

RESEARCH ARTICLE

Immunosuppressive activity on the murine immune responses of glycyrol from *Glycyrrhiza uralensis* via inhibition of calcineurin activity

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Abstract

Context: Calcineurin (CN), a unique protein phosphatase, plays an important role in immune regulation. Our laboratory has established an effective molecular drug-screening model based on CN activity.

Objective: Our aim is to search for an effective immunosuppressant from *Glycyrrhiza uralensis* (Leguminosae).

Materials and methods: As guided by CN inhibitory test, an active compound was purified and identified as glycyrol. Immunosuppressive activity of glycyrol *in vitro* was assayed by T lymphocytes proliferation and mixed lymphocyte reaction (MLR). In addition, delayed-type hypersensitivity reaction (DTH) and skin allograft test *in vivo* were also carried out. Further, we have investigated the effect of glycyrol on phorbol 12-myristate 13-acetate (PMA)/ionomycin (Io)-stimulated IL-2 expression in Jurkat cells.

Results: The enzymatic assay showed glycyrol ($IC_{50} = 84.6 \mu M$) inhibited calcineurin activity in a dose-dependent manner. Glycyrol, at the non-cytotoxic concentration, significantly inhibited proliferation of murine spleen T lymphocytes induced by Concanavalin A (Con A) and mixed lymphocyte reaction (MLR) *in vitro*. In addition, mice treated with glycyrol had shown the dose-dependent decrease in delayed type hypersensitivity (DTH) and prolonged the graft survival by 59% compared to the control group (* $p < 0.05$). RT-PCR showed glycyrol suppressed IL-2 production in a concentration-dependent manner.

Discussion and conclusion: Our results show the immunosuppressive activity of glycyrol and this activity should be due to its inhibitory effect on CN activity, thereby suppressing IL-2 production and regulating T lymphocytes. Thus, glycyrol could be a candidate for development as a novel immunomodulatory drug.

Keywords: Glycyrol; calcineurin; immunosuppressive; inhibit; T lymphocytes

Introduction

The phosphorylation-dephosphorylation of signal transduction protein plays a critical role in cell immune responses. Calcineurin (CN), a unique Ca^{2+} /calmodulin-dependent Ser/Thr protein phosphatase, is ubiquitously expressed in mammals, especially high in brain and immune system. It is a heterodimer consisting of the catalytic subunit A (CNA) and the regulatory subunit B

(CNB), which both play an important role on regulation of immunity (Rusnak & Mertz, 2000).

It is clear that CN could regulate the phosphorylation level of nuclear factor of activated T cells (NF-AT), thereby regulating the translocation of the cytosolic component of NF-AT to the nucleus. NF-AT is one of several transcription factors, which is required for T cell-specific expression, IL-2 gene activation, and protein synthesis. IL-2 is an essential factor for most T cells proliferation

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and differentiation. Therefore, CN is a critical signaling enzyme in the processes of T lymphocytes activation. It has been proved that cyclosporin A (CsA) and tacrolimus (FK506) are the common immunosuppressants through inhibiting the activity of calcineurin in this way above (Fruman et al., 1992). In other words, CN can serve as a common target enzyme for screening immunosuppressive drugs.

The immunosuppressive drugs CsA and FK506, which both inhibit calcineurin when complexed with their specific cytoplasmic receptors – cyclophilin and FK506-binding protein (FKBP), respectively (Bierer et al., 1993; Clipstone & Crabtree, 1993; Steffan et al., 1996), have revolutionized transplantation medicine, but their therapy effects were limited in large part by their side effects including nephrotoxicity and neurotoxicity (Hong & Kahan, 2000), and therefore the new immunosuppressive drugs are expected to be developed. Suppression with immune response from the natural products has becoming a hot issues recently at the traditional drug discovery pattern area (Hong et al., 2005; Cerqueira et al., 2003; Tae et al., 2006; Fumihide et al., 2007).

Our group had focused on the immunosuppressant screening of the natural products; especially on the diverse group of CN inhibitors which showed immunoregulatory activity as mediated through interaction with cell immune systems both directly and indirectly. In the preliminary study, the ethyl acetate (EtOAc) extract of *Glycyrrhiza uralensis* (Leguminosae) (Saitoh, et al., 1976; Toshio, et al., 2002) was characterized as a potent CN inhibitor. When EtOAc extracts were purified further, the inhibitory effect of obtained fractions on CN activity increased. As guided by CN inhibitory tests, the most active component was obtained and identified as glycyrol, a prenylated coumestan in *G. uralensis*, but no study on the immunomodulatory activity of glycyrol has been reported.

In this study we first tested the inhibitory effect of glycyrol on purified CN activity further. Then, to explore its immunomodulatory activity, we mainly investigated Con A-stimulated murine spleen lymphocytes proliferation, MLR *in vitro*, and also DTH, allogeneic skin graft rejection *in vivo*. Finally, we investigated the effect of glycyrol on PMA/Io-stimulated IL-2 expression in Jurkat cells to explain the mechanism of the immunosuppressive activity of glycyrol.

