcell Technologies) according to the manufacturer's manual with slight modifications. The serum EPO levels in healthy rats were measured using a sandwich immunoassay system (K150BQC; Meso Scale Diagnostics, LLC). The rat EPO concentration from BioLegend and Meso Scale Diagnostics were confirmed to be comparable. EPO levels below the detection limits were calculated as being zero.

Determination of plasma/serum TP0463518 concentration

The plasma/serum concentrations of TP0463518 were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) consisting of an LC-30AD HPLC system (Shimadzu Corporation) and a Triple Quad 5500 Mass Spectrometer (AB Sciex).

Protein binding

Human blood was obtained from healthy volunteers by venous puncture with EDTA-2K as anticoagulants for plasma or without anticoagulants for serum, as approved by the institutional ethics committee. The individual plasma or serum was obtained by centrifugation, and then mixed together to prepare a pooled plasma or serum. The pooled plasma of male SD rats and male beagle dogs, containing EDTA-2K as anticoagulants, were purchased from HAMRI Co., Ltd. Compounds (final concentration; 0.3 μg/mL for plasma and 1 μg/mL for serum) were added to plasma or serum, and dialyzed against 0.05 M sodium phosphate buffer (pH 7.4) containing 0.07 M sodium chloride. Dialysis was performed at 37 °C for 8 hours (plasma) or 4 hours (serum) using a 96-well equilibrium dialysis equipment. The concentrations of the test compounds in plasma, serum and buffer were analyzed by LC-MS/MS.

Metabolic stability in hepatocytes

The test compound (final concentration; 1.0 μM) was incubated with human hepatocytes (Bioreclamation In Vitro Technologies), SD rat hepatocytes (XenoTech, LLC), or beagle dog hepatocytes (XenoTech, LLC) at a final cell density of 0.5 million cells/mL at 37 °C for 1 hour. The reactions were terminated by the addition of 80%. Precipitated proteins were removed by centrifugation at 3974g for 10 min at 4 °C. Concentrations of the test compounds were determined using LC-MS/MS.

Pharmacokinetic profile

Pharmacokinetic profile of test compound was investigated in fasted or non-fasted male SD rats and male beagle dogs. After a single intravenous or oral administration of test compound, blood was taken from the tail vein for rats or from the cephalic vein for dogs at each sampling time point and centrifuged to prepare plasma. The quantitative analysis of the target analyte in plasma samples was performed using LC-MS/MS. Pharmacokinetic parameters were calculated by a non-compartmental analysis with Phoenix WinNonlin (pharmacokinetic analysis software).

Human PK parameters are predicted by single species scaling method as shown in following equations:

$$CL_{tot,human} = CL_{tot,animal} * \left(\frac{fu_{human}}{fu_{animal}}\right) * \left(\frac{BW_{human}}{BW_{animal}}\right)^{0.75} * \left(\frac{BW_{animal}}{BW_{human}}\right) \quad \text{equation 1-2}$$

BW_{animal}: body weight in animal (0.25 kg in rat and 10 kg in dog)

BW_{human}: body weight in human (60 kg)

fu: plasma or serum unbound fraction

$Vd_{ss,human} = Vd_{ss,animal} \times (fu_{human} / fu_{animal})$

Predicted human $t_{1/2} = Vd_{ss,human} / CL_{tot,human} \times 0.693$

Statistics

As the control group had values below the detection limits (monkeys) or there was an imbalance in variance among the groups (other species tested), a non-parametric Steel test was used for the statistical analysis (SAS 9.2). Data are shown as the mean ± S.E.M., if not otherwise specified. Statistical significance was defined as $P < 0.05$.

Results

Discovery of TP0463518

As some of known PHD inhibitors are glycinamide derivatives (Fig. 1-1), we focused on glycinamide structure and screened lots of compounds for the following unique character: IC_{50} <100 nM in *in vitro* inhibition activity, potential to induce EPO *in vivo*, expected to have once-daily regimen in clinical, and expected to have low urinary excretion rate. Finally, we found TP0463518 (Fig. 1-1). Profiles of TP0463518 was summarized in Table 1-1. TP0463518 powerfully inhibited both human and rat PHD2 (IC_{50}: 13 nM and 18 nM, respectively). The serum level of EPO in rats at 8 hours after oral administration of the compound at 40 mg/kg was 3003 pg/mL, which was considered sufficient to correct anemia from our preliminary experiments. The plasma protein bindings were 99.1% in humans and 98.9% in rats and dogs. The metabolism rates in the hepatocytes were 5.7% in humans, 0.9% in rats, and -0.3% in dogs. The urinary excretion ratios of the unchanged drug were 0.1% in rats and 15.2% in dogs. The bioavailability of TP0463518 was 82.8 to 52.8% in rats and dogs, respectively. The half-life in humans was predicted as 1.3 to 5.6 hours by analysis of the pharmacokinetic parameters in rats and dogs (Hosea et al., 2009).

From the results above, TP0463518 has a potential to be a good option for anemia treatment. Therefore, we investigated the effects of this novel PHD inhibitor, TP0463518, in detail in this chapter.

Inhibition of PHDs activity

To elucidate the inhibitory profiles of TP0463518, we evaluated the IC_{50} values for human PHD1 and PHD3 using HIF-1α peptide. TP0463518 inhibited PHD1 with an IC_{50} value of 18 nM (Table 1-2). Although TP0463518 also inhibited PHD3, the IC_{50} values were 3.5 and 4.8 times higher than those of PHD1/2. When using HIF-2α peptide as a substrate, TP0463518 inhibited all the PHDs at potencies comparable to those obtained using HIF-1α peptide as a substrate. The inhibitory profiles for other species were also investigated. TP0463518 inhibited monkey PHD2 with an IC_{50} value of 22 nM.

To elucidate the mode of inhibition, the maximum activity and *K*m values were measured at each of the TP0463518 concentrations. The maximum activities (mP values) were 46, 47 and 47 for 0, 20 and 40 nM of TP0463518, respectively. The *K*m values were 0.10 μM without TP0463518, whereas they were 0.32 and 0.61 μM for 20 and 40 nM of TP0463518, indicating competitive inhibition. Competitive inhibition was confirmed using a double-reciprocal plot (Fig. 1-2). Based on the competitive inhibition, the *K*i value of TP0463518 was calculated as 5.3 nM.

Effect of TP0463518 on serum EPO levels in healthy rodents

Next, to elucidate the EPO-producing effect of TP0463518 in rodents, single doses of TP0463518 were administered orally to healthy mice and rats. The serum EPO concentrations in the Balb/c mice

at 6 h after administration are shown in Fig. 1-3A. The serum EPO concentrations increased in a dose-dependent manner, and a significant EPO-producing effect was observed at a dose of 5 mg/kg or more. A pharmacokinetic-pharmacodynamic (PK/PD) analysis showed an excellent correlation between the plasma TP0463518 concentrations and the serum EPO levels with a correlation coefficient of 0.95 (Fig. 1-3B).

The serum EPO levels in SD rats were evaluated at 8 h after administration, at which time the maximum serum EPO concentration was obtained in preliminary experiments. A dose-dependent and significant EPO-producing effect was observed at a dose of 20 mg/kg or more (Fig. 1-4A). On the other hand, compared with the vehicle control group, a statistically significant decrease in serum EPO concentrations was observed in the 2.5 mg/kg group. The value was very close to that obtained in the vehicle group, and the decrease was not considered pharmacologically significant. A PK/PD analysis revealed a strong PK/PD correlation with a correlation coefficient of 0.92 (Fig. 1-4B).

Effect of TP0463518 on serum EPO levels in CKD model rats

To study the EPO-producing effect of TP0463518 in a CKD model, TP0463518 was administered to 5/6 Nx rats. As with the SD rats, the serum EPO levels were evaluated at the time when the maximum serum EPO concentration was obtained. The serum EPO levels increased significantly in a dose-dependent manner at a dose of 10 mg/kg or more (Fig. 1-5A). The serum TP0463518 concentration was also strongly correlated with the serum EPO concentration (Fig. 1-5B). The serum EPO concentration in the 5/6 Nx rats was comparable to that in the SD rats when the serum TP0463518 concentration was the same (Fig. 1-4B and Fig. 1-5B).

Effect of TP0463518 on serum EPO levels in monkeys

Finally, the effect of TP0463518 was studied in monkeys (*Macaca fascicularis*). The plasma TP0463518 concentrations peaked at 1.6 h after the administration of 20 mg/kg of TP0463518 and then decreased promptly during the distribution phase (Fig. 1-6A). The $T_{1/2}$ during the elimination phase was 5.2 h. The serum EPO concentration peaked at 8 h post-administration in all the dosing groups and then decreased at 24 h (Fig. 1-6B). The serum EPO AUC increased significantly at a dose of 5 mg/kg or more (Fig. 1-6C). The serum EPO AUC was correlated with the plasma TP0463518 AUC (Fig. 1-6D).

Table 1-1. Profiles of TP0463518

		human	rat	dog
Pharmacology				
	PHD2 IC_{50} (nM)	13	18	-
	serum EPO (40 mg/kg, po) (pg/mL)	-	3003	-
Pharmacokinetics				
(in vitro)				
	Plasma protein binding (%)	99.1	98.9	98.9
	Hep metabolized 4 h (%)	5.7	0.9	−0.3
(in vivo)				
iv	dose (mg/kg)	-	1	1
	CL_{tot} (mL/h/kg)	-	437	197
	Vd_{ss} (mL/kg)	-	904	231
	$t_{1/2}$ (h)	-	3.1	4.7
	UER (%)	-	0.1	15.2
po	dose (mg/kg)	-	10	5
	C_{max} (ng/mL)	-	10700	6980
	t_{max} (h)	-	0.25	0.75
	$t_{1/2}$ (h)	-	2.3	3.5
	BA (%)	-	82.8	52.8
	Predicted human $t_{1/2}$ (h)	-	5.6	1.3

Table 1-2. IC_{50} values of TP0463518 for PHD1/2/3.

IC_{50}	HIF	PHD1	PHD2	PHD3
human	HIF-1α	18 nM	13 nM	63 nM
	HIF-2α	20 nM	14 nM	62 nM
rat	HIF-1α	-	18 nM	-
monkey	HIF-1α	-	22 nM	-

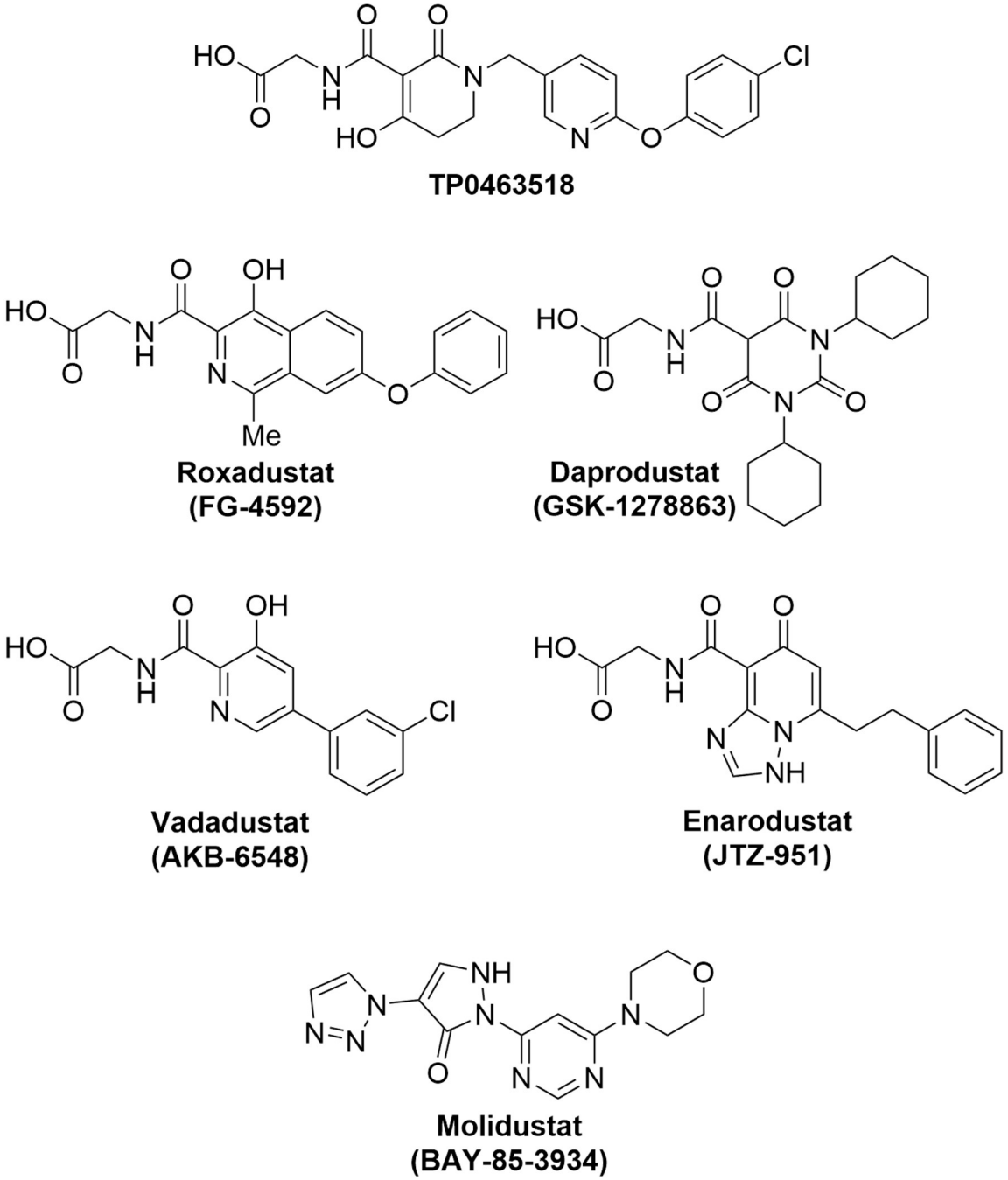


Fig. 1-1. Structure of PHD inhibitors.

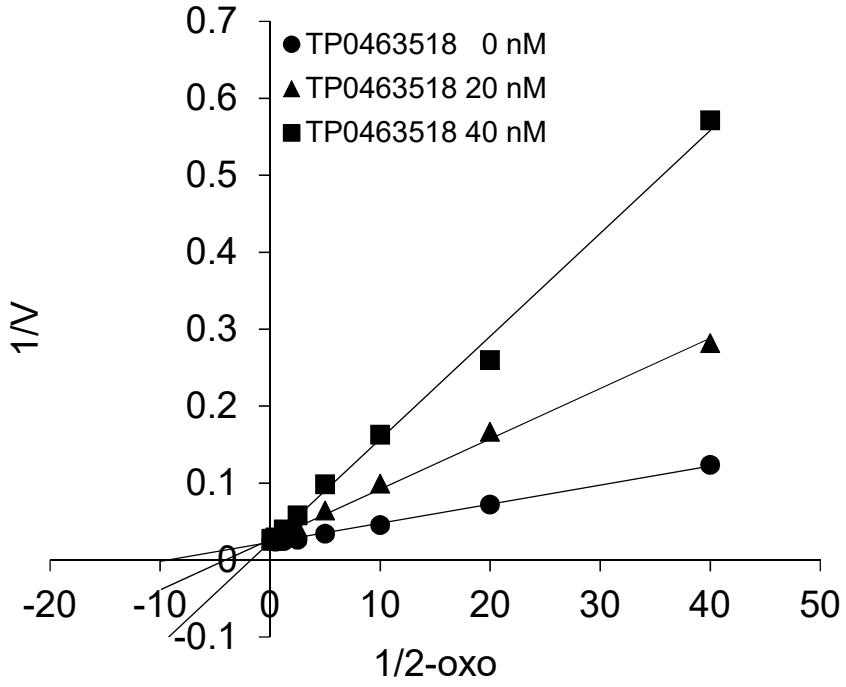


Fig. 1-2. Double reciprocal plot of PHD2 activity for 2-oxoglutarate concentration. 1/v was plotted against the corresponding 1/(2-oxoglutarate) for 0 (circle), 20 (triangle), or 40 (square) nM of TP0463518. The graph shows typical data for 3 replicated experiments.

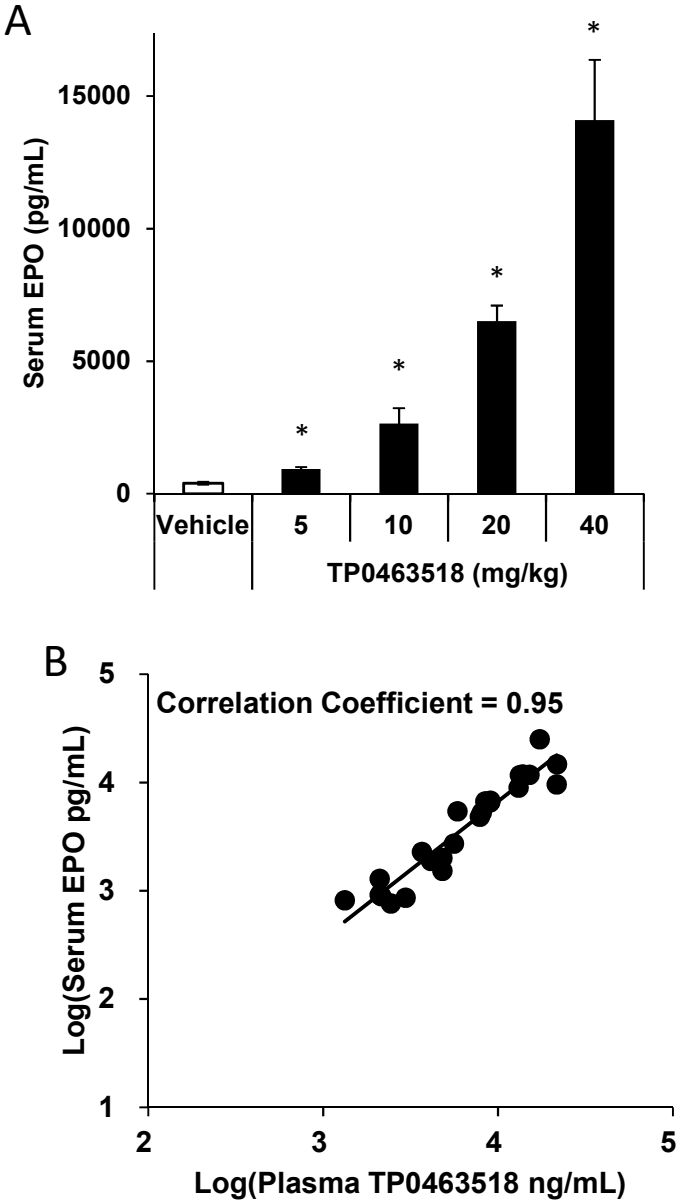


Fig. 1-3. Effect of TP0463518 on EPO production in Balb/c mice. (A) Serum EPO concentration at 6 h after a single administration of TP0463518. (B) PK/PD correlation. Logarithms of the EPO concentrations were plotted against logarithms of the plasma TP0463518 concentrations. The vehicle group was omitted from the plot. The correlation coefficient is shown in the upper left. Data are represented as the mean ± S.E.M. n = 4-6. Steel test was used to compare the TP0463518-treated groups and the corresponding vehicle-treated group. *P < 0.05.

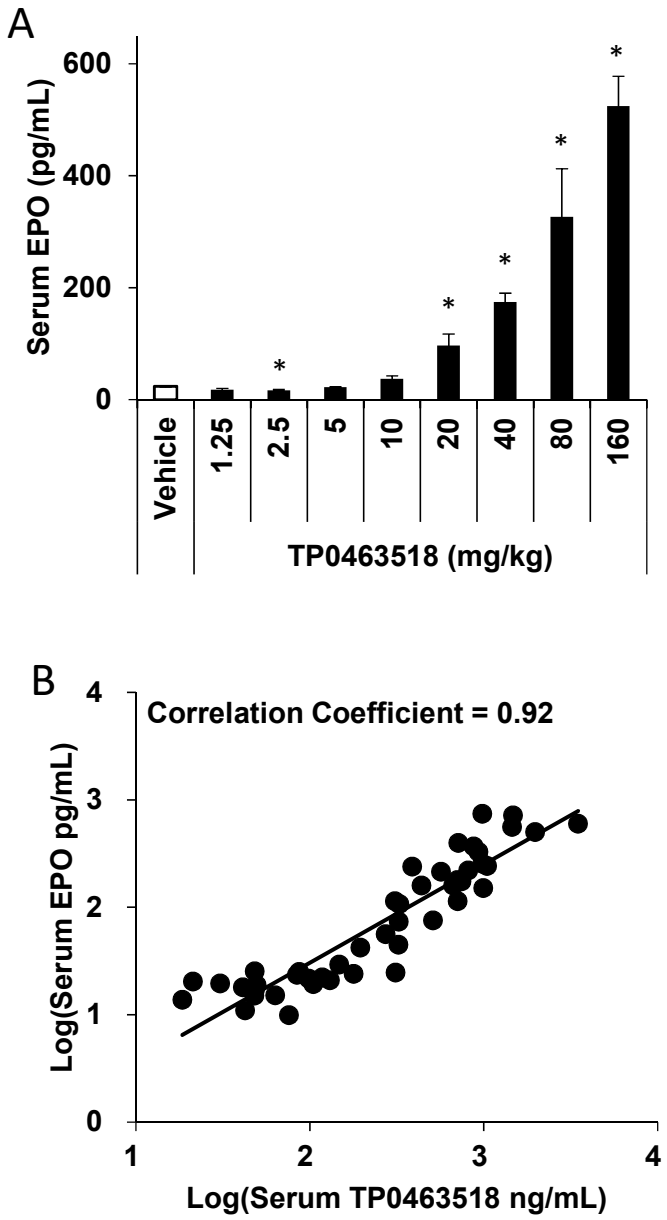


Fig. 1-4. Effect of TP0463518 on EPO production in healthy SD rats. (A) Serum EPO concentration at 8 h after a single administration of TP0463518. The maximum serum EPO concentration was obtained 8 h after administration in preliminary experiments. (B) PK/PD correlation. Logarithms of the EPO concentrations were plotted against logarithms of the serum TP0463518 concentrations. The vehicle group and 2 rats with exposure levels below the detection limits were omitted from the plot. The correlation coefficient is shown in the upper left. Data are represented as the mean ± S.E.M. n = 6. Steel test was used to compare the TP0463518-treated groups and the corresponding vehicle-treated group. *$P < 0.05$.

