Investigation Regarding the Properties of Protein Kinase Cθ Selective Inhibitor as a Novel T cell Immunosuppressant

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Investigation Regarding the Properties of Protein Kinase Cθ Selective Inhibitor as a Novel T cell Immunosuppressant

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Abbreviations

AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
CNIs	calcineurin inhibitors
Con A	concanavalin A
DAG	diacylglycerol
DMARD	disease-modifying anti-rheumatic drug
IC_{50}	the half maximal inhibitory concentration
IL	interleukin
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MST	median survival time
MTD	maximum tolerated dose
NHP	non-human primate
PBMCs	peripheral blood mononuclear cells
PKC	protein kinase C
RA	rheumatoid arthritis
SDF	solid dispersion formulation
S.E.M	standard error of the mean

TCMR	T cell-mediated rejection
TCR	T cell receptor
TNF	tumor necrosis factor

Chapter 1. Preface

The most of life science industry's activities are driven by R&D in the pharmaceutical sector, which accounts for about 85% of the industry's total R&D spending [1]. In such situation, pharmaceutical companies have created many revolutionary drugs by using application of life science to provide drastic change of social life. In addition, drugs sometimes contribute to advances of life science. In the case of tacrolimus, one of the most effective immunosuppressants, the detail mechanism of immunosuppression was not revealed at the time of discovery. Schreiber et al. elucidated that mechanism [2], and in the process, novel and important T cell activation signaling through calcineurin was found. These things show that drugs have deep relationships with life sciences, and pharmaceutical industry is the major piece on the global bio industry.

The immune system is the host defense system against pathogenic microorganisms, such as bacteria or viruses. The mammalian immune system consists of two components, called innate and adaptive immune system. Innate immunity is based on the pattern recognition, which detects molecular structures that are unique to microorganisms [3]. In contrast, the property of adaptive immunity is rigid antigen selectivity mediated by two types of antigen receptor, T-cell receptor and B-cell receptor [4]. The numerous diversities of antigen receptors are constructed by several mechanisms. Those diversities lead to recognize almost any antigenic determinant in a specific manner [5], and effective self-protection is achieved. T cells are central player in adaptive immune system, because T cells not only can secrete a number of cytokines or chemokines to

activate the other immune cells but also can directly attack pathogens or cancer cells with antigen specificity. Indeed, in the case of acquired immune deficiency syndrome (AIDS), the causative factor of the syndrome HIV infects and destroys the T cells, causing deficiency in the immunological surveillance and ultimately leading to AIDS [6]. The importance of T cell is clear in adaptive immunity, however abnormal T cell activation can result in an autoimmune response against self-antigens inducing several autoimmune diseases. Further, T cells also play a central role in allograft rejection on organ transplantation [7, 8]. In these cases, activation of T cells should be suppressed appropriately, thus T cells are an attractive drug target for the treatment of autoimmune diseases or prevention of allograft rejection. For example, the calcineurin inhibitors (CNIs) tacrolimus and cyclosporine A show potent inhibition of T cell activation and have been used to treat several severe autoimmune diseases and to prevent graft rejection in clinical settings. However, because these currently available agents exhibit adverse effects such as nephrotoxicity [9], I have focused to develop alternative therapeutic options that specifically inhibit T cell activation without adverse effects on other cell types and organs.

Protein kinase C (PKC) is a family of multi-modular ~80 kDa serine/threonine kinases that regulate cell growth, differentiation, apoptosis, and motility. All members of the PKC family consist of the N-terminal regulatory and C-terminal catalytic domains, connected by a proteolytically sensitive hinge region [10]. PKC consists of conventional PKCs (α, β, and γ), novel PKCs (δ, θ, η, and ε) and atypical PKCs (ζ and λ). Regarding activation, conventional PKCs are diacylglycerol (DAG)- and Ca^{2+}-dependent, novel PKCs are DAG-dependent but Ca^{2+}-independent, and atypical PKCs are DAG- and Ca^{2+}-independent [11-13]. Of

the PKC isoforms, PKCθ exhibits a selective pattern of expression in T cells, platelets and skeletal muscle [14, 15]. Further, PKCθ is required for signal transduction *via* the three major transcription factors NF-κB, AP-1, and NF-AT, particularly in CD3/CD28-induced T cell activation [16-21]. Although the proliferation and the cytokine production of murine PKC$\theta^{-/-}$ T cells is significantly impaired [17], PKCθ is not essential for antiviral immune responses *in vivo* [22]. Sotrastaurin (also known as AEB071) is a first-in-class, orally active and selective pan PKC inhibitor. This compound strongly inhibits PKCθ [23] and prevents allograft rejection in both rat and non-human primate (NHP) models of transplantation [24-26]. The immunosuppressive effects of sotrastaurin have also been confirmed in clinical trials for the treatment of psoriasis [27] and prevention of renal allograft rejection [28]. However, a near-linear relationship was noted between the dose of sotrastaurin and discontinuation of medication in a previous study, with the most frequent adverse effects being gastrointestinal disorders, particularly diarrhea [28]. Sotrastaurin has also been reported to inhibit not only PKCθ, but also PKCα, -β*1*, -δ, -η, and -ε [23]. Similarly, the PKC inhibitor enzastaurin inhibited PKCα, -β, -γ, -δ, -θ, and -ε [29] and also induced gastrointestinal disorders in clinical studies [30, 31]. Non-specific PKC inhibition might therefore cause adverse gastrointestinal effects. Considering the selective expression profile of PKCθ, the selective inhibition of this isoform might achieve a suitable balance between immunosuppression and the minimization of other systemic side effects.

AS2521780 was a novel PKCθ inhibitor synthesized by Astellas Pharma Inc., through chemical optimization investigation based on the structure-activity relationship approach [32]. Although it was revealed that knockout of the PKCθ

isoform in mice resulting in defective T cell activation, the outcome of selective inhibition of PKCθ by small molecule compounds on T cells has not been clarified yet. Gene knockout mice are effective for characterizing the physiological functions of target gene, but they might also express phenotypes due to unknown compensatory responses or loss of scaffold functions of these proteins. Further, species differences of target function cannot be assessed by only gene knockout mice approach. Pharmacological approaches using isoform-selective inhibitors might therefore clarify the precise functions of molecules across the species and their value as drug targets. Taken together, the aims of this investigation are to elucidate i) kinase inhibition profile of AS2521780, ii) importance of PKCθ in T cell activation signaling across species, and iii) whether PKCθ is an attractive drug target for T cell inhibition and for treating T cell-mediated autoimmune diseases or prevention of organ rejection through animal model evaluation (Fig. 1-1).

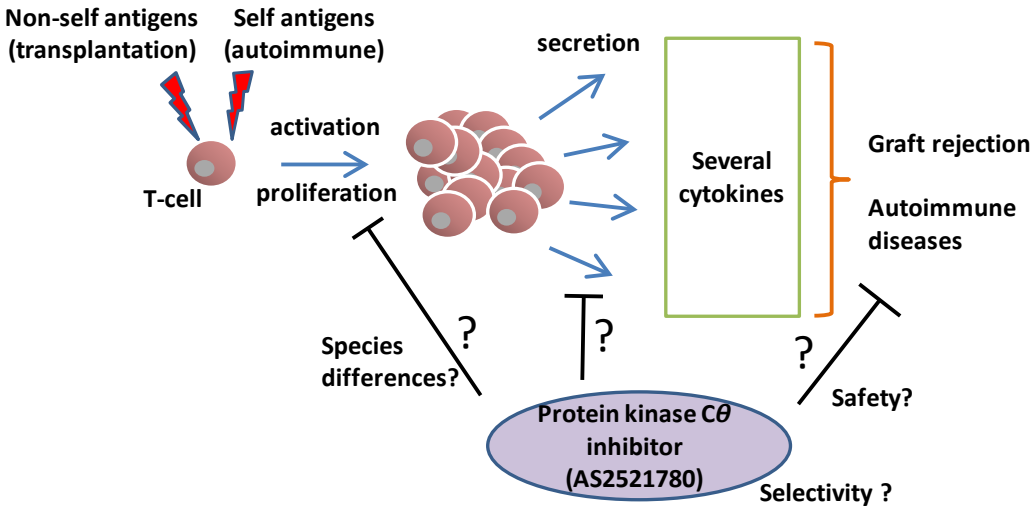

Fig. 1-1. The aims of investigation on this dissertation.

Chapter 2. Investigation regarding pharmacological effects of PKCθ inhibitor AS2521780 on T cell activation

2.1. Introduction

Protein kinases are enzymes which catalyze phosphorylation of hydroxyl group on serine/threonine or tyrosine residues in target proteins using adenosine triphosphate (ATP), and large number (approx. 500) of protein kinases are corded in human genome [33]. Since protein phosphorylation plays a prominent role in many signal transductions, protein kinases have been considered as an important drug targets, and a number of kinase inhibitors have been developed and launched especially as anti-cancer drug. Kinases have a homologous kinase domain (catalytic domain) in each structure [34], and ATP is used as common substrate , thus a kinase inhibitor (mainly ATP-competitive inhibitor) tends to inhibit multiple kinases. Such broad kinase inhibition spectrum is sometimes beneficial in cancer therapy. For example, regorafenib, a drug for metastatic colorectal cancer, inhibits VEGFR, PDGFR-β, KIT, RET, RAF-1, and B-RAF showing good efficacy clinically [35, 36]. However, of course broad spectrum causes unexpected adverse effects. Indeed, non-specific PKC inhibitor sotrastaurin and enzastaurin induced adverse gastrointestinal effects in clinical trials as mentioned previous chapter [26, 28, 29], and clinical development of sotrastaurin was terminated. Since T cell activation was impaired in PKC$\theta^{-/-}$ mice and the expression profile of PKCθ was selective, I hypothesized that the selective inhibition of PKCθ was enough to exert immunosuppressive effect and beneficial

to minimize systemic side effect. It was therefore very important to clarify the inhibition specificity of AS2521780 in this investigation.

In current study, I have focused to develop alternative therapeutic options that specifically inhibit T cell activation using a kinase inhibitor. In this regard, since T cells have multiple activation signaling and have multiple functions, it is important to check whether which signaling or which function is inhibited by the compound. This profiling process is a big help to consider the indication of the compound. Further, significant differences in the composition of the immune system in other species is known [37], and precise investigation regarding species differences can be conducted only by pharmacological approach. Thus, confirmation of species differences is the important evaluation item in this study. Moreover, oral bioavailability is the other important aspect on drug development. Poor metabolic instability or less membrane permeability would cause low bioavailability even if the compound has excellent *in vitro* effectiveness.

In this chapter, I investigated enzyme inhibition and pharmacological effects on T cell activation of AS2521780 through *in vitro* and *in vivo* studies, and described profile of AS2521780 and the importance of PKCθ in T cell activation signaling.

2.2. Materials and Methods

2.2.1. Animals

Male Lewis rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and housed in a specific pathogen-free room with free access to food and water. Male cynomolgus monkeys, free of simian immunodeficiency virus, simian retrovirus, salmonella bacteria, dysentery bacteria and B virus, were

purchased from Hamri Co., Ltd. (Ibaraki, Japan) and housed in individual cages. They were allowed free access to water and given food once a day. All animals were allowed at least 1 week to adapt to their environment before use.

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. The Tsukuba Research Center of Astellas Pharma Inc. is accredited by AAALAC International.

2.2.2. Test compound

AS2521780 (4-({[(1R,3R,4s,5S)-4-{[(*trans*-4-hydroxycyclohexyl)methyl] amino}adamantan-1-yl]methyl}amino)-2-({[2-(methylsulfanyl)pyridin-3-yl] methyl}amino)pyrimidine-5-carbonitrile) was synthesized at Astellas Pharma Inc. (Tsukuba, Japan). AS2521780 was dissolved in dimethyl sulfoxide for *in vitro* experiments and in propylene glycol for *in vivo* experiment.