Methods

Extraction and isolation of glycyrol

G. uralensis was purchased from Tongrentang Chinese Medicine Pharmaceutical Group (Beijing, China), and authenticated by Jing Luo of the Department of Biochemistry and Molecular Biology, Beijing Normal

University, where a voucher specimen has been deposited.

The powdered roots (1000g) were extracted with ethanol:H₂O (70:30, v:v), yielding 300g of extract. The ethanol extract was then partitioned between water and ethyl acetate, yielding 40g of ethyl acetate extract. The EtOAc soluble extract was purified subsequently by silica gel (800g) chromatogram with gradient petroleum ether-EtOAc (100% petroleum ether to 50% EtOAc), and 69 fractions were obtained. As guided by CN inhibitory test, fraction 26-28 (50mg) was the most active extract and then was recrystallized with methanol to yield glycyrol (20mg). The structure of glycyrol (3,9-dihydroxy-1-methoxy-2-isopentenylcoumestane) (Figure 1) was identified by comparing its spectral data (MS, ¹H- and ¹³C-NMR) with that reported in the literature (Saitoh & Shibata, 1969). Copies of the original spectra are obtainable from the correspondence author. Glycyrol was dissolved in a small volume of dimethyl sulfoxide (DMSO) for CN activity assay, lymphocytes proliferation assay and IL-2 mRNA expression assay *in vitro*. Glycyrol was dissolved in ethanol and polyoxyethylene castor oil (Cremophor EL), which is a solubilizer in a saline-alcoholic solution (Gelderblom, 2001) for *in vivo* assay.

Assay of phosphatase activity of purified calcineurin

The CN catalytic subunit CNA, regulatory subunit CNB and calmodulin (CaM), were obtained and purified following the described approach (Liu et al., 2005). The phosphatase activity of CN was detected in the assay buffer: 0.2 μM CNB, 0.6 μM CaM, 0.5 mM MnCl₂, 0.1 mM CaCl₂, 1 mM DTT, 20 μM phosphopeptide ³²P-RII in 50 mM Tris-HCl (pH 7.4 at 30°C). The reaction was initiated by mixing CNA (6.4 nM, 10 μL) and glycyrol (10 μL of different concentrations) for 10 min in an ice bath, then adding 10 μL assaying buffer with 10 μL phosphopeptide ³²P-RII and incubating in a water bath at 30°C for 20 min. The vehicle control was 2% DMSO, which didn't show any inhibitory effect. The subsequent procedures proceeded as previously described (Wang et al., 2007).

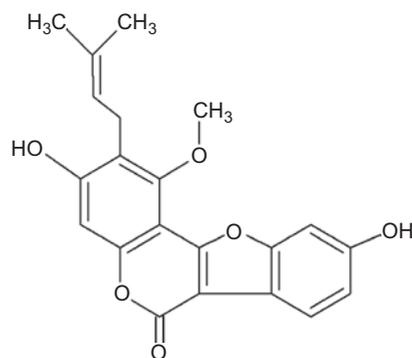


Figure 1. Chemical structure of glycyrol (MW = 366.367).

Preparation of murine spleen lymphocytes

BALB/c mice were obtained from the Department of Laboratory Animal Science (Peking University Health Science Center, China), about 6-8 weeks old (20 ± 2 g). Murine spleen lymphocytes were prepared as described previously with minor modification (Feng et al., 2003). BALB/c mice were sacrificed by cervical dislocation, and their spleens were collected in complete Roswell Park Memorial Institute (RPMI) RPMI 1640 (Sigma, St. Louis, MO), which was minced with surgical scissors and passed through a fine steel mesh to obtain the single cell suspension. The erythrocytes were lysed with ammonium chloride (0.75%, w/v). After centrifugation (1000 rpm at 4°C for 5 min), the resulting cells were washed three times with phosphate buffered saline (PBS, PH=7.4) and resuspended in complete RPMI 1640. Cell numbers were counted with a hemocytometer by Trypan blue dye exclusion technique, and cell viability exceeded 95%. The obtained cells were used in the lymphocytes proliferation experiment.

Effect of glycyrol on the proliferation of lymphocytes in vitro

Lymphocyte proliferation induced by T cell mitogen Con A ($0.2 \mu\text{M}$, Sigma) *in vitro* was determined by MTT assay. Spleen lymphocytes were seeded into 96-well plates at 2.5×10^7 cell/mL in 100 μL complete medium, thereafter Con A and RPMI 1640 medium containing various concentration glycyrol (3, 6, 12, 30, and 60 μM) were added giving a final volume of 200 μL . The control group was treated with RPMI 1640 medium containing only 0.1% DMSO, which did not cause any toxic effects. The plate was incubated for 72 h at 37°C in 5% CO₂ atmosphere, and then the cell viability rate was measured by standard MTT colorimetric assay (Mosmann, 1982).

