学位申請論文

Pharmacological studies on immunosuppressive effects of the novel JAK inhibitor on allograft rejection in pre-clinical transplantation models

（臓器移植拒絶反応に対する新規 JAK 阻害剤の免疫抑制効果に関する薬理学的研究）

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Contents

Abbreviation

AUC, area under the receiver operating characteristics (ROC) curve

CAV, cardiac allograft vasculopathy

CD, cluster of differentiation

CNI, calcineurin inhibitors

DSA, donor-specific antibody

ECM, extracellular matrix

EC-MPS, enteric-coated form of mycophenolate sodium

EMT, epithelial–mesenchymal transition

GFR, glomerular filtration rate

IC_{50}, the half maximal inhibitory concentration

IgG, immune globulin type G

IgM, immune globulin type M

IF/TA, interstitial fibrosis/tubular atrophy

IL, interleukin

JAK, janus kinase

KIM-1, kidney injury molecule-1

NHP, non-human primate

NFAT, nuclear factor of activated T cells

MCP-1, monocyte chemoattractant protein

MFI, median fluorescence intensity

MHC, major histocompatibility complex

MLR, mixed lymphocyte reaction

MMF, mycophenolate mofetil

MPA, mycophenolic acid

MST, median survival times

PBMC, peripheral blood mononuclear cell

RA, rheumatoid arthritis

STAT, signal transducer and activator of transcription

TAC, tacrolimus

THBS, thrombospondin

TIMP-1, tissue inhibitor of metalloproteinase-1

TGF, transforming growth factor

TYK2, tyrosine kinase 2

Introduction

Organ transplantation has enriched and prolonged the lives of many patients who otherwise would have died of organ failure. Many of these advances, which occurred in the later part of the 20th century, are due to improved techniques and pharmacological management [1,2]. Adaptive immune response is important to understand the pathogenesis of rejection and the mechanisms of action of different immunosuppressive drugs. The initiation of the adaptive immune response against the donor grafts begins with the recognition of an alloantigen by a naive T-cell, followed by T-cell activation, proliferation and differentiation. Various immunosuppressive drugs have been developed to suppress rejections occurring at the time of organ transplantation and engrafting grafts for a long time. When transplantation medication began, a combination therapy of corticosteroids and azathioprine, a purine nucleic acid synthesis inhibitor, was mainly used. However, these agents have not been satisfactory in terms of their efficacy for suppressing rejection. From the early 1980s, calcineurin inhibitors (CNIs) dramatically changed transplantation medication with significant reduction in acute rejection rates [2] (Fig. 0-1). Two commonly used CNIs, cyclosporin and tacrolimus inhibit the ability of calcineurin to dephosphorylate nuclear factor of activated T cells (NFAT), required for translocation from cytoplasm to nucleus, and prevent calcineurin-dependent gene transcription. The impressive results of CNIs in transplant recipients led to the treatment of a variety of autoimmune diseases as well. Mycophenolate mofetil (MMF), which inhibits proliferation of T cells and B cells by inhibiting the *de novo* purine synthesis present in lymphocytes, has been successfully used for the prevention of acute rejection. The current standard immunosuppressive

protocol consists of three drug groups, corticosteroids, CNIs, and MMF. Especially, CNIs are the most important cornerstone on the basis of their significant effectiveness. However, the use of CNIs is limited due to their side effects, such as nephrotoxicity [3,4]. Therefore, the discovery of promising new therapeutic drugs with different mechanisms of action from CNIs is of high clinical interest.

In addition, the improvement of long-term allograft survival by control of chronic rejection is another unmet medical need in transplantation. In chronic rejection, organ function is lost along with pathological changes represented by interstitial fibrosis, tubular atrophy, and intimal thickening [5-7]. While acute rejection occurs immediately after transplantation, chronic rejection occurs slowly over the time course of several months to several years after transplantation. With the widespread use of potent immunosuppressive drugs, early graft loss by acute rejection has decreased dramatically; however, current immunosuppressive drugs cannot control graft loss due to chronic rejection. Various factors associated with immunological and non-immunological systems contribute to the progression of chronic rejection (Fig. 0-2). The nephrotoxicity by CNIs is considered as one of the causes of chronic rejection. Thus, new drugs that can suppress chronic rejection are important to improve the long-term prognosis of transplanted graft.

Janus kinase/signal transducer and activator of transcription (JAK/STAT) families in mammals consist of four JAK members, namely JAK1, 2, 3 and tyrosine kinase 2 (TYK2). JAK families are tyrosine kinases that play an important role in cytokine signal transduction [8]. After cytokines bind to receptors, intracellular JAKs get activated and phosphorylate STAT proteins. Phosphorylated STAT proteins move into the nucleus and induce activation of target gene transcription [9] (Fig. 0-3). JAK1 and 3 are involved in T

cell differentiation and proliferation through signal transduction of γ-chain family cytokines such as IL-2, 7, 9, 15 and 21. JAK1, 2 and TYK2 are known to be involved in signal transduction of IL-6, and JAK2 is known to be involved in signal transduction of hematopoietic cytokines such as erythropoietin [10,11]. Accordingly, JAK families have been intensely studied as promising targets. JAK3 was the first to be focused on among JAK kinases. JAK3 has unique functions in the lymphoid system such that JAK3 ablation results in phenotypes resembling severe combined immunodeficiency syndrome [12,13]. However, functions other than the immune system are normal, suggesting that JAK3 inhibitors could be novel immunosuppressants. At present, several small compounds are being developed as JAK inhibitors (Fig. 0-4). Tofacitinib was originally reported as a JAK3 selective compound [14]. Subsequently, this compound was reported to exhibit inhibitory activity against other JAK families, and is currently recognized as a pan-JAK inhibitor that exhibits non-selective JAK inhibitory activity. Tofacitinib was launched as a drug for rheumatoid arthritis (RA), which is one of autoimmune diseases. Since then, other JAK inhibitors have been developed and approved for drugs of RA such as pefitcitinib and baricitinib which were a pan-JAK inhibitor and a JAK1/2 selective inhibitor, respectively [15-17].

In the phase 2 clinical trial in renal transplant patients, tofacitinib attained better kidney function and comparable efficacy to cyclosporin but also increased risks of certain adverse events [18]. The phase 3 clinical trial was not conducted in transplantation and tofacitinib was approved as a therapeutic agent for RA as described above. Post-hoc analysis exhibited below-median exposure to tofacitimib group had a similar overall safety profile to cyclosporin while preserving the advantage of better allograft function and histology [19]. These results suggest that the optimal exposure to JAK inhibitors can

achieve an acceptable safety profile without compromising efficacy in transplantation.

Several JAK inhibitors have demonstrated immunosuppressive efficacy against acute rejection but not chronic rejection in preclinical studies [14,20-24]. However, no studies have examined the effects of JAK inhibitors on chronic rejection in preclinical transplantation models, and it is still unknown whether JAK inhibitors have the therapeutic potential to prevent chronic rejection. Astellas Pharma Inc. generated AS2553627 as a novel and potent JAK inhibitor. Here, the aims of this investigation are to evaluate its efficacy against not only acute rejection but also chronic rejection in rat and monkey transplantation models and to investigate the potential as a therapeutic agent to achieve CNI-sparing for transplantation.

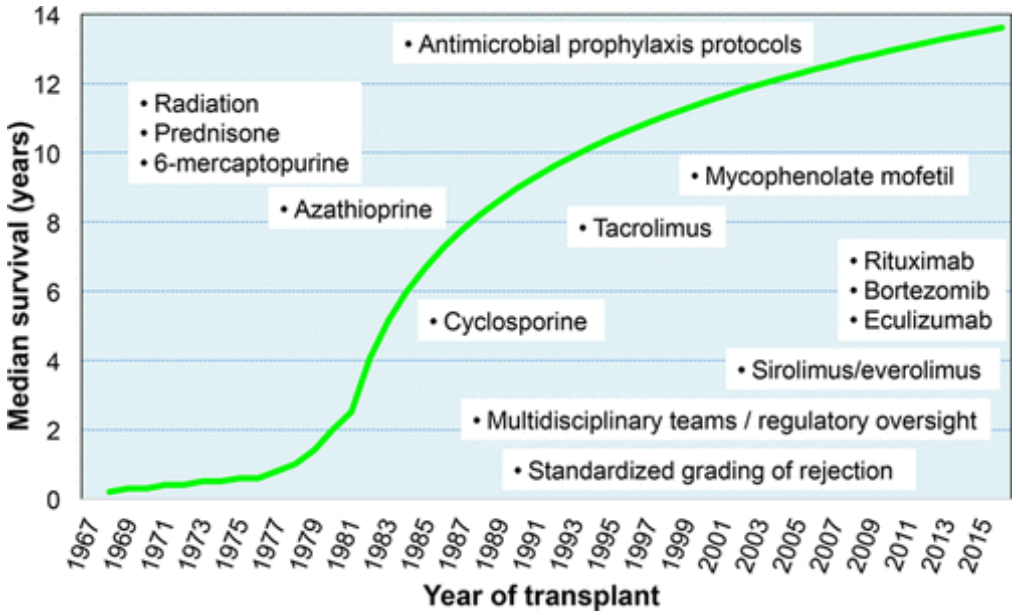


Figure 0-1. The improvement of graft survival after heart transplantation and history of key immunosuppressive drugs. (This figure is adapted from Ref. [2].)

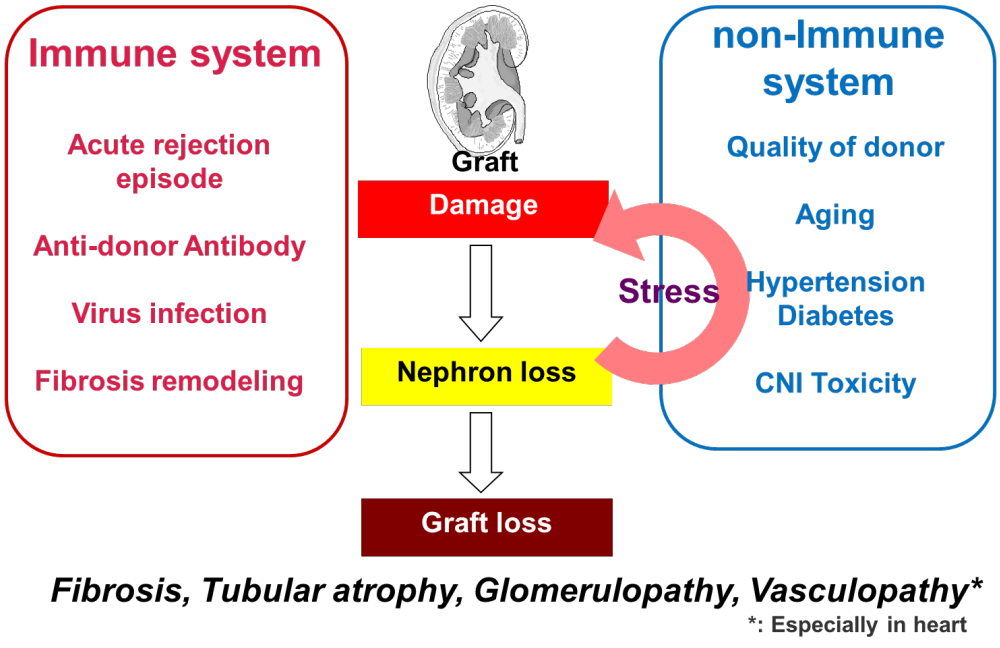


Figure 0-2. Risk factors in chronic rejection.

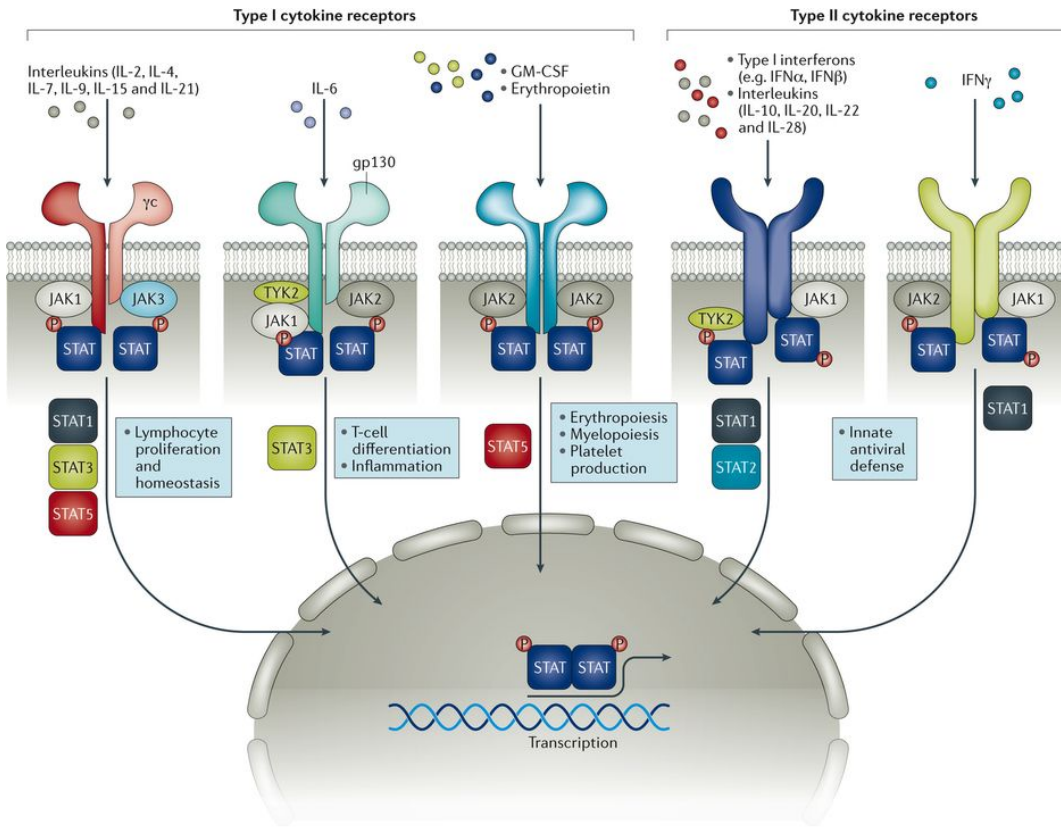
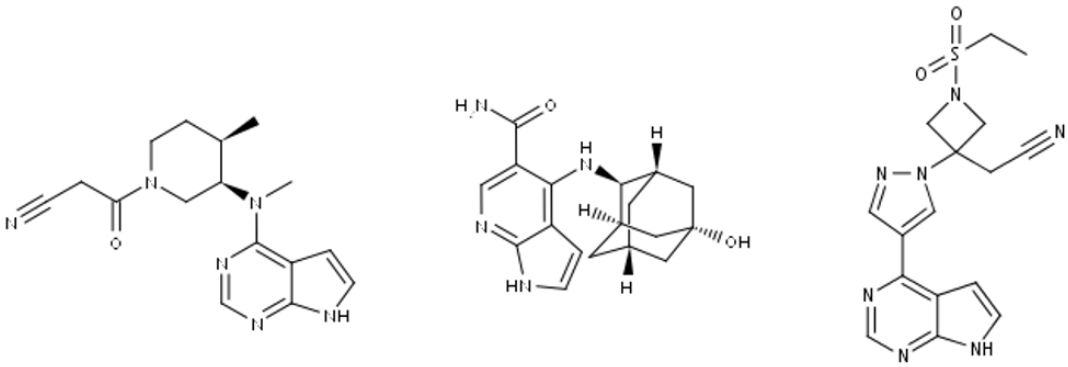


Figure 0-3. JAK/STAT signaling pathway. (This figure is adapted from Ref. [9].)



