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Title page

PO-322 has potent immunosuppressive activity *in vitro* and *in vivo* by selectively inhibiting SGK1 activity

Running title: The immunosuppressive activity of PO-322

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Abstract

BACKGROUND AND PURPOSE

Immunosuppressive drugs have shown great promise in treating the autoimmune diseases in recent years. A series of novel oxazole derivatives were screened for their immunosuppressive activity. PO-322 [1H-indole-2,3-dione 3-(1,3-benzoxazol-2-ylhydrazone)] was identified as the most effective of these compounds. The purpose of the current study was to investigate the potential mechanism of PO-322 in inhibiting T cell proliferation *in vitro*, as well as its effects on the delayed-type hypersensitivity response and imiquimod-induced dermatitis *in vivo*.

EXPERIMENTAL APPROACH

T cell proliferation and apoptosis were analysed with flow cytometry. Cell viability was assessed with a CCK-8 assay. Protein kinase activity was assessed by SelectScreen Kinase Profiling Services. The phosphorylation of signal-regulated molecules was measured by western blot. Cytokine levels were determined by ELISA. The effect of PO-322 on delayed-type hypersensitivity and imiquimod-induced dermatitis was evaluated in a mouse model.

KEY RESULTS

PO-322 inhibited human T cell proliferation with anti-CD3/anti-CD28 mAbs or alloantigen without significant cytotoxicity. Importantly, PO-322 was a selective SGK1 inhibitor and decreased NDRG1 phosphorylation but not p70S6K, STAT5, AKT or ERK 1/2 phosphorylation. Furthermore, PO-322 inhibited IFN-γ, IL-6 and IL-17 expression but not IL-10 expression. Finally, the administration of PO-322 was safe and effective for ameliorating the delayed-type hypersensitivity response and imiquimod-induced dermatitis.

CONCLUSION AND IMPLICATIONS

Taken together, PO-322 exerts immunosuppressive activity *in vitro* and *in vivo* by selectively inhibiting SGK1 activity. PO-322 represents a potential lead compound for the design and development of new drugs for the treatment of the autoimmune diseases.

Keywords: immunosuppressive activity; T cell proliferation; SGK1; PO-322

Abbreviations

CFSE, 5-carboxyfluorescein diacetate succinimide ester; DTH, delayed type hypersensitivity reaction; ELISA, enzyme-linked immunosorbent assay; JAK3, Janus protein tyrosine kinase; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; PBMCs, peripheral blood mononuclear cells; PI3K, phosphoinositide 3 kinase; RAPA, rapamycin; SGK1, the serum- and glucocorticoid-regulated kinase 1

Introduction

The important role of T cells in immune responses against pathogens has been clearly clarified, and aberrant T cell responses play a crucial role in mediating the immunorejection of transplanted organs (van Gelder *et al.*, 2014) and the pathogenesis of autoimmune diseases, including multiple sclerosis (Sabatino *et al.*, 2018), psoriasis (Hawkes *et al.*, 2018) and systemic lupus erythematosus (SLE) (He *et al.*, 2016). In recent years, immunosuppressants have been widely used in the clinic for treating these diseases, and advances in the understanding of the mechanisms of these diseases have suggested multiple novel drug targets (Wiseman, 2016). However, there are significant drug sensitivity differences amongst individual patients. Therefore, combination, rotation, or sequential therapies are often needed, especially when the response to single-drug therapy is not satisfactory. To provide more effective treatments for patients, it is important to identify new immunosuppressants and further understand the pathogenesis of these diseases.

TCR-mediated T cell signalling can induce T cell activation and proliferation through several signalling pathways, including the calcium/calcineurin, nuclear factor κB (NF-κB), phosphoinositide 3 kinase (PI3K)/Akt, mammalian target of rapamycin (mTOR)/p70S6K, Janus protein tyrosine kinase (JAK3)/STAT5, and mitogen activated protein kinase (MAPK) p38 signal pathways (Moulton *et al.*, 2015). Immunosuppressants that are the inhibitors of these pathways have been approved for clinical use, such as cyclosporin A, FK506, rapamycin (RAPA) and tofacitinib (Baker *et al.*, 2018). Recently, several new small molecule inhibitors of these pathways, such as apremilast (Nash *et al.*, 2018), peficitinib (Sands *et al.*, 2018) and baricitinib (Wallace *et al.*, 2018) have been advanced to late-stage clinical trials, highlighting the potential of these immunosuppressive approaches.

Serum- and glucocorticoid-regulated kinase 1 (SGK1) belongs to the AGC kinase family and mediates signals of cellular growth, proliferation and survival responses. SGK1 is activated by cAMP, IGF-1, insulin, steroids, TGF-β and especially IL-2 (Talarico *et al.*, 2016). SGK1 is a downstream target of mTOR, which integrates various signals to influence T cell proliferation and differentiation (Norton *et al.*, 2014). SGK1 inhibitors, such as GSK650394, EMD638683 and SI113, have been reported to have anticancerous activity (Abbruzzese *et al.*, 2017; Yuda *et al.*, 2018) and anti-hypertensive activity (Du *et al.*, 2018) and can prevent cardiac inflammation (Gan *et al.*, 2018).

Oxazole is a privileged structure motif present in a variety of bioactive agents, including chemotherapeutic (Lu *et al.*, 2016), antimicrobial (Zhang *et al.*, 2011), neuroprotective (Kaushal *et al.*, 2011), and analgesic agents (Payrits *et al.*, 2016). Oxazole derivatives are marketed for the treatment of cardiovascular disease (Dineen *et al.*, 2014) and as an inhibitor of carbonic anhydrase isoforms I and II (Krasavin *et al.*, 2015). In addition, oxazole derivatives exhibit anti-inflammatory activity by inhibiting GSK3β (Zhao *et al.*, 2018), MAPK p38α (Kalgutkar *et al.*, 2006) and cyclooxygenase-2 and cyclooxygenase-1 enzyme activities (Hashimoto *et al.*, 2002). Recently, we found an oxazole derivative, PO-296, that could inhibit T cell proliferation through the JAK3/STAT5 signalling pathway (Luo *et al.*, 2018). However, the immunosuppressive activity of oxazole derivatives through SGK1 inhibition has not been reported previously.

In the present study, a series of novel oxazole derivatives (Figure 1) were tested for their immunosuppressive activity, and PO-322 [1H-indole-2,3-dione 3-(1,3-benzoxazol-2-ylhydrazone)] was the most effective lead compound. PO-322 was identified as a selective SGK1 inhibitor and was investigated for its ability to inhibit T cell proliferation *in vitro*, as well as its effects on the DNFB-induced delayed type hypersensitivity (DTH) reaction and imiquimod-induced dermatitis *in vivo*.

