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Research Article
Stephanthraniline A and Cyclosporine A Synergize to Inhibit T-cell Response *in vitro* and *in vivo*

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Abstract

Background and Objective: Stephanthraniline A (STA), a natural C_{21} steroid isolated from *Stephanotis mucronata* (Blanco) Merr., is a potential immunosuppressant. The purpose of this study was to investigate the synergetic effects of STA and cyclosporine A (CsA) on T-cell response. **Materials and Methods:** First, the synergetic effects of STA and CsA were investigated *in vitro* on the cell proliferation, CD25 surface expression and cytokine IL-2 production of concanavalin A (Con A)-induced T-cells by MTT method, flow cytometric analysis and ELISA, respectively. Furthermore, 2,4-dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity (DTH), a T-cell-mediated response in mice was used to evaluate the synergetic effects of STA and CsA *in vivo*. Finally, the direct effect of STA on CYP3A4, which is the predominant enzyme for metabolism of CsA was determined using P450-Glo™ CYP3A4 screening system. **Results:** The results showed that Con A-induced T-cells proliferation, IL-2 production and CD25 expression were not inhibited by low-dose STA (1 μM) or CsA (1 nM) alone but was significantly reduced by the combination of STA and CsA. The DNFB-induced mice ear swelling, hyperplasia and infiltration of inflammatory cells were also significantly diminished by the combined treatment with non-therapeutic dose of STA (1 mg kg^{-1}) and CsA (0.5 mg kg^{-1}). In addition, STA at the concentration of more than 0.1 μM significantly decreased CYP3A4 activity. **Conclusion:** The STA synergized the inhibitory effects of CsA on T-cell response *in vitro* and *in vivo*. These effects were attributable to its different and complementary molecular mechanisms and may partly due to its increasing of the bioavailability of CsA via inhibiting CYP3A4.

Key words: Stephanthraniline A, cyclosporine A, T-cells, immunosuppressant, CYP3A4

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

T-cell response play critical role in controlling and mediating various types of immune responses, thus, T-cell targeting therapies are major treatment for autoimmune and inflammatory disorders, such as transplant rejection, systemic lupus erythematosus, multiple sclerosis and rheumatic arthritis. Cyclosporine A (CsA) and tacrolimus are calcineurin-inhibitors (CNIs), which inhibit the phosphatase activity of calcineurin and thereby suppresses translocation of NFAT and thus T-cell activation. The CNIs are the cornerstones of modern immunosuppressant. Since the launching of CsA in 1984, CNIs-based therapy is the main immunosuppressive protocol for almost all types of organ transplantations[1,2]. These drugs also greatly improved the outcomes for other autoimmune and inflammatory disorders[3,4]. However, because they have a narrow therapeutic window, they are associated with many side-effects, including nephrotoxicity, neurological disorders, hypertension, *de novo* cancers, new-onset diabetes and dyslipidemia with some being dose related[5]. Thus, there is a need to develop new therapeutic options with minimal side effects. Among these strategies, combination treatments which may reduce the dosages of CNIs and also the side effects while increasing the efficacy are practical.

Stephanthraniline A (STA, Fig. 1) is a natural C_{21} steroid isolated from *Stephanotis mucronata* (Blanco) Merr. Our previous studies suggested that STA is a potential immunosuppressant for use in the treatment of T-cell-mediated inflammatory and autoimmune diseases. The STA was found to inhibit CD3/CD28 cross-linking or Con A-induced T-cells activation and proliferation *in vitro* and suppress T-cell-mediated Delayed Type Hypersensitivity (DTH) in mice[6,7]. Recently, it was shown that pretreatment with STA reduced Con A-induced $CD4^+$ T-cells activation and aggregation in hepatic tissue and then attenuated hepatitis in mice[8]. The STA didn't induce T-cells apoptosis and activate the glucocorticoid receptor distinct from glucocorticoids[7]. In addition, it was proved that the inhibition of T-cells activation by STA didn't depend on proximal TCR signaling and Ca^{2+} signaling but through PKCθ and its downstream NFAT, NFκB and MAPK signaling cascades[8]. These results indicated that STA and CNIs possessed different and complementary molecular mechanisms and concomitant treatment of STA may reduce the dosages of CNIs to inhibit T-cell activation. Thus, the present study investigated the therapeutic potential of nontherapeutic dose of STA and CsA in combination on T-cell response *in vitro* and *in vivo.*