2.2.3. Protein kinase assays

Recombinant human PKC isoforms (*α*, *β1*, *γ*, *δ*, *ε*, *θ*, *η*, and *ζ*) were purchased from Carna Biosciences Inc. (Hyogo, Japan). Inhibition of PKC isoforms was measured using a HTRF KinEASE STK S1 kit (Cisbio Bioassays, Codolet, France) according to manufacturer's instructions. Briefly, each kinase was incubated in the presence or absence of multiple concentrations of AS2521780 (final; 0.03, 0.1, 0.3, 1, 3, 10, and 30 nM for PKC*θ*, 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 nM for others) and the STK substrate-biotin (final 250 nM). ATP was added to start the reaction. ATP concentration for each isoform was as follows: *α*, 10 μM; *β1*, 30 μM; *γ*, 3 μM; *δ*, 60 μM; *ε*, 10 μM; *θ*, 30 μM; *η*, 30 μM; and *ζ*, 10 μM. After 30-60 min incubation at room temperature, a

monoclonal phosphor-specific antibody labeled with Eu^{3+}-Cryptate and streptavidin-XL665 conjugates were added, and then TR-FRET signal which was proportional to the phosphorylation level of substrate was measured using HTRF plate reader (Artemis, Furuno, Hyogo, Japan).

Inhibition of 14 human tyrosine (ABL, BTK, CSK, EGFR, EphA1, FAK, HER2, IGF1R, ITK, SYK, TEC, TIE2, TRKA, and ZAP70) and 13 serine/threonine (AKT1, AurC, BMPR1A, CaMK2a, CDK2, CHK1, CK1d, GSK3b, JNK3, MLK1, p70S6K, RAF1, and ROCK1) kinases by 1 μM of AS2521780 were tested using a Mobility Shift Assay or ELISA, which were conducted at Carna Biosciences.

2.2.4. Reporter gene assay

Human Jurkat E6.1 T cells (ATCC TIB-152) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Biowest, Nuaillé, France) and 1% (v/v) penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Jurkat cells were transiently transfected with pGL3-Basic (Promega, Madison, WI, USA)-derived vector containing 445 bp of human IL-2 promoter region and seeded at 2 x 10^5 cells per well in 96-well plates. Multiple concentrations of AS2521780 (final 1, 3, 10, 30, 100, 300, and 1000 nM), anti-human CD3 (1 μg/mL, HIT3a; BD Biosciences Pharmingen, San Jose, CA, USA) and anti-human CD28 (1 μg/mL, CD28.2; BD Biosciences Pharmingen) were added to cells 16 h post-transfection and incubated for 8 h at 37°C in a CO_2 incubator. Luciferase activities indicating IL-2 transcription levels were detected using a Bright-Glo Luciferase Assay System (Promega).

2.2.5. T cell proliferation assay

Human peripheral T cells were purchased from AllCells LLC (Alameda, CA, USA). Cells (1 x 10^5) suspended in RPMI 1640 containing 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin were seeded in 96-well flat-bottomed plates pre-coated with anti-human CD3 (5 μg/mL, UCHT1; BD Biosciences Pharmingen). Multiple concentrations of AS2521780 (final 1, 3, 10, 30, 100, and 300 nM) and anti-human CD28 (0.5 μg/mL) were added to the wells. After 48 h incubation, cell proliferation was detected using intracellular ATP dependent CellTiter-Glo Luminescent Cell Viability Assay (Promega).

2.2.6. *In vitro* cytokine production assay

Splenocytes from rats and peripheral blood mononuclear cells (PBMCs) from cynomolgus monkeys were used as a source of IL-2. For optimal IL-2 production, rat splenocytes (2 x 10^5) were incubated with 3 μg/mL of concanavalin A (Con A; Funakoshi Corporation, Tokyo, Japan) in RPMI 1640 containing 10% fetal bovine serum, 50 μM β-mercaptoethanol and 1% (v/v) penicillin-streptomycin for 24 h and NHP PBMCs (2 x 10^5) were incubated with 25 μg/mL of Con A in RPMI 1640 containing 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin for 48 h. To assess the inhibitory effect, multiple concentrations of AS2521780 (final 0.3, 1, 3, 10, 30, 100, 300, and 1000 nM) were added to cells concurrently with Con A. The concentration of IL-2 in culture medium was determined using an ELISA kit (for rat splenocytes, R&D Systems, Minneapolis, MN, USA; and for NHP PMBCs, Bio Vender, Karásek, Czech Republic) according to manufacturer's instructions. Brief methods were as follows. Medium samples or standards were added in 96-well flat-bottomed plates

pre-coated with capture antibody. After 2 h incubation at room temperature, plates were washed, and detection antibody was added in each well. After 2 h (for rat) or 1 h (for NHP) incubation, plates were washed again, and streptavidin-HRP was added. Finally, TMB ELISA substrate was added, and then optical density was measured.

Splenocytes from rats were used as a source of IFN-γ, tumor necrosis factor (TNF)-α, and IL-17A. Rat splenocytes (2×10^5) were incubated with 5 μg/mL of Con A in RPMI 1640 containing 10% fetal bovine serum, 50 μM β-mercaptoethanol and 1% (v/v) penicillin-streptomycin for 48 h. Multiple concentrations of AS2521780 (final 0.3, 1, 3, 10, 30, 100, 300, and 1000 nM) were concurrently added to cells with Con A to assess its inhibition of cytokine production. Cytokine concentrations in culture medium were determined using an ELISA kit (eBioscience, San Diego, CA, USA for IL-17A and Invitrogen for others) according to manufacturer's instructions. Brief methods were as follows. Medium samples or standards were added in 96-well flat-bottomed plates pre-coated with capture antibody. After 2 h at 37°C (for IFN-γ) or 2 h at room temperature (for TNF-α and IL-17A) incubation, plates were washed, and detection antibody was added in each well. After 1 h at 37°C (for IFN-γ) or 1 h at room temperature (for TNF-α and IL-17A) incubation, plates were washed again, and streptavidin-HRP was added. Finally, TMB ELISA substrate was added, and then optical density was measured.

2.2.7. *In vivo* IL-2 production assay

Male Lewis rats aged 6 weeks and weighing 190-220 g were used. Interleukin (IL)-2 production was induced by injection of Con A dissolved in

saline at a dose of 15 mg/kg into the tail vein. AS2521780 (3, 10, and 30 mg/kg) or vehicle was orally administered to rats at 1 or 5 h pre-Con A administration. Blood was collected and IL-2 plasma concentration was determined using an ELISA kit (R&D systems) 3 h post-Con A administration (peak IL-2 production) according to manufacturer's instructions as described in 2.2.6.

2.2.8. Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Data were expressed as means ± standard error of the mean (S.E.M). The half maximal inhibitory concentration (IC_{50}) values were calculated using sigmoid-E_{max} non-linear regression analysis. Statistically significant differences were determined using Dunnett's multiple comparison test, and P-values less than 0.05 were considered significant.

2.3. Results

2.3.1. Highly selective inhibition of PKCθ by AS2521780

AS2521780 (Fig. 2-1) significantly inhibited PKCθ enzymatic activity with an IC_{50} value of 0.48 nM (Table 2-1). The inhibition of other conventional (α, $\beta 1$, and γ), novel (δ, ε, and η) and atypical (ζ) PKC isoforms by AS2521780 was also evaluated. The minimum IC_{50} value of the other PKC isoforms was 18 nM for PKCε (Table 2-1), which was approximately 30-fold greater than that of PKCθ, and IC_{50} values of the PKC isoforms except for PKCθ and PKCε were greater than 100 nM. Further, kinase panel assays against 14 tyrosine and 13 serine/threonine kinases (Table 2-2) showed inhibition of only CDK2 enzyme activity. However, this effect was moderate, and the IC_{50} value of 84 nM was

more than 100-fold greater than that on PKCθ. Enzyme activities of the other 26 kinases were not significantly inhibited by 1 μM of AS2521780. These results show that AS2521780 is a potent and highly selective inhibitor of PKCθ.

2.3.2. Inhibition of CD3/CD28-induced IL-2 transcription and cell proliferation by AS2521780 in human cells

To clarify the inhibition of T cell receptor (TCR) signal transduction by AS2521780, CD3/CD28-induced IL-2 expression in human Jurkat cells and cell proliferation in human peripheral T cells were evaluated. Luciferase activity reflecting IL-2 transcription was suppressed by AS2521780 in a concentration-dependent manner with an IC_{50} value of 14.0 nM (Fig. 2-2A). Further, AS2521780 suppressed CD3/CD28-induced *in vitro* proliferation of human T cells with an IC_{50} value of 17.0 nM (Fig. 2-2B). These results show that PKCθ selective inhibitor AS2521780 exerts potent inhibition of both cytokine production and cell proliferation induced by TCR signal activation in human cells.

2.3.3. Inhibition of *in vitro* and *in vivo* cytokine production by AS2521780 in rat and NHP

The T cell mitogen Con A induces cytokine production *in vitro* and *in vivo*. To evaluate the inhibition of IL-2 production *in vitro* by AS2521780, rat splenocytes and NHP PBMCs were stimulated by Con A in the presence of AS2521780. AS2521780 suppressed IL-2 production in a concentration-dependent manner with an IC_{50} value of 8.9 nM for rat splenocytes and 10.5 nM for NHP PMBCs (Fig. 2-3). Further, an *in vivo* IL-2 production assay using Lewis rats was conducted. AS2521780 was orally administered to rats

at doses of 3, 10, and 30 mg/kg at 1 or 5 h pre-Con A injection, and blood samples were collected 3 h post-Con A injection. AS2521780 inhibited Con A-induced IL-2 production in a dose-dependent manner (Fig. 2-4). Although a dose of 3 mg/kg of AS2521780 showed slight inhibitory effect of IL-2 production only for 1 h, a dose of 30 mg/kg of AS2521780 induced near-complete suppression for 5 h. Moreover, the effect of AS2521780 on Con A-induced IFN-γ, TNF-α, and IL-17A from rat splenocytes was evaluated and suppression of these cytokines observed with similar IC_{50} values against IL-2 (Fig. 2-5). IL-6 was not detected in this assay condition (data not shown). These results show that PKCθ selective inhibitor AS2521780 exerts potent inhibition of mitogen-induced production of multiple cytokines.

2.4. Discussion

Previous studies using genetically modified mice demonstrated that both PKCθ and PKCα have physiological functions in TCR activation. For example, CD3/CD28-induced cell proliferation and cytokine production (IL-2 and IFN-γ) was significantly impaired in PKC$\theta^{-/-}$ T cells [17]. Following the transplantation of cardiac allografts, graft survival times in PKC$\theta^{-/-}$ mice were significantly longer than those in wild type mice [38]. In the type II collagen-induced arthritis model, clinical scores and joint pathologies were significantly mitigated in PKC$\theta^{-/-}$ mice compared to wild type mice [39]. Regarding PKCα, CD3/CD28-induced proliferation was impaired in PKCα$^{-/-}$ T cells [40], and simultaneous genetic ablation of PKCα and PKCθ resulted in synergistic impairment of alloimmune responses [41]. Although these gene knockout mice are effective for characterizing the physiological functions of PKC isoforms, they

might also express phenotypes due to unknown compensatory responses or loss of scaffold functions of these proteins. Further, by gene knockout mice, the importance or function of PKC isoforms in T cell signaling can be clarified only in mice. Pharmacological approaches using isoform-selective inhibitors might therefore clarify the precise functions of molecules across the species and their value as drug targets, because function or effect in human should be clarified considering clinical application. In this regard, although sotrastaurin is a first-in-class and orally active selective PKC inhibitor, it strongly inhibits not only PKCθ but also PKCα, -β1, -δ, -η, and -ε. Therefore, sotrastaurin cannot be used to elucidate the specific physiological function of PKCθ in T cell signaling by pharmacological approach.