Methods

Experimental animals

Mice (BALB/c, 8-11 weeks, RRID:IMSR_ORNL:BALB/cRl) were purchased from Huaxi Laboratory Animal Center of Sichuan University (Chengdu, China) and kept under specific

pathogen-free conditions and provided with normal food and water. All experiments were approved by the Animal Ethics Committee of Chengdu Medical College on animal experiments. Animals were randomly assigned to treatment groups and the experimenter was blinded to drug treatment until data analysis has been performed.

Synthetic method of PO-322

PO-322 was synthesized in one step from commercially available 2-indolinone and 2-hydrazinobenzoxazole. A mixture of 2-indolinone (1.33 g, 10 mmol), 2-hydrazinobenzoxazole (1.49 g, 10 mmol) and acetic acid (1 mL) was stirred in ethanol (50 mL) at reflux temperature for 3 hours. After cooled down to room temperature, PO-322 (1 g, 36% yield) was collected as pale yellow powder. The compound was characterized by 1H NMR using Bruker Advance 600 spectrometer (chemical shifts expressed in ppm with reference to tetramethylsilane peak) and electrospray ionization mass spectrum using BioTOF-Q mass spectrometer. All reagents were obtained from J&K Chemical Co. Solvents were purchased from local suppliers.

Cell preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated as previously described (Liu *et al.*, 2013). PBMCs were cultured in RPMI 1640 supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). T cells were isolated using Pan T cell Isolation Kit II Human (Miltenyi Biotec, Bergisch Gladbach, Germany) with negative selection. The T cell purity of 95% was used for the following experiments.

Drug treatment

T cells (10^6 cells ml^{-1}) were treated with RAPA (0.1 μM, Sigma-Aldrich, St Louis, MO, USA), FK506 (0.1 μM, Sigma-Aldrich), GSK650394 (10 μM, MedChem Express, Monmouth Junction, NJ, USA), AG-490 (50 μM, Sigma-Aldrich), LY-294002 (50 μM, Promega, Madison, WI, USA), PD184352 (2 μM, Sigma-Aldrich) or different concentrations of PO-322 and activated by plate-bound anti-CD3 (2 μg ml^{-1}, HIT3a clone, BD PharMingen, San Diego, CA, USA) and soluble anti-CD28 (1 μg ml^{-1}, CD28.2 clone, BD PharMingen) as

previously described (Liu *et al.*, 2013). Cells were randomly assigned to treatment groups and the experimenter was blinded to drug treatment until data analysis has been performed.

CFSE labeling assay

T cell proliferation was measured by flow cytometry (Acurri C6, Becton Dickinson, San Jose, USA) with 5-carboxyfluorescein diacetate succinimide ester (CFSE, Molecular Probes, Eugene, OR, USA)-labeling as previously described (Liu *et al.*, 2015).

Cell apoptosis assay

Fluos-labeled Annexin V and PI dual staining kit (Roche, Indianapolis, IN, USA) was used to assess the cell apoptosis following the protocol and analyzed by flow cytometry (Acurri C6) as previously described (Luo *et al.*, 2018).

Cell viability assay

The CCK-8 assay kit (Dojindo, Kumamoto, Japan) was used to assess the viability of cell by detecting the O.D. values at 450 nm in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) as previously described (Liu *et al.*, 2013).

Protein kinase profiling

The effect of PO-322 against almost 100 kinases was assessed by SelectScreen Kinase Profiling Services (Thermo Fisher Scientific, Madison, WI, USA). PO-322 was dissolved in DMSO at 100 μm, and a final concentration of 1 μm was used for screening. Then, the inhibitory activity of PO-322 against SGK1 was measured with different concentrations of PO-322.

Western blot analysis

Cell pellets were treated with lysis buffer and clarified by centrifugation. Cell lysate proteins (20 μg lane^{-1}) were separated on 10 % SDS-polyacrylamide gel electrophoresis and transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked by PBS containing 5 % BSA and stained with

antibodies against N-Myc downstream-regulated gene 1 (NDRG1), phospho-NDRG1 (Cell Signaling Technology Cat# 5482, RRID:AB_10693451), Akt, phospho-Akt (Ser473), p70S6K, phospho-p70S6K, STAT5, phospho-STAT5, ERK1/2 or phospho-ERK 1/2 (CST Inc., Danvers, MA, USA) overnight at 4 °C followed by horseradish peroxidase (HRP)-conjugated second antibodies (Santa Cruz Biotech, Santa Cruz, USA) incubation. Finally, proteins were visualized by enhanced chemiluminescence (Millipore). All Western blotting procedures and analysis were conducted in accordance with current guidelines (Alexander *et al.*, 2018).

Cytokine ELISA assay

The levels of IL-2, IL-6, IL-10, IL-17A and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) as previously described (Liu *et al.*, 2015).

DNFB-induced DTH reaction in mice

The experiments of DNFB-induced DTH reaction in mice were performed. Individual BAL b/c mice were sensitized topically with 20 μl of 0.5% (v/v) DNFB (Sigma-Aldrich) in acetone:olive oil (4:1) onto each hind foot of mice on days 0 and 1. These mice were treated intraperitoneally with different doses of PO-322 (2.5, 10 and 40 mg kg^{-1}), RAPA (2 mg kg^{-1}) or vehicle alone beginning on day 6 for three consecutive days. The mice were challenged topically with 10 μl of 0.5% (v/v) DNFB on the inner and outer surfaces of the right ear on day 7. The thickness of both left and right ears and the weight of ear patches (8-mm punches) were measured 48 h post challenge.

Imiquimod-induced dermatitis in mice

The backs of female BALB/c mice (RRID:IMSR_ORNL:BALB/cRl) were shaved with an

electric clipper 1 d prior to treatment. Mice were treated intraperitoneally with 10 or 40 mg/kg PO-322 or vehicle 1 day prior to application of 62.5 mg 5 % imiquimod cream (Aldara, 3M Pharmaceuticals, St Paul, Minn) or control cream (Curel, Kao, Japan). Then, 10 or 40 mg/kg PO-322 or vehicle was given 0.5 h before treating with 62.5 mg 5 % imiquimod cream or control cream and this treatment was repeated for 6 consecutive days. Each day, the mice were assessed by the same researcher for redness and scaling on a 0-4 scale prior to handling, according to previously published guidelines (van der Fits *et al.*, 2009). On the sixth day, mice were sacrificed by cervical dislocation and photographs were taken. Back skin (3 mm diameter) was isolated and fixed in 10% formaldehyde. Then, fixed skin was paraffin embedded, sliced at 6 mm using a microtome (RM2235, Leica, Nussloch, Germany), and stained with haemotoxylin and eosin (H&E). H&E slides were blinded, and an overall score of severity was assessed (0-4) by a trained researcher. Sections of skin were photographed at an objective magnification of ×20 using a microscope (BX63, Olympus, Tokyo, Japan). Spleen was isolated and spleen mass was determined. Then, splenic mononuclear cells were prepared and resuspended in PBS. T cells in splenic mononuclear cells were stained with PE-anti-CD3 (BD PharMingen) and analyzed on a flow cytometry (Acurri C6).

