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RESEARCH ARTICLE

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A benzoxazole derivative PO-296 inhibits T lymphocyte proliferation by the JAK3/STAT5 signal pathway

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Abstract

Immunosuppressants have shown striking achievements in treating autoimmune diseases in recent years. It is urgent to develop more immunosuppressants to provide more options for patients. PO-296 [2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol] was identified as a novel benzoxazole derivative. We observed that it exhibits an obvious immunosuppressive activity to T lymphocytes. PO-296 significantly inhibited the proliferation of activated human T lymphocyte without cytotoxicity. Moreover, PO-296 did not affect the expression of cluster of differentiation (CD)-25 or CD69 but induced T lymphocyte cycle arrest in the G0/G1 phase. Furthermore, PO-296 inhibited interleukin (IL)-6, IL-17, and interferon gamma expression but had no effect on IL-2, IL-4, or IL-10. Yet, importantly, PO-296 inhibited the phosphorylation of signal transducer and activator of transcription 5 (STAT5), increased the phosphorylation of p70S6K, but did not affect the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mitogen-activated protein kinase pathway. In conclusion, these findings indicate that PO-296 inhibits human activated T-lymphocyte proliferation by affecting the janus kinase 3 (JAK3)/STAT5 pathway. PO-296 possesses a potential lead compound for the design and development of new immunosuppressants for the treatment of autoimmune diseases.

KEYWORDS

Immunosuppressive activity, [2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol] (PO-296), T lymphocyte proliferation

1 | INTRODUCTION

T-lymphocyte activation, proliferation, and differentiation play a crucial role in host immune response. Their function needs to be tightly regulated to ensure that immune responses are properly controlled. However, dysregulated T lymphocyte proliferation can lead to autoimmune diseases, including rheumatoid arthritis (RA),[1] systemic lupus erythematosus,[2] inflammatory bowel diseases,[3] and psoriasis.[4] Immunosuppressants are widely used in the treatment of

Xing-yan Luo and Hong Zhou contributed equally to this work.

these diseases, including rapamycin (RAPA), cyclosporine, FK506, and mycophenolate mofetil.[5] Nevertheless, there are significant differences in the pathogenesis and drug sensitivity of different patients. In the course of the treatment, if the effect of a single therapy is not obvious, combination, rotation, or sequential therapy should be used. Therefore, it is important to develop more immunosuppressants to help patients not responding to previous treatment.

Naïve T lymphocytes present characteristic changes to the reaction against specific antigens through activation, generous proliferation, and differentiation to effector T lymphocytes. During naive T lymphocytes activation and further differentiation, many signaling molecules are involved. T cell receptor (TCR) signal triggers naïve T lymphocytes activation after recognizing specific peptides presented by antigen presenting cells (APCs). TCR activates calcineurin, mitogen-activated protein kinase, and nuclear factor kappa B kinase (NF-κB) signal pathway through tyrosine kinase. These activated signal pathways promote the expression of multiple downstream effector molecules, including cytokines and cell surface molecules, such as interleukin (IL)-2 and cluster of differentiation (CD)-25, which make the cells into cell cycle directly or indirectly through the janus kinase 3 (JAK3)/signal transducer and activator of transcription 5 (STAT5)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mammalian target of rapamycin (mTOR)/p70S6K signaling pathways.[6] Immunosuppressants targeting these signaling molecules are widely used in the treatment of autoimmune diseases. In recent years, the JAK3/STAT5 signal pathway has received significant attention as a new target of potential immunosuppressants. Several immunosuppressants acting on the JAK3 signaling pathway are used as clinical trials, including CP690550 (tofacitinib) in psoriasis[7] and RA,[8] VX-509 (decernotinib) in RA.[9]

Previously, we reported that benzothiazole BD750[10] and BD926[11] have an effective immunosuppressive effect on T cell proliferation. However, compared with thiazole, oxazole is expected to be a better drug carrier due to its better skeleton structure.[12,13] Benzoxazole is a heterocyclic aromatic compound; benzoxazole and its derivatives have gained considerable attention in recent years because of their various biological properties, such as antimicrobial,[14] anticancer,[13] analgesic,[15] neuroprotective,[16] treatment of cardiovascular disease.[17] Our previous study demonstrated that the benzoxazole derivative K313 has significant anti-inflammatory properties.[18] We recently found that another novel benzoxazole derivative PO-296 (2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; Figure 1) exhibits an obvious immunosuppressive activity. PO-296 has not been reported to possess immunosuppressive activity previously, and it possesses the characteristics to be a leading compound to develop new immunosuppressants for autoimmune diseases treatment.

