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In vitro and *in vivo* characterization of AS2643361, a novel and highly potent inosine 5′-monophosphate dehydrogenase inhibitor

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ABSTRACT

Inosine 5′-monophosphate (IMP) dehydrogenase is a critical target in solid organ transplantation. To this end, the development of mycophenolate mofetil (MMF) represents a major advance in transplant medicine. Here, we investigated the *in vitro* and *in vivo* pharmacological effects of a novel IMP dehydrogenase inhibitor, AS2643361, in several immunological and non-immunological models. The *in vitro* inhibitory activity of AS2643361 on immune cell and endothelial cell proliferation and on antibody production from lipopolysaccharide-stimulated B cells, was significantly more potent than that of mycophenolic acid, the active form of MMF, despite the similar potency of these compounds on IMP dehydrogenase. In a rat heterotopic cardiac transplant model, monotherapy using orally administered AS2643361 at 10 or 20 mg/kg/day prolonged the median graft survival time from 6 to 16 and 19 days, respectively. In dinitrophenol-lipopolysaccharide stimulated rats, oral administration of AS2643361 at 2.5, 5 or 10 mg/kg/day resulted in suppression of antibody production. *In vivo* antibody production against alloantigen was also suppressed by AS2643361 treatment at 5 or 10 mg/kg/day. Furthermore, treatment with AS2543361 effectively inhibited balloon injury induced-intimal thickening, which is a major cause of late allograft loss. Overall, the *in vivo* activity of AS2643361 was over two-fold more potent than that of MMF. In addition, gastrointestinal toxicity, considered a dose-limiting factor for MMF, was reduced with AS2643361 treatment. These results suggest AS2643361 has higher potency and less toxicity than MMF, making it a potential candidate for treatment of acute and chronic rejection in transplant medicine.

1. Introduction

Inosine 5′-monophosphate (IMP) dehydrogenase (EC 1.1.1.205) catalyzes the NAD^+-dependent conversion of IMP to xanthosine 5′-monophosphate and is the rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides, which are also synthesized from guanine by a salvage reaction catalyzed by hypoxanthine-guanine phosphoribosyltransferase. IMP dehydrogenase is encoded by two distinct cDNAs that show 84% sequence similarity (Collart and Huberman, 1988; Natsumeda et al., 1990). However, the regulation of the two IMP dehydrogenase genes differs dramatically (Gu et al., 1997; Zimmermann et al., 1995). The increased IMP dehydrogenase activity observed in replicating or neoplastic cells is largely due to increased expression of IMP dehydrogenase Type II, whereas expression of IMP dehydrogenase Type I is relatively unaffected by cell proliferation or transformation (Nagai et al., 1991, 1992). The expression of both genes is, however, increased by mitogen activation of lymphocytes (Dayton et al., 1994). It has been suggested that the pharmacological inhibition of IMP dehydrogenase results in the depletion of guanosine nucleotides, leading to suppression of proliferation in cells such as lymphocytes. Indeed, lymphocyte proliferation is strongly inhibited by a potent and uncompetitive reversible inhibitor of human IMP dehydrogenase Types I and II, mycophenolic acid (MPA) (Allison and Eugui, 2000).

Mycophenolate mofetil (MMF) is the first pharmaceutical prodrug to be derived from MPA and is often used to prevent acute rejection in transplantation, where it dramatically improves patient outcome. Furthermore, several reports have suggested that MMF can reduce renal fibrosis (Ojo et al., 2000) and intimal thickening (Gibson and Hayden, 2007), two major morphological characteristics of chronic allograft nephropathy, compared to other immunosuppressants. Although IMP dehydrogenase inhibition can be beneficial for transplant outcome, MMF treatment shows a high incidence of gastrointestinal toxicity, leucopenia, anemia and opportunistic infections (European Mycophenolate Mofetil Cooperative Study Group, 1995; Salvadori et al., 2004). Where gastrointestinal toxicity necessitated MMF dose reduction, an increased risk of graft failure was reported (Bunnapradist et al., 2006). Enteric-coated mycophenolate sodium was designed to

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reduce the MPA-related gastrointestinal toxicity normally seen in MMF treatment (Chan et al., 2006). In *de novo* renal transplant patients, however, enteric-coated mycophenolate sodium showed no improvement (Ciancio et al., 2008; Salvadori et al., 2004). Therefore, an IMP dehydrogenase inhibitor that shows equivalent efficacy and greater safety than MMF and enteric-coated mycophenolate sodium is required.