Statistical analysis

Results are expressed as mean ± S.E.M. and the inhibitory concentration of the compound that reduced cell proliferation by 50% (IC_{50}) values were calculated using GraphPad Prism 6 (GraphPad Prism, RRID:SCR_002798). The sample size was n = 8 per group in animal experiments and n = 5 per group in other experiments. One-way analysis of variance (ANOVA) with Dunnett comparisons on post-tests were used to analyze data and compare groups. The post hoc tests were run only if F achieved $P < 0.05$ and there was no significant variance inhomogeneity. In each experiment, n represents the number of separate experiments (in vitro), and the number of mice (in vivo). Technical replicates were used to ensure the reliability of single values. A $P < 0.05$ was considered to be statistically significant. The data and statistical analyses comply with the recommendations and requirements on experimental design and analysis in pharmacology (Curtis *et al.*, 2018).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Synthesis and characterization of PO-322

PO-322 was synthesized in one step from 2-indolinone (1) and 2-hydrazinobenzoxazole (2), with 36% yield (Figure 2). ^{1}H NMR (300 MHz, DMSO-d6) δ(ppm) 12.64 (brs, 1H), 10.57 (brs, 1H), 8.34 (d, 1H, J=6.9 Hz), 7.66 (m,1H), 7.18-7.68 (m,4 H), 7.04 (m, 1H), 6.86 (m, 1H). ESI-MS: *m/z* 277 $[M - H]^-$.

PO-322 inhibits human T cell proliferation without obvious cytotoxicity in vitro

PO-322 and its analogues were screened for their immunosuppressive activity. Among these chemical compounds, PO-322, PO-324, PO-326, PO-327, PO-335, PO-341 and PO-342 showed significant inhibitory effects on T cell proliferation following anti-CD3 and anti-CD28 stimulation (Table 1). The most potent inhibitor, PO-322, was selected for further studies. PO-322 was found to inhibit human T cell proliferation after anti-CD3/anti-CD28 stimulation with an IC_{50} value of 0.7 ± 0.2 μM (Figure 3A, B) and alloantigen stimulation with an IC_{50} value of 0.6 ± 0.3 μM (Figure 3C). T cell proliferation was also inhibited by PO-322 following PHA or PMA/ionomycine stimulation (Figure S1).

To investigate the potential cytotoxicity of PO-322, human activated T cells treated with PO-322 were measured for apoptosis by flow cytometry. Resting T cells and IL-7-treated activated T cells both survived but did not proliferate under the conditions (Rathmell *et al.*, 2001). Additionally, PBMCs treated with PO-322 were assessed for cell viability with a CCK-8 assay. PO-322 treatment did not induce activated T cell apoptosis at 24 h or 48 h (Figure 3D) and had no significant impact on the relative viability of resting T cells (Figure 3E), IL-7-treated activated T cells (Figure 3F), or PBMCs at 72 h (Figure 3G), indicating that the activity of PO-322 was immunosuppressive rather than cytotoxic.

PO-322 is a highly selective inhibitor of SGK1

To identify the targets of PO-322, a protein kinase activity screen was carried out. Among 100 protein kinases screened, PO-322 (1,000 nM) showed a very high inhibitory activity towards SGK1 (98% inhibition), but not other kinases (inhibition < 50%) (Figure 4A). It was interesting that PO-322 inhibited SGK1 activity with an IC_{50} value of 54 ± 6 nM (Figure 4B). These results suggested that PO-322 was a highly selective SGK1 inhibitor.

To further reveal the signalling pathway affected by PO-322, the impact of PO-322 on the related kinase phosphorylation levels were analysed by western blot. The results showed that NDRG1 phosphorylation, but not its expression, was significantly inhibited by PO-322 (Figure 4C). Notably, the expression of p70S6K and STAT5 did not significantly change, but their phosphorylation levels were increased by PO-322 (Figure 4D, E). Finally, the Akt (Figure 4F) and ERK 1/2 (Figure 4G) expression and phosphorylation levels were not affected by PO-322. Phosphorylation of NDRG1 has previously been shown to be a specific SGK1 target (Inglis *et al.*, 2009), indicating that PO-322 inhibited T cell proliferation through the SGK1/NDRG1 signalling pathway.

PO-322 inhibits pro-inflammatory cytokines but does not affect anti-inflammatory cytokines

To understand the impact of PO-322 on pro-inflammatory and anti-inflammatory cytokine expression, the levels of IL-17, IFN-γ, IL-6 and IL-10 were measured by ELISA in the supernatants of activated T cells. The results showed that PO-322 significantly inhibited IL-17, IFN-γ and IL-6 expression, but did not affect IL-10 (Figure 5), indicating potential anti-inflammatory effects of PO-322.

PO-322 significantly mitigates DNFB-induced DTH reaction and ameliorates imiquimod-induced dermatitis in mice

Th1/Th17 responses are clearly related to DTH reaction (Sido *et al.*, 2016). We explored the activity of PO-322 on DNFB-induced DTH reaction in BAL b/c mice. We found that the administration of PO-322 markedly reduced ear swelling, including increases in ear thickness and patch weight, in a dose-dependent manner (Figure 6).

Finally, we explored the activity of PO-322 in imiquimod-induced dermatitis, a mouse model that closely resembles human psoriatic lesions (van der Fits *et al.*, 2009). We found that treatment with PO-322 markedly reduced scaling and redness compared with the vehicle-treated group (Figure 7A). In addition, the histopathological assessment of the skin on the back indicated that PO-322-treated mice showed significantly reduced histological scores, splenic mass and total T cells numbers in the spleen (Figure 7B, C and D), demonstrating that PO-322 can substantially reduce disease severity.

Furthermore, drug-related deaths, sickness or abnormal food and water intake were not observed in our experiments. These results suggested that PO-322 was safe and effective in ameliorating T cell-mediated DTH reaction and imiquimod-induced dermatitis *in vivo*.

