Biochemical Pharmacology 82 (2011) 260–268

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Mitochondria-dependent apoptosis of activated T lymphocytes induced by astin C, a plant cyclopeptide, for preventing murine experimental colitis

Yan Shen [a,1], Qiong Luo [a,1], Huimin Xu [b], Fangyuan Gong [a], Xiaobin Zhou [a], Yang Sun [a], Xuefeng Wu [a], Wen Liu [a], Guangzhi Zeng [b], Ninghua Tan [b,*], Qiang Xu [a,**]

[a] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 22 Han Kou Road, Nanjing 210093, China
[b] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

ARTICLE INFO

Article history:
Received 20 March 2011
Accepted 22 April 2011
Available online 5 May 2011

Keywords:
Astin C
T lymphocyte
Apoptosis
Mitochondria
Colitis

ABSTRACT

Facilitating T-cell apoptosis is implicated as an effective therapeutic strategy for treatment of T cell-mediated disease, including inflammatory bowel disease. Here, we report that astin C, a plant cyclopeptide isolated from the roots of *Aster tataricus* (Compositae), induced apoptosis of activated T cells in a mitochondria-dependent but Fas-independent manner in that such activity was still observed in T cells from Fas-mutated $MRL^{lpr/lpr}$ mice. Although caspase 8 was not activated, astin C treatment led to the cleavage of caspase 9 and caspase 3, the upregulation of Bad protein expression as well as release of cytochrome c in activated T cells. Astin C did not induce the expression of GRP78 and GADD153, excluding involvement of endoplasmic reticulum stress-mediated pathway. Moreover, oral administration of astin C protected mice against TNBS-induced colonic inflammation, as assessed by a reduced colonic weight/length ratio and histological scoring. Administering astin C significantly decreased serum levels of TNF-α, IL-4 and IL-17, accompanied with the induction of apoptosis in activated T cells *in vivo*. The results demonstrate, for the first time, the ability of astin C to induce apoptosis in activated T cells and its potential use in the treatment of colonic inflammation.

1. Introduction

Immune responses are frequently characterized by major expansions of antigen-specific T cells that have potent effector functions. To avoid overwhelming immune responses and to limit damage to healthy tissue, antigen-activated T cells die as a result of apoptosis during shutdown of such immune responses [1]. Thus, apoptosis has an essential role in T cell regulation and to reduce or even eliminate the unwanted activated T cells through facilitating apoptosis may provide a strategy for the treatment of T cell-dependent diseases.

Inflammatory bowel diseases (IBD) comprise Crohn's disease and ulcerative colitis, which are defined as chronically relapsing inflammations of the gastrointestinal tract [2]. Although the precise etiology of IBD still remains unclear, extensive studies have demonstrated that T cells are the main activated immune cells involved in IBD pathogenesis, which are characterized by enhanced proliferation, proinflammatory cytokine production and trafficking into the intestinal mucosa [3]. Moreover, T cells from patients with IBD exhibit resistance to multiple apoptotic pathways including Fas-mediated apoptosis, IL-2-deprivation-induced apoptosis as well as apoptosis mediated by nitric oxide [4,5]. T-cell resistance against apoptosis contributes to inappropriate T-cell accumulation and the perpetuation of chronic mucosal inflammation in IBD [6]. Therefore, new therapeutic strategies aim to restoring, or enhancing activated effector T-cell susceptibility to apoptosis in the gut [7]. Accumulating evidence in animal models of IBD has shown that various antibodies to proinflammatory cytokines and their receptors, such as IL-12, TNF-α and IL-6R, appear to suppress chronic intestinal inflammation by the induction of T-cell apoptosis [8–10]. In addition, many established immunosuppressive drugs such as azathioprine and sulfasalazine also seem to work partly by the induction of T cell apoptosis in patients with IBD [11,12]. Selective targeting of apoptosis emerges as a potential therapeutic approach for effective treatment of IBD.

Plant cyclopeptides are mainly cyclic compounds with the peptide bonds of 2–37 protein or non-protein amino acids and discovered in higher plants [13]. It has been demonstrated that plant cyclopeptides show several biological activities including antibiotic, sedative, antitumor, immunomodulatory activities. However, the mechanisms underlying are mostly unknown. Astin C is a member of natural cyclopentapeptide family isolated from

* Corresponding author. Tel.: +86 871 5223800; fax: +86 871 5219934.
** Corresponding author. Tel.: +86 25 8359 7620; fax: +86 25 8359 7620.
E-mail addresses: nhtan@mail.kib.ac.cn (N. Tan), molpharm@163.com (Q. Xu).
[1] These authors contributed equally to this work.

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.04.013

the roots of *Aster tataricus* (Compositae) that is commonly used in the traditional medicine, effective in the relief of cough. This class of compounds exhibit antitumor activities [14,15]. Recent studies have shown that synthesized cyclic astin analogues kill tumor cells via caspase-mediated induction of apoptosis [16]. In this study, we demonstrate for the first time that natural astin C has immunosuppressant property which is due to its induction of activated T-cell apoptosis. Furthermore, using a hapten-induced murine model of IBD, we demonstrate that astin C can attenuate colonic inflammation.

2. Materials and methods

2.1. Animals and agents

Female C57BL/6 (H-2b) mice, 6–8 weeks of age, were purchased from Experimental Animal Center of Jiangsu Province (Jiangsu, China). Eight-week-old female $MRL^{lpr/lpr}$ mice with a C57BL/6 background were purchased from Model Animal Research Center of Nanjing University. They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of Nanjing University. Concanavalin A (Con A), cyclosporin (CsA), quercetin, thapsigargin (TG), dexamethasone and 2, 4, 6-trinitrobenzenesulphonic acid (TNBS) were purchased from Sigma (St. Louis, MO). Annexin V-FITC/PI Kit was from Jingmei Biotech (Nanjing). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) Kit was from Calbiochem (La Jolla, CA). Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA), Ac-Ile-Glu-Thr-Asp-pNA (Ac-IETD-pNA) and Ac-Leu-Glu-His-Asp-pNA (Ac-LEHD-pNA) were from Alexis (San Diego, CA). PE-anti-mouse CD3 mAb was from BD pharmingen (San Diego). The primary Abs anti-poly(ADP-ribose) polymerase (PARP), cleaved caspase 3, cleaved caspase 9, cytochrome c, GRP78, GADD153 (all from Cell Signaling Technology, Beverly, MA), Bad, COX IV and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and Bcl-2 (BD pharmingen) for Western blots were used. Horseradish peroxidase-conjugated secondary Ab was from KPL (Gaithersburg, MD). All other chemicals were obtained from Sigma.