Recent experimental studies using rodent models have reported that graft rejection is effectively prevented by a number of IMP dehydrogenase inhibitors that have different structures from MPA (Decker et al., 2001; Jain et al., 2002; Nakanishi et al., 2010). We identified an indole derivative as a lead from the Astellas compound library and optimized to obtain more potent inhibitory activity. N-((4-fluorophenyl)(1-methyl-1H-imidazol-2-yl)methyl)-2-methyl-3-(1,2,4-thiadiazol-5-yl)-1H-indole-6-carboxamide (AS2643361, Fig. 1) is a novel and orally available low-molecular weight IMP dehydrogenase inhibitor. We investigated the *in vitro* effects of AS2643361 on the proliferation of lymphocytes and endothelial cells, and on antibody production. Further, we characterized the *in vivo* pharmacological efficacy of AS2643361 using rats models of cardiac transplantation, antibody production and balloon injury.

2. Materials and methods

2.1. Animals

Adult Lewis ($RT1^{l}$), and ACI ($RT1^{avl}$) and Sprague–Dawley rats were purchased from Charles River Japan Inc. (Kanagawa, Japan) and Japan SLC, Inc. (Shizuoka, Japan), respectively. All animals were used in accordance with the guidelines of the Committee for Animal Experiments of Astellas Pharma Inc.

2.2. Materials

AS2643361 and MMF were prepared at Astellas Pharma Inc. (Tokyo, Japan).

2.3. IMP dehydrogenase Types I and II enzymatic activity

The enzymatic activities of IMP dehydrogenase Types I and II were assayed by the method of Jain et al. (2002) with slight modifications. IMP dehydrogenase Types I and II were purified from *E. coli* expressing human enzymes. The assay was performed using a flat bottom, UV-transparent 96-well plate. The final 200 μl reaction mixture contained 0.1 M Tris, 0.1 M KCl, 3 mM EDTA pH 8.0, 2 mM DTT, and 40 nM of either IMP dehydrogenase Type I or Type II. AS2643361 was dissolved in dimethyl sulfoxide and added to the reaction mixture to a final concentration of 0.5%. The reaction was initiated by adding 400 μM NAD and 400 μM IMP, followed by incubation at 37 °C for 2.5 h. The reaction rate of the conversion of NAD to NADH was then measured based on the increase in absorbance at 340 nm. The assays were also performed in the presence of 50% human serum (Millipore, Corp., MA, USA) to estimate serum protein binding by different IMP dehydrogenase inhibitors.

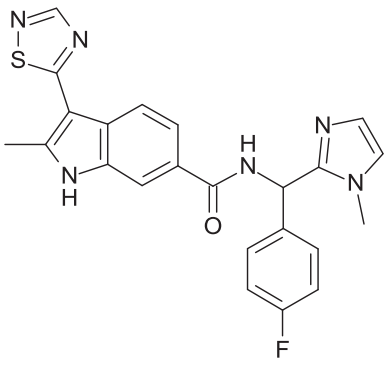

Fig. 1. Chemical structure of AS2643361.

2.4. T and B cell proliferation

The spleens of male Lewis rats aged eight weeks were aseptically removed and teased into single-cell suspensions and suspended in RPMI1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Assays were performed in flat-bottomed microtiter plates, with each well containing 1.5×10^5 splenocytes in 100 μl total volume. Splenocytes were incubated in medium containing either 1 μg/ml concanavalin A or lipopolysaccharide as either T or B cell mitogens along with various concentrations of AS2643361, at 37 °C for 48 h in a humidified atmosphere of 5% CO_2–95% air. During the final 6 h of incubation, cells were pulsed with 1 μCi of ^{3}H-thymidine/well, and harvested onto Perkin Elmer Unifilter GF/C plate (PerkinElmer, Inc., MA, USA) and counted in a liquid scintillation counter.

