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Plant alkaloid tetrandrine downregulates protein kinase C-dependent signaling pathway in T cells

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Abstract

Tetrandrine, a purified traditional Chinese medicinal herb that acts as an immunosuppressant and a Ca^{2+} channel blocker, has been clinically used to treat patients with arthritis, silicosis and hypertension. Since T cells play a critical role as autoreactive and pathogenic population in autoimmune diseases, in this study, we examined the immunosuppressive effect of tetrandrine on human peripheral blood T cells. We showed that tetrandrine inhibited phorbol 12-myristate 13-acetate (PMA) + ionomycin-induced T cell proliferation, interleukin-2 secretion and the expression of the T cell activation antigen, CD71. Further investigation of the molecular mechanism demonstrated that tetrandrine inhibited the expression of the protein kinase C-dependent interleukin-2 receptor alpha chain and CD69 but not the expression of the Ca^{2+}-dependent CD40 ligand and CD69. Interestingly, when tetrandrine and cyclosporin A were added together, significant synergism in the suppression of T cell activation was observed. Moreover, of the several tetrandrine analogues studied, hernandezine was the most potent inhibitor of protein kinase C signaling events. These results also suggest that the protein kinase C-inhibitory capacity of tetrandrine and its analogues may not be associated with their function as Ca^{2+} channel blockers. Lastly, we showed that, within therapeutic concentrations, tetrandrine and its analogues could induce cellular apoptosis, which is defective in autoimmune diseases. In conclusion, our findings provide novel information about the molecular mechanism of the immunosuppressive effect of tetrandrine and its analogues in human peripheral blood T cells.

Keywords: Tetrandrine; Apoptosis; T cell; Immunosuppression; Protein kinase C

1. Introduction

Han-Fang-Chi, a traditional Chinese anti-rheumatic remedy, is the dried tuberous root of the creeper *Stephania tetrandra* S. Moore. As one of the chemical constituents from the root of *S. tetrandra*, tetrandrine accounts for its major biological activities (Feng and Chen, 1985). Tetrandrine is a bis-benzylisoquinoline alkaloid with the formula $C_{38}H_{42}O_6N_2$ and a molecular weight of 622.73 Da (Fig. 1). Tetrandrine has been studied extensively as a Ca^{2+} channel blocker. Tetrandrine exerts a broad, non-selective inhibition of L-type, N-type, T-type and other poorly characterized voltage-dependent Ca^{2+} channels in different tissues (reviewed by Kwan, 1996). Several tetrandrine analogues also have similar effects as Ca^{2+} channel blockers, such as hernandezine, isolated from *Thalictrum glandulosissimum*, and berbamine, isolated from *Bereris soulieana*, which share a very similar structure with tetrandrine (Leung et al., 1994; Low et al., 1996; Leung et al., 1997) (Fig. 1).

Tetrandrine has been used in China for several decades to treat patients with arthritis and silicosis, diseases which are associated with the activation and infiltration of immune effector cells at lesion sites (Li et al., 1981; Castranova et al., 1991; Kang et al., 1992; Chang and But, 1987). Compatible to its effect in the treatment of silicosis, tetrandrine inhibits the proliferation of pulmonary fibroblasts, reduces mitochondrial ATP synthesis in alveolar type II cells, and kills alveolar macrophages through its cytotoxic effect (Reist et al., 1993; Miles et al., 1993; Pang and Hoult, 1997). The therapeutic effect of tetrandrine in rheumatic diseases is further supported by the observation

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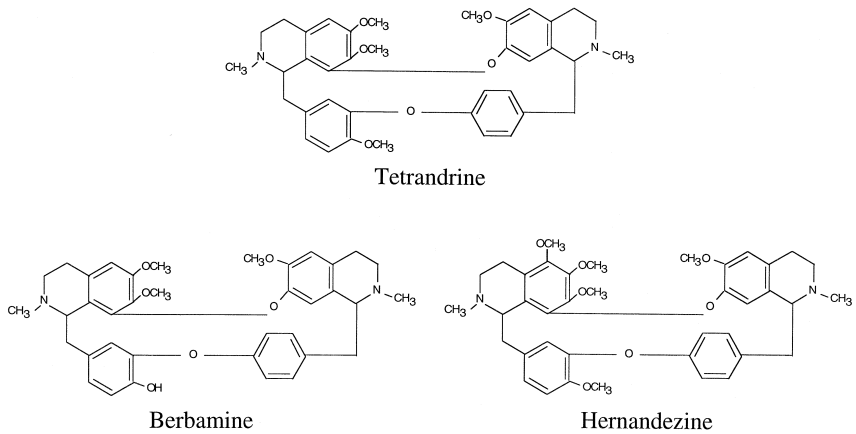

Fig. 1. Structure of tetrandrine and its analogues, hernandezine and berbamine.

that it has pluripotent suppressive effects on immune effector cells including monocytes, B cells, neutrophils, and mast cells (Seou et al., 1986, 1988; Teh et al., 1988; Ferrante et al., 1990; Chang et al., 1997). However, to our knowledge, there has been no study of the effect of tetrandrine on human peripheral blood T lymphocytes, a major cell population involved in the pathogenesis of autoimmune diseases.

Since T cells play a pivotal role in the pathogenesis of autoimmune diseases, the purpose of this study was to investigate the immunosuppressive effects of tetrandrine on human peripheral blood T cells. By using different tetrandrine analogues, we also examined their immunosuppressive effects and correlated these effects with the reported Ca^{2+} channel blocking effect of these drugs. We showed that tetrandrine was a potent immunosuppressive agent that inhibited the protein kinase C-dependent but not the Ca^{2+}-dependent signaling pathway in human peripheral blood T cells. In addition, our observations also suggest that the immunosuppressive effects of tetrandrine and its analogues may be independent of their inhibitory effects on Ca^{2+} channels.

