SM934, a water-soluble derivative of artemisinin, exerts immunosuppressive functions in vitro and in vivo

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ABSTRACT

In the present study, we investigated the immunosuppressive effects and underlying mechanisms of β-aminoarteether maleate (SM934), a derivative of artemisinin, against T cell activation in vitro and in vivo. In vitro, SM934 significantly inhibited the proliferation of splenocytes induced by concanavalin A (Con A), lipopolysaccharide (LPS), mixed lymphocyte reaction (MLR), and anti-CD3 plus anti-CD28 (anti-CD3/28). SM934 significantly inhibited interferon (IFN)-γ production and CD4+ T cell division stimulated by anti-CD3/28. SM934 also promoted apoptosis of CD69+ population in CD4+ T cells stimulated by anti-CD3/28. Furthermore, SM934 inhibited interleukin (IL)-2 mediated proliferation and survival through blocking Akt phosphorylation in activated T cells. In ovalbumin (OVA)-immunized mice, oral administration of SM934 suppressed OVA-specific T cell proliferation and IFN-γ production. SM934 treatment also significantly inhibited the sheep red blood cell (SRBC)-induced delayed type hypersensitivity (DTH) reactions in mice. Taken together, SM934 showed potent immunosuppressive activities in vitro and in vivo. Our results demonstrated that SM934 might be a potential therapeutic agent for immune-related diseases.

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1. Introduction

Derivatives of artemisinin are currently recommended as first-line antimalarial agent [1]. Besides having antimalarial activity, these drugs also exhibit potent immunosuppressive effects. In China, artemisinin derivatives have shown promising results when tested for treatment of autoimmune diseases, such as systemic lupus erythematosus (SLE) and allergic contact dermatitis [2–4]. However, the efficacy of clinically relevant artemisinin derivatives is limited, and their immunosuppressive mechanisms are still obscure. Therefore, we synthesized a series of new water-soluble and oil-soluble artemisinin derivatives and screened for a promising compound with potent immunosuppressive activity [5,6]. SM934, β-aminoarteether maleate (Fig. 1), was identified as a promising compound with higher bioavailability and drug likeability. In this study, we investigated the immunosuppressive effects of SM934 on T cell activation, both in vitro and in vivo, and explored its potential mode of action.

T cells play a pivotal role in acquired immune reaction and have been implicated in mediating many aspects of autoimmune diseases [7]. Firstly, TCR cross-linking drives T cells from G0 to G1 transition and subsequent secretion of T cell growth factor IL-2 and expression of high-affinity receptor IL-2Rα chain (CD25); secondly, through autocrine/paracrine proliferative loop, IL-2 induces clone expansion and maintains survival of activated T cells; thirdly, once there is successful clearance of the pathogen/autoantigen, the stimulus for cytokines production is lost and the majority of activated T cells enter apoptosis [8,9]. Whereas, in the circumstance of autoimmune diseases, persistent presence of autoantigen provides the survival signals for activated autoreactive lymphocytes to avoid apoptosis, which results in sustained tissue injury [10,11]. Abrogating IL-2 production by Cyclosporin A (CsA) has revolutionized the field of immune therapy [12]. The immunosuppressive action of CsA involves initial binding to cyclophilin, leading to reduced IL-2 production as part of the signal transduction pathway for the activation of T cells.

Actually, IL-2 often acts as a “double-edged sword” in immune responses. On one hand, IL-2 is a growth factor for T cells and plays important roles in the immune response. On the other hand, IL-2 is also critical for the development of immunosuppressive regulatory T cells, and IL-2 deficient mice develop severe autoimmune disorders [13,14]. And IL-2 secretion by T cells is decreased in some autoimmune diseases, especially systemic lupus erythematosus [15,16]. Thus, drugs affecting
IL-2-dependent T cell activation rather than IL-2 production should be of particular interest for their potential clinical uses.

