Prenylated quinolinecarboxylic acid derivative suppresses immune response through inhibition of PAK2

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Abstract

Development of new immunosuppressing agents is necessary in organ transplantation or immune diseases. Because Ppc-1 exhibits a suppressing effect on interleukin-2 (IL2) production in Jurkat cells, we synthesized and screened Ppc-1 derivatives that preserve prenylated quinolinecarboxylic acid (PQA) structure, and identified compound 18 (PQA-18) as a novel molecule with immunosuppressing effect. PQA-18 suppressed not only IL2 but also IL4, IL6, and tumor necrosis factor- α production in human peripheral lymphocytes without affecting cell viability.

Two-dimensional gel electrophoresis analysis and *in vitro* kinase assay revealed that PQA-18 inhibits kinase activity of p21-activated kinase 2 (PAK2). Administration of PQA-18 by intraperitoneal injection suppressed the population of a subset of regulatory T cells and the immunoglobulin (Ig) production against T cell-dependent antigens in mice. Treatment with the PQA-18 ointment on Nc/Nga mice, a model of human atopic dermatitis, improved skin lesions and serum IgE levels. These results suggest that PQA-18 is a unique PAK2 inhibitor with potent immunosuppressing effects *in vitro* and *in vivo*. PQA-18 may be a valuable lead for the development of novel immunosuppressants.

Keywords

cytokine, immune response, immunosuppressant, dermatitis, small molecule

Chemical compounds studied in this article

Ppc-1 (PubChem CID: 46910769); PQA-18 (PubChem CID: 73602831)

1. Introduction

New immunosuppressing agents that do not cause side effects are needed for the treatment of patients post transplantation, or those with autoimmune or allergic diseases. Interleukin-2 (IL2) producing Jurkat cells is often employed for selecting new immunosuppressing compounds, and contribute to discover potent immunosuppressants such as FK506 and cyclosporine A. FK506 and cyclosporine A bind to their specific immunophilins FKBP12 and cyclophilin, respectively, resulting in the inhibition of calcineurin, a key enzyme required for the functions of immune cells [1,2]. Both FK506 and cyclosporine A have been actually shown to be effective immunosuppressants in organ transplantation, autoimmune or allergic diseases such as atopic dermatitis (AD) [3-5]. Despite their effectiveness, long-term use of these immunosuppressants is associated with several adverse events including nephrotoxicity, diabetogenicity, neurotoxicity and liver toxicity, which impose serious problems in immunosuppressive therapy [6,7].

Cellular slime molds are soil microorganisms that produce many pharmacologically active molecules and are an important source of lead compounds for medical research. The slime mold *Dictyostelium discoideum* forms a fruiting body consisting of spores and a multicellular stalk at the end of its life cycle in response to differentiation inducing factor-1 (DIF-1), a putative morphogen that induces stalk cell differentiation [8]. The first metabolite produced during DIF-1 degradation is DIF-3, which, unlike DIF-1, has virtually no activity in the induction of stalk cell

differentiation [9]. Nevertheless, both DIF-1 and DIF-3 possess anti-tumor activity that suppresses mammalian cell proliferation and, in some cases, induces or promotes the differentiation of de-differentiated tumor cells *in vitro* [10-18]. In addition, derivatives of DIF-1 and DIF-3 promote IL2 production through enhancement of AP-1 activity [19,20]. Recently, we reported that Ppc-1, a secondary metabolite containing a unique structure that has prenylated quinolinecarboxylic acid (PQA), derived from *Polysphondylium pseudo-candidum*, enhances mitochondrial oxygen consumption and induces weight loss in mice without lesional changes in kidney or liver tissues and without tumor formation [21-23]. Ppc-1 also shows an inhibitory effect on concanavalin A (ConA)-induced IL2 production.

In the present study, we synthesized and screened a series of Ppc-1 derivatives, and demonstrated that compound 18 (PQA-18) strongly suppresses IL2, IL4, IL6, and tumor necrosis factor- α (TNF α) production through inhibition of the activity of p21-activated kinase 2 (PAK2). Administration of PQA-18 suppressed the population of a subset of regulatory T cells and antigen-specific immune response in mice, as well as alleviated dermatitis in a mouse model of human AD. These findings suggest that PQA-18 is a novel PAK2 inhibitor with potent immunosuppressing effects.