2.2. Extraction and isolation

Aster tataricus (Compositae) were purchased from Kunming Medicinal Material Co. (Kunming, China) and identified by Dr. Guangzhi Zeng. Powered roots (50 kg) of *A. tataricus* was extracted three times with 95% MeOH under reflux. The crude extract was concentrated *in vacuo*, and then partitioned with EtOAc and n-BuOH successively to yield EtOAc (2 kg) and n-BuOH (5 kg) extracts, respectively. The EtOAc extract (2 kg) was subjected to silica gel column chromatography, eluted with $CHCl_3$–MeOH gradient (100:0 to 0:100) to obtain 8 fractions. Fraction 3 was further chromatographed on a silica gel column with petroleum ether–acetone (1:1 to 1:2) to obtain 2 subfractions, Fr.3-1 and Fr.3-2. Fr.3-2 was subjected to silica gel column chromatography, eluted with $CHCl_3$–EtOAc gradient (1:3 to 1:5) and further separated on Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) to yield astin C (8 g, MW 570). The structure was confirmed by comparison of MS, ^{1}H NMR and physical data with those reported in the literature [14]. For testing, astin C was dissolved in DMSO and diluted to the desired concentrations in medium. DMSO final concentrations were kept below 0.1% and used as vehicle control.

2.3. HPLC analysis and structural elucidation

HPLC analysis was applied on a Waters 600 series HPLC system consisting of a Waters 600 pump, a 2487 UV detector, an online degasser and a LC Work Station equipped with Empower TM software. Astin C was applied to YMC-pack Pro C18 column (5 μm, 150 mm × 4.6 mm, YMC Co. Ltd., Japan) and eluted with acetonitrile–water (23:77, v/v). The effluents were detected at 200 nm. Column temperature was set up at 25 °C and the flow rate was 1 mL/min. The mobile phase was degassed by ultrasonic and filtered though a 0.22 μm membrane filter (Advantec, Tokyo Roshi Kaisha Ltd., Japan). Before sample analysis, the column was stabilized with mobile phase for at least 30 min. NMR and ES-MS were used for structure elucidation. The ^{1}H NMR measurements were carried out in Bruker DPX-300 spectrometer operating at 300 MHz. ES-MS experiments were recorded on ABI Mariner ESI-TOF mass spectrometer.

2.4. Cell proliferation

Lymph node cells isolated from mice were activated by Con A (5 μg/mL) for 24 h, which were indicated as activated cells, whereas those without Con A stimulation were used as nonactivated cells. The cells were further incubated in 96 well-plate with or without various concentrations of astin C at a density of 3×10^5 cells/well for 24 h. For MTT assay, 20 μL of MTT (Sigma; 4 mg/mL in PBS) was added per well 4 h before the end of the incubation. MTT formazan production was dissolved by DMSO replacing the medium. The optical density at 540 nm (OD_{540}) was measured by a microplate reader.

2.5. Cell apoptosis assay

Cell apoptosis was determined by two methods. For annexin V/PI staining, the cells were measured by flow cytometry after addition of FITC-conjugated annexin V and PI as previously described [17]. Annexin V^+/PI^- and Annexin V^+/PI^+ were considered as apoptotic cells in the early and late phase, respectively. For TUNEL assay, DNA Fragmentation Detection Kit was used according to the manufacturer's instruction, followed by flow cytometric analysis. Samples were analyzed by flow cytometry on a FACScan (Becton Dickinson).

2.6. Western blotting

In brief, cells were washed with PBS and lysed. Mitochondrion protein and cytosolic protein were isolated using Mitochondrial Fractionation Kit (Active Motif, Carlsbad, Calif) according to the manufacturer's instructions. Proteins were quantified using a BCA™ protein assay kit (Pierce, Rochford, IL). The protein lysates were separated by 10% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% nonfat milk for 1–2 h at room temperature. The blocked membrane was incubated with the indicated primary Abs, and then incubated with a horseradish peroxidase-conjugated secondary Ab. Protein bands were visualized using Western blotting detection system according to the manufacturer's instructions (Cell Signaling Technology).

2.7. Caspase activity assay

Cells were lysed in the lysis buffer containing 10 mmol/L HEPES, 5 mmol/L dithiothreitol, 2 mmol/L EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% CHAPS, pH7.4. After 12,000 × *g* centrifugation for 15 min, the protein concentration in the supernatant was determined by a BCA™ protein assay kit. Caspases 3, 8 and 9 activities were tested in duplicate experiments by measuring the proteolytic cleavage of specific chromogenic substrates: Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, respectively [18].

2.8. TNBS-induced colitis in mice and treatment

Colitis was induced by intrarectal injection of TNBS as previously described [18]. Briefly, mice were fasted for 24 h with free access to drinking water. They were anesthetized by sodium pentobarbital (50 mg/kg, i.p.). Next, 100 μL of 2 mg TNBS in 50% ethanol solution was injected intrarectally through a flexible catheter of 3.5 cm length. To ensure distribution of the agent within the entire colon and cecum, mice were held upside down in a vertical position for 30 s. The sham group received 100 μL of 50% ethanol alone through the same technique. In the drug treatment group, astin C (2, 4 mg/kg) and dexamethasone (2 mg/kg) were given orally and intraperitoneally, respectively. The administration was performed on a daily basis after the day of immunization (day 0). The same solvent (0.5% methylcellulose in normal saline) was administered orally to both groups sham and model. In all protocol studies, mice were monitored for loss of body weight and overall mortality for ten days after TNBS administration.

2.9. Colitis severity

As TNBS-induced acute colitis is self-limiting and displays a high level of colonic damage 3 days after colitis induction [19], the mice were sacrificed three days after TNBS administration in some experiment. The colon was removed by cutting at the pubis symphysis and at the cecum and gently washed with 0.9% saline solution. The colon was blotted dry, measured and weighed. Colonic length and weight were measured as gross indicators of colitis. For histological morphometry, colonic tissues were embedded in paraffin, and cut into 5 μm of sections and stained with hematoxylin and eosin. Histopathological grading system was used in a blinded manner [20]: Grade 0: histological findings identical to normal mice; Grade 1: mild mucosal and/or submucosal inflammatory infiltrate and edema, punctuate mucosal erosions often associated with capillary proliferation, muscularis mucosae intact; Grade 2: 50% of the specimen display Grade 1 changes; Grade 3: prominent inflammatory infiltrate and edema frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa, rare inflammatory cells invading the muscularis propria but without muscle necrosis; Grade 4: 50% of the specimen display Grade 3 changes; Grade 5: extensive ulceration with coagulative necrosis bordered underneath by numerous neutrophils and lesser numbers of mononuclear cells, necrosis extends deeply into the muscularis mucosae; Grade 6: 50% of the specimen display Grade 5 changes.