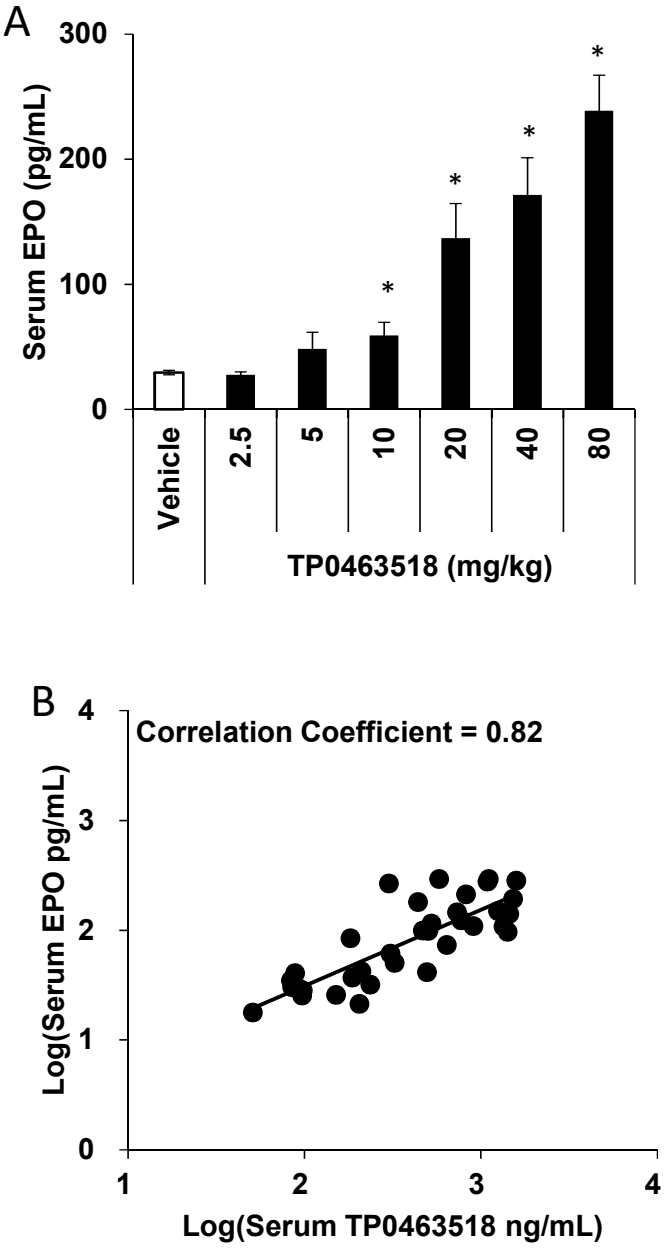


Fig. 1-5. Effect of TP0463518 on EPO production in 5/6 Nx SD rats. (A) Serum EPO concentration at 4 h after a single administration of TP0463518. The maximum serum EPO concentration was obtained 4 h after administration in preliminary experiments. (B) PK/PD correlation. Logarithms of the EPO concentrations were plotted against logarithms of the plasma TP0463518 concentrations. The vehicle group was omitted from the plot. The correlation coefficient is shown in the upper left. Data are represented as the mean ± S.E.M. n = 5-6. Steel test was used to compare the TP0463518-treated groups and the corresponding vehicle-treated group. *P < 0.05.

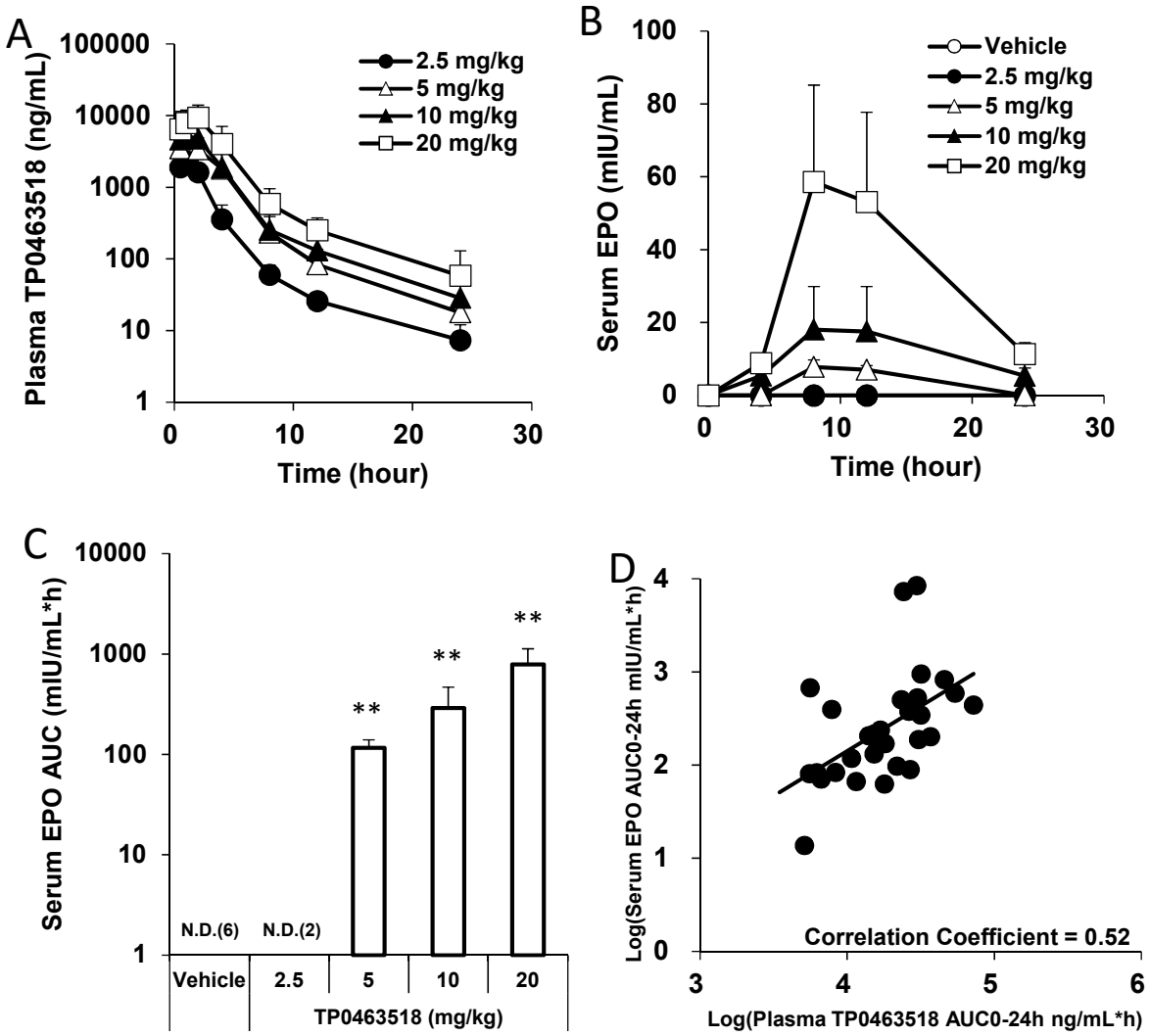


Fig. 1-6. Effect of TP0463518 on EPO production in *Macaca fascicularis*. Time course of plasma TP0463518 concentration (A) and serum EPO levels (B). (C) Twenty-four-hour AUC of serum EPO. ND means "not detected." The number of animals with values below the detection limits are shown in parentheses. (D) PK/PD correlation. Logarithms of the EPO AUC were plotted against logarithms of the plasma TP0463518 AUC. The vehicle group and 2 monkeys with EPO levels below the detection limits at all time points were omitted from the plot. The correlation coefficient is shown in the lower right. The plasma TP0463518 concentration is represented as the mean ± S.D. The serum EPO levels and the AUC were represented as the geometric mean ± S.E.M. due to outliers. n = 8. Steel test was used to compare the TP0463518-treated groups and the corresponding vehicle-treated group. **P < 0.01.

Discussion

PHD inhibitors protect HIFα from proteasomal degradation by inhibiting HIFα hydroxylation (Schmid and Jelkmann, 2016). Subsequently, *EPO*, which is located downstream of the HIF response element, is upregulated and induces hematopoiesis (Haase, 2006; Percy et al., 2008b). Recently, PHD inhibitors have been developed in clinical studies to ameliorate renal anemia, and a series of results showing a clinical proof-of-concept were reported from some companies (Provenzano et al., 2016; Akizawa et al., 2017; Martin et al., 2017). TP0463518 is a glycineamide-type PHDs inhibitor (Hamada et al., 2018) that is presently being examined in a clinical trial. In this chapter, we summarized the characteristics of TP0463518 in in vitro and in vivo studies. TP0463518 inhibited all human PHD1/2/3 on HIF-1α, and it also inhibited rat and monkey PHD2. TP0463518 is a competitive inhibitor to 2-oxoglutarate, and its *K*i value for human PHD2 was 5.3 nM. These findings suggest that the potency of TP0463518 is similar to daprodustat which is now in phase 3 trial (Ariazi et al., 2017). The IC_{50} value of TP0463518 for PHD3 was 3.5 and 4.8 times higher than those for PHD1/2, suggesting that TP0463518 is preferable to PHD1/2. Although TP0463518 had a preference for PHD1/2, the TP0463518 Cmax of monkey was much higher than the IC_{50} values (i.e., IC_{50} for human PHD3 63 nM was 27 ng/mL), so TP0463518 was considered to inhibit all the PHDs. TP0463518 also inhibited PHD2 when the substrate was HIF2-α. As HIF-2α plays an important role in EPO production (Appelhoff et al., 2004; Kapitsinou et al., 2010), the effects of TP0463518 on EPO production were then investigated in in vivo studies.

TP0463518 showed a significant EPO-inducing effect in healthy mice and rats from doses of 5 and 20 mg/kg, respectively, with an excellent PK/PD correlation. In renal anemia, EPO production in response to hypoxia is impaired. To investigate the EPO-inducing effect of TP0463518 in renal anemic model animals, 5/6 Nx rats were dosed with TP0463518. TP0463518 induced EPO production with a strong PK/PD correlation. The serum EPO concentration in 5/6 Nx rats was comparable to that in healthy SD rats at the same exposure level. The number of renal EPO-producing (REP) cells in 5/6 Nx rats was estimated to be one sixth of those in healthy SD rats. Therefore, some mechanisms were assumed to increase the serum EPO levels to a certain level after TP0463518 administration regardless of the amount of remnant kidney. The following three possibilities could explain these mechanisms.

First, TP0463518 induced an approximately 6-times higher level of EPO production in the remnant damaged kidney in 5/6 Nx rats. When a unilateral ureteral obstruction was created in knockout mice lacking *Phd1/2/3* in EPO-producing cells, the *Epo* mRNA levels in the damaged kidney were reportedly higher than those in the healthy kidney (Souma et al., 2016). In the paper, myofibroblasts, which had been REP cells before transformation, had the potency to express EPO in response to *Phd* deficiency. So, in our experiment, damaged REP cells in 5/6 Nx rats could have produced more EPO than normal REP cells. A second possibility is that TP0463518 induced more EPO under hypoxic

conditions in 5/6 Nx rats. One paper reported that hypoxia and ciclopirox (CPX), which is an iron chelator, synergistically increased reporter gene expressions via EPO HRE (Wanner et al., 2000). Iron chelators deprive iron from the enzyme and seems to inhibit the first step of the reaction (Hoffart et al., 2006). TP0463518 competed with 2-oxoglutarate and also seems to inhibit the first step of the reaction. Therefore, as in the case for CPX, a synergistic effect of TP0463518 and hypoxia might be expected in our experiments. Finally, TP0463518 possibly increased extra-renal EPO production without enhancing EPO production in the kidney. Liver-specific *Phd 1/2/3* triple knockout mice are known to have elevated liver *Epo* production (Minamishima and Kaelin, 2010). TP0463518 is a PHD1/2/3 pan-inhibitor, though the potency is slightly weak in PHD3. In this case, TP0463518 would not have reached REP cells since EPO production in the kidney is up-regulated by single suppression of PHD2 (Takeda et al., 2008). We are now investigating which organ is the main source of EPO and all these possibilities will be examined in future studies.

Next, the EPO-producing effect of TP0463518 was investigated in monkeys (*Macaca fascicularis*). The serum EPO AUC was correlated with the plasma TP0463518 AUC and increased significantly at a dose of 5 mg/kg or more. The EPO AUC, and not the EPO Cmax, is important for increasing blood hemoglobin levels (Masunaga et al., 1989). Because high levels of hemoglobin increase the risks of cardiovascular disease and stroke (Singh et al., 2006; Pfeffer et al., 2009), controlling the EPO AUC is very important for maintaining adequate levels of hemoglobin. Unlike exogenous erythropoiesis stimulating agent, as PHD inhibitor regulates endogenous EPO levels, a strong PK/PD correlation would lead to desirable hemoglobin control.

A previous report suggested that a high dose of recombinant EPO used to treat anemia patients, which greatly exceeded normal physiologic ranges of EPO, might increase the risk of a cardiovascular event independent to blood pressure rise (Szczech et al., 2008; Inrig et al., 2012). In our experiment in monkeys, the serum EPO increased to 60 mU/mL at a dose of 20 mg/kg. This increase is comparable to the physiologic increase in endogenous EPO observed at high altitudes (Klausen et al., 1996) and is sufficient to ameliorate anemia when administered once daily in not only monkeys but also human (Flamme et al., 2014; Holdstock et al., 2016; Akizawa et al., 2017). As Flamme et al. discussed, erythropoiesis stimulating agents therapy, which leads serum EPO concentration over normal physiologic range, has a potential of long-term safety concern and a therapy with PHD inhibitors might not need such a high exposure of EPO. Therefore, TP0463518, which induced effective levels but not excess normal physiologic ranges of EPO, could ameliorate anemia with a lower risk of cardiovascular events that that observed for recombinant EPO.

The systemic conditional knockout of *Phd2* increases the serum VEGF concentration (Takeda et al., 2007). To reduce mechanism-based adverse effects, an interval during which the PHDs inhibitor does not work is considered important. The half-life of TP0463518 ($T_{1/2}$) in monkeys was 5.2 h. This value is very close to the predicted human $T_{1/2}$ of 1.3-5.6 h estimated from pharmacokinetic

parameters obtained in rats and dogs (Hamada et al., 2018). A $T_{1/2}$ of 5h might be a sufficient interval, since 2.5 mg/kg, which is one eighth of the effective dose of 20 mg/kg, was ineffective in the monkey study. Based on these results, clinical trials of TP0463518 are now being conducted as a once-daily preparation. Since PHDs inhibitors regulate a wide range of gene expressions, we believe that it is important to address concerns about mechanism-based side effects, especially VEGF induction. In the daprodustat, where the inhibitory activity is close to TP0463518 and the preparation is once-daily, a trend of VEGF was not clearly apparent (Holdstock et al., 2016; Akizawa et al., 2017). To conduct clinical trials in safe, we carefully titrated the dose and monitored VEGF in first-in-human study with healthy volunteers.

Hypertension is a well-known adverse event observed in erythropoiesis therapy. Since TP0463518 induced EPO not exceeding the normal physiologic range, we believe that the risk of hypertension is low. Actually, there were no observed trends in blood pressure in phase 2 clinical study in vadadustat, which induces EPO not exceeding the normal physiologic range (Pergola et al., 2016; Martin et al., 2017), and hypertension was only observed in few patients in daprodustat, whose potency is close to TP0463518 (Akizawa et al., 2017). It will be soon reported that TP0463518 does not affect vital signs including blood pressure after single administration (Shinfuku et al., 2018). Based on these information, we believe that the risk of hypertension is low in TP0463518. Nevertheless, we plan to carefully monitor blood pressure in future clinical trials.

In summary, TP0463518 competitively inhibited human PHDs and also inhibited rat and monkey PHD2. TP0463518 increased serum EPO levels not only in healthy rodents, but also in anemic rats and monkeys. The serum EPO concentrations were well correlated with TP0463518 exposure in all the animals tested. TP0463518 is now being examined in a clinical trial with a once-daily dose regimen, and a clinical proof-of-concept for TP0463518 will be available in the future. TP0463518 is expected to become a new therapeutic option for the easy control of hemoglobin levels in renal anemia patients.

Chapter 2

TP0463518, a novel prolyl hydroxylase inhibitor, specifically induces erythropoietin production in the liver

Introduction

EPO is a hematopoietic factor that acts on the EPO receptor to activate the Janus Activating Kinase 2 signaling cascade and stimulate the differentiation of erythroid progenitor cells into erythrocytes (Kuhrt and Wojchowski, 2015; Koury and Haase, 2015). In neonates and infants, EPO is mainly synthesized in the liver (Zanjani et al., 1981; Dame et al., 1998). During development, the main site of EPO production shifts from the fetal liver to the adult kidney (Rankin et al., 2007; Kapitsinou et al., 2010). The kidney thus becomes the main source of EPO in adults and is considered to be the most sensitive organ to hypoxia (Haase, 2017). Importantly, in nephropathy, renal EPO-producing (REP) cells transform to myofibroblasts and lose their EPO-producing capacity (Asada et al., 2011; Souma et al., 2013). As a result, patients with severe kidney diseases cannot produce sufficient EPO to maintain the hemoglobin level, and consequently develop renal anemia.

Expression of EPO is regulated by HIF-2α, a master transcriptional regulator of the response to hypoxia. Under the normoxic condition, proline residues of HIF-2α are hydroxylated by PHD, which utilizes oxygen as a substrate (Hirsilä et al., 2003; Dao et al., 2009; Pappalardi et al., 2011). Hydroxylated HIF-2α is recognized by a ubiquitin ligase, VHL, and degraded through the ubiquitin-proteasome pathway (Maxwell et al., 1999; Jaakkola et al., 2001). Under the hypoxic condition, the PHD activity is suppressed due to depletion of oxygen, and HIF-2α escapes hydroxylation and subsequent degradation. Stabilized HIF-2α binds to the hypoxia response element together with CBP/p300 and the constitutively active HIFβ to upregulate *EPO* in both the kidney and liver (Haase, 2006). Three isoforms of PHD have been recognized: PHD1, PHD2, and PHD3. Genetically, deletion of the *Phd2* gene alone increases the renal EPO production and hemoglobin levels (Takeda et al., 2008; Minamishima et al., 2009), and individuals with loss-of-function mutations in *PHD2* show elevated blood hemoglobin levels (Percy et al., 2006, 2007; Ladroue et al., 2008). On the other hand, triple-knockout of *Phd1/2/3* dramatically increases hepatic *Epo* expression (Minamishima and Kaelin, 2010; Tojo et al., 2015). Therefore, it is thought that PHD2 is involved in renal EPO production, while all the PHDs function cooperatively to regulate hepatic EPO expression.

Based on the finding that PHD inhibitors exert erythropoietic effects via inducing HIF-2α stabilization and EPO production, phase III trials of PHD inhibitors as alternative erythropoiesis-stimulating agents are under way. All the PHD inhibitors under clinical trials so far inhibit all three PHD isoforms and potentially induce EPO in both the kidney and liver (Flamme et al., 2014; Ariazi et al., 2017; Kato et al., 2018). However, the effects of the PHD inhibitors on the renal EPO production seem to vary depending on the type of PHD inhibitor used and the experimental conditions of nephropathy. When unilateral ureteral obstruction was induced in knockout mice lacking *Phd1/2/3*, myofibroblast-transformed REP cells proliferated in the damaged kidney and higher levels of *Epo* mRNA were found in the damaged kidney than in the healthy kidney (Souma et al., 2016). In contrast,

the increase of the renal *Epo* mRNA expression induced by a PHD inhibitor was significantly lower in gentamicin-induced nephropathic rats as compared to healthy control rats, possibly due to the loss of the kidney parenchyma (Flamme et al., 2014). On the other hand, the amount of liver-derived EPO induced by PHD inhibitors remains unchanged regardless of the stage of nephropathy. Therefore, we considered it important to conduct a detailed investigation of whether an inhibitor would act mainly on the kidney or on the liver.

TP0463518 is a competitive PHD1/2/3 pan-inhibitor and increases the serum EPO levels in mice, rats, and monkeys (Kato et al., 2018). Since our previous study indicated that the serum EPO levels in 5/6 Nx SD rats were comparable to those in healthy SD rats for the same plasma TP0463518 concentrations, we hypothesized that TP0463518 could increase hepatic EPO production without increasing EPO production in the kidney. In this study, we examined the production of EPO after administration of TP0463518 using healthy and BNx rats, and also examined whether the liver-derived EPO improved the anemia in 5/6 Nx rats. Our study demonstrated that TP0463518 stabilized HIF-2α only in the liver, and induced liver-derived EPO production, irrespective of the stage of nephropathy. Based on the present findings, we propose that TP0463518 could be developed as a new therapeutic alternative for patients with renal anemia.

Materials and Methods

Compound

DMOG was purchased from Tokyo Chemical Industry Co., Ltd.

Animal Protocol

All animal protocols in this chapter were approved by the Animal Committee of Taisho Pharmaceutical Co., Ltd., and all the animal experiments were conducted under the approval of the committee (approval number: 93118, 141007, 143006, 153010 and AN12941). The room temperature and humidity were maintained at 23 ± 3˚C and 50% ± 20%, respectively, with a light-dark cycle of 12/12 hours. Food and water were made freely available to the animals.

Seven-week-old SD rats (Japan SLC, Hamamatsu, Shizuoka, Japan) received oral TP0463518 (suspended with 0.5%MC) administration or intraperitoneal DMOG (dissolved in saline) injection. The rats were anesthetized, and blood samples were collected at each time-point. Then, the rats were euthanized, and the kidneys and liver were removed. The right kidney of the SD rats was divided into the inferior half and superior half. The inferior half (whole kidney) was cut into 3-mm-thick slices. All of the sliced whole kidney, specimens of the left kidney cortex, and specimens of the liver were immersed in RNAlater solution overnight at 4°C and then stored at -80°C until the mRNA extraction. The superior half of the right kidney and specimens of the liver were stored at -80°C to determine the organ TP0463518 exposure. Specimens of the left kidney cortex and of the liver were snap-frozen in liquid N_2 and stored at -80°C until measurement of the organ levels of HIF-2α. The blood samples were mixed with EDTA. The samples were then centrifuged (4˚C, 2130 xg, 10 min) to obtain plasma.