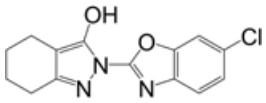

FIGURE 1 Chemical structure of PO-296. PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol

2 | MATERIAL AND METHODS

2.1 | Cell preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Lymphoprep™ (Axis-Shield, Oslo, Norway) density gradient centrifugation, as previously described.[10] Untouched naive pan T lymphocytes from PBMCs were separated by using the Naive Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, T lymphocytes were stained with PE-anti-CD3 (BD PharMingen, San Diego, CA) and analyzed by flow cytometry (Acurri C6; Becton Dickinson, San Jose). More than 95% of T-lymphocyte purity was used in the further experiments. PBMCs and T lymphocytes were cultured in complete Roswell Park Memorial Institute medium (RPMI) 1640 media (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 100 U/mL Penicillin-Streptomycin (Invitrogen). Fibroblast-like synoviocytes (FLS) were isolated from synovial tissues of three patients undergoing arthroplasty. FLS preparation was carried out as described by Rosengren et al.[19] FLS was cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin.

2.2 | 5-Carboxyfluorescein diacetate succinimide ester labeling assay

The proliferation of T lymphocyte was measured by flow cytometry with 5-carboxyfluorescein diacetate succinimide ester (CFSE; Molecular Probes, Eugene, OR) labeling, as previously described.[11] PO-296 (Figure 1) was purchased from ChemBridge Corp (San Diego, CA) and dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) as 40 mM stock solution. Simply, human naïve T lymphocytes (10^6 cells/mL), activated T lymphocytes (10^6 cells/mL), or PBMCs (10^6 cells/mL) were dyed with 2.5 μM CFSE at 37°C for 10 minutes, washed twice with phosphate-buffered saline (PBS), and then resuspended in complete RPMI 1640 medium. Then, the labeled naïve T lymphocytes (10^6 cells/mL) were stimulated by plate-coated 2 μg/mL anti-CD3 antibody (HIT3a clone; BD PharMingen) and soluble 1 μg/mL anti-CD28 antibody (CD28.2 clone; BD PharMingen),

phytohaemagglutinin (PHA) (Sigma-Aldrich) or phorbol 12-myristate 13-acetate (PMA)/ionomycine (Sigma-Aldrich). The labeled activated T lymphocytes (10^6 cells/mL) were induced by 10 ng/mL IL-2 (PeproTech, Rocky Hill, New Jersey). The labeled PBMCs (10^6 cells/mL) were stimulated with an equal number of PBMCs irradiated with 3000 rad from another donor. Subsequently, the proliferation of T lymphocytes stimulated by anti-CD3/CD28 antibodies or alloantigen was analyzed by flow cytometry after stimulation for 72 hours incubated with or without different concentrations of PO-296. A negative control was the cells without stimulator and drug while the positive control was cells with a stimulator, but without drugs.

2.3 | Cell apoptosis assay

The effect of PO-296 on the cell apoptosis of activated T lymphocytes was detected by flow cytometry using a Fluos-labeled Annexin V and PI dual staining kit (Annexin V-Fluos staining kit; Roche, Indianapolis, IN). The activated T lymphocytes (10^6 cells/mL) were treated with 1.25, 5, and 20 μM PO-296, 0.1 μM RAPA (Sigma) or vehicle and activated with 2 μg/mL anti-CD3 antibody and 1 μg/mL anti-CD28 antibody for 24 hours or 48 hours. Subsequently, the cells were collected and assessed by flow cytometry with Annexin V and PI dual staining.

2.4 | Cell viability assay

Cell viability was measured by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). T lymphocytes were stimulated with anti-CD3/CD28 antibodies monoclonal antibodies for 72 hours, washed with 1640, and treated with 10 ng/mL IL-4 (PeproTech) for 48 hours. Then, naïve T lymphocytes (10^6 cells/mL), IL-4 treated activated T lymphocytes (10^6 cells/mL), and FLS (5×10^4 cells/mL) were treated with 5, 10, 20, 40, and 80 μM PO-296 or vehicle for 72 hours. The CCK-8 assay kit was used to evaluate cell viability by detecting the optical density values at 450 nm in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

2.5 | Determination of CD25 and CD69 expression

T lymphocytes (10^6 cells/mL) were collected after 2 μg/mL anti-CD3 antibody and 1 μg/mL anti-CD28 antibody stimulated 24 hours in the presence of 1.25, 5, and 20 μM PO-296, 0.1 μM FK506 (Sigma-Aldrich) or vehicle. The activated T lymphocytes were then stained with PE-anti-CD25 or APC-anti-CD69 (BD PharMingen) at 4°C for 30 minutes. The cells were then washed with PBS and analyzed on a flow cytometer.

2.6 | Cell cycle progression assay

T lymphocytes (10^6 cells/mL) were treated with 1.25, 5, and 20 μM PO-296, 0.1 μM RAPA, or vehicle and stimulated with, or without 2 μg/mL anti-CD3 antibody and 1 μg/mL anti-CD28 antibody for 72 hours. Subsequently, the cells were collected, washed, and analyzed on a flow cytometer using Cycletest Plus DNA Reagent Kit (BD PharMingen) following the manufacturer's protocol.