Mixed lymphocyte reaction in vitro

BALB/c mice and C57BL/6 mice were sacrificed for spleen lymphocytes preparation as described above. BALB/c spleen lymphocytes were cultured in 96-well plates at 3.2×10^6 cells/well in the absence or in the presence of C57BL/6 spleen lymphocytes at 3.2×10^6 cells/well, which was pretreated with Mitomycin-C for 40 min at 37°C, plus different glycyrol preparations (3, 6, 12, 30, and 60 μM), and RPMI 1640 medium with 0.1% DMSO as the control group. After 72 h of incubation, proliferation was determined by MTT assay as described above.

Delayed-type hypersensitivity in vivo

Delayed-type hypersensitivity (DTH) response was evaluated by priming the BALB/c mice with sheep red blood capsule (sRBC)/20g body weight (b.w.) injected

subcutaneously in the neck on day 0. A group of 50 mice were divided into five groups and were injected intraperitoneally with glycyrol (10, 20, and 40 mg/kg), CsA (40 mg/kg) or vehicle (saline solution containing ethanol and Cremophor EL) on day 0. Then, sensitized mice were challenged with 1×10^9 sRBC/20g b.w. injected subcutaneously on the left hind footpad on day 5, with an equal volume of saline on the right hind footpad as control. The increase in the footpad swelling was measured after 24 h using Vernier Calipers (Beijing, China). The results were expressed as the increase of footpad thickness. After the experiment, the mice were sacrificed as described above, and their spleen lymphocytes numbers were counted.

Effect on skin allograft survival in a murine model in vivo

Male mice (BALB/c) were engrafted with skin grafts from male donor mice (C57BL/6) according to an adaptation of the methods of Billingham and Medawar (1951). After the skin allograft, the mice were injected intraperitoneally daily for 10 days with glycyrol (20 mg/kg), CsA (20 mg/kg) or vehicle (saline solution containing ethanol and Cremophor EL), and graft rejection time (GRT) was recorded by daily observation of epithelial skin layer survival.

Effect on general behavior and maximum dose tolerance in mice

In the preliminary experiment, mice treated with glycyrol at a maximum drug concentration of 20 mg/mL did not show mortality, so the maximum dose tolerance (MDT) test was carried out subsequently. Forty mice were divided into two groups, and administered intraperitoneally (i.p.) with glycyrol (400 mg/kg, three times on day 1) or vehicle (saline solution containing ethanol and Cremophor EL), respectively. The mice were observed for changes in reactivity, gait, motor activity, respiration rate, etc., especially mortality was recorded over a one-week period.

Reverse transcriptase (RT)-polymerase chain reaction (PCR) for interleukin (IL)-2 mRNA expression assay in Jurkat cells

Jurkat cells (human T-lymphocyte line) were used in this study, which has been proved to express CN, largely in the recent report (David et al., 1996). They were cultured in RPMI 1640 at 37°C (5% CO₂) as described above. The cells were pre-incubated in a cell culture flask filled with 3 mL RPMI 1640 (at 4×10^6 cells/flask) in the presence of various concentrations of glycyrol (20, 40 and 60 μM) or 1 μM CsA (positive group) for 6 h. The control group was treated with RPMI 1640 medium only containing 0.1%

DMSO, which did not cause any toxic effects. Phorbol 12-myristate 13-acetate (PMA, 25 ng/mL) and ionomycin (Io, 1 µg/mL) were then added to the media, and cells were further incubated for 4 h and collected by centrifugation (1000 rpm, 5 min).

Total RNA was prepared from collected cells, and 2 µg of total RNA was reversely transcribed into cDNA according to the manufacturer's instructions (TaKaRa, Japan). PCR primers used were designed according to the recent report (Wang et al., 2008). Briefly, β-actin forward, 5-CCT CTGACGTCCATCATCTA-3; β-actin reverse, 5-ATCTTCTGCTGCCGTC GCTT-3; IL-2 forward, 5-GT-CACAAACAGTGCACCTACTTC-3; IL-2 reverse, 5-TGATATTGCTGATTAAGTCCCTG-3. Thirty cycles of PCR were performed, with each cycle consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR products were then separated by 1% agarose gel electrophoresis and visualized with ethidium bromide. The gel image was captured on an Image Analysis System (Bio-Rad, Hercules, CA, America).

Statistical analysis

All the results were presented as the mean ± SD. The OriginPro7.0 statistical software was employed to analyze the data and draw plots. One-way analysis of variance (ANOVA) and subsequent Dunnett's two-sided post hoc tests were used to determine the statistical significance of differences between means by SPSS13.0.

Results

Inhibition of calcineurin activity by glycyrol

The inhibition of CN activity by glycyrol was investigated by using phosphopeptide ³²P-RII as a substrate in this work. The results showed glycyrol at the concentration of 10-150 µM inhibited CN in a dose-dependent manner in the enzymatic assay (IC₅₀ = 84.6 µM, Figure 2). The result demonstrated glycyrol had an inhibitory effect on calcineurin activity, which suggested glycyrol may have influence on immune reaction.