The *in vitro* immune response of alloantigen-specific T cells was evaluated as a proliferation response by using a one-way mixed lymphocyte reaction assay. Mesenteric lymph nodes cells from male Lewis rats and splenocytes from male ACI rats aged eight weeks were used as responder and stimulator cells, respectively. Single-cell suspensions were prepared, and the responder cells (2.5×10^5 cells) were cultured with irradiated (20 Gy) stimulator cells (2.5×10^5 cells) in RPMI1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in 96-well plates (U-bottom) and in the presence of various concentrations of AS2643361 (total volume: 200 μl, 37 °C, 5% CO_2 humidified atmosphere, 72 h). As a negative control, responder cells were cultured with irradiated splenocytes from Lewis rats. Cell proliferation was quantified by the uptake of ^{3}H-thymidine, as described above.

2.5. In vitro antibody production

Assays were performed in flat-bottomed microtiter plates, with each well containing 1.0×10^5 splenocytes in a total medium volume of 200 μl. Lewis rat splenocytes were incubated in medium containing 1 μg/ml lipopolysaccharide and various concentrations of AS2643361 at 37 °C for 72 h. After incubation, culture supernatants were collected and IgM concentrations were detected using a commercially available ELISA kit (Bethyl Laboratories, Inc., TX, USA). Each data point of the dose–response curve represents the mean of triplicate assays.

2.6. Human umbilical vein endothelial cell (HUVEC) proliferation

HUVECs (Cascade Biologics, OR, USA) were maintained in M200-LSGS medium (Cascade Biologics) containing 2% fetal calf serum, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 1 μg/ml heparin. Cells were used between passages 2 and 4 for each experiment. Assays were performed in flat-bottomed microtiter plates, with each well containing 5×10^3 HUVEC in a total volume of 200 μl. HUVECs were incubated with various concentrations of MPA or AS2643361 at 37 °C for 72 h in a humidified atmosphere of 5% CO_2–95% air. Cell proliferation was quantified by the uptake of ^{3}H-thymidine, as described above.

2.7. Cardiac transplantation

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 (Ono and Lindsey, 1969). AS2643361 (2.5, 5 and 10 mg/kg) was dissolved in propylene glycol. Beginning on the day of operation, AS2643361 was orally administered twice daily for 14 consecutive days. After transplantation, cardiac allograft function was assessed by daily palpation for 28 days, and graft rejection was defined as the cessation of palpable cardiac graft beats.

2.8. In vivo antibody production

On day 0, male Lewis rats aged six weeks were intravenously injected with 50 μg dinitrophenol-lipopolysaccharide. From day 0, AS2643361 prepared as described above was orally administered twice daily. Rats administered with propylene glycol were used as controls. On day 4 after dinitrophenol-lipopolysaccharide injection, rats were bled and plasma anti-dinitrophenol antibody (IgM) levels were determined by ELISA in a 96-well plate, in which immobilized dinitrophenol-bovine serum albumin (Cosmo Bio Co., Ltd., Tokyo, Japan) was incubated with plasma, followed by the addition of horse radish peroxidase-conjugated goat anti-rat IgM (Cosmo Bio Co., Ltd.). A peroxidase substrate (TMB microwell peroxidase substrate system, KPL Inc., MD., USA) was then added to each well, and the peroxidase reaction was stopped by adding 2 M sulfuric acid. The amount of anti-dinitrophenol IgM was determined based on absorbance measurements at 450 nm.

Male Lewis rats aged 6 weeks were infused intraperitoneally with splenocytes (4×10^7 cells/rat) derived from male ACI rats aged 6 weeks on day 0. From day 0, AS2643361 was orally administered twice daily. Rats administered with propylene glycol were used as negative controls. Rats were bled on day 7 after immunization to determine anti-allo IgM and IgG1/2a. Each serum was incubated with splenocytes (5×10^5 cells/well) from ACI rats for 30 min prior to the addition of FITC-conjugated anti rat IgM or IgG1/2a (BD Biosciences Pharmingen, Tokyo, Japan). The mean fluorescence intensity was detected by flow cytometry.