2.7 | Cytokines enzyme-linked immunosorbent assay

The levels of IL-2, IL-4, IL-6, IL-10, IL-17A, and interferon gamma (IFN-γ) in the supernatants of cells culture were determined by using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA). T lymphocytes (10^6 cells/mL) were treated with 1.25, 5, 20 μM PO-296, 0.1 μM FK506, 50 μM LY-294002 (Sigma-Aldrich), or vehicle and stimulated by 2 μg/mL anti-CD3 antibody and 1 μg/mL anti-CD28 antibody for 24 hours or 48 hours. The supernatants were harvested to determine the levels of cytokines in accordance with the standard curves of recombinant cytokines by ELISA.

2.8 | Western blotting assay

T lymphocytes (10^6 cells/mL) were washed and incubated alone for 6 hours after 72 hours of 2 μg/mL anti-CD3 antibody and 1 μg/mL anti-CD28 antibody stimulation. T lymphocytes were treated with 5, 10, or 20 μM PO-296, 1 μM CP690550 (Tofacitinib citrate) (Sigma-Aldrich), 0.1 μM RAPA, 50 μM LY294002, 2 μM PD184352 (Sigma-Aldrich) or vehicle for another 6 hours and stimulated with 10 ng/mL IL-2 for 30 minutes. Subsequently, cell pellets were treated with lysis buffer and clarified by centrifugation. 20 μg/lane cell lysate proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked by PBS containing 5% bovine serum albumin (BSA) and stained with antibodies against STAT5, p-STAT5(Tyr694), JAK3, p-JAK3(Tyr980/Tyr981), p70S6K, p-p70S6K(Thr389), Akt, p-Akt(Ser473), extracellular signal-regulated kinase (ERK)1/2 or p-ERK 1/2(Thr202/Tyr204) (CST Inc, Danvers, MA) overnight at 4°C followed by horseradish peroxidase-conjugated second antibodies (Santa Cruz Biotech, Santa Cruz) incubation. Finally, proteins were visualized by enhanced chemiluminescence (Millipore).

2.9 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, San Diego, CA). The data of cell proliferation, cytokine analysis were compared by one-way analysis of variance and Dunnett comparisons on post-tests were used to analyze data and compare groups. Comparisons were considered to be significant at P values less than 0.05. The result data are expressed as the mean ± standard error of the mean.

3 | RESULTS

3.1 | PO-296 inhibits T lymphocyte proliferation without obvious cytotoxicity *in vitro*

To discover the immunosuppressive activity of the lead compound, a large number of benzoxazole derivatives were screened with CFSE-labeling on human T lymphocyte proliferation by flow cytometry. The results showed PO-296 had significant activity. T lymphocyte proliferation was inhibited by PO-296 after anti-CD3/CD28 antibody stimulation with an IC_{50} value of $2.67 \pm 0.71\ \mu M$ (Figure 2A and 2B) and alloantigen stimulation with an IC_{50} value of $2.73 \pm 1.20\ \mu M$ (Figure 2C). T lymphocyte proliferation was also inhibited by PO-296 followed PHA (Figure 2D) or PMA/ionomycine (Figure 2E) stimulation with IC_{50} values of $2.77 \pm 0.93\ \mu M$ and $2.84 \pm 1.14\ \mu M$, respectively.

To test whether the activity of PO-296 was immunosuppressive, not cytotoxicity, human activated T lymphocytes treated with PO-296 were measured for cell apoptosis by flow cytometry and naïve T lymphocytes, IL-4 treated activated T lymphocytes, and FLS treated with PO-296 were assessed for viability of cells by the CCK-8 assay. IL-4 treated activated T lymphocytes did not proliferate, but could maintain, cell survival[20] and FLS severed as the nonlymphocyte control.[21] The results showed that PO-296 treatment did not induce activated T lymphocytes apoptosis in 24 hours and 48 hours (Figure 3A) and there was no significant impact of the relative viability on naïve T lymphocytes (Figure 3B), IL-4 treated activated T lymphocytes (Figure 3C), and FLS in 72 hours (Figure 3D). Collectively, these data indicated that the activity of PO-296 was immunosuppressive, not cytotoxic.