Effect of glycyrol on the inhibition of Con A-stimulated proliferation and MLR

We investigated the effect of glycyrol on spleen lymphocytes *in vitro* by measuring their proliferation level. As expected, Con A-stimulated lymphocyte proliferation was several fold above the baseline control (data not shown). Glycyrol at the concentration of 60 µM and lower had no toxic effect on the lymphocytes in the absence of mitogen after 72 h cell culture, and Trypan blue assay test showed that the cell viability exceeded 95%. As shown in Table 1, the Con A-induced lymphocyte proliferation

was suppressed by glycyrol, and inhibitory effect was in a dose-dependent manner. Glycyrol at the non-cytotoxic concentration of 60 µM significantly inhibited proliferation of spleen T lymphocytes induced by Con A (9–79%), which suggested that glycyrol had a strong effect on T cell proliferation *in vitro* (Table 1). As shown in Table 2, lymphocyte proliferation in mixed lymphocyte reaction (MLR) had demonstrated cultures of spleen cells of BALB/c mice stimulated with C57BL cells were decreased by 21.51%, 73.12%, and 78.19% at the concentration of 12, 30, and 60 µM, respectively. The IC₅₀ value of glycyrol for inhibiting Con A-induced lymphocyte proliferation and MLR, determined by a logistic regression analysis, were 18.8 and 23.3 µM, respectively.

Effect of glycyrol on DTH response to sRBC

The effect of glycyrol on cellular immune response *in vivo* is shown in Figure 3A. The body weight and the absolute weights of thymus, spleen and inguinal lymph nodes were also determined in these groups. All organs showed

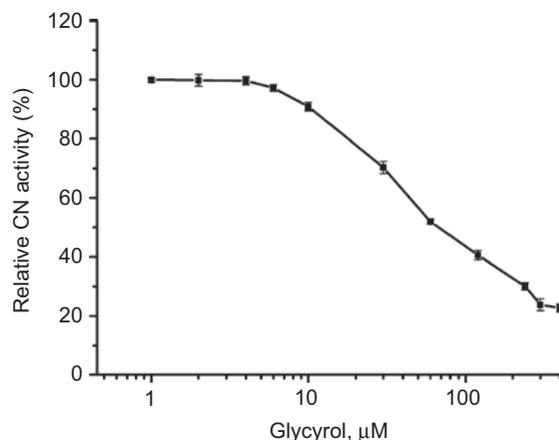


Figure 2. Inhibition of CN activity by glycyrol. The phosphopeptide ³²P-RII was used as a substrate. The CN activity is shown as percentage of control group in the normal condition. The data represent mean ± SD of three independent experiments. The degree of freedom between groups is 10, as well as 22 within groups.

Table 1. Inhibition of Con A-induced lymphocyte proliferation by glycyrol *in vitro*.

Groups	Concentration (µM)	OD ₅₇₀ value	Inhibitory rate (%)
control	0	0.262 ± 0.004	—
+glycyrol	3	0.250 ± 0.006	—
	6	0.236 ± 0.004*	9.92
	12	0.134 ± 0.013**	48.96
	30	0.094 ± 0.008**	64.21
	60	0.055 ± 0.003**	79.01

Data are the mean ± SD of three separate wells. *p < 0.05, **p < 0.001 versus control group. The degree of growth inhibition was calculated by the following equation: Inhibitory rate (%) = [(OD₅₇₀ of glycyrol group - OD₅₇₀ of control group) / OD₅₇₀ of control group] × 100. The degree of freedom between groups is 5, as well as 12 within groups.

no reduction in their weights after treatment with glycyrol (data not shown). The results showed that glycyrol had a dose-dependent effect on DTH response against sheep red blood capsule (sRBC), and mice treated with 40 mg/kg glycyrol have shown a similar decrease in DTH ($***p < 0.001$) at 40 mg/kg CsA ($***p < 0.001$). After the experiment, the mice were sacrificed, and their spleen lymphocyte number was counted. The results (Figure 3B) showed glycyrol also inhibited spleen lymphocyte number in sRBC-treated mice *in vivo*, and the effect was also dose-dependent. Therefore, it was suggested that glycyrol had strong inhibitory effect on cellular immune response DTH through decreasing spleen lymphocyte number.

Inhibition of the allogeneic effect in a mouse model

We have studied the inhibition of the allogeneic effect in a mouse model of rejection of skin transplant. Glycyrol prolonged significantly the graft survival by 59% with respect to the control group ($*p < 0.05$, Figure 4). As a positive control, the immunosuppressant CsA was

Table 2. Inhibition of lymphocyte proliferation by glycyrol in MLR *in vitro*.