2. Materials and methods

2.1. Preparation of tetrandrine and its analogues

The powder of tetrandrine, $C_{38}H_{42}O_6N_2$, with a purity of more than 98% was obtained from Yichang Pharmaceutical, Hubei Province, the People's Republic of China, and dissolved in 0.1 N HCl. The tetrandrine analogues, hernandezine and berbamine, were kindly provided by Dr. C.Y. Kwan (McMaster University, Canada) and were dissolved in 0.1 N HCl. For stimulation, the required concentrations of tetrandrine and its analogues were made by further dilution of the concentrated stock solution with culture medium. At concentrations equal to or less than 10 μM, tetrandrine and its analogues did not show any significant cytotoxic effect on T cells, as determined in trypan blue exclusion assays. In addition, the final concentrations of HCl in the experiments did not have any effect on cell proliferation and viability.

2.2. Cells, reagents, antibodies and cell stimulation

Human peripheral blood T cells were purified from whole blood by negative selection as described previously (Lai et al., 1995). Briefly, whole blood (20–40 ml) from normal donors (more than 30 participants in this study) was mixed with Ficoll-Hypaque and, after centrifugation, the layer of mononuclear cells was collected. After lysis of red blood cells, the peripheral blood mononuclear cells were plated out on Petri dishes to remove adherent cells and then incubated with antibodies including L243 (anti-DR; ATCC, Rockville, Md), OKM1 (anti-CD11b; ATCC), and LM2 (anti-Mac1; ATCC) for 30 min at 4°C. The cells were then washed with medium containing 10% fetal bovine serum and incubated with magnetic beads conjugated with goat anti-mouse immunoglobulin-G (IgG) (R&D). The antibody-stained cells were then removed with a magnet. After the above procedures were repeated, the purity of the T cells was shown to be more than 98%, as determined by the percentage of CD3+ cells in flow cytometry (Beckton Dickinson). The median CD45RA+ naive/CD45RO+ memory T cell ratio for four donors was 1.36, which was similar to data reported by Ullum et al. (1997) but slightly higher than data reported by Aukrust et al. (1996) (data not shown). For the stimulation experiments, the stimuli used included PMA (phorbol 12-myristate 13-acetate, Sigma) and ionomycin (Sigma) at the concentrations described in the figure legends. Another protein kinase C activator, 1-oleoyl-2-acetyl-glycerol, was purchased from Calbiochem. Cyclosporin A was purchased from Sandos.

2.3. Cell proliferation and viability examinations

The T cell proliferation assay has been described previously (Bryan et al., 1994). In brief, T cells in 200 μl were cultured in triplicate samples in 96-well flat-bottom microtiter plates (Costar) at 1×10^5 cells/well in RPMI medium containing 10% fetal bovine serum. Before stimu-

lation, the T cells were incubated with tetrandrine for 24 h. After stimulation with appropriate stimuli for 3 days, cell proliferation was measured in a liquid scintillation counter (Beckman) after the cells were incubated with 1 μCi/well of [^{3}H]thymidine for the last 16 h of stimulation. The standard error of the mean was less than 10% for each sample. The cell viability was evaluated in trypan blue exclusion assays.

2.4. DNA fragmentation assays

Briefly, 1×10^6 human peripheral blood T cells were treated with tetrandrine for various times and then lysed in 50 μl of NTE buffer containing 100 mM NaCl, 40 mM Tris–HCl (pH 7.4), 20 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS). The lysates were heated at 65°C for 10 min and then incubated with 0.5 mg/ml proteinase K for 2 h. After this, the mixtures were incubated with 0.2 mg/ml RNase A at 50°C for 2 h. Finally, the samples were analyzed on a 1.8% agarose gel and stained with ethidium bromide.

2.5. Interleukin-2 ELISA assay

The determination of interleukin-2 concentration was performed according to the manufacturer's instructions (R&D) with some modifications. Briefly, a 96-well flat-bottom plate was coated with anti-interleukin-2 monoclonal antibody (100 μl at 4 μg/ml) in phosphate-buffered saline (PBS) pH 7.3 at room temperature overnight. Then, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) three times. After this, the plate was incubated with a blocking solution containing 1% bovine serum albumin, 5% sucrose and 0.05% NaN_3 in PBS for more than 1 h. After a wash with PBS-T, 100 μl supernatant was then added to each well for 24 h. After 24 h, the plates were washed with PBS-T three times and then incubated with biotinylated anti-interleukin-2 detection antibodies