Here, I examined the inhibitory effect of AS2521780 on PKCθ, 7 other PKC isoforms, 14 tyrosine and 13 serine/threonine kinases (Tables 2-1 and 2-2). These results showed that AS2521780 was a highly selective PKCθ inhibitor. Although AS2521780 exhibited relatively potent inhibition of PKCε, as PKCε does not appear to be involved in T cell activation signaling [42] and its inhibition was approximately 30-fold weaker than that on PKCθ, its impact on T cell activation considered to be negligible. Moreover, in contrast to sotrastaurin, AS2521780 did not exhibit strong inhibition of PKCα, which is potentially engaged in T cell signaling on the basis of gene knockout mice data. Taken together, these findings suggest that AS2521780 can be used to investigate the precise physiological functions of PKCθ in T cell signaling by pharmacological approach.

On the basis of these characteristics, I conducted several *in vitro* experiments and found that AS2521780 completely suppressed CD3/CD28-induced T cell proliferation or IL-2 production in human cells, and Con A-induced production of

IL-2, IFN-γ, TNF-α, and IL-17A (Figs. 2-2, 2-3, and 2-5) in rat and NHP cells. Interestingly, IC_{50} values of these experiments were approximately the same regardless of species and stimulus. These results show that PKCθ has fundamental roles in a broad range of T cell activation signaling, which is consistent with the phenotype of PKC$\theta^{-/-}$ mice. Further, although previous studies using PKC$\alpha^{-/-}$ mice [40, 41] showed that PKCα contributed to T cell signaling in mice, my results demonstrate that selective PKCθ inhibition without an effect on PKCα is sufficient for the complete prevention of T cell activation in human, rat or NHP. This finding is an important achievement in my pharmacology-based investigation. Since the expression of PKCθ is limited compared to that of PKCα [14, 15], selective PKCθ inhibition might be a favorable strategy in achieving a suitable balance between T cell inhibition and the minimization of other systemic effects.

To investigate the oral activity of AS2521780, I also tested an *in vivo* Con A-induced rat model of IL-2 production. AS2521780 significantly reduced plasma IL-2 levels in a dose-dependent manner, with 30 mg/kg of AS2521780 showing near-complete suppression for 5 h (Fig. 2-4). This result indicates that AS2521780 has good oral activity and this compound can be administered per orally for evaluation of pre-clinical animal models.

As summary of this chapter, I show that AS2521780 is highly selective PKCθ inhibitor, and reveal the importance of PKCθ in T cell activation signaling across several species *via* a pharmacological approach using AS2521780. As a next step, evaluation of AS2521780 in pre-clinical animal models is needed to confirm attractiveness of PKCθ selective inhibition as a drug target for T cell immunosuppression.

2.5. Tables and Figures

Table 2-1. PKC selectivity of AS2521780.

PKCs	IC_{50} (nM)
PKCθ	0.48
PKCα	160
PKCβ1	>840
PKCγ	>1000
PKCδ	160
PKCε	18
PKCη	>1000
PKCζ	>1000

Table 2-2. Inhibition of non-PKC kinases by AS2521780.

Tyrosine kinases	% inhibition by 1 μM of AS2521780	Serine/threonine kinases	% inhibition by 1 μM of AS2521780
ABL	4.4	AKT1	0.9
BTK	−15.4	AurC	4.0
CSK	−1.5	BMPR1A	−4.4
EGFR	−2.5	CaMK2a	0.7
EphA1	1.6	CDK2	92.8[a]
FAK	−0.5	CHK1	2.2
HER2	29.0	CK1d	4.7
IGF1R	1.3	GSK3b	6.4
ITK	−0.6	JNK3	−0.5
SYK	4.6	MLK1	5.6
TEC	−0.3	p70S6K	16.1
TIE2	4.3	RAF1	−15.1
TRKA	6.5	ROCK1	3.2
ZAP70	−0.9		

[a] IC_{50} value: 84 nM.

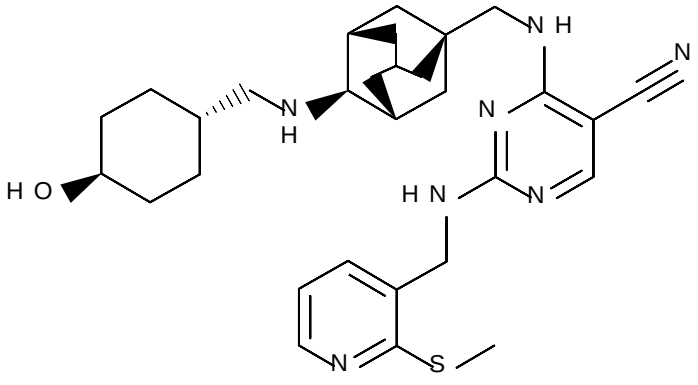

Fig. 2-1. Structure of AS2521780.

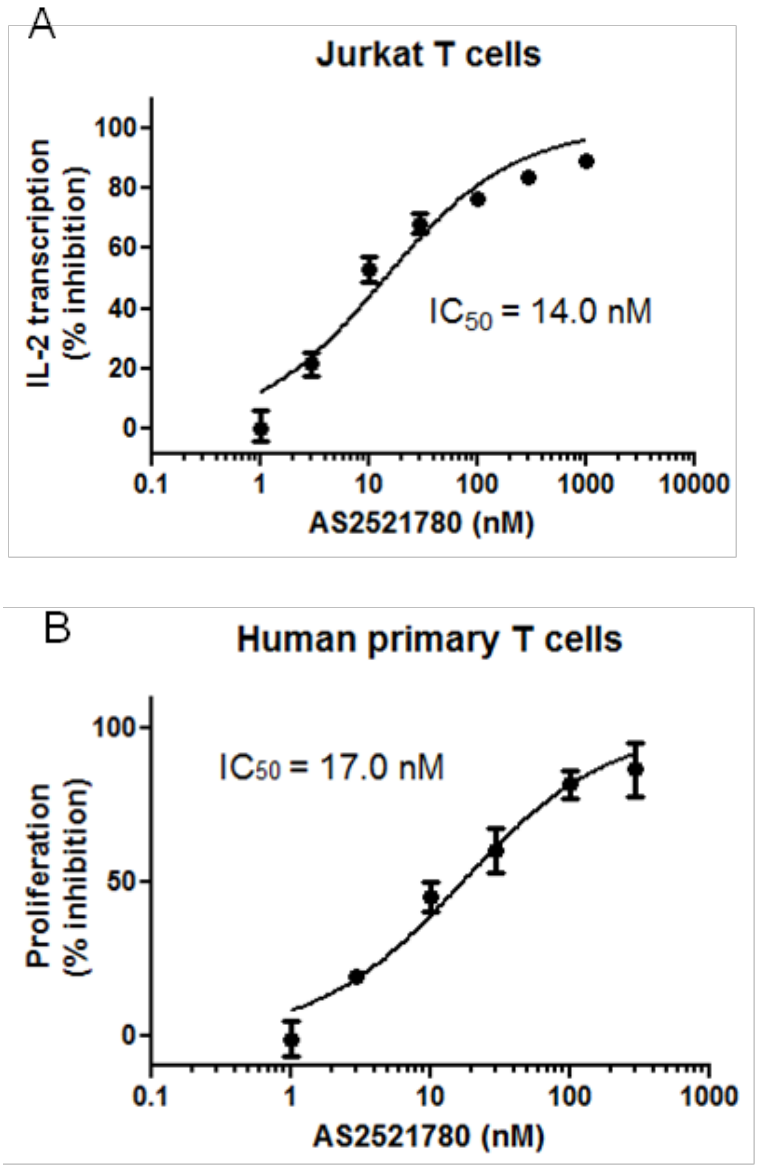

Fig. 2-2. Effect of AS2521780 on CD3/CD28-induced IL-2 transcription and cell proliferation.

Luciferase reporter-transfected Jurkat cells were cultured with AS2521780 and anti-CD3/CD28 antibodies. After 8 h incubation, luciferase activities reflecting IL-2 transcription were measured. Expressed values are mean ± S.E.M of five independent experiments conducted in duplicate (A). Human primary T cells were cultured with AS2521780 and anti-CD3/CD28 antibodies. After 48 h incubation, cell proliferation was detected by a CellTiter-Glo Luminescent Cell Viability Assay. Values are expressed as mean ± S.E.M of three independent experiments conducted in duplicate. (B). IC_{50} values were calculated using sigmoid-E_{max} non-linear regression analysis.

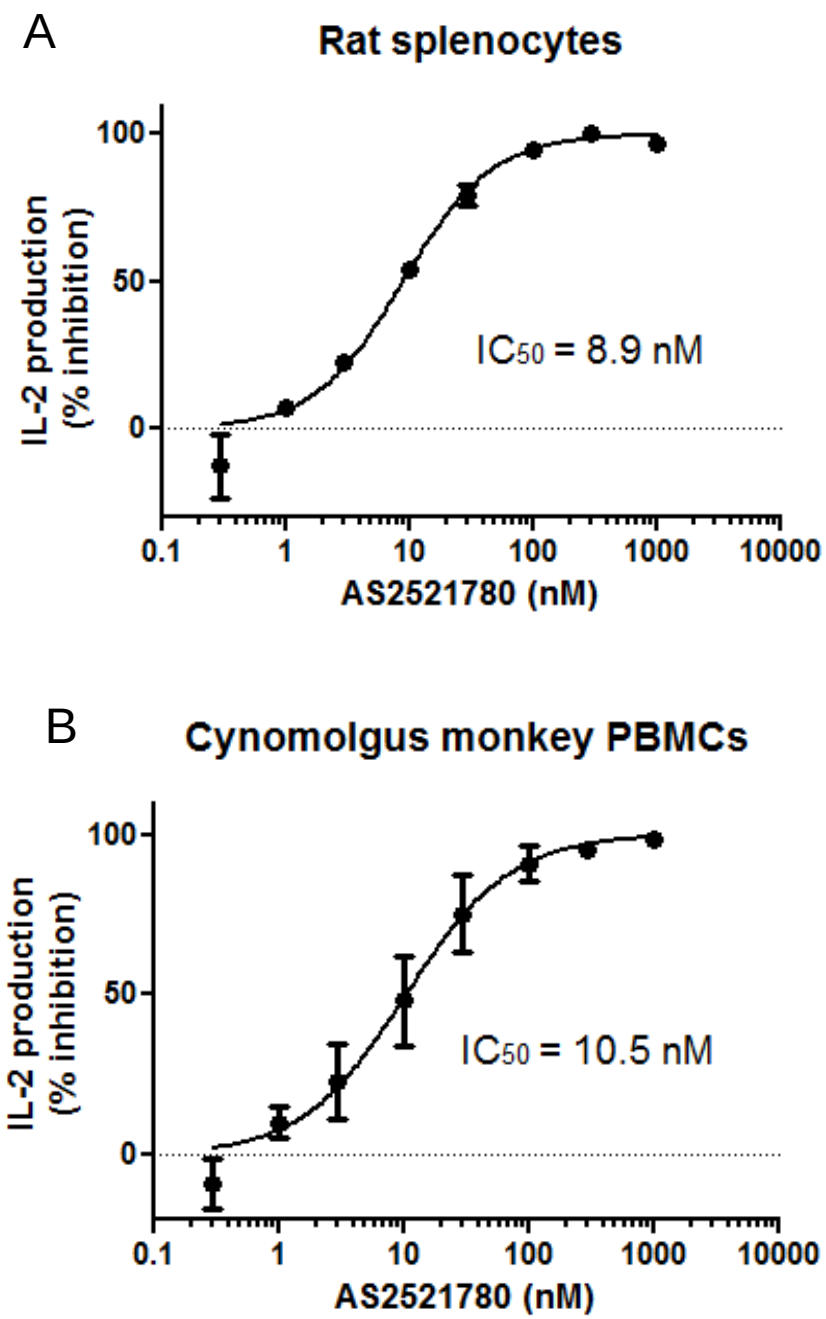

Fig. 2-3. Effect of AS2521780 on *in vitro* Con A-induced IL-2 production.
Rat splenocytes (A) or PBMCs from cynomolgus monkeys (B) cultured with AS2521780 and Con A. After 24 h incubation for rat splenocytes or 48 h incubation for NHP PBMCs, concentration of IL-2 in supernatant measured by ELISA. Values are expressed as mean ± S.E.M of three independent experiments conducted in duplicate. IC_{50} values were calculated using sigmoid-E_{max} non-linear regression analysis. Con A, concanavalin A.