2. Materials and methods

2.1. Antibodies and Chemicals

The PQA compounds used in this study were synthesized and purified as previously described [23], and the structure and purity were confirmed by ^1H and ^{13}C NMR spectroscopy and high-resolution mass spectroscopy. The purities of all compounds were greater than 98%. Mouse anti-FLAG M2 monoclonal antibody (mAb), mouse anti- β -actin mAb, dimethyl sulfoxide (DMSO), glyceryl trioctanoate, and ConA were purchased from Sigma-Aldrich (St. Louis, MO); rabbit anti-PAK2 polyclonal antibody, rabbit anti-phospho-PAK2 (Ser141) polyclonal antibody, and FK506 were obtained from Cell Signaling Technology (Beverly, MA); recombinant human histone H4 protein was from New England Biolabs (Beverly, MA); sequencing grade modified trypsin and DNase I were from Promega (Madison, WI); Imject Alum Adjuvant, human IL2 enzyme-linked immunosorbent assay (ELISA) kit, and SuperScript Reverse Transcriptase III were from Thermo Fisher Scientific (Waltham, MA); mouse IgE ELISA kit, human IL4, IL6, and TNF α ELISA kit, and Propidium iodide (PI) were from eBioscience (San Diego, CA); vaseline and Lys-C were from Wako (Tokyo, Japan); ISOGEN was from NIPPON GENE Co. (Tokyo, Japan); $\gamma[^{32}\text{P}]\text{ATP}$ was from PerkinElmer (Waltham, MA); horseradish peroxidase-conjugated anti-mouse IgM, IgG $_1$, or IgG $_3$ antibodies were from SouthernBiotech (Birmingham, AL);

SureBlue was from KPL (Gaithersburg, MD); (4-hydroxy-3-nitrophenyl)acetyl-conjugated chicken γ -globulin (NP-CGG) and NP-bovine serum albumin (NP-BSA) were from Biosearch Technologies (Petaluma, CA); Immobilized pH gradient (IPG) gel strips (pI 4-7, pI 3-10, 18 cm) were from GE Healthcare (Waukesha, WI). All other chemicals and reagents were of the highest grade commercially available.

2.2. Cell cultures

Human T cell leukemia Jurkat cells were cultivated in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 4 mM L-glutamine (Thermo Fisher Scientific) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Human peripheral lymphocytes were prepared from healthy volunteers by Lymphoprep (AXS-Shield, Oslo, Norway) according to the manufacturer's recommended protocol and cultivated in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS and 4 mM L-glutamine.

2.3. Measurement of human cytokines

Jurkat cells and peripheral lymphocytes were cultivated in 96-well plates in the presence of ConA at 40 μ g/mL and at 10 μ g/mL, respectively, and treated with either 0.1% DMSO, PQA-18, or the

derivatives [24]. After 24 h, supernatants were collected and the IL2, IL4, IL6, and TNF α levels were determined by human ELISA kits according to the manufacturer's recommended protocol.

2.4. Quantitative PCR

Total RNA was extracted from Jurkat cells using the standard ISOGEN procedure. First-strand cDNA synthesis was performed with 10 μ g RNA using SuperScript Reverse Transcriptase III and random hexamer primers (Thermo Fisher Scientific). The resulting cDNA was amplified using TaqMan probes and the ABI Prism Sequence Detection System 7000 (Thermo Fisher Scientific). Three replicates were run for each real-time PCR reaction. Results were normalized to the endogenous β -actin within the log-linear phase of the amplification curve obtained for each primer using the comparative Ct method. The TaqMan gene expression assays used were Hs00174114_m1 (IL2) and Hs99999903_m1 (β -actin).

2.5. Flow cytometric analysis

To analyze the number of regulatory T cells, single-cell suspensions from mouse spleen were obtained by depletion of erythrocytes by osmotic stress and aliquots of cells suspensions (10^6 cells) were stained with anti-Foxp3-PE (FJK-16s) mAb, anti-CD4-FITC (RM4-5) mAb, and anti-CD25-PE-Cy7 (PC61.5) mAb (eBioscience) and analyzed with FACSCanto II (BD

Biosciences, San Jose, CA). Cell viability was evaluated with PI (5 µg/ml) staining in Jurkat cells after treatment with either PQA-18 at 20 µM or calcium ionophore A23187 at 1 µM for 24 h.