Groups	Concentration (μM)	OD ₅₇₀ value	Inhibitory Rate (%)
control	0	0.093 \pm 0.003	—
+glycyrol	3	0.09 \pm 0.008	—
	6	0.089 \pm 0.07*	4.3
	12	0.073 \pm 0.001*	21.51
	30	0.025 \pm 0.003**	73.12
	60	0.02 \pm 0.001**	78.49

Data are the mean \pm SD of three separate wells. $*p < 0.05$, $**p < 0.01$ versus control group. The degree of growth inhibition was calculated by the following equation: Inhibitory rate (%) = [(OD₅₇₀ of glycyrol group - OD₅₇₀ of control group) / OD₅₇₀ of control group] \times 100. The degree of freedom between groups is 5, as well as 12 within groups.

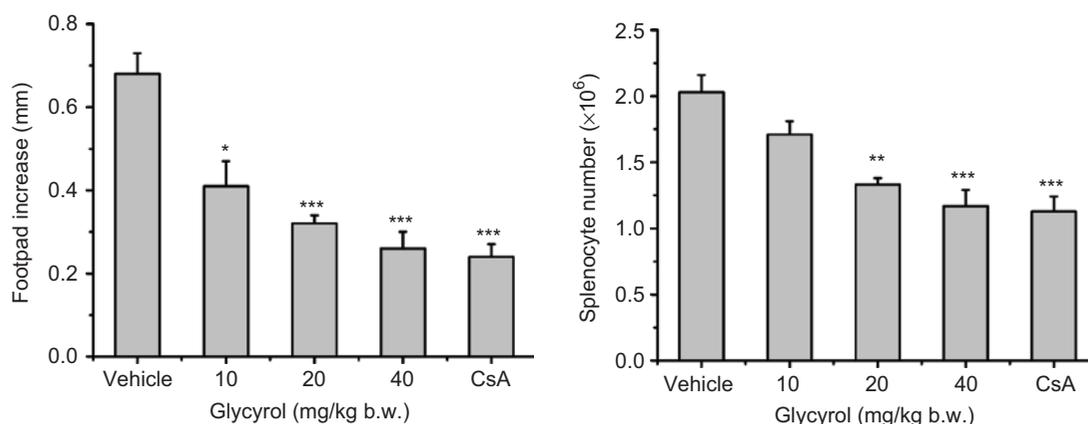


Figure 3. Effects of glycyrol on DTH response to sRBC. (A) Delayed type hypersensitivity (DTH) response was induced by immunization with 1×10^8 cells/20 g body weight (b.w.), s.c. in the neck at day 0, challenge with 1×10^8 cells/20 g b.w., s.c. in the left hind paw at day 5 and evaluation of footpad thickness (Vernier caliper) 24 h after challenge. On day 0, mice were treated intraperitoneally and daily for five days with vehicle, glycyrol (10, 20, and 40 mg/kg), or CsA (40 mg/kg). (B) The mice were sacrificed, and their spleen lymphocyte number was counted. Values represent mean \pm SD of three independent experiments ($n = 8-10$ /group in each experiment). $**p < 0.01$ and $***p < 0.001$ versus vehicle.

employed, which increased graft survival time by 41% in graft survival similarly ($*p < 0.05$). The results showed glycyrol had a slightly stronger effect on the allograft survival than CsA.

Effect on general behavior and maximum dose tolerance in mice

Compared with the control group, mice treated with glycyrol at a maximum tolerance dose of 400 mg/kg (i.p.) did not show any difference in gross general behavior as described previously, and also no mortality was observed over observation period of seven days. Based on the results, it was suggested that the maximum tolerance dose of glycyrol is greater than 400 mg/kg (i.p.).

Effect of glycyrol on PMA/Io-stimulated IL-2 production

PMA (protein kinase C activator) and ionomycin (calcium ionophore) can stimulate IL-2 production in T cells by mimicking the signals via T-cell receptors (Truneh et al., 1985; Astoul et al., 2001). We examined the effect of glycyrol on PMA/Io-stimulated IL-2 mRNA expression in Jurkat cells and found that glycyrol suppressed IL-2 expression in a concentration-dependent manner, particularly, glycyrol at 40 and 60 μM (Figure 5). The result indicated that the addition of glycyrol resulted in inhibition somewhere downstream of the Ca^{2+} increase and/or protein kinase C activation, thereby suppressing IL-2 production.

Discussion

Traditional Chinese medicine (TCM) is a very rich potential source for finding new drugs. In the course of

our screening efforts to discover novel, less-toxic immunotherapeutic agents derived from TCM targeted calcineurin, the bioactive prenylated coumestane-glycyrol was isolated from *G. uralensis*. Like other isoflavonoids in the Leguminosae, such as isoflavone, pterocarpene, rotenoid, and coumaronochromone, coumestan possesses pronounced pharmacological activities (Shaw et al., 2002). For example, coumestane is one kind of phytoestrogen whose chemical structure presents similarities with estradiol. Some *in vitro* experiments had

demonstrated the estrogenic activities of coumestrol (one coumestane) capable of binding to blood steroid-binding proteins and to estrogen receptors of target cells (Verdeal et al., 1980) and produce a protective effect against osteoporosis (Tsutsumi, 1995). It was generally agreed that at least one phenolic group and prenyl group were required for the antifungal/antimicrobial activity of flavonoid compounds (Denis & Ragai, 1996). Moreover, the presence of a prenyl group at the C-2 or C-4 of pterocarpanes increased the anticancer properties (Gardenia et al., 2007). Although no particular pattern for structure-activity relationship had been established in the previous studies, the presence of a prenyl group seemed to be an important structural requirement for biological activities. However, considering the first study on the immunosuppressive activity of glycyrol, further research should be devoted to the evaluation of their immunomodulatory effect of coumestane compounds which have similar structure to glycyrol.