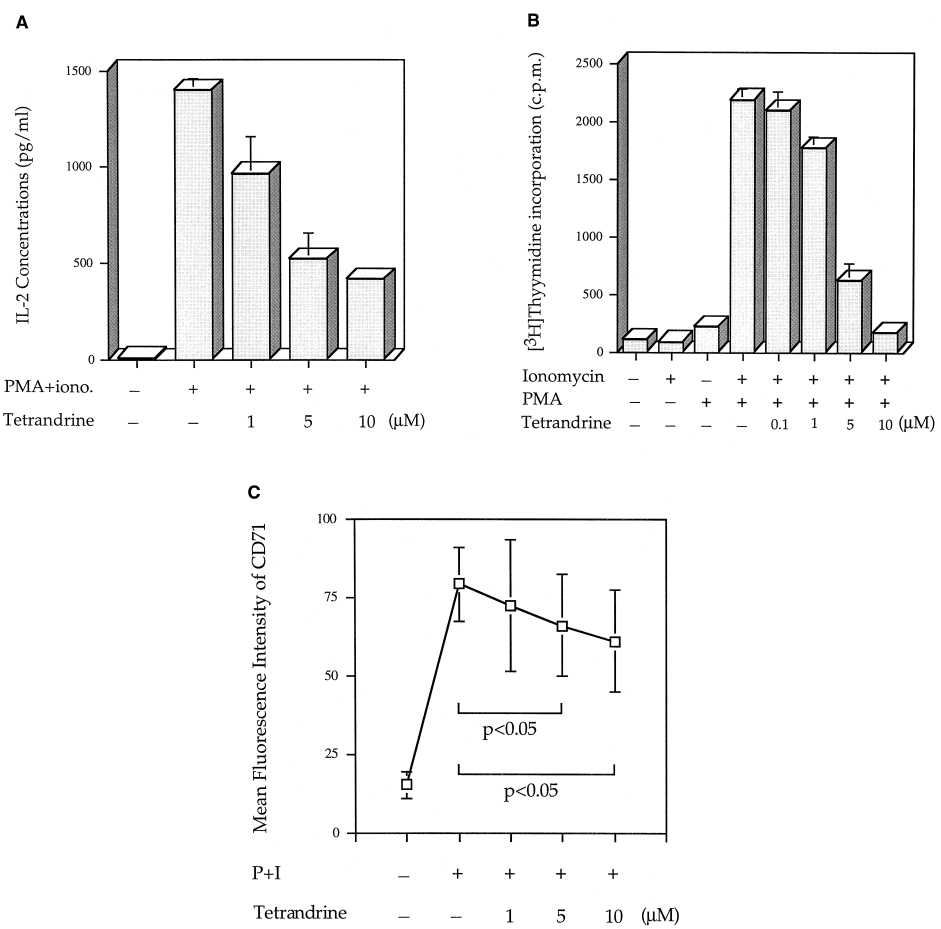

Fig. 2. Tetrandrine inhibits T cell activation. (A) Purified human peripheral blood T cells at 5×10^5/ml were treated in triplicate with various concentrations of tetrandrine for 24 h and then stimulated with PMA 5 ng/ml + ionomycin 1 μM for another 24 h. The supernatants were collected for interleukin-2 measurements. (B) T cells at 1×10^5/well in triplicate were pretreated with various concentrations of tetrandrine for 24 h and stimulated with PMA 1 ng/ml + ionomycin 0.3 μM for 3 days. [^{3}H]Thymidine was added to the cells for the last 16 h of stimulation and the thymidine incorporation was measured with a liquid scintillation counter. (C) T cells at 5×10^5/ml were treated with various concentrations of tetrandrine for 24 h and then stimulated with PMA 5 ng/ml + ionomycin 1 μM for another 72 h. The expression of CD71 was measured by flow cytometry. Data shown are the means ± S.D. of mean fluorescence intensity from four different donors.

(100 μl at 12.5 ng/ml) for 2 h at room temperature. After a single wash step, 100 μl of streptavidin horseradish peroxidase (1:2000 dilution of a 1.25 mg/ml solution) was added and incubated for 20 min at room temperature. After a triple wash step, 100 μl of substrate solution containing 1:1 mixture of H_2O_2 and tetramethylbenzidine was added and incubated for another 20 min at room temperature. The reaction was stopped by adding stop solution, and the interleukin-2 concentrations were measured with a microplate reader (Dynatech).

2.6. Measurement of cell surface molecule expression

Human peripheral blood T cells at a concentration of 5×10^5/ml were preincubated with tetrandrine for 24 h. After stimulation with various stimuli, T cells were collected and washed with PBS. After the wash step, T cells were stained with either phycoerythrin-conjugated anti-interleukin-2 receptor alpha monoclonal antibody, fluorescein isothiocyanate-conjugated CD69 or CD71 monoclonal antibody (Becton Dickinson), or fluorescein isothiocyanate-conjugated anti-CD40 ligand monoclonal antibody (Calbiochem), and the expression of these surface molecules was determined with a FACSTAR (Becton Dickinson) as described by the manufacturer. The phycoerythrin- or fluorescein isothiocyanate-conjugated isotype-matched monoclonal antibody was used as a control. The mean fluorescence intensity of each surface molecule expressed was used to evaluate the drug effect.

2.7. Statistics

The results are expressed as means ± S.D. A paired or unpaired Student's *t*-test was used to determine the significance of differences; a value of $P < 0.05$ was considered statistically significant.