Tofacitinib Peficitinib Baricitinib

Figure 0-4. Chemical structures of tofacitinib, peficitinib and barcitinib.

Chapter 1 Pharmacological characterization of AS2553627 as a JAK inhibitor

1.1. Introduction

Allograft rejection in transplantation is initiated by recognition of the protein derived from the graft, which is an external antigen, as an alloantigen by antigen-presenting cells and antigen presentation to helper T cells [25]. Helper T cells activated by antigen presentation produce IL-2 and promotes helper T cell differentiation and proliferation. Differentiated helper T cells activate cytotoxic T cells, B cells and macrophages by producing various cytokines. Activated immune cells attack the graft by cytotoxic activity, antibody production and phagocytosis, causing graft dysfunction [26,27]. Accordingly, acute rejection involves the production of IL-2 and the differentiation and proliferation of helper T cells by IL-2 signaling. CNIs, the main agent of existing immunosuppressive therapy, exhibit strong immunosuppressive effects by suppressing IL-2 production of T cells [28]. Thus, immunomodulators that can inhibit IL-2 signaling are recognized to be effective to suppress rejection in organ transplantation [27](Fig.1-1).

The IL-2 receptor (IL-2R) α subunit primarily increases the affinity of ligand binding and is not known to contain a signaling domain, whereas the β and γc subunits participate in both ligand binding and signal transduction. The protein tyrosine kinases JAK1/3, which are associated with the IL-2R β and γc subunits, respectively, are activated after binding of IL-2 to its receptor [29]. Subsequently, JAK1/3 phosphorylated STAT5. Phosphorylated STAT5 forms a dimer, and the translocation of this dimer into the nucleus causes transcriptional activation of gene expression, leading to T cell differentiation and proliferation [30]. The roles of JAK1/3 in IL-2-mediated proliferation

are well established. In this chapter, we conducted *in vitro* and *in vivo* studies and compared AS2553627 with the existing JAK inhibitors tofacitinib and peficitinib to elucidate the pharmacological characteristics of AS2553627 as a novel JAK inhibitor. The inhibitory activity on the JAK kinases family and IL-2 stimulated T cell proliferation was examined.

CNIs such as cyclosporin and tacrolimus have been the cornerstones of modern immunosuppressive agents for more than 30 years because they have resulted in a significant improvement in graft survival. However, CNIs have been associated with chronic nephrotoxicity, and CNI-sparing strategies are important for improvements in long-term allograft survival [3,31]. Considering the above issues in transplantation medicine, CNI-sparing the inhibitory effect on acute rejection with a sub-optimal dose of tacrolimus was also evaluated by the survival prolongation effect in a rat cardiac transplantation model.

1.2. Material & Methods

1.2.1. Animals

Lewis ($RT1^{l}$) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), and ACI ($RT1^{avl}$) rats from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained in a temperature- and humidity-controlled room with a 12-h light-dark cycle. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. The Kashima and Tsukuba Research Centers of Astellas Pharma Inc. have been awarded Accreditation Status by AAALAC International.

1.2.2. Reagents

AS2553627 (3-[(3R,4R)-3-(dipyrrolo[2,3-b:2',3'-d]pyridin-1(6H)-yl)-4-methylpiperidin-1-yl]-3-oxopropanenitrile) and tacrolimus (Prograf® injection) were prepared at Astellas Pharma Inc. (Tokyo, Japan).

1.2.3. Kinase assays

The human JAK1, 2, 3 and TYK2 kinase-domains were purchased from Carna Biosciences Inc. (Kobe, Japan). Assays were performed using streptavidin-coated 96-well plates. The final concentrations of kinases were 3, 1, 0.8 and 3.2 ng/well for JAK1, 2, 3 and TYK2, respectively. A reaction mixture contained 15 mM Tris-HCl (pH 7.5), 0.01% Tween 20, 2 mM DTT, 10 mM $MgCl_2$, 250 nM Biotin-Lyn-Substrate-2 (for JAK1, 2 and 3, Peptide Institute, Inc., Osaka, Japan) or Biotin-IRS1-Substrate (for TYK2, Invitrogen, Carlsbad, CA, USA) and ATP. The final concentrations of ATP were 200, 10, 8, and 4 μM for JAK1, 2, 3 and TYK2, respectively. AS2553627, tofacitinib or baricitinib was dissolved in dimethyl sulfoxide. The reaction was initiated by adding the kinase domain, followed by incubation at room temperature for 1 hour. Kinase activity was measured as the rate of phosphorylation of Biotin-Lyn-Substrate-2 or Biotin-IRS1-Substrate using HRP-conjugated anti-phosphotyrosine antibody (HRP-PY-20; Santa Cruz Biotechnology, Incyclosporinnta Cruz, CA, USA) by using phosphotyrosine-specific ELISA. Twenty-nine other human kinase assays were performed at Carna Biosciences Inc. using standard conditions.

1.2.4. IL-2-stimulated T cell proliferation assays

Splenocytes from male Lewis rats were suspended in RPMI1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Biowest, Nuaillé, France), 1% (v/v)

penicillin-streptomycin and 50 μM 2-mercaptoethanol at 2.0×10^6 cells/mL. Rat splenocytes were cultured with concanavalin A (1 μg/mL, Sigma) for approximately 24 hours at 37°C to induce the expression of IL-2 receptors. The cells were washed and then suspended in culture medium at 2.4×10^4 cells/well in 96-well plates. Human peripheral blood was collected from healthy volunteers and subjected to density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to purify the mononuclear cells in the peripheral blood. After washing, the cells (5×10^5 cells/mL) were suspended in RPMI1640 containing 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin, and cultured with phytohemagglutinin (10 μg/mL, Sigma) for 5 days at 37°C in a 5% CO_2 incubator. The cells were washed and then suspended in culture medium at 2×10^4 cells/well in 96-well plates. Rat splenocytes and human peripheral blood mononuclear cells were incubated with IL-2 (BD Biosciences, San Diego, CA, USA) and AS2553627, tofacitinib or baricitinib at designated concentrations. The final concentrations of IL-2 were 1 and 3 ng/ml for rat and human, respectively. After incubation for 3 days, alamarBlue® (Life Technologies, Carlsbad, CA, USA) was added to each of the test wells. After several hours, Fluorescence intensity was measured at an excitation wavelength of 545 nm and an emission wavelength of 590 nm.

1.2.5. Rat heterotopic cardiac transplantation

Male ACI and Lewis rats aged 6–10 weeks were used as cardiac donors and recipients, respectively. All procedures were performed under aseptic conditions. Rats were intraperitoneally anesthetized with pentobarbital (40 mg/kg). Abdominal vascularized heterotopic cardiac transplantation was performed as previously described

[32,33]. Briefly, cardiac grafts were transplanted from male ACI donors into male Lewis recipients. The donor aorta and pulmonary artery were anastomosed, end to side, to the recipient abdominal aorta and inferior vena cava, respectively. The day of transplantation was designated as day 0.

1.2.6. Acute rejection evaluation by graft survival

AS2553627 (5, 10 and 20 mg/kg as monotherapy, 0.1, 0.25 and 0.5 mg/kg as combination therapy) was dissolved in propylene glycol solvent. Tofacitinib (0.5, 1, 2 and 3 mg/kg as combination therapy) was dissolved in PEG400 solvent and was orally administered. Tacrolimus (0.02 mg/kg) was diluted with saline and intramuscularly administered. Beginning on the operation day, all test compounds were administered once a day for 14 consecutive days. Cardiac allograft function was assessed by daily palpation for 28 days, and graft rejection was defined as the cessation of palpable cardiac graft beats.

1.2.7. Statistical analysis

Analysis for statistical significant difference was done using Graphpad Prism 8 software (Graphpad Software, La Jolla, CA). The results of *in vitro* experiments were expressed as the mean. The IC_{50} value of each kinase and cell proliferation assay was calculated using linear regression analysis. Median survival times (MST) of the grafts in the transplant study were analyzed by comparing groups using the log-rank test. P values of less than 0.05 were considered statistically significant.

1.3. Results

1.3.1. Enzymatic inhibitory activity of AS2553627

The structure of AS2553627 is shown in Fig. 1-2. AS2553627 inhibited JAK kinases activity and exhibited the most potent inhibitory activity on JAK3. The IC_{50} values were 0.46 nM (JAK1), 0.30 nM (JAK2), 0.14 nM (JAK3), and 2.0 nM (TYK2) (Table 1). Under the same assay conditions, tofacitinib and peficitinib inhibited JAK kinases activity, but showed the less potent inhibition than AS2553627. A kinase panel assay revealed that AS2553627 has no inhibitory activity on any other kinases, including SYK and ZAP70, at concentrations of up to 1 μM (Table 1-1).

1.3.2. Inhibitory effect of AS2553627 on T cell proliferation

IL-2 is a T cell-derived cytokine that plays an important role in regulation of the growth and differentiation of T cells. IL-2 induces tyrosine phosphorylation and activation of JAK1 and 3. To determine inhibitory activity in T cells, the effects of AS2553627 in an IL-2-stimulated T cell proliferation assay were analyzed. AS2553627 inhibited proliferation of IL-2 stimulated human and rat T cells, with IC_{50} values of 2.4 and 4.3 nM, respectively (Table 1-2). We also examined the inhibitory effects of other JAK inhibitors on IL-2 stimulated human and rat T cells. The IC_{50} value of tofacitinib was 24nM. Peficitinib also inhibited IL-2-induced rat and human T cell proliferation with similar potency (IC_{50}=10 and 18 nM, respectively), which were several fold less potent than the inhibitory effect of AS2553627 on IL-2 stimulated human or rat T cell proliferation.

1.3.3. Acute rejection: graft survival in cardiac transplantation

The preventive effect of acute rejection by orally administered AS2553627 was assessed in an ACI-to-Lewis rat heterotopic cardiac transplant model. Because the maximum tolerated dose of AS2553627 is 30 mg/kg, AS2553627 at 5, 10 and 20 mg/kg was orally administered in monotherapy studies. Compared to MST of 5.5 days in rats administered the vehicle control, rats treated with AS2553627 at 5, 10 and 20 mg/kg showed significantly improved graft survival, with MSTs of 13, 16 and 17 days, respectively (Table 1-3). To determine the concomitant efficacy of AS2553627 and a calcineurin inhibitor, AS2553627 was administered orally in combination with intramuscular injection of a sub-optimal dose of tacrolimus (0.02 mg/kg). Compared to MST of 10 days for recipients administered with vehicle plus tacrolimus, those administered AS2553627 at 0.25 or 0.5 mg/kg plus tacrolimus showed a significant prolongation of graft survival, with MST of 21 days for both (Table 1-4). In addition, we also evaluated the efficacy of tofacitinib on allograft prolongation. Daily administration of 1, 2 and 3 mg/kg tofacitinib showed significantly improved graft survival, with MSTs of 19, 20.5 and 20 days, respectively (Table 1-5). These results indicate that AS2553627 has the potent JAK inhibitor with synergistic activities in combination with tacrolimus against acute rejection.

1.4. Discussion

In this chapter, we compared the *in vitro* pharmacological profiles of AS2553627 with those of the existing JAK inhibitors tofacitinib and peficitinib to reveal the characteristics of AS2553627 as a JAK inhibitor. AS2553627 showed potent inhibition of all JAK kinases in cell-free enzymatic assays, whereas this the most potently

inhibited JAK3. The IC_{50} values for JAK kinases activity of AS2553627 were lower than those of tofacitinib and peficitinib [34]. These results indicate that AS2553627 was a potent pan-JAK inhibitor. JAK1/3 are essential for the IL-2 response [35]. We therefore examined the effect of each JAK inhibitor on IL-2-induced T cell proliferation and found that the AS2553627 potently inhibited T cell proliferation compared with tofacitinib and peficitinib [34]. This result is consistent with the inhibitory effects of AS2553627 on JAK families revealed by the kinase enzymatic assay data. In contrast, AS2553627 did not exert inhibitory effects on other kinases, including T cell receptor-associated kinases such as SYK and ZAP70. Taken together, these results suggest that AS2553627 is a potent and selective JAK inhibitor.

Next we compared the *in vivo* efficacy of JAK inhibitors on acute rejection in rat cardiac transplantation models. In graft survival evaluation in ACI to LEW cardiac transplantation, we previously demonstrated the occurrence of acute cellular rejection with severe mononuclear cell infiltration and damaged myocardium [36]. AS2553627 at 5 mg/kg significantly prolonged the graft survival time when administered as a monotherapy. Since the maximum tolerated dose was 30 mg/kg, it was shown that AS2553627 is a potent drug that can sufficiently suppress acute rejection as a monotherapy. This compound at 0.25 mg/kg also exerted synergistic effects in combination with a sub- optimal dose of tacrolimus. The efficacy of AS2553627 at 0.25 mg/kg was comparable to that of tofacitinib at 1 mg/kg, indicating that AS2553627 exhibited 4-fold potent *in vivo* efficacy. In IL-2 stimulated rat T cell proliferation assay, the IC_{50} value of AS2553627 was approximately 5-fold potent than that of tofacitinib. Taken together, these results suggest the close correlation between inhibition on IL-2 stimulated T cell proliferation and graft survival prolongation.

The synergistic outcome of JAK inhibitors and tacrolimus combination therapy may be due to differences in their mechanisms of action. CNIs inhibit the calcium/calmodulin-dependent protein phosphatase calcineurin, and prevents the translocation of NFAT into the nucleus, consequently blocking the transcription of several genes related to T-cell activation, including IL-2 [37]. JAK inhibitors can directly prevent IL-2 signaling though the JAK-STAT pathway, as shown in IL-2-stimulated T cell proliferation assay. Renal failure is one of common causes of late mortality following cardiac transplantation and surviving patients may eventually require dialysis and/or a renal transplant. CNIs have the potential risk for nephrotoxicity and optimizing the dose of these drugs is important for the long-term graft preservation [4,5]. Our findings suggest that AS2553627 inhibits acute rejection in cardiac transplantation, and also may help to optimize the exposure to CNIs. Collectively, AS2553627 is a novel oral small molecule compound with efficacy that is JAK selective and superior to existing JAK inhibitors.

1.5. Tables and Figures

Table 1-1. Inhibitory activity of JAK inhibitors on human JAK 1, 2, 3 and TYK2 activity.

IC_{50} (nM)	JAK1	JAK2	JAK3	TYK2	Other kinases[a]
AS2553627	0.46	0.30	0.14	2.0	>1000
Tofacitinib[b]	3.7	3.1	0.8	16	-
Peficitinib[b]	3.9	5.0	0.7	4.8	-

[a] ABL, BTK, CSK, EGFR, EphA2, FAK, IGF1R, ITK, SRC, SYK, TIE2, TRKA, ZAP70, AKT1, AurC, BMPR1A, CaMK2a, CDK2, CHK1, CK1d, GSK3b, IKKb, JNK1, MAP2K1, MAP3K1, MAPKAPK2, p38b, p70S6K, RAF1

[b] Data of tofacitinib and peficitinib were previously reported by our laboratory [15].