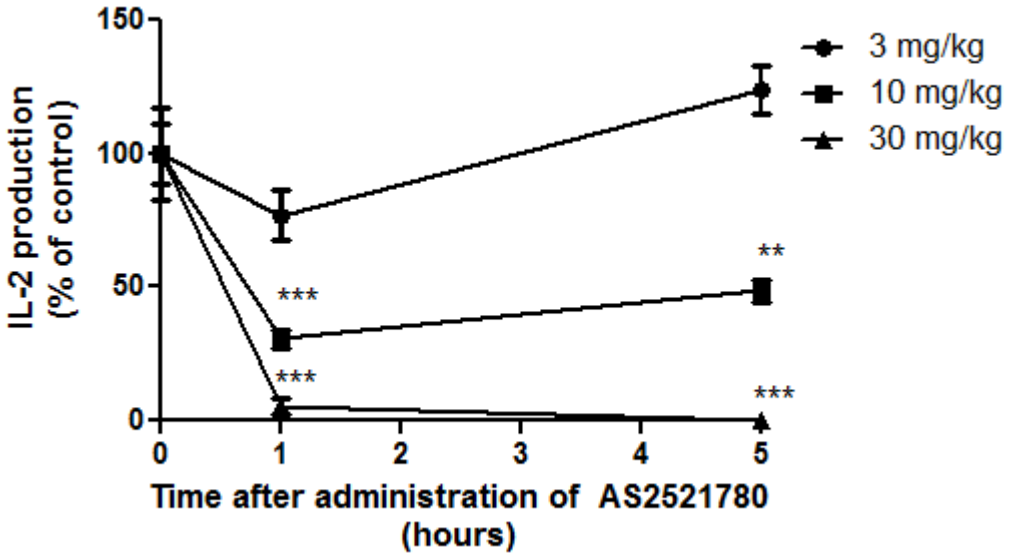

Fig. 2-4. Effect of AS2521780 on *in vivo* Con A-induced IL-2 production.
Male Lewis rats (n=4–5) were given a single oral administration of AS2521780 at a dose of 3, 10, or 30 mg/kg, or vehicle (propylene glycol). At 1 or 5 h post-administration, Con A (15 mg/kg) was injected into the tail vein to induce IL-2 production. At 3 h post-Con A injection, blood samples were collected and plasma IL-2 concentrations were determined by ELISA. Mean concentration of plasma IL-2 in vehicle-administered animals was defined as the control value. Values are presented as a mean percentage of the control with S.E.M., and significant differences vs. each control shown as ***P <0.001 and **P <0.01 by Dunnett's multiple comparison test. Con A, concanavalin A.

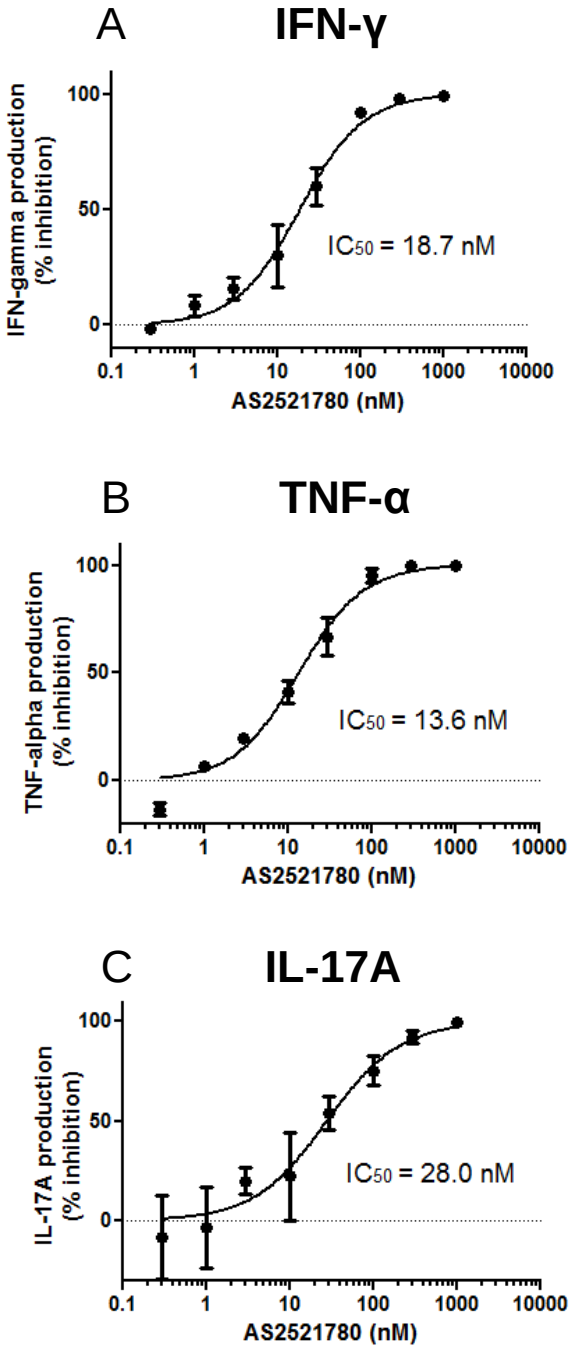

Fig. 2-5. Effect of AS2521780 on *in vitro* Con A-induced cytokine production.
Rat splenocytes cultured with AS2521780 and Con A. After 48 h incubation, concentrations of IFN-γ (A), TNF-α (B), and IL-17A (C) in supernatant measured by ELISA. Values are expressed as the mean ± S.E.M of three independent experiments conducted in duplicate. IC_{50} values were calculated using sigmoid-E_{max} non-linear regression analysis. Con A, concanavalin A.

Chapter 3. Investigation regarding pharmacological effects of AS2521780 on rat adjuvant-induced arthritis model

3.1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized with chronic inflammatory synovitis, progressive joint destruction and multiple organ manifestations that causes severe disability and mortality. Auto-reactive T cells and inflammatory cytokines, such as tumor necrosis factor (TNF)-α, play a pivotal role in the pathological processes of RA through the accumulation of immune cells and the self-perpetuation of inflammation [43]. There were few treatment options with poor outcomes previously, however, the combined use of recently developed drugs such as synthetic disease-modifying anti-rheumatic drug (DMARD) and a biological DMARD targeting TNF-α has revolutionized treatment of RA [43]. Especially, biological DMARDs show quite effective anti-inflammatory activities and can induce clinical remission on patients, but administration routes of these agents are limited in intravenous or subcutaneous. Thus, orally active and effective new agents are still needed.

Rat adjuvant-induced arthritis model has been used as reliable, reproducible, and predictive non-clinical model of RA to evaluate potentially effective drug candidates [44, 45]. For example, the CNI tacrolimus have been used for RA treatment in clinical settings, and showed significant efficacy in rat adjuvant-induced arthritis model through inhibition of CD4+ T cell-dependent immune response [46, 47]. Further, tofacitinib, a newly approved Janus kinase

inhibitor, also showed dose-dependent inhibition of adjuvant-induced arthritis as indicated by decreased paw volume or clinical score [48, 49].

As shown in chapter 2, PKCθ selective inhibitor AS2521780 showed potent inhibitory effects on T cell activation, including proliferation and cytokine production. In this chapter, I investigated pharmacological effects of AS2521780 on rat adjuvant-induced arthritis model to elucidate the potential of PKCθ selective inhibitor as a promising drug candidate for RA treatment.

3.2. Materials and Methods

3.2.1. Test compound

AS2521780 was synthesized at Astellas Pharma Inc. and dissolved in propylene glycol for oral administration.

3.2.2. Rat adjuvant-induced arthritis model

Female Lewis rats (Charles River Laboratories Japan, Inc.), aged 7 weeks and weighing 140-170 g, were used. Arthritis was induced by injection of 0.5 mg of adjuvant (*Mycobacterium tuberculosis* H37RA, Difco Laboratories, Detroit, MI, USA) in 50 μL of liquid paraffin into the right hind footpad (Day 0). Untreated rats were used as negative controls. Following arthritis induction, the left hind paw volume was measured on Days 9, 15, 22, and 25 by a water displacement method using a plethysmometer (MK-550; Muromachi Kikai Co., Ltd., Tokyo, Japan). Paw swelling was presented as a change in hind paw volume from Day 0. AS2521780 (1, 3, 10, and 30 mg/kg) or vehicle was orally administered b.i.d. (twice per day) from Day 1 to 24. On Day 25, the left hind paw of each rat was dissected above the ankle joint and fixed in 10% neutral

buffered formalin. After decalcification, the paws were embedded in paraffin, sectioned longitudinally and stained with hematoxylin and eosin. Plasma IL-6 concentrations on normal, arthritis control and 30 mg/kg b.i.d. of AS2521780 treated group were measured in samples collected on Days 8, 13, 20, and 25 using an ELISA kit (Invitrogen) according to manufacturer's instructions. Brief methods were as follows. Medium samples or standards were added in 96-well flat-bottomed plates pre-coated with capture antibody. After 2 h incubation at 37°C, plates were washed, and detection antibody was added in each well. After 1.5 h incubation at room temperature, plates were washed again, and streptavidin-HRP was added. Finally, TMB ELISA substrate was added, and then optical density was measured.

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc.

3.2.3. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software. Data were expressed as means ± S.E.M. Statistically significant differences were determined using Dunnett's multiple comparison test or Student's t-test, and P-values less than 0.05 were considered significant.

3.3. Results

After injection of adjuvant, right hind paw (injected side) of animals immediately showed inflammatory reaction such as skin redness and edema (data not shown). After calming down these first inflammatory responses, left hind paw (non-injected side) of animals in the arthritis control group exhibited swelling.

Swelling was confirmed at Day 15 post-adjuvant injection and that plateaued by Day 25 (Fig. 3-1A). In histopathological analysis at Day 25, severe bone destruction and cartilage destruction were observed on all animals of arthritis control group (Fig. 3-2B).

AS2521780 inhibited this left hind paw swelling in a dose-dependent manner, and significant inhibition was observed with 3 to 30 mg/kg b.i.d. at Day 25 (Fig. 3-1A). In particular, AS2521780 at 30 mg/kg b.i.d. almost completely suppressed paw swelling and all histopathological change (Fig. 3-2C). Further, AS2521780 at 30 mg/kg b.i.d. completely prevented plasma IL-6 elevation (Fig. 3-1B). Moreover, although the mean body weight of arthritis control rats was markedly lower than that of normal rats due to severe systemic inflammation, this weight loss was recovered by AS2521780 in a dose-dependent manner (Fig. 3-1C), and statistically significance was confirmed in 30 mg/kg b.i.d. treatment group. Of the cytokines tested, only IL-6 was detected in the plasma of rats with adjuvant-induced arthritis (data not shown).

3.4. Discussion

In this rat adjuvant-induced arthritis model, AS2521780 significantly prevented paw swelling associated with arthritis in a dose-dependent manner. In particular, the highest dose of 30 mg/kg b.i.d. of AS2521780 almost completely suppressed paw swelling and histological tissue damage without severe systemic side effects on the basis of body weight gain. As these anti-arthritic effects at each dose highly correlated with those of the Con A-induced rat model of IL-2 production (Fig. 2-4). For example, 10 mg/kg of AS2521780 showed more than 50% inhibition of IL-2 production, and paw swelling was also prevented more

than 50%. In addition, 30 mg/kg of AS2521780 completely suppressed both IL-2 production and paw edema. In addition to IL-2, AS2521780 completely inhibited the production of IL-17A and TNF-α from T cells *in vitro* (Fig. 2-5), and suppressed systemic IL-6 production in adjuvant-induced arthritis model (Fig. 3-1B). These cytokines would be involved in arthritis pathogenesis because biological DMARDs targeting these cytokines exert clear therapeutic effects in clinical settings [50-52]. I therefore consider AS2521780 to exert an anti-arthritic effect *via* inhibition of T cell activation and its induced cytokine production.