2.6. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) analysis

Jurkat cells were solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer, 1 mM benzamidine, 25 µg/ml leupeptin, 20 µg/ml pepstatin-A, 20 µg/ml aprotinin, 1 mM Na₃VO₄, 1 µM microcystin-LR, 20 mM dithiothreitol), and the lysate was clarified by centrifugation at 100,000 g for 30 min. After protein determination by a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA), the supernatants (200 µg) were processed for isoelectric focusing as described previously [25] using IPG gel strips and 12.5% SDS-PAGE. 2-DE gels were silver stained using methanol fixation (Silver stain MS kit, Wako, Tokyo, Japan). Gels were analyzed using a Melanie III Viewer (GeneBio, Geneva, Switzerland) to detect changes in protein spot intensities on the gel images. Responsive proteins were identified by in-gel digestion from wet gels using both trypsin and Lys-C, and desalted on C18 spin column (Thermo Fishier Scientific). The resulting peptides were analyzed by Orbitrap Elite mass spectrometer (Thermo Fishier Scientific) [26]. Proteome Discoverer 1.4 software (Thermo Fishier Scientific) was used to generate the peak lists of all acquired MS/MS spectra, and all peptides were then searched against the human SWISS-PROT protein sequence database

(Sprot.human.57.fasta; 545,388 entries) using the Mascot 2.4 searching program.

2.7. Western blotting

2-DE gels were removed from glass plates and proteins were transferred to PVDF filter membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with primary antibodies. Blots were probed with goat anti-rabbit antibody coupled to HRP (Bio-Rad Laboratories), and the positive signals were visualized by ECL (PerkinElmer).

2.8. *In vitro* kinase assay

PAK2 proteins recovered by anti-PAK2 antibody from Jurkat cells were incubated with histone H4 proteins in kinase buffer consisting of 20 mM Hepes-NaOH, pH 7.4, 10 mM MgCl₂, 200 μ M ATP, 0.01% BSA, and 92.5 kBq γ [³²P]ATP in a total volume of 40 μ l at 30°C for 20 min as previously described [26]. Phosphorylation of the H4 proteins was assessed by SDS/PAGE followed by autoradiography, and quantified using a densitograph.

2.9. Ethics Statement

All experiments were conducted in accordance with the guidelines of the National Institutes of

Health, as well as those of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and were approved by the Fukushima Medical University Animal Studies Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

2.10. Immunization and determination of immunoglobulin (Ig) titers

C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan), and housed at 21°C with a 12:12-h light/dark cycle with free access to water and a commercial diet. Male C57BL/6J mice (10 weeks old) were immunized by intraperitoneal injection of 50 µg NP-CGG pretreated with Imject Alum Adjuvant (Thermo Fisher Scientific). Vehicle (0.1% DMSO), PQA-18 (0.5 mg/kg), or FK506 (1.0 mg/kg) was administered by intraperitoneal injection three times a week, and blood was taken once a week from the tail. The serum levels of anti-NP antibodies were determined by ELISA. In brief, 96-well flat-bottom plates were coated with 2 µg/ml NP-BSA followed by blocking with 0.5% BSA in phosphate-buffered saline (PBS). Serially diluted sera were incubated in the plates at 4°C for 24 h. After washing with PBS, horseradish peroxidase-conjugated anti-mouse IgM, IgG₁, or IgG₃ antibodies were added to each well. SureBlue was used as substrate, and absorbance at 450 nm was measured using a spectrofluorophotometer (Varioskan Flash; Thermo Fisher Scientific). For tissue examination,

animals were treated with vehicle or PQA-18 (1.0 mg/kg) for six weeks and then euthanized to collect liver, kidney, and serum samples. Serum components including urea-N (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -GTP, and lactate dehydrogenase (LDH) were measured. The liver and kidney samples were fixed in 10% formalin solution. A block of these tissues was removed and embedded in paraffin by a conventional method. A 5- μ m section was stained with hematoxylin-eosin.

2.11. Evaluation of PQA-18 ointment

Nc/Nga mice (12 weeks old; Japan SLC, Shizuoka, Japan) with spontaneous dermatitis were used. After preliminary breeding for one week, the mice were divided into five groups and received no ointment treatment or an application of 100 mg of one of the following vaseline ointments to the skin of the ear, face, neck, and rostral back three times a week: vehicle (0.1% DMSO), 0.05% PQA-18, 0.1% PQA-18, or 0.1% FK506. The severity of dermatitis was assessed once a week by evaluating skin conditions such as skin dryness, eruption, and wound, using the following grades (no symptoms, 0; mild, 1; moderate, 2; severe, 3) [27]. Blood was taken once a week from the tail, and the serum IgE levels were measured by mouse IgE ELISA kit according to the manufacturer's recommended protocol. After four weeks of the ointment treatment, the animals were euthanized to collect skin samples, followed by fixation with 10% formalin. A block of the

skin specimen was removed and embedded in paraffin by the conventional method, and 5- μ m sections were stained with Toluidine blue or Luna. Thickness of the skin and the number of mast cells and eosinophils were determined in five fields for each sample.