2.10. Cytokine analysis by ELISA and CBA assay

Blood was obtained from mice at the indicated time points and immediately centrifuged at 12,000 × *g* for 10 min. Serum samples were stored at −70 °C until ready for use. Serum TNF-α levels were measured by a specific ELISA kit from eBioscience (San Diego, CA) according to the manufacturer's instruction. Cytokine levels of IL-2, IFN-γ, TNF-α, IL-4, IL-6, IL-10 and IL-17 were determined using Cytometric Bead Array (CBA) cytokine assay kit according to the manufacture as recommended by BD pharmingen.

2.11. Preparation of lamina propria mononuclear cells isolated from colonic tissue

Colon lamina propria mononuclear cells (LPMC) were isolated as previously described with some modification [18]. Briefly, colon tissue was opened longitudinally, cut into 5-mm pieces, and incubated in 0.5 mM EDTA in calcium- and magnesium-free Hank's balanced salt solution for 20 min at 37 °C. After repeated washing, tissue was incubated for 60 min at 37 °C in digestion solution containing 4% fetal calf serum, 0.5 mg/mL each of type IV collagenase (Sigma) and DNase I (Sigma) as well as 50 U/mL Dispase (Roche, Indianapolis, IN). Then the tissue was subjected to further mechanical disruption using a 1-mL syringe. The product was layered onto a 40:80% gradient Percoll column (Sigma). Cells were spun at 2200 × *g* for 20 min to obtain the leukocyte-enriched population (LPMC) at the 40–80% interface.

2.12. Statistical analysis

Data are expressed as means ± SEM from at least three independent experiments. Statistical analyzes were performed using one-way analysis of variance (ANOVA), followed by Student two-tailed *t* test. The data of colon histologic changes were analyzed using non-parametric test, as applicable. $P < 0.05$ was considered significant.

3. Results

3.1. Astin C inhibited proliferation and induced apoptosis in activated T cells

Astin C isolated from the roots of *A. tataricus* was subjected to HPLC analysis and structure determination. The purity of astin C was confirmed to be over 95% by HPLC (Fig. 1A). The structure of astin C was identified by MS and NMR spectral analyzes and compared with the reported data [14].

To test the immunomodulatory activity of astin C, lymph node cells from C57BL/6 mice were either unactivated or activated with Con A for 24 h and subsequently incubated with various concentrations of astin C (5–20 μM) for 24 h. As shown in Fig. 1B, astin C dose-dependently inhibited the proliferation of Con A-activated T cells, but demonstrated little effect on unactivated cells. The well-known immunosuppressant CsA was used as a positive control, inhibiting cell proliferation regardless of activation. A similar observation was made with lymph node cells activated by anti-CD3 plus anti-CD28 antibodies (data not shown). The data obtained from Annexin V/PI staining showed that T cells activated with Con A and exposed to astin C underwent apoptosis in a dose-dependent manner (Fig. 1C and D). And 20 μM of astin C displayed comparable activity to 50 μM of quercetin, a positive control which exerts a high apoptotic potential through several pathways [21,22].

3.2. Astin C also induced apoptosis in activated T cells with Fas gene mutation

The induction of apoptosis by astin C was confirmed by TUNEL assay (Fig. 2, left). Astin C dose-dependently induced apoptosis of Con A-activated T cells from wild type mice. However, following activation the T cells from $MRL^{lpr/lpr}$ mice with mutation of Fas gene demonstrated a similar susceptibility to astin C-induced apoptosis (Fig. 2, right), excluding the involvement of Fas/FasL system. In the context of quercetin, remarkably impaired apoptosis was observed in the T cells from $MRL^{lpr/lpr}$ mice compared with those from wild type mice.

3.3. Astin C triggered the changes of relevant molecules in mitochondria pathway

To further investigate the molecular mechanism of astin C-induced apoptosis, we monitored the changes in apoptotic molecules related to mitochondria pathway in activated T cells. Western blotting analysis showed that astin C treatment caused the cleavage of the 116-kDa PARP into an 89-kDa fragment (Fig. 3A). And the active fragments of caspase 3 and caspase 9

