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# Immunomodulatory activity of a chymotrypsin inhibitor from *Momordica cochinchinensis* seeds

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**Abstract:** Serine protease inhibitors are widely distributed in the plant kingdom. Many of them have been purified and characterized from different species. While the physicochemical properties of these protease inhibitors have been extensively investigated, their biological effects, e.g. immunomodulatory effect, remain relatively unexplored. Recently, we isolated a chymotrypsin-specific inhibitor (MCoCI) from the seeds of *Momordica cochinchinensis* (Lour) Spreng (Family Cucurbitaceae), the traditional Chinese medicine known as *Mubiezhi*, which has been used as an antiinflammatory agent. In the present study, the effects of MCoCI on different types of cells of the immune system, including splenocytes, splenic lymphocytes, neutrophils, bone marrow cells and macrophages, were investigated. MCoCI was shown to possess immuno-enhancing and antiinflammatory effects. MCoCI could stimulate the proliferation of different cells of the immune system, e.g. splenocytes, splenic lymphocytes and bone marrow cells, in a manner comparable to that of Concanavalin A. Moreover, MCoCI could also suppress the formation of hydrogen peroxide in neutrophils and macrophages. These immunomodulatory effects may explain some of the therapeutic actions of Mubiezhi. 

**Keywords:** antiinflammatory; chymotrypsin inhibitor; immunomodulatory; *Momordica cochinchinensis*

## INTRODUCTION

Immunomodulation can be defined as any type of therapeutic intervention aimed to restore the normal function of the immune system. Immunomodulators can have either a positive (immunostimulation) or a negative (immunosuppression) effect on a specific immune function. The clinical use of immunomodulators includes reconstitution of immune deficiency and suppression of normal or excessive immune functions in the treatment of graft rejection or autoimmune diseases [1,2].

There is worldwide interest in the search for drugs with immunomodulatory effects. Since a number of proteases are involved in immune action, its inhibitors represent a potential source of immunomodulatory agents. *N*-Tosyllysine chloromethyl ketone (TLCK), a serine protease inhibitor, can diminish phytohemagglutinin (PHA)-induced lymphocyte stimulation and interleukin-2 (IL-2) production, which are responsible for different stages of cell growth [3]. For neutrophils, proteolytic activation is thought to play a major role in unwanted tissue destruction, such as in emphysema. Consequently, numerous medical investigations have been carried out to search for protease inhibitors that can antagonize such protease-associated pathological conditions. In this regard, it is noted that the human secretory leukocyte protease inhibitor (SLPI) can inhibit the activity of the proinflammatory serine proteases elastase and cathepsin G [4].

SLPI may also affect the functioning of macrophages. Macrophages transfected with the SLPI gene could resist the lipopolysaccharide (LPS)-induced activation of NF-$\kappa$B, causing a decrease in the production of tumor necrosis factor (TNF)-$\alpha$ and nitric oxide, suggesting that SLPI has antiinflammatory activities [5]. However, a recent report showed that the LPS-hyporesponsiveness of macrophages is not due to the overexpression of SLPI [6]. Neutral protease has also been shown to play an important role in the random migration of inflammatory macrophages [7].

The Chinese medicinal material, Mubiezhi, is the seed of *Momordica cochinchinensis* (Family Cucurbitaceae). It has a long history of use for its antiinflammatory activities [8]. Recently, we have isolated a chymotrypsin-specific protease inhibitor MCoCI from this traditional Chinese medicine [9] and demonstrated that it possesses antioxidant properties [10]. As there is a close relationship between protease inhibitors and the immune system, it would be of interest to investigate whether MCoCI has any effect on the different types of cells of the immune system, which may account for the antiinflammatory activities of Mubiezhi from which it is isolated.

## MATERIALS AND METHODS

### Materials

BALB/c mice (6-week old) and blood samples were obtained from the Laboratory Animal Services Centre at the Chinese University of Hong Kong. Cell culture materials including

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fetal calf serum (FCS), L-glutamine, RPMI-1640 medium, penicillin and streptomycin sulfate were purchased from Invitrogen. Concanavalin A (Con A), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), LPS, soybean trypsin inhibitor (SBTI) and horseradish peroxidase were purchased from Sigma Chemical Company. [$^3$H-Methyl]-thymidine ($^3$H-TdR) was purchased from Amersham Biosciences.