To gain insight into the molecular mechanism of action of SM934, we investigated the effects of SM934 on TCR cross-linking induced proliferation and cytokine production in T cells, which were mimicked by anti-CD3/28 stimulation. The results showed that SM934 significantly inhibited T cell proliferation without much influence on IL-2 production. Further studies revealed that SM934 inhibited IL-2 mediated proliferation and survival through blocking Akt phosphorylation in activated T cells. Using OVA-immunized mice and SRBC-induced DTH reaction, we demonstrated that SM934 also had the potent immunosuppressive effects in vivo. These results indicated that SM934 possessed potent immunosuppressive activities in vitro and in vivo. SM934 might be a novel immunosuppressive agent.

2. Materials and methods

2.1. SM934

SM934, β-aminoarteether maleate, was synthesized from β-hydroxyarteether at Shanghai Institute of Materia Medica. Before use, SM934 was dissolved in phosphate buffered saline (PBS) or saline as a stock solution, and stored at −20 °C. The chemical structure is shown in Fig. 1.

2.2. Mice

Female BALB/c and C57BL/6 mice (6 to 8-week old) were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Certificate No.2002-0010). The mice were housed under specific pathogen-free conditions. All mice were fed standard laboratory chow and water ad libitum. The environment was maintained at 22 ± 1 °C with a 12-h light and dark cycle. All mice were allowed to acclimatize in our facility for 1 week before any experiments were started. Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Animal Ethics Committee of the Shanghai Institute of Materia Medica.

2.3. Splenocyte preparation

Mice were sacrificed and their spleens were removed aseptically. The spleens were pressed against the bottom of the petri dish with the plunger of a 6-ml syringe. A single cell suspension was prepared and cell debris and clumps were removed. Erythrocytes were lysed with Tris-buffered ammonium chloride (0.155 M NH₄Cl and 16.5 mM Tris, pH 7.2). Cells were washed and resuspended in RPMI 1640 media (containing 10% FBS) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Cells were counted by trypan blue exclusion. The viability of splenocytes was consistently >99%.

2.4. MTT assay

Cytotoxicity was assessed by the MTT assay. Briefly, splenocytes were cultured in triplicate for 48 h with SM934. The cells cultured with media alone were used as controls. MTT (5 mg/ml) reagent was added 4 h before the end of culture, and then cells were lysed with 10% sodium dodecyl sulfate (SDS), 50% N, N-dimethyl formamide, pH 7.2. O.D. values were read at 570 nm, and the percentage of cell death was calculated. Experiments were conducted at least three times.

2.5. Con A and LPS-induced proliferation assay

Splenocytes were cultured in triplicate for 48 h with 5 μg/ml of Con A or 10 μg/ml of LPS plus SM934. Cells were pulsed with 0.5 μCi/well of [³H]thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta TriLux, PerkinElmer Life Sciences, Boston, MA). Experiments were conducted at least three times.

2.6. Mixed lymphocyte reaction assay

BALB/c splenocytes (3 × 10⁵ cells/well, stimulator cells) were pre-treated with 30 Gy γ-irradiation (Gammacell 3000, Ottawa, ON, Canada) and then co-cultured in triplicate with C57BL/6 splenocytes (3 × 10⁵ cells/well, responder cells) in the presence or absence of SM934. After 72 h, cells were pulsed with 1 μCi/well of [³H]-thymidine and incubated for another 24 h. Cells were harvested onto glass fiber filters and incorporated radioactivity was counted using a Beta Scintillation Counter.

To determine cytokine levels, the cultures were incubated for 72 h and the supernatants were collected by centrifugation at 300g for 5 min and stored at −20 °C. Mouse IL-2 and IFN-γ level in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences and Pharmingen, San Diego, CA, USA) following the manufacturer’s instruction. Experiments were conducted at least three times.

2.7. CD4⁺ T cell isolation

Purified CD4⁺ T cells from BALB/c mice were prepared by using immunomagnetic negative selection to deplete CD8⁺ T cells, B-cells, NK T cells and I-A⁺ APCs as described previously with slight modification [17]. Lymphocytes were reacted with anti-I-Ad/b, anti-B220, anti-Mac1, anti-CD8, and anti-NK1.1 (mAbs used here were all purified from ascites using agarose-conjugated-protein G column in our lab) and then incubated with magnetic particles bound to goat anti-rat and goat anti-mouse Ig (Advanced Magnectics). Purity of the resulting CD4⁺ T cell populations was examined by FACS Calibur (Becton Dickinson, San Jose, CA, USA), and was consistently >93%.