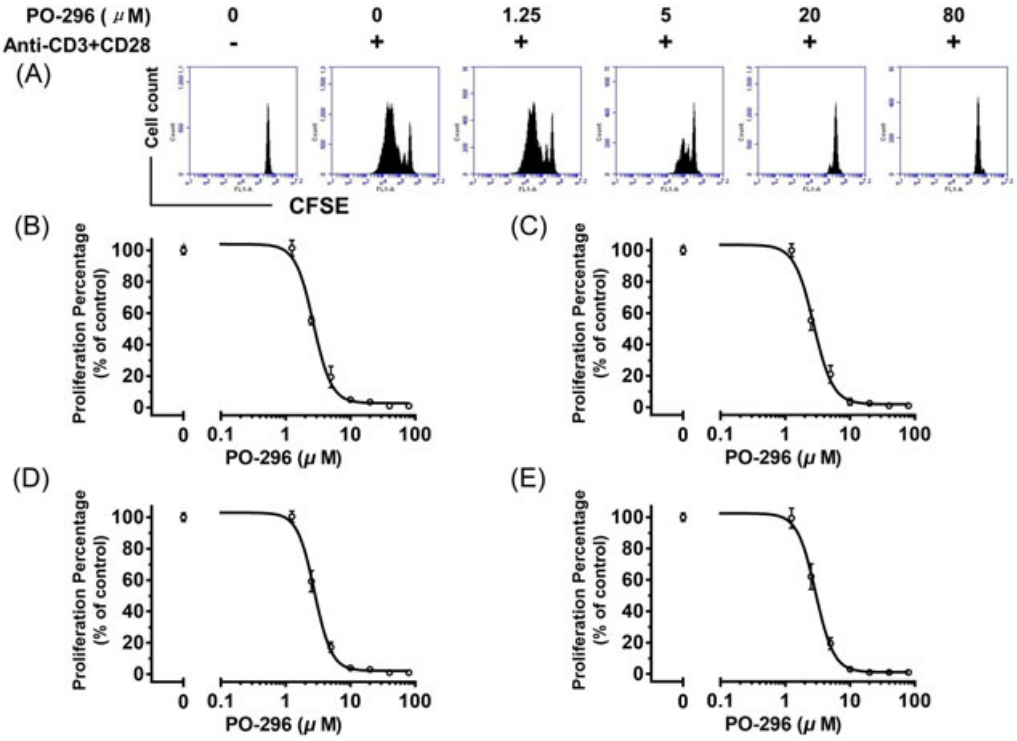

FIGURE 2 PO-296 inhibits T-cell proliferation in vitro. The CFSE-labeled T cells were treated with 1.25, 2.5, 5, 10, 20, 40, and 80 μM PO-296 and activated with anti-CD3/CD28 antibodies (A,B), allogeneic PBMCs (C), PHA (D) or PMA/ionomycine (E) for 72 hours. Cell proliferation was measured by flow cytometry. The cells without stimulator and PO-296 served as negative control (0%) while the cells with a stimulator, but without PO-296, served as the positive control (100%). The results are presented as mean ± SEM (n = 5 per group) from three independent experiments. CFSE, 5-carboxyfluorescein diacetate succinimide ester; PBMCs, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; PMA, phorbol 12-myristate 13-acetate; PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; SEM, standard error of the mean

3.2 | PO-296 does not inhibit T lymphocyte activation

The expression of CD25 and CD69 and the secretion of IL-2 were induced by activated T lymphocytes.[22] The effect of PO-296 on T-lymphocyte activation was assessed through the expression of CD25 and CD69 and the secretion of IL-2. While FK506 inhibited CD25, CD69 expression and IL-2 secretion, similar to a previous report,[23] PO-296 had no obvious effect on them (Figure 4A-C), indicating that PO-296 did not affect T-lymphocyte activation.

3.3 | PO-296 induces T lymphocyte cycle arrest in the G0/G1 phase

Cell cycle progression plays an important role in cell proliferation.[24] To understand the impact of PO-296 on T-lymphocyte cycle progression, the DNA content of T lymphocytes was characterized by flow cytometry. The result indicated that PO-296 increased the percentages in the G0/G1 phase of T lymphocyte cycle, similar to RAPA treatment (Figure 5A and 5B), indicating that PO-296 induced cell cycle arrest at the G0/G1 phase in activated T cells.

3.4 | PO-296 inhibits proinflammatory cytokines production but not anti-inflammatory cytokines in activated T lymphocytes

To understand the impact of PO-296 on the proinflammatory and anti-inflammatory cytokines, the levels of IFN-γ, IL-6, IL-17, IL-2, IL-4, and IL-10 were measured by ELISA in the supernatants of activated T lymphocytes. As shown in Figure 6, PO-296 significantly inhibited IFN-γ (A), IL-17 (B), and IL-6 (C), but did not decrease the levels of IL-2 (D), IL-4 (E), and IL-10 (F) in activated T lymphocytes, indicating that the PO-296 did not affect anti-inflammatory cytokine release from regulatory T lymphocytes but mainly inhibited proinflammatory cytokine release from Th1/17 cells.

3.5 | PO-296 blocks JAK3/STAT5 signaling pathway in IL-2-stimulated activated T lymphocytes

To further reveal the mechanism of PO-296 on T-lymphocyte proliferation, the effect of PO-296 on IL-2-induced T-lymphocyte proliferation and the related signal pathways were analyzed by flow cytometry or