Table 1-2. Inhibitory effects of JAK inhibitors on IL-2-induced T cell proliferation.

IC_{50} (nM)	Human T cells	Rat T cells
AS2553627	2.4	4.3
Tofacitinib[a]	-	24
Peficitinib[a]	18	10

[a] Data of tofacitinib and peficitinib were previously reported by our laboratory [15,38].

Table 1-3. Effect of AS2553627 monotherapy on graft survival time in rat cardiac transplantation.

AS2553627 (mg/kg, p.o.)	n	Graft survival time (days)	MST (days)	P- value[a]
Vehicle	8	5, 5, 5, 5, 6, 6, 6, 7	5.5	-
5 mg/kg	7	7, 8, 10, 13, 15, 20, >28	13	P < 0.001
10 mg/kg	11	7, 8, 8, 9, 10, 16, 18, 21, 22, 22, >28	16	P < 0.001
20 mg/kg	7	7, 15, 17, 17, 17, 20, >28	17	P < 0.001

[a] P-value calculated by the log-rank test compared to the vehicle group.

MST = median survival time

Table 1-4. Effect of combination therapy with AS2553627 and tacrolimus on graft survival time in rat cardiac transplantation.

AS2553627 (mg/kg, p.o.)	Tacrolimus (mg/kg. i.m.)	n	Graft survival time (days)	MST (days)	P- value[a]
Vehicle	0.02	7	6, 7, 9, 10, 10, 11, 12	10	-
0.1 mg/kg	0.02	8	6, 7, 7, 8, 10, 10, 14, >28	9	-
0.25 mg/kg	0.02	7	12, 12, 15, 21, 22, 22, >28	21	$P < 0.001$
0.5 mg/kg	0.02	7	8, 8, 16, 21, 22, 22, 24	21	$P < 0.05$

[a] P-value calculated by log-rank test compared to combination therapy with vehicle plus tacrolimus group.

MST = median survival time

Table 1-5. Effect of combination therapy with tofacitinib and tacrolimus on graft survival time in rat cardiac transplantation.

Tofacitinib (mg/kg, p.o.)	Tacrolimus (mg/kg. i.m.)	n	Graft survival time (days)	MST (days)	P- value[a]
Vehicle	0.02	15	5, 5, 6, 6, 6, 7, 7, 8, 9, 9, 9, 10, 11, 11, 20	8	-
0.5 mg/kg	0.02	9	6, 6, 6, 7, 13, 19, 19, 22, >28	13	-
1 mg/kg	0.02	16	5, 6, 7, 7, 7, 9, 12, 19, 19, 20, 20, 22, 22, 22, 23, 23	19	P < 0.01
2 mg/kg	0.02	8	6, 7, 17, 20, 21, 23, 23, >28	20.5	P < 0.01
3 mg/kg	0.02	5	11, 16, 20, 21, 25	20	P < 0.01

[a] P-value calculated by log-rank test compared to combination therapy with vehicle plus tacrolimus group.

MST = median survival time

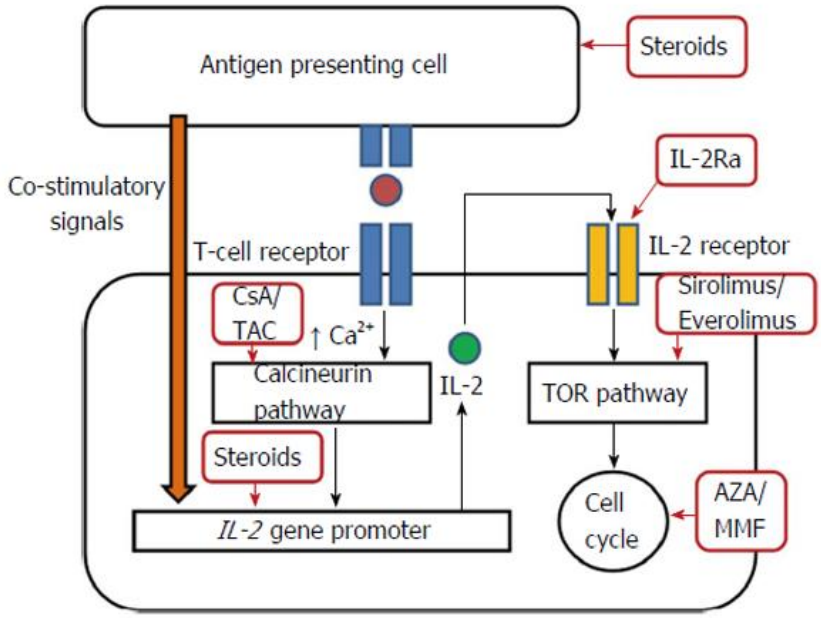


Figure 1-1. The cellular site of action of the immunosuppressive drugs commonly used in solid organ transplantation. (This figure is adapted from Ref. [27].)

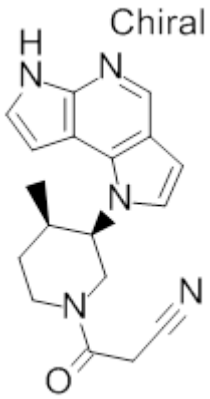


Figure 1-2. Chemical structure of AS2553627.

Chapter 2 Pharmacological evaluation of AS2553627 on chronic allograft rejection in rat transplantation models

2.1. Introduction

In chapter 1, we revealed that AS2553627 has stronger inhibitory activity on JAK kinases and IL-2 stimulated T cell proliferation than existing JAK inhibitors. In addition, AS2553627 prolonged strong graft survival, and also showed a synergistic effect of a sub-optimal dose with tacrolimus, suggesting the possibility to reduce CNI exposure.

CNIs dramatically have reduced graft failure by acute rejection following transplantation [5], however, chronic graft rejection remains prominent. In cardiac transplantation, cardiac allograft vasculopathy (CAV) is the major chronic allograft rejection which is an accelerated form of the intimal hyperplasia of the coronary arteries. After immunological damage, proliferation of vascular smooth muscle cells occurs and subsequent diffuse progressive luminal narrowing. Renal transplantation is the most common among all solid organ transplantation. In renal transplantation, the major pathological features of chronic allograft rejection are interstitial fibrosis/tubular atrophy (IF/TA) and glomerular lesions [6,7]. Chronic rejection limits long-term graft survival after transplantation and subsequently lead to graft failure. Currently, various drug targets have been studied for their potential to prevent chronic rejection. However, no effective or therapeutic strategies for chronic rejection have been developed [7,39-42]. Therefore, the discovery of promising new therapeutic targets that prevent chronic rejection is desired.

The previous chapter revealed the efficacy of AS2553627 for acute rejection in the short-term treatment. In this chapter, we examined whether chronic rejection such as vasculopathy, IF/TA and glomerular lesions was observed in a rat cardiac or renal transplantation model that was long-term engrafted with an optimal dose of tacrolimus. Moreover, it was also clarified whether co-administration of AS2553627 suppressed chronic allograft rejection.

2.2. Material & Methods

2.2.1. Animals

Lewis ($RT1^{l}$) and Brown Norway (BN, $RT1^{n}$) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), and ACI ($RT1^{avl}$) rats from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained in a temperature- and humidity-controlled room with a 12-hour light-dark cycle. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. The Kashima and Tsukuba Research Centers of Astellas Pharma Inc. have been awarded Accreditation Status by AAALAC International.

2.2.2. Reagents

AS2553627 (3-[(3R,4R)-3-(dipyrrolo[2,3-b:2',3'-d]pyridin-1(6H)-yl)-4-methylpiperidin-1-yl]-3-oxopropanenitrile) and tacrolimus (Prograf® solid dispersion formulation [SDF] and injection) were prepared at Astellas Pharma Inc. (Tokyo, Japan).

2.2.3. Evaluation of AS2553627 in a rat cardiac chronic allograft rejection model

2.2.3.1. Heterotopic cardiac transplantation and drug treatments

Male ACI and Lewis rats aged 6–10 weeks were used as cardiac donors and recipients, respectively. All procedures were performed under aseptic conditions. Rats were intraperitoneally anesthetized with pentobarbital (40 mg/kg). Abdominal vascularized heterotopic cardiac transplantation was performed as previously described using ACI to ACI (isograft) or ACI to Lewis (allograft) rats [32,33]. Briefly, Cardiac grafts were transplanted from donors into recipients. The donor aorta and pulmonary artery were anastomosed, end to side, to the recipient abdominal aorta and inferior vena cava, respectively. The day of transplantation was designated as day 0. AS2553627 (0.3 and 1 mg/kg) was dissolved in propylene glycol solvent and orally administrated. Tacrolimus (3.2 mg/kg) was suspended in sterile water and orally administered more than 6 hours after the treatment with AS2553627. Beginning on the operation day, all test compounds were administered once a day for 90 consecutive days.

2.2.3.2. Histopathological analysis of cardiac graft rejection

At the end of the study, each recipient was euthanized, and heart specimens were collected for histopathological evaluation. Collected cardiac specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Two coronal sections, 2 and 4 mm from cardiac apex, were each stained with elastica van Gieson (EVG), Azan and hematoxylin-eosin (HE). In this study, chronic rejection was deduced from the occurrence of histological changes including vasculopathy and fibrosis. Acute cellular rejection was deduced from the occurrence of mononuclear cell infiltration. These rejections were diagnosed according to the Working Formation of the International Society for Heart and Lung Transplantation (ISHLT-WF) guidelines, clinical, and

experimental studies [5,41]. Vasculopathy was evaluated based on the ratio of EVG stained epicardial and intramyocardial coronary arteries showing intimal thickening of more than 100 μm in diameter. The extents of fibrosis and mononuclear cell infiltration were assessed by Azan and HE staining, respectively. Fibrosis was scored as follows: 0 = none, 1 = focal deposition of Azan positive matrix, 2 = Azan positive matrix less than 50%, 3 = Azan positive matrix greater than 50%. Mononuclear cell infiltration was scored as follows: 0 = none, 1 = focal infiltration, 2 = multifocal infiltration, 3 = diffuse infiltration.

2.2.4. Evaluation of AS2553627 in a rat renal chronic allograft rejection model

2.2.4.1. Renal transplantation and drug treatments

Male LEW and BN rats aged 7-8 weeks were used as renal donors and recipients, respectively. All procedures were performed under aseptic conditions. Rats were intraperitoneally anesthetized with pentobarbital (40 mg/kg). LEW to LEW(isograft) and LEW to LEW(allograft) abdominal vascularized heterotopic renal transplantations were performed as described previously [43]. Briefly, the donor renal artery and vein were anastomosed to the recipient abdominal aorta and inferior vena cava, respectively. The ureter reconstruction was performed with ureter-to-ureter anastomosis. Immediately after transplantation, contralateral nephrectomy was performed. The day of transplantation was designated as day 0. Tacrolimus (0.1 mg/kg) was diluted with saline and administered intramuscularly once a day for 10 consecutive days starting on the day of transplantation (days 0 to 9) to prevent initial acute rejection. After discontinuation of tacrolimus treatment from days 10 to 28, AS2553627 (1 and 10 mg/kg) was dissolved in propylene glycol (vehicle) and orally administrated with tacrolimus (0.1 mg/kg, intramuscularly) until 13 weeks after renal transplantation. All data for the isograft

(n=4) and vehicle plus tacrolimus-treated allograft groups (n=7) were obtained from animals used in our previous study [43]. All parameters were obtained from recipients that survived until the end of the study.

2.2.4.2. Plasma creatinine, urinary protein and kidney injury biomarkers

Post transplantation, plasma samples were collected at days 10 and 28, and then once a week until the end of the study, when animals were euthanized. Twenty-four-hour pooled urine samples were collected at two-week intervals using a metabolic cage system. Levels of creatinine in plasma were determined by the Fuji DRI-CHEM system (Fuji Film Ltd, Tokyo, Japan). Urinary protein was measured using a Bio Rad Protein Assay™ (Bio Rad Lab., Inc., Hercules, CA, USA). Kidney injury molecule-1 (KIM-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels were measured using a WideScreen™ Rat Kidney Toxicity Panel (Merck, Millipore, MA, USA).

2.2.4.3. Histopathological analysis of renal graft rejection

Renal graft samples were harvested at 13 weeks, fixed with 10% neutral buffered formalin, and then embedded in paraffin wax. Three-micrometer-thick sections were stained with HE, Periodic acid-Schiff (PAS), and Azan. We adapted the Banff classification (Banff 2007) [44] of renal pathology to semi-quantitatively score tubular atrophy, interstitial fibrosis, and glomerulosclerosis. Graft samples were graded from 0 to 3, with a score of 3 indicating the most severe histological characteristics.

2.2.4.4. Gene expression in renal intragraft

RNA was isolated from renal grafts using a QuantiGene Sample Processing kit (Affymetrix Inc., Fremont, CA, US) according to the manufacturer's instructions.

mRNA was analyzed using a QuantiGene 2.0 Plex Assay kit (Affymetrix Inc., Fremont, CA, US) according to the manufacturer's protocol. Data were normalized against the housekeeping gene TBP and expressed as fold change compared with isografts.

2.2.4.5. Plasma donor-specific antibody levels

Donor-specific antibody (DSA) levels were analyzed using flow cytometry. Plasma samples from recipient animals were incubated with 0.9×10^7 splenocytes from LEW rats, followed by FITC-anti-rat IgM or IgG (BD Biosciences, San Jose, CA, USA). The cells were fixed and analyzed using Guava® easyCyte flow cytometer (Merck Millipore, Darmstadt, Germany). The results were expressed as mean fluorescence intensity (MFI).

2.2.5. Statistical analysis

Analysis for statistical significant difference was done using Graphpad Prism 8 software and SAS (SAS Institute Japan, Tokyo, Japan). Body weight changes were expressed as the mean ± S.E.M. Significant differences among all time-points and treatment groups were determined using Tukey's multiple comparison test. Vasculopathy data were expressed as the mean. Significant differences between two groups were determined using Student's t-test, and those between more than two groups were assessed using Dunnett's multiple comparison test. Other histological data were expressed as the median. Significant differences between two groups were determined using Mann-Whitney U-test, while those between more than two groups were assessed using Steel test. Other data were expressed as the mean ± S.E.M or geometric means. Significant differences between two groups were determined using Student's t-test, and

those between more than two groups using a Dunnett's multiple comparisons test. P values of less than 0.05 were considered statistically significant.

2.3. Results

2.3.1. Chronic histopathological changes in long-surviving cardiac allografts

To investigate the effect of AS2553627 on chronic allograft rejection in cardiac transplantation model, AS2553627 was administered for 90 consecutive days from the day of operation, in combination with an optimal dose of tacrolimus (3.2mg/kg). Body weight gain in the AS2553627 plus tacrolimus-treated group was comparable to that in the vehicle plus tacrolimus-treated group (Fig. 2-1). No obvious signs of discomfort or gastrointestinal symptoms such as diarrhea were observed in any animal during the treatment period. Heartbeats were maintained in all recipients treated with tacrolimus throughout the 90-day treatment period, and mononuclear cell infiltration was absent or mild (Fig. 2-2A and Fig. 2-3A), indicating that acute rejection was enough controlled by tacrolimus. Mononuclear cell infiltration was completely suppressed by AS2553627 at 1 mg/kg plus tacrolimus (Fig. 2-2A vs. B and Fig. 2-3A).