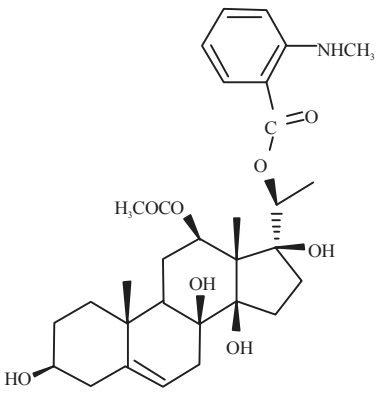

Fig. 1: Chemical structure of stephanthraniline A (STA)

MATERIALS AND METHODS

Chemicals and reagents: The 12-O-acetyl-20-O-(N-methyl)-anthraniloylsarcostin (STA, $C_{31}H_{43}NO_8$) was previously isolated from the stems of *S. mucronata* and identified on basis of chemical and spectroscopic evidence including HR-ESI-MS and two-dimensional NMR spectroscopy[9]. The purity of STA was determined to be more than 98% by HPLC analysis. The stock solution of STA in DMSO was prepared and then diluted as desired with RPMI 1640 medium. The final concentration of DMSO in the assays was less than 0.1% in all experiments and did not show any detectable effect on cell growth. The Con A, MTT and ketoconazole were purchased from Sigma Chemical Co. (St. Louis, MO, USA); cyclosporine A (CsA) was provided by Hangzhou Huadong Medicine Co. Ltd., Zhejiang, China; mouse cytokine IL-2 detecting ELISA kits were from Wuhan Boster Biological Technology Ltd., Hubei, China, FITC-anti-CD3 and PE-anti-CD25 were purchased from eBioscience Inc. (San Diego, CA, USA), RPMI 1640 medium was purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA); Fetal Bovine Serum (FBS) was purchased by Hangzhou Sijiqing Corp., Zhejiang, China; 2,4-dinitrofluorobenzene (DNFB) was purchased from BD Biosciences Pharmingen (CA, USA), P450-Glo™ CYP3A4 Screening System was purchased from Promege (Madison, WI, USA). All other chemicals were of highest purity and analytical grade.

Experimental animals: Female C57BL/6 and ICR mice (6 weeks old) were purchased from Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China and acclimatized for 7 days before use. All the procedures were in strict accordance with the P.R. China legislation on the use and care of laboratory animals and with the guidelines established by the Experimental Animals Center of Zhejiang Province and were approved by the Animal Care and Use Committee of Zhejiang Academy of Medical Sciences, China.

T-cells proliferation assay: Splenocytes prepared from C57BL/6 mice as previously described[7] were seeded into 4 wells of a 96-well flat-bottom plate at 5×10^6 cells mL^{-1} in 100 mL complete medium, thereafter Con A (3 mg mL^{-1}) or medium with STA and CsA were added giving a final volume of 200 mL. The plates were incubated at 37°C in a humid atmosphere with 5% CO_2. Cells were cultured for 48 h and splenocytes proliferation was detected by MTT assay. The Proliferation Index (PI) was calculated based on the following: PI is the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures[8].

Cytotoxicity analysis: Splenocytes were seeded into 4 wells of a 96-well flat-bottom plate at 5×10^6 cells mL^{-1} in 100 mL complete medium, thereafter STA or CsA were added giving a final volume of 200 mL. The plates were incubated at 37°C in a humid atmosphere with 5% CO_2. Cells were cultured for 48 h and cell viability was detected by MTT assay.

Analysis of CD25 cell surface expression: Splenocytes were seeded into 24-well flat-bottom plate at 5×10^6 cells mL^{-1} in 1 mL complete medium, thereafter Con A (3 mg mL^{-1}) or medium with STA and CsA were added giving a final volume of 2 mL. After incubation for 24 h, the cells were collected and stained with FITC-anti-CD3 plus PE-anti-CD25. After washed with PBS, samples were immediately detected by a FACScan flow cytometer[10].