Discussion and Conclusions

In this study, a series of new oxazole derivatives, including PO-322, were tested for immunosuppressive activity. PO-322 was identified as the lead molecule with the highest activity among these derivatives. PO-322 significantly inhibited T cell proliferation after anti-CD3/anti-CD28 mAbs, alloantigen, PHA or PMA/ionomycine stimulation. Notably, PO-322 did not induce activated T cells apoptosis and had no significant cytotoxicity on resting T cells, IL-7-treated activated T cells or PBMCs, indicating that the activity of PO-322 was primarily immunosuppressive rather than cytotoxic. Therefore, further exploration of the possible mechanism of action for PO-322 and its immunosuppressive effects *in vivo* is warranted.

Next, to identify the targets of PO-322, a protein kinase activity screen was carried out. We found that PO-322 was a selective SGK1 inhibitor. Several pathways are involved in T cell proliferation, such as the mTOR/p70S6K, JAK3/STAT5, PI3K/Akt and p38 MAPK pathways. Furthermore, IL-2 engagement with its receptor can induce SGK1 activation, which is a downstream target of mTOR (Talarico *et al.*, 2016). Western blotting results of PO-322 on these signalling pathways showed that the PI3K/Akt and p38 MAPK pathways were not affected by PO-322 treatment, but the SGK1/NDRG1 pathway was significantly inhibited under the same conditions in IL-2-induced T cells. Therefore, PO-322 inhibited activated T cell proliferation by targeting SGK1 activity. Interestingly, p70S6K and STAT5

phosphorylation was increased by PO-322. The mTOR/SGK1 pathway inhibition may selectively expand regulatory T cells and promote de novo generation of $Foxp3^+$ regulatory T cells through the upregulation of the STAT5 phosphorylation (Shan *et al.*, 2015). This implies a potential role of PO-322 in inducing tolerance of immune responses though regulating the balance between regulatory T cells and effector T cells.

SGK1 is activated by IL-2 in T cells and induces T cell proliferation and differentiation. Furthermore, SGK1 regulates TH1 and TH2 cell differentiation, as well as TH17 cell development, and improves pro-inflammatory cytokines levels (Norton *et al.*, 2014). The loss of SGK1 in T cells leads to a selective defect in pathogenic Th17 differentiation (Heikamp *et al.*, 2014). SGK1 has been shown to be critical for the development of autoimmune diseases, such as experimental autoimmune encephalomyelitis (Wang *et al.*, 2017) and ulcerative colitis (Spagnuolo *et al.*, 2018). PO-322, as a SGK1 inhibitor, significantly inhibited IFN-γ, IL-6 and IL-17 release from activated T cells. It has been well established that pro-inflammatory cytokines, including IFN-γ, IL-6 and IL-17, play a critical role in autoimmune diseases (Hawkes *et al.*, 2018; McGeachy *et al.*, 2019). Therefore, PO-322 may have beneficial anti-inflammatory effects by regulating Th cell differentiation in treating autoimmune diseases.

The mouse model experiments also showed that PO-322 was effective and safe in inhibiting T cell-mediated inflammation *in vivo*. We found that the administration of PO-322 significantly reduced DNFB-induced DTH reaction and substantially reduced disease severity in an imiquimod-induced dermatitis mouse model, which implicates the anti-psoriatic properties of PO-322 *in vivo*. However, the potential mechanism of PO-322 in treating DTH and psoriasis remains to be investigated in further studies.

While highly specific and potent biological drugs targeting the components of the immune system have received much attention in recent years, small-molecule chemical therapies can be administered orally and are often less expensive to manufacture, resulting in better patient compliance and affordability. Nevertheless, the structure of PO-322 is significantly different from other known inhibitors of SGK1, as well as from other immunosuppressants. It would be interesting to identify the mechanism of PO-322 in autoimmune diseases, which could help to further elucidate the pathogenesis of autoimmune diseases and to guide the design of

new drugs and would ultimately benefit patients with safer and more effective therapeutic options for these diseases.

In summary, we synthesized and identified a novel oxazole derivative, PO-322, as a potent immunosuppressant. PO-322 inhibits T cell proliferation by selectively inhibiting SGK1 activity and relieving both the DNFB-induced DTH reaction and imiquimod-induced dermatitis *in vivo*. Further structure and activity relationship (SAR) studies and the optimization of PO-322 are warranted to improve the potency and help elucidate the mechanism of action for this promising lead compound for the treatment of the immunorejection of transplanted organs and autoimmune diseases.

Author contributions

Y. L. and Y. W. conceived and designed the experiments; Y. L., X. L., H. Z., S. W. and J. X. performed the experiments; Y. L. and X. L. analyzed the data; C. M., H. G., Y. W., S. Y. and L. L. contributed reagents/materials/analysis tools; Y. L. and Y. L. wrote the paper.