## Purification of MCoCI from the Seeds of *Momordica cochinchinensis*

The seeds of *M. cochinchinensis* (Lour) Spreng were purchased from a local vendor. Decoated seeds (100 g) were cut into small pieces, soaked overnight in 3 M NaCl, 50 mM Tris-HCl, pH 8.0 at 4 °C, homogenized and then centrifuged at 12 000 g for 1 h at 4 °C. The clear supernatant was dialyzed (MWCO: 1000) against the extraction buffer overnight and then loaded onto a chymotrypsin-Sepharose 4B column (15 × 150 mm) equilibrated with the same buffer at a flow rate of 0.8 ml/min. After washing, the bound proteins were eluted with 0.05 M HCl. Fractions with chymotrypsin-inhibitory activity were pooled, dialyzed against Milli-Q water, and lyophilized. The samples were then ready for fractionation by reversed-phase HPLC using a Prep Nova-Pack HR C18 HPLC column (19 × 300 mm) equilibrated with 0.1% trifluoroacetic acid in 12% acetonitrile. Elution was carried out with two successive linear gradients of acetonitrile, from 12 to 27% in 10 min and then 27 to 39% in 90 min, at a flow rate of 3 ml/min. The peak eluted at 33% showed potent chymotrypsin-inhibitory activity. The homogeneity of this preparation was confirmed by the presence of a single protein band in SDS-PAGE using the tricine buffer system. The molecular mass of MCoCI, determined by mass spectrometry, was 7514 daltons. Amino acid sequencing of a peptide fragment of MCoCI generated by digestion with lysyl endopeptidase revealed a sequence of 23 amino acids. Comparison of this sequence and the molecular mass with those of other protease inhibitors suggests that MCoCI belongs to the potato I inhibitor family [9].

## Isolation of Different types of Cells of the Immune System

The different types of cells of the immune system, including splenocytes, splenic lymphocytes, bone marrow cells, neutrophils and macrophages, were isolated according to Chu and Ng [11].

*Splenocytes*: After the mice were sacrificed by cervical dislocation, the spleens were removed aseptically. They were then cut into small pieces and pressed gently through a 200-gauge stainless steel sieve. After washing with RPMI medium, low-speed centrifugation (100 g for 30 s) was carried out to remove adipose tissue and connective tissue. The cell suspension was further centrifuged twice at 400 g for 5 min and any observable white debris embedded in the cell pellet was removed. The resulting cell pellet was resuspended in RPMI medium supplemented with 10% FCS.

*Splenic lymphocytes*: The suspension of splenocytes was slowly layered onto the surface of an equal volume of Ficoll-paque gradient. The mixture was then centrifuged at 800 g for 20 min at 20 °C. Viable lymphocytes were collected at the interface between the two layers. The cells were then washed twice with plain RPMI medium and resuspended in RPMI complete medium.

*Bone marrow cells*: The femurs of BALB/c mice were removed and put into a Petri dish containing cold RPMI complete medium. The marrow plugs were flushed with RPMI complete medium using a 2-ml syringe with a 25 gauge needle. The cells were spun down at 400 g for 5 min and the red cells were removed by Ficoll-paque gradient centrifugation. The bone marrow cells were washed twice with plain RPMI medium and resuspended in complete RPMI medium.

*Neutrophils*: Pellet cells from the plasma were prepared by centrifugation at 250 *g* for 10 min at 4 °C. The pellet cells were washed twice and resuspended in a balanced salt solution (BSS) containing 137 mM NaCl, 5 mM KCl, 8.5 mM $Na_2HPO_4$, 0.8 mM $MgSO_4$ and 5 mM glucose, pH 7.4. About one-fifth volume of the cell suspension of Ficoll-paque solution was introduced beneath the cell suspension. After centrifugation at 1400 rpm for 40 min the top layer in the centrifugation tube was aspirated leaving the neutrophils/red blood cell pellet. To remove residual red blood cells, the cells were subjected to hypotonic lysis by resuspending the pellet in cold 0.2% NaCl for 30 s. Then the cells were resuspended and centrifuged until the cell pellet appeared to be free of red blood cells.

*Macrophages*: Three days after thioglycollate injections, macrophages from the mice were obtained by peritoneal lavage with Hanks' balanced salt solution (HBSS) containing 10 mM Hepes (pH 7.4) and 3.5 mM $NaHCO_3$. The cells were washed once with HBSS and suspended in RPMI medium containing 2 mM glutamine, 12.5 units/ml penicillin, 6.5 mg/ml streptomycin and 5% FCS. The cells were plated at a density of $4 \times 10^5$ cells/well into a 96-well plate and incubated for 2 h at 37 °C in a humidified $CO_2$ incubator.