2.12. Data analysis

The statistical significance of differences was determined using the one-way analysis of variance with Turkey-Kramer post-hoc comparisons and Student's *t*-test. Data are expressed as means and SD (**, $p < 0.01$; *, $p < 0.05$).

3. Results

3.1. Selection of PQA-18 as immunosuppressing agent

Our preliminary observation demonstrated that Ppc-1 has a weak but significant inhibitory effect on IL2 production in Jurkat cells (IC_{50} : 4 μ M) (Fig. 1A and 1B). In order to obtain more potent inhibitors for IL2 production than Ppc-1, we synthesized a number of Ppc-1 derivatives [23] and evaluated their effects on IL2 production. Assays were carried out using Jurkat cells treated with 40 μ g/mL of ConA for 24 h, and IL2 contents in culture supernatants were determined with ELISA. Among Ppc-1 derivatives that had previously been reported [23], the most potent inhibition was detected with PQA-18 (IC_{50} : 400 nM) (Fig. 1A and 1C); therefore, we chose this compound for further analyses. To understand the underlying mechanism of the inhibition of IL2 production by PQA-18, we examined its effect on IL2 expression in Jurkat cells. The cells were stimulated with ConA for 6 h in the presence of PQA-18 at different concentrations, followed by determination of IL2 mRNA levels. As shown in Fig. 1D, a marked increase in IL2 mRNA level was observed in ConA-stimulated Jurkat cells, and that was suppressed by treatment with PQA-18. This effect was rather weak; only ~50% of IL2 transcripts were affected by the treatment with a high dose (10 μ M) of PQA-18. These results suggest that PQA-18 may perturb post-transcriptional signaling pathways in Jurkat cells. PQA-18 did not affect cell viability (Fig.

1E).

We further examined the effect of PQA-18 on cytokines production in human peripheral lymphocytes. We selected IL2, IL4, IL6, and TNF α as typical cytokines for evaluation of its effects on Ig production, and allergy and inflammation. The cells were stimulated with 10 μ g/mL of ConA for 24 h in the presence of PQA-18 or FK506, and IL2, IL4, IL6, and TNF α contents in culture supernatants were determined with ELISA. PQA-18 markedly inhibited ConA-induced production of IL2 (IC₅₀: 450 nM), IL4 (IC₅₀: 650 nM), IL6 (IC₅₀: 580 nM), and TNF α (IC₅₀: 460 nM) in peripheral lymphocytes in a dose-dependent manner (Fig. 2). Strong inhibition was observed in cells treated with FK506 (Fig. 2). These results confirm the inhibitory effect of PQA-18 on cytokines production in human peripheral lymphocytes.

3.2. Inhibition of PAK2 activity by PQA-18

Effects of PQA-18 on protein levels were studied on 2-DE. Cellular proteins prepared from Jurkat cells with stimulation of ConA for 10 min in the presence or absence of 10 μ M PQA-18 were compared on 2-DE. Two proteins, cofilin-1 and histone H4 were identified as specific proteins reproducibly altered by treatment with PQA-18 by peptide sequencing by MS analysis. As shown in Fig. 3A, acidic form of cofilin-1 is significantly reduced by treatment with PQA-18, while its basic form is significantly increased. In addition, phosphorylation at Ser3 was detected

in acidic form of cofilin-1 (cofilin-1-pSer3), but not in the basic form by MS analysis. Similar results were obtained in both acidic and basic forms of histone H4 (Fig. 3B), although phosphorylation sites were not yet identified. These results suggest that PQA-18 attenuates phosphorylation of these proteins by the inhibition of corresponding kinase. Recent studies demonstrated that PAK2 phosphorylates cofilin-1 at Ser3 and histone H4 at Ser47 [28,29]. Therefore, PAK2 was analyzed by Western blotting on 2-DE using anti-PAK2 antibody. As shown in Fig. 3C, PAK2 formed a line of four spots (spot 1-4 in Fig. 3C) with distinct pI in non-treated control and PQA-18-treated cells. The intensities of acidic spots 1 and 2 were significantly reduced by treatment with PQA-18, while those of basic spots 3 and 4 were significantly increased, suggesting that PQA-18 may affect phosphorylation status of PAK2. Since autophosphorylation of PAK2 at Ser141 plays a key role for maintaining the protein kinase in an active conformation [30], the spots was re-evaluated with an antibody specific for Ser141-phosphorylation (PAK2-pSer141). The majority of positive signal was detected in acidic spot 1 in control cells, while weak signal was detected in the spot 2 in PQA-18-treated cells (Fig. 3D). These results suggest that PQA-18 inhibits PAK2 activity by its autophosphorylation at Ser141.