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Conflicts of Interest

The authors state no conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for

Design & Analysis, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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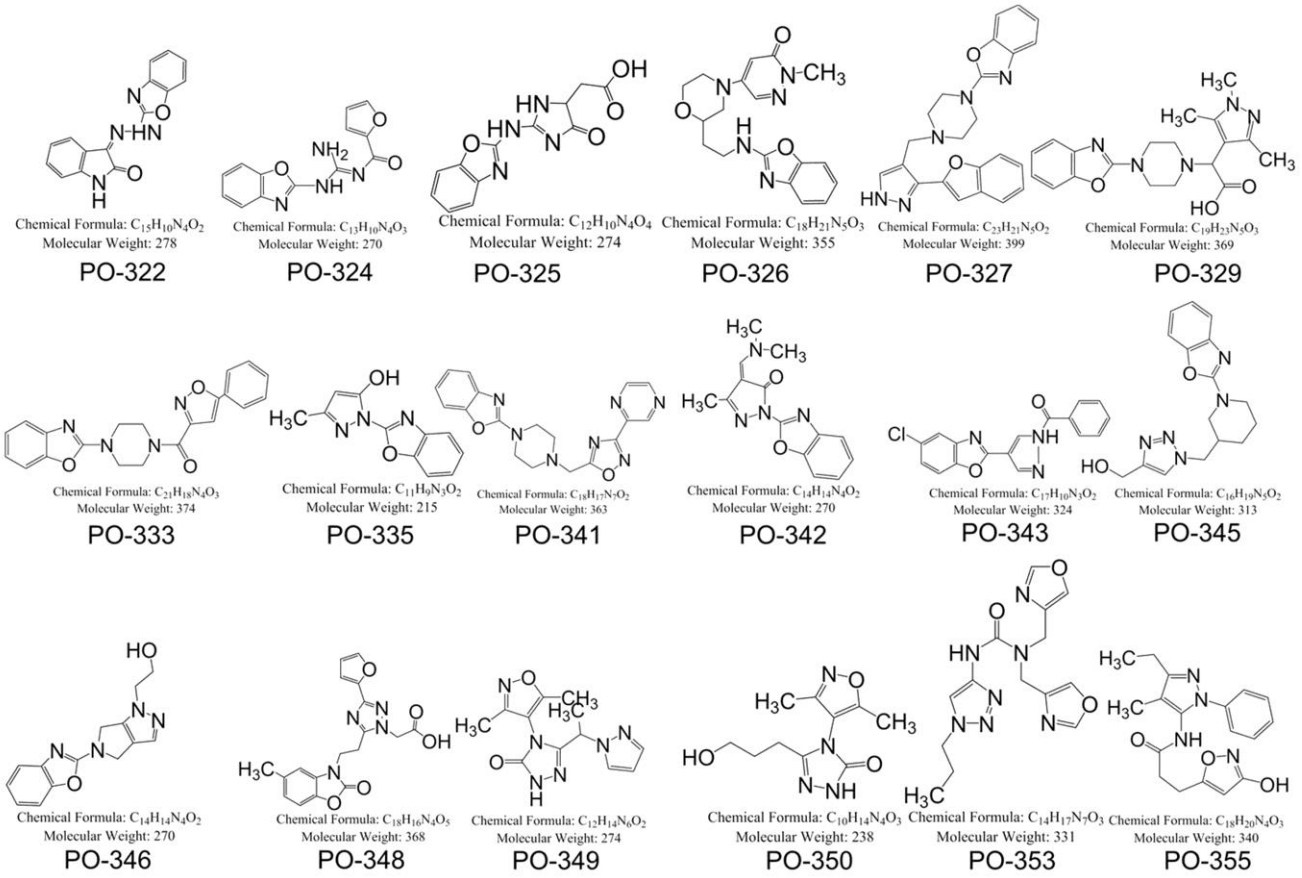

Figure 1. Chemical structure of oxazol derivatives.

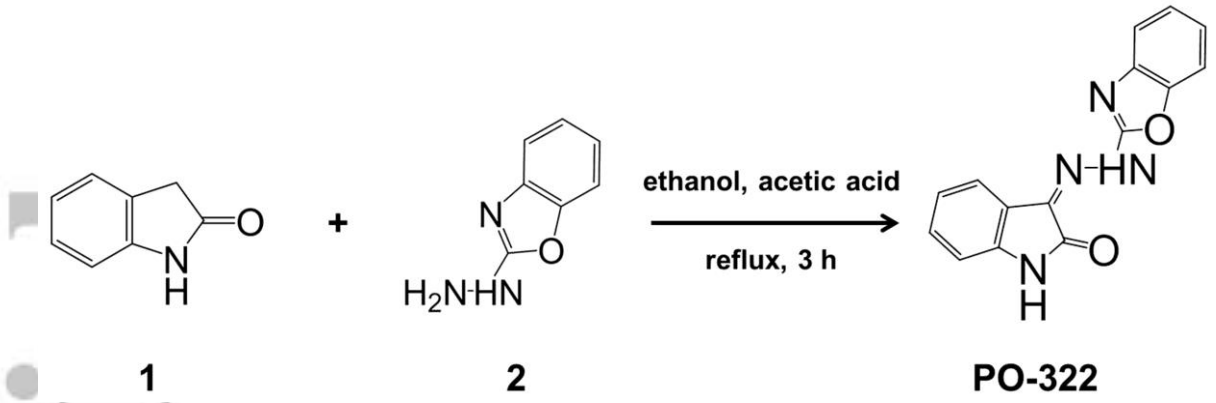

Figure 2. Synthesis of PO-322. A mixture of compound 1 (2-indolinone, 1.33 g, 10 mmol), compound 2 (2-hydrazinobenzoxazole, 1.49 g, 10 mmol) and acetic acid (1 mL) was stirred in ethanol (50 mL) at reflux temperature for 3 hours. After cooled down to room temperature, PO-322 (1 g, 36% yield) was collected as pale yellow powder.