2.8. Anti-CD3/28 mAb mediated primary CD4⁺ T cells activation

Primary CD4⁺ T cells (2 × 10⁵ cells/well) were cultured with anti-CD28 mAb (1 μg/ml) in 96-well flat-bottom plates coated with anti-CD3 mAb (5 μg/ml). SM934 (1 and 10 μM) was added simultaneously. CD4⁺ T cells were cultured for 48 h to assess [³H]thymidine incorporation and to determine IL-2 and IFN-γ productions. For activated marker expression and apoptosis determination, cells were cultured for 24 h. Experiments were conducted at least three times.
2.9. Flow cytometry tests of activated markers

Cells were collected and blocked with rat-anti-mCD16/CD32 (clone 2.4G2, purified from ascites using agarose-conjugated-protein G column in our lab) and then stained with FITC-, phycoerythrin-, or biotin-conjugated monoclonal antibody followed by fluorescein-conjugated streptavidin: rat anti-mouse-CD4, anti-CD25, anti-CD69, anti-CD44, and anti-CD62L (all from Becton Dickinson) respectively and analyzed by FACS Calibur with CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.10. Activated T cells preparation and IL-2 mediated activation

Splenocytes were stimulated with anti-CD3 (5 μg/ml pre-coated) and recombinant IL-2 (100 U/ml, Boehringer-Mannheim, Germany) and expanded for 5 days. Then the cells were washed three times and starved for an additional 24 h in 0.1% FBS-1640 medium. And then dead cells were removed by one-step gradient using Histopaque 1.077. Cell purity was determined by flow cytometry to be 98% Thy1.2+, ~68% CD4+, and ~26% CD8+.

Activated T cells were co-cultured with IL-2 in the presence or absence of SM934 for 24 h. To determine the effects of SM934 on IL-2-mediated proliferation, the culture supernatants were pulsed with 0.5 μCi/well [3H]thymidine for 8 h and assessed for [3H]thymidine incorporation at the end of incubations. To determine the effects of SM934 on IL-2-mediated survival of activated T cells, the cultured cells were collected after 24 h incubation and examined for apoptosis. Experiments were conducted at least three times.

2.11. CFSE labeling

Purified primary CD4+ T cells were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 μM) at 37 °C for 5 min. The staining was stopped by the addition of FBS to reach a concentration of 20%. The cells were then extensively washed with PBS. Labeled cells were counted and then cultured with anti-CD28 mAb (1 μg/ml) in 96-well flat-bottom plates coated with anti-CD3 mAb (5 μg/ml) in the presence or absence of SM934 (1 and 10 μM) for 72 h. The sequential loss of CFSE fluorescence was used to measure cell division by flow cytometry. Division numbers were obtained from an interval analysis of the CFSE histogram by using CellQuest software. Experiments were conducted at least three times.

2.12. DNA contents assay

Cells were collected and washed with cold PBS and fixed with 70% ethanol for 2 h at 4 °C. The fixed cells were stained with 20 μg/ml of propidium iodide (PI) containing 10 μg/ml of RNase A at room temperature for 20 min in a dark room. The DNA contents were analyzed by flow cytometry with Modfit software (Becton Dickinson, San Jose, CA, USA). Experiments were conducted at least three times.

2.13. Apoptosis analysis

Cells were collected and washed and resuspended in 100 μl Annexin V labeling buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl and 2 mM CaCl2). The final concentration of 1 μg/ml of FITC-conjugated Annexin V (556419, BD Pharmingen) or PE-Cy5-conjugated Annexin V (ab14158, Abcam) was added. To track early and late stages of cell death, 5 μg/ml of PI was used before analysis. Samples were analyzed by flow cytometry with CellQuest software.

To measure apoptosis of primary CD4+ T cells stimulated with anti-CD3/28, cells were simultaneously stained with PE-a-mCD69 and FITC-Annexin V on ice for 30 min. In such situation, 0.5% BSA was added into the Annexin V labeling buffer.