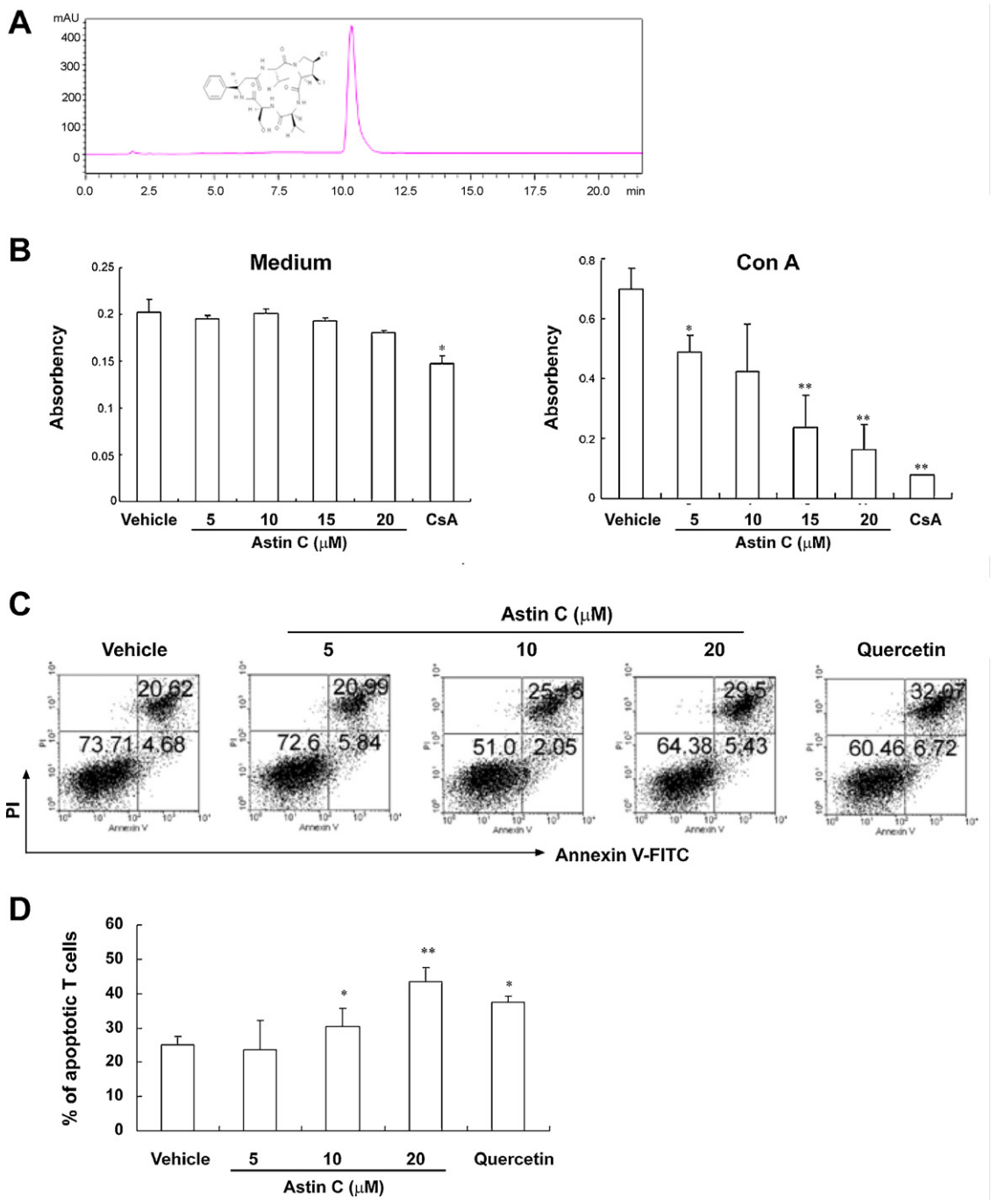

Fig. 1. Inhibition of cell proliferation and induction of apoptosis in activated T cells by astin C. (A) The chemical structure and HPLC analysis of astin C. The purity of astin C was confirmed to be over 95%. Lymph node cells isolated from C57BL/6 mice were incubated in medium or in the presence of Con A for 24 h. Then the cells were further incubated with or without various concentrations of astin C for 24 h. (B) MTT method was used to detect cell proliferation. Data are shown as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle controls. (C and D) Cells were co-stained with Annexin V/PI to determine apoptosis by flow cytometry. C demonstrates a representative experiment for apoptosis and *D* shows means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle controls.

appeared more intensive in astin C-treated cells than vehicle-treated cells. In addition, astin C treatment caused increase in the expression of pro-apoptotic gene Bad whereas there was little change in the expression of anti-apoptotic gene Bcl-2. When the levels of cytochrome *c* were monitored in both mitochondrion and cytosolic fraction, dose- and time- dependent release of cytochrome *c* from mitochondrial to cytosol was detected in activated T cells upon exposure to astin C (Fig. 3B and C). COX IV (cytochrome *c* oxidase IV), as a control, remained in the mitochondrion fractions.

Next, we analyzed caspase enzymatic activities using substrates specific for caspases 3, 8, and 9. Significantly increased caspase activities for caspase 3 and caspase 9 were observed in astin C-treated, Con A-activated T cells compared with vehicle-treated T cells, whereas no caspase 8 activation was detected in these cells (Fig. 3D). By contrast, quercetin treatment caused significant increase in all three caspase activities.

3.4. Astin C failed to induce apoptosis in activated T cells through endoplasmic reticulum stress

As another potential mechanism underlying the apoptosis induced by natural products, the intrinsic endoplasmic reticulum

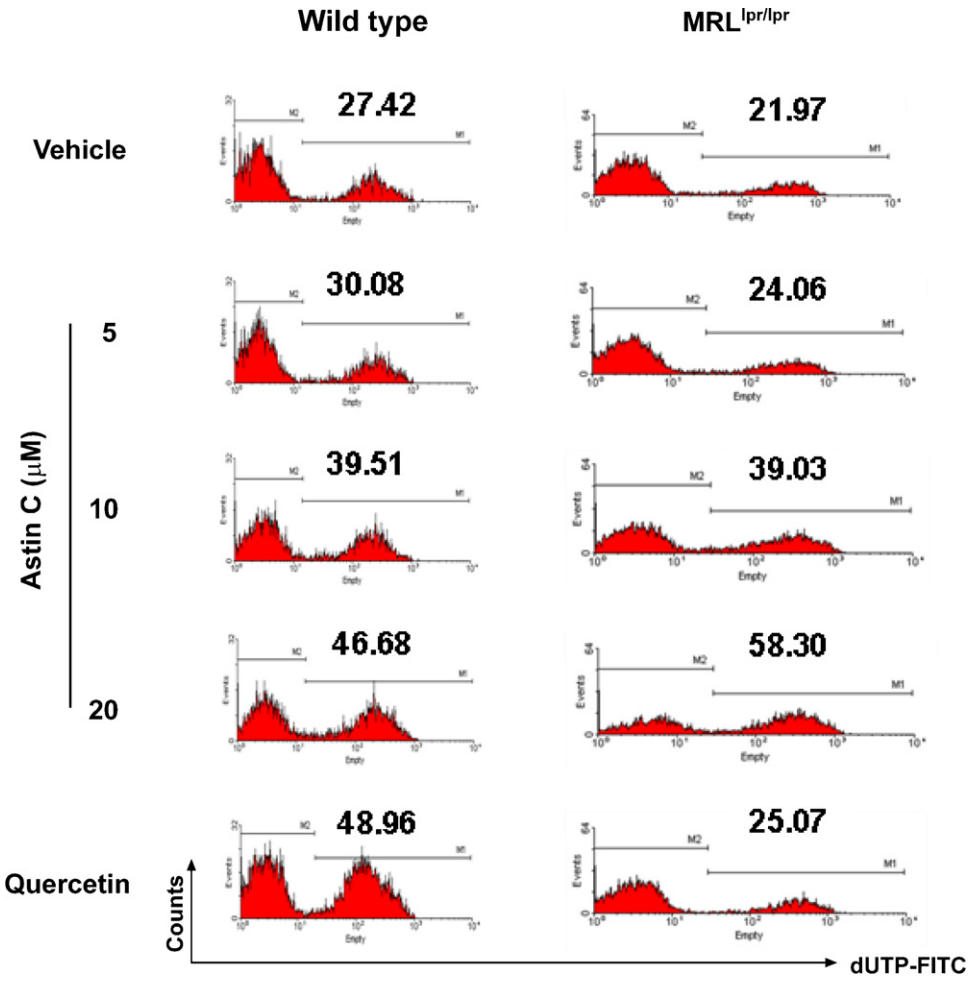

Fig. 2. Induction of apoptosis in activated T cells with Fas mutation by astin C. Lymph node cells isolated from C57BL/6 wild type mice or MRL$^{lpr/lpr}$ mice were activated by Con A for 24 h. Then the cells were further incubated with or without various concentrations of astin C for 24 h. Percentages of apoptotic T cells were determined by TUNEL assay. Numbers in each histogram refer to the percentage of cells above the threshold of normal controls. One result representative of three experiments was shown.