Measurement of cytokine IL-2 production: Splenocytes were seeded into 24-well flat-bottom plate at 5×10^6 cells mL^{-1} in 1 mL complete medium, thereafter Con A (3 mg mL^{-1}) or medium with STA and CsA were added giving a final volume of 2 mL. After incubation for 24 h, the cultured supernatants were collected and the concentration of IL-2 were assayed by commercial ELISA kits[10].

Assessment of DNFB-induced DTH: The ICR mice were divided into four groups, each consisting of 10 mice. Animals were initially sensitized with 50 µL of 1% DNFB dissolved in acetone/olive oil 1:1 on the shaved abdominal skin of recipients. Beginning on the day of immunization, mice were treated with STA at the doses of 1 mg kg^{-1} (i.g.) or CsA at a dose of 0.5 mg kg^{-1} (i.g.) for 5 days once daily. The control groups received the same volume of saline. After 5 days, the DTH was elicited by smearing 10 µL of 1% DNFB on both sides of the left ear. About 24 h later, the DTH to DNFB was evaluated by measuring the weight difference of right and left ear with an analytical balance[7]. Slices of left ear were fixed with 10% formalin in phosphate buffered saline for 24 h and embedded in paraffin. Sections of 5 µm thickness were made using a microtome and stained with haematoxylin-eosin[11]. The histopathological changes in the ear were observed under an IX73 microscope (Olympus, Japan). Photographs of each of the slides were taken at 40x magnification.

CYP3A4 activity assay: The effect of STA on CYP3A4 activity was determined by P450-Glo™ CYP3A4 screening system. In brief, 25 µL of STA or ketoconazole solution and CYP3A4 reaction mixture were incubated in the wells of white opaque 96-well plate at 37°C for 10 min. Next, 50 µL of NADPH regeneration system was added to initiate the CYP reactions. After incubating for 30 min, the reconstituted luciferin detection reagent was added to stop the CYP reaction and initiates luminescence. At last, the luminescence was record in a plate-reading luminometer (BioTek, USA).

Statistical analysis: The data were expressed as Mean±Standard Deviation (SD) and examined for their statistical significance of difference with ANOVA and the standard's t-test. The $p<0.05$ were considered to be statistically significant.

RESULTS

STA and CsA synergize to inhibit T-cell response *in vitro*: In previous studies, STA and CsA significantly inhibited T-cell activation and proliferation at the concentration of 5 µM and 30 nM, respectively. Therefore, to investigate their synergetic immunosuppressive effects *in vitro*, the present study used nontherapeutic dose of STA (1 µM) and CsA (1 nM) to determine their effects on Con A-induced T-cell response. Figure 2a shows that Con A significantly induced T-cells proliferation. This elevated proliferation wasn't reduced by STA (1 µM) or CsA (1 nM) alone at the low-dose but was significantly reduced by the combination of STA and CsA at the same dose ($p<0.05$). The production of IL-2, an important cytokine involved in T-cells activation was also measured by ELISA. In accordance with above findings, the result (Fig. 2b) showed that Con A-induced IL-2 production in T-cell wasn't effected by STA or CsA alone ($p>0.05$) but was significantly inhibited by their combination treatment ($p<0.05$). The CD25 is usually considered as the activation marker expressed in the early phase after T-cell activation. In this study, flow cytometric analysis showed (Fig. 2c, d) that the surface expression levels of CD25 was up-regulated in T-cells incubated with