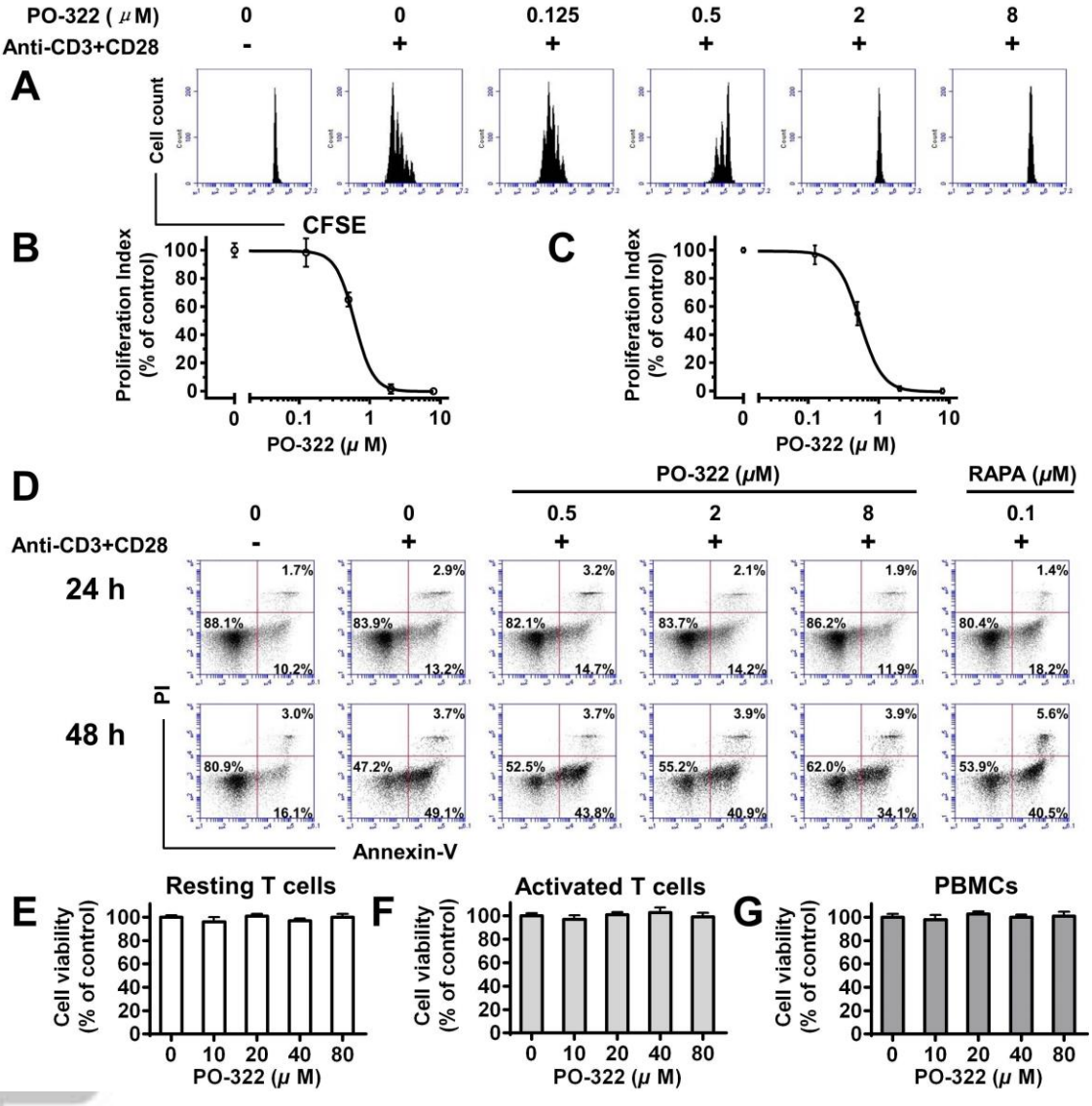

Figure 3. PO-322 inhibits human T cell proliferation without obvious cytotoxicity. CFSE-labelled T cells were treated with PO-322 (0.125, 0.5, 2 and 8 μM) and activated with anti-CD3/anti-CD28 (A, B) or allogeneic PBMCs (C) for 72 h. Cell proliferation was measured by flow cytometry. Cells without stimulation and PO-322 treatment served as negative controls (0%), while the cells with stimulation but without PO-322 treatment served as positive controls (100%). T cells were treated with PO-322 (0.5, 2 and 8 μM), Rapa (0.1 μM) or vehicle and activated with anti-CD3/anti-CD28 for 24 h or 48 h. Cell apoptosis was assessed by flow cytometry with Annexin V and PI dual staining (D). Resting T cells (E), IL-7-treated activated T cells (F) and PBMCs (G) were treated with PO-322 (10, 20, 40 and 80 μM) or vehicle for 72 h. The CCK-8 assay kit was used to assess cell viability. Cells without drug treatment served as controls (100%). "+" and "-" indicate with or without anti-CD3/anti-CD28 stimulation, respectively. RAPA is an abbreviation for rapamycin. The results are presented as the mean ± S.E.M., n = 5 for each experimental group.