(ER) stress-mediated pathway is likely to be implicated [23,24]. We assessed the effect of astin C on the expression of GRP78 and GADD153, which are the major markers of ER stress. As shown in Fig. 4, astin C treatment did not increase their levels whereas treatment with the positive control TG resulted in a significant increase as early as 12 h in activated T cells. Astin C also had no effect on the cytosolic calcium levels (data not shown).

3.5. Astin C ameliorated TNBS-induced colitis in mice

Because facilitating apoptosis of activated effector T cells in the gut may be an ideal strategy for increasing the elimination of pathogenic T cells, and thus be useful in the treatment of IBD, we investigated the therapeutic efficacy of astin C in TNBS-induced T-cell mediated murine colitis. As shown in Fig. 5A, oral administration of astin C (2 mg/kg/day) prevented the reduction of body weights, and the protective effect became overt at day 6 post TNBS immunization and persisted over the course of colitis. Next, we collected the colon tissues at day 3 post TNBS immunization when colitis severity frequently reaches the peak. A significantly increased weight/length ratio of the mice colon, an indicator of inflammation, was observed in TNBS-treated model mice compared with sham mice (Fig. 5B, left). Astin C at 2 and 4 mg/kg/day or dexamethasone at 2 mg/kg significantly reduced the weight/length ratio. TNBS induced mucosal thickening, crypt damage, increase of lymphoid follicle size and loss of goblet cells. The data obtained from histologic examination of mice colon showed that astin C at high dose significantly ameliorated the sign of colitis compared with the TNBS model group (Fig. 5B, right). As shown in Fig. 5C, the colon tissues from the model group displayed serious mucosal injury characterized by necrosis of the epithelium, destruction of mucosal architecture and diffuse infiltration of lymphocytes and neutrophils in the mucosa and submucosa. A marked decrease in the colonic inflammation was shown in astin C-treated mice.

To test the effect of astin C on colitis-related pro-inflammatory cytokines, CBA assay was used to determine serum cytokine levels at day 10 post TNBS immunization. Dramatic reduction in TNF-α and a modest decrease in IL-4 and IL-17 were observed in the sera from astin C-treated mice compared with the model mice (Fig. 5D). Since TNF-α plays a critical role in colonic inflammation and the therapy targeting TNF-α is currently used with considerable success in the treatment of IBD [25], we also examined serum TNF-α level at day 3 post TNBS immunization. Consistently, astin C at high dose caused a significant decrease in TNF-α level (Fig. 5E).

3.6. Astin C induced apoptosis of T cells from lamina propria of mice with TNBS-induced colitis

Because augmented lamina propria T-cell activation, and thus resistance to apoptosis, has been identified as a key factor in IBD pathogenesis, apoptosis measurement was made by Annexin V/PI

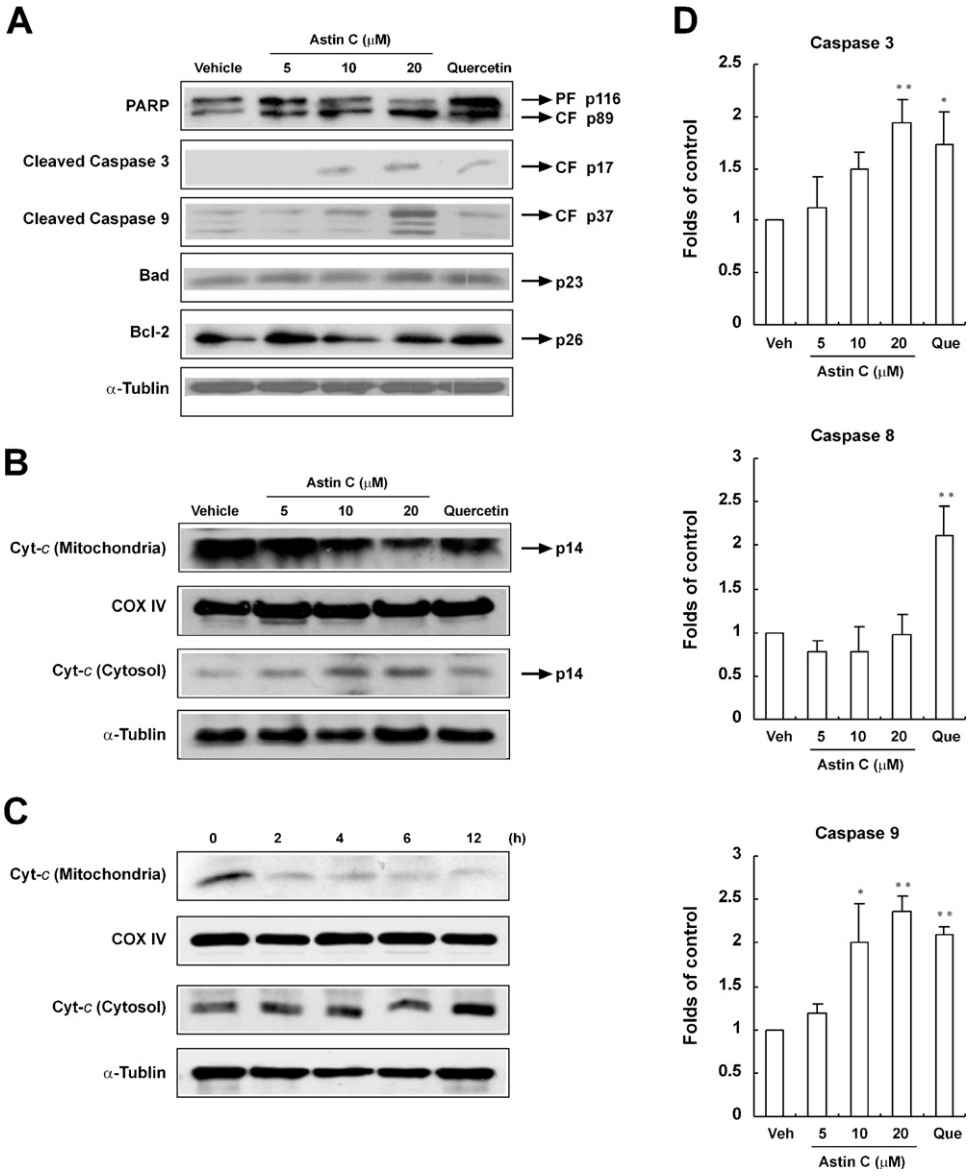

Fig. 3. Effects of astin C on relevant molecules in mitochondrial-dependent pathway. Lymph node cells isolated from C57BL/6 mice were activated by Con A for 24 h. Then the cells were further incubated with or without various concentrations of astin C for 24 h. (A) Protein levels of PARP, cleaved caspases 3 and 9, Bad and Bcl-2 were analyzed by western blotting. (B) Dose-dependent release of cytochrome *c* was analyzed by western blotting. (C) Time-dependent release of cytochrome *c* was analyzed by western blotting. Activated cells were incubated 20 μM of astin C for indicated time. These blots are the representative result of three independent experiments. (D) Enzymatic activities of caspases 3, 8 and 9 were determined in Con A-activated T cells 10 h after exposure to astin C. The exposure time was selected according to pilot experiments. Data are shown as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle controls.