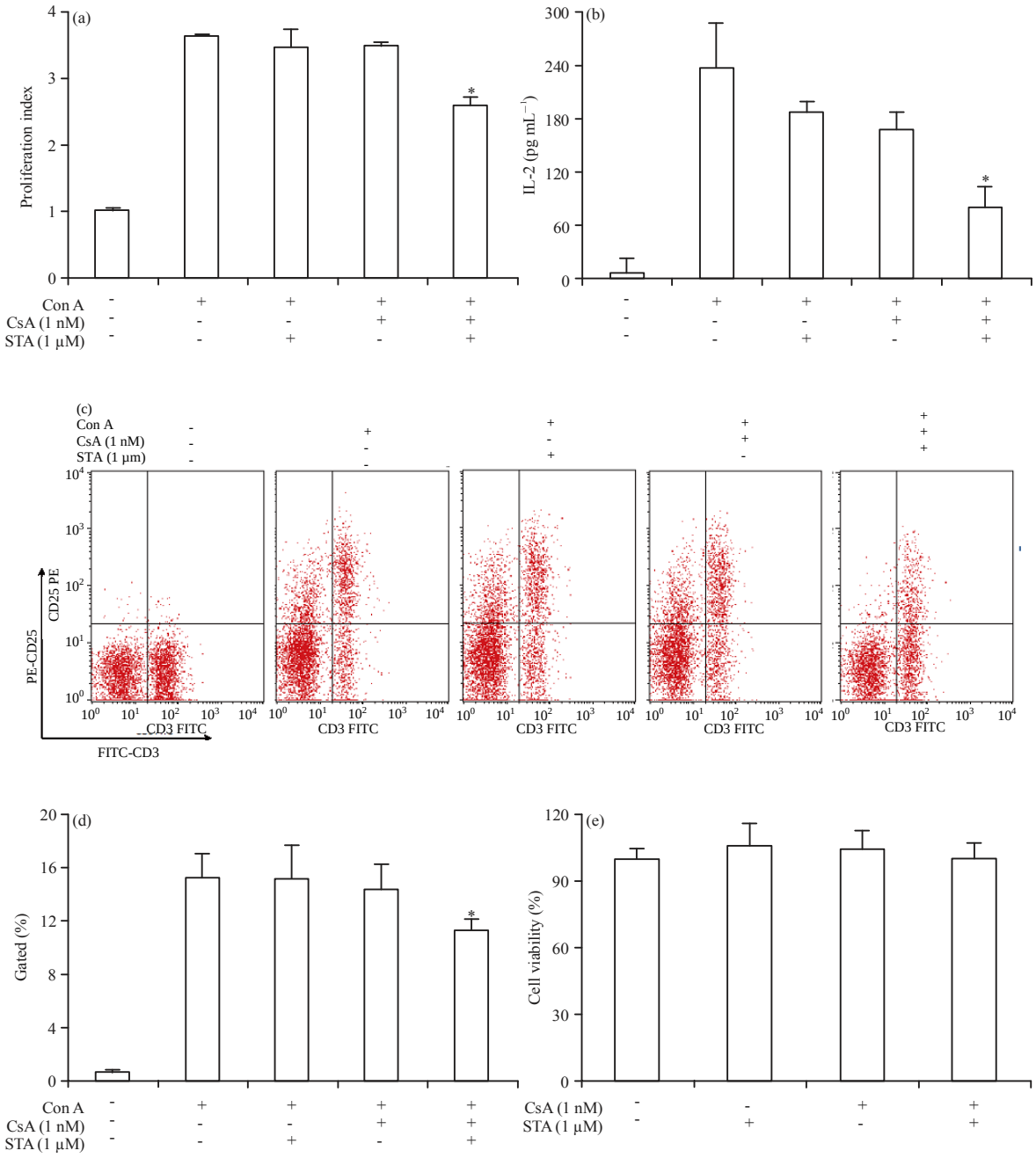

Fig. 2(a-e): STA and CsA synergize to inhibit T-cell response *in vitro,* (a) Con A (3 mg mL^{-1}) stimulated splenocytes were incubated with STA or/and CsA for 48 h. Cell proliferation was detected by MTT assay and calculated as proliferation index, (b) Con A-stimulated splenocytes were incubated with STA or/and CsA for 24 h. The cultured supernatants were collected and the concentration of IL-2 was assayed by commercial ELISA kits, (c-d) Con A-stimulated splenocytes were incubated with STA or/and CsA for 24 h. The cells were collected and stained with FITC-anti-CD3 plus PE-anti-CD25. The expressions of cell surface molecule CD25 was analyzed by flow cytometry and (e) Splenocytes were incubated with STA or/and CsA for 48 h, the cell viability was determined by MTT assay. The values are presented as Means±SD (n = 3). Significant differences with all other groups were designated as *p<0.05