To consider clinical application, effective dose is an important aspect. In current study, significant inhibition of paw swelling was observed with 3 to 30 mg/kg b.i.d. of AS2521780. If effective dose for human RA is equal to that on rat arthritis model, doses of 3 to 30 mg/kg b.i.d. may be high for oral administration. However, in the case of tofacitinib, complete inhibition of paw swelling in rat adjuvant-induced arthritis models (while detail protocols were different from my experiment) were achieved at doses of 15 to 20 mg/kg per day [48, 49], and clinically used (this meant effective) dose was only 5 mg b.i.d. (approx. 0.2 mg/kg per day). This discrepancy may be made by the difference of oral availability between rats and humans, or difference of pathology between rat model and actual human disease. Further, methotrexate, one of effective DMARDs, is sometimes concomitantly administered with an investigational drug to RA patients [53], and dose of that investigational drug may be reduced through synergistic function with methotrexate. Therefore, PKCθ selective inhibitors remain an intriguing possibility as drug for RA.

Taken together, current investigation revealed that PKCθ selective inhibitors might be a quite effective therapeutic option for RA as a multiple cytokine signal

inhibitor. In addition, T cells or T cell-secreted cytokines are involved in the pathogenesis of the other autoimmune diseases such as systemic sclerosis, inflammatory bowel disease or psoriasis [54-57], hence PKCθ selective inhibitors have a potential to treat these T cell-mediated autoimmune diseases. Indeed, sotrastaurin showed effectiveness in clinical trials for the treatment of psoriasis [27].

3.5. Figures

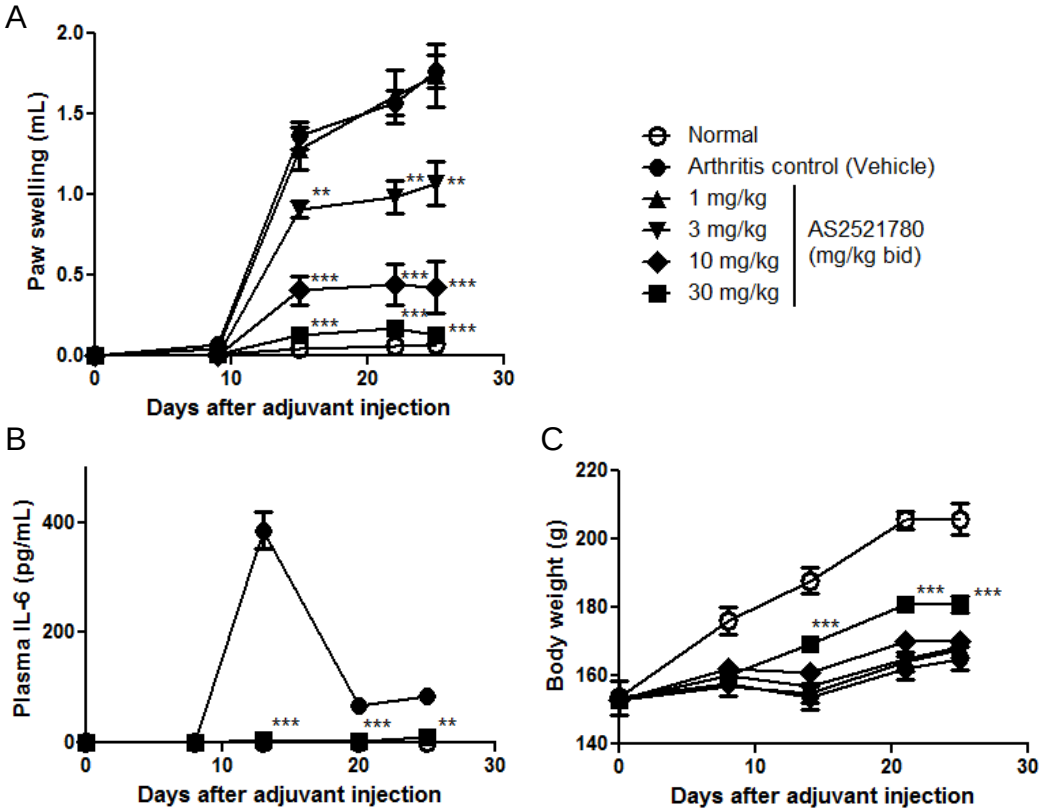

Fig. 3-1. Effect of AS2521780 on adjuvant-induced arthritis in rats.
Female Lewis rats received an injection of adjuvant-containing liquid paraffin into the right foot hind pad (Day 0). Vehicle (propylene glycol) or AS2521780 (1, 3, 10, 30 mg/kg b.i.d.) was administered from Day 1 to 24. The left hind paw (non-injected side) volume (A), concentration of plasma IL-6 (B), and body weight (C) are shown. The left hind paw volume was measured by water displacement method using a plethysmometer. Plasma IL-6 concentrations were measured only on normal, arthritis control and 30 mg/kg b.i.d. of AS2521780 treated group by ELISA. Data expressed as mean ± S.E.M (n=8). Significant differences between arthritis control (vehicle) group shown as ***P <0.001 and **P <0.01 by Dunnett's multiple comparison test. For IL-6 concentration, Significant differences between arthritis control (vehicle) group and AS2521780 treated group shown as ***P <0.001 and **P <0.01 by Student's t-test.

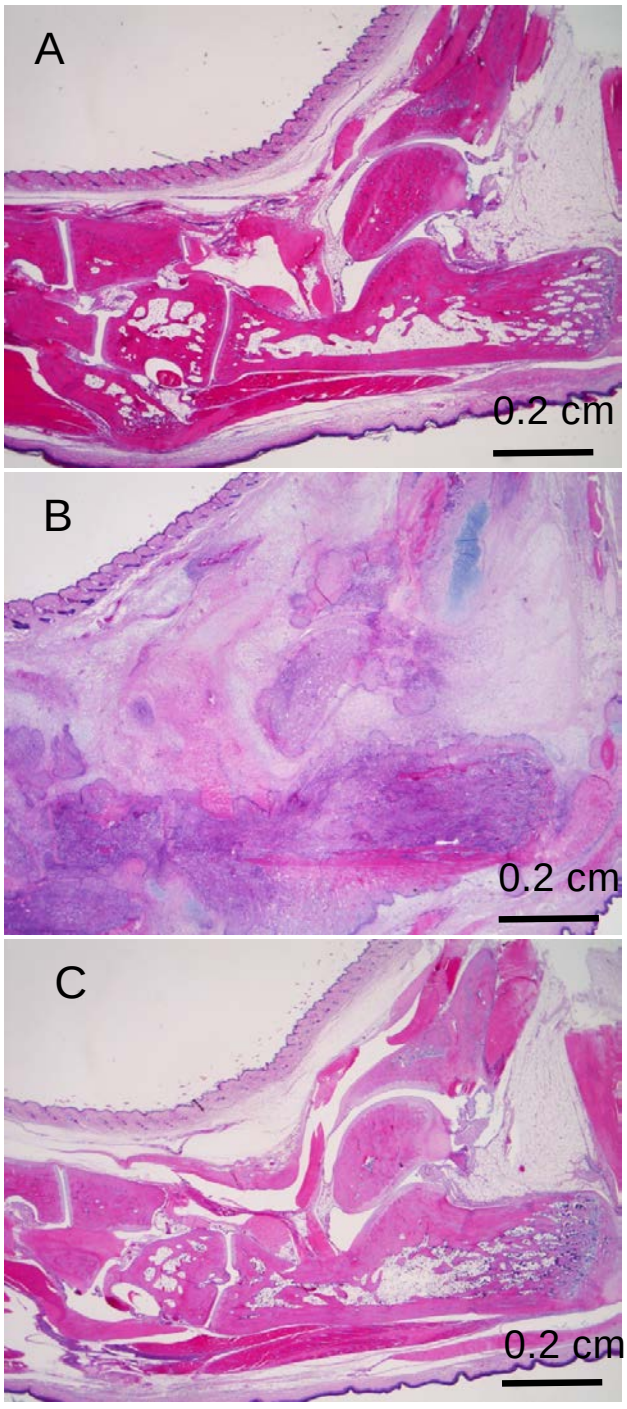

Fig. 3-2. Representative micrographs of calcaneal region in rats with adjuvant-induced arthritis.

Figures show hematoxylin and eosin-stained calcaneal regions of left hind paws (non-injected side) in a rat with adjuvant-induced arthritis. Bone destruction and cartilage destruction were indicated. Representative images of normal (A), arthritis control (B), and AS2521780 30 mg/kg b.i.d.-treated rats (C).

Chapter 4. Investigation regarding pharmacological effects of AS2521780 on acute rejection in rat and non-human primate models of transplantation

4.1. Introduction

Organ transplantation is the only procedure at this moment to reverse organ functions for patients with end-organ failure on such as kidney, heart, liver, lung and so on. Although transplanted organs are provided from living or cadaveric donor, in either case transplanted organs are allo-derived (same species, non-self), and rejection reaction occurs as a result of normal immune-response against non-self-antigens. Since T cells are playing a central role in graft rejection following solid organ transplantation [7, 58], immunosuppression of T cells is necessary to ensure proper graft function by impairment of rejection reaction. There were no effective methods to interrupt T cell activation in past days [59], thus graft survival time was extremely limited. From the 1950s onwards, azathioprine (DNA replication inhibitor), mycophenolate mofetil (MMF; lymphocyte proliferation inhibitor), and CNIs were developed [59], and graft survival was drastically improved [60]. Consequently, organ transplantation became an essential medical procedure. In currently available drugs for transplantation, CNIs are the most important anchor-drugs on the basis of their significant effectiveness. However, CNIs exhibit adverse effects such as nephrotoxicity [9] due to broad expression profile of calcineurin. In addition, although CNI minimization protocols that still maintain immunosuppression are being developed [61], alternative therapies that specifically inhibit T cell

activation without adverse effects on other cell types and organs are still required for further improvement of medical transplantation.

As shown in chapter 2, PKCθ selective inhibitor AS2521780 showed potent inhibitory effects on T cell activation, and the possibility was confirmed that selective PKCθ inhibition might be a favorable strategy in achieving a suitable balance between T cell inhibition and the minimization of other systemic effects. In this chapter, I investigated the effect of AS2521780 mono- and combination therapy on acute rejection in rat cardiac and NHP renal transplantation models to determine the efficacy of the selective inhibition of PKCθ and to elucidate the potential of PKCθ selective inhibitor as a promising drug candidate for organ transplantation.

4.2. Materials and Methods

4.2.1. Animals

Male Lewis ($RT1^{l}$) rats were purchased from Charles River Laboratories Japan, Inc., and ACI ($RT1^{avl}$) rats from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in a specific pathogen-free room with free access to food and water. Male cynomolgus monkeys (*Macaca fascicularis*) free of simian immunodeficiency virus, simian retrovirus, salmonella bacteria, dysentery bacteria, and B virus, were purchased from Hamri Co., Ltd. and housed in individual cages. Monkeys were allowed water *ad libitum* and food once a day. All animals were allowed at least 1 week to adapt to their environment before experiments.

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc.

4.2.2. Test compounds

AS2521780, sotrastaurin, tacrolimus (Prograf® injection or solid dispersion formulation [SDF]), and MMF were synthesized at Astellas Pharma Inc.

4.2.3. ACI-to-Lewis rat heterotopic cardiac transplantation

ACI and Lewis rats aged 8 to 9 weeks were used as cardiac donors and recipients, respectively. Heterotopic abdominal cardiac transplantation was performed as previously described [62, 63]. Briefly, hearts were transplanted from male ACI donors into male Lewis recipients. The donor aorta and pulmonary artery were anastomosed, end to side, to the recipient's abdominal aorta and inferior vena cava, respectively. The day of transplantation was designated as Day 0.