In contrast to mononuclear cell infiltration, cardiac allografts treated with vehicle plus tacrolimus showed typical vasculopathy, which was characterized by intimal hyperplasia. The internal elastic membrane was focally disrupted and luminal narrowing was observed (Fig. 2-2C). In combination with tacrolimus, AS2553627 at 0.3 and 1 mg/kg significantly prevented vasculopathy in cardiac allografts (Fig. 2-2C vs. D and Fig. 2-3B). Moreover, cardiac allografts treated with vehicle plus tacrolimus exhibited mild or moderate fibrosis ($p=0.058$ vs. tacrolimus-treated isografts). AS2553627 at 1 mg/kg plus tacrolimus significantly inhibited fibrosis (Fig. 2-2E vs. F and Fig. 2-3C).

These data suggest that AS2553627 inhibits not only acute rejection, but also chronic rejection that an optimal dose of tacrolimus cannot suppress completely.

2.3.2. Plasma creatinine, urinary protein and kidney injury biomarkers in long-surviving recipients

Next, we evaluated the effect of AS2553627 on chronic rejection such as IF/TA and glomerulosclerosis in a rat renal transplantation model and conducted multilateral analysis to reveal the detailed mechanism of action of AS2553627 on chronic rejection. In this model, we evaluated two dosages of AS2553627: 1 mg/kg because it is the effective dose used in combination with tacrolimus for reducing chronic rejection in the rat cardiac transplantation model, and 10 mg/kg because it is the effective dose used in monotherapies for graft survival prolongation. Plasma creatinine levels after transplantation are shown in Fig. 2-4A. Plasma creatinine significantly increased following discontinuation of tacrolimus from days 10 to 28. Resumption of tacrolimus treatment in the vehicle plus tacrolimus group until 13 weeks immediately decreased plasma creatinine levels, but it remained high compared with that of the isograft group and gradually increased over time despite resumption of treatment. In contrast, AS2553627 (1 and 10 mg/kg) plus tacrolimus-treated animals maintained low plasma creatinine levels. Furthermore, the increase in total protein levels in 24-hour urine samples from recipients was suppressed in the AS2553627 plus tacrolimus treated-group throughout the duration of the experiment (Fig. 2-4B, C). Urinary TIMP-1 and KIM-1, known biomarkers of renal dysfunction [45-47], were also suppressed by AS2553627 plus tacrolimus treatment to a level comparable with those of the isograft group (Fig. 2-4D). These results suggest that AS2553627 sustained renal function for a long time after transplantation.

2.3.3. Chronic histopathological changes in long-surviving renal allografts

We analyzed renal grafts by histology at 13 weeks after transplantation to examine whether AS2553627 inhibited chronic rejection. Allografts in vehicle-treated recipients under tacrolimus displayed histopathological changes including segmental/global glomerulosclerosis (Fig. 2-5A), tubular atrophy (Fig. 2-5B), and interstitial fibrosis (Fig. 2-5C), indicating that these animals developed chronic rejection features. AS2553627 plus tacrolimus treatment significantly prevented the formation of glomerulosclerosis (Fig. 2-5D and G) and tubular atrophy (Fig. 2-5E and H) in a dose-dependent manner: no animals developed moderate or severe glomerulosclerosis (Fig. 2-5G) or tubular atrophy (Fig. 2-5H) in the 10 mg/kg AS2553627 group, with more than half of the animals showing no histological signs of glomerulosclerosis. Furthermore, allografts of AS2553627-treated animals showed no moderate changes in interstitial fibrosis (Fig. 2-5F and I). These data indicate that AS2553627 inhibited chronic allograft rejection after renal transplantation.

2.3.4. Gene expression in renal intragraft

To further reveal the effect of AS2553627 on chronic rejection, intragraft mRNA expression of cell surface markers, fibrosis/epithelial-mesenchymal transition (EMT) and inflammation-related factors were examined at 13 weeks (Fig. 2-6). The gene expressions of CD4, CD8 and CD14 which were T cells and monocytes/macrophages cell surface markers were upregulated in allografts from vehicle plus tacrolimus-treated animals compared with isografts, whereas CD8 mRNA was significantly reduced by AS2553627 plus tacrolimus treatment, indicating that T cell infiltration was inhibited in renal allografts. EMT has become widely accepted as a

mechanism by which injured renal tubular cells transform into mesenchymal cells and contribute to the development of fibrosis in chronic renal failure [48]. Fibrosis/EMT and inflammation-related genes such as transforming growth factor (TGF)-β1, collagen I, vimentin, thrombospondin (THBS) 2, monocyte chemoattractant protein (MCP)-1, and IL-6 mRNA levels were elevated more than 4-fold in tacrolimus plus vehicle-treated allografts compared with isografts. AS2553627 reduced the expression of these genes, especially IL-6. These results indicate that AS2553627 inhibited the expression of genes associated with fibrosis and inflammation in renal allografts.

2.3.5. Plasma donor-specific antibody in renal transplantation

Alloantibody-mediated chronic allograft injury is characteristically observed as transplant glomerulopathy in kidney biopsies [49]. To determine the effect of AS2553627 on the production of DSAs, plasma anti-donor IgM and IgG levels were analyzed by flow cytometry. Anti-donor IgM (Fig. 2-7A) and IgG levels (Fig. 2-7B) were decreased but remained at a detectable level upon resumption of tacrolimus treatment. Seven weeks after transplantation, anti-donor IgM in the AS2553627 plus tacrolimus-treated group decreased compared with the vehicle plus tacrolimus-treated group and was equal to that of the isograft group. Recipients treated with 10 mg/kg AS2553627 plus tacrolimus showed slightly reduced anti-donor IgG levels compared to the vehicle plus tacrolimus-treated allograft group.

2.4. Discussion

In the present chapter, we evaluated the therapeutic potential of AS2553627 for chronic rejection in the long-term surviving recipients which were not inhibited by

tacrolimus at its optimal dose in rat cardiac or renal transplantation models. Oral administration of AS2553627 did not affect body weight gain and was well tolerated through the experiment period. These results indicate that AS2553627 has a favorable safety profile in the long term treatment. AS2553627 plus tacrolimus treatment prevented chronic histopathological changes of CAV, glomerulosclerosis and IF/TA which were observed 13 weeks after transplantation. In a renal transplantation model, AS2553627 treatment maintained renal function such as plasma creatinine levels, urinary protein, and kidney injury biomarkers. AS2553627 also reduced the upregulation of intragraft gene expressions related with fibrosis/EMT and inflammation. Our results are consistent with clinical evidence indicating that treatment with the JAK inhibitor, tofacitinib, can lower the incidence of chronic rejection outcomes such as IF/TA in kidney transplant patients [18]. The efficacy of tofacitinib on glomerulosclerosis in kidney transplants was not previously evaluated in clinical trials since glomerular damage often occurs after a few years, compared to the earlier manifestation of IF/TA, which develops within the first 12 months [50]. In addition, there is no clinical evidence of JAK inhibitor for CAV after cardiac transplantation. Information on the efficacy of JAK inhibitors against chronic allograft rejection in transplantation has been also lacking in experimental transplantation models, whereas the efficacy of JAK inhibitors on graft survival and acute rejection has been evaluated [14,20-24]. Our present study is therefore the first to present data showing the therapeutic potential of a JAK inhibitor in preventing chronic rejection following transplantation in preclinical models.

The molecular composition of urine is highly reflective of intrarenal events. Levels of KIM-1, a type I transmembrane protein, increase in biopsies with IF/TA [51]. TIMP-1 is an important cytokine for extracellular matrix (ECM) synthesis and degradation; excess

accumulation of ECM is the main pathological mechanism of fibrosis. In renal transplant patients with IF/TA, the expression of TIMP-1 was positively correlated with serum creatinine levels [46]. While urinary KIM-1 and TIMP-1 were detected in the vehicle plus tacrolimus-treated allograft group, AS2553627 plus tacrolimus treatment significantly reduced these biomarkers following improvement of chronic rejection. These results suggest that AS2553627 can inhibit kidney injury in terms of biomarkers.

Various mechanisms have been linked to chronic rejection progression, including calcineurin inhibitor toxicity, cellular or antibody-mediated injury, inflammation and EMT [48]. EMT has been reported to contribute to the process of fibrosis in various organs, including the kidneys. Several studies have shown that epithelial cells have an altered phenotype in transplanted kidneys with IF/TA [48]. Intragraft fibrosis/EMT-related genes such as TGF-β1, collagen I, THBS2 and vimentin are elevated in kidney transplant patients [52,53]. Similarly, the present study indicates that these gene markers in intragraft were upregulated and AS2553627 showed tends to inhibit them.

T cell infiltration into allografts induces tissue injury and plays important roles in the development of chronic rejection [54,55]. Measurement of intragraft gene expression showed that upregulation of CD8 mRNA was significantly inhibited by AS2553627 plus tacrolimus treatment, indicating that AS2553627 decreased T cell-mediated rejection in allografts. This result is consistent with our findings in chapter 1 that AS2553627 suppressed IL-2-induced T cell proliferation [56]. Clinical studies in cardiac transplantation have shown that acute cellular rejection episodes were risks for CAV development, and experimental studies in rodents have also shown that depletion of $CD4^+$T cells prevented CAV [57]. In our study, AS2553627 (1mg/kg) in combination with tacrolimus suppressed mononuclear cell infiltration, which tacrolimus alone could not

completely inhibit, indicating that AS2553627 may prevent CAV by inhibiting acute cellular rejection. However, a low dose of AS2553627 (0.3mg/kg), which did not reduce mononuclear cell infiltration, also inhibited CAV. These results imply that AS2553627, in addition to inhibiting acute cellular rejection, could potentially prevent CAV by other mechanisms.

The upregulation of IL-6 mRNA expression in allografts treated with vehicle plus tacrolimus compared with isografts was significantly reduced by AS2553627 treatment. IL-6 is a cytokine that mediates JAK1, 2 and TYK2/STAT3 signaling and regulates inflammation, antibody production, and fibrosis [58]. Studies have shown that IL-6 promoted vascular smooth muscle cell motility, and blockade of gp130/STAT3 signaling attenuated smooth muscle cell migration from media to intima, thereby decreasing neointima formation in a rat balloon injury model [59]. Moreover, this cytokine can enhance TGF-β/Smad pathway and accelerate the TGF-β-induced EMT [60]. A STAT3 inhibitor attenuated renal interstitial fibroblast activation and interstitial fibrosis in obstructive nephropathy [61]. Tocilizumab, an anti-IL-6 receptor monoclonal antibody, recently provided good long-term outcomes in transplant patients ransplant glomerulopathy in whom baseline immunosuppression consisted of TAC, MMF, and prednisone [62]. AS2553627 has the potential to prevent IL-6 signaling because this compound inhibits JAK kinases potently and broadly including JAK1/2 and TYK2 [56]. Moreover, tofacitinib has been shown to inhibit IL-6 production via anti-inflammatory effects, as well as IL-6-mediated signaling [63,64]. This evidence is consistent with our data showing that AS2553627 significantly inhibited IL-6 mRNA expression in allografts. Given the wide ranging activity of IL-6 in various signaling pathways, the

efficacy of AS2553627 in chronic rejection progression may be partially mediated by its inhibition of T cell-mediated rejection and control of IL-6 signaling.

A critical function of the kidney is glomerular filtration. When a glomerulus is completely obliterated, the related tubular apparatus is destroyed and the intraglomerular pressure in the remaining functioning glomeruli is increased, causing proteins to leak into the urine [7]. Thus, the suppression of urinary proteins by AS2553627 treatment is considered as the result from its significant inhibition of glomerulosclerosis. DSA plays critical roles in the pathogenesis of antibody-mediated rejection (AMR) such as transplant glomerulopathy, and is associated with impaired allograft survival in renal transplant recipients [65]. In our study, resumption of the optimal dose of tacrolimus alone led to immediate and significant reductions in plasma DSA levels. Consequently, it was difficult to detect the effect of AS2553627 on DSA, although the AS2553627 plus tacrolimus-treated group had reduced DSA levels compared with the vehicle plus tacrolimus-treated group. It is likely that AS2553627 functions through other additional mechanisms to prevent glomerular lesions. AS2553627 may be due to its direct action on kidney function since the JAK-STAT pathway plays a major role in various kidney functions and the development of pathologies such as diabetic kidney disease (DKD). JAK1, 2, and 3 are expressed at higher levels in the glomeruli of patients with DKD [66]. In a mouse diabetic model, enhanced expression of JAK2 selectively in glomerular podocytes increased the pathological features of DKD including albuminuria, mesangial expansion, and glomerulosclerosis. These features were improved by the treatment with a JAK1 and JAK2 inhibitor [67]. Recently, the phase 2 clinical trial demonstrated that baricitinib, a selective JAK1/2 inhibitor decreased albuminuria in participants with Type 2 diabetes

and DKD [68]. Therefore, AS2553627 might suppress urinary protein and glomerulosclerosis by the direct kidney protective effect in addition to inhibition of allograft rejection.

Taken together, we have demonstrated that AS2553627 was effective not only in acute rejection but also in reducing chronic rejection in rodent transplantation models to improve long-term allograft survival, without adverse effects. In the next chapter, we evaluate the effect of AS2553627 renal allograft rejection in monkey.

2.5. Tables and Figures

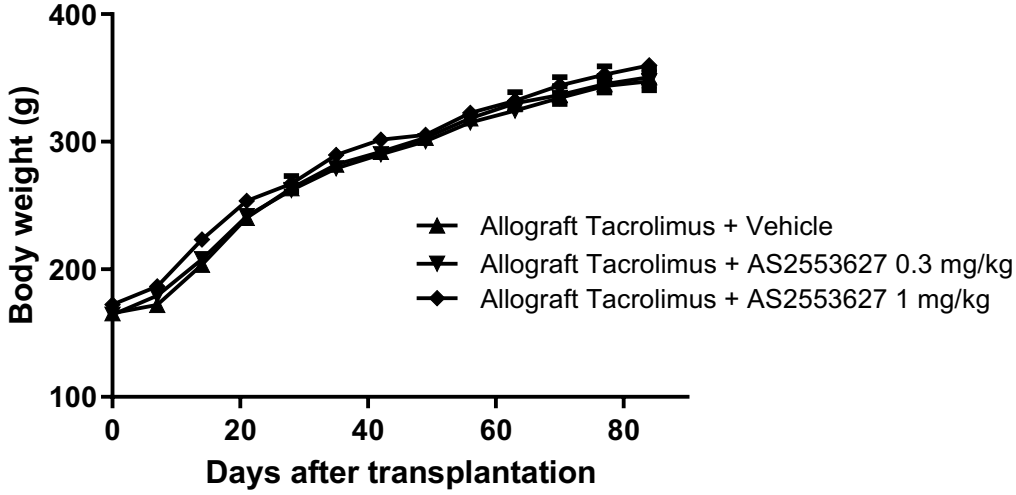


Figure 2-1. Changes in body weight after long-term treatment with AS2553627 in a rat cardiac transplantation model.