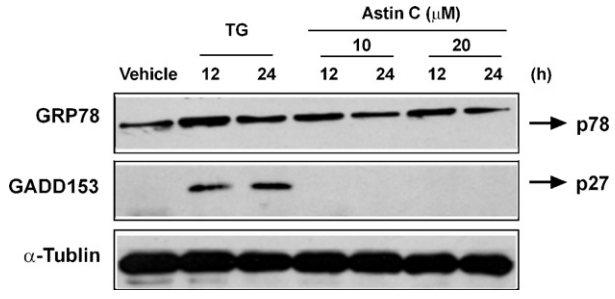

Fig. 4. Effects of astin C on relevant molecules in ER stress-mediated pathway. Lymph node cells isolated from C57BL/6 mice were activated by Con A for 24 h. Then the cells were further incubated with 200 nM of TG or various concentrations of astin C for indicated time. Protein levels of GRP78 and GADD153 were analyzed by western blotting. These blots are the representative result of three independent experiments.

staining on lamina propria T cells freshly isolated from the mice at day 3 post TNBS immunization. Compared with the model mice, the percentages of apoptotic T cells among gated $CD3^{+}$ cells were increased in astin C-treated mice, whereas no change was observed in dexamethasone-treated mice (Fig. 6A and B).

4. Discussion

Immunosuppressants are required for an array of medical purpose, such as the treatment of inflammatory diseases. CsA, a cyclopeptide derived from microorganisms, is considered as a milestone in immunosuppression despite its adverse effects on renal function and cardiovascular disease [26]. In this study, we found that astin C, a plant cytopeptide, has immunosuppressive activity, protective potential against TNBS-induced colitis, and the immunosuppressive mechanism may be different from CsA. Astin C reduced the proliferative response of Con A-activated T cells

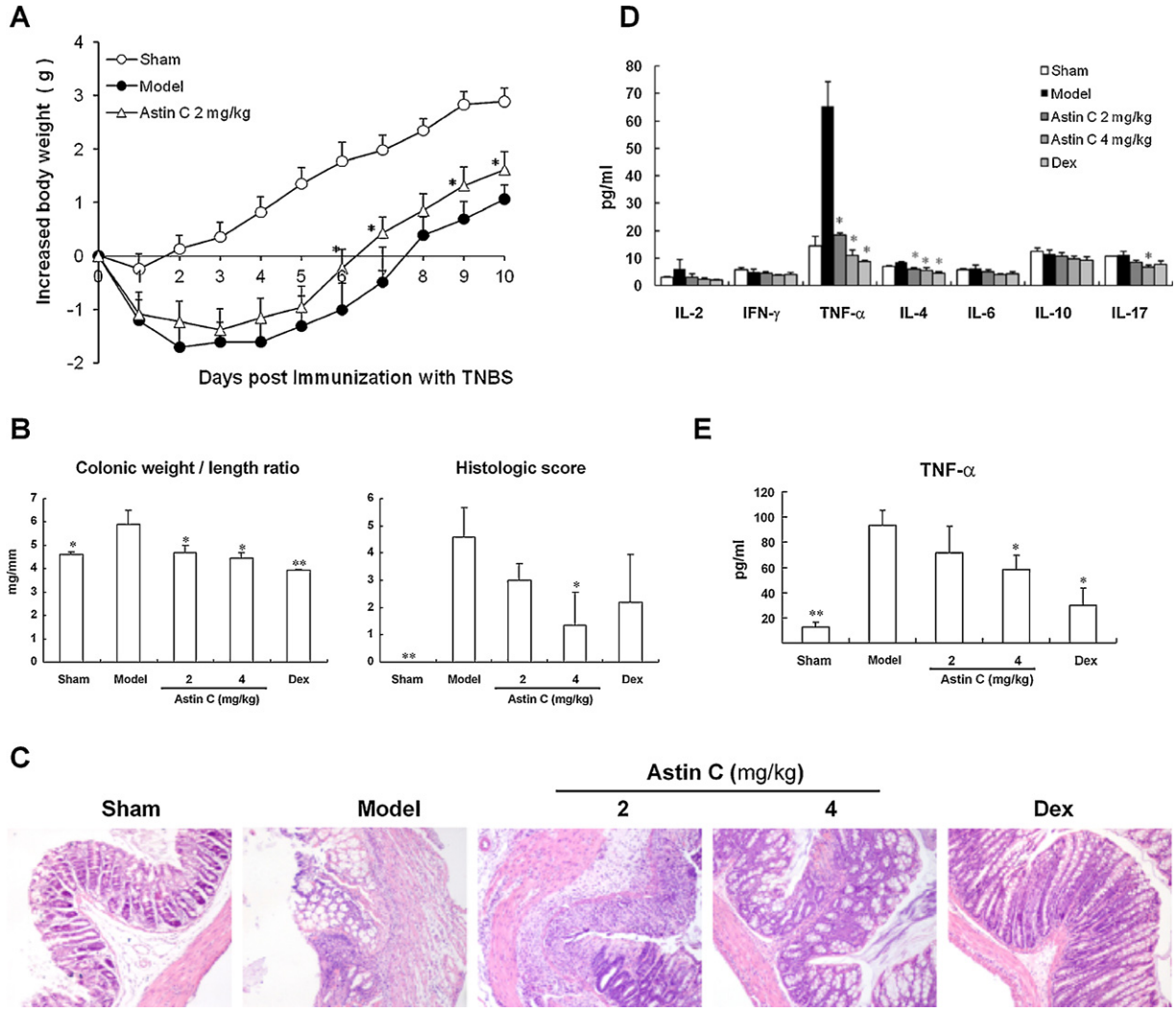

Fig. 5. Protective effects of astin C against TNBS-induced colitis in mice. (A) Body weight change. (B) Weight/length ratio (left) and histologic score (right). (C) Representative photograghs of H&E-stained colon sections. Original magnification 100×. (D) Serum cytokine levels were measured by CBA assay at day 10 post TNBS immunization. (E) TNF-α serum level was measured by ELISA at day 3 post TNBS immunization. Data are shown as means ± SEM of three independent experiments. $n = 8–10$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs model control.

without any effects on nonactivated cells. Unlike CsA, astin C did not prevent upregulation of Con A-induced activation markers CD25 and CD69 (Fig. 1S), suggesting that astin C cannot interfere with naïve T cell activation. In fact, astin C induced activated T cell-apoptosis in a dose-dependent manner as demonstrated by Annexin/PI staining and TUNEL assay.