We next examined the effect of PQA-18 on PAK2 kinase activity. For this assessment, immunoprecipitates with anti-PAK2 antibody prepared from Jurkat cells and recombinant histone

H4 protein were employed as an enzyme source and a substrate, respectively [29].

Phosphorylation of H4 by PAK2 was linearly increased with incubation time (Fig. 4A) and was inhibited by the addition of PQA-18 in a concentration-dependent manner (IC_{50} : 10 nM) (Fig. 4B). When the inhibition kinetics was analyzed by the Lineweaver-Burk plot method, the K_m values were 1.9 μ M and 2.1 μ M, and the V_{max} values were 4.1 pmol/min and 3.2 pmol/min for vehicle and PQA-18, respectively (Fig. 4C). These results suggest that PQA-18 inhibits PAK2 kinase activity in a non-competitive manner.

3.3. Suppression of immune response by PQA-18

To assess the immunosuppressive effect of PQA-18 *in vivo*, C57BL/6J mice were immunized with NP-CGG to elicit T cell-dependent immune response [31], and treated with vehicle (0.1% DMSO), PQA-18 (0.5 mg/kg) or FK506 (1.0 mg/kg) three times a week by intraperitoneal administration. FK506 was used as a positive control. Peripheral blood was taken from each animal once a week, and the levels of antigen-specific IgM, IgG₁, and IgG₃ were determined by ELISA assays. The NP-specific responses were significantly suppressed in all Ig classes tested in mice treated with PQA-18 or FK506 compared with vehicle (Fig. 5A). Significant suppression was observed at all time points in the PQA-18-treated animals. We further examined the effect of PQA-18 on the regulatory T cells. C57BL/6J mice were immunized with NP-CGG, and treated

with vehicle (0.1% DMSO) or PQA-18 (0.5 mg/kg) three times a week by intraperitoneal administration. After a week of immunization, the Foxp3⁺CD4⁺CD25⁺ regulatory T cells in the spleen of each animal were analyzed by a multiparameter flow cytometric analysis. The number of Foxp3⁺CD4⁺CD25⁺ cells was increased by the immunization, which was significantly suppressed by the treatment with PQA-18 (Fig. 5B). These findings indicate that PQA-18 displays immunosuppressing activity *in vivo*. On the other hand, no abnormal changes were detected in liver and kidney tissues (Fig. 5C) or in the serum levels of AST, ALT, γ -GTP, BUN, creatinine, and LDH derived from mice administered PQA-18 (1.0 mg/kg) for six weeks (Table 1).

3.4. Improvement of dermatitis by treatment with PQA-18 ointment

To test the applicability of PQA-18 to allergic diseases *in vivo*, we used Nc/Nga mice as a model for AD [32]. Nc/Nga mice spontaneously develop severe eczema with aging under pathogen-free conditions; the histology of its dermatitis mimics human AD. Moreover, the serum IgE level is elevated after the onset of dermatitis. Nc/Nga mice (13 weeks of age) were divided into five groups (nine animals in each group) then treated without or with the ointment (100 mg/mouse) containing vehicle (0.01% DMSO), 0.05% PQA-18, 0.1% PQA-18, or 0.1% FK506 three times a week. While typical symptoms of severe dermatitis were observed in the mice with vehicle and

no treatment for four weeks, skin conditions, appearance, and severity scores were remarkably improved in all animals treated with the PQA-18 or FK506 ointment (Fig. 6A). In addition, improvement of serum IgE levels was also observed in the mice treated with 0.1% PQA-18 or 0.1% FK506 ointment (Fig. 6B). The histological assessment revealed that thickening of the epidermis, prominent hyperkeratosis, and infiltration of inflammatory cells were observed in mice treated with vehicle or no treatment at the age of 17 weeks (Fig. 6C). Morphological assessments revealed staining-identified infiltrates in the dermis were degranulated mast cells (Fig. 7A) and eosinophils (Fig. 7B). Application of the PQA-18 and FK506 ointment significantly reduced epidermis hypertrophy, hyperkeratosis (Fig. 6C), as well as excess infiltration of mast cells (Fig. 7A), and eosinophils (Fig. 7B). These results show that treatment with the PQA-18 ointment improves dermatitis and IgE levels in Nc/Nga mice.