Vehicle or AS2553627 (0.3 and 1 mg/kg) in combination with tacrolimus (3.2 mg/kg) was orally administrated for 90 consecutive days from the day of cardiac transplantation. Data represent the mean ± S.E.M. No significant differences were noted using Tukey's multiple comparison test among all time-points and treatment groups.

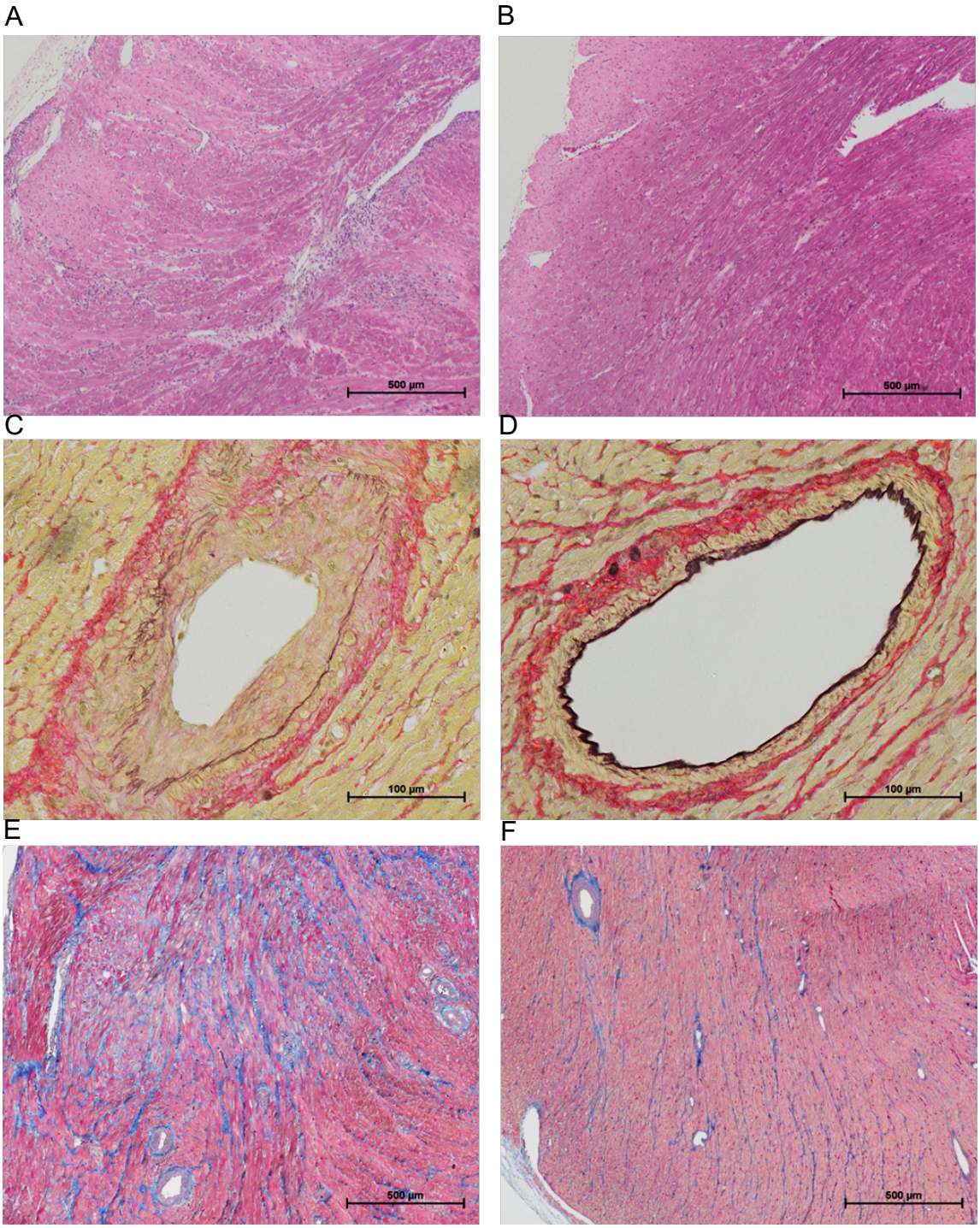


Figure 2-2. Typical microscopic images of heterotopic cardiac grafts after long-term treatment with AS2553627 in a rat cardiac transplantation model.

Heterotopic cardiac transplantation was performed using ACI to ACI (isograft) or ACI to Lewis (allograft) rats. Cardiac grafts were harvested 90 days after transplantation and stained by HE (A and B), EVG (C and D) and Azan (E and F). (A) Vehicle plus tacrolimus-treated allograft group, (B) AS2553627 1 mg/kg plus tacrolimus-treated allograft group, HE-stained. Arrows in (A) indicate infiltration of mononuclear cells. (C) Vehicle plus tacrolimus-treated allograft group. Arrows indicate focal disruption of the internal elastic membrane. (D) AS2553627 0.3 mg/kg plus tacrolimus-treated allograft group, EVG-stained. (E) Vehicle plus tacrolimus-treated allograft group, (F) AS2553627 0.3 mg/kg plus tacrolimus-treated allograft group, Azan-stained.

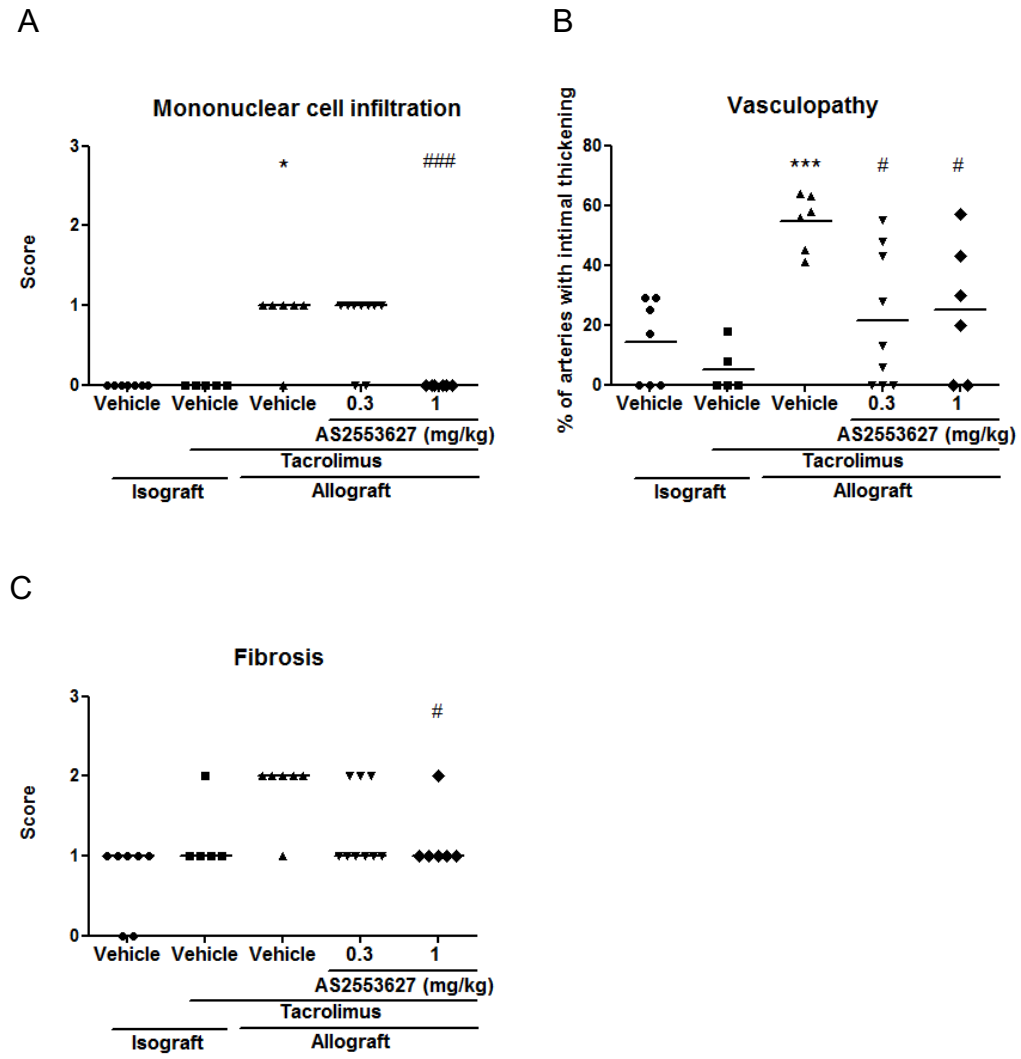


Figure. 2-3. Quantitative assessment of histological changes in heterotopic cardiac grafts after long-term treatment with AS2553627 in a rat cardiac transplantation model.

(A) Mononuclear cell infiltration score was evaluated by HE stain. Data represent the median (n=5-9). *P<0.05 vs. vehicle plus tacrolimus-treated isograft group (Mann-Whitney U-test). ###P<0.001 vs. vehicle plus tacrolimus-treated allograft group (Steel test). (B) Vasculopathy was expressed as the ratio of arteries showing intimal thickening among more than 100 μm in diameter arteries on EVG stain. Data represent the mean (n=5-9). ***P<0.001 vs. vehicle plus tacrolimus-treated isograft group

(Student's t-test). #P<0.05 vs. vehicle plus tacrolimus-treated allograft group (Dunnett's multiple comparison test). (C) Fibrosis score was evaluated using Azan stain. Data represent the median (n=5-9). #P<0.05 vs. vehicle plus tacrolimus-treated allograft group (Steel test).

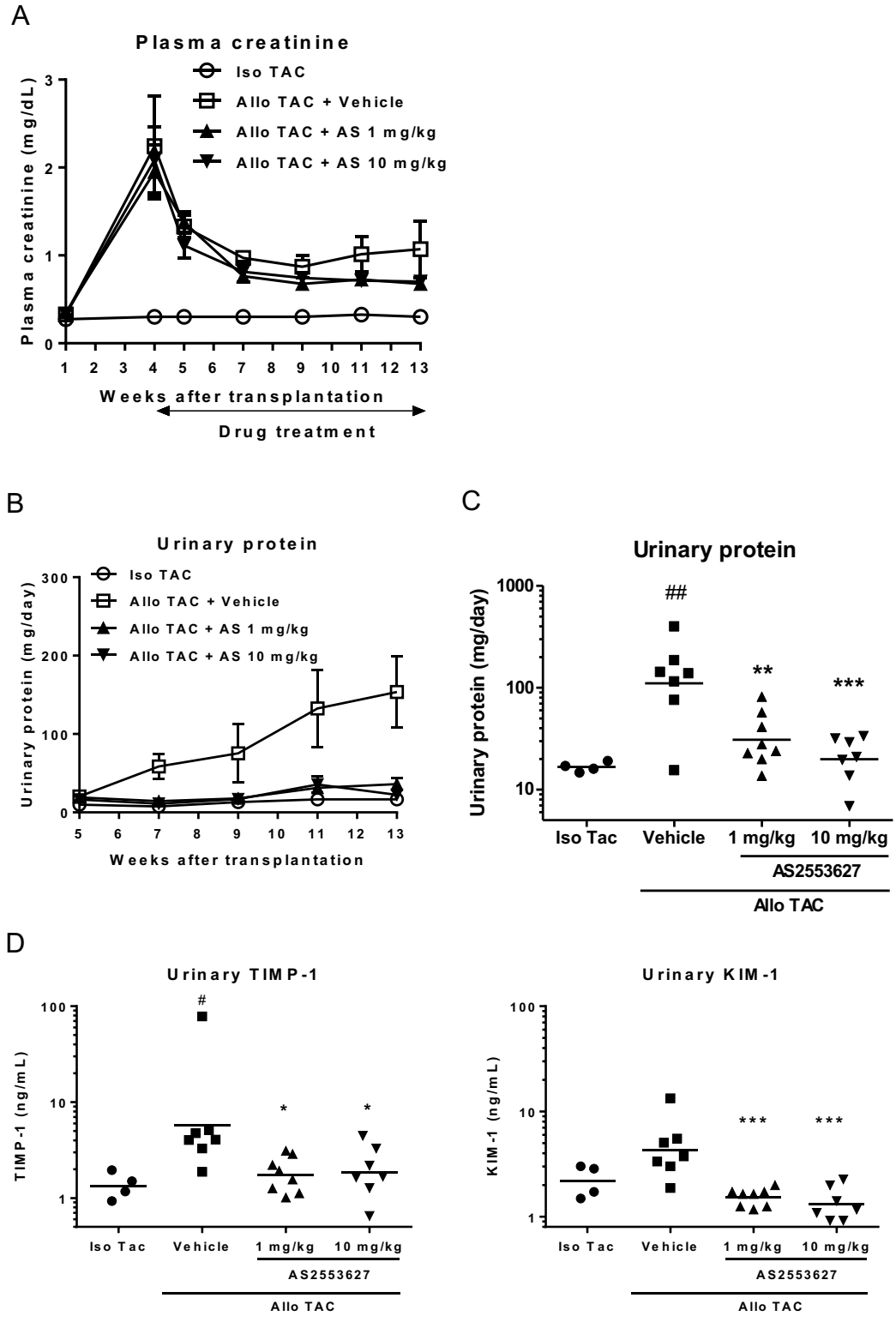
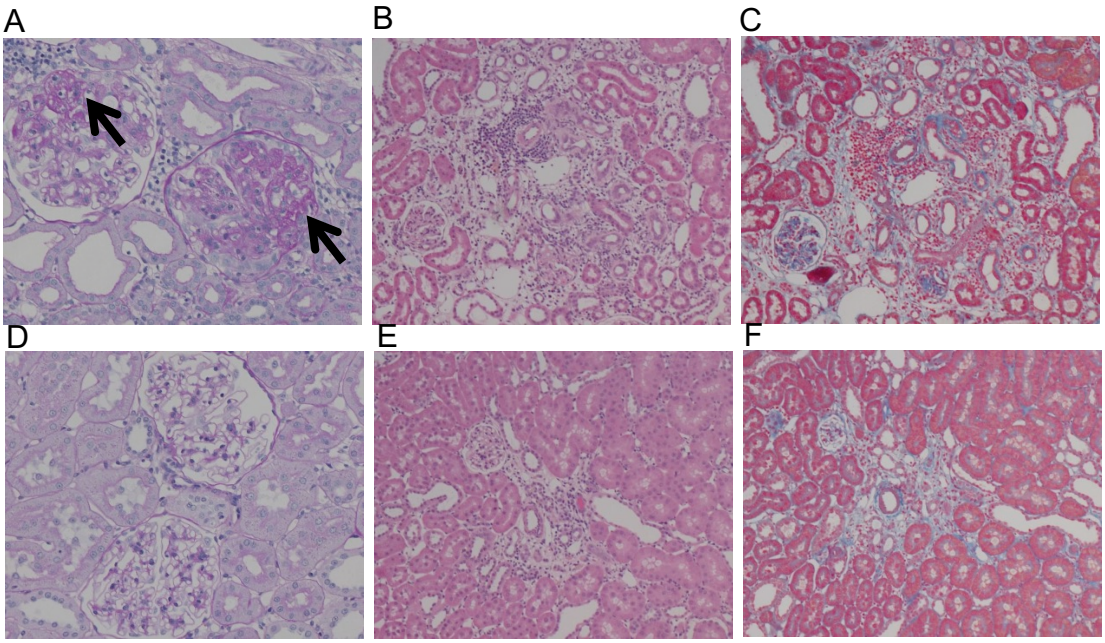


Figure 2-4. Effect of AS2553627 on plasma creatinine, urinary proteins, and urinary biomarkers in a rat renal transplantation model.