As shown in the results section, glycyrol at non-cytotoxic concentration, suppressed T-cell proliferation in spleen lymphocytes induced by the T cell mitogen Con A and allotypic antigen in the mixed lymphocyte reaction. Glycyrol also had an inhibitory effect on sRBC-DTH via influencing on spleen lymphocytes possibly *in vivo*, which was used for assessment of the effects of drugs on specific cell-mediated immunity. However, glycyrol had a weak effect on LPS-stimulated B lymphocyte proliferation *in vitro* and humoral antibody response to sRBC *in vivo* (data not shown). Previous studies (Rusnak & Mertz, 2000) have shown calcineurin plays a critical role in the

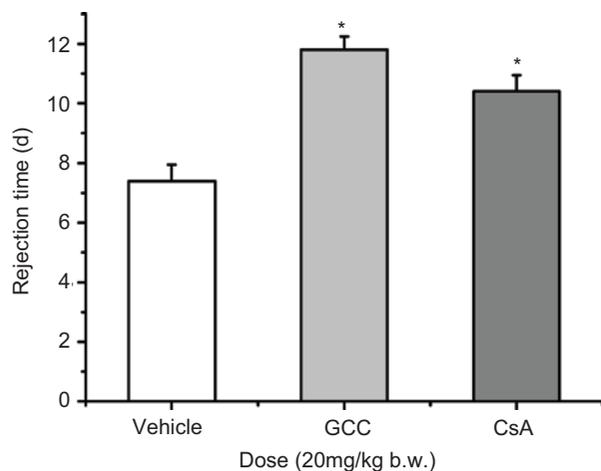


Figure 4. Effect of glycyrol on allograft rejection in mice. The data represent mean \pm SD of three independent experiments with ten animals per groups. * $p < 0.05$ versus the control group. Graft survival time (%) = [(Graft rejection time of treated group – Graft rejection time of untreated group)/Graft rejection time of untreated group] \times 100.

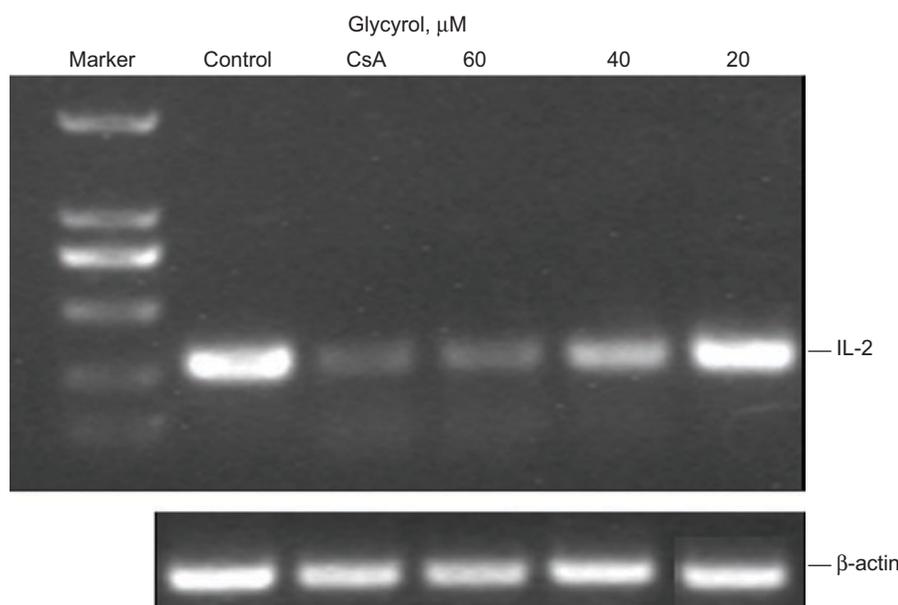


Figure 5. Effect of glycyrol on PMA/Io-induced IL-2 mRNA expression in Jurkat cells. Cells were pre-incubated with glycyrol (20, 40, and 60 μ M) or 1 μ M CsA for 6 h, and after addition of PMA (25 ng/mL) and Io (1 μ g/mL), they were further incubated for 4 h (note that all the cultures contain 0.1% DMSO as control). RNAs were prepared from the cells, and RT-PCR was performed for IL-2 and β -actin expression. After RT-PCR, amplified product was run on 1% agarose gel.

process of T lymphocyte activation, it is reasonable to presume that glycyrol had immunosuppressive activity *in vitro* and *in vivo* mainly because of its suppression of T lymphocytes through its inhibitory effect on CN.