4. Discussion

In this study, we have identified PQA-18 as a novel immunosuppressing agent by screening the PQA compounds structurally related to Ppc-1 (Fig. 1). PQA-18 inhibits cytokines production in ConA-stimulated human peripheral lymphocytes (Fig. 2). Comparison studies of protein levels in PQA-18-treated and non-treated cells have suggested that PAK2 activity might be a target of PQA-18 (Fig. 3); PQA-18 actually inhibits kinase activity of PAK2 in a non-competitive manner *in vitro* (Fig. 4). PAKs, effector molecules of Rac and Cdc42, are serine/threonine kinases that phosphorylate multiple substrates, including those that are involved in cytoskeletal reorganization, cell proliferation, and survival [30,33,34]. The PAK family is divided into two groups (Group I and Group II) based on sequence and structural homology, PAK2 is the major PAK expressed in T cells and has been implicated in T cell function, development, and maturation [35]. A recent report has suggested that PAK2 kinase activity is involved in TCR signaling and NF-AT activation [34]. Here, we have demonstrated that the PAK2 inhibitor PQA-18 significantly suppresses immune responses to T cell-dependent antigens (Fig. 5) and improves established dermatitis (Fig. 6 and 7). Based on these results, we conclude that PQA-18 attenuates T cell activation through the inhibition of PAK2 activity, which in turn suppresses immune responses and alleviates AD. To our knowledge, this is the first direct demonstration of a new type of immunosuppressant with inhibition of PAK2 kinase.

Recently, several small molecules, including IPA-3, which selectively inhibits Group I PAKs in a non-competitive manner, were discovered to be inhibitors of PAK functions [33,36,37]. Substantial data link the expression and hyperactivity of PAKs to tumorigenesis and metastasis, and elevated PAK expression is associated with various human tumors such as breast cancer, neurofibromatosis, colon cancer, and lung cancer [33,34]. PAKs maintain cell transformation by promoting a number of hallmark processes including cell proliferation, survival, motility, and angiogenesis. Therefore, PAK inhibition is of high interest as a therapeutic strategy. In addition, recent reports have suggested that the highly conserved HIV protein, Nef, specifically associates with and activates PAK2, but not PAK1, in T cells [38]. Nef plays an essential role in the pathogenesis of HIV and subsequent development of AIDS by promoting efficient viral budding and enhancing viral particle infectivity. However, no PAK inhibitors are yet available for clinical treatment, and efforts to identify small molecule inhibitors have been ongoing. Thus, it may be possible that PQA-18 improves the pathogenesis of various diseases, including cancers and HIV, by interfering with PAK2 activation.

Development of immunosuppressants that causes no adverse effects has been indispensable for the improvement of quality of life in patients post transplantation, or those with autoimmune or allergic diseases [6,7]. Currently, several types of immunosuppressive drugs have been developed for clinical use in transplantation. These include small-molecule, antibody, and

glucocorticoid drugs. Azathioprine and glucocorticoids, which are the primary immunosuppressive drugs, inhibit purine biosynthesis and the expression of proinflammatory genes, respectively, as well as allowing the transfer of organs between genetically disparate donors and recipients. On the other hand, the clinical use of calcineurin inhibitors, cyclosporine A, and FK506 also improves graft outcome. In addition, mTOR inhibitor sirolimus, which is known as rapamycin and blocks cell cycle, is also used as another type of immunosuppressive drug. Because the mechanistic basis for their anti-inflammatory properties is diverse, their clinical applications can be limited by the possible appearance of serious side effects, such as hypertension, hyperlipidaemia and diabetes. To minimize their toxic effects, these drugs are used at low doses in combination with other types of drugs for clinical treatment. In this study, we revealed that PQA-18 treatment exhibits therapeutic potential for AD symptoms (Fig. 6 and 7). Our histological analysis also indicated that dorsal skin thickness and infiltration of mast cells and eosinophils were significantly reduced by the application of PQA-18 (Fig. 6C, Fig 7A and 7B). Because long-term use of PQA-18 at a high dose (1.0 mg/kg over a period of six weeks) exhibits no abnormal changes in serum component (Table 1) and histological features in kidney and liver in mice (Fig. 5C), PQA-18 is likely to be a safer immunosuppressant than other drugs for the treatment of human AD. PQA-18 may be a lead compound useful for the development of safer immunosuppressant drugs.