Bilateral nephrectomy was performed in 7-week-old SD rats under isoflurane and xylocaine anesthesia. A midline incision was made and the pedicle of the left kidney was ligated at two sites. The left kidney was removed by cutting between the two ligated sites. Then, the right kidney was also removed in the same manner as the left kidney. The cecum and small intestine were returned to their original positions. Then, after suturing the fascia, the skin incision was closed with Aron Alpha. The rats were laid on a heat pad at 37˚C during and after the surgery and observed until they regained consciousness. The sham rats were operated in the same way, except that the steps from ligation of the renal pedicle to removal of the kidneys were skipped. At 16 hours after the operation, the rats were administered TP0463518. In the first study, the BNx rats were euthanized at 4 hours after administration, and the kidneys (Sham rats) and liver (Sham and BNx rats) were removed to measure the *Epo* mRNA expression level. In the second study, blood was collected at 8 hours after administration from the subclavian vein under anesthesia. The samples were then centrifuged (RT, 2130 xg, 10 min) to obtain serum.

In the 5/6 Nx rats, two-thirds of the left kidneys of the SD rats were resected when the rats were 4-week-old, and the right kidney was removed when the animals were 5-week-old. As they became 10-week-old, the 5/6 Nx rats were randomly assigned to the experimental groups, while ensuring that the variance and mean hemoglobin levels remained balanced among the groups. In the first study, the 5/6 Nx rats were euthanized at 2 hours after administration of TP0463518, and the remnant kidney and liver were removed. Specimens of the kidney cortex and liver were stored in RNAlater solution to measure the *Epo* mRNA. In the second study, the 5/6 Nx rats received oral vehicle or TP0463518 administration once daily for 14 days. Blood was collected from the tail vein on days 0, 7 and 14. The blood samples were mixed with EDTA and analyzed using ADVIA 120 (Siemens Healthcare Diagnostics, Tokyo, Japan).

Cellular Assay

HepG2 and H4-II-E-C3 carcinoma cell lines were seeded in 48-well plate at 2.0×10^5 cells/well or 1.2×10^5 cells/well, respectively, in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Twenty-four hours after seeding, the medium was changed to 100 μL of opti-MEM (GIBCO) containing various concentrations of TP0463518. The cells were incubated for 24 hours and then the mRNA were extracted to measure *EPO* mRNA expression. Another plate of cells were incubated for 72 hours and then the media were collected to measure EPO concentration in the media.

Measurement of EPO mRNA

The mRNA in the cells, renal cortex and liver were extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's manual. The mRNA from the whole kidney was prepared as follows. The inferior half of the right kidney was homogenized with Tissue Lyser in 1200 μL of buffer RLT. Then, 150 μL of the homogenate was added to 500 μL of buffer RLT. The remaining steps were carried out according to the manual. Reverse-transcription and real-time quantitative PCR were carried out according to the standard methods (Tea et al., 2009; Wigestrand et al., 2011). The primer sequences are listed in Table 2-1. In the case of *Epo* expression below the detection limit (3 cases in liver in all the experiments), the *Epo* expression was calculated by substituting 40 for the Ct value.

Relative *Epo* mRNA expressions in the cells, renal cortex and liver were calculated as the ratios to those in the vehicle group. The total *Epo* mRNA expressions in the whole organs were calculated using a modification of the method described by Eckardt et al. (1992). The total *Epo* mRNA expressions were calculated as shown in formula (1).

$$\text{Total } \textit{Epo} \text{ mRNA expression} = \frac{\text{(total) } \textit{Epo} \text{ mRNA}}{\text{(total) } \textit{Hprt} \text{ mRNA}} \times \frac{\textit{Hprt} \text{ mRNA}}{\text{RNA amount}} \times \frac{\text{RNA amount}}{\text{organ weight}} \times \text{organ weight} \quad (1)$$

For "*Epo* mRNA/*Hprt* mRNA" and "organ weight", measured values were substituted. For "RNA amount/organ weight", the values were cited from the previous study (Eckardt et al., 1992). Our preliminary data indicated that the "*Hprt* mRNA/RNA amount" was almost the same between the kidney and liver, as reported previously (Vandesompele et al., 2002). Therefore, formula (1) could be converted to formula (2), as follows, where k is a constant, such that the total *Epo* mRNA expression in the kidney is 1.

$$\text{Total } \textit{Epo} \text{ mRNA expression} = \frac{\textit{Epo} \text{ mRNA}}{\textit{Hprt} \text{ mRNA}} \times \frac{\text{RNA amount}}{\text{organ weight}} \times \text{organ weight} \times k \quad (2)$$

Determination of HIF-2α Expression in the Kidney Cortex and Liver

The kidney cortices and livers were homogenized in 10 volumes of protein extraction buffer (20 mM Tris-HCl pH8.0, 1.5 mM $MgCl_2$, 420 mM NaCl, 25 vol% glycerol, 0.2 mM EDTA, 0.5 vol% IGEPAL CA-630, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and proteinase inhibitor cocktail) and the homogenates were centrifuged (4˚C, 15,000 xg, 10 min) to prepare the protein extracts. The extracts were mixed with sample buffer and heated at 96˚C for 5 minutes. Then, 140 μg of the protein extracts were applied to SDS-PAGE. The gels were stained with CBB and an area of 90-120 kDa was cut out and dried. Cysteine residues in the protein of the gel were reduced at 56˚C for 60 minutes with reduction buffer (10 mM DTT and 25 mM ammonium bicarbonate), and then alkylated at room temperature for 45 minutes, protected from light, with alkylation buffer (55 mM iodoacetamide and 25 mM ammonium bicarbonate). Then, the gels were dried again, and the proteins in the gels were digested with 200 ng/mL of trypsin at 37˚C for 15 hours. After the digestion, peptides were extracted from the gels with extraction buffer (50% acetonitrile and 1% formic acid). Stable isotope-labeled peptides (GQVVS[Gly($^{13}C_2$;^{15}N)]QYR and LAISF[Leu($^{13}C_6$;^{15}N)]R) were added to the peptide extracts as an external standard. The peptide extracts were separated with DiNa nano-LC system (KYA Technologies, Tokyo, Japan) and analyzed with QTRAP 5500 (AB Sciex LLC, Framingham, MA). A standard curve was prepared with unlabeled peptides.

Determination of EPO in Serum and Medium

The EPO levels in serum and medium were measured using a commercially available EPO ELISA kit (rat: BioLegend, San Diego, CA; human: Stemcell Technologies, Vancouver, Canada) according to the manufacturer's manual. EPO levels below the detection limits were considered as zero for the purpose

of analysis.

Determination of the TP0463518 Concentration in the Kidney, Liver and Plasma

The TP0463518 concentrations in the kidney, liver and plasma were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), using the LC-20AD HPLC system (Shimadzu Corporation, Kyoto, Japan) and API4000 (AB Sciex LLC).

Statistics

Data are shown as the means ± S.E.M. or means ± S.D. As the *Epo* mRNA expression levels increased exponentially, the means and S.E.M. were calculated for logarithmically transformed values of *Epo* mRNA. The statistical significances of differences were determined using the Student's t test or Dunnett's test, using SAS 9.2. Statistical significance was defined as $P < 0.05$.

Results

TP0463518 and DMOG Only Slightly Increase Renal EPO Production in Healthy Rats

To examine whether TP0463518 exerts EPO-producing effect in the kidney, we addressed the effect of TP0463518 on the HIF-2α and *Epo* mRNA expression levels in the kidney cortex after administration of 20 mg/kg of TP0463518. TP0463518 failed to increase the HIF-2α level in the kidney cortex for 24 hours (Fig. 2-1A). The *Epo* mRNA expression in the kidney cortex increased slightly at 2 and 4 hours after the TP0463518 administration, and returned to the baseline level thereafter (Fig. 2-1B). Analysis of the dose dependence of the effect of TP0463518 on the HIF-2α and *Epo* mRNA expressions at 4 hours showed that TP0463518 did not change the HIF-2α levels up to 40 mg/kg (Fig. 2-1C). TP0463518 did not change the *Epo* mRNA levels up to 10 mg/kg, but thereafter, slightly, but significantly increased the *Epo* mRNA expression level at 20 mg/kg or more (Fig. 2-1D). These results indicated that TP0463518 only slightly induced *Epo* mRNA expression in the kidney cortex but this effect was transient and HIF2α-independent.

We also addressed the effect of DMOG, another PHD inhibitor, on the *Epo* mRNA expression in the kidney cortex at 4 hours after administration of 60 and 600 mg/kg. The *Epo* mRNA levels did not change at 60 mg/kg of DMOG but slightly increased at 600 mg/kg (Fig. 2-2A).

TP0463518 and DMOG Increases Hepatic EPO Production in Healthy Rats

We next measured the HIF-2α and *Epo* mRNA expression levels in the liver to examine whether TP0463518 exerts EPO-producing effect in the liver. HIF-2α markedly increased in the liver at 1 hour after TP0463518 administration and peaked at 2 hours (Fig. 2-3A). At 2 hours post administration TP0463518 significantly increased liver HIF-2α at 5 mg/kg or more in a dose-dependent manner; the increase in HIF-2α expression at 40 mg/kg of TP0463518 was 5.7-fold higher from 0.27 fmol/mg in the vehicle-treated group to 1.53 fmol/mg in the 40 mg/kg of TP0463518-treated group (Fig. 2-3C). Remarkably, the *Epo* mRNA expression level increased dramatically following administration of TP0463518 and remained high for over 24 hours, with the peak at 4 hours post TP0463518 administration (Fig. 2-3B). At 4 hours post administration, the increase of the *Epo* mRNA level induced by TP0463518 was dose-dependent, and the expression level in the 40 mg/kg of TP0463518-treated group was 1300-fold higher than that in the vehicle-treated group (Fig. 2-3D).

DMOG dose-dependently increased *Epo* mRNA levels in the liver at 4 hours post administration (Fig. 2-2B). The increase of *Epo* mRNA was 7800-fold higher in the 600 mg/kg of DMOG-treated group than that in the vehicle-treated group.

Total *Epo* mRNA Expression Levels in the Whole Liver are Higher than Those in the Whole Kidney

Since *Epo* mRNA expression in the adult rat liver is very low (Eckardt et al., 1992; Tan et al., 1992),

the marked increase in the relative *Epo* mRNA expression level could be a result of overestimation of the mRNA levels after TP0463518 administration. We therefore estimated the total *Epo* mRNA expression levels in the whole liver and whole kidney by multiplying *Epo* mRNA levels in the liver and kidney by the organ weight and total mRNA expression amount in each organ. The total *Epo* mRNA expressions in each organ after TP0463518 administration were then compared in reference to the renal *Epo* mRNA level in the vehicle-treated group set as 1 (see Materials and Methods). In the kidney, the total *Epo* mRNA expression level failed to increase up to 10 mg/kg of TP0463518, and then slightly increased at doses of 20 mg/kg and 40 mg/kg (Fig. 2-4A). In the vehicle-treated group, the total *Epo* mRNA expression level in the whole liver was 0.29-fold to that in the whole kidney. Importantly, this expression became 18-fold higher in the group treated with 10 mg/kg of TP0463518. Furthermore, at 20 mg/kg of TP0463518, the total *Epo* mRNA in the whole liver was 22-fold higher than that in the whole kidney, even though the total *Epo* mRNA expression in the whole kidney was slightly increased under this condition. These results explicitly demonstrated that TP0463518 was considerably more potent at inducing *Epo* production in the liver than in the kidney.

In the 600 mg/kg of DMOG-treated group, the total *Epo* mRNA expression levels in the whole liver was 79-fold higher than that in the whole kidney (Fig. 2-4B).

TP0463518 Increases the Serum EPO Levels in BNx Rats

We next examined whether the rise in the serum EPO levels after TP0463518 administration could reflect the increased *Epo* mRNA expression levels in the liver. We have shown in the previous chapter that TP0463518 increased the serum EPO levels in 5/6 Nx rats. However, since 5/6 Nx rats have a remnant kidney, we could not exclude the effect of TP463518 on the remaining kidney in the rise of the serum EPO levels. Therefore, we examined the EPO-producing effect of TP0463518 in BNx rats. Animal experiments using BNx rats (by 48 hours post operation) have been conducted in Europe and the United States, where animal welfare standards are higher than Japan (Subramanian et al., 2016; Mair et al., 2019; Kovalčíková et al., 2020). In our preliminary experiments, no rats died by 24 hours post operation. TP0463518 at 10 and 20 mg/kg did not increase the *Epo* mRNA expression in the kidney in the sham rats (Fig. 2-5A), indicating that the kidney did not contribute to the rise of the serum EPO levels after TP0463518 administration in the sham rats. TP0463518 at the doses of 10 and 20 mg/kg increased the *Epo* mRNA expression levels in the liver of the sham rats by 56 and 388-fold, respectively (Fig. 2-5B). TP0463518 at the same doses also raised the *Epo* mRNA expression levels in the livers of the BNx rats by 127 and 753-fold, respectively, indicating that TP0463518 induces *Epo* mRNA expression increase in the liver even in BNx rats.

The serum EPO concentration in the BNx rats was below the detection limit at 24 hours after nephrectomy (Fig. 2-5C), because BNx rats do not have EPO secretion from the kidney. In normal rats, TP0463518 increased the serum EPO concentration in a dose-dependent manner, as shown in

chapter 2. In the sham rats, TP0463518 increased the serum EPO concentrations from 18 pg/mL in the vehicle-treated group to 71 pg/mL in the 20 mg/kg of TP0463518-treated group. In the BNx rats, TP0463518 at 10 and 20 mg/kg also raised the serum EPO concentrations from 0 pg/mL (below detection limit) to 28 and 180 pg/mL, respectively. Therefore, consistent with the effective increase in the hepatic *Epo* mRNA levels induced by TP0463518 administration, the serum EPO concentrations were higher in the BNx rats than in the sham rats. These results demonstrated that TP0463518 induced hepatic EPO production, and in turn, increased liver-derived EPO concentrations in the serum.

TP0463518 Has a Higher EPO-inducing Potency in the Liver than in the Kidney

In order to investigate whether the differential actions of TP0463518 on *Epo* expression between the liver and kidney are attributable to the difference in the exposure levels of TP0463518 between these organs, we measured the TP0463518 concentrations in each of these organs. In healthy rats, the TP0463518 concentrations at 4 hours post administration were 5.3-6.4 and 17.5-23.5 times higher in the kidney and the liver compared to plasma, respectively (Table 2-2). To investigate the relationship between the TP0463518 concentrations and *Epo* mRNA expression levels, we plotted the *Epo* mRNA levels against the corresponding TP0463518 concentrations in the kidneys and liver. *Epo* mRNA expression in the liver increased in an exposure-dependent manner (Fig. 2-6). In contrast, the *Epo* mRNA expression level in the kidney scarcely increased despite the increase of the TP0463518 concentration. At the same TP0463518 concentrations that yielded 5940 ng/g in the liver (10 mg/kg) and 5910 ng/g in the kidney (40 mg/kg), the *Epo* mRNA expressions increased by 185-fold and 3.0-fold, respectively. These results demonstrated that TP0463518 exerted its EPO-producing effect almost exclusively in the liver, and greatly contributed to augmentation of EPO levels in the serum.

Liver-Derived EPO Ameliorates Anemia

We next addressed whether liver-derived EPO induced by TP0463518 has ameliorating effects on anemia, we administrated TP0463518 to 5/6 Nx rats, which is a model of renal anemia. While a single administration of TP0463518 at 10 or 20 mg/kg to 5/6 Nx rats failed to increase the renal *Epo* mRNA expression, it markedly increased the hepatic *Epo* mRNA expression by 670 and 1800-fold, respectively, as compared to the level in the vehicle-treated group (Fig. 2-7). Therefore, in 5/6 Nx rats, in which most of the kidney tissue has been removed, TP0463518 was not effective in inducing renal *Epo* production up to 20 mg/kg, unlike in normal rats.

In the 5/6 Nx rats, while the reticulocyte count was similar to that in the sham rats (263 ± 8 vs. 255 ± 12 billion cells/L), the hemoglobin concentration was significantly lower than that in the sham rats (15.8 ± 0.1 vs. 13.4 ± 0.3 g/dL), indicating that the 5/6 Nx rats had anemia. After once-daily administration of TP0463518 for a week to these rats, the reticulocyte count increased in a dose-dependent manner, and the count was 481 ± 23 billion cells/L at 10 mg/kg (Fig. 2-8A). The hemoglobin

concentration in the vehicle-treated group was still low as compared to that in the sham rats after 2 weeks of repeated administration (16.6 ± 0.2 vs. 14.2 ± 0.4 g/dL). However, the hemoglobin concentration in the TP0463518-treated groups increased in a dose-dependent manner, and the concentration reached 16.8 ± 0.4 g/dL at 10 mg/kg of TP0463518, which was comparable to the hemoglobin concentration in the sham rats (Fig. 2-8B). The hematocrit increased from 41.4 ± 1.2 in the vehicle-treated group to 51.4 ± 1.2% at 10 mg/kg, becoming comparable to that in the sham rats, (49.1 ± 0.7%) (Fig. 2-8C). The red blood cell count also increased from 7.40 ± 0.24 to 8.80 ± 0.20 million cells/μL (9.09 ± 0.14 million cells/μL in the sham rats, Fig. 2-8D).

TP0463518 Increases EPO Expression in Human and Rat Cell Lines

To examine whether TP0463518 exerts EPO-producing effect in the liver cells, we addressed the effect of TP0463518 on *EPO* mRNA expression and EPO secretion in the human and rat liver cell lines, HepG2 and H4-II-E-C3. After the treatment of the cells with TP0463518 for 24 hours, *EPO* mRNA levels in both HepG2 and H4-II-E-C3 cells increased at 10 μM or more; the increase at 30 μM were 4.04 and 3.47-fold higher than control group, respectively (Fig. 2-9A, B). EPO concentration in the medium of HepG2 cells treated with TP0463518 for 72 hours increased from 38.7 mU/mL in control wells to 74.0 mU/mL at 30 μM (Fig. 2-9C). EPO concentration in medium of H4-II-E-C3 cells treated with TP0463518 for 72 hours also increased from 79.1 pg/mL in control wells to 254.9 pg/mL at 30 μM (Fig. 2-9D). These results indicated that TP0463518 increased EPO expression not only in rat liver but also in human liver.

Table 2-1. Primer sequences.

	gene	Sequence (5' to 3')
human	*RNR1*	GTAACCCGTTGAACCCCATT
		CCATCCAATCGGTAGTAGCG
	EPO	GAGGCCGAGAATATCACGACGGG
		TGCCCGACCTCCATCCTCTTCCAG
rat	*Hprt*	TTGTTGGATATGCCCTTGACT
		CCGCTGTCTTTTAGGCTTTG
	Epo	ACCAGAGAGTCTTCAGCTTCA
		GAGGCGACATCAATTCCTTC

Table 2-2. TP0463518 concentrations in the plasma, liver and kidney at 4 hours after administration. L/P or K/P indicate the liver-plasma or kidney-plasma ratio of the TP0463518 concentrations. Mean ± S.D., n = 6.

Matrix	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg
Plasma	166 ± 53.4	337 ± 120	669 ± 160	1130 ± 357
Liver	3810 ± 1090	5940 ± 1130	11700 ± 1120	20300 ± 9270
Kidney	1040 ± 324	2040 ± 790	3810 ± 1130	5910 ± 1690
Ratio (L/P)	23.5 ± 5.3	18.8 ± 5.9	18.1 ± 3.5	17.5 ± 2.8
Ratio (K/P)	6.4 ± 0.6	6.0 ± 0.7	5.7 ± 0.8	5.3 ± 0.4

Concentration (ng/mL plasma or g tissue)

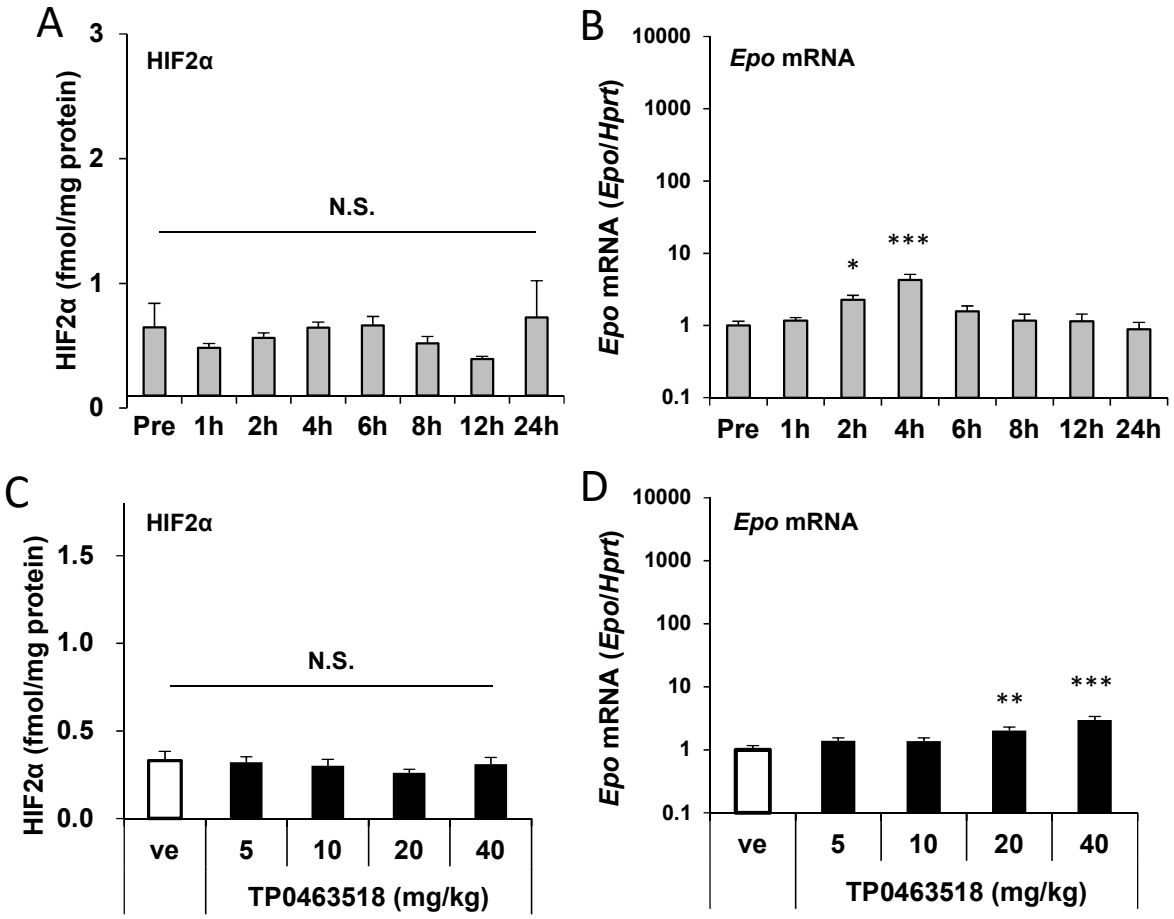


Fig. 2-1. TP0463518 scarcely increases the expressions of HIF-2α and *Epo* mRNA in the kidney cortex. (A, B) Time-course study of HIF-2α stabilization and *Epo* mRNA expression after administration of 20 mg/kg of TP0463518. TP0463518 failed to increase the HIF-2α level in the kidney cortex up to 24 hours (A). A slight and transient increase of the *Epo* mRNA expression level was observed at 2 and 4 hours post administration (B). (C, D) Dose-titration study of HIF-2α stabilization and EPO mRNA expression at 4 hours post administration. TP0463518 failed to increase the HIF-2α level in the kidney cortex up to 40 mg/kg (C). *Epo* mRNA expression remained stable up to 10 mg/kg, with slight, but significant increase at 20 mg/kg or more (D). Data are represented as the means ± S.E.M. n = 5-6. Dunnett's multiple comparison test was used to compare the TP0463518-treated groups and the corresponding control groups (the non-treated Pre group in the time-course study and the vehicle-treated group in the dose-dependent study). N.S., not statistically significant; *P < 0.05; **P < 0.01; ***P < 0.001.