## Determination of Cell Proliferation

The cells were plated in flat-bottomed 96-well culture plates at a density of $2 \times 10^5$ cells/well in RPMI medium. After incubation with MCoCI or Con A, the cells in each well were pulsed with 0.5 μCi $^3$H-TdR and then harvested 8 h later. The radioactivity incorporated was determined by counting in a liquid scintillation counter [11].

## Determination of $H_2O_2$ Formation

The cells ($4 \times 10^4$) suspended in BSS were stimulated with 10 μg/ml LPS for 6 h. After preincubation, the cells were washed twice with phosphate buffered saline (PBS). Different concentrations (0–100 μg/ml) of MCoCI, aprotinin or SBTI were added and then incubated together with 25 nM phenol red and 50 μg/ml horseradish peroxidase at 37 °C for 2 h. The amount of oxidized phenol red, which is a measure of $H_2O_2$ produced by the cells, was determined by measuring the absorbance at 590 nm.

## Assay of Interleukin-2

IL-2 activity was assayed by the ability of the culture medium to maintain the lymphoproliferation of Con A blast [12]. Con A blast was prepared from a 3-day culture of BALB/c splenocytes ($10^7$) in 10 ml RPMI medium supplemented with 10% FCS; 1 μg/ml Con A in a 25 cm$^2$ culture flask. Con A blast thus obtained was freed from residual Con A by

washing it twice with the medium. Viable cells were suspended at $4 \times 10^5$ cells/ml. Fifty microliters of the suspension of blast cells were then added to the 96-well flat-bottomed microtiter plates containing 50 µl of different concentrations (0–10 µg/ml) of MCoCI. The plate was incubated for 16–24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$ in air. The cells in each well were pulsed with 0.5 µCi $^3$H-TdR and then harvested 8 h later. The radioactivity incorporated was determined by counting in a liquid scintillation counter.

### Determination of Cell Viability

The cells were seeded onto a 96-well plate. After incubation with different concentrations (0–50 µg/ml) of MCoCI, the cells were washed with PBS twice and 400 µl of diluted MTT solution (0.5 mg/ml in serum-free RPMI medium) was added to each well. The formazan crystals formed in active metabolic cells were extracted with 400 µl 10% sodium dodecyl sulfate in 10 mM HCl after 2 h of incubation at 37 °C. The absorbance was determined at 540 nm.

### Statistical Analysis

Results obtained were expressed as mean ± SD ($n = 3$). Statistical analysis was performed by Student's $t$-test with one-way analysis of variance. Significant difference was taken as $p < 0.05$.

## RESULTS

### Effect of MCoCI on Splenocytes

MCoCI stimulated the proliferation of splenocytes by 44% at a concentration of 100 µg/ml. On the other hand, Con A, a well-known mitogen for splenocytes, enhanced the proliferation of splenocytes by 2.7-fold at a concentration of 25 µg/ml. The effect decreased slightly at higher Con A concentrations (Figure 1(A)). As shown in Figure 1(B), MCoCI increased the production of IL-2 in splenocytes. The effect was more potent than that of Con A. At a concentration of 2.5 µg/ml, IL-2 production was increased by 3.2-fold and 2.3-fold for MCoCI and Con A, respectively.

### Effect of MCoCI on Lymphocytes

MCoCI exerted a positive effect on the proliferation of lymphocytes. The maximal stimulation of lymphocyte growth by MCoCI was comparable to that induced by Con A, although the potency was lower (Figure 2(A)). For Con A, maximal stimulation on growth was observed at 2 µg/ml while 20 µg/ml of MCoCI was needed for maximal stimulation. Beyond the optimal concentration, the effect of growth stimulation for both MCoCI and Con A gradually diminished and returned to the control level. Similar to the effect on splenocytes, MCoCI also brought about an increase in IL-2 production, with a 4.9-fold increase at 10 µg/ml (Figure 2(B)).

### Effect of MCoCI on Bone Marrow Cells

MCoCI significantly stimulated the proliferation of murine bone marrow cells. Maximal stimulation was seen at a concentration of 1.5 µg/ml MCoCI. The effect declined with a further increase in MCoCI concentration. MCoCI was comparable to Con A in both the maximal stimulatory effect as well as the potency (Figure 3).