2.9. Balloon injury

Sprague–Dawley rats aged 11 weeks were anesthetized with pentobarbital (40 mg/kg). After dissecting the left carotid artery, the injury was performed by three passages of an inflated 2F Fogarty embolectomy catheter (Edwards Lifesciences, CA, USA). All procedures were performed under aseptic conditions. AS2643361 prepared as described above was orally administered twice daily from the operation for 14 consecutive days. MMF (40 mg/kg) suspended in 0.5% methylcellulose water was orally administered once daily for 14 consecutive days. The aortic samples were harvested 14 days after balloon injury, and analyzed in paraffin-embedded sample sections prepared and stained using the orcein staining method to stain the internal elastic lamina. Intimal and medial areas were calculated using computer planimetry. Intimal thickening was expressed as intimal/medial ratios.

2.10. Gastrointestinal toxicity

Normal Lewis rats aged seven weeks were administered escalating doses of AS2643361 twice daily. On day 4 after initiation of drug administration, the stool status for each rat was monitored and scored on a 0–3 scale (0: normal stool, 1: stool with soft surface, 2: soft stool, 3: watery stool). The mean score of each stool sample voided during a 6 h observation period was calculated for each rat.

2.11. Statistical analysis

All *in vitro* experiments were performed in duplicate or triplicate. IC_{50} values were determined by Sigmoid-Emax non-linear regression analysis. All analyses were performed using SAS software (SAS Institute Inc., NC, USA). Differences in *in vivo* antibody production and intimal thickening induced by balloon injury between control and dosed rats were compared using one-way analysis of variance followed by the Dunnett Multiple Comparison test. Median survival times of the grafts in the transplant study and median scores in the gastrointestinal toxicity study were analyzed by comparing groups using the log rank and Steel tests, respectively. Statistical analysis was performed using SAS software.

3. Results

3.1. In vitro activity of AS2643361

Our results showed that AS2643361 inhibited the activity of purified recombinant human IMP dehydrogenase Types I and II (Table 1). Further, the IC_{50} values for AS2643361 (51 and 19 nM for Types I and II, respectively) were similar to those for MPA (39 and 27 nM). However, with an IC_{50} of 71 nM in the presence of 50% human serum AS2643361 had seven to eight times the potency of MPA ($IC_{50}=540$ nM) which suggests lower serum protein binding by AS2643361. To determine the inhibitory activity on IMP dehydrogenase in cell-based assays, the effects of AS2643361 on the proliferation of immune cells were also investigated. The results showed that AS2643361 inhibited the proliferation of concanavalin A-stimulated T cells and lipopolysaccharide-stimulated B cells in a concentration-dependent manner with IC_{50} values of 19 nM and 20 nM, respectively (Table 1). AS2643361 also inhibited the proliferation of mixed lymphocyte reaction ($IC_{50}=9.0$ nM, Table 1). The results of immune cell proliferation assays showed a five- to six-fold increase in AS2643361 potency over MPA. The results also showed concentration-dependent inhibition of IgM production from lipopolysaccharide-stimulated B cells ($IC_{50}=22$ nM). Regarding the effect of AS2643361 on the proliferation of human endothelial cells, Fig. 2 shows that AS2643361 causes concentration-dependent inhibition of HUVEC proliferation with an $IC_{50}=45$ nM, making it three times more potent than MPA (140 nM).

3.2. Cardiac transplantation

The prevention of allograft rejection by orally administered AS2643361 was assessed in an ACI-to-Lewis rat heterotopic cardiac transplant model. Compared to a graft survival period of four to six days for rats administered with a vehicle control, treatment with 10 or 20 mg/kg/day AS2643361 resulted in a significant prolongation of graft survival, with median survival times of 16 and 19 days, respectively (Table 2). Treatment with 5 mg/kg/day AS2643361, however, showed no improvement in graft survival.

Table 1
Inhibitory activity of AS2643361 on IMP dehydrogenase Types I and II, and immune cell function (IC_{50} nM ± S.E.M.).