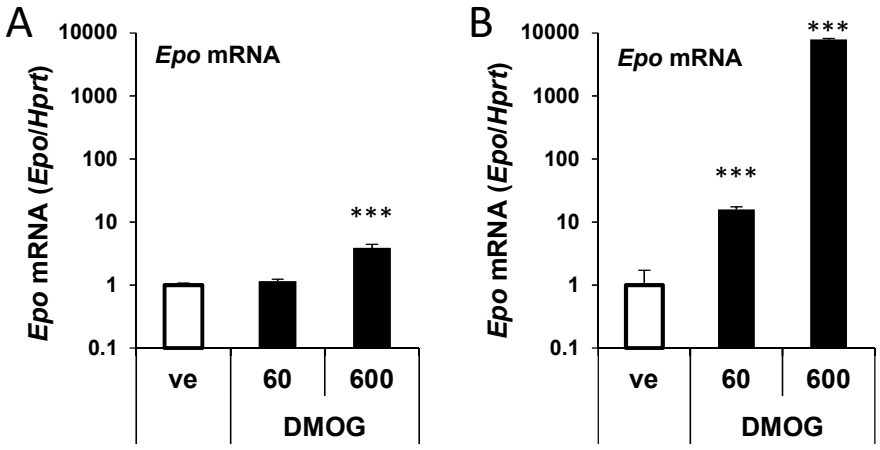


Fig. 2-2. DMOG scarcely increases the expressions of *Epo* mRNA in the kidney cortex, whereas it markedly increases the expressions of *Epo* mRNA in the liver. (A) *Epo* mRNA expression in the kidney cortex at 4 hours post administration slightly, but significantly increased at 600 mg/kg. (B) DMOG caused a dramatic increase of the *Epo* mRNA expression in the liver at 4 hours post administration at 60 mg/kg or more. Data are represented as the means ± S.E.M. n = 6. Dunnett's multiple comparison test was used to compare the DMOG-treated groups and the corresponding vehicle-treated groups. ***P < 0.001.

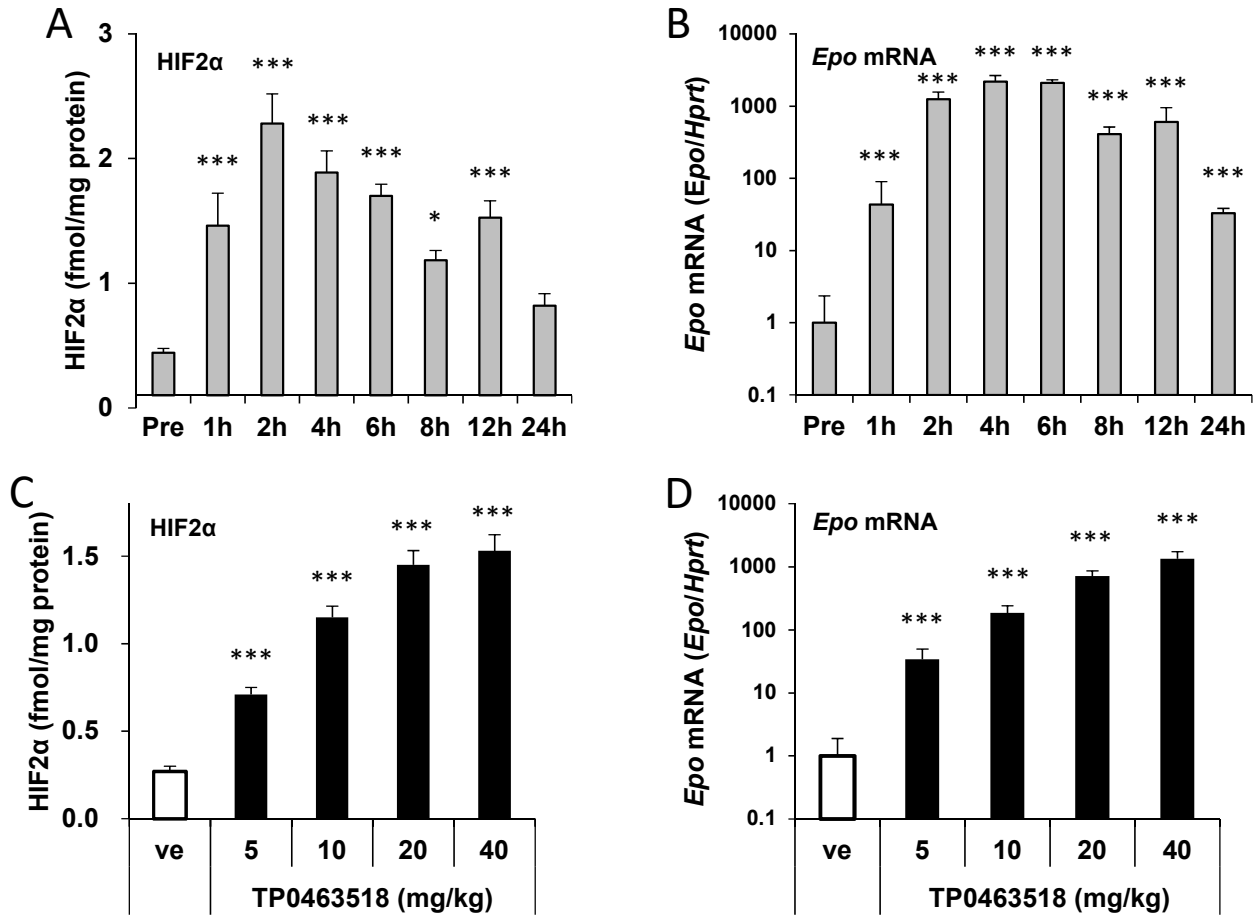


Fig. 2-3. TP0463518 markedly increases the expressions of HIF-2α and *Epo* mRNA in the liver. (A, B) Time-course study of HIF-2α stabilization and *Epo* mRNA expression after administration of 20 mg/kg of TP0463518. The HIF-2α level markedly increased in the liver and peaked at 2 hours post administration (A). *Epo* mRNA expression dramatically increased post TP0463518 administration and peaked at 4 hours post administration (B). (C, D) Dose-titration study of HIF-2α stabilization and *Epo* mRNA expression. TP0463518 administration at 5 mg/kg or more significantly increased the HIF-2α level at 2 hours post administration (C). TP0463518 caused a dramatic increase of the *Epo* mRNA expression in a dose-dependent manner at 4 hours post administration (D). Data are represented as the means ± S.E.M. n = 5-6. Dunnett's multiple comparison test was used to compare the TP0463518-treated groups and the corresponding control groups (the non-treated Pre group in the time-course study and the vehicle-treated group in the dose-dependent study). *P < 0.05; ***P < 0.001.

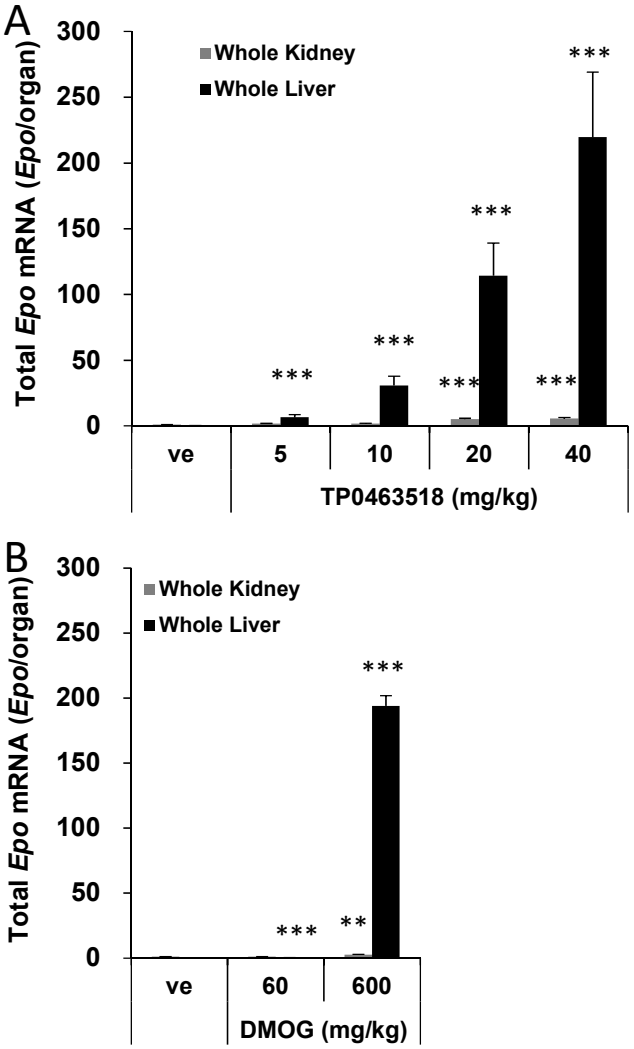


Fig. 2-4. Total *Epo* mRNA expression levels in the whole liver are higher than those in the whole kidney. Relative *Epo* mRNA expression at 4 hours post (A) TP0463518 or (B) DMOG administration was multiplied by the organ weight and the total mRNA expression amount, to determine the total *Epo* mRNA expression levels in the whole kidney (gray bar) and whole liver (black bar). The total *Epo* mRNA expression level in each organ was normalized by the renal *Epo* mRNA expression level in the vehicle-treated group. The total *Epo* mRNA expression in the whole liver was dramatically increased, whereas that in the whole kidney was scarcely increased. Data are represented as the means ± S.E.M. n = 6. Dunnett's multiple comparison test was used to compare the TP0463518 or DMOG-treated groups and the vehicle-treated groups. **P < 0.01; ***P < 0.001.

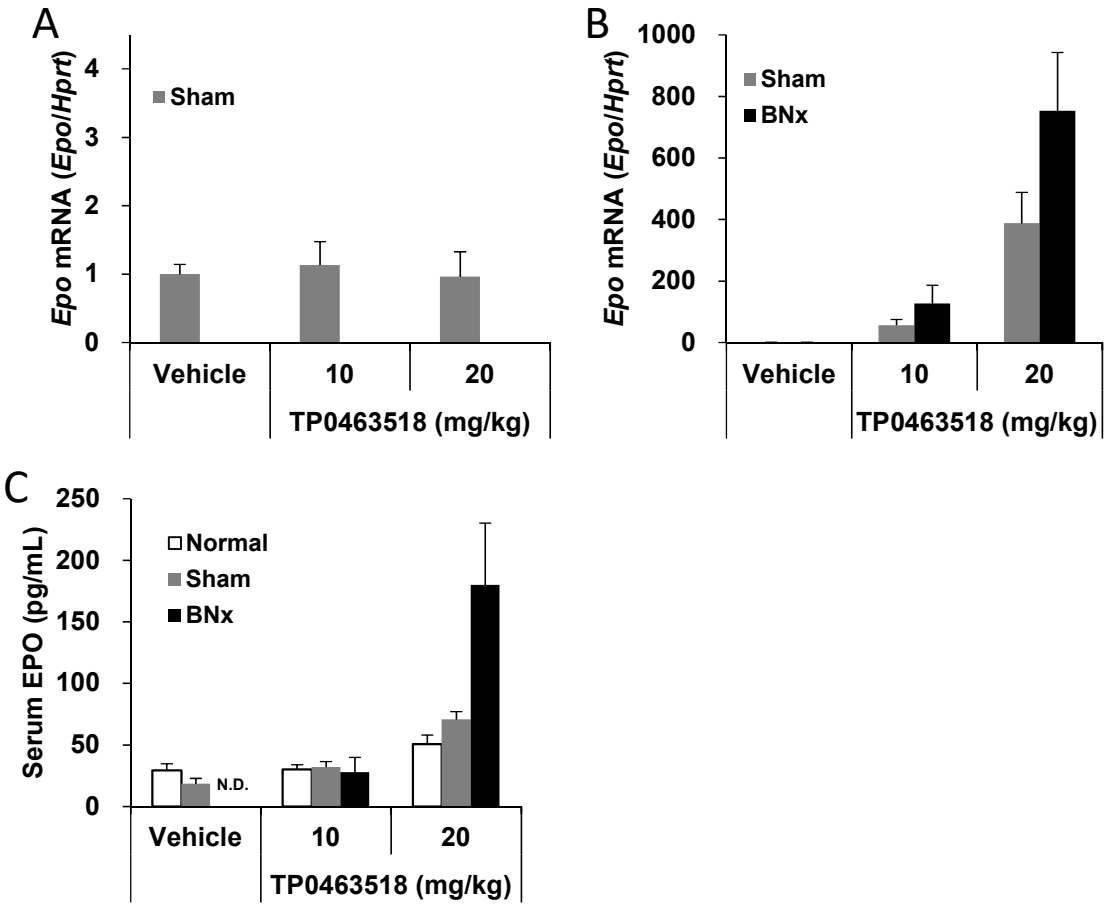


Fig. 2-5. The increase in the serum EPO after TP0463518 administration is mainly attributable to increased hepatic EPO production. (A, B) Renal and hepatic *Epo* mRNA expression levels in the sham and BNx rats. Real-time PCR analysis of the kidney and the liver was performed at 4 hours post administration. (A) Renal *Epo* mRNA expression in the sham rats remained stable up to 20 mg/kg of TP0463518. (B) Hepatic *Epo* mRNA expression levels in the sham and BNx rats increased in a dose-dependent manner. (C) Serum EPO concentrations in the normal, sham and BNx rats. Elevated serum EPO concentrations in a dose-dependent manner were observed in each group at 8 hours post administration. White bar, normal control rats; gray bar, sham rats; black bar, BNx rats. Data are represented as the means ± S.E.M. n = 8-14 (mRNA) or 6-8 (serum EPO). Serum EPO concentrations below detection limit were regarded as zero. BNx, bilaterally nephrectomized rats; N.D., not detected.

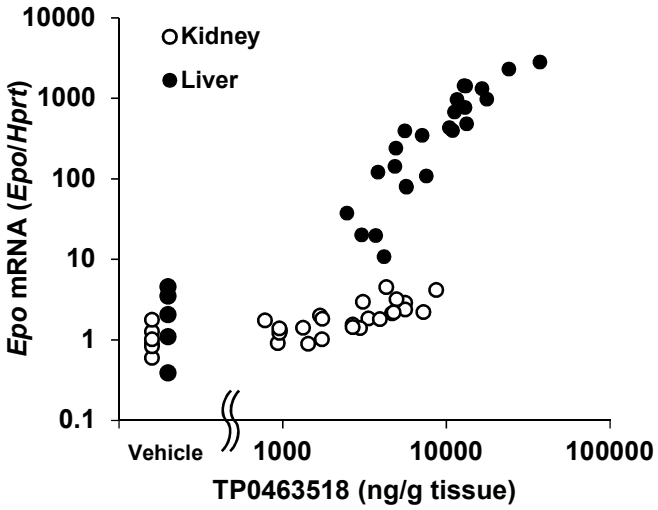


Fig. 2-6. TP0463518 increases *Epo* mRNA expression exclusively in the liver. Scatter plot of *Epo* mRNA expression levels against the TP0463518 concentrations. Individual *Epo* mRNA expression levels in the liver and kidney are plotted against the corresponding plasma TP0463518 concentrations (n = 24 in each organ). The *Epo* mRNA expression levels in the vehicle-treated group are plotted on the left (n = 6 in each organ). While *Epo* mRNA expression in the liver increased in an exposure-dependent manner, the *Epo* mRNA expression level in the kidney scarcely increased despite the increase of the TP0463518 concentration. Open circles, kidney; filled circles, liver.

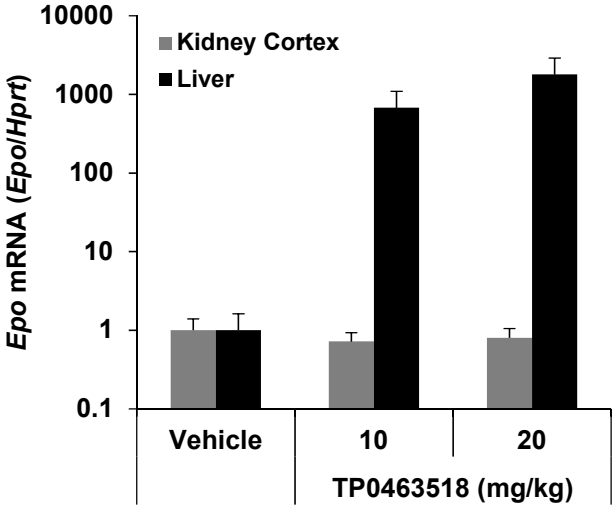


Fig. 2-7. Renal and hepatic *Epo* mRNA expressions in the 5/6 Nx rats. Real-time PCR analysis of the kidney cortex and liver was performed at 2 hours post administration. Renocortical *Epo* mRNA expression in the 5/6 Nx rats remained stable up to 20 mg/kg of TP0463518, whereas the hepatic *Epo* mRNA levels were elevated in a dose-dependent manner. Gray bar, kidney cortex; black bar, liver. Data are represented as the means ± S.E.M. n = 5.

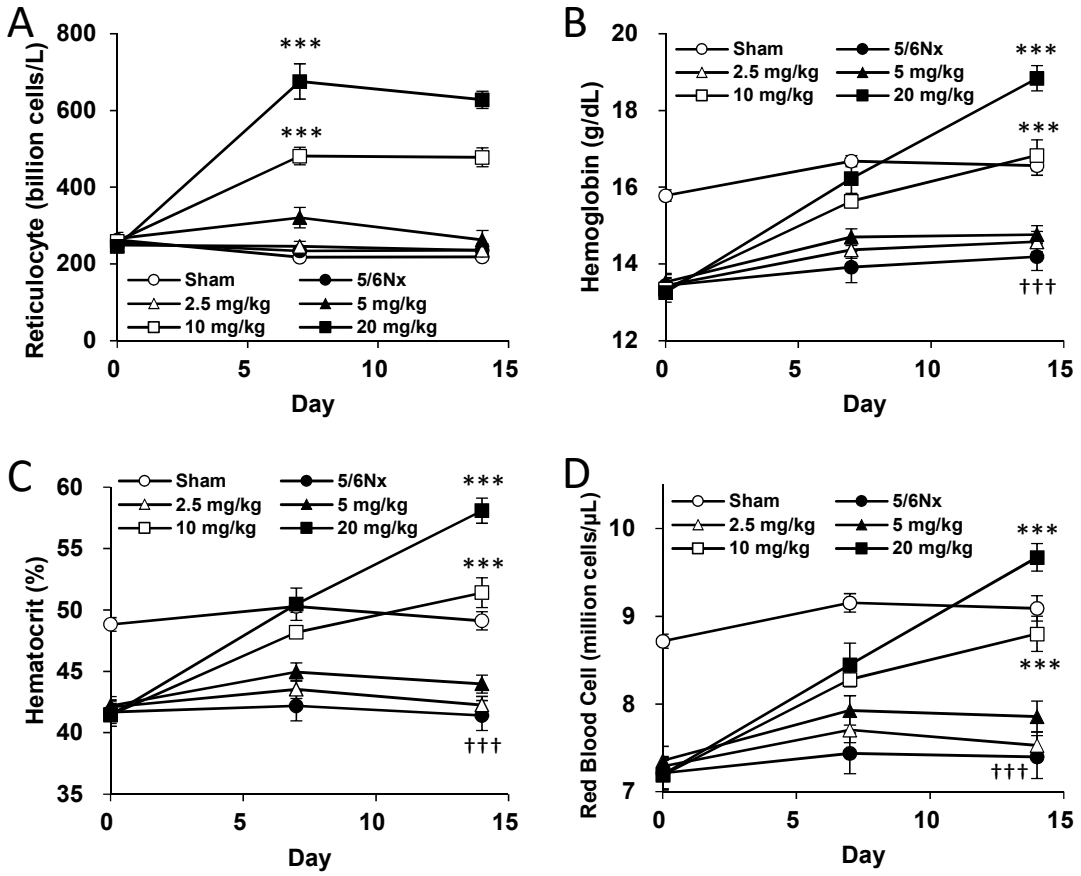


Fig. 2-8. TP0463518 ameliorates renal anemia. The reticulocyte count (A), hemoglobin level (B), hematocrit (C) and red blood cell count were measured on days 0, 7 and 14 of treatment. TP0463518 raised all the parameters in a dose-dependent manner in the 5/6 Nx rats. Data are represented as the means ± S.E.M. n = 9-10. Student's t-test was performed to compare the sham rats and 5/6 Nx rats. †††$P < 0.001$. Then, Dunnett's multiple comparison test was used to compare the vehicle-treated 5/6 Nx rats and TP0463518-treated groups (closed testing procedure). ***$P < 0.001$.