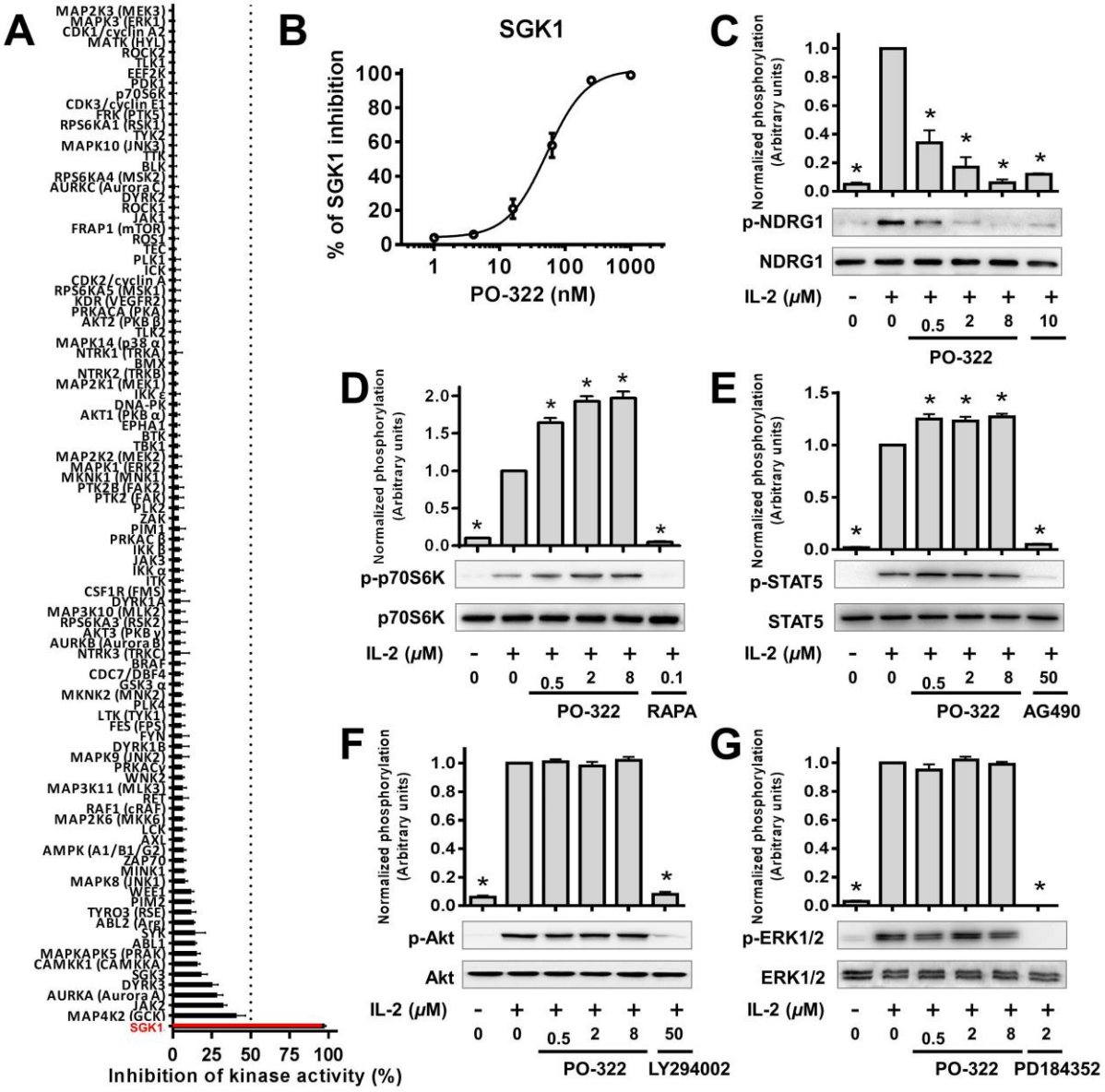

Figure 4. PO-322 is a highly selective inhibitor of SGK1. The effect of PO-322 against almost 100 kinases was assessed by SelectScreen Kinase Profiling Services. PO-322 was dissolved in DMSO at 100 μM, and a final concentration of 1 μM was used for screening (A). Then, the inhibitory activity of PO-322 against SGK1 was measured with different concentrations of PO-322 (B). T cells were incubated alone for 6 h after 72 h of anti-CD3/anti-CD28 stimulation. T cells were then treated with PO-322 (0.5, 2 or 8 μM), GSK650394 (10 μM), RAPA (0.1 μM), AG-490 (50 μM), LY294002 (50 μM), PD184352 (2 μM) or vehicle for another 6 h. Subsequently, T cells were induced by IL-2 for 30 min, and the relative phosphorylation and expression levels of NDRG1 (C), p70S6K (D), STAT5 (E), Akt (F) and ERK 1/2 (G) were assessed by western blot analysis. "+" and "-" indicate experiments with or without IL-2, respectively. The results are presented as the mean ± S.E.M., n = 5 for each experimental group. *P < 0.05 vs. the activated group without drug.

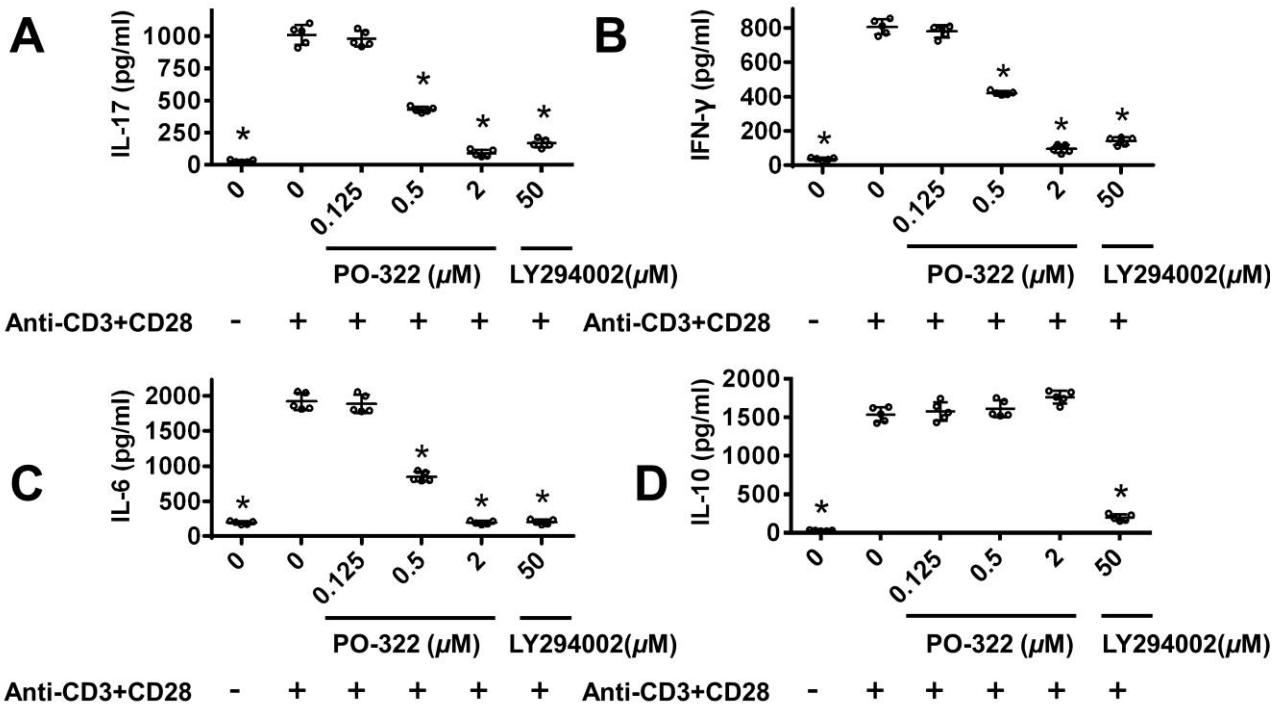

Figure 5. PO-322 inhibits pro-inflammatory cytokine expression and does not affect anti-inflammatory cytokine expression. T cells were treated with PO-322 (0.125, 0.5 and 2 μM), LY-294002 (50 μM) or vehicle and activated with anti-CD3/anti-CD28 for 48 h. The supernatants were collected and the levels of IL-17A (A), IFN-γ (B), IL-6 (C) and IL-10 (D) were measured by ELISA. "+" and "-" indicate experiments with or without anti-CD3/anti-CD28, respectively. The results are presented as the mean ± S.E.M., n = 5 for each experimental group. *P < 0.05 vs. the activated group without drug.

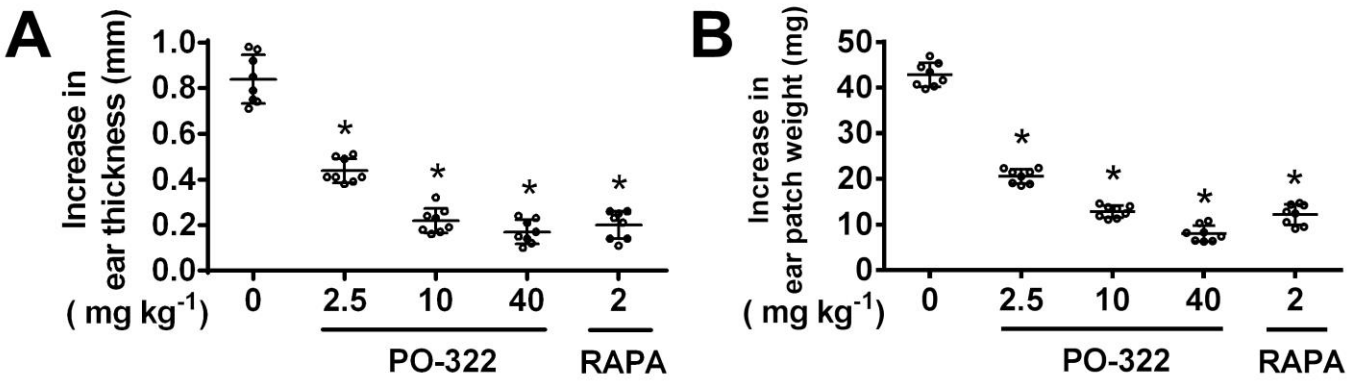

Figure 6. PO-322 mitigates DNFB-induced DTH reaction in mice. BALB/c mice were sensitized with DNFB on days 0 and 1 and treated intraperitoneally with different doses of PO-322 (2.5, 10 and 40 mg kg^{-1}), RAPA (2 mg kg^{-1}) or vehicle alone beginning on day 6 for three consecutive days. These mice were challenged with DNFB on day 7. The thickness of both the left and right ears and the ear patch weights were measured 48 h post challenge. Ear swelling was calculated as an increase in ear thickness (A) and ear patch weight (B) between the left (DNFB-untreated) and right (DNFB-treated) ears. The results are presented as the mean ± S.E.M., n = 8 for each experimental group. *$P < 0.05$ vs. the group without drug.