Three different death signaling pathways lead to apoptosis, such as the extrinsic death receptor-dependent pathway, the intrinsic mitochondria-dependent pathway and the intrinsic ER stress-mediated pathway. These pathways work together to regulate the function of T lymphocytes [27]. Although activated T cells express Fas ligand (FasL), and Fas-mediated signaling plays important parts in the induction of lymphocyte apoptosis [28], astin C is still capable of inducing apoptosis in activated T cells from $MRL^{lpr/lpr}$ mice which cannot mediate apoptosis via the Fas pathway. Furthermore, astin C did not demonstrate any effect on the upregulation of FasL expression on the surface of activated T cells (data not shown). These results indicate that Fas-dependent pathway may not be involved in astin C-induced apoptosis, which is also supported by evidence that no activation of caspase 8 occurred whereas caspases 3 and 9 were active upon exposure to astin C. It is not likely that ER stress-mediated pathway was involved in astin C-induced apoptosis, because astin C treatment failed to induce the expression of ER stress-related proteins GRP78 and GADD153 in activated T cells. By contrast, astin C treatment significantly increased cleaved caspases 3, 9, Bad protein levels and caused cytochrome *c* release. Depolarization of the mitochondrial membrane potential was also observed in astin C-treated, activated T cells (Fig. 2S), suggesting that in activated T cells astin C-induced apoptosis occurs predominantly through the activation of the intrinsic mitochondrial pathway. However, it was reported that a synthesized cyclic astin analogue activated an apoptotic pathway involving the sequential activation of caspases 8, 9 and 3 in NPA cells, which were derived from human papillary thyroid carcinomas [16]. It is possible that the underlying mechanisms of apoptosis-inducing actions of cyclic astins are associated with compound structures and cell types.

It was well documented that intestinal T cells exhibit resistance to multiple apoptotic signals in experimental models of colitis, as well as in IBD patients. This broad resistance to apoptosis accords with the fact that T cells in inflamed tissue express increased levels of Bcl-2 and thus may be resistant to a range of apoptotic mechanisms that involve mitochondrial activity [4,5,8]. The colitis-protective effects mediated by many agents, such as natural

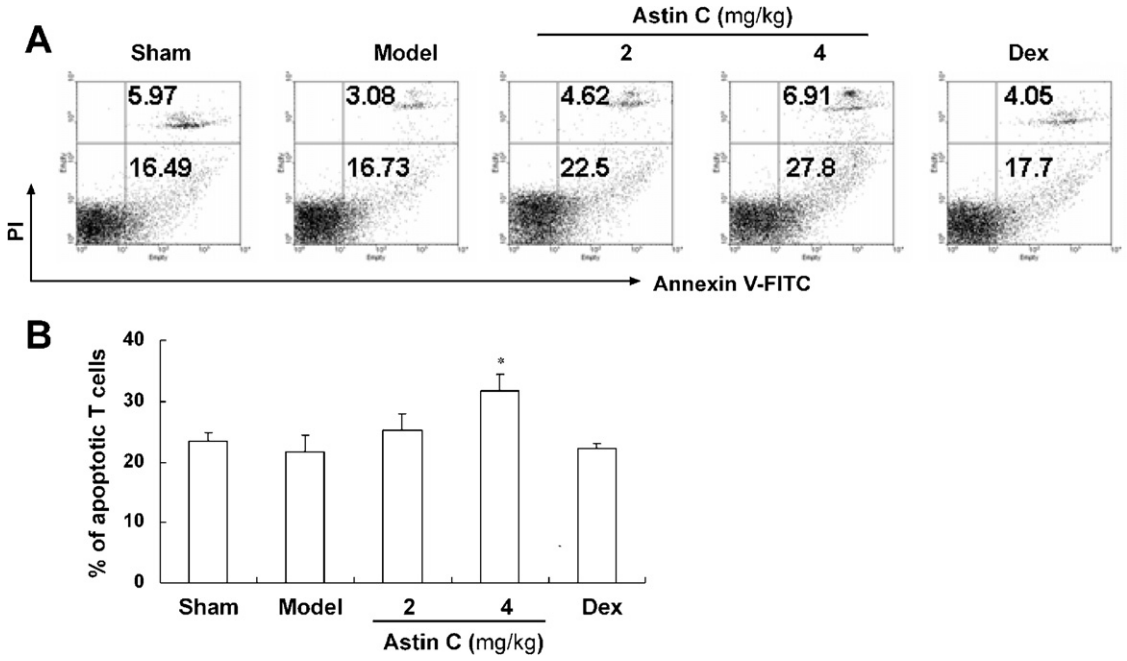

Fig. 6. Induction of apoptosis in lamina propria T cells from mice with TNBS-induced colitis by astin C. (A and B) Lamina propria mononuclear cells were isolated from the mice at day 3 post TNBS immunization. Percentages of apoptotic cells among gated $CD3^+$ cells were determined by Annexin V/PI staining (A) is a representative of three independent experiments and (B) shows means ± SEM of three independent experiments. $^*P < 0.05$ vs model control.

compound garcilin and anti-IL-6R antibodies, involve reduction of Bcl-2 expression and induction of lymphocyte apoptosis [8,29]. In the current study, oral administration of astin C potentially protected mice against TNBS-induced inflammation, reducing the colonic weight/length ratio and histological scores. As expected, astin C treatment caused apoptosis of lamina propria $CD3^+$ T cells in a dose-dependent manner. Astin C treatment also reduced serum TNF-α levels and this inhibitory effect seemed to persist over the course of colitis. It is noteworthy that even 2 mg/kg of astin C improved the reduction of body weight post TNBS immunization. In agreement with this finding, previous studies indicate that administering astin C at a dose of 5 mg/kg/day inhibited the tumor growth by 45% *in vivo*, although its IC_{50} values of killing tumor cells ranged from 7 to 15 μg/mL *in vitro* [30,31]. Further studies are in progress to determine the pharmacokinetic properties of this compound.