	AS2643361	MPA
Enzymatic activity		
IMP dehydrogenase Type I	51 ± 5	39 ± 10[a]
IMP dehydrogenase Type II	19 ± 5	27 ± 4[a]
IMP dehydrogenase Type II (w/human serum)[b]	71 ± 15	540 ± 49
Cellular activity		
Concanavalin A-stimulated T cell proliferation	19 ± 6	100 ± 26[a]
Lipopolysaccharide-stimulated B cell proliferation	20 ± 4	120 ± 29[a]
Mixed lymphocyte reaction	9.0 ± 4	51 ± 6[a]
In vitro IgM production	22 ± 5	110 ± 15[a]

[a] Previous data (Nakanishi et al., 2010).
[b] IMP dehydrogenase Type II inhibition activity in the presence of 50% human serum.

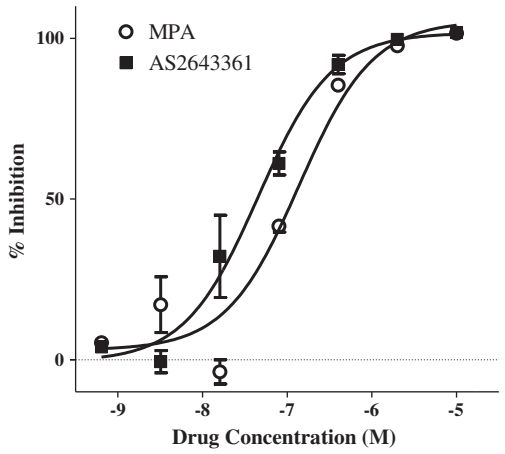

Fig. 2. Effect of MPA and AS2643361 on HUVEC proliferation. HUVECs were incubated with MPA (○) or AS2643361 (■). After 72 h of incubation, cell proliferation was quantified by the uptake of ^{3}H-thymidine. Each value represents the mean percent ± S.E.M. of triplicate cultures.

3.3. In vivo antibody production

The effect of AS2643361 on *in vivo* antibody production of plasma anti-dinitrophenol IgM levels in Lewis rats was evaluated on day 4 after dinitrophenol-lipopolysaccharide stimulation, the peak level of antibody production (Fig. 3A). AS2643361 showed a significant and dose-dependent suppressive effect on the production of anti-dinitrophenol antibodies, with 95% maximum inhibition at the highest concentration studied (10 mg/kg/day). AS2643361 were also evaluated in an *in vivo* alloantigen-specific antibody production model in which Lewis rats were immunized with splenocytes from ACI rats. Both IgM and IgG1/2a production were detectable using flow cytometry on day 7 after immunization. Administration of AS2643361 showed a significant suppressive effect on the production of alloantigen-reactive antibody (Fig. 3B). Results showed that AS2643361 at 5 mg/kg/day and 10 mg/kg/day inhibited IgM by 85% and 93%, and IgG1/2a production by 86% and 99%, respectively.

3.4. Balloon injury

To examine the effect of AS2643361 on intimal thickening formation, the area of newly formed intima was measured in a rat model of balloon-induced arterial vascular injury. Typical images of orcein staining and mean intimal/medial ratios of each group are shown in Fig. 4. After 14 days of balloon injury, intimal thickening was commonly observed in the aortic sample of rats administered no drug (Fig. 4A). Administration of AS2643361 showed statistically significant and dose-dependent suppressive effects on balloon-injury induced intimal thickening (Fig. 4B–E). Treatment with 20 mg/kg/day AS2643361 and 40 mg/kg/day MMF showed identical efficacy (Fig. 4E).

Table 2
Effect of AS2643361 on graft survival in ACI-to-Lewis rat cardiac transplantation.

Treatment	N	Graft survival (days)	MST[a]
Vehicle	4	4, 6, 6, 6	6
AS2643361 5 mg/kg/day	3	7, 7, 10	7
AS2643361 10 mg/kg/day	4	14, 14, 18, 18	16[b]
AS2643361 20 mg/kg/day	4	12, 18, 20, 20	19[b]

[a] MST: Median survival times (days).
[b] Significant differences from vehicle control rats according to Bonferroni multiple correction ($P<0.0167$), after median survival times of the grafts were analyzed by comparing groups using the log rank test.