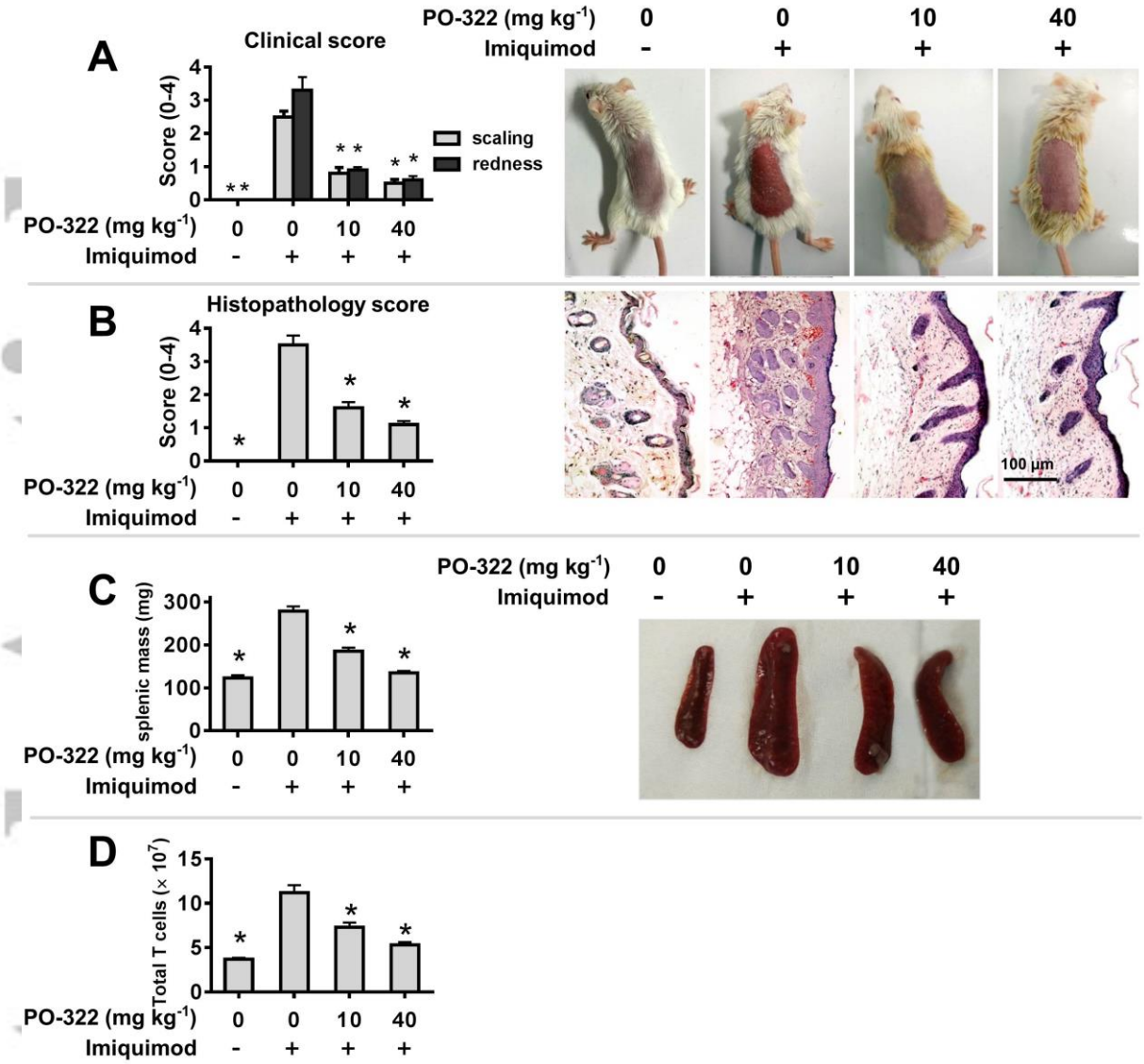

Figure 7. PO-322 relieves imiquimod-induced psoriasis-like dermatitis. BALB/c mice were treated intraperitoneally with 10 or 40 mg/kg PO-322 or vehicle 1 day prior to the application of imiquimod or control cream. Then, PO-322 or vehicle was given 0.5 h before treatment with imiquimod cream, and this treatment was repeated for 6 consecutive days. Clinical scores for redness and scaling (A) and H&E-stained slides (B) were scored (0 - 4) for disease severity. Mice were sacrificed and splenic masses were determined (C). The numbers of T cells were measured by flow cytometry (D). "+" and "-" indicate runs with or without imiquimod, respectively. The results are presented as the mean ± S.E.M., n = 8 for each experimental group. *P < 0.05 vs. the imiquimod-induced group without drug.

Table 1. Immunosuppressive activity of oxazole derivatives

Compound	IC_{50} (μM)[a]	Compound	IC_{50} (μM)
PO-322	0.7 ± 0.2	PO-342	28.9 ± 3.9
PO-324	31.6 ± 2.3	PO-343	> 100
PO-325	> 100	PO-345	> 100
PO-326	25.3 ± 3.1	PO-346	> 100
PO-327	15.1 ± 3.9	PO-348	> 100
PO-329	> 100	PO-349	> 100
PO-333	> 100	PO-350	> 100
PO-335	32.4 ± 4.2	PO-353	> 100
PO-341	24.5 ± 3.5	PO-355	> 100

[a] IC_{50} of inhibitory effect on cell proliferation. In the experiment, CFSE-labeled T cells were treated with a series of oxazole derivatives following anti-CD3 and anti-CD28 mAbs stimulation for 72 h. Cell proliferation was analyzed on a flow cytometry using proliferation index. The results are presented as the mean ± S.E.M., n = 5 for each experimental group.