Renal transplantation was performed in LEW to LEW (isograft) or LEW to BN (allograft) rats. Tacrolimus (0.1 mg/kg) was intramuscularly administered from days 0 to 9 after renal transplantation. After discontinuation of tacrolimus treatment from days 10 to 28, treatment was resumed in combination with oral administration of AS2553627. (A) Plasma creatinine and (B) urinary protein excretion in 24 hours were measured at each time point. Data represent the mean ± S.E.M (n=4-8). (C) Urinary protein excretion, and (D) urinary TIMP-1 and KIM-1 were measured at 13 weeks after renal transplantation. Data represent the geometric mean (n=4-8). #P<0.05, ##P<0.01 vs. vehicle plus tacrolimus-treated isograft group (Student's t-test). *P<0.05, **P<0.01, ***P<0.001 vs. vehicle plus tacrolimus-treated allograft group (Dunnett's multiple comparison test). Iso = isograft, Allo = allograft, TAC = tacrolimus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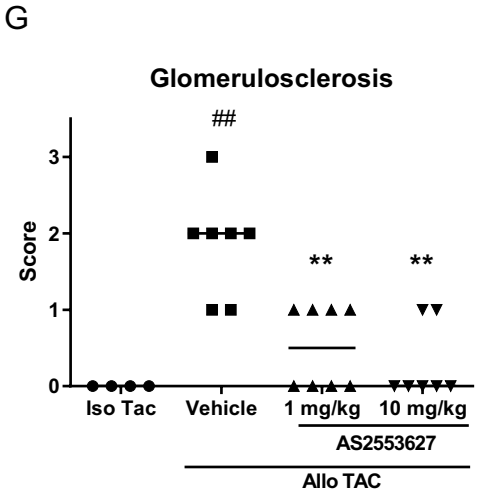
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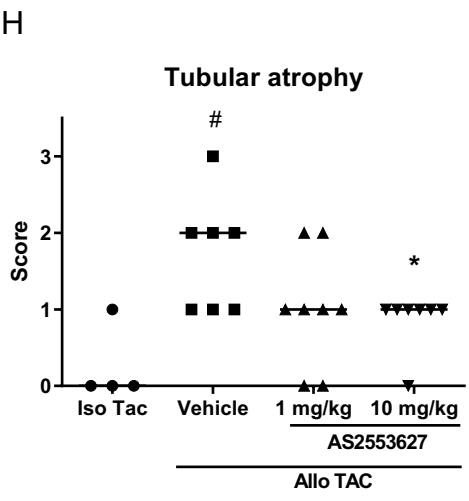
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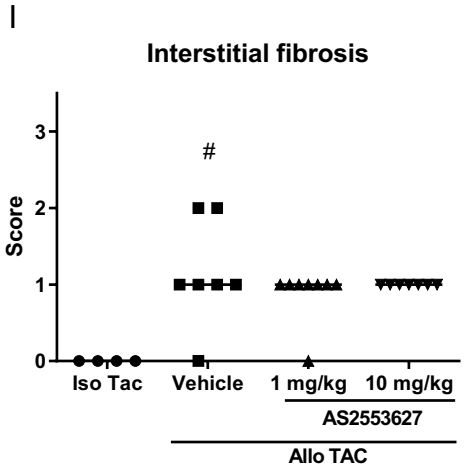
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Figure 2-5. Effect of AS2553627 on renal histopathological changes in a rat renal transplantation model.

Renal grafts were harvested at 13 weeks after transplantation and stained by PAS (A and D), HE (B and E) and Azan (C and F). Representative micrographs from the different groups are shown (A-F). (A-C): Allografts from the vehicle plus tacrolimus -treated group. (D-F): Allografts from the AS2553627 10 mg/kg plus tacrolimus -treated group. (PAS, × 200; HE, × 100; Azan, × 100). Arrows in A indicate glomerulosclerosis. (G) Glomerulosclerosis score was evaluated from PAS-stain. (H) Tubular atrophy score was evaluated from HE-stain. (I) Interstitial fibrosis score was evaluated from Azan stain. Data represent the median (n=4-8). #P<0.05, ##P<0.01 vs. vehicle plus tacrolimus-treated isograft group (Mann-Whitney U-test). *P<0.05, **P<0.01 vs. vehicle plus tacrolimus-treated allograft group (Steel test). Iso = isograft, Allo = allograft, TAC = tacrolimus

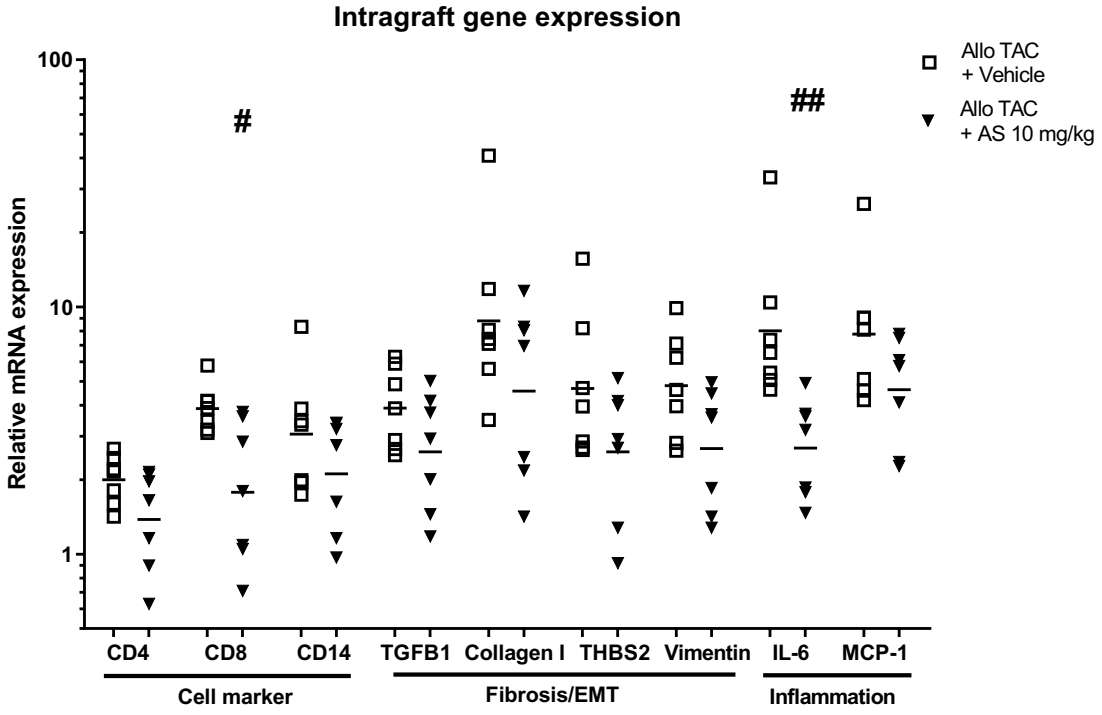

Figure 2-6. Effect of AS2553627 on intragraft mRNA expression in a rat renal transplantation model.

Intragraft mRNA expression of cell surface, fibrosis/epithelial-mesenchymal transition (EMT), and inflammation-related markers were analyzed at 13 weeks after renal transplantation. Data are shown as relative expression compared to isograft group values. Data represent the geometric mean (n=7). #P<0.05, ##P<0.01 vs. vehicle plus tacrolimus-treated allograft group (Student's t-test). Allo = allograft, TAC = tacrolimus.

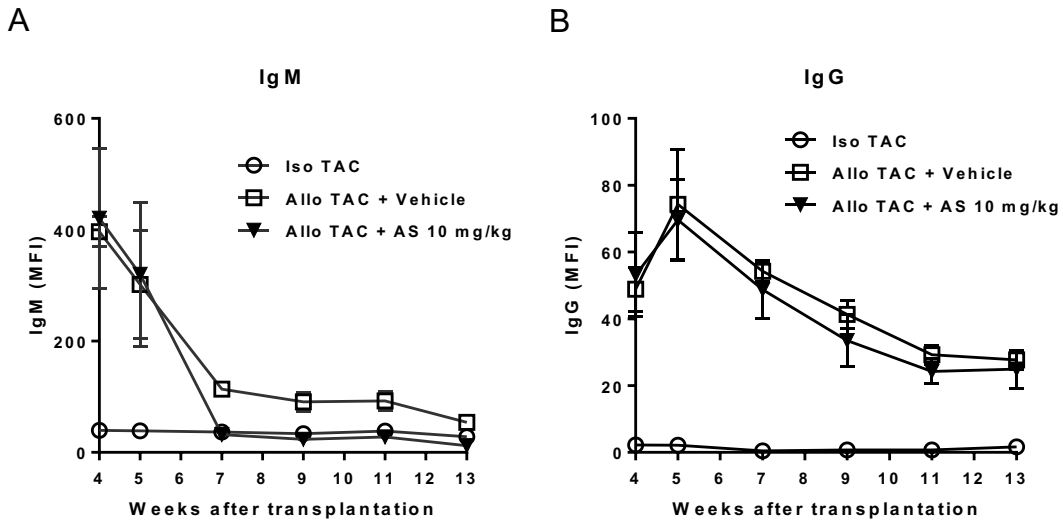

Figure 2-7. Effect of AS2553627 on plasma donor-specific antibody levels in a rat renal transplantation model.

After renal transplantation, plasma samples from recipient animals were assessed for circulating anti-donor IgM (A) and IgG (B) levels by monitoring their binding to splenocytes from donor rats by flow cytometry. Results are expressed as mean fluorescence intensity for IgM and IgG. Data represent the mean ± S.E.M (n=4-8). Iso = isograft, Allo = allograft, TAC = tacrolimus

Chapter 3 Pharmacological evaluation of AS2553627 in a monkey renal transplantation model

3.1. Introduction

In chapter 1 and 2, rat transplantation model studies demonstrated that AS2553627 significantly prolonged graft survival and reduced chronic histopathological changes in allografts. These data suggest the therapeutic potential of AS2553627 to prevent the development of acute and chronic rejection and improve long-term allograft survival after transplantation. Rodent transplantation models are useful for clarifying the effects of drugs on the immune system. However, these models contain major limitations, as they do not fully reflect the human immune system. Organ transplantation in non-human primates (NHPs) has been extensively used to test new immunosuppressants because immune system and physiological features in NHPs closely resemble those in humans [14,69,70]. In this chapter, we investigated the efficacy of AS2553627 in combination with a sub-optimal dose of tacrolimus on allograft rejection in a monkey renal transplantation model.

MMF is a prodrug of MPA (mycophenolic acid), which potently inhibits inosine-5'-monophosphate dehydrogenase (IMPDH), thereby *de novo* purine synthesis. Because lymphocytes proliferation depend on *de novo* synthesis, this compound potently inhibit the function of T cells and B cells [71]. MMF has increasingly replaced azathioprine in organ transplantation and widely been used as a standard immunosuppressive therapy in combination with CNIs. Transplant patients usually receive a 1-2 g daily dose of MMF (0.5-1 g, b.i.d.), where the clinically accepted target $AUC_{0\text{-}12h}$ of MPA is in the range of 30-60 μg • h/ml [72,73]. MMF often causes

gastrointestinal adverse effects. This requires dose reduction or discontinuation in a high proportion of cases, resulting in increased risk of rejection [74-76]. Thus, new immunosuppressive drugs that can replace MMF in combination therapy with low-dose CNIs are needed to improve long-term graft survival. In this chapter, we also elucidated the possibility of replacing MMF with AS2553627 in renal allograft rejection in clinical settings by comparing with the efficacy of a clinically relevant dose of MMF.

3.2. Material & Methods

3.2.1. Animals

Purpose-bred male cynomolgus monkeys (*Macaca fascicularis*) weighing 3-6 kg were obtained from Hamri Co., Ltd. (Ibaraki, Japan). They were all seronegative for simian immunodeficiency virus, *Salmonella* spp., dysentery bacteria, and B virus. All monkeys were housed in individual cages, allowed free access to water, and fed once a day. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Astellas Pharma Inc..

3.2.2. Reagents

Mycophenolic acid (MPA) was purchased from Sigma Aldrich Japan (Tokyo, Japan). Tacrolimus (Prograf® solid dispersion formulation [SDF]), AS2553627 (3-[(3R,4R)-3-(dipyrrolo[2,3-b:2',3'-d]pyridin-1(6H)-yl)-4-methylpiperidin-1-yl]-3-oxo propanenitrile) and mycophenolate mofetil (MMF) were prepared at Astellas Pharma Inc. (Tokyo, Japan).

3.2.3. IL-2-stimulated monkey T cell proliferation assays

PBMCs were isolated from whole blood of normal cynomolgus monkeys using a gradient technique (Ficoll-Paque, GE Healthcare Bio-Sciences AB). After washing, the cells (5×10^5 cells/ml) were suspended in RPMI1640 (Thermo Fisher Scientific Inc, Waltham MA, USA) containing 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin and cultured with phytohemagglutinin (10 μg/ml) for 5 days at 37°C in a 5% CO_2 incubator. After washing, suspended PBMCs (5×10^4 cells/well) were incubated with 3 ng/ml human recombinant IL-2 and AS2553627 at designated concentrations in 96-well tissue culture plates. After incubation for 3 days, a alamarBlue® was added to each of the test wells. After several hours, fluorescence intensity was measured at an excitation wavelength of 545 nm and an emission wavelength of 590 nm.

3.2.4. Mixed lymphocyte reaction (MLR) with monkey PBMCs

PBMCs were isolated from the whole blood of normal cynomolgus monkeys using the gradient technique and were resuspended in RPMI medium containing 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin, and MPA. Responder PBMCs (1.5×10^5/well) were cultured in duplicate with an equal number of irradiated (20 Gy) stimulator PBMCs in 96-well plates for 4 days at 37°C with 5% CO_2. The cells were pulsed with H^3-thymidine (37 kBq/well) 6 hours before the termination of culture, and activity was assessed by H^3-thymidine incorporation using a scintillation counter.

3.2.5. Renal transplantation and drug treatments

Donor and recipient pairs were selected by matching ABO blood type and mismatching according to the results of a one-way MLR assay to elicit a stimulation

index > 2.5 prior to transplant. Each animal was then used as both a donor and recipient, with one kidney from each animal being transplanted to the other in the pair. The method of renal transplantation was the same as reported previously [33,77]. Briefly, the donor renal artery and vein were anastomosed to the recipient distal aorta and vena cava, respectively. The ureter reconstruction was performed with ureter-to-ureter anastomosis using a microsurgical technique with a ureteric stent. The remaining native kidney was removed immediately by nephrectomy or vascular ligation after implantation of the transplanted allograft. Failure of the renal allograft was defined as a plasma creatinine value > 10 mg/dl and rejection was confirmed by histological examination of the transplanted kidney at necropsy. When abrupt reduction of urine was observed along with detecting dilation of the renal pelvis by ultrasound, then replacement of the ureteric stent was surgically performed and any associated temporary elevations in creatinine levels were excluded from the analysis. Study groups were as follows: Group 1, no treatment (n = 4); Group 2, tacrolimus 1 mg/kg/day (n = 7); Group 3, tacrolimus 2 mg/kg/day (n = 7); Group 4, MMF 20 mg/kg/day with tacrolimus 1 mg/kg/day (n = 7); and Group 5, AS2553627 0.24 mg/kg/day with tacrolimus 1 mg/kg/day (n = 4). MMF (20 mg/kg/day) was suspended in 0.5% methylcellulose solution and orally administered once daily. AS2553627 was dissolved in propylene glycol solvent and orally administrated twice daily (0.24 mg/kg/day, 8-/16-h intervals). Tacrolimus (1 mg/kg/day) was suspended in sterile water and orally administered > 2 hours after the administration of MMF or AS2553627. Beginning on the day of surgery, drug administration continued until the time of graft rejection or 90 days after operation. Each animal was euthanized when it became moribund, its plasma creatinine elevated to > 10 mg/dl, or it was 90 days after transplantation, according to the study design.