DNA fragment analysis was also conducted to detect apoptosis using a DNA ladder kit purchased from BioVision Inc.


To examine the influence of SM934 on IL-2-induced phosphorylation of Jak1 and Jak3 protein, activated T cells were co-cultured with SM934 (1 and 10 μM) for 6 h and then were stimulated with IL-2 (25 U/ml) for 20 min. Then, the cells were collected and lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue) and boiled for 5 min at 100 °C. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to the nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and blocked with 5% BSA-TBST buffer (TBS containing 0.1% Tween20) for 1 h at room temperature. The membranes were incubated overnight at 4 °C with a 1:1000 dilution of one of the polyclonal antibodies against phospho-Akt, phospho-Erk1/2 and phospho-Stat5 (Cell Signal Technology, Danvers, MA, USA). The blots were rinsed three times with TBST buffer for 15 min each. The washed blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Biotechnology Company, Shanghai, China) for 1 h and then washed three times with the TBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Respective band intensities were measured using Quantity One (version 4.62; Bio-Rad), P-Akt, p-Erk1/2, and p-Stat5 from different groups were compared and expressed as ratios, relative to the corresponding GAPDH. P-Jak1 and p-Jak3 were compared and expressed as ratios to the corresponding Jak1 or Jak3 levels. Experiments were conducted at least three times.

2.15. Western blotting

To examine the influence of SM934 on IL-2-induced phosphorylation of Erk1/2, Akt and Stat5 protein, activated T cells were co-cultured with SM934 (1 and 10 μM) for 1 h and then were stimulated with IL-2 (25 U/ml) for 20 min. Then, the cells were collected and lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue) and boiled for 5 min at 100 °C. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to the nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and blocked with 5% BSA-TBST buffer (TBS containing 0.1% Tween20) for 1 h at room temperature. The membranes were incubated overnight at 4 °C with a 1:1000 dilution of one of the polyclonal antibodies against phospho-Akt, phospho-Erk1/2 and phospho-Stat5 (Cell Signal Technology, Danvers, MA, USA). The blots were rinsed three times with TBST buffer for 15 min each. The washed blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-technology Company, Shanghai, China) for 1 h and then washed three times with the TBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Respective band intensities were measured using Quantity One (version 4.62; Bio-Rad), P-Akt, p-Erk1/2, and p-Stat5 from different groups were compared and expressed as ratios, relative to the corresponding GAPDH. P-Jak1 and p-Jak3 were compared and expressed as ratios to the corresponding Jak1 or Jak3 levels. Experiments were conducted at least three times.

2.16. OVA specific T cell immune responses

OVA at 2 mg/ml in PBS was emulsified in an equal volume of complete Freund’s adjuvant (CFA). The emulsion (200 μl containing 200 μg OVA) was injected subcutaneously into the backs of BALB/c mice on day 1. OVA-immunized mice were treated orally with saline or SM934 (1, 3 and 10 mg/kg), once a day for 14 consecutive days. On day 14, splenocytes (5 × 10⁶ cells/well) of OVA-immunized or normal mice were stimulated with OVA (100 μg/ml). Cells were pulsed with 0.5 μCi [3H]thymidine per well for 8 h before harvesting and assessed for [3H] thymidine incorporation at 72 h. To determine cytokines, the supernatants were harvested at 48 h and the levels of IL-2 and IFN-γ were measured by ELISA assay.

2.17. SRBC-induced delayed type hypersensitivity (DTH) response

The experiments for studying SRBC-induced DTH reaction were performed as described previously [18]. The sensitization was induced by subcutaneous injection (s.c.) of 50 μl of SRBC (2.5 × 10⁹/ml) into the
right hind food pad. Five days later, the mice were challenged with 50 μl of SRBC (2.5×10^9/ml) into the left hind food pad. The difference between the thickness of left and right food pads was measured at 24 h after challenge by using an engineer’s micrometer (Harbin Measuring & Cutting Tool Works). Saline, SM934 (1, 3 and 10 mg/kg) and prednisone acetates (PNS, 2 mg/kg), served as golden controls, were orally administered for six consecutive days from sensitization to challenge. To exclude that the foot edema was induced by non-specific inflammation, ten mice were injected with PBS as sensitization and then were also challenged with SRBC.