Con A for 24 h and this up-regulation was also obviously inhibited by combined treatment with nontherapeutic dose of STA and CsA ($p<0.05$). To exclude from the cytotoxicity of combination therapies responsible for above

immunosuppressive effects, we also measured their effects on the viability of splenocytes using the MTT method. The results showed that neither monotherapy nor combination therapies with STA and CsA affected the cell viability (Fig. 2e). All these data suggested that STA and CsA synergized to inhibit T-cell response *in vitro* and these synergetic immunosuppressive effects were not due to their cytotoxicities.

STA and CsA synergize to inhibit T-cell-mediated DTH in mice: The DTH is a T-cell-mediated pathologic reaction. To test the synergetic effects of STA and CsA on T-cells *in vivo*, we analyzed their effects at the nontherapeutic dose on DNFB-induced DTH in ICR mice. Figure 3a shows that low-dose STA (1 mg kg^{-1}) or CsA (0.5 mg kg^{-1}) alone didn't reduce the ear swelling in DNFB-induce mice compared with the control group ($p>0.05$) but their combination at the nontherapeutic dose significantly inhibited DNFB-induce ear swelling in mice. The histological observations also supported these results. Figure 3b shows the epidermis in the DNFB-treated only mice displayed hyperplasia and significant edema and infiltration of inflammatory cells were observed. The STA (1 mg kg^{-1}) or CsA (0.5 mg kg^{-1}) monotherapy slightly diminished the development of histopathological changes. However, the combined treatment with STA and CsA significantly diminished hyperplasia, edema and infiltration of inflammatory cells in the ear of DNFB-stimulated mice. These data suggested that STA and CsA synergized to inhibit T-cell response *in vivo*.

STA inhibited CYP3A4 activity: The CsA is predominantly metabolised by CYP3A4. Any modulator of CYP3A4 may alter the pharmacokinetics and pharmacodynamics of CsA. To investigate the drug-drug interactions between STA and CsA *in vivo*, the direct effect of STA on CYP3A4 activity was determined *in vitro* using P450-Glo™ CYP3A4 screening system. Figure 4 shows that as the positive control of CYP3A4 inhibitor, ketoconazole, STA significantly decreased CYP3A4 activity at above the concentration of 0.1 μM. These results indicated that STA is a CYP3A4 inhibitor.

DISCUSSION

T-cell is the potential target for treatment of autoimmune diseases and inflammatory disorders. Its activation involves the expression of new cell surface molecules, the secretion of cytokines and the induction of mitotic activity resulting in clonal proliferation. Con A directly triggers T-cells activation and proliferation by interacting with diverse receptors including sugars, glycoproteins, or glycolipids[12] and by stimulating the energy metabolism in T-cells[13]. In this study, Con A-induced T-cells proliferation, IL-2 production and CD25 expression weren't inhibited by low-dose STA (1 μM) or CsA (1 nM) alone but was significantly reduced by the combination STA and CsA (Fig. 2). These results indicated that STA and CsA synergized to directly inhibit T-cell activation *in vitro*. The CsA is a calcineurin inhibitor, which inhibits T-cell activation through calcineurin-NFAT pathway[1]. However, recent findings indicated that distinct from CsA, STA inhibited T-cell activation through PKCθ and its downstream NFAT, NFκB and MAPK signaling cascades[8]. In addition, the present results showed the combination STA with CsA didn't induce cytotoxicity on splenocytes (Fig. 2e). Therefore, it could be concluded that the synergetic immunosuppressive effects of STA and CsA *in vitro* were not due to their cytotoxicities but attributable to their different and complementary molecular mechanisms.