AS2521780 (10 and 30 mg/kg as monotherapy, 0.25, 0.5, 1, 3, and 5 mg/kg as combination therapy) and sotrastaurin (10, 30, and 50 mg/kg) were dissolved in 0.1 M HCl containing-propylene glycol and orally administered at 2.5 mL/kg b.i.d. (8/16 h intervals). Tacrolimus (Prograf® injection) was diluted with saline and intramuscularly administered at 0.02 mg/1 mL/kg q.d. (once daily). MMF was suspended in 0.5% methylcellulose solution and orally administered at 15 mg/5 mL/kg q.d. All test compounds were administered for 14 consecutive days immediately after operation. Graft survival was monitored by daily palpation until 29 days post-transplantation, and graft rejection was defined as cessation of palpable cardiac graft beats.

4.2.4. Renal transplantation in non-human primates

Donor and recipient pairs were established by matching ABO blood type and un-matching major histocompatibility complex (MHC) indicated by a one-way mixed lymphocyte reaction (stimulation index >2.5). Each animal was then used as both a donor and recipient, with one kidney from either animal being exchanged between pairs. Renal transplantation was performed using standard microsurgical techniques as previously described [64]. Briefly, all donor and recipient monkeys were anesthetized and the left kidney of the donor was mobilized and excised, along with a long segment of the renal vessels and ureter. The renal allograft was then perfused with Euro-Collins solution (4°C) and stored in cold saline while the recipients were prepared. The graft kidney was transplanted into the lower part of abdomen *via* end-to-side anastomoses of the renal artery to the aorta and the renal vein to the vena cava. End-to-end anastomosis of the donor and recipient ureters *via* ureteral stent was also done using 8-0 nylon sutures. Finally, the remaining kidney of each animal was removed. To anesthetize animals, tiletamine and zolazepam (USP, Rockville, MD, USA) were used for induction, and vaporized isoflurane (Pfizer, New York, NY, USA) was used for maintenance. For postoperative care, an analgesic (0.02 mg/body buprenorphine, Otsuka Pharmaceutical, Tokyo, Japan), antibiotic (250 mg/body cefazolin, Astellas Pharma Inc.), gastroprokinetic agent (1 mg/body metoclopramide, Astellas Pharma Inc.), and histamine H2-receptor antagonist as gastric acid suppressant (5 mg/body famotidine, Astellas Pharma Inc.) were subcutaneously administered twice daily for 3 days post-transplantation. Function of transplanted kidneys was monitored *via* measurement of plasma creatinine levels twice per week. Graft rejection was diagnosed based on deterioration of

kidney function, which was defined as creatinine level exceeding 10 mg/dL. When ultrasonography indicated that creatinine elevation was due to occlusion of the ureteral stent, the stent was replaced, and temporarily elevated creatinine levels were excluded from the analysis.

Six previously untreated cynomolgus monkeys were used in the present study. AS2521780 (3 mg/2 mL/kg b.i.d., 8/16 h intervals) and tacrolimus (SDF form, 1 mg/2 mL/kg q.d.) were orally administered to recipients immediately before operation, and daily administration continued until the time of graft rejection or 90 days after operation. At the end of the study, each recipient was euthanized, and kidney specimens were collected for histopathological evaluation. Collected kidneys were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, and rejection was graded according to Banff-97 classification [65] with Banff-09 modifications [66].

4.2.5. Statistical analysis

Graft survival times for both rat cardiac and NHP renal transplantation were compared between groups using the log-rank test (Prism 5; GraphPad Software Inc.), and P-values less than 0.05 were considered significant. Body weight changes in rat transplantation were expressed as mean ± S.E.M, and statistical analysis was conducted using Tukey’s multiple comparison test.

4.3. Results

4.3.1. Effect of AS2521780 monotherapy on rat cardiac transplantation

In the ACI-to-Lewis, MHC (RT1) fully-mismatched, rat heterotopic cardiac transplantation model, grafts only survived until 5 or 6 days post-transplantation

in the non-treated group (Table 4-1). Administration of AS2521780 significantly prolonged graft survival time, and median survival time (MST) was 14 days for the 10 mg/kg b.i.d. group and 20 days for the 30 mg/kg b.i.d. group. In particular, in the 30 mg/kg b.i.d. group, heart beats were maintained for all recipients throughout the 14-day treatment period, and body weight gain was comparable to that of non-treated animals (Fig. 4-1). Administration of sotrastaurin also significantly prolonged graft survival time, with an MST of 11 days for the 10 mg/kg b.i.d. group and 14 days for the 30 mg/kg b.i.d. group. However, 6 of the 7 recipients in the 50 mg/kg b.i.d. group died with an aggravated general condition by 3 days post-transplantation.

4.3.2. Combinational effects of AS2521780 on eat cardiac transplantation

The effect of AS2521780 in combination with tacrolimus and MMF on cardiac graft survival was investigated in rats. AS2521780 was administered to recipient rats concomitantly with each of these drugs at suboptimal dose levels where slight prolongation of graft survival was observed.

In combination with tacrolimus (suboptimal dose: 0.02 mg/kg, MST = 7 days), AS2521780 1 mg/kg b.i.d. drastically reduced graft rejection (Table 4-2), and the MST (20 days) was comparable to that of AS2521780 alone at 30 mg/kg b.i.d. (Table 4-1). Further, all grafts survived for more than 20 days in the group receiving AS2521780 at 5 mg/kg b.i.d. in combination with tacrolimus, despite drug treatment being withdrawn at 13 days post-transplantation in accordance with study design (Table 4-2).

Treatment of recipients using AS2521780 3 mg/kg b.i.d. in combination with MMF 15 mg/kg (suboptimal dose, MST = 8 days) significantly prolonged graft

survival compared to MMF monotherapy (Table 4-3). Differences in MST between monotherapy with each concomitant drug and combination therapy with AS2521780 at 3 mg/kg b.i.d. were 16 days for tacromilus and 13 days for MMF.

4.3.3. Effect of AS2521780 combined with tacrolimus on NHP model of renal transplantation

My laboratory previously reported the effect of tacrolimus on renal transplantation in cynomolgus monkeys for graft survival time, graft function (plasma creatinine), and histopathology [64]. That report demonstrated that MST in vehicle-treated monkeys was 6 days, and that tacrolimus at 1 mg/kg significantly prolonged MST to 21 days.

In the present study, I investigated the effect of AS2521780 at 3 mg/kg b.i.d. in combination with tacrolimus at 1 mg/kg in an NHP model of renal transplantation using the same protocol as previously described [64]. Six cynomolgus monkeys that received renal transplantation followed by immunosuppressive therapy with AS2521780 and tacrolimus showed prolonged graft survival. MST was 56 days and significantly improved compared to that with tacrolimus monotherapy (Table 4-4). In particular, two monkeys survived throughout the experimental period (90 days post-transplantation) with well-maintained kidney function (Fig. 4-2A). Further, renal histopathological evaluation at autopsy revealed that while 6 of the 7 monkeys in the tacrolimus monotherapy group exhibited grade IIA, IIB, or III acute T cell-mediated rejection (TCMR), 5 of the 6 monkeys in the AS2521780-treated group did not exceed grade I acute TCMR (Fig. 4-3, Table 4-4). Regarding safety, body weights remained relatively stable (Fig. 4-2B), and no marked changes in levels of plasma

hepatic enzymes (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase) were observed (data not shown) in the AS2521780-treated group throughout the experimental period. These results demonstrate that concomitant use of tacrolimus and AS2521780 potently inhibits T cell-mediated rejection in the NHP model of renal transplantation.

4.4. Discussion

Here, I demonstrated that the selective PKCθ inhibitor AS2521780 prevents allograft rejection in both ACI-to-Lewis rat cardiac and NHP renal transplantation models. In the ACI-to-Lewis rat cardiac transplantation model, both AS2521780 and sotrastaurin monotherapy exhibited significantly prolonged graft survival. In my rat model, all grafts in the non-treated group survived for only 5 or 6 days post-transplantation, and test drug was withdrawn in the treated group at 13 days post-transplantation. This graft survival exceeding 20 days therefore indicates that acute rejection is nearly completely prevented during the drug-treatment period. Six of the 10 grafts survived for more than 20 days in the AS2521780 30 mg/kg group. In contrast, graft survival did not exceed 20 days in the sotrastaurin 30 mg/kg group, which is the maximum tolerated dose (MTD). As shown in chapter 2, a dose of 30 mg/kg of AS2521780 induced near-complete suppression of Con A-induced IL-2 production (Fig. 2-4). Basiliximab, a chimeric monoclonal antibody directed against the alpha chain of IL-2 receptor, can significantly reduce the incidence of acute rejection in clinical settings [67], this therefore indicates IL-2 significantly contribute to rejection reaction. I therefore consider this strong inhibitory effect induced by AS2521780 on IL-2 production contribute to the marked efficacy observed in the rat transplantation model, at least partially. My

group also previously reported the efficacy of MMF [68], which is one of the major immunosuppressants currently used to treat transplant rejection and autoimmune diseases [69], on rat cardiac allograft rejection using the same experimental conditions as the present study. The MST of rats administered 40 mg/kg (MTD) of MMF monotherapy was 18.5 days, and survival time of half the grafts exceeded 20 days, indicating that the effect of AS2521780 30 mg/kg b.i.d. is comparable to that of MMF 40 mg/kg in a rat cardiac transplantation model. These results suggest that immunosuppression *via* PKCθ inhibition with an isotype-specific small-molecule inhibitor is comparable to current immunosuppressive therapy in transplant patients.

In current clinical settings, the combination of immunosuppressive medications such as tacrolimus and MMF is widely used as a maintenance therapy to achieve appropriate immunosuppression with limited toxicity after kidney transplantation [69]. In the present study, I investigated the efficacy of AS2521780 with a suboptimal dose of tacrolimus (0.02 mg/kg) in rat cardiac transplantation to consider the clinical application of PKCθ-selective inhibition. My results demonstrate the efficacy of AS2521780 with tacrolimus and that the MST of rats concomitantly treated with 1 mg/kg of AS2521780 and a suboptimal dose of tacrolimus was comparable to that of MMF 20 mg/kg and tacrolimus [68]. I also confirmed that 1 mg/kg of AS2521780 did not affect the plasma concentration of tacrolimus (data not shown), thus this additive effect would exert *via* biologically. Regarding monotherapy, AS2521780 at 30 mg/kg b.i.d. was required to exert an effect comparable to MMF at 40 mg/kg. However, in the case of combination therapy with tacrolimus, only AS2521780 at 1 mg/kg b.i.d. exerted an effect comparable to MMF at 20 mg/kg. These results suggest that the

efficacy of a PKCθ selective inhibitor and tacrolimus is more effective than that of MMF and tacrolimus.

To identify other immunosuppressive regimens using the selective inhibition of PKCθ, I further investigated the combination of AS2521780 with MMF in a rat model of cardiac transplantation. These results showed that concomitant administration of 3 mg/kg b.i.d. of AS2521780 with a suboptimal dose of MMF (15 mg/kg) significantly prolonged graft survival compared to MMF monotherapy. MST in rats with a suboptimal dose of tacrolimus (7 days) was comparable to that in rats with a suboptimal dose of MMF (8 days), and MST in rats treated with 3 mg/kg b.i.d. of AS2521780 combined with tacrolimus or MMF were also comparable at 23 or 21 days, respectively. As the combination of a selective PKCθ inhibitor with tacrolimus or MMF is clearly effective, selective PKCθ inhibitors might be versatile and useful in clinical settings.

Rat models of transplantation extremely 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 [70]. Recently, NHP transplant models have become increasingly common for the evaluation of new immunosuppressive agents before moving to clinical trials. In the present study, 6 cynomolgus monkeys were used to evaluate the combination effect of AS2521780 and tacrolimus in an NHP model of renal transplantation. These results demonstrated that concomitant use of tacrolimus and AS2521780 exerts potent inhibition of graft rejection, particularly for T cell-mediated rejection, with histopathological confirmation.