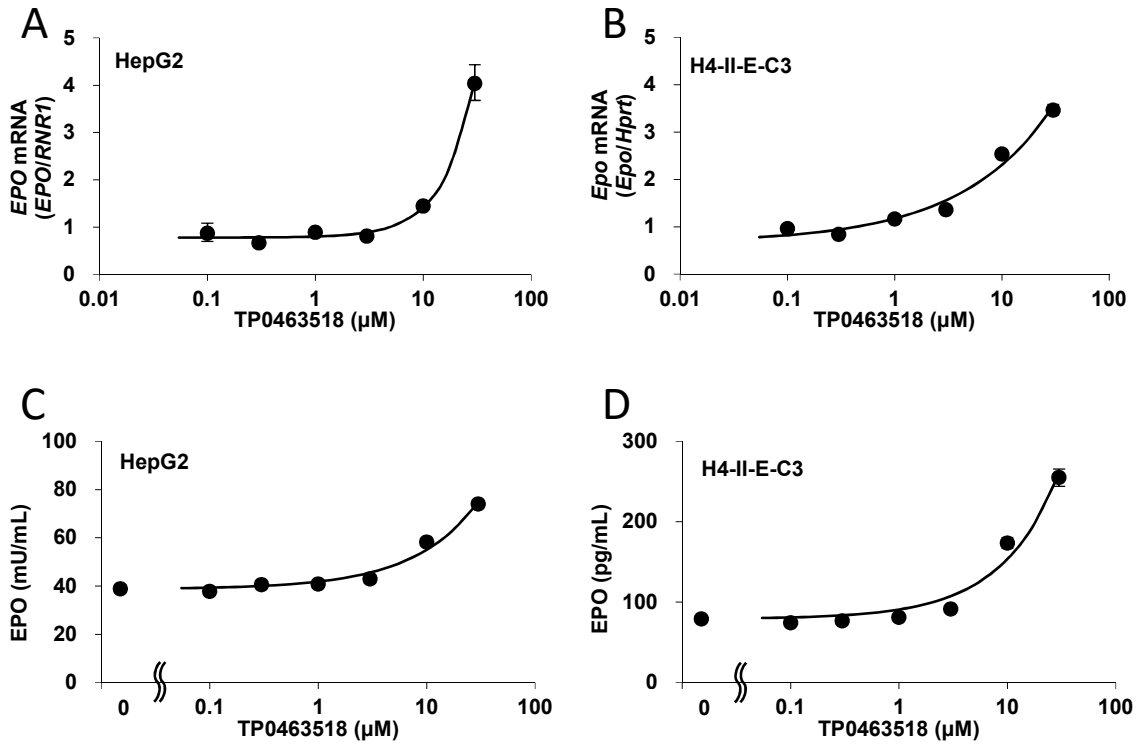


Fig. 2-9. TP0463518 increases the *EPO* mRNA expression and EPO concentration in the medium in HepG2 and H4-II-E-C3 cell lines. (A, B) *EPO* mRNA expression levels after the treatment with TP0463518 for 24 hours. TP0463518 increased the *EPO* mRNA expression levels in both (A) HepG2 and (B) H4-II-E-C3 cells at 10 μM or more. (C, D) EPO concentration in medium after the treatment with TP0463518 for 72 hours. TP0463518 increased the EPO concentration in both (C) HepG2 and (D) H4-II-E-C3 cells at 10 μM or more. Data are represented as the means ± S.E.M. n = 3.

Discussion

TP0463518 is a PHD1/2/3 pan-inhibitor and now under phase 1 clinical trials for renal anemia (Kato et al., 2018; Shinfuku et al., 2018). Based on previous findings, in this study, we investigated whether TP0463518 specifically induced EPO production in the liver. This study indicated that while TP0463518 did not induce HIF-2α-dependent *Epo* mRNA expression in the kidney, it markedly induced HIF-2α and *Epo* mRNA expression in the liver. The different action of TP0463518 could not be ascribed to different TP0463518 concentrations between the kidney and liver. Consistent with the findings that TP0463518 increased hepatic *Epo* mRNA expression, the serum EPO concentrations in BNx rats increased to an extent comparable to or more than that in the sham rats. Furthermore, TP0463518 induced *Epo* mRNA expression only in the liver in 5/6 Nx rats. These results thus clearly demonstrate that TP0463518 enhanced EPO production much more potent in the liver and increased the serum concentrations of EPO. We further demonstrated that liver-derived EPO induced by TP0463518 was pharmacologically effective in ameliorating nephrectomy-induced anemia. Repeated administration of TP0463518 at 20 mg/kg, while not increasing the renal *Epo* mRNA expression, greatly improved the hemoglobin levels in the 5/6 Nx rats to the same degree or more than that in the sham rats in a dose-dependent manner. Therefore, TP0463518 is a liver-specific PHD inhibitor at a therapeutic dose and has the potent ability to induce sufficient hepatic EPO production to ameliorate anemia. Additionally, TP0463518 increased EPO expression in human and rat cell lines, HepG2 and H4-II-E-C3. These results indicated that TP0463518 increased EPO expression not only in rat liver but also in human liver.

A number of studies have so far investigated the effects of genetic or pharmacological PHD suppression in animal models of nephropathy (Flamme et al., 2014; Souma et al., 2016). When unilateral ureteral obstruction was induced in knockout mice lacking *Phd1/2/3*, the *Epo* mRNA levels in the damaged kidney were higher than those in the healthy kidney (Souma et al., 2016). In contrast to this findings, the increase in renal *Epo* mRNA expression induced by a PHD inhibitor was significantly lower in gentamicin-induced nephropathic rats as compared to healthy control rats (Flamme et al., 2014). These findings suggest that the effects of a PHD inhibitor on the renal EPO production vary depending on the stage of nephropathy. Interestingly, hepatic *Epo* expression induced by a PHD inhibitor was reportedly almost comparable between gentamicin-induced nephropathic rats and healthy control rats (Flamme et al., 2014). There is no explicit evidence until date indicating that PHD inhibitors have the ability to stabilize HIF-2α specifically in the liver and increase liver-derived serum EPO concentrations. FG-2216 increased the serum EPO levels in anephric hemodialysis patients, but the increase of the serum EPO was smaller than that in nephric hemodialysis patients (Bernhardt et al., 2010). Molidustat was reported to increase the renal *Epo* mRNA expression in rats by several hundred fold, but raised hepatic *Epo* mRNA expression by only several ten times (Flamme

et al., 2014). Three other PHD inhibitors are now under phase 3 clinical trials (Coyne et al., 2017), but the main organ source of EPO production after administration of these inhibitors remains unknown. Therefore, to the best of our knowledge, TP0463518 is the only clinical compound inducing EPO specifically in the liver to ameliorate anemia.

TP0463518 scarcely induced EPO production in the kidney cortex despite the increase of the TP0463518 concentration. As kidney is a highly heterogeneous organ, the localization of the compound should be considered. The urinary excretion rates of FG-2216 and molidustat, which induced renal EPO, were 11% and 1.4-3.6%, respectively (Bernhardt et al., 2010; Böttcher et al., 2018). On the other hands, the urinary excretion rate of TP0463518 was almost zero and DMOG, which dominantly induced hepatic EPO, was metabolized in the liver (Hamada et al., 2018; Singh et al., 2019). Taking these points into consideration, a compound in the tubular might be delivered to the tubulointerstitium, and then the compound would exert EPO-producing effect in the kidney.

It is also noteworthy that liver-specific *Phd1/2/3* triple-knockout mice reportedly increased hepatic *Epo* expression and developed polycythemia (Minamishima and Kaelin, 2010; Tojo et al., 2015). Furthermore, liver-derived EPO was also reported to cause polycythemia in liver-specific *Vhl* knockout mice (Haase et al., 2001; Rankin et al., 2007). Higher hemoglobin level is associated with increased risk of stroke, cardiovascular events and dialysis (Drüeke et al., 2006; Singh et al., 2006; Pfeffer et al., 2009). Therefore, it is crucial to ameliorate anemia without causing polycythemia. TP0463518 increased hemoglobin levels in the correction phase with a clear dose-response from 2.5 mg/kg, at which TP0463518 was ineffective, to 20 mg/kg, at which TP0463518 was excessive. This result suggests that an adequate dose adjustment of TP0463518 could control hemoglobin levels within desirable range in the maintenance phase. TP0463518 is now being examined in a clinical trial, and the clinical proof-of-concept will be available in the future.

The present study thus revealed a very unique feature of TP0463518, in that TP0463518 stabilized HIF-2α and induced EPO specifically in the liver at a therapeutic dose. Furthermore, TP0463518 improved the hemoglobin level to within normal range with a good dose response, and was expected to allow hemoglobin levels to be maintained in the normal range over the long term without causing polycythemia. Therefore, the characteristic feature of TP0463518 would lead to not only more a detailed understanding of the PHD-HIF2-EPO pathway in erythropoiesis, but also a new therapeutic alternative for renal anemia in patients with chronic kidney disease.

Chapter 3

TP0463518 (TS-143) ameliorates peptidoglycan-polysaccharide induced anemia of inflammation in rats

Introduction

Iron deficiency anemia can occur as a result of absolute iron deficiency or functional iron deficiency (Lopez et al., 2016). 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 IL-1β and IL-6 induce hepcidin, a master regulator of iron metabolism (Lee et al., 2005a). Hepcidin internalizes ferroportin (Nemeth et al., 2004; Ramey et al., 2010), 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 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.

HIF-2α regulates iron metabolism. The expression level of HIF-2α is tightly controlled by 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 (Maxwell et al., 1999; Jaakkola et al., 2001). 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., *DMT1* and *DCYTB*) (Mastrogiannaki et al., 2009; Anderson et al., 2011). HIF-2α also indirectly downregulates hepcidin expression via induction of cyclin and EPO expressions (Pinto et al., 2008; Anderson et al., 2012; Liu et al., 2012; Mastrogiannaki et al., 2012). 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 (Flamme et al., 2014; Brigandi et al., 2016; Martin et al., 2017; Akizawa et al., 2019a, 2019b; Chen et al., 2019a, 2019b). 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 (Kato et al., 2018, 2019; Shinfuku et al., 2018). While TP0463518 has been shown to improve renal anemia in 5/6 nephrectomized rats (Kato et al., 2019), 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. These findings suggest that TP0463518 can potentially ameliorate anemia of chronic inflammation.

Materials and Methods

Reagents

PG-PS was purchased from Becton, Dickinson and Company.

Animal experiment protocols

All animal experiment protocols in this chapter were approved by the Animal Committee of Taisho Pharmaceutical Co., Ltd., and all the animal experiments were conducted with the approval of the committee (approval number: 153011). The animals were reared in cages maintained at a room temperature and humidity level of 23°C ± 3°C and 50% ± 20%, respectively, under a light-dark cycle of 12/12 hours. 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 received oral administration of 10 mg/kg of TP0463518 once daily for 6 weeks. Blood samples were collected from the tail vein every week into tubes containing EDTA, and the reticulocyte counts, hematocrit values, hemoglobin levels, red blood cell counts, and mean corpuscular volume (MCV) in the samples were measured using ADVIA 2120i (Siemens Healthcare Diagnostics). At week 6, the rats were anesthetized with isoflurane and blood samples were collected from the abdominal vein. While a part of each blood sample was mixed into an aliquot containing EDTA, the remaining part was left to stand at room temperature to allow clot formation. The blood samples were then centrifuged (2130 xg, 10 min, 4°C or room temperature) to obtain the plasma and serum samples. Then, the rats were euthanized by exsanguination and the liver and duodenum were removed. Small pieces of the organs were immersed in RNAlater solution overnight at 4°C, then stored at -80°C until the mRNA extraction.

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.

Measurement of mRNA

The pieces of liver and duodenum were homogenized using Tissue Lyser (Qiagen) 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). 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 3-1. The PCR conditions were as follows: 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The expression level of each target gene was normalized by that of an internal standard gene (*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.

Western blotting

The duodenal tissue samples were homogenized in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail, and the homogenates were sonicated for 20 seconds and left to stand on ice for 30 minutes. The homogenates were then centrifuged (14500 xg, 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 minutes 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, and anti-dCYTb and anti-vinculin antibodies from Abcam) overnight at 4°C, followed by incubation with the secondary antibody conjugated with horseradish peroxidase (Abcam) for 1 hour at room temperature. The membranes were visualized and quantified using an enhanced-chemiluminescence (Cytiva) and FUSION imaging system (Vilber-Lourmat).

ELISA

The TNFα and IL-6 levels in the liver were measured according to a following method. The liver tissue specimens were homogenized in 5 volumes of tris-buffered saline containing 5 mM EDTA, 1% Triton X100, and a protease inhibitor cocktail. The homogenates were centrifuged at 11000 g for 5 minutes at 4°C, and the TNFα and IL-6 levels in the supernatants were measured using ELISA kits (R&D Systems).

Plasma ferritin levels were measured using the rat ferritin ELISA kit (Kamiya biomedical company), and the serum EPO and hepcidin levels were measured using the rat EPO and hepcidin ELISA kits (BioLegend and Novus Biologicals), respectively, in accordance with the instructions in the respective manufacturers' manuals.

Statistics

Data shown are the means ± 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. For the time-course studies, two-way repeated measures ANOVA with closed testing procedure was used. In this method, a comparison between healthy control group and vehicle-treated group was performed first. Then, if the first analysis was significant, second comparison between vehicle-treated group and TP0463518-treated group was performed. If the first analysis was not significant, second comparison was not performed. For the endpoint studies, protected LSD procedure was used. First, one-way ANOVA was performed. If the one-way ANOVA was significant, Student's t tests were performed to compare healthy control group vs vehicle-treated group and vehicle-treated group vs TP0463518-treated group. All statistics were performed using SAS 9.4. 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 Figure 3-1A. There were no significant differences in the body weight measured after TP0463518 treatment among the groups (Fig. 3-1B). 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 (Fig. 3-1D-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. 3-1C). The hematocrit value in the TP0463518-treated group increased significantly from 32.8% ± 0.8% prior to TP0463518 treatment to 44.5% ± 2.1% after 6 weeks of treatment, which was comparable to the hematocrit value in the healthy control group (Fig. 3-1D). 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 (Fig. 3-1E, 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. 3-1G). 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 (Fig. 3-2A, B), and increased the plasma ferritin levels (Fig.

3-2C). 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 (Fig. 3-3A, 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 vehicle-treated group than in the healthy control group, and significantly increased in the TP0463518-treated group (Fig. 3-3B). This trend was also observed for the DMT1 protein level (Fig. 3-3E). 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. 3-3C).

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. 3-3G). Neither PG-PS treatment nor TP0463518 exerted any effects on the mRNA expression levels of hepcidin (*Hamp*) and ferroportin (*Fpn1*, Fig. 3-3H, I). The serum hepcidin concentration also remained unchanged in all the groups (Fig. 3-3F).

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 *Mcp1*, *Il1b*, *Tnfa*, and *Tgfb1* in the liver were higher in the vehicle-treated group than in the healthy control group (Fig. 3-4A-D). No changes in the mRNA expression levels of *Mcp1*, *Il1b*, *Tnfa* and *Tgfb1* 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 (Fig. 3-4E, F). TP0463518 treatment had no effect on the expression levels of TNFα and IL-6.

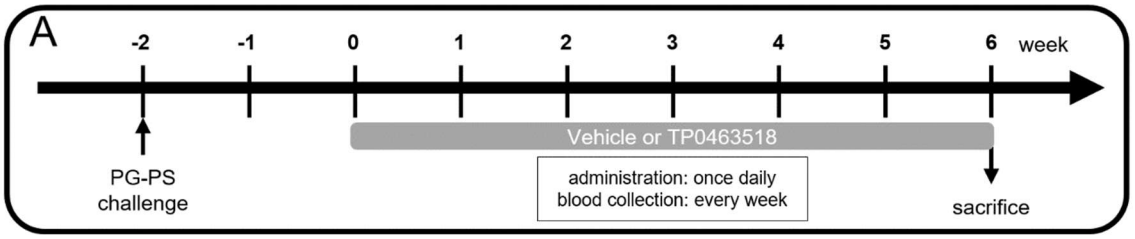
Effect of TP0463518 on the expression level of EPO

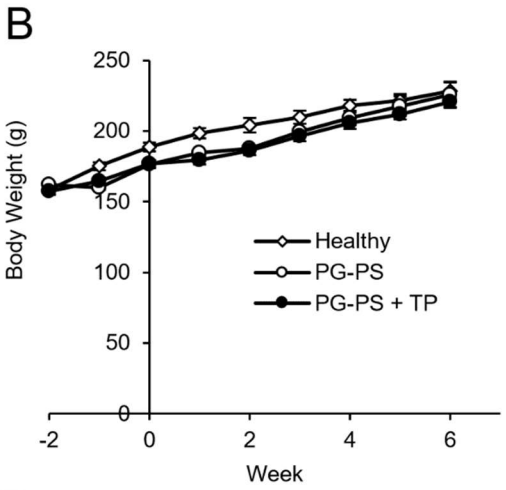
TP0463518 treatment had no effects on the expression levels of either genes involved in iron metabolism or those involved in inflammation in the liver in this study, whereas in chapter 2, we showed that TP0463518 induced EPO expression in the liver. Therefore, to examine the effect of TP0463518 treatment 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

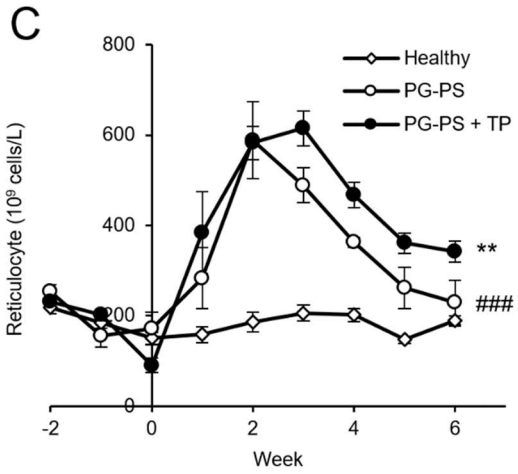
compared to the level in the healthy control rats (Fig. 3-5). TP0463518 treatment further increased the *Epo* mRNA expression in the liver.

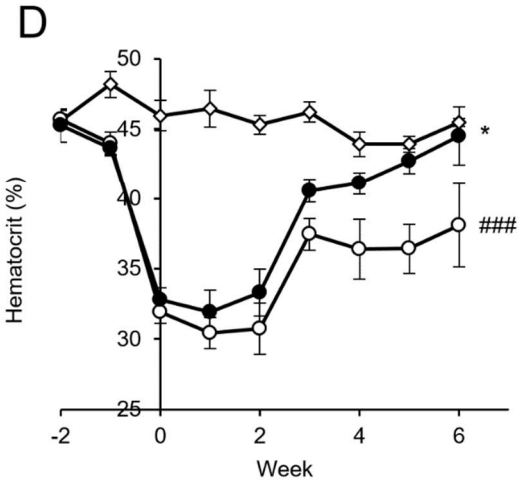
Table 3-1. Primer sequences.

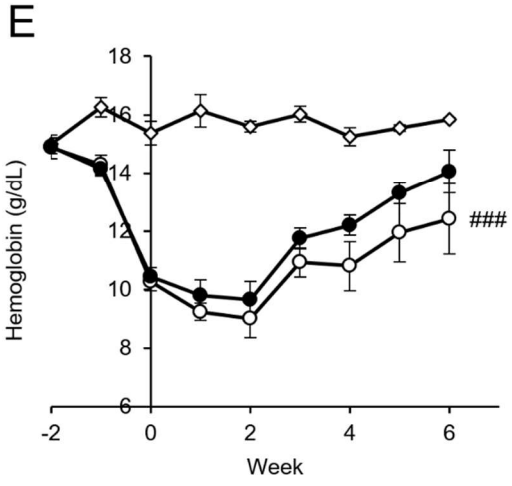
	gene	Sequence (5' to 3')
rat	*Hprt*	TTGTTGGATATGCCCTTGACT
		CCGCTGTCTTTTAGGCTTTG
	Dcytb	TCTGGACTCCTCCTCTTTGG
		TCTGGTGGGAATGAATGGT
	Dmt1	GCTGAGCGAAGATACCAGCG
		TGTGCAACGGCACATACTTG
	Fpn1	TTGCAGGAGTCATTGCTGCTA
		TGGAGTTCTGCACACCATTGAT
	Hamp	GAAGGCAAGATGGCACTAAGCA
		TCTCGTCTGTTGCCGGAGATAG
	Mcp1	AGCATCCACGTGCTGTCTC
		GATCATCTTGCCAGTGAATGAG
	Il1b	TGTGATGAAAGACGGCACAC
		CTTCTTCTTTGGGTATTGTTTGG
	Tnfa	TCAGTTCCATGGCCCAGAC
		GTTGTCTTTGAGATCCATGCCATT
	Tgfb1	GCTGAACCAAGGAGACGGAATA
		ACCTCGACGTTTGGGACTGA
	Epo	ACCAGAGAGTCTTCAGCTTCA
		GAGGCGACATCAATTCCTTC

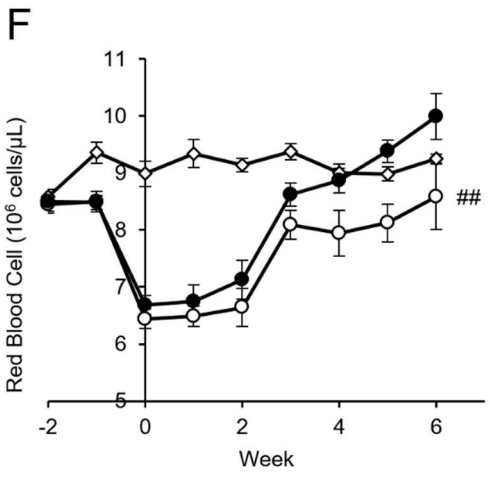

A
-2
-1
0
1
2
3
4
5
6
week
Vehicle or TP0463518
PG-PS
challenge
administration: once daily
blood collection: every week
sacrifice


B
Body Weight (g)
250
200
150
100
50
0
-2
0
2
4
6
Week
Healthy
PG-PS
PG-PS + TP


C
Reticulocyte (10⁹ cells/L)
800
600
400
200
0
-2
0
2
4
6
Week
Healthy
PG-PS
PG-PS + TP
**
###


D
Hematocrit (%)
50
45
40
35
30
25
-2
0
2
4
6
Week
*
###


E
Hemoglobin (g/dL)
18
16
14
12
10
8
6
-2
0
2
4
6
Week
###


F
Red Blood Cell (10⁶ cells/μL)
11
10
9
8
7
6
5
-2
0
2
4
6
Week
##

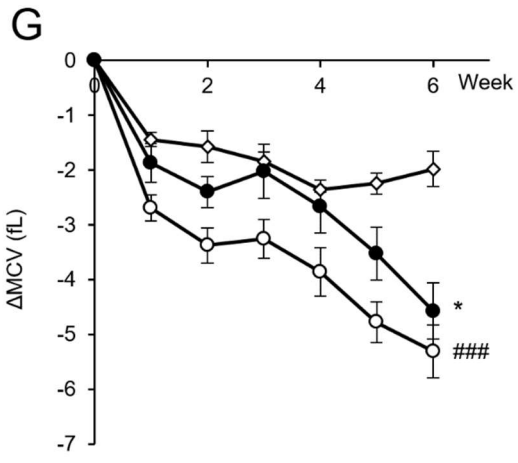

G
ΔMCV (fL)
0
-1
-2
-3
-4
-5
-6
-7
2
4
6
Week
*
###

Fig. 3-1. A scheme of the experiments (A) and effects of TP0463518 on the (B) body weight, (C) reticulocyte count, (D) hematocrit, (E) hemoglobin, (F) red blood cell count, and (G) ΔMCV in the PG-PS rats. Diamonds, healthy rats; open circles, vehicle-treated PG-PS rats; filled circles, 10 mg/kg of TP0463518-treated PG-PS rats. Data are presented as the mean values ± S.E.M. n = 5-6. Two-way repeated measures ANOVA with closed testing procedure was performed. ##P <0.01 and ###P <0.001 as compared to the corresponding time-courses in the healthy control rats; *P <0.05 and **P <0.01 as compared to the corresponding time-courses in the vehicle-treated PG-PS rats.