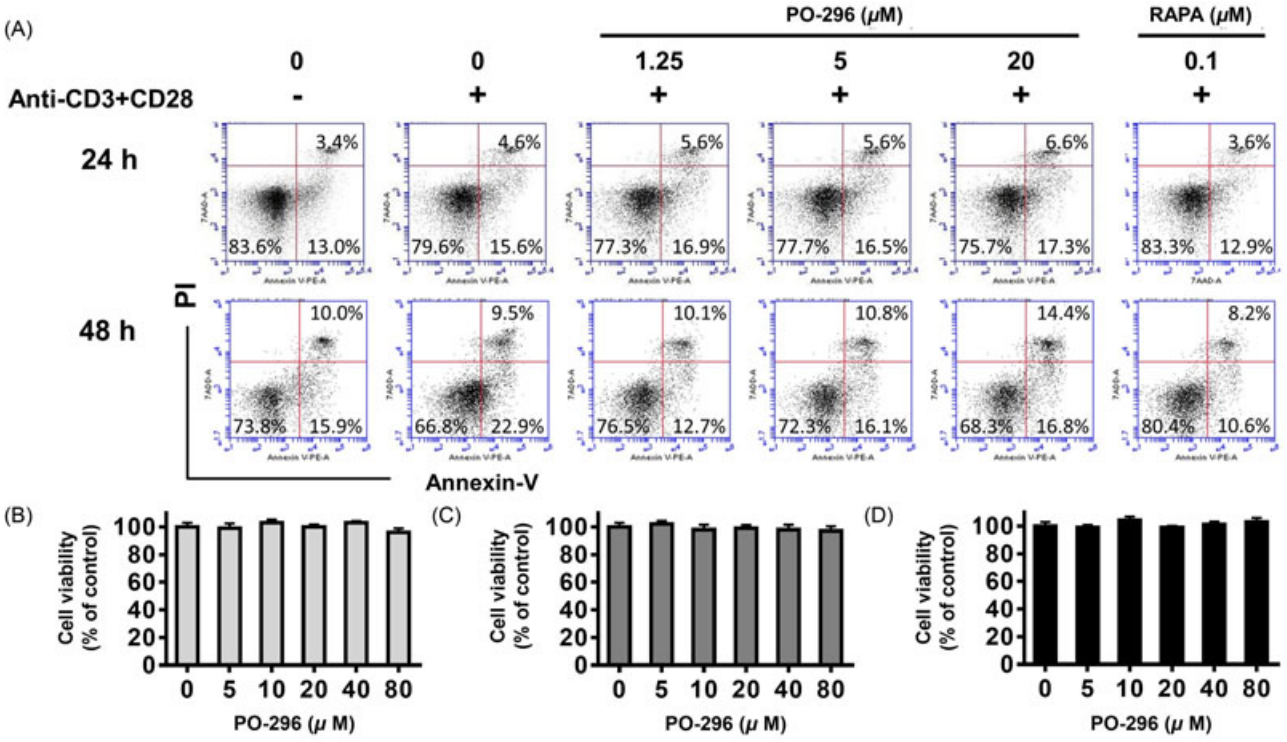

FIGURE 3 PO-296 had no significant cytotoxicity in vitro. T cells were treated with 1.25, 5, and 20 μM PO-296, 0.1 μM RAPA or vehicle and activated with anti-CD3/CD28 antibodies for 24 hours or 48 hours. Cell apoptosis was assessed by flow cytometry with Annexin V and propidium iodide (PI) dual staining (A). Naïve T cells (B), IL-4 treated activated T cells (C) and FLS (D) was treated with 5, 10, 20, 40, and 80 μM PO-296 or vehicle for 72 hours. The CCK-8 assay kit was used to assess the viability of cell. The cells without drugs served as control (100%). The results are presented as mean ± SEM (n = 5 per group) from three independent experiments. CCK-8, Cell Counting Kit-8; FLS, fibroblast-like synoviocytes; IL-4, interleukin 4; PI, propidium iodide; PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; RAPA, rapamycin; SEM, standard error of the mean