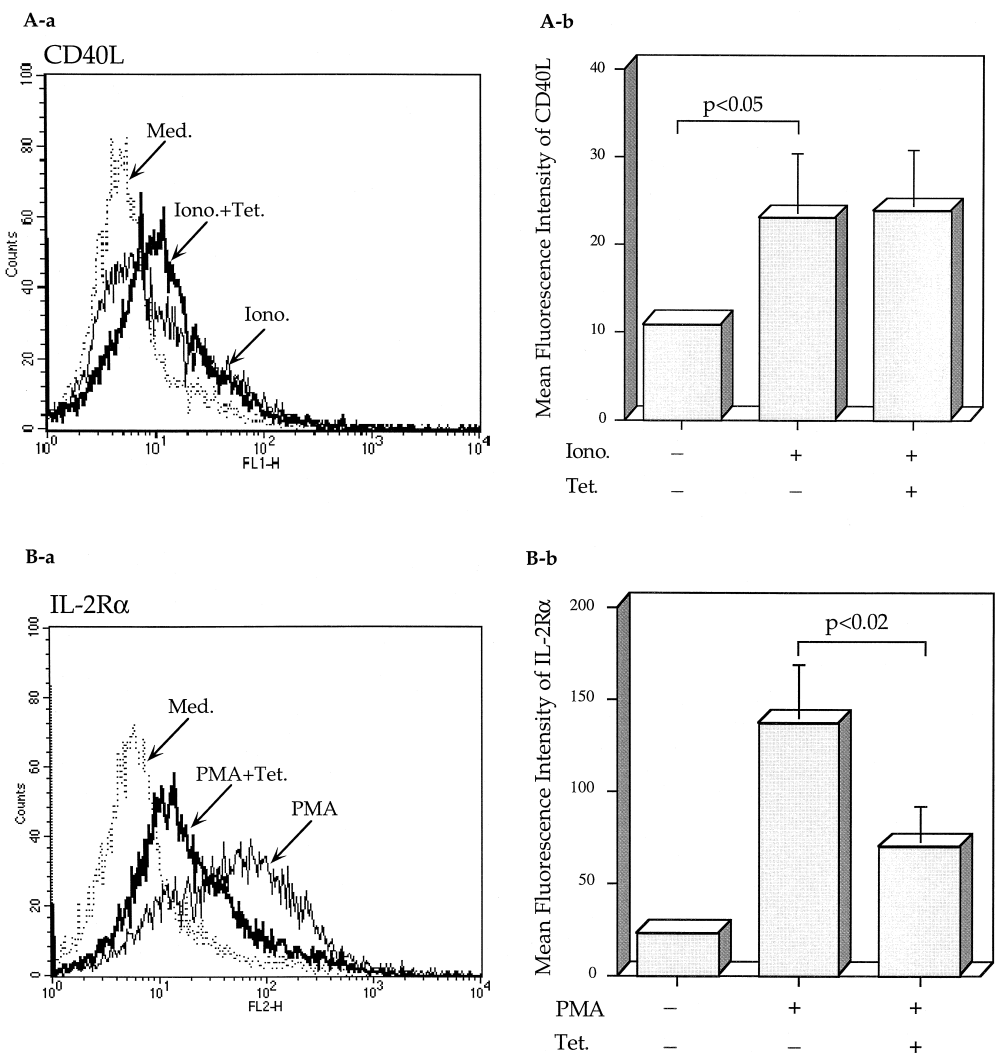

Fig. 3. Tetrandrine inhibits PMA-induced interleukin-2 receptor alpha chain but not Ca^{2+}-dependent CD40 ligand expression. Purified human peripheral blood T cells at 5×10^5/ml were either untreated or treated with tetrandrine at 10 μM for 24 h and then stimulated with ionomycin 2 μM for 6 h (A) or PMA 5 ng/ml for 24 h (B), respectively, and the expression of CD40 ligand (A) and interleukin-2 receptor alpha chain (B) was measured with flow cytometry. a (A–a and B–a) shows the representative data and b (A–b and B–b) shows the statistical analysis of the results for cells from four different donors. Med. = medium; Iono. = ionomycin; Tet. = tetrandrine.

3. Results

3.1. Tetrandrine inhibits PMA + ionomycin-induced T cell activation

Binding of antigen to the T cell receptor induces the activation of phospholipase C, resulting in an increase in cytosolic Ca^{2+} concentration and in protein kinase C activity. The immunosuppressive effect of tetrandrine was examined with PMA, a protein kinase C activator, and ionomycin, a Ca^{2+}-ionophore, as stimuli which mimic the T cell receptor signaling events (Truneh et al., 1985). Fig. 2A shows that tetrandrine inhibited the PMA + ionomycin-induced interleukin-2 secretion in a concentration-dependent manner. The cell proliferation and the expression of CD71, measured as indicators of late-phase T cell activation, were also inhibited by tetrandrine (Fig. 2B and C).

3.2. Tetrandrine inhibits protein kinase C- but not Ca^{2+}-dependent signaling event

The inhibition of PMA + ionomycin-induced T cell activation by tetrandrine was further investigated. In order to examine whether tetrandrine inhibits the Ca^{2+}-dependent signaling pathway, the expression of CD40 ligand was used as a parameter because its expression is induced by the Ca^{2+}-dependent but not by the protein kinase C-dependent signaling pathway (Nusslein et al., 1996). As shown in Fig. 3A, although the induction was not very strong, the difference in mean fluorescence intensity of CD40 ligand