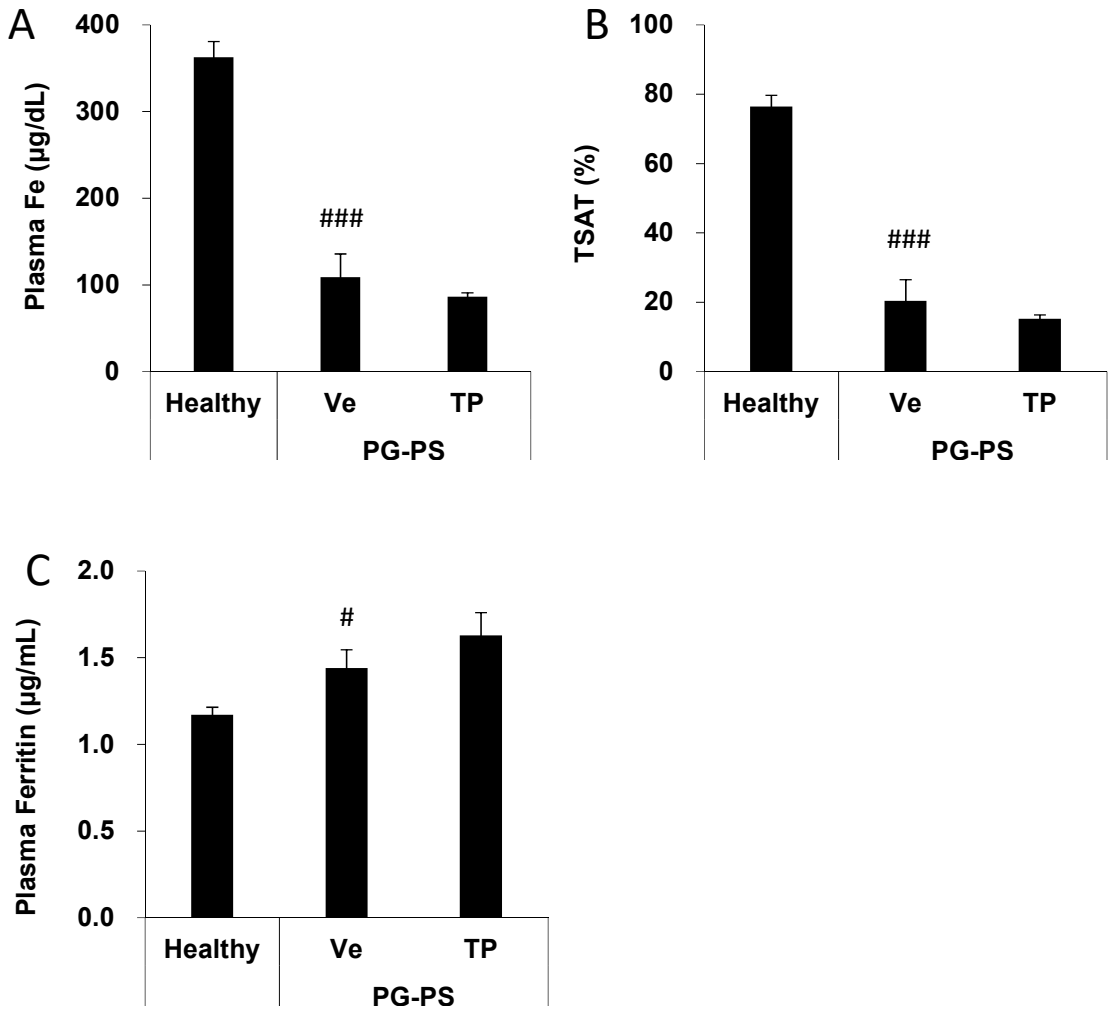
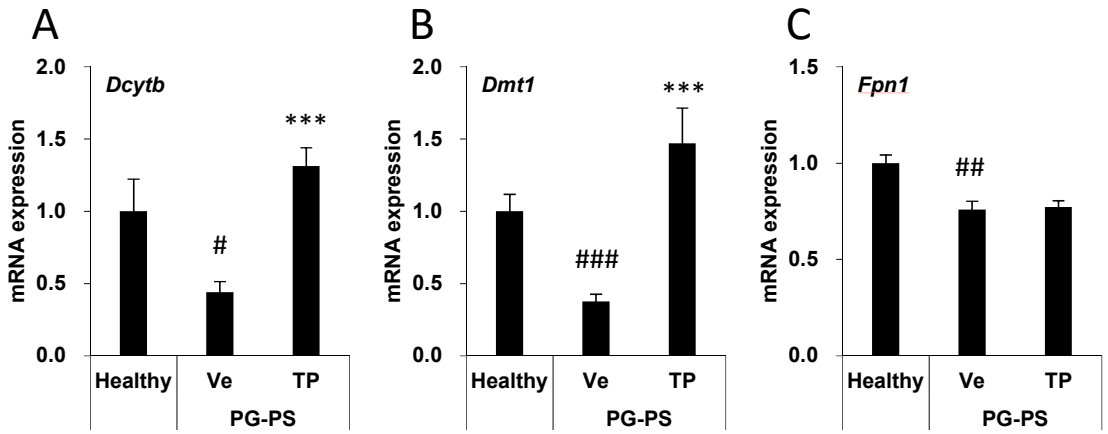
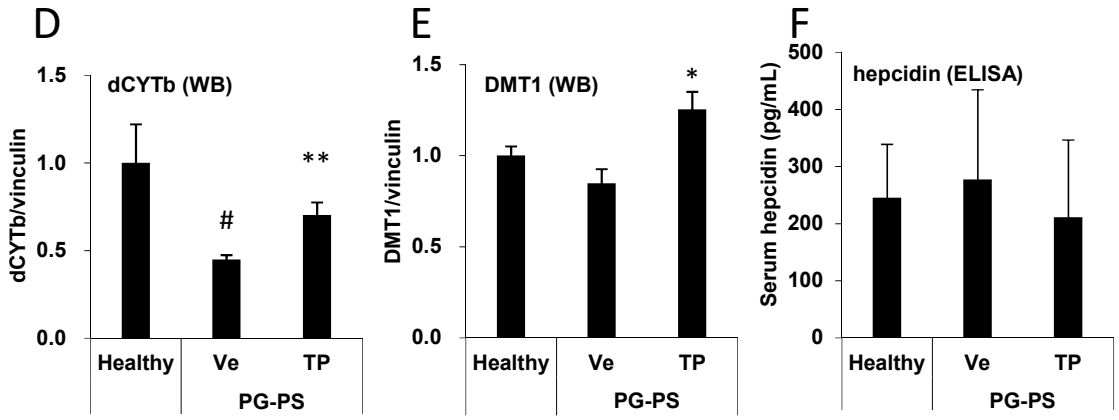


Fig. 3-2. Effects of TP0463518 on the (A) Plasma iron, (B) TSAT, and (C) plasma ferritin in the PG-PS rats. Data are presented as the mean values ± S.E.M. n = 5-6. One-way ANOVA was performed between 3 groups. When the one-way ANOVA was significant, Student's t tests were performed to compare healthy control group vs vehicle-treated group and vehicle-treated group vs TP0463518-treated group. #P <0.05 and ###P <0.001 as compared with the corresponding values in the healthy control rats.

Duodenum



Serum



Liver

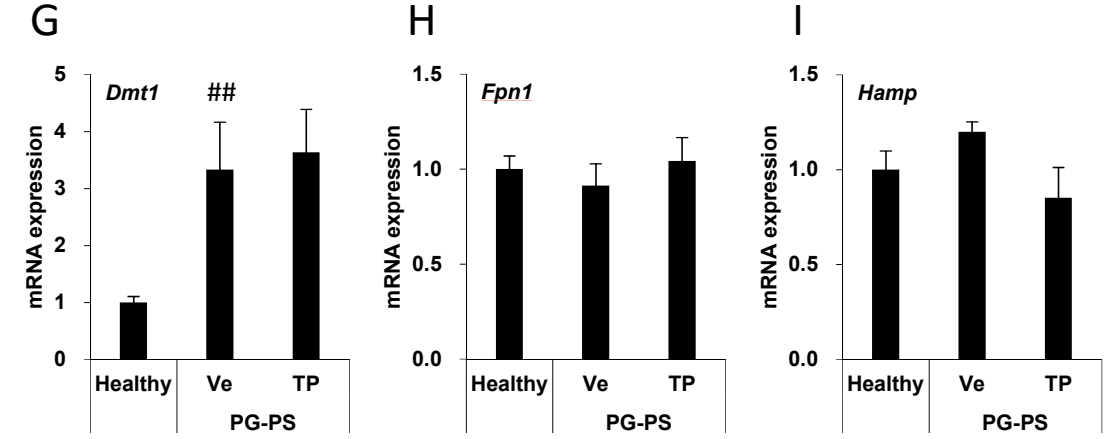


Fig. 3-3. Effects of TP0463518 on the expression levels of genes involved in iron metabolism in the duodenum and the liver. The mRNA expression levels of (A) *Dcytb*, (B) *Dmt1*, and (C) *Fpn1*, and the protein expression levels of (D) dCYTb and (E) DMT1 were analyzed in the duodenum. (F) Serum hepcidin concentration. The mRNA expression levels of (G) *Dmt1*, (H) *Fpn1*, and (I) *Hamp* were analyzed in the liver. Data are presented as the mean values ± S.E.M. n = 5-6. One-way ANOVA was performed between 3 groups. When the one-way ANOVA was significant, Student's t tests were performed to compare healthy control group vs vehicle-treated group and vehicle-treated group vs TP0463518-treated group. #P <0.05, ##P <0.01 and ###P <0.001 as compared with the corresponding values in the healthy control rats. *P <0.05, **P <0.01 and ***P <0.001 as compared with the corresponding values in the vehicle-treated PG-PS rats.

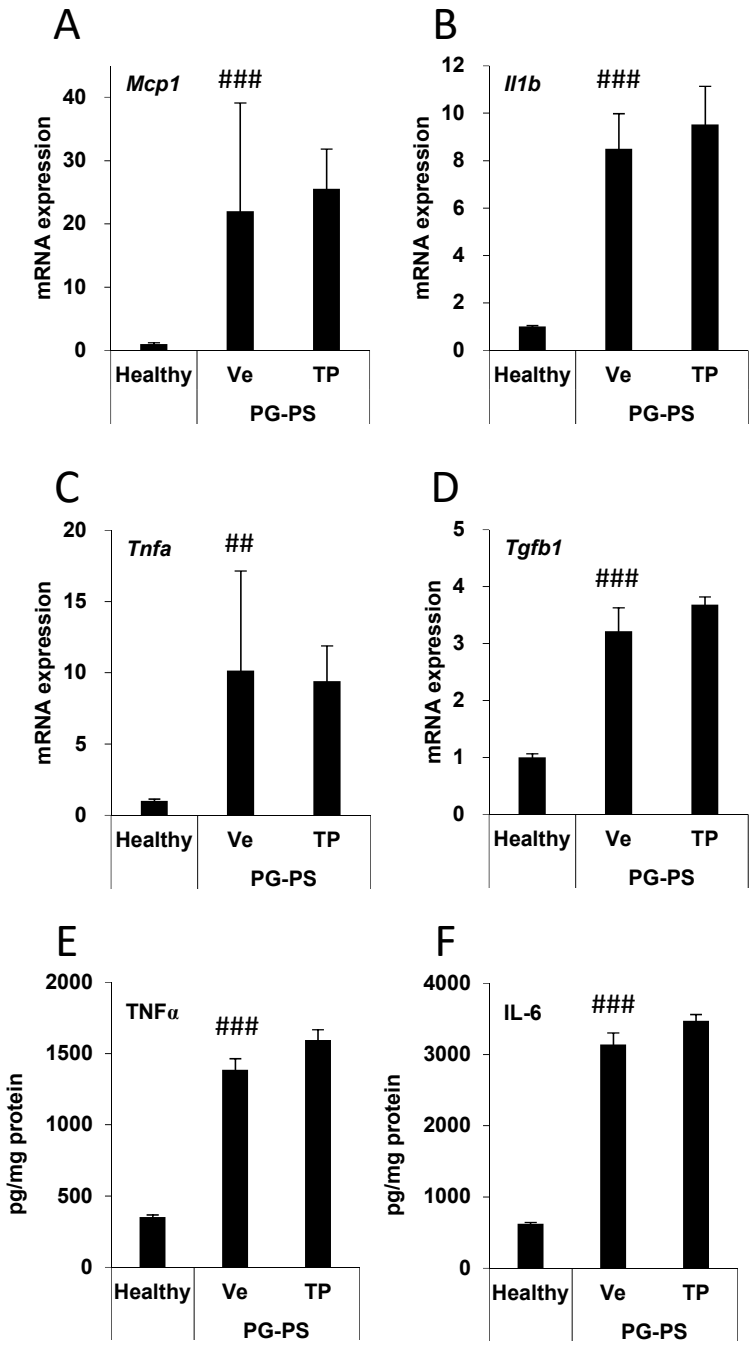


Fig. 3-4. Effects of TP0463518 on the (A) *Mcp1*, (B) *Il1b*, (C) *Tnfa*, and (D) *Tgfb1* gene expressions, and on the (E) TNFα and (F) IL-6 protein levels in the liver. Data are presented as the mean values ± S.E.M. n = 5-6. One-way ANOVA was performed between 3 groups. When the one-way ANOVA was significant, Student's t tests were performed to compare healthy control group vs vehicle-treated group and vehicle-treated group vs TP0463518-treated group. ##P <0.01 and ###P <0.001 as compared with the corresponding values in the healthy control rats.

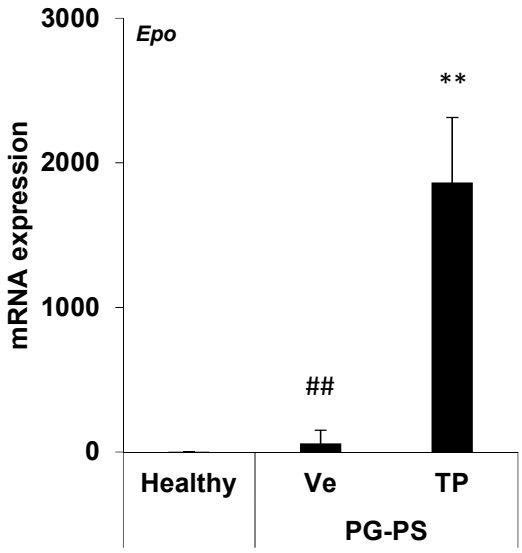


Fig. 3-5. Effect of TP0463518 on the *Epo* expression in the liver. Data are presented as the mean values ± S.E.M. n = 5-6. One-way ANOVA was performed between 3 groups. When the one-way ANOVA was significant, Student's t tests were performed to compare healthy control group vs vehicle-treated group and vehicle-treated group vs TP0463518-treated group. ##P <0.01 as compared with the corresponding value in the healthy control rats. **P <0.01 as compared with the corresponding value in the vehicle-treated PG-PS rats.

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 (Kato et al., 2019). 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 (Sartor et al., 1989; Coccia et al., 2001). 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 (Barrett et al., 2011; Flamme et al., 2014). 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 (Shah et al., 2009). Intestine-specific deletion of *Hif2a* decreased the *Dmt1* and *Dcytb* expressions (Mastrogiannaki et al., 2009; Anderson et al., 2011). In contrast, intestine-specific *Vhl*-knockout mice showed protection against HIF-2α degradation and increased *Dmt1* and *Dcytb* gene expressions (Shah et al., 2009). 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 (Gunshin et al., 2005). dCYTb is a ferric reductase expressed only in the duodenal mucosa. Deletion of *Dcytb* decreased the reticulocyte hemoglobin level (Choi et al., 2012). 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 (Wang and Babitt, 2016). Increased hepcidin not only suppresses *Fpn1* mRNA expression, but also facilitates internalization of ferroportin (Nemeth et al., 2004; Yeh et al., 2004). Schwartz et al. reported that

intestine-specific *Fpn1* conditional-knockout mice showed increased iron retention in the enterocytes (Schwartz et al., 2019). 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 (Flamme et al., 2014). In addition, it has been reported that the serum hepcidin concentration increases up to 2 weeks after PG-PS administration, to start decreasing thereafter (Asshoff et al., 2017). Interestingly, Schwartz et al. also found that iron deficiency suppressed liver hepcidin expression (Schwartz et al., 2019). 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 (Kuriyama et al., 2020). 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 (Akizawa et al., 2019a, 2019b; Chen et al., 2019a, 2019b). 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 (Besarab et al., 2016; Provenzano et al., 2016). 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 *Phd1/2/3* in the liver has been reported to be associated with severe hepatotoxicity (Minamishima et al., 2009; Taniguchi et al., 2013). Although TP0463518 did not induce hepatotoxicity in our previous studies performed using healthy and 5/6 nephrectomized rats (Kato et al., 2018, 2019), 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 (Lichtman et al., 1994; Flamme et al., 2014). 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 (Taniguchi et al., 2013). 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 (Kato et al., 2019). 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 hours of the administration of TP0463518 (Kato et al., 2019). 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.

Chapter 4

DMOG, a prolyl hydroxylase inhibitor, increases hemoglobin levels without exacerbating hypertension and renal injury in salt-sensitive hypertensive rats

Introduction

EPO acts on its receptor to activate the Janus Activating Kinase 2 signaling cascade to stimulate the differentiation of erythroid progenitor cells into erythrocytes (Koury and Haase, 2015; Kuhrt and Wojchowski, 2015). EPO is produced in EPO producing cells found in the renal cortical interstitium (Obara et al., 2008; Paliege et al., 2010). In CKD, these cells transform into myofibroblasts and lose the ability to produce EPO (Asada et al., 2011; Souma et al., 2013). Consequently, CKD patients develop anemia. The prevalence of anemia is 20% in patients with stage 3 CKD, and 60% and 75% in patients with stage 4 or 5 CKD (McFarlane et al., 2008).

Recombinant human EPO (rHuEPO) is widely used to compensate for the deficiency in EPO production to treat anemia and improves the quality of life in CKD patients (Evans et al., 1990). On the other hand, some issues are associated with the use of rHuEPO for the treatment of anemia in CKD patients. rHuEPO is given by chronic injections and is painful for non-dialysis patients. Chronic administration of exogenous rHuEPO promotes the development of anti-rHuEPO antibodies, which neutralizes endogenous EPO (Means, 2016). Treatment of rHuEPO has also been reported to promote the development of hypertension, perhaps by increasing blood viscosity (Letcher et al., 1981; Raine, 1988; Steffen et al., 1989). Furthermore, 3 pivotal clinical studies have raised concerns about the safety of high dose rHuEPO for the treatment of anemia. In the CREATE and the TREAT studies, the risks of hypertension, dialysis, and stroke were higher when the target hemoglobin level was high (Drüeke et al., 2006; Pfeffer et al., 2009). In the CHOIR study, high doses of rHuEPO increased the risk of cardiovascular events independent of the target hemoglobin level (Singh et al., 2006; Szczech et al., 2008).

Potential alternatives to rHuEPO for the treatment of anemia are PHD inhibitors. PHD hydroxylates proline residues of HIFα, which is a master regulator of the hypoxic response (Epstein et al., 2001). Hydroxylated HIFα is recognized by a ubiquitin ligase, VHL, and degraded through the ubiquitin-proteasome pathway (Jaakkola et al., 2001). In hypoxic conditions, the PHD activity is reduced, and HIFα escapes from hydroxylation and subsequent degradation. Once HIFα is stabilized, HIFα binds to the hypoxia response element together with CBP/p300 and constitutively active HIFβ to upregulate the expression of target genes (Haase, 2006). As a consequence, vascularity is increased through HIF-1α, and hemoglobin levels are elevated through the actions of HIF-2α (Elson et al., 2001; Percy et al., 2008b). PHD inhibitors are under the investigation in ongoing phase 3 clinical trials to treat renal anemia in both non-dialysis and dialysis patients (Cernaro et al., 2019). Recently, roxadustat was approved for the treatment of anemia in CKD in China (Dhillon, 2019). PHD inhibitors are orally active (Flamme et al., 2014; Kato et al., 2018), and upregulate the formation of endogenous EPO and other renoprotective factors such as VEGF. Therefore, it is important to compare the safety profile of PHD inhibitors and rHuEPO in models of renal disease.

Several studies have investigated the effects of rHuEPO on renal function in several CKD models in normotensive strains of rats and mice (Lee et al., 2005b; Katavetin et al., 2007; Toba et al., 2009; Cañadillas et al., 2010; Rjiba-Touati et al., 2012). In these studies, rHuEPO reduced renal inflammation and fibrosis regardless of hematopoietic effect. On the other hand, hypertension and diabetes are the primary risk factors for the development of CKD, and these patients may be more susceptible to potential adverse effects of rHuEPO on blood pressure and hypertension-induced renal injury. To explore this possibility, we compared the effects of rHuEPO and DMOG, an injectable PHD 1/2/3 pan-inhibitor (Epstein et al., 2001), on the development of hypertension and renal injury in Dahl S rats that are highly susceptible to the development of salt-sensitive hypertension and renal injury. This study demonstrated that DMOG markedly attenuated the development of hypertension and renal injury in this model, whereas rHuEPO had the opposite effect. These findings suggest that PHD inhibitors may provide a safer therapeutic option for the treatment of anemia in diabetic and hypertension-induced CKD.