Sotrastaurin has been reported to prolong the survival times of kidney allografts in cynomolgus monkeys [24], and immunosuppression *via* sotrastaurin

has been confirmed in clinical trials for anti-rejection therapy following kidney transplantation [28]. In this clinical trial, however, a near-linear relationship was noted between the dose of sotrastaurin and discontinuation of study medication. Thus, the adverse effects associated with sotrastaurin might prevent its full efficacy from being reached. Indeed, in my rat experiments, 30 mg/kg b.i.d. of sotrastaurin significantly prolonged graft survival. However, 50 mg/kg b.i.d. was lethal, and the maximum prolongation of graft survival following administration of sotrastaurin was significantly shorter than that of AS2521780 (Table 4-1). AS2521780 was well tolerated in the present study (Figs. 4-1 and 4-2B). Further, in the preliminary 2-week toxicological study using cynomolgus monkeys, toxicological findings were not observed when AS2521780 was administered at 10 mg/kg (data not shown). These findings suggest that compounds with selective PKCθ inhibition might exhibit improved efficacy and safety profiles compared to sotrastaurin.

In summary of this chapter, the investigation regarding AS2521780 in a rat model of cardiac transplantation and NHP model of renal transplantation demonstrates the potential of PKCθ as a new drug target for organ transplantation. As AS2521780 was well tolerated and the dose of tacrolimus or mycophenolate mofetil can be reduced when used in combination with this drug, immunosuppressive regimens containing selective inhibitors of PKCθ might have good safety profiles.

4.5. Tables and Figures

Table 4-1. Effect of AS2521780 and sotrastaurin monotherapy on ACI-to-Lewis rat cardiac transplantation.

Test compound	Dose (mg/kg b.i.d.)	n	Graft survival (days)	MST[a]	*P*-value
–	0[b]	7	5, 5, 5, 5, 6, 6, 6	5	–
AS2521780	10	9	5, 6, 7, 7, 14, 20, 20, 22, 25	14	$P < 0.01$[c]
	30	10	14, 14, 16, 16, 20, 20, 20, 20, 21, 22	20	$P < 0.001$[c] $P < 0.01$[d]
Sotrastaurin	10	6	6, 6, 11, 11, 12, 14	11	$P < 0.01$[c]
	30	6	14, 14, 14, 14, 15, 17	14	$P < 0.001$[c]
	50	7	1[e], 1[e], 1[e], 2[e], 3[e], 3[e], 16	–	–

[a]MST; median survival time

[b]Propylene glycol was administered.

[c]P-value was calculated by log-rank test compared to non-treated group.

[d]P-value was calculated by log-rank test compared to 30 mg/kg b.i.d. of sotrastaurin-treated group.

[e]Died with functioning graft.

Table 4-2. Effect of AS2521780 concomitant treatment with tacrolimus on ACI-to-Lewis rat model of cardiac transplantation.

AS2521780 (mg/kg b.i.d.)	Tacrolimus (mg/kg)	n	Graft survival (days)	MST[a]	*P*-value[b]
0[c]	0.02	11	5, 5, 6, 6, 6, 7, 7, 7, 9, 11, 11	7	–
0.25	0.02	7	5, 6, 7, 9, 12, 12, 22	9	–
0.5	0.02	15	6, 6, 6, 6, 7, 7, 9, 10, 11, 11, 16, 17, 18, 23, 24	10	$P < 0.05$
1	0.02	9	9, 11, 13, 17, 20, 23, 24, 24, 26	20	$P < 0.001$
3	0.02	8	16, 16, 19, 23, 23, 24, 24, 28	23	$P < 0.001$
5	0.02	6	20, 21, 24, >28, >28, >28	>26	$P < 0.001$

[a]MST; median survival time

[b]P-value was calculated by log-rank test compared to tacrolimus monotherapy.

[c]Propylene glycol was administered.

Table 4-3. Effect of AS2521780 concomitant treatment with mycophenolate mofetil (MMF) on ACI-to-Lewis rat model of cardiac transplantation.

AS2521780 (mg/kg b.i.d.)	MMF (mg/kg)	n	Graft survival (days)	MST[a]	*P*-value[b]
0[c]	15	7	7, 7, 7, 8, 8, 13, 14	8	–
3	15	8	14, 18, 21, 21, 21, 21, 22, >28	21	$P < 0.001$

[a]MST; median survival time

[b]P-value was calculated by log-rank test compared to MMF monotherapy.

[c]Propylene glycol was administered.

Table 4-4. Effect of AS2521780 concomitant treatment with tacrolimus on NHP model of renal transplantation.

AS2521780 (mg/kg b.i.d.)	Tacrolimus (mg/kg)	N	Graft survival (days)	Histopathology (Banff classification)	MST[a]	*P*-value[b]
–	–	4	5[c]	Other (infarction)	6	–
			6[c]	Acute TCMR III (i1)		
			6[c]	Acute TCMR III (i2)		
			7[c]	Acute TCMR III (i2)		
–	1	7	8[c]	Acute TCMR III (i2)	21	–
			18[c]	Acute TCMR IA (i2)		
			19[c]	Acute TCMR IIA (v1, i3)		
			21[c]	Acute TCMR IIB (v1, i2)		
			21[c]	Acute TCMR III (v1, i3)		
			24[c]	Acute TCMR IIA (v1, i3)		
			39[c]	Acute TCMR IIB (v1, i2), Chronic active TCMR		
3	1	6	9	Acute TCMR III (v2, i2)	56	$P < 0.05$
			25	Acute TCMR IA (i1), chronic active TCMR, IF/TA II		
			35	Acute TCMR IA (i2), chronic active TCMR, IF/TA II		
			77	Acute TCMR IA (i3), chronic active TCMR, IF/TA III		
			>90	Chronic active TCMR, IF/TA I		
			>90	Chronic active TCMR, IF/TA II		

NHP; non-human primate, TCMR; T cell-mediated rejection, IF/TA; interstitial fibrosis/tubular atrophy

[a]MST; median survival time

[b]P-value was calculated by log-rank test compared to 1 mg/kg of tacrolimus-treated group.

[c]Data of graft survival on these animals were previously reported from my laboratory [64]. Histopathological analysis using Banff classification was newly conducted.

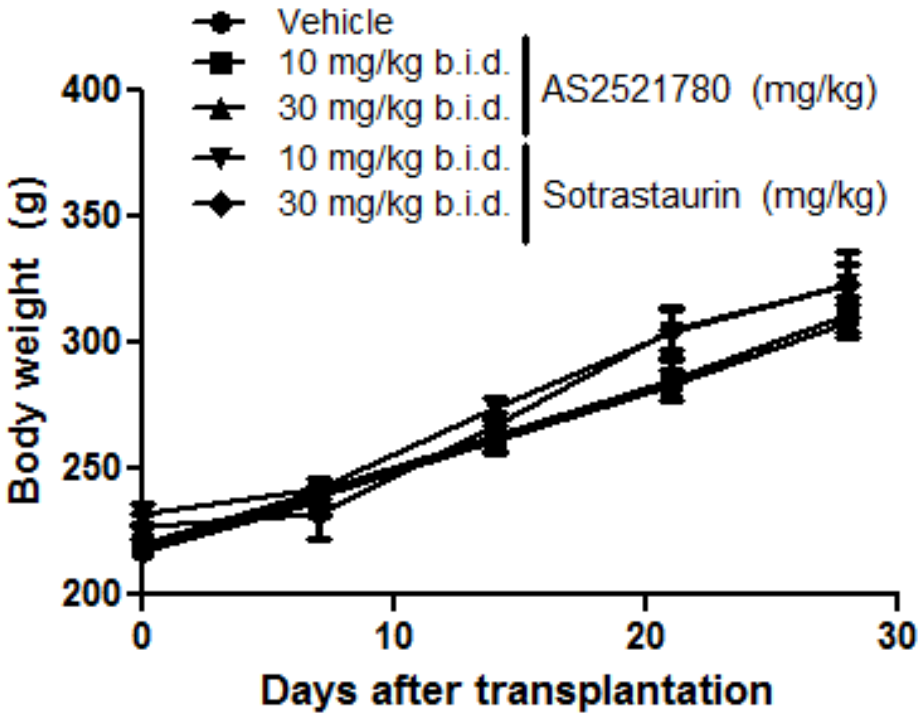

Fig. 4-1. Changes in body weight in rat model of cardiac transplantation.
Graft survival times of animals are shown in Table 4-1. Data are expressed as mean ± S.E.M. No significant differences were noted using Tukey's multiple comparison test among all time-points and treatment groups.

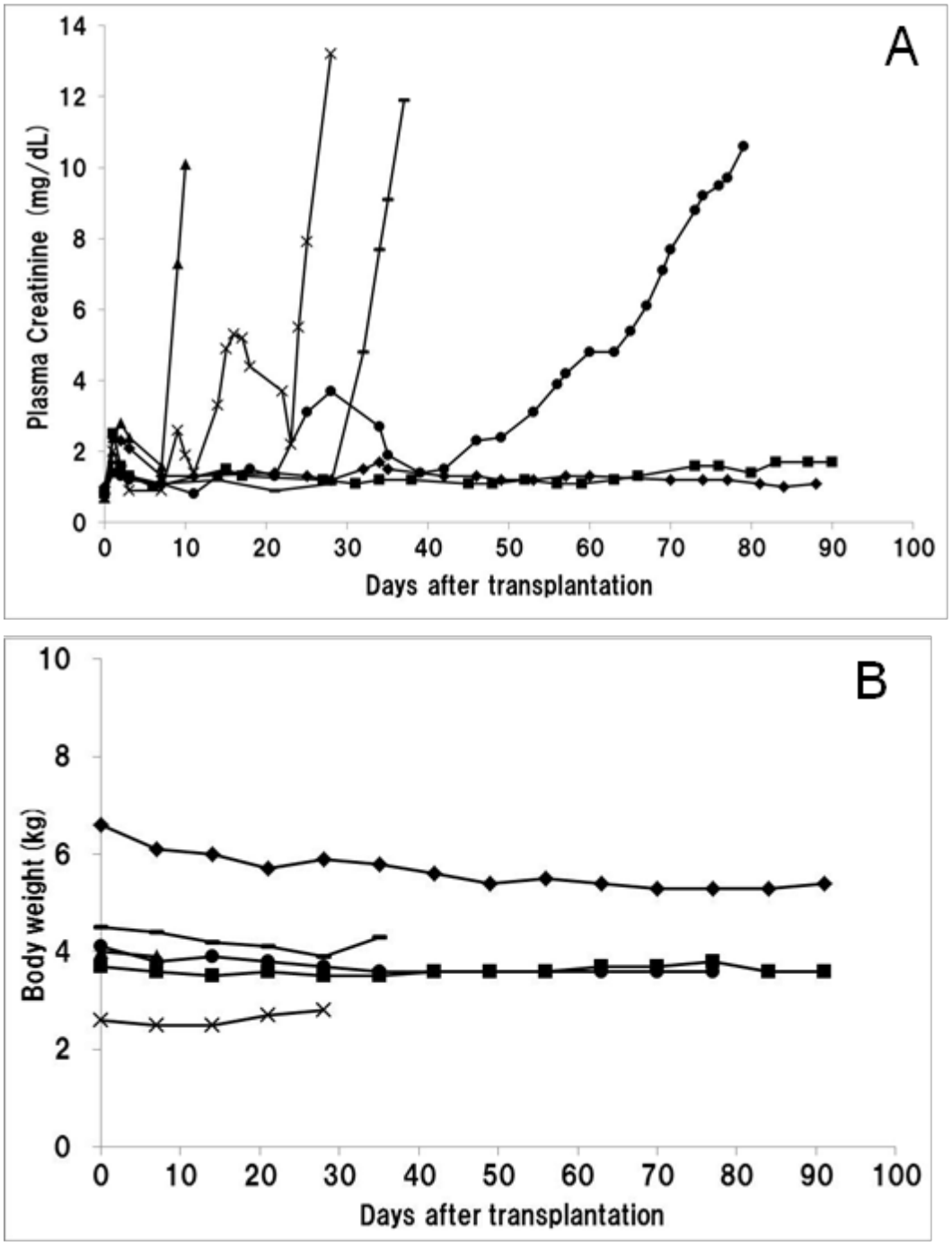

Fig. 4-2. Changes of parameters in NHP model of renal transplantation.
Figures show plasma creatinine concentration (A) and body weight (B) of renal-transplanted cynomolgus monkeys, which were concomitantly treated with 3 mg/kg of AS2521780 and 1 mg/kg of tacrolimus. Plasma creatinine levels were measured twice per week and body weights were measured once per week. Matching symbols indicate the same individuals across two figures. NHP; non-human primate.