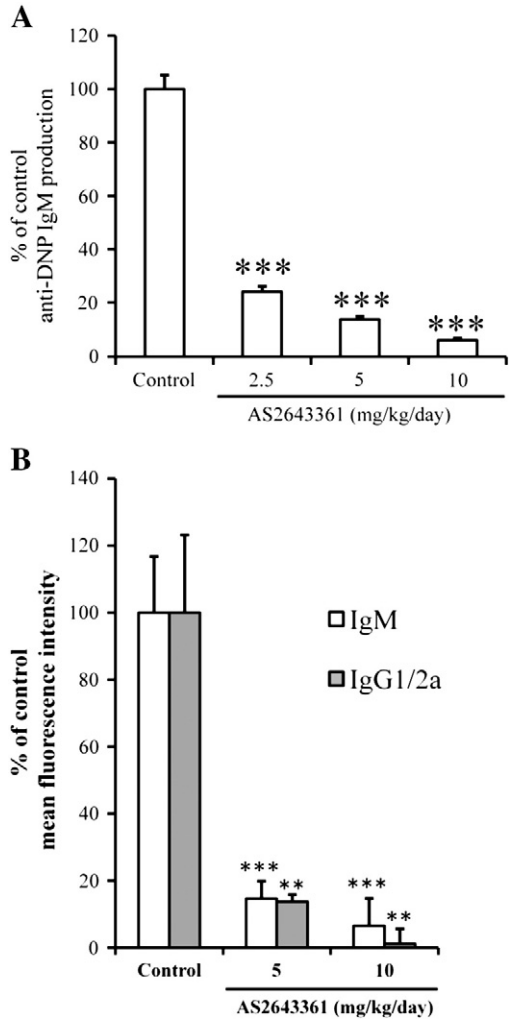

Fig. 3. Effect of AS2643361 on *in vivo* anti-dinitrophenol IgM production (A) and *in vivo* alloantigen-specific IgM and IgG1/2a production (B). n = 4 for all groups. Differences between control and dosed rats were compared using one-way analysis of variance followed by the Dunnett Multiple Comparison test. $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) were considered significant.

3.5. Gastrointestinal toxicity

To determine the gastrointestinal toxicity of AS2643361, the stool status of each rat was monitored and scored. Our previous study showed MMF at doses of over 60 mg/kg/day in the same model induced severe diarrhea, including watery stools on day 4 after the start of administration (Nakanishi et al., 2010). Our present results show no deleterious effect of AS2643361 even at doses of 60 mg/kg/day, indicating that it exerts less gastrointestinal toxicity than MMF.

4. Discussion

These results show that the *in vitro* inhibitory activity of a novel IMP dehydrogenase inhibitor, AS2643361, on T and B cell proliferation and antibody production is more potent than that of other IMP dehydrogenase inhibitors such as MPA and BMS-566419, whose activities we reported in our previous study (Nakanishi et al., 2010). The higher activity of AS264331 observed in cell-based assays may be an effect of higher cell permeability or lower binding to fetal calf serum in assay medium, possibilities that are supported by its higher inhibitory activity on IMP dehydrogenase Type II in the presence of human serum. We also found that AS2643361 prolonged graft survival in an ACI-to-Lewis rat heterotopic cardiac transplant model. Comparing our results, the *in*