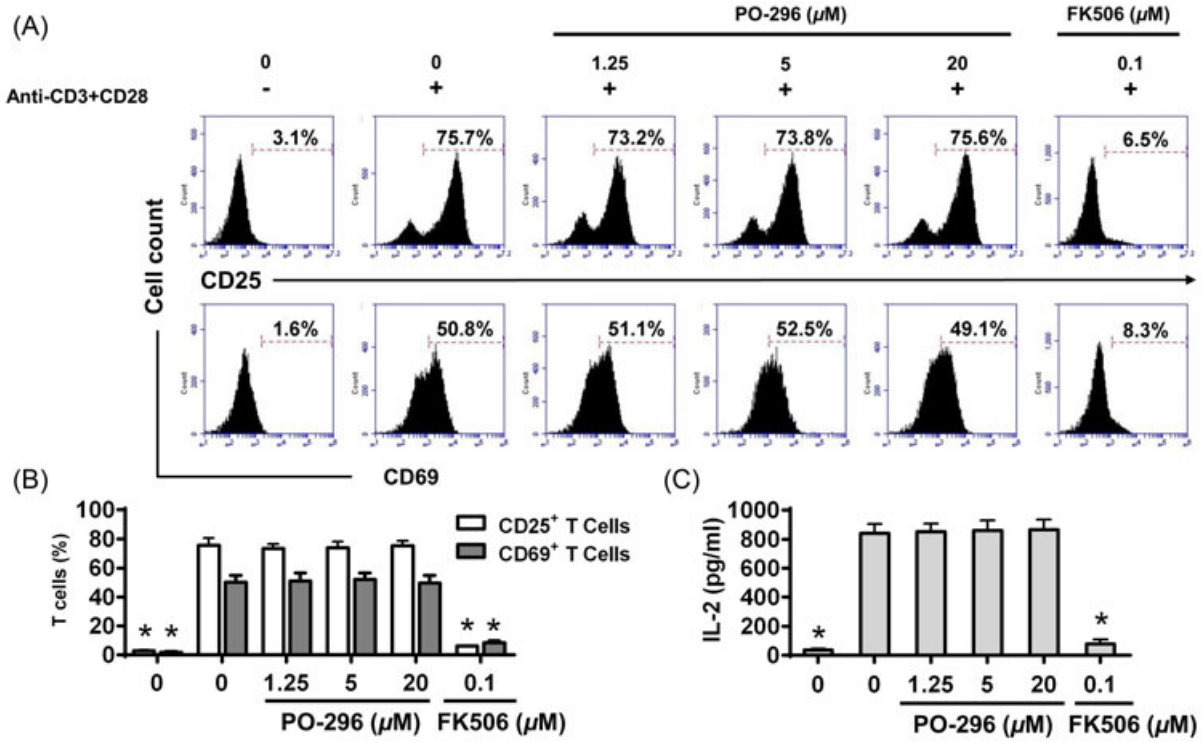

FIGURE 4 PO-296 does not inhibit T cell activation. T cells were treated with 1.25, 5, and 20 μM PO-296, 0.1 μM FK506 or vehicle and activated with anti-CD3/CD28 antibodies for 24 hours. The expression of CD25 and CD69 was analyzed on a flow cytometer (A,B). The supernatant was harvested, and the level of IL-2 was assessed by ELISA (C). The results of flow cytometry are representative histograms and data of ELISA are presented as mean ± SEM (n = 5 per group) from three independent experiments. *$P < 0.05$ versus the group of activated without drug. ELISA, enzyme-linked immunosorbent assay; IL-2, interleukin 2; PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; SEM, standard error of the mean

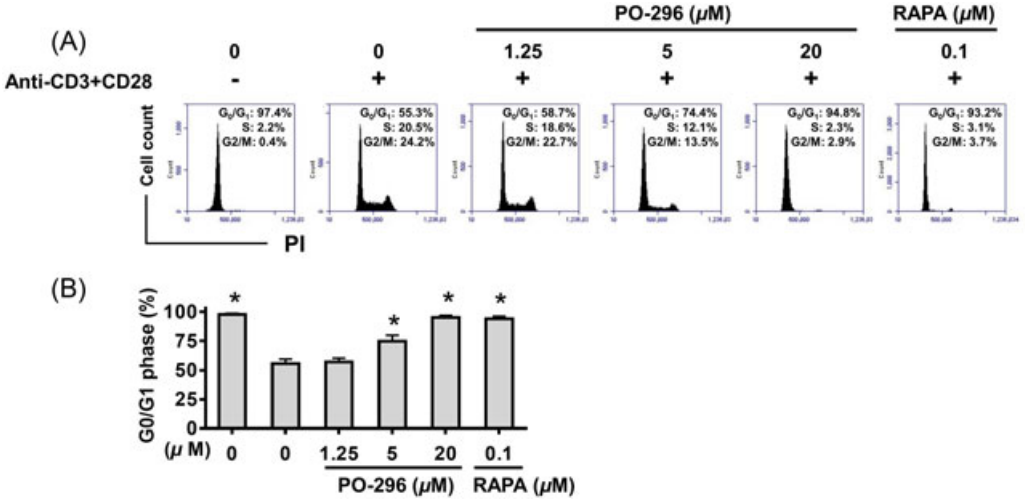

FIGURE 5 PO-296 induces T-cell cycle arrest in the G0/G1 phase. T cells were treated with 1.25, 5, and 20 μM PO-296 or 0.1 μM RAPA and activated with anti-CD3/anti-CD28 antibodies for 72 hours. Cell cycle progression was analyzed by flow cytometry. The results of flow cytometry are representative histograms with mean ± SEM (n = 5 per group) from three independent experiments. Representative flow cytometry histograms (A), Quantitative analysis of the percentages of cells in the G0/G1 phase (B). *$P < 0.05$ versus the group of activated without drug. PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; RAPA, rapamycin; SEM, standard error of the mean