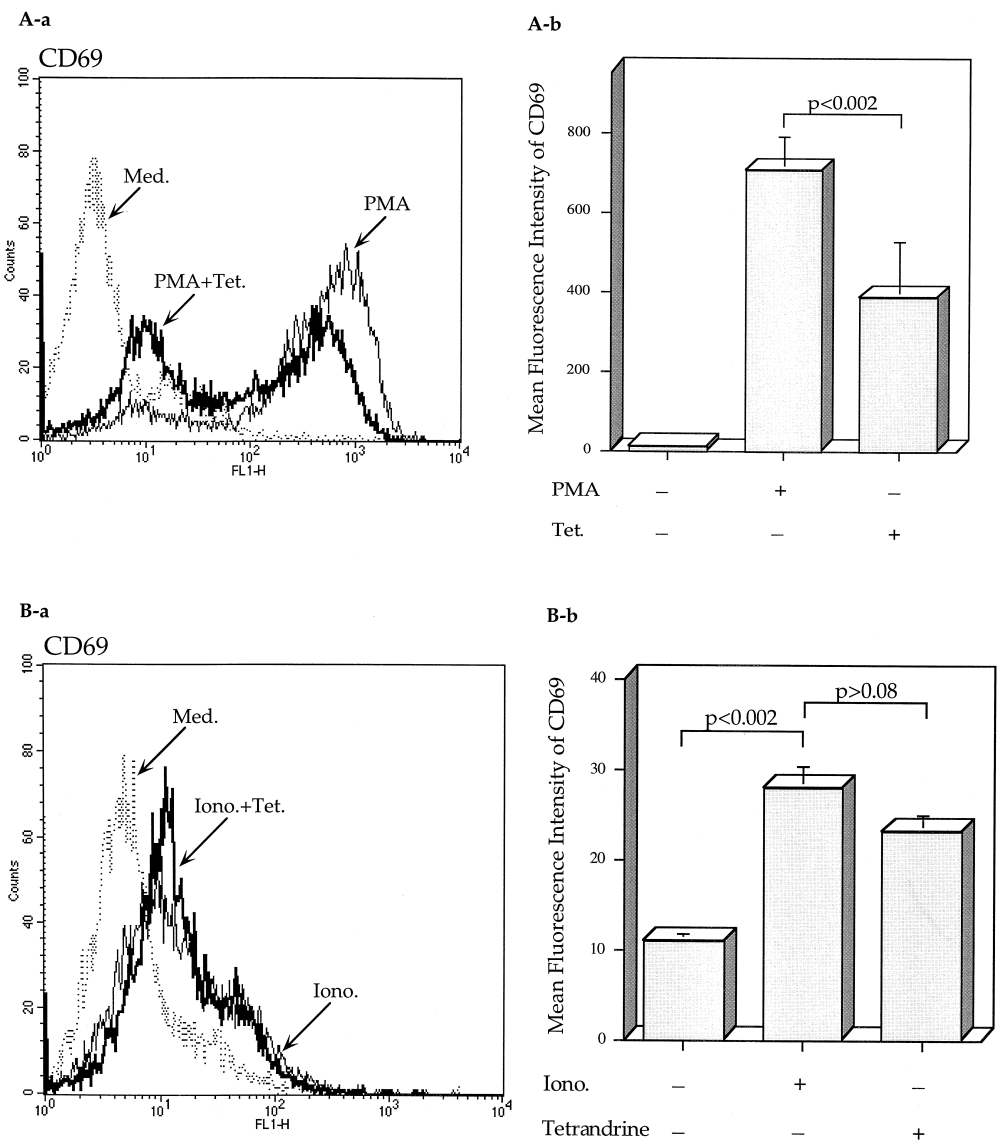

Fig. 4. Tetrandrine inhibits PMA-mediated but not calcium-dependent CD69 expression. Human peripheral blood T cells at 5×10^5/ml were treated with 10 μM tetrandrine for 24 h and then stimulated with PMA 5 ng/ml (A) or ionomycin 2 μM (B) for another 12 h. The expression of CD69 was measured by flow cytometry. a (A–a and B–a) shows the representative data and b (A–b and B–b) shows the statistical analysis of the results for cells from six (A) or four (B) different donors. Med. = medium; Iono. = ionomycin; Tet. = tetrandrine.

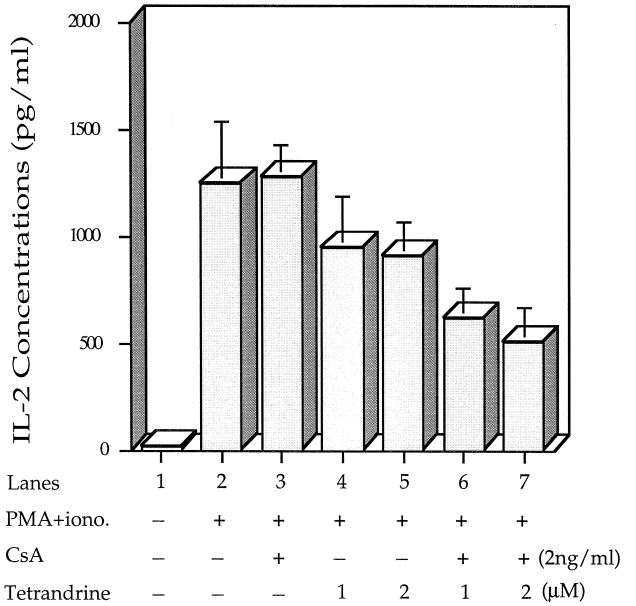

Fig. 5. Synergism between tetrandrine and cyclosporin A in inhibiting T cell activation. Human peripheral blood T cells at 5×10^5/ml were treated in triplicate with various concentrations of tetrandrine in the presence or absence of cyclosporin A (2 ng/ml) for 24 h and then stimulated with PMA 5 ng/ml + Ionomycin 1 μM for another 24 h. The interleukin-2 concentrations in the supernatants were determined.

expression between unstimulated and ionomycin-stimulated cells was statistically significant ($P < 0.05$). In the presence of 10 μM tetrandrine, ionomycin-induced CD40 ligand expression was not reduced. We then examined the expression of interleukin-2 receptor alpha chain, which is induced by the protein kinase C-dependent but not by the Ca^{2+}-dependent signaling pathway (Depper et al., 1984; Mills et al., 1985; Bryan et al., 1994). Fig. 3B shows that tetrandrine inhibited the PMA-induced expression of interleukin-2 receptor alpha chain. The protein kinase C-dependent selectivity of tetrandrine was further investigated by examining the expression of another activation antigen, CD69, which can be induced by both the protein kinase C-dependent and the calcium-dependent signaling pathways through different mechanisms (Taylor-Fishwick and Siegel, 1995). Fig. 4 shows that tetrandrine significantly inhibited the PMA-mediated expression of CD69 (4A) but not the ionomycin-mediated expression of CD69 (4B). Moreover, tetrandrine potently inhibited the expression of CD69 induced by another protein kinase C activator, 1-oleoyl-2-acetyl-glycerol, which is structurally different from PMA (Hirobe, 1994) (data not shown). This observation also argued against the possibility that the inhibition of the effect of PMA by tetrandrine was mediated by structural hindrance.