2.18. Statistical analysis

Data are presented as the mean±s.e.m. Comparisons between treated and control groups were made by Student t test. Statistical difference was accepted at P below 0.05.

3. Results

3.1. SM934 inhibited mitogen-induced splenocyte proliferation

The in vitro immunosuppressive activity of SM934 was evaluated on splenocyte proliferation induced by Con A and LPS. The results shows, (in Table 1) that the inhibitory effects of SM934 were on Con A and LPS-induced proliferation of splenocytes, with the IC50 (the inhibitory concentration of the compound that reduces cell proliferation by 50%) value of 1.2±0.5 μM and 2.6±1.4 μM, respectively. The cytotoxicity of SM934 was examined by MTT assay. The data showed that SM934 had a low toxicity relative to its anti-proliferation activity, with the CC50 (the cytotoxic concentration of the compound that reduces cell viability by 50%) value of 67.3±32.7 μM. These results indicated that SM934 had significant inhibitory effects on splenocyte proliferation, and its activities observed here were not due to compound toxicity. In the following in vitro studies, 1 and 10 μM of SM934 were tested for its immunosuppressive activity.

3.2. SM934 suppressed alloantigen-induced splenocytes proliferation and IFN-γ production

The mixed lymphocyte culture reaction (MLR) is a model of T cell response to alloantigenic peptides complexed with major histocompatibility (MHC) proteins on APC. The results shows that (in Fig. 2A) SM934 at concentrations of 1 and 10 μM significantly inhibited T cell proliferation and IFN-γ production induced by MLR.

3.3. SM934 suppressed TCR cross-linking-induced CD4+ T cell proliferation and IFN-γ production

In vitro stimulation of T cells with anti-CD3/28 served to mimic the physiologic cross-linking of TCR. Primary T cells were tested for anti-CD3/28-induced proliferative responses. The results shows (in Fig. 2B) SM934 inhibited primary CD4+ T cell proliferation and IFN-γ production induced by anti-CD3/28 stimulation. IL-2 and IFN-γ are produced and released upon T cell activation. Interestingly, SM934 showed minor effects on IL-2 secretion in MLR and anti-CD3/28-stimulation (data not shown). SM934 also showed no influence on the varied expression of activation markers including CD25, CD44 and CD62L on the surface of primary CD4+ T cells stimulated with anti-CD3/28 for 24 h (data not shown).

3.4. SM934 inhibited anti-CD3/28-induced CD4+ T cell division

To monitor the effect of SM934 on cell division, primary CD4+ T cells were pre-labeled with CFSE. As the cell divides, the retained CFSE label is distributed to each daughter cell. The results shows (in Fig. 3) CFSE-
labeled cells divided extensively after stimulation with anti-CD3/28 for 72 h. SM934 (1 and 10 μM) significantly inhibited anti-CD3/28-stimulated primary CD4+ T cell division. SM934 at 10 μM almost completely blocked cell division. Because SM934 inhibited cell division, we then analyzed the effects of SM934 on cell cycle distribution by staining DNA content (PI staining). Results showed SM934 significantly inhibited the cell cycle progression. After 16 h anti-CD3/28 stimulation, 24% of stimulated CD4+ T cells were at S phases, while 16% and 7.4% of the cells co-cultured with SM934 at 1 and 10 μM were at S phases, respectively (data not shown).

3.5. SM934 promoted apoptosis of CD69+ population in CD4+ T cells stimulated by anti-CD3/28

To assess the effect of SM934 on activated T cells, primary CD4+ T cells were stimulated with anti-CD3/28 and analyzed with flow cytometry. The expression levels of CD69 indicated the activation state of CD4+ T cells. The results showed (in Fig. 4A) that anti-CD3/28 stimulation for 24 h induced the great up-regulation of CD69 expression with 58±11% positive on primary CD4+ T cells. There was no significant influence in CD69 expression on CD4+ T cells with SM934 treatment (1 and 10 μM) that obtained 54±12% and 52±11% positive expressions, respectively.