3.2.6. Histopathological analysis of renal graft rejection

The donor kidney with at least 5 mm of renal artery and vein was removed immediately after euthanasia for histopathological analysis. The kidney sample was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into longitudinal sections, and stained with hematoxylin-eosin. The degree of rejection was evaluated according to the Banff 97 classification [78] with Banff 09 modifications [79].

3.2.7. Determination of drug concentration in plasma

Plasma samples were collected from animals in stable condition with surviving grafts at 2, 4, 8, 9 and 12 weeks after renal transplantation to measure plasma MPA levels. $AUC_{0\text{-}24h}$ at each week was calculated from plasma MPA levels at 0, 0.5, 1, 2, 4, 6, 8 and 24 hours after MMF administration using HPLC-Tandem Mass Spectrometry. Finally, $AUC_{0\text{-}24h}$ was expressed as the mean of $AUC_{0\text{-}24h}$ calculated from plasma MPA levels at each time point until graft failure.

3.2.8. Statistical analysis

Analysis for statistical significant difference was done using Graphpad Prism 8 software. The IC_{50} values of the *in vitro* experiments and $AUC_{0\text{-}24h}$ values of plasma MPA levels were expressed as the mean. The IC_{50} values of the *in vitro* experiments were calculated using linear regression analysis. MSTs of the grafts in the transplant study were analyzed by comparing groups using the log-rank test. Histological data are expressed as the median. Significant differences between two groups were determined using the Mann-Whitney U-test. P values of less than 0.05 were considered statistically significant.

3.3. Results

3.3.1. Inhibitory effects of AS2553627 and MPA on lymphocyte proliferation

In chapter 1, we showed that AS2553627 exhibited an inhibitory effect on IL-2-stimulated human and rat T cell proliferation (IC_{50}; 2.4 and 4.3 nM, respectively). Consistent with those findings, AS2553627 inhibited proliferation of IL-2-stimulated monkey T cells with an IC_{50} value of 7.5 nM. To determine the inhibitory effect of MPA on monkey lymphocyte proliferation, we performed MLR with normal cynomolgus monkey PBMCs. We measured lymphocyte proliferation using H^3-thymidine incorporation. MPA inhibited the proliferation of PBMCs stimulated with an allogeneic stimulator with an IC_{50} of 54 nM, which was approximately equal to the IC_{50} of MPA in human MLR [80]. These results suggest that there was little species difference between cynomolgus monkeys and humans in the *in vitro* inhibitory activity on lymphocyte proliferation of MPA.

3.3.2 Comparison of AS2553627 and MMF on monkey renal allograft rejection in tacrolimus combination therapy

To elucidate the potential of AS2553627 for CNI-sparing, we assessed the efficacy of AS2553627 and MMF along with a low-dose tacrolimus regimen. Our group previously reported that all untreated control animals rejected the renal allograft within 8 days (MST = 6 days), whereas oral treatment with tacrolimus administration prolonged animal survival in a dose-dependent manner, and MST was 11, 21, and >90 days for the 0.5, 1.0, and 2.0 mg/kg tacrolimus groups, respectively (Table 3-2) [81]. Based on these results, we used 1 mg/kg of tacrolimus a sub- optimal dose for combination therapy.

Clinically relevant dose of MMF was determined based on the pharmacokinetic profile of oral treatment with MMF. The target of $AUC_{0\text{-}24h}$ is in the range of 60-120 μg • h/ml,

which was calculated from the clinically authorized target $AUC_{0\text{-}12h}$ (doubled from the target $AUC_{0\text{-}12h}$: 30-60 μg • h/ml) [72]. The pharmacokinetic analysis indicated that 20 mg/kg MMF achieved a mean $AUC_{0\text{-}24h}$ of 85.0 μg • h/ml, which fell into the extrapolated target range of $AUC_{0\text{-}24h}$ 60-120 μg • h/ml (Table 3-1). As shown in Table 3-2, tacrolimus monotherapy at 1 mg/kg/day prolonged graft MST to 21 days, subsequent to which all allografts were rejected by 40 days post-transplantation. The combination of 20 mg/kg of MMF and tacrolimus did not show statistically significant prolongation compared with tacrolimus alone, with MST of 24 days, whereas two of seven monkeys achieving a graft survival time of > 90 days after transplantation with high plasma creatinine levels (Table 3-2, 3-3, and Fig. 3-1A). Oral administration of AS2553627 0.24 mg/kg/day in combination with tacrolimus significantly prolonged renal allograft survival to MST of > 90 days with low plasma creatinine levels (Table 3-2, 3-3, and Fig. 3-1B). In histopathological analysis, all renal allografts in MMF in combination with tacrolimus exhibited acute T cell-mediated rejection (TCMR): 6 of 7 monkeys showed a vasculitis score of ≥ 2 (Table 3-3, Fig. 3-2A and 3-3A) and all monkeys showed interstitial mononuclear infiltration scores ≥ 2 (Table 3-3, Fig. 3-2B and 3-3B). AS2553627 in combination with tacrolimus significantly prevented TCMR: 2 of 4 monkeys had borderline changes and all monkeys had vasculitis and interstitial mononuclear infiltration scores ≤ 2 (Table 3-3, Fig. 3-2D, E and 3-3A, B). Chronic histological changes of IF/TA were noticeable in the two MMF-treated monkeys surviving > 90 days, with severe IF/TA scores of 3 (Table 3-3, Fig. 3-2C and 3-3C). In contrast, all three monkeys with a survival time of > 90 days in the AS2553627-treated group exhibited IF/TA scores ≤ 1 (Table 3-3, Fig. 3-2F and 3-3C).

3.4. Discussion

In this chapter, we compared the efficacy of AS2553627 with MMF in combination with a sub-optimal dose of tacrolimus on allograft rejection in a monkey renal transplantation model. Firstly, we evaluated the pharmacokinetic profile of MPA for those dose levels in monkeys undergoing renal transplantation. In clinical solid organ transplantation, the clinically accepted target $AUC_{0\text{-}12h}$ of MPA is in the range of 30-60 μg • h/ml [72,73]. Based on this, we targeted the $AUC_{0\text{-}24h}$ of MPA in monkey as 60-120 μg • h/ml and confirmed that 20 mg/kg/day of MMF treatment reached the extrapolated target $AUC_{0\text{-}24h}$ of MPA exposure on average. Two of seven monkeys (ID = No.3 and 4) treated with 20 mg/kg MMF in combination with tacrolimus treatment had prolonged graft survival of > 90 days with $AUC_{0\text{-}24h}$ > 60 μg • h/ml. In monkeys undergoing rapid rejection within 30 days in a combination treatment group, ID = No.2 and 6, MPA exposure was under the $AUC_{0\text{-}24h}$ of 60 μg • h/ml. The clinical evidence indicated that MPA exposure of $AUC_{0\text{-}12h}$ < 30 μg • h/ml ($AUC_{0\text{-}24h}$ of 60 μg • h/ml) identified 79% of patients rejecting within 3 months [82]. Thus, the early rejections can be explained by the lack of MPA exposure and our results indicate that pharmacological effects and pharmacokinetic profiles in NHP reflect clinical settings. Toxicological studies in monkeys showed that oral administration of MMF at 45 mg/kg led to gastrointestinal toxicity such as diarrhea within 28 days and 15 mg/kg was determined as the well-tolerated dose (Roche, data on file). Accordingly, MMF 20 mg/kg/day is also supported from the toxicological point of view. We showed the equivalent *in vitro* inhibitory activity of MPA on lymphocyte proliferation suggesting no species difference between cynomolgus monkeys and humans. Taken together, 20 mg/kg/day of MMF with oral administration was the most clinical relevant dose regimen at a fixed-dose

strategy in cynomolgus monkeys.

AS2553627 also inhibited IL-2-stimulated monkey T cell proliferation. The IC_{50} value of monkey T cell proliferation was comparable to that of rat T cell proliferation shown in chapter 1 (7.5 and 4.3 nM, respectively), indicating no species difference. In chapter 1, we demonstrated that AS2553627 0.25 mg/kg/day with a sub-optimal dose of tacrolimus effectively prolonged allograft survival in a rat cardiac transplantation model. Based on the effective dose in rodent transplantation models, AS2553627 0.24 mg/kg/day was administered as the optimal dose to achieve both adequate efficacy and safety. According to monkey toxicity studies, main toxic finding of this compound was hypocellularity in bone marrows (data not shown). AS2553627 0.24 mg/kg/day showed the significant synergistic effect with a sub-optimal dose of tacrolimus in a monkey renal transplantation model and this dosage did not exert hypocellularity in bone marrows, suggesting that 0.24 mg/kg/day is the well-tolerated dose with the safety margin. The efficacy of AS2553627 with a sub-optimal tacrolimus on allograft rejection was also comparable to that of 2 mg/kg/day of tacrolimus monotherapy, which was considered an optimal dose in the cynomolgus monkey renal transplantation model (MST > 90 days) [81]. In addition, we confirmed that AS2553627 did not have the potential to affect the pharmacokinetics of tacrolimus (data not shown). Accordingly, we demonstrated that AS2553627 had the potential to achieve CNI-sparing which is one of the current issues in clinical settings in not only rodent but also monkey transplantation models.

Gastrointestinal adverse events associated with MMF are frequent, and lead to MMF dose reduction or withdrawal in 40-50% of cases. MMF reduction or discontinuation make short- and long-term outcome worse [74-76]. An enteric-coated form of

mycophenolate sodium (EC-MPS) that delayed the absorption of MPA in the small intestine was developed to reduce gastrointestinal symptoms with MMF despite high MPA exposure. However, high MPA exposure by EC-MPS has been achieved only in limited numbers of transplant patients [83]. Oral administration of AS2553627 in combination with tacrolimus significantly prolonged allograft survival to MST of > 90 days with low plasma creatinine levels, whereas MMF prolonged graft survival with MST 24 days. Histopathological analysis provided that acute T cell-mediated rejection events such as vasculitis and interstitial mononuclear cell infiltration were significantly inhibited by AS2553627 compared with MMF. Furthermore, we compared the efficacy of AS2553627 on IF/TA with MMF in animals with a survival time of > 90 days. As our group reported, short-term graft failure was mainly due to T cell-mediated acute rejection because histopathological analysis showed that vasculitis and interstitial mononuclear infiltration were mainly observed rather than IF/TA [33]. On the other hand, monkeys with long-term graft survival showed IF/TA which were also observed in renal transplant patients as shown in this study. The AS2553627-treated group exhibited less IF/TA compared with the MMF-treated group. These results indicate that AS2553627 combined with CNIs can exert superior effects compared to MMF, suggesting the possibility of replacing MMF with a JAK inhibitor with the low-dose CNIs regimen.

In conclusion, we demonstrated the superior efficacy of AS2553627 to the clinical relevant dose of MMF on preventing allograft rejection by graft survival times, plasma creatinine levels, and histological analysis in combination treatment with a sub-optimal dose of tacrolimus. Taken together, we revealed that AS2553627 can be an attractive candidate to prevent renal allograft rejection in CNI-sparing strategies with MMF replacement.

3.5. Tables and Figures

Table 3-1. Pharmacokinetics profile of mycophenolic acid (MPA) in cynomolgus monkeys transplanted with renal allografts in tacrolimus combination therapy.

Treatment (mg/kg/day p.o.)	Animal ID	Graft survival time (days)	$AUC_{0\text{-}24h}$[a] (μg • h/ml)	$AUC_{0\text{-}24h}$ (μg • h/ml) Mean
MMF 20	No.2	21	38.0	85.0
+ Tacrolimus 1	No.3	> 90	143.8	
	No.4	> 90	79.9	
	No.6	11	37.6	
	No.7	46	125.5	

[a] $AUC_{0\text{-}24h}$ shows the mean of $AUC_{0\text{-}24h}$ calculated from plasma MPA levels at 2, 4, 8, 9 and 12 weeks until graft failure.

AUC = area under the receiver operating characteristics (ROC) curve, MMF = mycophenolate mofetil

Table 3-2. Effects of AS2553627 or mycophenolate mofetil (MMF) on renal allograft survival of cynomolgus monkeys in tacrolimus combination therapy.

Treatment (mg/kg/day p.o.)	N	Graft survival time (days)	MST (days)	P- value[a]
[b]Untreated	4	5, 6, 6, 7	6	-
[b]Tacrolimus 1	7	8, 18, 19, 21, 21, 24, 39	21	-
[b]Tacrolimus 2	7	24, 82, >90, >90, >90, >90	> 90	-
MMF 20 + Tacrolimus 1	7	10, 11, 21, 24, 46, > 90, > 90	24	P = 0.14
AS2553627 0.24 + Tacrolimus 1	4	78, > 90, > 90, > 90	> 90	P < 0.01

[a] P-value calculated by the log-rank test compared to the tacrolimus (1 mg/kg)-treated group.

[b] Data of graft survival on these animals were previously reported by our laboratory [81].

MST = median survival time

Table 3-3. Histopathology of cynomolgus monkeys transplanted with renal allografts.

Treatment (mg/kg/day p.o.)	Animal ID	Graft survival (days)	Plasma creatinine at the endpoint (mg/dl)	Histopathology (Banff classification[a])
MMF 20	No.1	24	-	Acute TCMR IIB (v2,i2), IF/TA I
+ Tacrolimus 1	No.2	21	-	Acute TCMR IIB (v2,i3), IF/TA II
	No.3	> 90	7.4	Acute TCMR IIA (v1,i3), IF/TA III
	No.4	> 90	9.0	Acute TCMR III (v3,i3), IF/TA III
	No.5	10	-	Acute TCMR III (v3,i2), IF/TA I
	No.6	11	-	Acute TCMR III (v3,i2), IF/TA I
	No.7	46	-	Acute TCMR IIB (v2,i2), IF/TA III
AS2553627 0.24	No.1	> 90	1.3	Borderline changes (v0, i1)
+ Tacrolimus 1	No.2	> 90	1.3	Acute TCMR IIA (v1, i1), IF/TA I
	No.3	78	-	Acute TCMR IIB (v2, i2), IF/TA II
	No.4	> 90	1.2	Borderline changes (v0, i1), IF/TA I

[a] The Banff 09 classification of kidney pathology was used as a reference for the degree of T cell mediated rejection (TCMR), and interstitial fibrosis and tubular atrophy (IF/TA).