3.3. Synergism between tetrandrine and cyclosporin A in suppression of T cell activation

Lieberman et al. (1993) reported synergism between tetrandrine and tacrolimus (FK506) in the prevention of diabetes in BB rats. Although cyclosporin A and FK506 are not structurally similar, they both downregulate the activity of the same target, calcineurin, in suppressing T cell activation (Schreiber and Crabtree, 1992). In order to investigate whether tetrandrine and cyclosporin A act synergistically to inhibit T cell activation, the effect of various concentrations of tetrandrine with or without cyclosporin A was examined. As shown in Fig. 5, cyclosporin A at 2

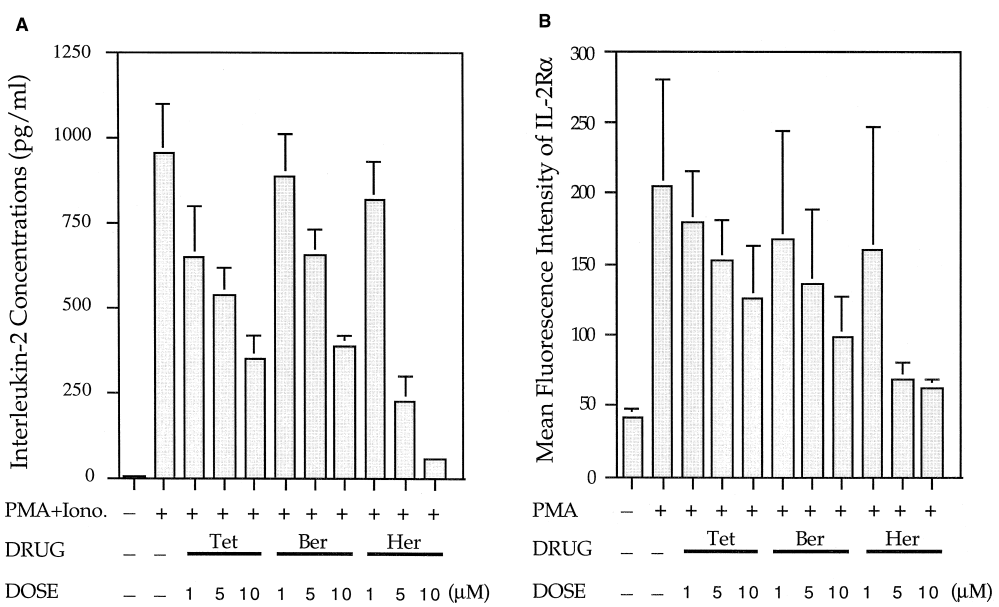

Fig. 6. Comparison of the immunosuppressive effects of tetrandrine and its analogues. (A) and (B) show the results comparing the inhibitory effects of tetrandrine (Tet), berbamine (Ber) and hernandezine (Her) on PMA + ionomycin-induced interleukin-2 secretion (A) or PMA-induced interleukin-2 receptor alpha chain (IL-2Rα) expression (B) as described in figure legends of Figs. 2 and 3.

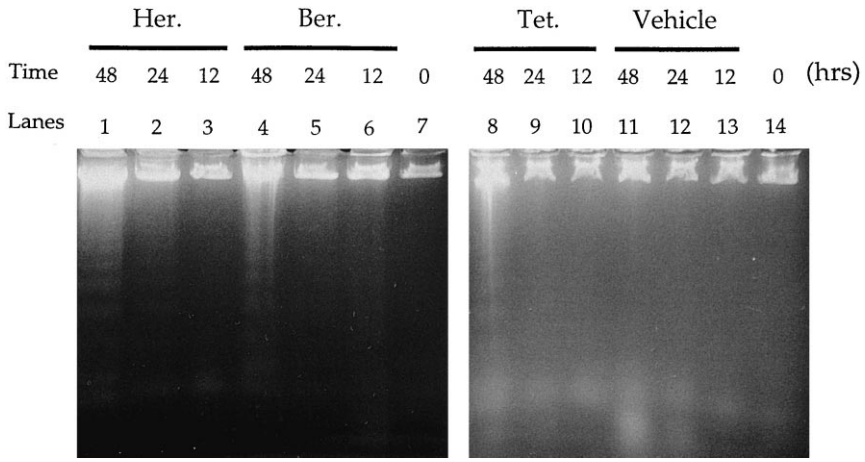

Fig. 7. The induction of apoptosis by tetrandrine and its analogues. Human peripheral blood T cells at 1×10^6/ml were treated with vehicle or 10 μM of tetrandrine or its analogues for various times and DNA fragmentation assays were performed as described in Section 2. Tet. = tetrandrine; Ber. = berbamine; Her. = hernandezine.

ng/ml concentration did not significantly inhibit PMA + ionomycin-induced interleukin-2 secretion from T cells (lane 3). However, in the presence of a suboptimal noninhibitory concentration of cyclosporin A, the suppressive effect of tetrandrine was moderately enhanced (compare lanes 6 and 7 to 4 and 5, respectively). The synergism was not so pronounced when both drugs were used at suboptimal noninhibitory concentrations (data not shown).