The DNFB-induced DTH is a T-cell-mediated pathologic reaction, which is also known as contact hypersensitivity skin disease[14]. The DTH occurs in two phases: sensitization and elicitation phase. In the sensitization phase, the first contact of the skin with a hapten (such as DNFB) leads to binding of the hapten to an endogenous protein in the skin where they form immunogenic hapten-carrier complexes. The hapten-carrier complex is taken up by Langerhans cells and dermal dendritic cells and transported from the epidermis to the draining lymph node. Here, they present the haptenated peptides to naive T-cells which are subsequently activated. The newly activated T-cells proliferate and migrate out of the lymph node and into circulation. In the elicitation phase, re-exposure of the skin to the hapten activates the specific T-cells in the dermis and triggers the inflammatory process responsible for the cutaneous lesions[15]. In this study, the ear swelling, hyperplasia and infiltration of inflammatory cells in the mice induced by DNFB were significantly diminished by the combined treatment with nontherapeutic dose of STA and CsA (Fig. 3), which indicated that STA and CsA synergized to inhibit T-cell response *in vivo*.

However, it should be noted that several factors may influence the drug-drug interactions between CsA and other drugs[16]. The CsA is metabolized by the hepatic and gastrointestinal cytochrome P-450 system. The studies have shown that CYP3A4 is the predominant enzyme for metabolism of CsA[17]. Any modulator of CYP3A4 may alter the pharmacokinetics and pharmacodynamics of CsA. In an open-label, prospective observational clinical trial, it was found that the combination low dose of ketoconazole, a