MMF = mycophenolate mofetil, v = vasculitis, i = interstitial mononuclear cell infiltration

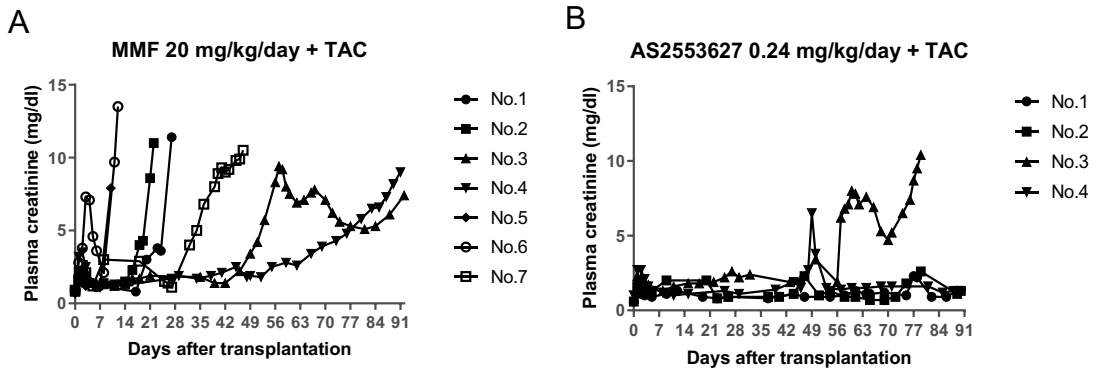

Fig. 3-1 Effects of AS2553627 or mycophenolate mofetil (MMF) on plasma creatinine levels of recipients in tacrolimus (TAC) combination therapy in a monkey renal transplantation model.

After renal transplantation, plasma creatinine concentration was measured at each time point in recipients treated with (A) MMF 20 mg/kg/day plus tacrolimus and (B) AS2553627 0.24 mg/kg/day plus tacrolimus.

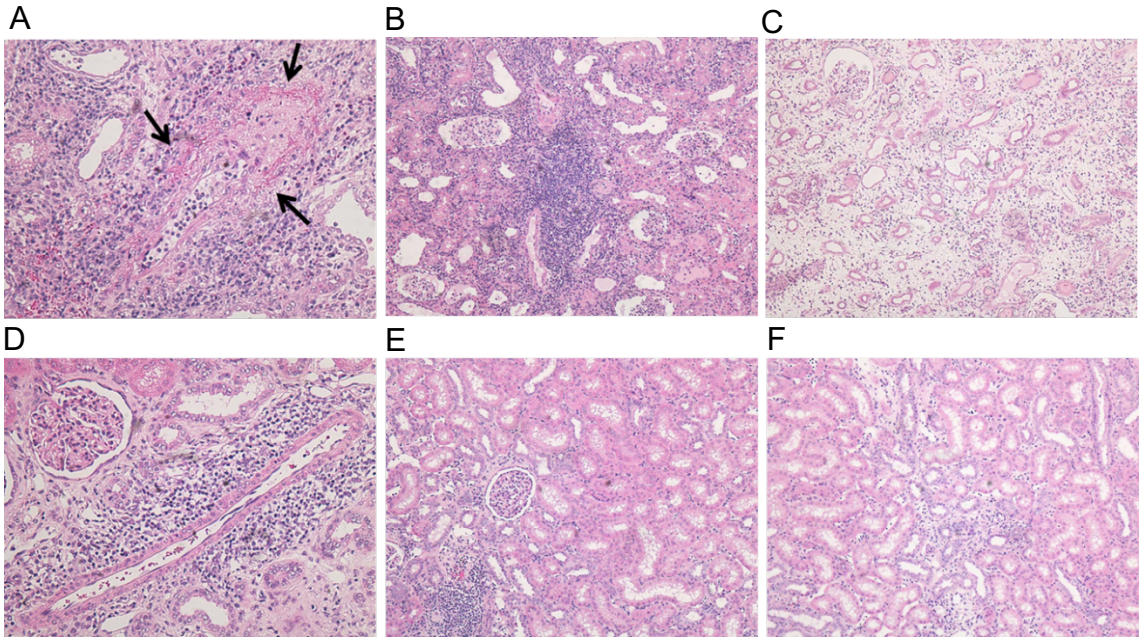

Fig. 3-2 Typical microscopic images of renal allografts after AS2553627 or mycophenolate mofetil (MMF) with tacrolimus in a monkey renal transplantation model.

Renal grafts were harvested at autopsy after transplantation and stained by HE. (A-F) Histology of renal allografts treated with MMF 20 mg/kg/day plus tacrolimus 1 mg/kg/day. Each microscopic image of graft sections shows (A) vasculitis; v3, (B) interstitial mononuclear cell infiltration; i2, and (C) interstitial fibrosis and tubular atrophy (IF/TA) grade III. Arrows in (A) indicate an artery with severe vasculitis. (D-F) Histology of renal allografts treated with AS2553627 0.24 mg/kg/day plus tacrolimus 1 mg/kg/day. Each microscopic image of graft sections shows (D) an artery without vasculitis; v0, (E) interstitial mononuclear cell infiltration; i1, and (F) IF/TA grade I. (A, D): magnification × 100, (B, C, E, F): magnification × 200

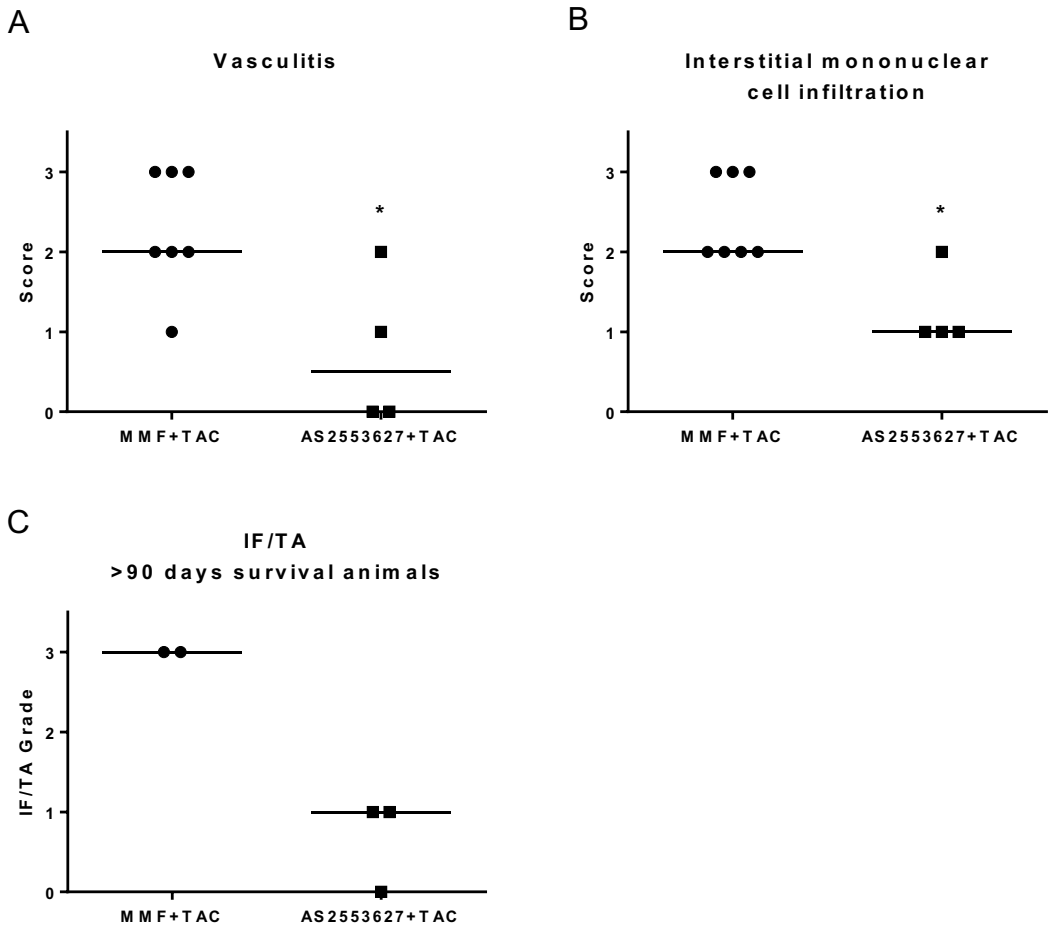

Fig.3-3 Effects of AS2553627 or mycophenolate mofetil (MMF) on histopathology of recipients in tacrolimus (TAC) combination therapy in a monkey renal transplantation model.

(A) Vasculitis, (B) interstitial mononuclear cell infiltration, and (C) interstitial fibrosis and tubular atrophy (IF/TA) score were evaluated from HE-stain, according to the Banff classification. Data represent the median (n = 2-7). *P<0.05, vs. MMF plus tacrolimus-treated allograft group (Mann-Whitney U-test).

Conclusion

We generated AS2553627 as a novel and potent JAK inhibitor. Here, the aims of this investigation are to evaluate its efficacy against not only acute rejection but also chronic rejection in rat and monkey transplantation models and to investigate the potential as a therapeutic agent to achieve CNI-sparing for transplantation.

As described chapter 1, we first compared the *in vitro* pharmacological profiles of AS2553627 with those of the existing JAK inhibitors tofacitinib and peficitinib to reveal the characteristics of AS2553627 as a JAK inhibitor. AS2553627 inhibited JAK kinases activity without inhibitory effects on any other kinases. The IC_{50} values for JAK kinases activity of AS2553627 were lower than those of tofacitinib and peficitinib. AS2553627 potently inhibited rat and human T cell proliferation by IL-2 stimulation compared with tofacitinib and peficitinib. Next we evaluated the preventive effect on acute rejection in a rat cardiac transplant model. Oral administration for 14 days of AS2553627 alone or co-administration with a sub-therapeutic dose of tacrolimus effectively prolonged allograft survival times, suggesting the synergistic effect with tacrolimus on acute rejection.

In chapter 2, the effect of AS2553627 on chronic rejection was investigated in long-term surviving allografts in rat transplantation models. In a cardiac transplantation model, recipients were administered a therapeutic dose of tacrolimus for 90 days. In combination with tacrolimus, AS2553627 significantly reduced allograft vasculopathy and fibrosis that optimal dose of tacrolimus could not inhibit. In a renal transplantation model, we evaluated the effect of AS2553627 on chronic rejection and conducted multilateral analysis to reveal the detailed mechanism of action of AS2553627 on

chronic rejection. AS2553627 in combination with tacrolimus exhibited low plasma creatinine and a marked decrease in urinary protein and kidney injury markers. At 13 weeks after transplantation, AS2553627 also inhibited chronic allograft histopathological changes such as glomerulosclerosis, interstitial fibrosis and tubular atrophy. In addition, upregulation of cell surface markers, fibrosis and inflammation-related genes were reduced by AS2553672, particularly CD8 and IL-6 mRNAs, indicating that AS2553627 prevented cell infiltration and inflammation in renal allografts. These data in rodent transplantation models suggest the therapeutic potential of AS2553627 to prevent the development of acute and chronic rejection and improve long-term allograft survival after transplantation.

In chapter 3, we investigated whether AS2553627 had the potential to achieve CNI-sparing in a monkey renal transplantation model because of the similarities of their immune system and physiological features to their human counterparts. MMF has been used as a standard immunosuppressive therapy as well as tacrolimus, but often causes gastrointestinal adverse effects. To improve long-term graft survival, new immunosuppressive drugs that can replace MMF in combination therapy with low-dose CNIs are needed. Thus, we also investigated the possibility of replacing MMF with AS2553627 by comparing with the efficacy of a clinically relevant dose of MMF. In combination therapy with tacrolimus, pharmacokinetic analysis indicated that MMF 20 mg/kg/day achieved the clinical target exposure and prolonged allograft survival, with MST of 24 days. Oral administration of AS2553627 in combination with tacrolimus significantly prolonged allograft survival to MST of > 90 days with low plasma creatinine levels. Histopathological analysis indicated that acute T cell-mediated rejection events such as vasculitis and interstitial mononuclear cell infiltration were

significantly inhibited by AS2553627 compared with MMF. All AS2553627-treated monkeys surviving > 90 days exhibited less interstitial fibrosis and tubular atrophy than MMF-treated monkeys. These results suggest that AS2553627 replacing MMF is an attractive CNI-sparing strategy to prevent renal allograft rejection. In term of MMF evaluation in NHPs, no reports have discussed the relationship of the dose level of MMF in an animal model to that for clinical use in humans. Our study revealed pharmacological and pharmacokinetic profiles of MMF in cynomolgus monkey renal transplantation. We hope that our findings can contribute to compare new immunosuppressants with MMF in the future.

In the present study, we generated a novel JAK inhibitor, AS2553627, which has potent inhibitory activities on JAK kinases and the suppressive effect on T cell activation compared with existing JAK inhibitors. AS2553627 monotherapy or combination with sub-optimal dose of tacrolimus strongly prevented acute rejection in rat cardiac and monkey renal transplantation models. These results suggest that AS2553627 can achieve CNI-sparing to reduce the CNI-toxicity which is currently a problem in the clinical settings. Furthermore, AS2553627 also inhibited chronic allograft rejection such as vasculopathy, fibrosis, tubular atrophy and glomerulosclerosis which were found in long-term survived animals. Taken together, AS2553627, a novel JAK inhibitor has the therapeutic potential to meet current unmet medical needs in transplantation medicine.

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List of original articles

1) AS2553627, a novel JAK inhibitor, prevents chronic rejection in rat cardiac allografts.

Koji Nakamura, Masamichi Inami, Hiroki Morio, Kenji Okuma, Misato Ito, Takahisa Noto, Shohei Shirakami, Jun Hirose, and Tatsuaki Morokata

European Journal of Pharmacology 796 (2017) 69‑75

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2) Prevention of chronic renal allograft rejection by AS2553627, a novel JAK inhibitor, in a rat transplantation model

Koji Nakamura, Yuka Kawato, Yoko Kaneko, Kaori Hanaoka, Kaori Kubo, Tomonori Nakanishi, Masashi Maeda, Hidehiko Fukahori, Misato Ito, Takahisa Noto, Masamichi Inami, Jun Hirose and Tatsuaki Morokata.

Transplant Immunology 46 (2018) 14‑20

DOI: 10.1016/j.trim.2017.10.001

3) Replacement of mycophenolate mofetil with a JAK inhibitor, AS2553627, in combination with low-dose tacrolimus, for renal allograft rejection in non-human primates

Koji Nakamura, Shinsuke Oshima, Masashi Maeda, Hiroki Morio, Hidehiko Fukahori, Tomonori Nakanishi, Susumu Tsujimoto, Jun Hirose, Takahisa Noto, Nozomu Hamakawa, Masamichi Inami, Tatsuaki Morokata

International Immunopharmacology 64 (2018) 201‑207

DOI: 10.1016/j.intimp.2018.08.029

Supportive article

4) A chronic renal rejection model with a fully MHC-mismatched rat strain combination under immunosuppressive therapy

Kaori Hanaoka, Yuka Kawato, Kaori Kubo, Tomonori Nakanishi, Masashi Maeda, **Koji Nakamura**, Jun Hirose, Takahisa Noto, Hidehiko Fukahori, Akihiko Fujikawa, Sosuke Miyoshi, Shoji Takakura, Tatsuaki Morokata, Yasuyuki Higashi

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