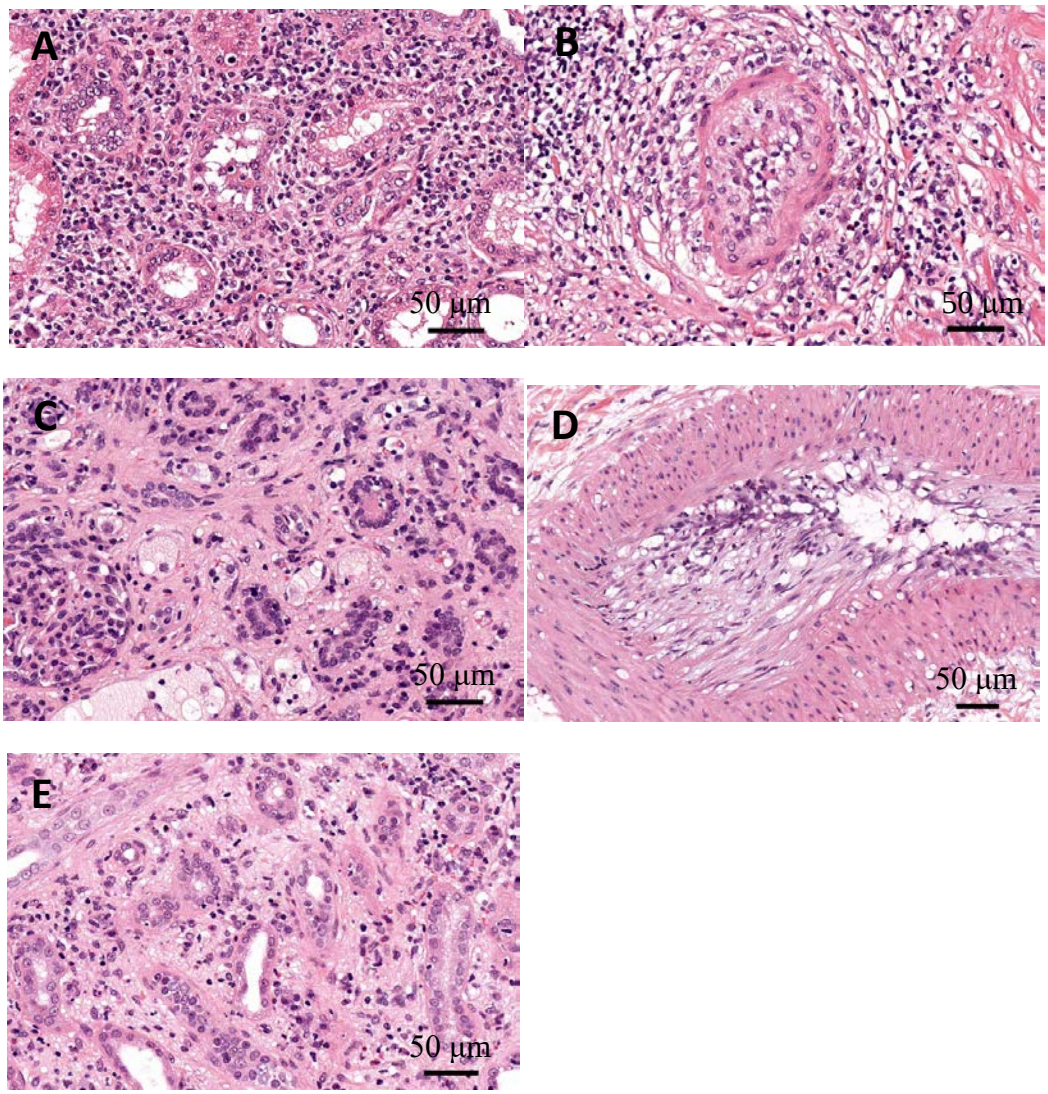

Fig. 4-3. Representative micrographs of transplanted kidneys in AS2521780 and tacrolimus-treated monkeys at autopsy.

(A-C) Histology of a monkey that survived 77 days. Each photo shows acute TCMR grade IA with mononuclear cell interstitial infiltration (A), chronic active TCMR with arterial intimal hypertrophy (B), and IF/TA grade III (C).

(D, E) Histology of a monkey that survived 90 days. Each photo shows chronic active TCMR with arterial intimal hypertrophy (D), and IF/TA grade II (E). Scale bars indicate 50 μm. TCMR; T cell-mediated rejection, IF/TA; interstitial fibrosis/tubular atrophy.

Chapter 5. Concluding remarks

To provide better immunosuppressant into clinical settings, I have focused to develop alternative therapeutic options that specifically inhibit T cell activation without adverse effects on other cell types and organs. In this regard, the aims of this investigation are to elucidate i) kinase inhibition profile of AS2521780, ii) importance of PKCθ in T cell activation signaling across species, and iii) whether PKCθ is an attractive drug target for T cell inhibition and for treating T cell-mediated autoimmune diseases or prevention of organ rejection through animal model evaluation.

As described in chapter 2, I first examined kinase selectivity of AS2521780, a newly synthesized PKCθ inhibitor, against 7 other PKC isoforms and 14 tyrosine and 13 serine/threonine kinases to confirm whether this compound could be used as a PKCθ selective inhibitor for further investigations. Results showed that AS2521780 was a highly selective PKCθ inhibitor and indicates that AS2521780 can be used to investigate the physiological functions of PKCθ in T cell signaling. On the basis of these characteristics, I conducted several *in vitro* experiments and found that AS2521780 completely suppressed CD3/CD28-induced T cell proliferation or IL-2 production in human cells, and Con A-induced production of several cytokines in rat and NHP cells. IC_{50} values of these experiments were approximately the same regardless of species and stimulus. My investigation *via* pharmacological approach shows that PKCθ has fundamental roles in a broad range of T cell activation signaling across several species. In addition, since my results demonstrate that selective PKCθ inhibition without an effect on PKCα is

sufficient for the complete prevention of T cell activation, selective PKCθ inhibition might be a favorable strategy in achieving a suitable balance between T cell inhibition and the minimization of other systemic effects.

In chapters 3 and 4, effects of AS2521780 in pre-clinical animal models was investigated to confirm attractiveness of PKCθ as a drug target for T cell immunosuppression. In chapter 3, I investigated effects of AS2521780 on adjuvant-induced arthritis in rats, which is a model of RA, to investigate whether PKCθ selective inhibition can potentially ameliorate T cell-mediated autoimmune diseases. As a result, AS2521780 inhibited paw swelling in a dose-dependent manner, and significant inhibition was observed with 3 to 30 mg/kg b.i.d. These results highlight the potential of a PKCθ selective inhibitor in the treatment of RA or other T cell-mediated autoimmune diseases. Further, to elucidate the possibility of PKCθ selective inhibitors for other therapeutic indication, I investigated the effect of the PKCθ selective inhibitor AS2521780 in mono- and combination therapies on acute rejection in ACI-to-Lewis rat cardiac and NHP renal transplantation models, and results were described in the chapter 4. Data demonstrate that the potential of PKCθ as a new drug target for organ transplantation with good safety profiles.

As summary of this dissertation, I summarize important findings in current investigation as follows; i) AS2521780 is a potent and selective PKCθ inhibitor, ii) PKCθ has fundamental roles in a broad range of T cell activation signaling across several species, iii) PKCθ selective inhibitor effectively prevents development of rat adjuvant-induced arthritis with safety, and iv) PKCθ selective inhibitor effectively prevents rejection reaction on rat and NHP models of transplantation with safety (Fig. 5-1). Taken together, these findings show that the

developmental possibility of PKCθ selective inhibitors as effective novel immunosuppressants, and this is the first experimental output regarding the potential clinical application of PKCθ selective inhibitor. Although many researchers have made effort to explore novel immunosuppressant, there are only a few successes. In particular, limited to selective kinase inhibitor, only 2 drugs Janus kinase inhibitor tofacitinib and mTOR inhibitor rapamaycin have been launched. Thus, my current finding, PKCθ selective inhibitor can exert strong immunosuppression without toxicity, are quite noteworthy and I believe my investigation clearly contribute to the progress of biotechnology and bioindustrial sciences. Further, after my publication, the other researchers on pharmaceutical companies reported profiles of their PKCθ selective inhibitors with different chemical structures, and they showed similar T cell inhibitory effects and anti-arthritic effect in rat models [71, 72]. These findings support my discovery that PKCθ is a valuable drug target of immunosuppressants. Moreover, although I focused PKCθ as a target for immunosuppressant, recent investigations revealed the potential of the other clinical indications of PKCθ inhibitors, such as anti-cancer or anti- human immunodeficiency virus [73, 74]. I strongly hope my investigation will lead to development of new specific drug, and that drug will provide drastic change of social life.

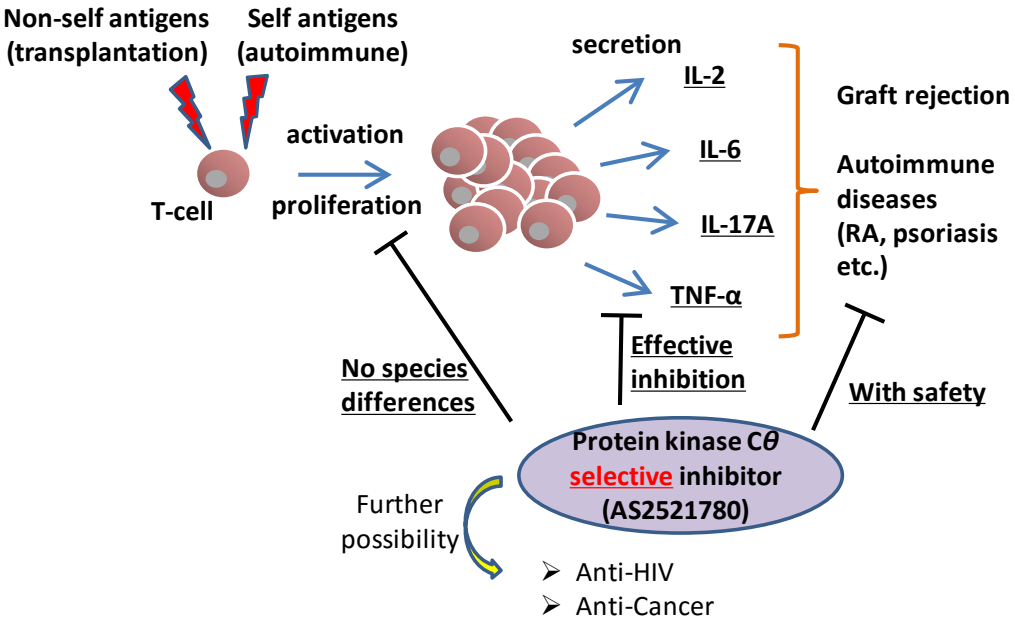

Fig. 5-1. Summary of findings on this dissertation.

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List of publications

Effect of AS2521780, a novel PKCθ selective inhibitor, on T cell-mediated immunity.

Hidehiko Fukahori, Noboru Chida, Masashi Maeda, Mamoru Tasaki, Tomoko Kawashima, Hideaki Matsuoka, Keiko Suzuki, Takeshi Ishikawa, Akira Tanaka, Yasuyuki Higashi

Eur. J. Pharmacol. 2014;745:217-22.

Effect of novel PKCθ selective inhibitor AS2521780 on acute rejection in rat and non-human primate models of transplantation.

Hidehiko Fukahori, Noboru Chida, Masashi Maeda, Mamoru Tasaki, Tomoko Kawashima, Takahisa Noto, Susumu Tsujimoto, Koji Nakamura, Shinsuke Oshima, Jun Hirose, Yasuyuki Higashi, Tatsuaki Morokata

Int Immunopharmacol. 2015;27(2):232-7.