Materials and Methods

General

DMOG was synthesized by Medical Chemistry Laboratories in Taisho Pharmaceutical Co., Ltd. Recombinant human EPO (rHuEPO, PROCRIT®) was purchased from Centocor Ortho Biotech Products, L.P. These experiments were performed using male Dahl S rats obtained from inbred colonies maintained in University of Mississippi Medical Center. They were maintained on a 0.4% salt diet from weaning to the start of the experiments. All the experiments were approved by the Animal Care Committee of University of Mississippi Medical Center.

Effect of DMOG on EPO expression

These experiments were performed on male 9 week-old Dahl S rats. A control blood sample was collected from the jugular vein, and the rats received intraperitoneal injection of saline or DMOG at a dose of 600 mg/kg. Blood samples were collected from jugular vein, 4, 8, and 24 hours later. The samples were centrifuged at 2130 g for 10 min, and serum EPO levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN). Additional groups of rats were given DMOG 600 mg/kg, and the kidneys were removed before (0h) and 1, 2, and 4 hours after administration. mRNA was extracted from the renal cortex using TRIZOL (Life Technologies, Grand Island, NY). The samples were reverse transcribed using a poly t and random hexamer primers. *Epo* mRNA levels were determined using real-time quantitative PCR. The primer sequences are listed in Table 4-1.

Time course of the effects of DMOG and rHuEPO

Male 9 week-old Dahl S rats were randomly assigned to 4 groups and treated with vehicle, DMOG at 60 mg/kg, DMOG at 600 mg/kg or rHuEPO at 100 units/kg. DMOG was dissolved in 0.9% NaCl solution and rHuEPO was diluted with a 0.5% BSA in a 0.9% NaCl solution. DMOG was given intraperitoneally while rHuEPO was given subcutaneously, 3 times per week starting from day 1.

Baseline urinary protein excretion, blood pressure, hemoglobin, and hematocrit were measured during a control period while the rats were fed a normal salt diet containing 0.4% NaCl. Then, they were switched to a high salt diet (8.0% NaCl), and DMOG or EPO were administered for 3 weeks, while samples were collected weekly. Urine samples were collected using metabolic cages, and urine protein concentrations were determined by Bradford method (Bio-Rad Laboratories, Hercules, CA). Blood pressure was measured using a tail-cuff device (Hatteras Instruments, Cary, NC). Hemoglobin levels were determined in 10 μL of blood using HemoCue Hb 201+ device (HemoCue, Brea, CA) and hematocrit was determined in 80 μL blood samples collected in a hematocrit tube from tail vein.

Histology

After 3 weeks of treatment with vehicle, DMOG or rHuEPO, the rats were sacrificed and the left kidneys were fixed in 10% buffered formalin. Paraffin sections (3 μm) were stained with Masson's trichrome and analyzed for the degree of glomerulosclerosis, glomerular and renal interstitial fibrosis. Glomerulosclerosis was scored on a scale from 0 to 4, where 0 represents a normal glomerulus, 1 represents 1-25% loss of capillary area, 2 represents 26-50% loss, 3 represents 51-75% loss and 4 represents >75% loss. The images were captured using a Nikon Eclipse 55i microscope equipped with a Nikon DS-Fi1 color camera (Nikon Instruments Inc., Melville, NY). The degree of renal interstitial fibrosis was calculated as the percentage of area stained blue using the NIS Elements 3.0 software (Nikon Instruments Inc.). Protein casts were determined as the percentage of area stained red in the sections (Muroya et al., 2015).

Measurement of renal injury biomarkers

BUN levels were determined using a BUN detection kit (Arbor Assays, Ann Arbor, MI). Serum creatinine levels were determined using a LabAssay Creatinine kit (Wako Pure Chemical Industries, Osaka, Japan). Urinary NAG and KIM-1 were determined using a NAG assay kit (SIGMA, St. Louis, MO) and a rat KIM-1 ELISA kit (R&D Systems).

Measurement of renal inflammatory markers and VEGF levels

The right kidneys were separated into cortex and medulla, and they were homogenized in a Tris buffer containing 5 mM EDTA, 1 mM EGTA, 1% Triton X100 and a proteinase inhibitor cocktail. The homogenate was centrifuged at 11000 g for 5 minutes at 4˚C, and the MCP-1, IL-1β, TGFβ1 and VEGF levels in the supernatant were measured using ELISA kits (R&D Systems). Samples for measurement of TGFβ1 were first activated by acidification for 5 minutes and then measured using an ELISA kit from R&D systems.

Statistics

Data are presented as mean values ± S.E.M. The statistical significances of differences were determined using the Student's t-test for comparisons between two groups, a one-way ANOVA followed by Holm-Sidak test for multiple comparisons or a two-way ANOVA for repeated measures followed by Holm-Sidak test for the time course studies (Sigma Plot 11, Systat Software, San Jose, CA).

Results

Effect of DMOG on EPO expression

We first addressed the effect of DMOG on renal *Epo* mRNA expression and serum EPO concentration after administration of 600 mg/kg of DMOG. *Epo* mRNA levels in the renal cortex started to increase 1 hour after the administration of DMOG. *Epo* mRNA levels at 4 hours were 70-fold higher than that seen at baseline (Fig. 4-1A). Serum EPO concentration in the vehicle-treated group was below detection, 24 hours after administration. The EPO concentration in the 600 mg/kg of DMOG-treated group increased to 890 pg/mL 4 hours after administration. The levels returned to the baseline, 24 hours after the administration of DMOG (Fig. 4-1B).

Comparison of the hematopoietic effects of chronic DMOG and rHuEPO administration

The hematopoietic effects of DMOG (60 or 600 mg/kg, intraperitoneally) or rHuEPO (100 U/kg, subcutaneously) given 3 times per week were studied in Dahl S rats fed an 8% NaCl diet for 3 weeks. There was no difference in food intake or body weight among the groups (data not shown), indicating that these drugs were well tolerated. Hemoglobin and hematocrit levels in the vehicle and the 60 mg/kg of DMOG-treated groups remained unchanged throughout the study. The hemoglobin and hematocrit levels in the 600 mg/kg of DMOG-treated group increased from 16.3 ± 0.2 g/dL and 56.1 ± 2.0% to 18.3 ± 0.4 g/dL and 63.1 ± 1.4% after 3 weeks of treatment. These values were comparable to the rise in hemoglobin and hematocrit levels seen in the rHuEPO-treated group (18.8 ± 0.4 g/dL and 63.3 ± 1.8%, respectively) (Fig. 4-2A, B).

Effects of DMOG rHuEPO on the development of hypertension and renal injury

A comparison of the effects of DMOG or rHuEPO treatment on blood pressure and urinary protein excretion in Dahl S rats fed a high salt diet are presented in Figure 4-3. Baseline systolic blood pressure averaged 161 ± 3 mmHg in the vehicle treated-group and rose to 226 ± 4 mmHg after 3 weeks on a high salt diet (Fig. 4-3A). Blood pressure and proteinuria were not different in the vehicle-treated and 60 mg/kg of DMOG-treated groups (Fig. 4-3A, B). Blood pressure and proteinuria increased to a greater extent in rats treated with rHuEPO. Chronic treatment with 600 mg/kg of DMOG attenuated the development of hypertension and proteinuria and systolic blood pressure only increased to 189 ± 8 mmHg over the 3-week course of the study.

Effects of DMOG and rHuEPO on renal injury

At baseline, glomerular injury and fibrosis were rare (Fig. 4-4A, B). The degree of glomerulosclerosis and fibrosis was markedly enhanced in the vehicle-treated group fed a high salt diet for 3 weeks (Fig. 4-4C). They also exhibited some degree of renal interstitial fibrosis (Fig. 4-4D). The degree of

glomerulosclerosis and renal interstitial fibrosis was markedly enhanced in rats receiving rHuEPO (Fig. 4-4G, H). In contrast, the degree of glomerulosclerosis and glomerular fibrosis, as well as renal interstitial fibrosis, were markedly reduced in rats treated with 600 mg/kg of DMOG (Fig. 4-4E, F).

A comparison of the glomerulosclerosis score and degree of renal fibrosis is presented in Figures 4-4I, J, and K. The glomerular sclerosis index and degree of glomerular fibrosis were similar in rats treated with vehicle versus rHuEPO. However, rHuEPO markedly enhanced the development of renal interstitial fibrosis. The degree of glomerulosclerosis and fibrosis and renal interstitial fibrosis was markedly attenuated in a dose-dependent manner in rats treated with DMOG.

A comparison of plasma BUN and serum creatinine levels among the groups is presented in Table 4-2. Plasma BUN and creatinine levels increased similarly in all the groups following 3 weeks on a high salt diet. Urinary NAG and KIM-1 excretion increased following the development of hypertension in the vehicle-treated rats. rHuEPO increased urinary KIM-1 excretion to a greater extent than that seen in the vehicle-treated group. The increases in NAG and KIM-1 excretion were markedly attenuated in the rats treated with DMOG (Fig. 4-5A, B).

The degree of fibrosis in the outer medulla of the kidney was markedly elevated in vehicle-treated rats fed a high salt diet for 3 weeks. rHuEPO aggravated renal medullary fibrosis (Fig. 4-6G), while it was completely prevented in rats treated with DMOG (Fig. 4-6E). The formation of protein casts was increased dramatically following the development of hypertension in the vehicle-treated group. (Fig. 4-6B, D). DMOG, as well as rHuEPO, attenuated the formation of protein casts. (Fig. 4-6F, H, J). Quantitative analysis revealed that DMOG prevented the renal medullary fibrosis and protein cast formation in a dose-dependent manner (Fig. 4-6I, J).

Effects of DMOG and rHuEPO on renal inflammatory markers and growth factors

High-salt treatment increased renal cortical and medullary IL-1β and MCP-1 compared to baseline levels (Fig. 4-7A). rHuEPO had no significant effect on the increase in renal IL-1β and MCP-1 levels. In contrast, DMOG treatment prevented the increase of IL-1β and MCP-1 levels in a dose-dependent manner.

Exposure to a high salt treatment increased TGFβ1 levels in the medulla in the vehicle-treated group, and rHuEPO further increased the TGFβ1 levels. DMOG-treatment had no significant effect on the rise in TGFβ1 expression.

The levels of VEGF in both cortex and medulla fell following 3 weeks on a high salt diet in the vehicle-treated group. rHuEPO had no effect on the fall in VEGF expression (Fig. 4-8). In contrast, DMOG dose-dependently increased renal VEGF levels by 7-fold in cortex and 60-fold in medulla compared to values seen in the vehicle-treated group.

Table 4-1. Primer sequences.

	gene	Sequence (5' to 3')
rat	*Actb*	CCTCTATGCCAACACAGTGC
		GTACTCCTGCTTGCTGATCC
	Epo	GCTCCAATCTTTGTGGCATCT
		TGGCTTCGTGACCCTCTGT

Table 4-2. BUN and serum creatinine.

		n	BUN (mg/dL)	sCre (mg/dL)
	Baseline	8-10	10.8 ± 0.4	0.56 ± 0.02
	Vehicle	8	25.6 ± 1.9*	1.07 ± 0.12*
DMOG	60 mg/kg	6	31.6 ± 3.2	0.96 ± 0.10
	600 mg/kg	8	36.2 ± 1.8	0.89 ± 0.03
EPO	100 U/kg	7	33.6 ± 4.1	0.97 ± 0.05

Mean values ± S.E.M. are presented. * indicate $P<0.05$ from the pooled baseline value.

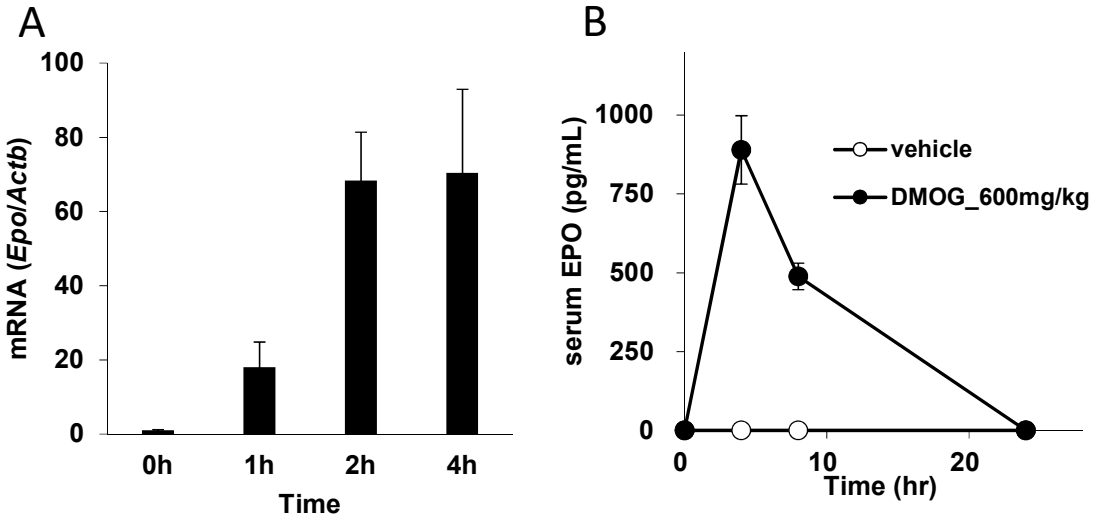


Fig. 4-1. Time-course study of the effects of DMOG (600 mg/kg) on *Epo* mRNA expression and serum EPO concentration. (A) DMOG increased *Epo* mRNA expression level in renal cortex. (B) Serum EPO concentration increased 4 hours after the DMOG administration, and returned to the baseline level thereafter. Open circles represent serum EPO concentration in vehicle-treated animals and closed circles represent levels in DMOG-treated animals. Data are represented as mean values ± S.E.M. from n = 3 animals per group.

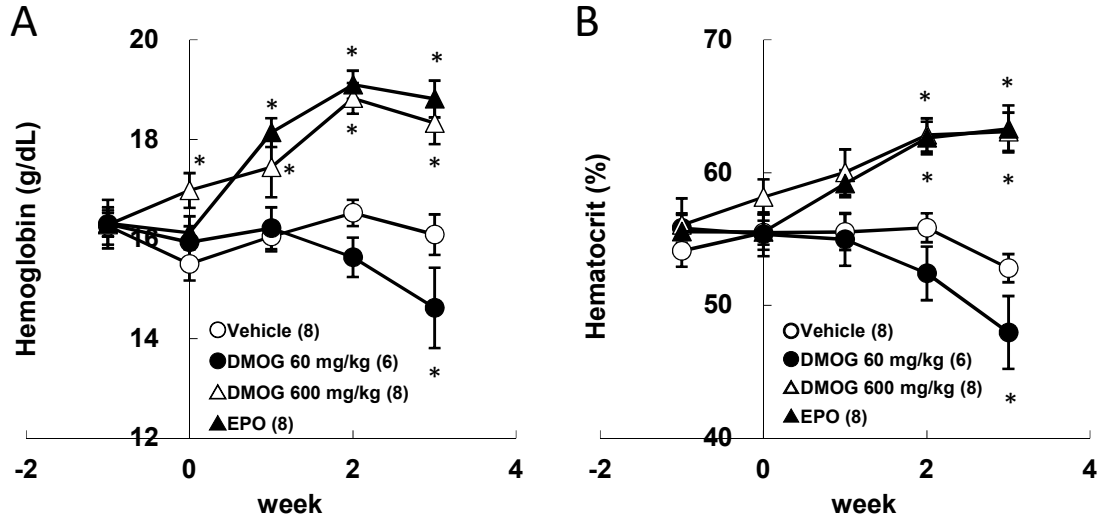


Fig. 4-2. Effect of DMOG and rHuEPO. (A) Hemoglobin and (B) hematocrit in high salt-fed Dahl S rats before and after chronic administration of vehicle (open circle), 60 mg/kg of DMOG (filled circle), 600 mg/kg of DMOG (open triangle) and 100 U/kg of rHuEPO (filled triangle). The high salt diet and the drug administration were started together on day 1 after the control period. Hemoglobin and hematocrit were measured at 1 week before the start of the study through at week 3. Data are presented as the mean values ± S.E.M. The numbers of animals studied in each group are shown in parentheses. * indicates a significant difference from the corresponding value in vehicle-treated rats.

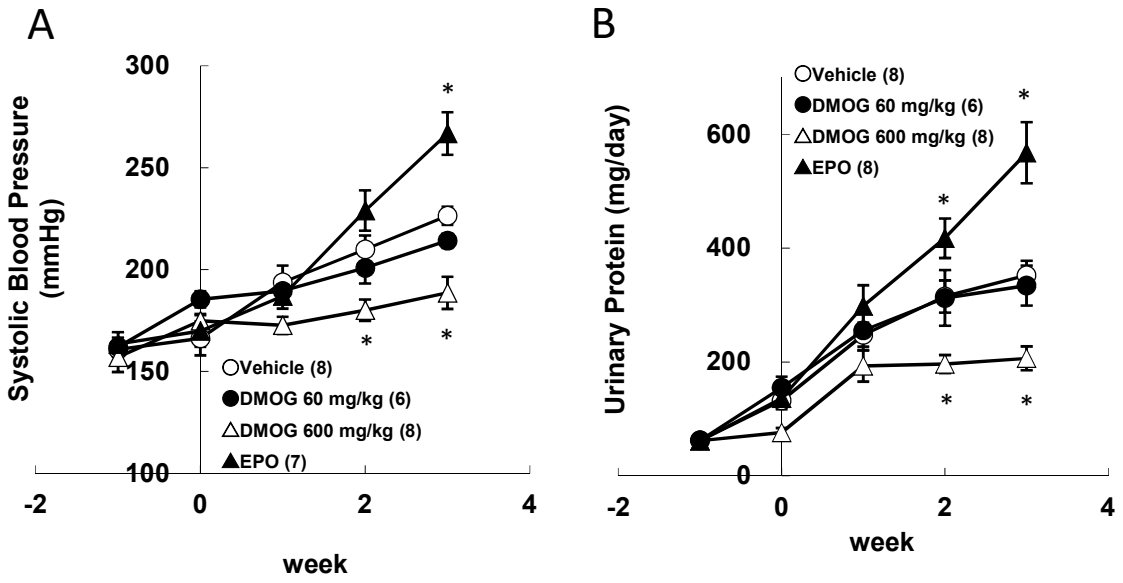
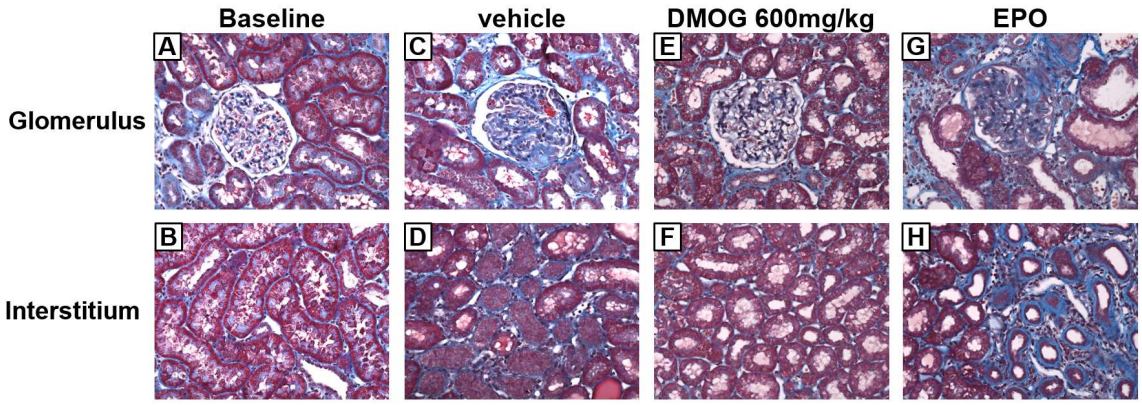
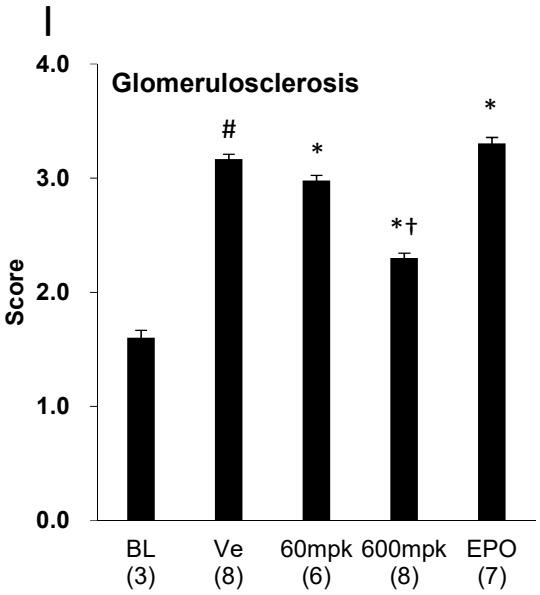


Fig. 4-3. Effect of DMOG and rHuEPO. (A) Systolic blood pressure and (B) urinary protein excretion in Dahl S rats fed a high salt diet before and after chronic administration of vehicle (open circle), 60 mg/kg of DMOG (filled circle), 600 mg/kg of DMOG (open triangle) and 100 U/kg of rHuEPO (filled triangle). The high salt diet and the drug administration were started together on day 1 after the control period. The drugs were given 3 times per week. Systolic blood pressure and urinary protein excretion were measured at 1 week before the start of the study through at week 3. Data are presented as the mean values ± S.E.M. The numbers of animals studied per group are shown in parentheses. * indicates a significant difference from the corresponding value in vehicle-treated rats.