It is interesting to understand molecular basis underlying PQA-18 inhibits PAK2 activity in non-competitive manner. Although regulation of PAK2 activity is not fully understood, autoinhibitory and GTPase-binding domains are involved in the control of PAK2 activation [39]. PAK2 has the autoinhibitory domain, which overlaps the GTPase binding domain. Inactive form of PAK2 exists as dimers, where the autoinhibitory domain binds *in trans* to the PAK kinase domain. This interaction prevents autophosphorylation and subsequent activation of PAK2 kinase. When Cdc42/Rac, along with lipids, binds to the GTPase binding domain, the interaction between the autoinhibitory domain and dimerizing PAK2 is disrupted, causing monomerization and autophosphorylation of PAK2 [39,40]. There have been reported several autophosphorylation sites including Ser141, Ser165 and Thr402, and phosphorylation of Ser141 in autoinhibitory domain is required for maintaining the protein kinase in an active state by inhibiting the autoinhibitory interaction [30]. Our results suggest phosphorylation of multiple sites on PAK2 (Fig. 3C), and its highly phosphorylated form seems to be most active. PQA-18 inhibits PAK2 activity (Fig. 4C), and also suppresses autophosphorylation of PAK2 at Ser141 and other sites (Fig. 3D). In this context, autophosphorylation of PAK2 at Ser141 may be a crucial for the full activation of PAK2, and PQA-18 may disrupt active conformation of PAK2 by association with its autoinhibitory domain. Further studies are required to clarify precise mechanisms by which PQA-18 inhibits PAK2 kinase activity.

In summary, we have identified PQA-18, a PQA compound, as a potent inhibitor of cytokine production and demonstrated that PQA-18 inhibits PAK2 activity. We have further demonstrated that PQA-18 exhibits a suppressive effect on the population of a subset of regulatory T cells and Ig production induced in normal mice, and therapeutic effects on dermatitis in Nc/Nga mice without any adverse changes in the liver and kidney. These results suggest that PQA-18 may be a feasible lead compound for the development of novel and safe immunosuppressants. Future studies on PQA-18 will provide additional insights into therapeutic approaches.

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Figure legends

Fig. 1. Inhibition of IL2 production by PQA-18 in Jurkat T cells.

Structures of Ppc-1 and PQA-18 (A). IL2 production was determined by ELISA using Jurkat cells cultured without or with ConA (40 µg/ml) for 24 h in the presence of Ppc-1 (B), PQA-18 (C) at indicated concentrations. IL2 mRNA expression was determined by quantitative RT-PCR using Jurkat cells cultured without or with ConA for 6 h in the presence of PQA-18 (D). Cell viability was determined by PI staining using Jurkat cells cultured with PQA-18 at 20 µM, A23187 at 1 µM for 24 h (E). PI positive cells were determined by flow cytometric analysis. Calcium ionophore A23187 is used as an apoptosis inducer of Jurkat cells. The experiments were repeated three times.

Fig. 2. Inhibition of cytokines production by PQA-18 in human peripheral lymphocytes.

Human peripheral lymphocytes cultured without or with ConA (10 µg/ml) for 24 h in the presence of PQA-18, FK506 at indicated concentrations, production of IL2 (A), IL4 (B), IL6 (C), and TNFα (D) was determined by ELISA. The experiments were repeated three times.

Fig. 3. Inhibition of PAK2 phosphorylation by PQA-18.

2-DE gel images show cofilin-1 (A) and histone H4 (B) changes in cellular protein prepared from Jurkat cells cultured with ConA (40 $\mu\text{g/ml}$) for 10 min in the presence (upper right) or absence (upper left) of PQA-18 at 10 μM . Arrowheads represent protein spots identified as cofilin-1 (A) and histone H4 (B) by MS analysis. The protein spots are quantified using densitograph (lower). (C) Cellular proteins prepared from Jurkat cells cultured with ConA in the presence (upper right) or absence (upper left) of PQA-18 were separated by 2-DE, and the blots were probed with anti-PAK2 antibody. (D) The blots were re-probed with anti-PAK2-pSer141 antibody. Immunoreactive spots for PAK2 are indicated with numbers, and their intensity was quantified (lower).