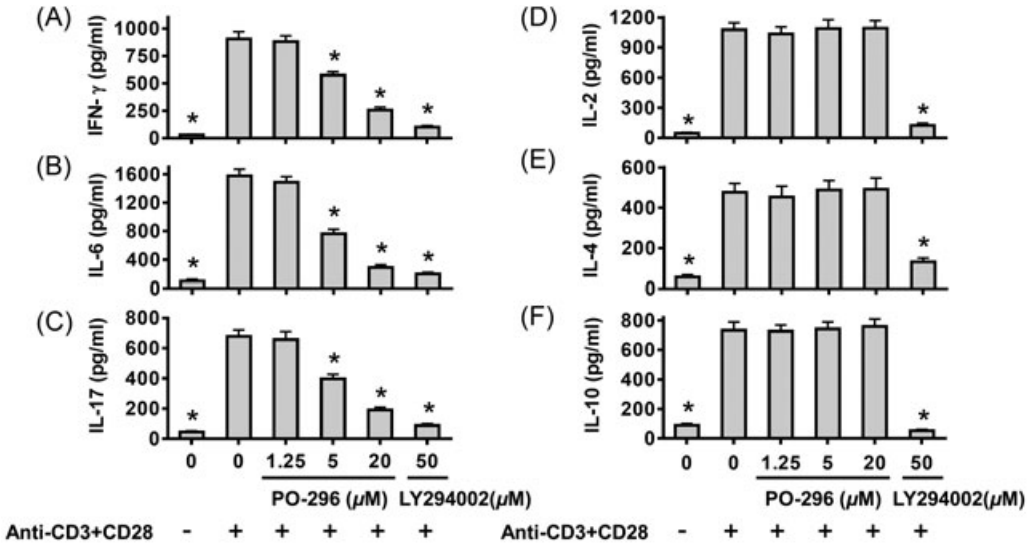

FIGURE 6 PO-296 inhibits proinflammatory cytokines production but not anti-inflammatory cytokines in activated T cells. T cells were treated with 1.25, 5, 20 μM PO-296, 50 μM LY-294002 or vehicle and activated with anti-CD3/CD28 antibodies for 24 hours or 48 hours. The supernatants were collected in 24 hours, and the levels of IFN-γ (A), IL-2 (D) were measured by ELISA. The 48 hours supernatants were measured the cytokines of IL-6 (B), IL-17 (C), IL-4 (E), and IL-10 (F). Results are presented as mean ± SEM (n = 5 per group) from three independent experiments. $^{*}P < 0.05$ versus the group of activated without drug. ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; IL-2, interleukin 2; PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; SEM, standard error of the mean

Western blot, respectively. As shown in Figure 7, PO-296 inhibited IL-2-induced T lymphocyte proliferation with IC_{50} values of 2.53 ± 0.49 μM (A). The phosphorylation of STAT5 was significantly reduced (B), but the phosphorylation of p70S6K was increased by PO-296 (C), not both of their expression with the same treatment, in activated T lymphocytes induced by IL-2. Furthermore, PO-296 did not affect the levels of p-JAK3, p-Akt, or p-ERK 1/2 expression and phosphorylation (BDE). The above results suggested that PO-296 inhibited T lymphocyte

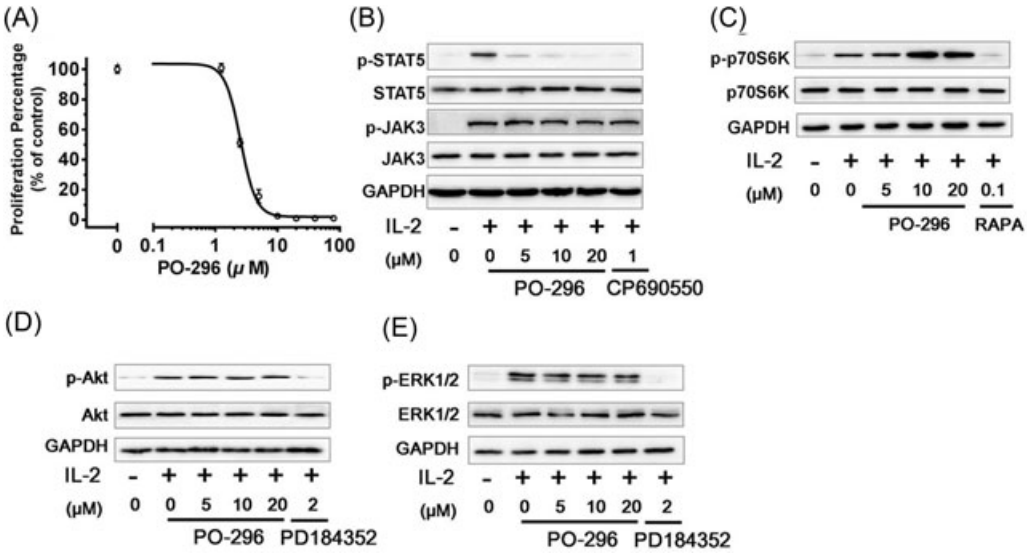

FIGURE 7 PO-296 affects the JAK3/STAT5 signaling pathway in IL-2-stimulated activated T cells. The CFSE-labeled activated T cells were treated with PO-296 and stimulated by IL-2 for 72 hours. Cell proliferation was measured by flow cytometry (A). T cells were incubated alone for 6 hours after 72 hours of anti-CD3/CD28 antibodies monoclonal antibody stimulation. T cells were then treated with 5, 10, or 20 μM PO-296, 1 μM CP690550, 0.1 μM RAPA, 50 μM LY294002, 2 μM PD184352 or vehicle for another 6 hours. Subsequently, T cells were induced by IL-2 for 30 minutes, and the relative phosphorylation and expression levels of STAT5 and JAK3(B), p70S6K (C), Akt (D), and ERK1/2 (E) were assessed by Western blot analysis. The results of flow cytometry are presented as the mean ± SEM (n = 5 per group) and data of Western blot analysis are representative images. CFSE, 5-carboxyfluorescein diacetate succinimide ester; ERK, extracellular signal-regulated kinase; IL-2, interleukin 2; JAK3, janus kinase 3; PO-296, 2-(6-chlorobenzo[d]oxazol-2-yl)-4,5,6,7-tetrahydro-2H-indazol-3-ol; RAPA, rapamycin; SEM, standard error of the mean; STAT5, signal transducer and activator of transcription 5

proliferation through mainly blocking the JAK3/STAT5 signaling pathway.