Baseline
vehicle
DMOG 600mg/kg
EPO
Glomerulus
Interstitium
A
B
C
D
E
F
G
H


I
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BL (3)
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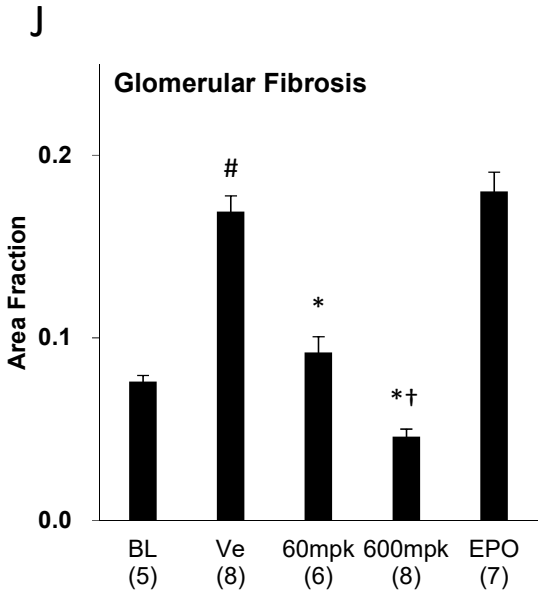
EPO (7)


J
Glomerular Fibrosis
Area Fraction
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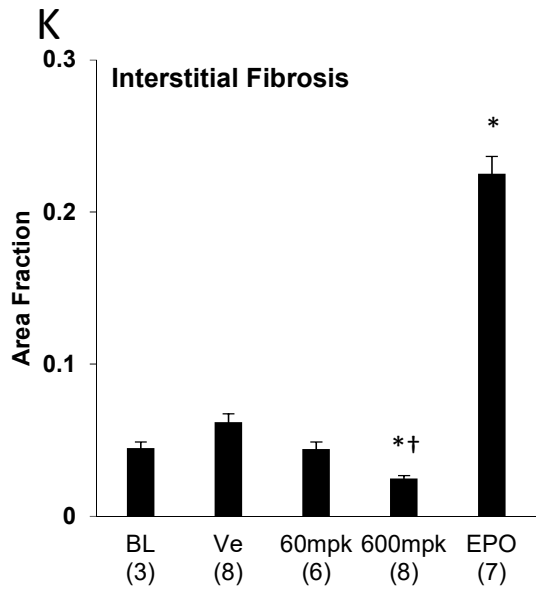
K
Interstitial Fibrosis
Area Fraction
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EPO (7)

Fig. 4-4. Effect of DMOG and rHuEPO on glomerular injury and renal interstitial fibrosis in the renal cortex. Representative micrographs of the baseline (A and B), vehicle (C and D), 600 mg/kg of DMOG (E and F), or rHuEPO (G and H) treated rats are shown. A, C, E, and G are micrographs of glomerulus and B, D, F, and H are micrographs of the interstitium. Magnification is 200X. Glomerulosclerosis (I), glomerular fibrosis (J), and interstitial fibrosis (K) are quantified and shown in the bar graphs. Thirty glomeruli were scored for the degree of glomerulosclerosis and ten fields were quantified for fibrosis in each animal. The number of animals studied per group are presented on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

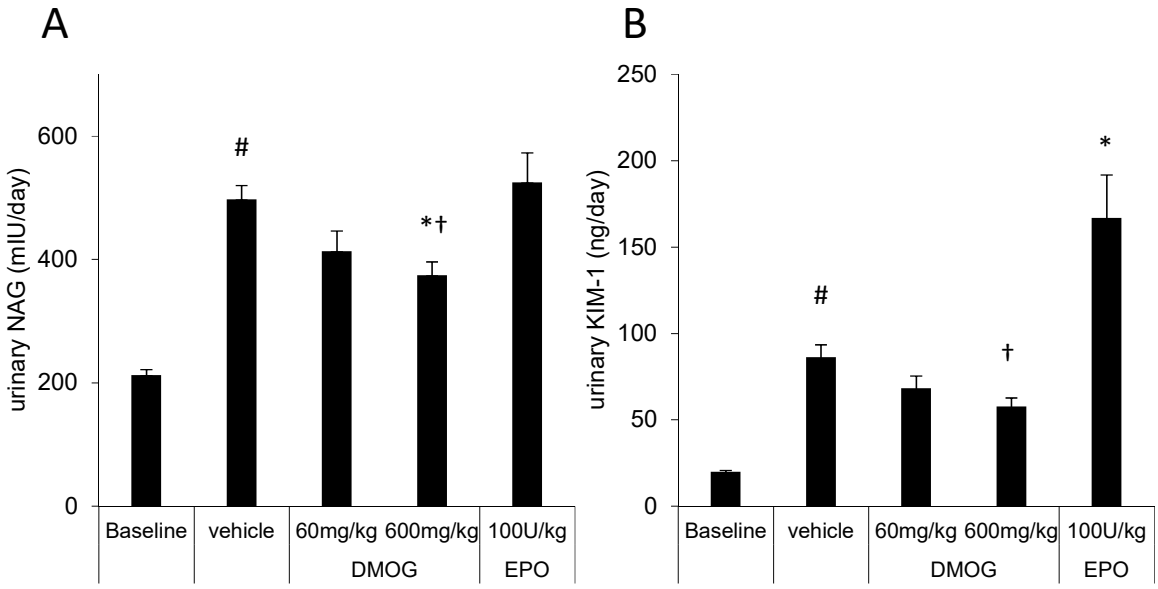

Fig. 4- 5. Effect of DMOG and rHuEPO. (A) Urinary NAG and (B) KIM-1 excretion in Dahl S rats fed a high salt diet. The numbers of animals studied in each group are shown on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

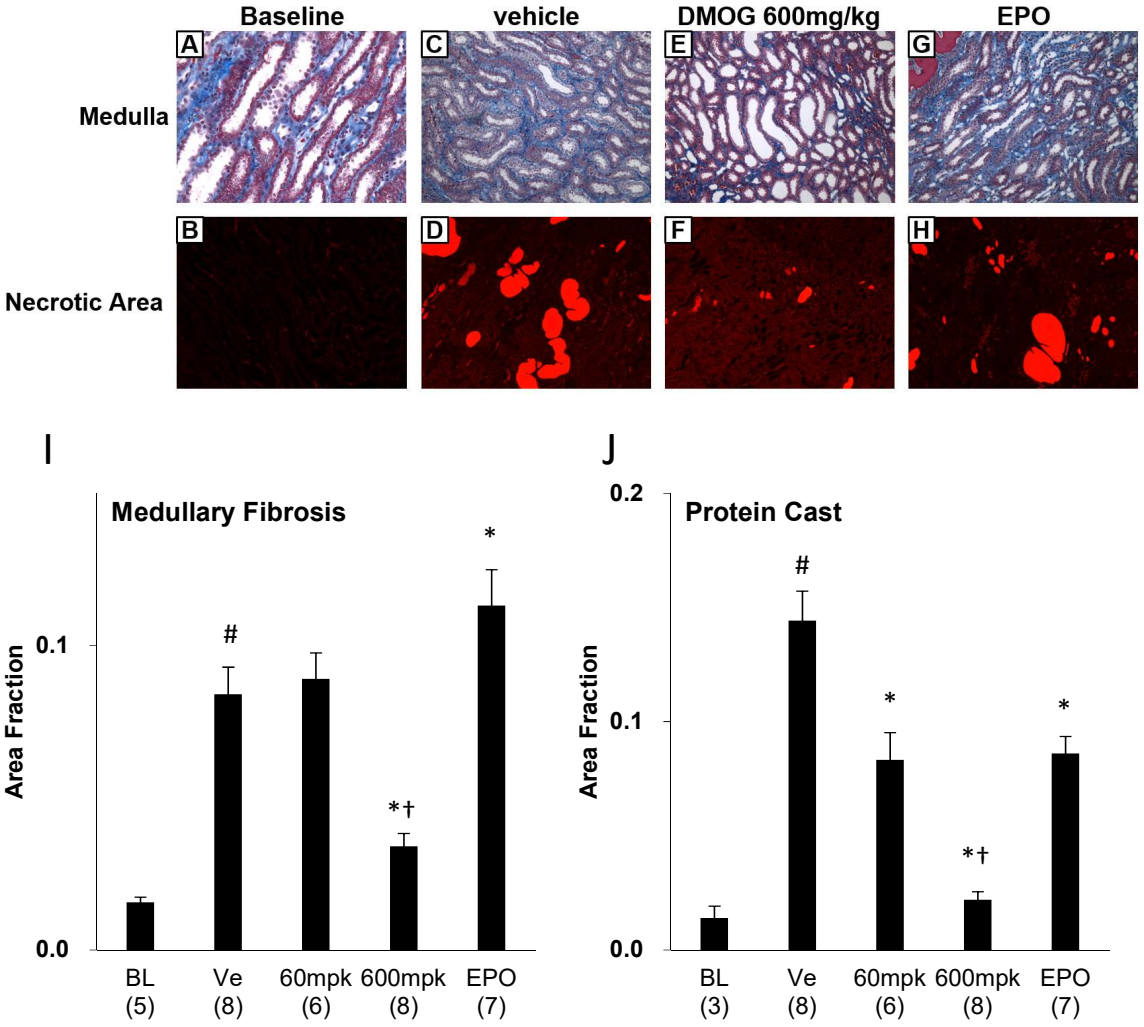

Fig. 4-6. Effect of DMOG and rHuEPO on renal injury in the medulla. Representative micrographs of the baseline (A and B), vehicle (C and D), 600 mg/kg of DMOG (E and F), or 100 U/kg of rHuEPO (G and H) treated rats are shown. A, C, E, and G are micrographs of medullas and B, D, F, and H are micrographs of protein casts. Magnification is 100X. Medullary fibrosis (I) and protein cast area (J) are quantified and shown in the bar graphs. Ten fields were quantified for fibrosis and five fields were quantified for protein cast area in each animal. The number of animals studied per group are shown on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

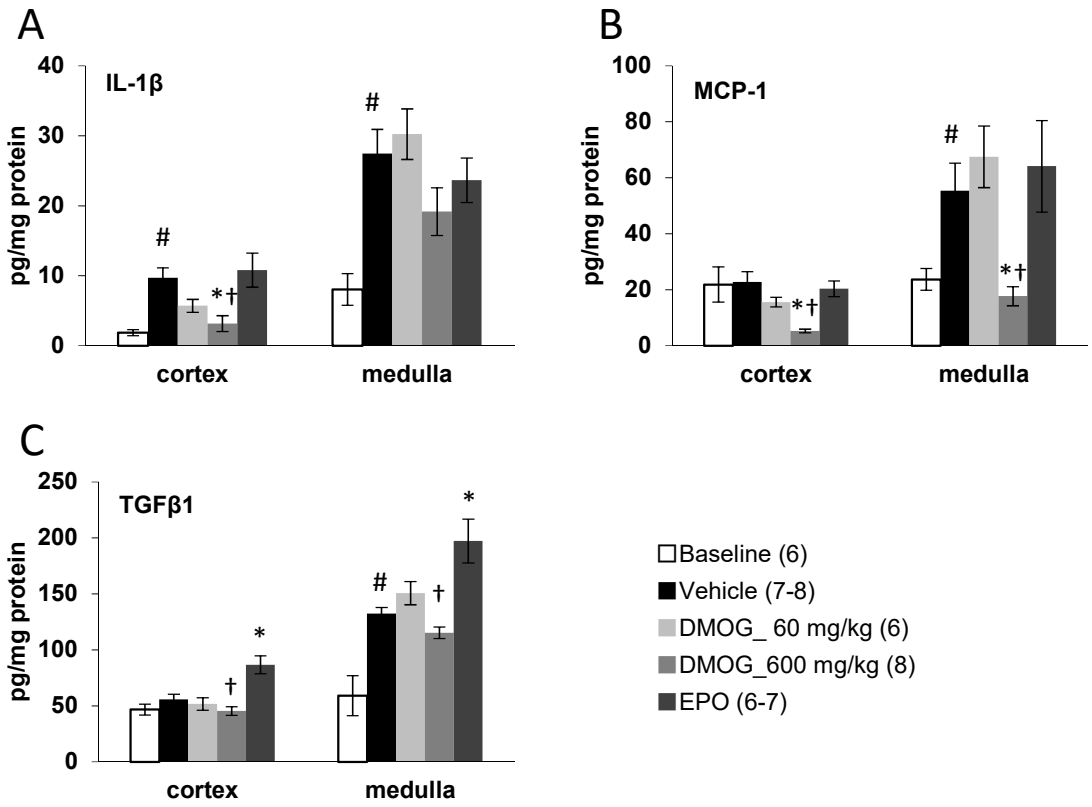

Fig. 4-7. Effect of DMOG and rHuEPO. (A) IL-1β, (B) MCP-1, and (C) TGFβ1 levels in the renal cortex and medulla in Dahl S rats fed a high salt diet. The numbers of animals studied per group are shown on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

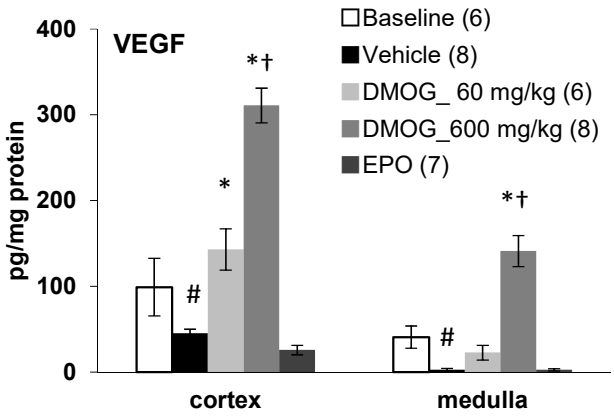

Fig. 4-8. Effect of DMOG and rHuEPO on VEGF expression in the renal cortex and medulla. The numbers of animals studied in each group are shown on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

Discussion

Recombinant human EPO is widely used to treat anemia and improves the quality of life in CKD patients (Evans et al., 1990). Several studies have explored the effects of rHuEPO on renal function in a variety of experimental models of CKD (Lee et al., 2005b; Katavetin et al., 2007; Toba et al., 2009; Cañadillas et al., 2010; Rjiba-Touati et al., 2012). In general, these studies indicate that rHuEPO reduced renal inflammation and fibrosis regardless of its hematopoietic effect. However, subsequent clinical trials indicate that the use of rHuEPO increased the risks of hypertension, stroke, cardiovascular events and dialysis in CKD patients (Drüeke et al., 2006; Singh et al., 2006; Szczech et al., 2008; Pfeffer et al., 2009). Since hypertension and diabetes are the primary risk factors for the development of CKD; it is possible that these patients may be more susceptible to potential adverse effects of rHuEPO on blood pressure and hypertension-induced renal injury. To explore this possibility, we compared the effects of rHuEPO and DMOG, an injectable PHD inhibitor, on the development of hypertension and renal injury in Dahl S rats that is an experimental model that is highly susceptible to the development of salt-sensitive hypertension and renal injury.

Treatment of Dahl S rats fed a high salt with rHuEPO increased hemoglobin levels, but it augmented the degree of hypertension. The treatment of the rats with rHuEPO also aggravated proteinuria and fibrosis in the renal cortex and medulla. These effects were associated with increased expression of TGFβ1. These results are consistent with the clinical findings that stimulation of hematopoiesis by rHuEPO treatment in CKD patients is associated with increased risk of hypertension and renal dysfunction (Raine, 1988; Drüeke et al., 2006). Interestingly, in other experimental models of renal disease, such as cyclosporine-induced nephropathy, unilateral ureter obstruction, and diabetic nephropathy, and in normotensive strains of rats and mice that are not salt-sensitive, blood pressure did not increase following administration of rHuEPO. Moreover, rHuEPO suppressed TGFβ1 expression and reduced renal inflammation and fibrosis in several of these studies (Lee et al., 2005b; Park et al., 2007; Toba et al., 2009). Since the blockage of TGFβ1 attenuates hypertension and renal fibrosis (Dahly et al., 2002; Murphy et al., 2012), these results suggest that in models in which rHuEPO does not increase blood pressure, rHuEPO can attenuate renal interstitial fibrosis by reducing TGFβ1 expression. In contrast, our results indicate that when rHuEPO increased both hemoglobin levels and blood pressure, the rise in blood pressure may increase renal fibrosis.

DMOG is a PHD 1/2/3 pan-inhibitor that stimulates erythropoiesis by stabilizing HIFs (Epstein et al., 2001; Barrett et al., 2011). We compared the effects of rHuEPO and that of DMOG on blood pressure and renal function. In our experiments, 600 mg/kg of DMOG increased endogenous EPO concentration in serum and increased hemoglobin levels as potently as rHuEPO. However, in sharp contrast to rHuEPO, DMOG attenuated the development of hypertension and proteinuria in the Dahl S rats. Furthermore, DMOG prevented the increase of renal inflammatory markers in the kidney

along with renal fibrosis and medullary protein cast formation. These results are consistent with the previous findings that the activation of HIF-1α attenuated the rise of blood pressure in high-salt treated Dahl S rats (Zhu et al., 2012; Zhu et al., 2014). However, unlike the results of the previous study, we could not confirm an increase in HO-1 expression in the kidney of Dahl S rats (data not shown). We instead found that renal VEGF levels were very low in high salt-treated Dahl S rats and that it increased in the rats treated with DMOG. The renoprotective effects of VEGF have been reported elsewhere (Kang et al., 2001; Suga et al., 2001; Ma et al., 2011; Sivaskandarajah et al., 2012). Suppression of VEGF signaling using a humanized monoclonal antibody, genetic knockout of VEGF, or pharmacological blockade (Eremina et al., 2008; Lankhorst et al., 2017) has been shown to increase blood pressure. Interestingly, Eremina et al. (2008) found that glomerular injury proceeded the development of hypertension in VEGF knockout mice. Their observations are consistent with the present results that renal fibrosis and injury were attenuated in the 60 mg/kg of DMOG-treated group whereas the hemoglobin levels and blood pressure were similar to the vehicle-treated group. Overall, these findings suggest that the reduction of renal VEGF expression could be one of the mechanisms contributing to the development of hypertension and renal injury in Dahl S rats. Based on this mechanism, DMOG could oppose the development of nephropathy by induction of renal VEGF expression and the attenuation of hypertension.

HIF-1α induces CD73 (Ecto-5-prime-nucleotidase) (Synnestvedt et al., 2002) and increases adenosine signaling. In the kidney, adenosine is a vasoconstrictor involved in tubuloglomerular feedback responses (Schnermann, 2015; Romero and Carretero, 2019). Upregulation of CD73 has been reported to protect the kidney from ischemia-reperfusion injury and diabetic nephropathy (Tak et al., 2014; Sung et al., 2017). Therefore, PHD inhibitors may protect the kidney from salt-sensitive hypertension and diabetic induced renal injury by enhancing tubuloglomerular feedback responsiveness and reducing transmission of systemic pressure to the glomerulus.

Currently, PHD inhibitors are under the investigation of phase 3 clinical trials to treat renal anemia in non-dialysis and dialysis patients (Cernaro et al., 2019), and roxadustat was first approved for the treatment of anemia in China (Dhillon, 2019). The potential adverse events of three PHD inhibitors, molidustat, daprodustat, and vadadustat have been investigated in recent trials. While the administration of molidustat was as effective as rHuEPO in increasing hemoglobin in CKD patients, the incidence of hypertension was lower in molidustat-treated group (Macdougall et al., 2019). Daprodustat was also effective in increasing hemoglobin levels by more than 2 g/dL, but it did not affect blood pressure (Brigandi et al., 2016). Additionally, vadadustat increased hemoglobin levels by 1.4 g/dL without altering blood pressure rise in a 6-week study (Martin et al., 2017); however, the number of patients with hypertension was higher in Vadadustat-treated group in a 20-week study (Pergola et al., 2016). Although the statistical power in these clinical trials was insufficient to detect the change in blood pressure, it is noteworthy that the results from these clinical trials suggest that

treatment of anemia with PHD inhibitors did not raise blood pressure. In the present study, we found that the blood pressure was lower in DMOG-treated group than in the vehicle-treated group. This result is consistent with the results of the previous clinical trials and contrasts with the pro-hypertensive effects seen in rats treated with rHuEPO.

DMOG has been reported to increase VEGF expression in the eye (Safran et al., 2006). Thus, there is a risk of retinopathy. However, in the present study, we did not observe any adverse effects in the Dahl S rats chronically treated with 60 or 600 mg/kg/day of DMOG.

In summary, rHuEPO and DMOG are equally effective in increasing hemoglobin levels in Dahl S rats fed a high salt diet. However, rHuEPO aggravated the degree of hypertension, proteinuria and renal injury. This was associated with induction of the expression of TGFβ1. In contrast, DMOG attenuated the development of hypertension and renal injury through induction of renal VEGF expression. These results suggest that PHD inhibitors may provide an alternative and safer therapeutic option for the treatment of anemia in patients with diabetic and hypertension-induced CKD.

Summary

Chapter 1

The pharmacological profiles of TP0463518 was investigated in vivo and in vitro. TP0463518 was identified as a novel potent and competitive PHD inhibitor. TP0463518 increased serum EPO levels in healthy mice, rats and monkey, and also in 5/6 Nx rats with a good PK-PD profile. These results suggest that TP0463518 contribute to desirable hemoglobin control in patients with renal anemia.

Chapter 2

The effects of TP0463518 on the EPO production in the kidney and liver were investigated by measuring HIF-2α protein, *Epo* mRNA, and serum EPO levels in normal and bilaterally nephrectomized rats. Furthermore, the effects of liver-derived EPO on anemia in 5/6 Nx rats were also investigated. As results, TP0463518 specifically induced EPO production in the liver, and that the liver-derived EPO was pharmacologically effective. These findings suggest that investigation of the effects of TP0463518 may pave the way for the development of a new therapeutic alternative for renal anemia patients.

Chapter 3

The effects of TP0463518 on anemia of inflammation using PG-PS treated Lewis rats were investigated. TP0463518 increased the expressions of genes involved in iron metabolism in the duodenum and improved anemia of inflammation without exacerbating liver inflammation. These findings suggest that TP0463518 appear to have an acceptable safety profile and could become a useful new therapeutic option for anemia of inflammation.

Chapter 4

The effects of DMOG and rHuEPO on the development of hypertension and renal injury were compared using Dahl salt-sensitive rats fed an 8% salt diet for 3 weeks. DMOG and rHuEPO were equally effective in increasing hemoglobin levels in Dahl S rats; however, rHuEPO aggravated hypertension and renal injury, whereas DMOG attenuated the development of hypertension and prevented renal injury. These findings suggest that PHD inhibitors provide a safer therapeutic option for the treatment of anemia in CKD.

List of publications

This doctoral dissertation is based on the following original publications.

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