There has been growing interest to explore novel compounds with anti-inflammatory or immunomodulatory properties from herbal medicine. Such natural compounds can be purified, synthesized and modified in chemical structure for new drug design, and often have a low toxicity profile. In the case presented here, astin C, a plant cyclopeptide isolated from the traditional medicine *A. tataricus*, induced activated T-cell apoptosis via mitochondria-mediated pathway. Its targeting of apoptosis in activated effector T cells contributes to a chemopreventive potential in inflammatory intestinal diseases.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Nos. 90913023, 30973920, 30973546), Science Fund for Creative Research Groups (No. 30821006) and Natural Science Foundation of Jiangsu Province (BK2008022, BK2009238).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.04.013.

References

[1] Strasser A, Pellegrini M. T-lymphocyte death during shutdown of an immune response. Trends Immunol 2004;25:610–5.
[2] Podolsky DK. Inflammatory bowel disease (1). N Engl J Med 1991;325:928–37.
[3] Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182–205.
[4] Boirivant M, Marini M, Di Felice G, Pronio AM, Montesani C, Tersigni R, et al. Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. Gastroenterology 1999;116:557–65.
[5] Ina K, Itoh J, Fukushima K, Kusugami K, Yamaguchi T, Kyokane K, et al. Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. J Immunol 1999;163:1081–90.
[6] Neurath MF, Finotto S, Fuss I, Boirivant M, Galle PR, Strober W. Regulation of T-cell apoptosis in inflammatory bowel disease: to die or not to die, that is the mucosal question. Trends Immunol 2001;22:21–6.
[7] Atreya R, Neurath MF. New therapeutic strategies for treatment of inflammatory bowel disease. Mucosal Immunol 2008;1:175–82.
[8] Atreya R, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat Med 2000;6:583–8.
[9] Stallmach A, Marth T, Weiss B, Wittig BM, Hombach A, Schmidt C, et al. An interleukin 12 p40-IgG2b fusion protein abrogates T cell mediated inflammation: anti-inflammatory activity in Crohn's disease and experimental colitis in vivo. Gut 2004;53:339–45.
[10] van Deventer SJ. Transmembrane TNF-alpha, induction of apoptosis, and the efficacy of TNF-targeting therapies in Crohn's disease. Gastroenterology 2001;121:1242–6.
[11] Doering J, Begue B, Lentze MJ, Rieux-Laucat F, Goulet O, Schmitz J, et al. Induction of T lymphocyte apoptosis by sulphasalazine in patients with Crohn's disease. Gut 2004;53:1632–8.
[12] Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, et al. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human $CD4^+$ T lymphocytes. J Clin Invest 2003;111:1133–45.
[13] Tan NH, Zhou J. Plant cyclopeptides. Chem Rev 2006;106:840–95.
[14] Morita H, Nagashima S, Takeya K, Itokawa H. Astins A and B, antitumor cyclic pentapeptides from Aster tataricus. Chem Pharm Bull (Tokyo) 1993;41:992–3.

[15] Rossi F, Zanotti G, Saviano M, Iacovino R, Palladino P, Saviano G, et al. New antitumour cyclic astin analogues: synthesis, conformation and bioactivity. J Pept Sci 2004;10:92–102.
[16] Cozzolino R, Palladino P, Rossi F, Cali G, Benedetti E, Laccetti P. Antineoplastic cyclic astin analogues kill tumour cells via caspase-mediated induction of apoptosis. Carcinogenesis 2005;26:733–9.
[17] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995;184:39–51.
[18] Sun Y, Wu XX, Yin Y, Gong FY, Shen Y, Cai TT, et al. Novel immunomodulatory properties of cirsilineol through selective inhibition of IFN-gamma signaling in a murine model of inflammatory bowel disease. Biochem Pharmacol 2010;79:229–38.
[19] Ruyssers NE, De Winter BY, De Man JG, Loukas A, Pearson MS, Weinstock JV, et al. Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice. Inflamm Bowel Dis 2009;15:491–500.
[20] Busserolles J, Paya M, D'Auria MV, Gomez-Paloma L, Alcaraz MJ. Protection against 2,4,6-trinitrobenzenesulphonic acid-induced colonic inflammation in mice by the marine products bolinaquinone and petrosaspongiolide M. Biochem Pharmacol 2005;69:1433–40.
[21] Chen D, Daniel KG, Chen MS, Kuhn DJ, Landis-Piwowar KR, Dou QP. Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. Biochem Pharmacol 2005;69:1421–32.
[22] Chien SY, Wu YC, Chung JG, Yang JS, Lu HF, Tsou MF, et al. Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. Hum Exp Toxicol 2009;28:493–503.
[23] Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 2000;403:98–103.
[24] Kim SM, Park HS, Jun DY, Woo HJ, Woo MH, Yang CH, et al. Mollugin induces apoptosis in human Jurkat T cells through endoplasmic reticulum stress-mediated activation of JNK and caspase-12 and subsequent activation of mitochondria-dependent caspase cascade regulated by Bcl-xL. Toxicol Appl Pharmacol 2009;241:210–20.
[25] Peyrin-Biroulet L. Anti-TNF therapy in inflammatory bowel diseases: a huge review. Minerva Gastroenterol Dietol 2010;56:233–43.
[26] Calne R. Cyclosporine as a milestone in immunosuppression. Transplant Proc 2004;36:13S–5S.
[27] Opferman JT, Korsmeyer SJ. Apoptosis in the development and maintenance of the immune system. Nat Immunol 2003;4:410–5.
[28] Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992;356:314–7.
[29] Xu XM, Yu JP, He XF, Li JH, Yu LL, Yu HG. Effects of garlicin on apoptosis in rat model of colitis. World J Gastroenterol 2005;11:4579–82.
[30] Morita H, Nagashima S, Uchiumi Y, Kuroki O, Takeya K, Itokawa H. Cyclic peptides from higher plants XXVIII. Antitumor activity and hepatic microsomal biotransformation of cyclic pentapeptides, astins, from Aster tataricus. Chem Pharm Bull (Tokyo) 1996;44:1026–32.
[31] Itokawa H, Takeya K, Hitotsuyanagi Y, Morita H. Antitumor compounds isolated from higher plant. J Biochem Mol Biol Biophys 2000;4:213–22.
[32] Sun Y, Qin Y, Gong FY, Wu XF, Hua ZC, Chen T, et al. Selective triggering of apoptosis of concanavalin A-activated T cells by fraxinellone for the treatment of T-cell-dependent hepatitis in mice. Biochem Pharmacol 2009;77:1717–24.