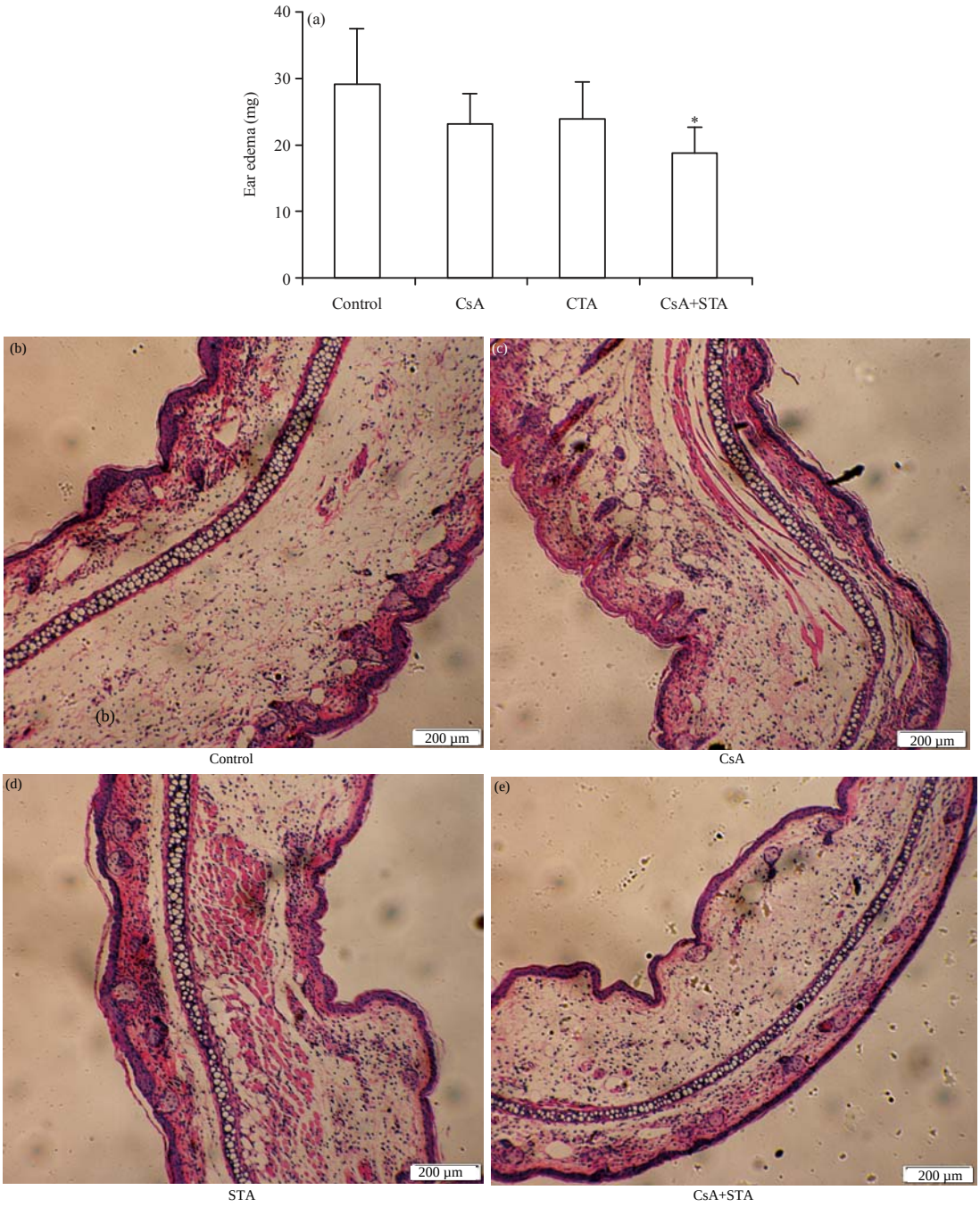

Fig. 3(a-e): STA and CsA synergize to inhibit T-cell-mediated DTH in mice. The ICR mice were initially sensitized with 50 μL of 1% DNFB and then were orally administered with STA or/and CsA for 5 days once daily. After 5 days, the DTH was elicited by smearing 10 μL of 1% DNFB on both sides of the left ear. About 24 h later, the DTH to DNFB was evaluated by measuring the weight difference of right and left ear with an anal. Balance, (a) The values are presented as Means±SD (n = 10). Significant differences with all other groups were designated as *p<0.05. (b-e) Slices of ear were stained with hematoxylin and eosin for histopathological analysis (original magnification x40)

well-known CYP3A4 inhibitor, with CsA in low immunological risk kidney transplant recipients is feasible, effective, safe and affordable even in the long term. Ketoconazole saved 80% dose of CsA[18]. Likewise, capsaicin pretreatment decreased the total clearance and increased the bioavailability of CsA in rats partly through inhibiting CYP3A[19]. In contrast, resveratrol and

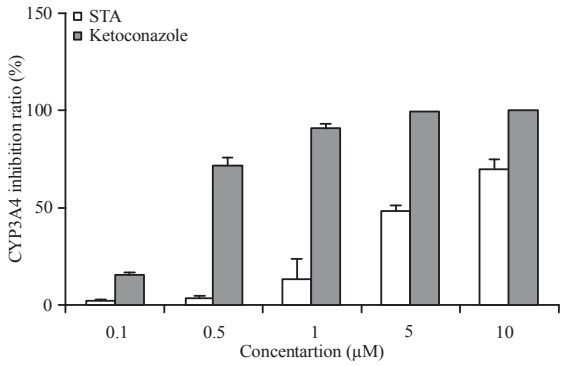

Fig. 4: STA inhibited CYP3A4 activity. The CYP3A4 activity was determined by CYP3A4-Glo™ Kinase assay. The values are presented as Means±SD (n = 3). Ketoconazole was used as positive control

glycyrrhetic acid, served as CYP3A4 activator, decreased the AUC_{0-t}, C_{max} and the oral bioavailability of CsA[20,21]. The present study showed that STA as well as ketoconazole is a CYP3A4 inhibitor (Fig. 4). These results suggested that STA enhanced the activity of CsA *in vivo* may partly through increasing the bioavailability and altering the pharmacokinetics of CsA. However, this hypothesis required to be confirmed in further research.

CONCLUSION

In summary, it was concluded that the combination of STA and CsA at the nontherapeutic dose could significantly inhibit T-cell response *in vitro* and *in vivo.* The STA synergized the effects of CsA was attributable to its different and complementary molecular mechanisms and may partly due to its increasing the bioavailability of CsA via inhibiting CYP3A4. These findings highlight the potential of STA as an effective leading compound for use in combination with CsA in the treatment of T-cell-mediated inflammatory and autoimmune diseases.

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