3.4. The potency of protein kinase C-inhibitory effect of tetrandrine and its analogues

In order to appreciate whether the immunosuppressive effect of tetrandrine is correlated with its effect as a Ca^{2+} channel blocker, the protein kinase C-inhibitory effect was also investigated of two other tetrandrine analogues, hernandezine and berbamine. Although these drugs are derived from different plant sources, they have very similar structures (Fig. 1). In comparison with the extensive studies on their Ca^{2+} channel blocking effect, studies to compare the immunosuppressive activities of these drugs are very limited. Both interleukin-2 secretion induced by PMA + ionomycin stimulation and interleukin-2 receptor alpha chain expression induced by PMA stimulation were used to measure the protein kinase C-inhibitory effect of these drugs. As shown in Fig. 6A and B, all tetrandrine analogues were able to significantly inhibit protein kinase C signaling events. Among them, hernandezine was the most potent; tetrandrine and berbamine appeared to have similar potency.

3.5. Induction of apoptosis by tetrandrine and its analogues

Tetrandrine and its analogues did not significantly affect T cell proliferation or survival when the concentrations used were equal to or less than 10 μM, a concentration that is attained in tetrandrine-treated silicosis patients (Li et al., 1981; data not shown). Since several anti-rheumatic drugs have been shown to cause apoptosis, which is defective in autoimmune diseases, this potential therapeutic effect was examined in human peripheral blood T cells incubated with tetrandrine and its analogues. T cells were treated with vehicle or 10 μM tetrandrine or its analogues for various times and the cells were collected and DNA samples were analyzed on agarose gels. Fig. 7 shows that tetrandrine and its analogues but not vehicle caused fragmentation of DNA, which is typical of apoptosis (compare lane 1, 4 and 8 to 11). The DNA ladders were prominent when cells were treated for 48 h.

4. Discussion

4.1. Tetrandrine as a pluripotent immunosuppressant which targets protein kinase C-dependent signaling pathway

Tetrandrine is a pluripotent immunosuppressive drug both in vitro and in vivo. In vitro, tetrandrine was shown to inhibit the production of nitric oxide, a critical mediator of inflammation, in lipopolysaccharide-activated macrophages (Kondo et al., 1993b), the release of histamine from rat mast cells induced by various stimuli (Teh et al., 1988), the secretion of tumor necrosis factor-α (TNF-α) by activated monocytes (Ferrante et al., 1990) and the bacterial endotoxin- or silica-induced activation of alveolar macrophages (Chen et al., 1997). In addition, we showed that tetrandrine could also inhibit cytokine secretion by activated macrophages, antibody production by activated B cells and the phagocytic activity of neutrophils (Chang et al., 1997). In animal models, tetrandrine was

shown to reduce the severity of adjuvant-induced arthritis in rats, to inhibit interleukin-1- or endotoxin-induced uveitis in rats, and to suppress lipopolysaccharide-induced fulminant hepatitis in mice (Whitehouse et al., 1994; Xiao and Chiou, 1996; Kondo et al., 1993a). The therapeutic effect of tetrandrine was also demonstrated in silicosis patients (Li et al., 1981). In the current study, through the determination of cell proliferation, cytokine production and cell surface molecule expression, we provided evidence that tetrandrine also exerted inhibitory effects on T cell activation. Interestingly, the immunosuppressive effect of tetrandrine was relatively specific because it only targeted the protein kinase C-dependent but not the Ca^{2+}-dependent signaling pathway (Figs. 3 and 4). There are at least 11 identified protein kinase C isoenzymes and most of them respond to phorbol ester stimulation (Newton, 1995). Whether tetrandrine targets one or several protein kinase C isoenzymes or their downstream signaling pathways is currently not known. It is a mystery why there are so many protein kinase C isoenzymes with similar substrate specificity and sensitivity to different activators, although different compartmentalization of the proteins may be an explanation (Mochly-Rosen and Gordon, 1998). It would help to unravel this mystery if tetrandrine were to specifically target one protein kinase C isoenzyme, as some compounds can do (Hofmann, 1997). Furthermore, since protein kinase C isoenzymes are extensively shared by many activation signaling pathways in a variety of immune and non-immune effector cells, our observations provide support for the broad-spectrum anti-inflammatory properties of tetrandrine (Nesic et al., 1997 and reviewed in Lu and Durkin, 1997; Hofmann, 1997).

4.2. Implication of tetrandrine-induced apoptosis in autoimmune diseases

Recently, tetrandrine has been shown to inhibit cell survival through the induction of apoptosis in human peripheral blood lymphocytes (Dong et al., 1997). Identical results were also obtained when human peripheral blood T cells were examined (Fig. 7). Since the apoptotic process is defective in autoimmune diseases (Nakajima et al., 1995; Mountz et al., 1994 and reviewed in Vaishnaw et al., 1997), tetrandrine-induced apoptosis in T cells may represent an additional way to control the population of activated T cells. Moreover, several Western anti-rheumatic drugs, such as corticosteroid, nonsteroidal anti-inflammatory drugs and hydroxychloroquine, and a Chinese anti-rheumatic drug, tripterygium wilfordii Hook f, have been shown to cause apoptosis (Cohen and Duke, 1984; Meng et al., 1997; Lu et al., 1995; Ho et al., 1998). These results suggest that apoptosis may represent a possible common mechanism for anti-rheumatic drugs. Finally, in conjunction with our observation for human peripheral blood T cells, the accumulated data support the notion that tetrandrine can be a potentially useful drug for the treatment of autoimmune diseases.