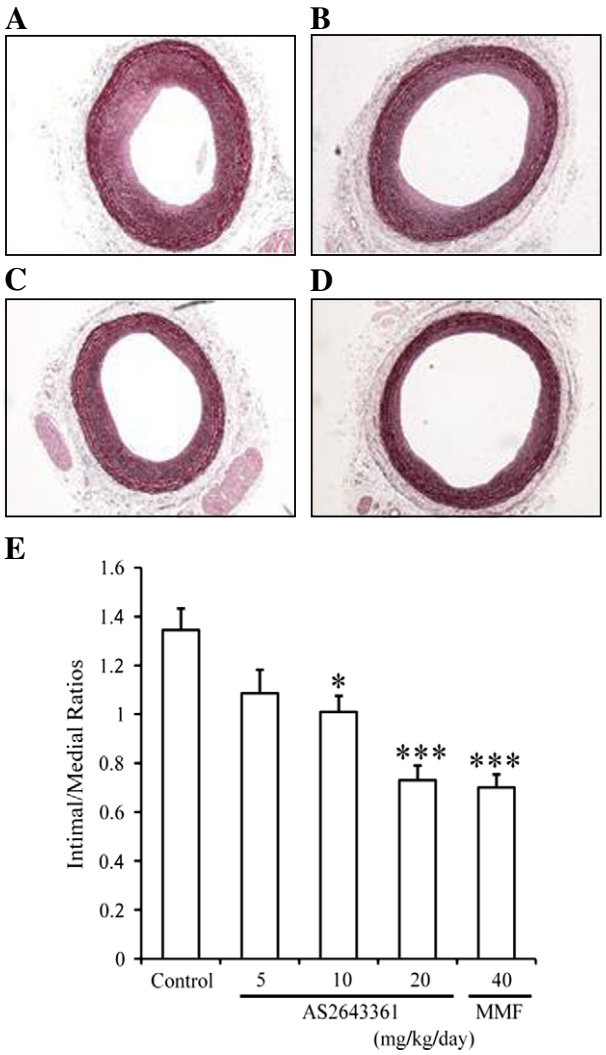

Fig. 4. Typical microscopic images of rat aorta following balloon injury stained by the orcein staining method in the vehicle control group (A), 5 mg/kg/day AS2643361 group (B), 10 mg/kg/day AS2643361 group (C), and 20 mg/kg AS2643361 group (D). Intimal thickening was expressed as intimal/medial ratio and calculated using computer planimetry (E). Differences between control and dosed rats were compared using one-way analysis of variance followed by the Dunnett Multiple Comparison test. $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) were considered significant.

vivo inhibitory potency of AS2643361 on allograft rejection is two to four times higher than that of either MMF or BMS-566419 (Nakanishi et al., 2010). Compared to MMF, which requires relatively high dosing from 500 to 2000 mg/day (Budde et al., 2007; Patel et al., 2007; Pawinski et al., 2006), these findings suggest that AS2643361 is more effective at lower doses, and could offer increased medical compliance and adherence for the prevention of graft rejection in clinical practice.

Three mechanisms for graft rejection prevention by MPA have been proposed to explain IMP dehydrogenase inhibitor efficacy (Allison and Eugui, 2000): induction of activated T cell apoptosis; suppression of glycosylation and expression of adhesion molecules by guanosine nucleotide depletion; and depletion of tetrahydrobiopterin, a cofactor involved in the inducible form of nitric oxide synthase. Because our cardiac transplant studies showed identical efficacy between AS2643361 and MMF, we propose that AS2643361 uses one or a combination of these mechanisms, although further studies are needed.

Currently, standard immunosuppressive protocols consist of three drug groups, calcineurin inhibitors, corticosteroids, and antiproliferative agents, among which MMF is the most widely used agent. Our previous study in rat transplant models showed that the combination of IMP dehydrogenase inhibitors with FK506 significantly prolongs graft survival (Nakanishi et al., 2010), suggesting that the two operate *via* different pharmacological mechanisms. IMP dehydrogenase inhibitors do not interrupt interleukin-2 signaling (Eugui et al., 1991), or interfere with signaling pathways such as those involving extracellular signal-related kinase 2 or signal transduction and activation of transcription 5 (STAT5) phosphorylation (Quemeneur et al., 2002). They do, however, inhibit the cleavage of cyclin-dependent kinase inhibitor $p27^{Kip1}$ as well as induce cell cycle inhibition (Quemeneur et al., 2002). In general, the pharmacological profile of IMP dehydrogenase inhibition is significantly distinct from the profiles of other immunosuppressive drugs that inhibit interleukin-2 production, such as FK506 and cyclosporine A, or those that interfere with interleukin-2 receptor signaling events, such as rapamycin and anti-CD25 monoclonal antibodies. The results of the present study, which show that AS2643361 exerts significant efficacy against allograft rejection, suggest that combination therapy using AS2643361, calcineurin inhibitors, and corticosteroids could be optimal for clinical transplantation.