IL-2 production in T-cells is an index of immune power. When T cells are stimulated by PMA plus ionomycin, protein kinase C (PKC) and Ca^{2+} /calcineurin-dependent pathways are activated (Truneh et al., 1985; Diehn et al., 2002). These molecules subsequently activate various downstream protein kinases, leading to activation of transcription factors, such as NF-AT which is responsible for IL-2 gene expression. In the present study, we have investigated the effect of glycyrol on PMA/Io-stimulated IL-2 production in Jurkat cells and found that glycyrol indeed suppressed IL-2 expression (Figure 5), indicating that the action point of glycyrol should reside at least in the downstream of PKC activation or Ca^{2+} increase in the cells. Furthermore, since glycyrol inhibited the activity of a purified CN *in vitro* (Figure 2) and CN can regulate the phosphorylation level of NF-AT, the inhibitory effect of glycyrol on IL-2 production should be exerted via the inhibition of CN activity, thereby possibly regulating the activity of NF-AT.

Licorice (the roots and stolons of *Glycyrrhiza* species) has been used by human beings for at least 4000 years not only as sweetening agents, as flavor in chewing gum and as a depigmentation agent in cosmetics, but also in traditional medicine against ulcer, hepatitis and as an antitussive, etc. (Nomura & Fukai, 1998). Licorice isoflavonoids (including glycyrol) have shown potent antibacterial action against upper airway respiratory tract pathogens (Tanaka et al., 2001). In this work, it is the first time to report that glycyrol was an effective CN inhibitor showing immunosuppressive activity. What's more, glycyrol significantly suppressed allogeneic skin graft rejection time in mice treated with glycyrol, as slightly stronger than CsA. It is possible that glycyrol could be beneficial for treating Graft-Versus-Host Disease (GVHD).

In Japanese patents (Azuma et al., 1994), it was confirmed that glycyrol and other derivatives were important components of skin-lightening agents and cosmetics, which suggested glycyrol could be expected to be relatively safe for application to patients. Again, MDT tests showed that the maximum tolerance dose of glycyrol was more than 400 mg/kg which was 40 times as high as the lowest doses required to show pharmacological effects as described in *in vivo* tests. The result demonstrated glycyrol is weakly toxic in experimental treatment. However, more information about its toxicity and safety should be understood in long-term toxicity test and clinical trial before glycyrol is used as a new immunosuppressant.

Our findings that glycyrol, an effective CN inhibitor from natural products, has shown immunosuppressive activity *in vitro* and *in vivo*, can make it an attractive

drug lead for searching for a novel class of immunosuppressant. At this point there are still some limitations in using glycyrol for clinical treatment, which include: 1) the dosage of glycyrol isolated from licorice is still relatively low; 2) the solubility of glycyrol is not very good in aqueous media (such as blood serum) because of its low polarity; 3) the apparent safety over long-term administration of glycyrol hasn't been tested. Thus, further chemical synthesis and modification of glycyrol are required to increase its dosage and solubility to generate a fully useful immunosuppressive drug. Alternatively, glycyrol could be used as a supplementary immunosuppressive drug in combination with other immunosuppressants.

Conclusion

The results from this study established that glycyrol exhibited low toxicity and compelling immunosuppressive effects, which might be attributed to its suppressing IL-2 production and regulating T lymphocytes via inhibiting phosphatase activity of calcineurin. Further, better understanding of action of glycyrol may contribute to its exploitation as a therapeutic molecule in autoimmune and allergic diseases.

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Declaration of interest

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References

- Astoul E, Edmunds C, Cantrell DA, Ward SG (2001): PI 3-K and T cell activation: Limitations of T-leukemic cell lines as signaling model. *Trends Immunol* 22: 490-496.
- Azuma S, Yada Y, Imokawa G, Shibuya J, Nishizawa Y (1994): Skin-lightening agents and cosmetics containing coumestrol derivatives. Patent, JP93-11490419930517.
- Bierer B, Holländer G, Fruman D, Burakoff S (1993): Cyclosporin A and FK506: Molecular mechanisms of immunosuppression and probes for transplantation biology. *Curr Opin Immunol* 5: 763-773.
- Billingham, RE, Medawar, PB (1951): The technique of free skin grafting in animals. *J Exp Bio* 28: 385-402.
- Corqueira F, Cordeiro-Da-Silva A, Araujo N, Cidade H, Kijjoa A (2003): Inhibition of lymphocyte proliferation by prenylated flavones: Artelastin as a potent inhibitor. *Life Sci* 73: 2321-2334.