4.3. Therapeutic potential of the combination of tetrandrine and cyclosporin A

Another interesting finding is the observation of synergism between tetrandrine and cyclosporin A in the inhibition of T cell activation in human peripheral blood T cells. This observation further indicates that the cellular signaling pathway targeted by tetrandrine is different from that targeted by cyclosporin A, which inhibits Ca^{2+}-dependent calcineurin activity (Liu et al., 1991). Our finding also suggests that a combination of both tetrandrine and cyclosporin A may have great potential to treat diseases in which T cell activation is detrimental. Importantly, the concentration of cyclosporin A can be kept much lower and the general therapeutic effect can still be obtained by the addition of tetrandrine, which has fewer side effects (Li et al., 1981).

4.4. Protein kinase C-inhibitory effect and Ca^{2+} channel blocking effect of tetrandrine are independent mechanisms

It is possible that the inhibition of protein kinase C activity by tetrandrine is secondary to its Ca^{2+} channel blocking effect. However, our results and the accumulated findings in the literature do not support this assumption. Firstly, we showed that, compared to both tetrandrine and berbamine which were equipotent, hernandezine had a more potent protein kinase C-inhibitory effect (Fig. 6A and B). In contrast to their effects on protein kinase C activity, hernandezine is equipotent to tetrandrine but much more potent than berbamine as a Ca^{2+} channel blocker in HL-60 cells (Leung et al., 1994, 1996). Similar results were also obtained when vascular endothelial cells were examined (Low et al., 1996). Secondly, with rat glioma C6 cells, Imoto et al. (1997) showed that tetrandrine is 10-fold more potent than hernandezine in inhibiting the increase in intracellular Ca^{2+} induced by bombesin or tharsigargin, a microsomal Ca^{2+}-ATPase inhibitor. Thirdly, in human leukemic T cell line Jurkat, 100 μM tetrandrine does not inhibit the anti-CD3 monoclonal antibody-induced increase in intracellular Ca^{2+} concentration (Takemura et al., 1996). We also showed that tetrandrine did not inhibit the ionomycin-induced expression of CD40 ligand and CD69 (Fig. 3A and Fig. 4B). Finally, the induction of interleukin-2 receptor alpha chain expression is not associated with Ca^{2+} influx and can be induced by protein kinase C activation alone (Depper et al., 1984; Mills et al., 1985) (Figs. 3 and 6). The activation of protein kinase C, presumably the βI isoenzyme, by phorbol ester results in a reduction of Ca^{2+} influx in Jurkat T cells (Haverstick et al., 1997). This effect is similar to that of tetrandrine, which mainly blocks the entry of Ca^{2+} into cells (Leung et al., 1994; Xuan et al., 1996; Low et al., 1996). Taken together, these findings suggest that the protein kinase C-inhibitory effects of tetrandrine and its analogues may not be associated with their Ca^{2+}-antagonistic effects.

4.5. Methoxyl group at the R2 position might be critical for protein kinase C-inhibitory potency

Based upon the structural similarity among tetrandrine, hernandezine and berbamine, we further demonstrated that the methoxyl group at the R2 position of these drugs might be very important in determining their different protein kinase C-inhibitory activities (Figs. 1 and 6A and B). To our knowledge, so far, there has been no report comparing the immunosuppressive activity of these three drugs in the same assay system. This is the first report showing that, in human peripheral blood T cells, hernandezine appeared to have the most potent protein kinase C-inhibitory effect among the drugs tested. Regarding tetrandrine and berbamine, the published reports seem to indicate that the immunosuppressive effect of tetrandrine is similar or slightly stronger than that of berbamine. For example, both tetrandrine and berbamine are equally potent in inhibiting leukocyte infiltration into an air pouch induced by interleukin-1 and tumor necrosis factor-α stimulation (Wong et al., 1992). Tetrandrine but not berbamine inhibits the chronic inflammation of adjuvant-induced arthritis in rats (Whitehouse et al., 1994). Nevertheless, based upon limited information, it may be too naive to draw any conclusion on the relationship between the structure and the immunosuppressive activity of these drugs. We are currently investigating this issue with more tetrandrine analogues and comparing their effects with several in vitro and in vivo assays. It is possible that modulation of the structure of tetrandrine and of its analogues may enhance their immunosuppressive activity, Ca^{2+}-antagonistic potency or reduce side effects.

4.6. Important questions remain to be answered

Since the protein kinase C-inhibitory effects of tetrandrine and its analogues were measured in a large pool of CD3 + T cells, several concerns are raised. For example, do tetrandrine and its analogues have a preferential inhibitory effect on certain T cell populations such as CD45RO + memory T cells, CD45RA + naive T cells, CD4 + T cells or CD8 + T cells? As indicated by Schwinzer and Siefken (1996), both CD45RO + and CD45RA + T cells may have different susceptibility to the inhibitory effect of cyclosporin A. In addition, commercially available tetrandrine contains 2% impurities and the biological activity of the impurities has not yet been studied. At present, one of the most important issues will be to investigate whether tetrandrine and its analogues specifically target one protein kinase C isoenzyme. Moreover, whether the inhibitory effect of tetrandrine is mediated by direct interaction with protein kinase C isoenzyme or through suppression of its downstream signaling molecules has to be determined. These questions are being investigated in our laboratory.

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