Donor-reactive antibodies are known to be produced during the post-transplant period and these contribute significantly to the pathology of both acute and chronic allograft rejection. Theruvath et al. (2001) reported that antibody responses are reduced when MMF is co-administered with FK506 in a transplant setting, while Mannami and Mitsuhata (2005) reported that MMF decreased humoral rejection and improved transplant outcomes. Our results show that *in vivo* anti-dinitrophenol antibody production and alloantigen-specific alloantibody production is significantly suppressed by AS2643361 at a higher potency than other IMP dehydrogenase inhibitors (Nakanishi et al., 2010). Overall, these results suggest that AS2643361 strongly suppresses alloantibody production as well as reduces antibody-mediated rejection in clinical transplantation.

Despite the introduction of calcineurin inhibitors and significant improvements in short-term graft survival, chronic allograft nephropathy remains a major cause of late renal allograft loss. Intimal thickening and interstitial fibrosis are morphological characteristics of chronic allograft nephropathy, and are major impediments to long-term graft survival (Nankivell et al., 2001). Furthermore, intimal thickening produced by graft vascular disease is also a major complication that limits the long-term survival of cardiac transplantation (Miller, 1992). We therefore induced intimal thickening using non-immunological balloon injury, a common animal model for intimal thickening (Li et al., 2010; Wang et al., 2009), to examine if drugs that inhibit intimal thickening also have the potential to attenuate the progress of chronic allograft rejection. Indeed, vascular smooth muscle cell proliferation is a major component in intimal thickening of both allografts and balloon injury models (Li et al., 2010; Michael, 2003). Consistent with previous studies (Gregory et al., 1995), our results showed that MMF attenuated the progression of balloon injury-induced intimal thickening. Further, several reports have suggested that MMF has anti-intimal thickening properties in both animal and human allografts (Jolicoeur et al., 2003; Kobashigawa et al., 2006). In the present study, we found that AS2643361 inhibited balloon-induced intimal thickening with an even higher potency than MMF. Because endothelial cells play multiple and important roles in transplant immunology and stimulate the migration and proliferation of smooth muscle cells (McDonald et al., 1999), we also investigated the effect of AS2643361 on HUVEC proliferation. The inhibitory effect shown in this study suggests that AS2643361 is a potent and potentially beneficial new drug for the treatment of both acute rejection and intimal thickening, or chronic rejection, in transplantation medicine.

A number of clinical studies have reported that MMF shows dose-limiting gastrointestinal toxicity, including gastritis, diarrhea, anorexia, nausea, abdominal pain and vomiting (European Mycophenolate Mofetil Cooperative Study Group, 1995). Possible etiologies for MPA-related gastroenteropathy include direct gut toxicity by an MMF metabolite, systemic or local antiproliferative effects due to IMP dehydrogenase inhibition, combination toxicity with calcineurin inhibitors, opportunistic

infectious gastroenteritis, modulation of local immune responses, and local toxicity of acyl-mycophenolic acid glucuronide, a metabolite of MPA (Arns, 2007). Another study also reported that gastrointestinal toxicity is related to the enterohepatic recirculation properties of MPA rather than plasma MPA levels (Sugioka et al., 2006). Furthermore, other studies reported that BMS-566419, whose chemical structure differs from MPA and MMF, shows less gastrointestinal toxicity and a wider therapeutic window than MMF (Nakanishi et al., 2010; Watterson et al., 2007). Our present results showed that AS2643361 has no obvious gastrointestinal toxicity at up to 60 mg/kg/day, in contrast to MMF, which induced severe diarrhea at 60 mg/kg/day (Nakanishi et al., 2010). Based on the similar median survival times prolongations, our transplant study shows that AS2643361 is two to four times more potent than MMF. Our results also suggest that AS2643361 has four- to eight-fold lower gastrointestinal toxicity than MMF. Details of the mechanism of this lower gastrointestinal toxicity remain unclear, although it is possibly related to the pharmacokinetic profile of AS2643361, which may not exhibit enterohepatic recirculation or produce gastrointestinal toxic metabolites, unlike MPA and MMF. Further investigation of toxicity other than gastrointestinal symptoms is also required before AS2644361 can be used clinically.

5. Conclusions

In this study, we show that the pharmacological effects of AS2643361 *in vitro* and *in vivo* are much more potent than those of either MPA or MMF, and that AS2643361 has lower gastrointestinal toxicity than MMF in animal experiments. These findings suggest that AS2643361 is a potential candidate for acute and chronic rejection in transplant medicine.

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