4 | DISCUSSION

Considerable advances in the treatment of autoimmune diseases have been made after the advent of immunosuppressants. However, the long-term use of one drug can lead to parts of patients losing their primary response. The future looks bright for patients as many new drugs are being developed. In this study, we found that the benzoxazole derivative PO-296 significantly inhibited the proliferation of activated human T lymphocyte in vitro. Most importantly, no obvious cytotoxic effects of PO-296 were observed on resting T lymphocytes, IL-4-treated T lymphocytes, activated T lymphocytes, and FLS. Therefore, PO-296 selectively inhibits T-lymphocyte proliferation without significant cytotoxicity.

The expression of CD25, CD69 and the secretion of IL-2 are markers of T-lymphocyte activation in vitro.[22] PO-296 did not inhibit IL-2 secretion and affect $CD25^+$ or $CD69^+$ T lymphocytes with anti-CD3/CD28 antibodies stimulation, suggesting that PO-296 did not affect the activation of T lymphocytes. To further explore the mechanism of PO-296 inhibition, we found that the cell cycle of T lymphocytes stimulated with anti-CD3/CD28 antibodies was blocked by PO-296 in the G0/G1 phase compared with vehicle-treated T lymphocytes, which may be the reason why T lymphocyte proliferation was inhibited.

Cytokines play a pivotal role in the development of autoimmune diseases. We found that proinflammatory cytokine IFN-γ,[25] IL-6,[26] and IL-17[27] production was inhibited in a dose-dependent manner, whereas anti-inflammatory cytokine IL-2,[28] IL-4,[29] and IL-10[30] secretion was not affected significantly. The results indicated that PO-296 affected the differentiation of T lymphocytes into Th1/17 cells but did not affect the differentiation of T lymphocytes into regulatory T lymphocytes.[31] Thus, PO-296 may selectively inhibit the differentiation of T lymphocytes.

PO-296 inhibited the proliferation of IL-2-activated T lymphocytes. IL-2 stimulates activated T-lymphocytes proliferation through the JAK3/STAT5, PI3K/Akt, mTOR/p70S6K, and mitogen-activated protein kinase (MARK) signal pathways.[32,33] To investigate which signal pathway was affected by PO-296, the phosphorylation of JAK3, STAT5, Akt, p70S6K, and ERK1/2 was measured by Western blot analysis. We found that the phosphorylation of STAT5 decreased with the increase of concentration, but not JAK3 phosphorylation. These results implied that PO-296 might directly target the SH2 domain of JAK3 to prevent STAT5 phosphorylation, not JAK3 phosphorylation in T cells. Because the JAK3/STAT5 signal pathway is a key mediator for activating T-lymphocyte proliferation,[11] PO-296 may inhibit activated T-lymphocytes proliferation through the JAK3/STAT5 signal pathway.

Recently, JAK inhibitors have been developed as a new class of immunosuppressants that inhibit related intracellular signaling of various cytokines and growth factors. The JAK3/STAT5 signaling pathway is a key intracellular cascade for intracellular signaling conduction from cytokines.[34] JAK3 mainly expresses in hematopoietic tissues and regulates T-lymphocyte proliferation and differentiation.[35] Mice and humans with defected JAK3 gene show immune deficiency, inhibiting the function of T lymphocytes.[36,37] And JAK3 gene mutation may cause natural killer-cell lymphoma[38,39] and T-lymphocyte prolymphocytic leukemia.[40,41] Thus, the restricted expression and function of JAK3 make it a potential therapeutic target.

JAK inhibitor therapy is witnessing an unprecedented speed of development and entering a significant new era in autoimmune diseases. For example, the current evidence indicates that oral tofacitinib is a useful option for the treatment of patients with RA[42] and solid organ transplantation.[43] T-cell proliferation inhibitory activity of PO-296 is similar to CP690550, but the structure of PO-296 is different from CP690550 and other JAK3 inhibitors. PO-296 might target JAK3 as CP690550 or might target other proteins. We will select about 100 kinds of kinases related to cell proliferation by kinase assay to find the target proteins of PO-296 in our future experiments.

In conclusion, our results showed that the potential anti-proliferation mechanisms of PO-296 inhibit the STAT5 phosphorylation levels. Our data also indicated that PO-296 preferably inhibited IFN-γ, IL-6, and IL-17 production of the activated T cells. Thus, PO-296 is a potential lead agent to develop new immunosuppressive agents. This discovery is expected to improve patients' quality of life.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

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