We then investigated whether SM934 influenced the apoptosis of activated CD4+ T cells. The proportion of Annexin V+ was analyzed on the gated CD69+ and CD69− population. The results showed (in Fig. 4B) that SM934 treatment only significantly promoted the CD69+ population into early stage of apoptosis. The proportion of Annexin V+ was 7.8±3.2% in CD69+ population from CD4+ T cells stimulated by anti-CD3/28. While SM934 treatment (1 and 10 μM) significantly increased the proportion of Annexin V+ with 24.9±2.6% and 37.9±7.4%, respectively. The pro-apoptotic effects of SM934 seemed to be stringently presented on activated cells, and there were no effects on CD69− cells (Fig. 4C).

3.6. SM934 suppressed IL-2-mediated proliferation and survival of activated T cells

IL-2 is a growth factor for T cells and plays important roles in the immune response. To know about the effect of SM934 on IL-2-mediated proliferation, activated T cells after one day starving culture were re-cultured with IL-2 (5 and 25 U/ml) in the presence or absence of SM934 (1 and 10 μM) for additional 24 h incubation. The results showed (in Fig. 5A) that SM934 significantly suppressed IL-2-mediated proliferation. Cells were also stained with Annexin V and PI to determine early and later apoptosis. SM934 alone did not induced activated T cells into apoptosis (Fig. 5B), but it was significantly impeding IL-2-mediated survival (Fig. 5C).

3.7. SM934 inhibited IL-2 mediated Akt phosphorylation in activated T cells

To observe the effects of SM934 on the IL-2 related signal transduction pathway, activated T cells were pre-incubated with SM934 for 6 h and then stimulated with IL-2 (25 U/ml). Cells were collected and directly lysed to monitor Akt, Erk1/2, and Stat5 activation by western

Fig. 3. SM934 inhibits the T cell division stimulated with anti-CD3/28. CFSE-labeled primary CD4+ T cells were stimulated with anti-CD3/28 for 72 h to achieve the full cycling in the presence or absence of SM934 (1 and 10 μM). Three experiments were performed with similar results.

Fig. 4. SM934 promotes apoptosis of CD69+ population in CD4+ T cells stimulated by anti-CD3/28. Primary CD4+ T cells (2×10^5/well) stimulated with anti-CD3/28 in the presence or absence of SM934 (1 and 10 μM) for 24 h, and then stained with CD69 and Annexin V for activation (A) and apoptosis analysis (B and C) in flow cytometry. The results were expressed as mean ± s.e.m with three independent experiments. ** P<0.01, versus the medium-treated stimulated group.
blotting 20 min after IL-2 stimulation. To determine the effects of SM934 on activation of Jak1 and Jak3, cells were collected 5 min after IL-2-stimulation. The results showed (in Fig. 6A) that SM934 had no influence on the downstream signaling pathways of IL-2R involving Erk1/2, Stat5, Jak1, and Jak3, but it obviously suppressed the phosphorylation of Akt.

To confirm that SM934 could impede IL-2-mediated survival of activated T cells, we conducted DNA ladder analysis. LY294002 (an inhibitor of Akt), PD98059 (an inhibitor of Erk1/2), and rapamycin, which could block IL-2-mediated T cell cycle progression, whereas preserve the T cell survival [19], were used as controls. Activated T cells were co-cultured with rapamycin, LY294002, PD98059, or SM934 in the presence of IL-2 for 24 h, then DNAs were extracted for fragment analysis. Results showed (in Fig. 6B) that LY294002, PD98059 and SM934 all significantly induced the DNA fragment in activated T cells re-stimulated with IL-2 for 24 h. In contrast, rapamycin did not inhibit IL-2 mediated survival, which was consistent with a previous report [19].

3.8. SM934 inhibited OVA-specific T cell activation

To further illustrate the in vivo immunosuppressive property of SM934, the splenocytes from OVA-immunized mice were assessed for their capacity to proliferate and cytokine production to OVA ex vivo. The results showed (in Fig. 7) that compared with splenocytes from naïve mice, splenocytes from OVA-immunized mice exhibited a prominent antigen-specific recall response to OVA. Oral treatment with SM934 significantly inhibited the OVA-specific T cell proliferation (Fig. 7A) and IFN-γ production (Fig. 7B). Interestingly, SM934 treatment exerted no influence on IL-2 production of splenocytes re-stimulated with OVA (data not shown).