- Clipstone N, Crabtree G (1993): Calcineurin is a key signaling enzyme in T lymphocyte activation and the target of the immunosuppressive drugs cyclosporin A and FK506. *Ann NY Acad Sci* 696: 20-30.
- David A, Fruman, Sung YP, Claude BK (1996): Measurement of calcineurin phosphatase activity in cell extracts. *Methods: A Companion to Methods in Enzymology* 9: 146-154.
- Denis B, Ragai KI (1996): Isoprenylated flavonoids – a survey. *Phytochemistry* 43: 921-982.
- Diehn M, Alizadeh AA, Rando OJ, Liu CL, Stankunas K, Botstein D, Crabtree GR, Brown PO (2002): Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc Natl Acad Sci USA* 99: 11796-11801.
- Feng YH, Zhou WL, Wu QL, Li XY, Zhao WM, Zou JP (2003): Low dose of resveratrol enhanced response of mice. *Acta Pharmacol Sin* 23: 893-897.
- Fruman D, Mather P, Burakoff S, Bierer B (1992): Correlation of calcineurin phosphatase activity and programmed cell death murine T cell hybridomas. *Eur J Immunol* 22: 2513-2517.
- Fumihide T, Masaya Y, Satoko T, Satoko S, Nobuo Y, Tomoya T, Tomihisa O (2007): Capsicum ethanol extracts and capsaicin enhance interleukin-2 and interferon-gamma production in cultured murine Peyer's patch cells *ex vivo*. *Life Sci* 80:1553-1563.
- Gardenia CG, Militão SM, Pinheiro IN (2007): Bioassay-guided fractionation of pterocarpanes from roots of *Harpalyce brasiliensis*. *Bioorg Med Chem* 15: 6687-6691.
- Gelderblom H, Verweij J, Nooter K, Sparreboom A (2001): Cremophor EL: The drawbacks and advantages of vehicle selection for drug formulation. *Eur J Cancer* 37:1590-1598.
- Hong JC, Kahan BD (2000): Immunosuppressive agents in organ transplantation: Past, present, and future. *Semin Nephrol* 20: 108-125.
- Hong XS, Feng Q, Yuan JP (2005): *In vitro* and *in vivo* immunosuppressive activity of *Spica* ethanol extract on the immune responses in mice. *J Ethnopharmacol* 101: 31-36.
- Liu P, Huang C, Jiang ZC, Yu DY, Wei Q (2005): Non-catalytic domains of subunit A negatively regulate the activity of calcineurin. *Biochimie* 87: 215-221.
- Mosmann T (1982): Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-59.
- Nomura T, Fukai T (1998). Phenolic constituents of licorice (*Glycyrrhiza* species), in: Herz W, Kirby GW, Moore RE, Steglich W, Tamm C, eds, *Progress in the Chemistry of Organic Natural Products*. Springer, Vienna, pp. 1-140.
- Rusnak F, Mertz P (2000): Calcineurin: Form and function. *Phys Rev* 80: 1483-1521.
- Saitoh T, Shibata S (1969): Some new constituents of licorice root: Glycyrol, 5-O-methylglycyrol and isoglycyrol. *Chem Pharm Bull* 17: 729-734.
- Saitoh T, Kinoshita T, Shibata S (1976): Flavonols from licorice root. *Chem Pharm Bull* 24: 1242-1245.
- Shaw W, Sayo U, Yuriko K (2002): Isoflavones for prevention of cancer, cardiovascular diseases, gynecological problems and possible immune potentiation. *Biomed Pharmacother* 56: 302-312.
- Steffan H, Neil C, Luika T, Jeffrey N (1996): The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol* 80: S40-45.
- Tae WJ, Chun HJ, Sang KL, In HJ, Ghee HK, Dong JL (2006): Immunosuppressive effects of rutaecarpine in female BALB/c mice. *Toxicol Lett* 164: 155-166.
- Tanaka Y, Kikuzaki H, Fukuda S, Nakatani N (2001): Antibacterial compounds of licorice against upper airway respiratory tract pathogens. *J Nutr Sci Vitaminol* 47: 270-273.
- Truneh A, Albert E, Golstein P, Schmitt-Verhulst AM (1985): Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature* 313: 318-320.
- Toshio F, Ai M, Kiyoshi K, Toshihisa K (2002): Anti-*Helicobacter pylori* flavonoids from licorice extract. *Life Sci* 71: 1449-1463.
- Tsutsumi N (1995): Effect of coumestrol on bone metabolism in organ culture. *Biol Pharm Bull* 18: 1012-1015.
- Verdeal K, Brown RR, Richardson T, Ryan DS (1980): Affinity of phytoestrogens for estradiol-binding proteins and effect of coumestrol on growth of 7, 12-dimethylbenz(a)anthracene-induced rat mammary tumors. *J Natl Cancer Inst* 64: 285-290.
- Wang HL, Yao SY, Lin W L, Du YW, Xiang BQ, He S, Huang C, Wei Q (2007): Different roles of Loop 7 in inhibition of calcineurin. *Biochem Biophys Res Commun* 362: 263-268.
- Wang H, Zhou CL, Lei H, Zhang SD, Zhen JQ, Wei Q (2008): Kaempferol: A new immunosuppressant of calcineurin. *IUBMB Life* 60: 549-554.