Fig. 4. Inhibition of PAK2 activity by PQA-18.

(A) An aliquot of histone H4 protein (1 μg) was reacted with anti-PAK2 immunoprecipitates prepared from Jurkat cells in kinase buffer containing $\gamma[^{32}\text{P}]\text{ATP}$ at 30°C for the indicated periods. After separating on SDS/PAGE, proteins were visualized by Coomassie Brilliant Blue staining (middle), and phosphoproteins were detected by autoradiography (upper). Quantitative data are shown (lower). (B) Inhibition of PAK2 activity was determined in the presence of the indicated concentrations of PQA-18. (C) Phosphorylation of H4 protein by PAK2 is measured in the presence of 0.1, 0.2, 0.5 or 1.0 μg of H4 and PQA-18. Lineweaver-Burk analysis was used for the

assessment of PQA-18.

Fig. 5. Suppression of antigen-specific immune response by PQA-18.

(A) Mice were immunized with NP-CGG and treated with vehicle (0.1% DMSO), PQA-18 (0.5 mg/kg), or FK506 (1.0 mg/kg) by intraperitoneal injection three times a week, and serum levels of IgM, IgG₁, and IgG₃ were determined (n = 8 each). (B) Mice were divided into four groups, and two groups were immunized with NP-CGG. One non-immunized and one immunized groups were treated with 0.5 mg/kg PQA-18 by ip injection three times a week. After a week, splenic regulatory T cells were analyzed, and the number of Foxp3⁺CD4⁺CD25⁺ cells was determined (n = 5 each). The determination was repeated three times, and quantitative data are shown. (C) Cross sections were prepared of kidney and liver tissues from mice treated with vehicle or PQA-18 (1.0 mg/kg) for six weeks and stained with hematoxylin-eosin. Representative images are shown. No abnormal changes were detected in the two groups.

Fig. 6. Improvement of dermatitis and IgE levels of Nc/Nga mice by PQA-18.

(A) Nc/Nga mice (13 weeks old, n = 9 each) were treated without or with 100 mg of vaseline ointment containing either vehicle, 0.05% PQA-18, 0.1% PQA-18, or 0.1% FK506 three times a week. The dermatitis scores were assessed once a week based on the following criteria: no lesion,

0; minor hair loss or wound without bleeding, 1; wound bleeding in parts, 2; broad area of serious wounds, 3. (B) Serum IgE levels were measured, and the quantitative data at 17 weeks old are shown. (C) Sections were prepared from skin samples of Nc/Nga mice treated without or with 100 mg of vaseline ointment containing either vehicle, 0.05% PQA-18, 0.1% PQA-18, or 0.1% FK506 and stained by hematoxylin-eosin. The representative images are shown on the left, and quantitative data of skin thickness are shown on the right.

Fig. 7. Histological features of skin lesions in Nc/Nga mice.

Sections were prepared from skin samples of Nc/Nga mice treated for four weeks with vehicle, 0.05% PQA-18, 0.1% PQA-18, or 0.1% FK506 ointment and stained by Toluidine blue (A) or Luna's solution (B). Arrows indicate the purple-red granular staining of mast cells (A) and red staining of eosinophils (B). The representative images are shown on the left, and quantitative data of the number of mast cells and eosinophils are shown on the right.

Table 1. Profiling of serum factors.

PQA-18	BUN (mg/l)		Creatinine (mg/l)		AST (IU/l)		ALT (IU/l)		γ -GTP (IU/l)		LDH (IU/l)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	-	Mean	SD
Vehicle	211	27	1.1	0.3	72.3	15.4	28.0	15.6	<3		891	191
1.0 mg/kg	262	27	1.5	0.2	80.3	24.5	35.8	13.3	<3		696	40

Figure 1

Figure 2

Figure 3

A

B

C

D

Figure 4

A **B** **C**

Figure 5

A

—□— Vehicle
—▲— PQA-18 (0.5 mg/kg)
—◆— FK506 (1 mg/kg)

B

□ Vehicle
■ PQA-18 (0.5 mg/kg)
▒ NP-CGG
■ NP-CGG + PQA-18 (0.5 mg/kg)

C

100 μm

Figure 6

Figure 7

A

B

Graphical abstract

PAK2

Cytokine production

IL2

IL4

IL6

TNF α

Immunoglobulin production

IgM

IgG3

IgG1

Atopic dermatitis

Skin lesions

IgE