3.9. SM934 inhibited SRBC-induced DTH response

The DTH reaction is based on a cell-mediated pathologic response involved with CD4+ T cell activation. We analyzed the effects of SM934 on DTH responses in BALB/c mice. The results showed (in Fig. 8) that oral administration of SM934 inhibited SRBC-induced DTH. The foot pad volume of normal mice was 81±50 mm. The foot pad swelling was observed in SRBC sensitized mice with 475±269 mm. Oral administration of SM934 (1, 3, 10 mg/kg) significantly suppressed SRBC-induced foot pad swelling to 196±74 mm, 203±101 mm, and 225±30 mm, respectively.

4. Discussion

Artemisinin and its derivatives exhibit potent immunosuppressive activity, and they have been used for the treatment of autoimmune diseases with promising results. In previous studies, we synthesized and examined a series of new artemisinin derivatives and demonstrated several of the compounds with potent immunosuppressive effects.
However, most of these derivatives possess poor water solubility or low bioavailability. Here we report a water-soluble artemisinin derivative SM934 with higher bioavailability and drug likeability. In the present study, we demonstrated the potent immunosuppressive activity of SM934, both in vitro and in vivo, and explored the mechanism of action.

SM934 exhibits the potent immunosuppressive effects in vitro, including suppression of splenocyte proliferations induced by mitogen and MLR, and suppression of CD4⁺ T cell proliferation induced by anti-CD3/CD28. SM934 also inhibited IFN-γ production induced by MLR and anti-CD3/CD28, while showing no effects on IL-2 production.

Previous studies from different laboratories reported that the effects of artemisinin derivatives on IL-2 production remained to be variable and elusive[20–24]. It may be due to the fact that derivatives differ from each other in their side chain groups, which vary in structure, size, and electric charge, and which influence the mode of action. Since IL-2 plays a dual role in acquired immune response, the mode of action of SM934 on IL-2 and IL-2 related immune response events is still attractive. In this study, SM934 exerted potent immunosuppressive activity both in vitro and in vivo, showing no direct inhibitory effect on IL-2 production.

We demonstrated that SM934 significantly inhibited T cell division induced by TCR cross-linking, which was in accord with our previous studies[21,22]. In addition, our study revealed that SM934 could induce CD69⁺ population of CD4⁺ T cells into the apoptosis, but no effects on the activation of CD4⁺ T cells with CD69 expression. Also the results indicated such pro-apoptotic effects of SM934 seemed to be stringently occurring on CD69⁺ population, but not on the CD69⁻ population. This study was the first to demonstrate that the derivative of artemisinin, SM934, could enhance activated T cells going to apoptosis. Such mechanism might be shared with artemisinin and its derivatives to exert immunosuppressive effects.
Proliferation and survival of T cells induced by TCR cross-linking are mostly dependent on IL-2 signaling [25]. Having ruled out the influence on IL-2 production, we shift focus to the effects of SM934 on the downstream events of IL-2. IL-2 binds to a heterotrimeric receptor complex consisting of IL-2Rα, IL-2Rγ, and γ chains [26]. Heterodimerization of IL-2Rγ and γ chains results in activation of Jak1 and Jak3 protein, which are followed in turn by tyrosine phosphorylation of Stat5[26,27].The pathway [25,26,28]

IL-2 receptor also activates the Ras/Erk pathway and PI3K/Akt pathway [25,26,28,30]. Extensive reports revealed that PI3K/Akt activation and progression associated with SM934, a water-soluble derivative of artemisinin. The results appear to show that SM934 has potent immunosuppressive effects in vitro by inhibiting T cell proliferation, division, IFN-γ production; promoting activated T cells into apoptosis via blocking Akt phosphorylation. Further, SM934 appeared to exert similar immunosuppressive activity, in vivo. Collectively, these data suggest that SM934 may be a potential therapeutic for the treatment of select autoimmune diseases such as rheumatoid arthritis or SLE.

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