### Effect of MCoCI on Neutrophils

MCoCI exerted 82% inhibition on $H_2O_2$ formation after activation of neutrophils by LPS. The effect of MCoCI

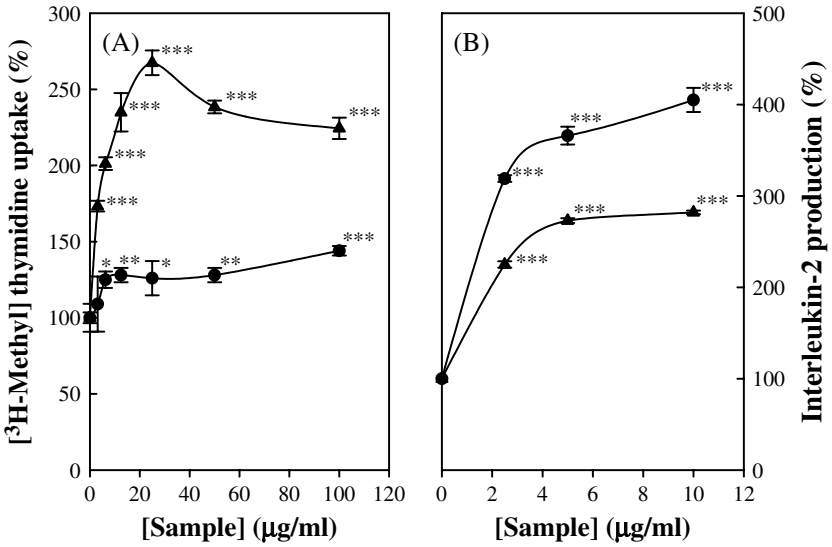


**Figure 1** Effects of MCoCI and Con A on splenocytes. BALB/c splenocytes ($2 \times 10^5$ cells/well) were incubated with various concentrations (0–100 µg/ml for cell proliferation experiment and 0–10 µg/ml for IL-2 production experiment) of MCoCI (●) or Con A (▲) at 37 °C for 48 h. (A) Cell proliferation. Cultures were pulsed with 0.5 µCi of $^3$H-TdR for 8 h before harvest. (B) IL-2 production. Each value represents mean ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, when compared with the control.

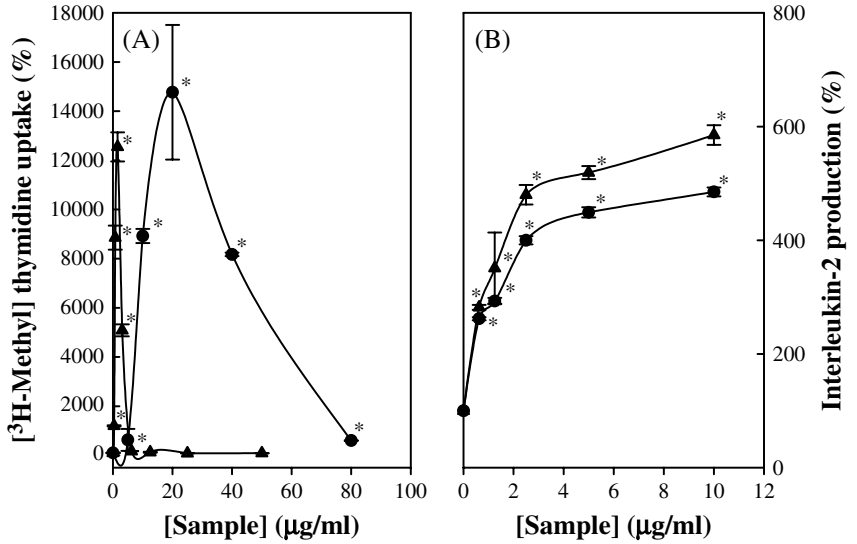


**Figure 2** Effects of MCoCI and Con A on lymphocytes. BALB/c lymphocytes ($2 \times 10^5$ cells/well) were incubated with various concentrations of MCoCI (●, 0–80 μg/ml for cell proliferation experiment and 0–10 μg/ml for IL-2 production experiment) or Con A (▲, 0–50 μg/ml for cell proliferation experiment and 0–10 μg/ml for IL-2 production experiment) at 37 °C for 48 h. (A) Cell proliferation. Cultures were pulsed with 0.5 μCi of $^3$H-TdR for 8 h before harvest. (B) IL-2 production. Each value represents mean ± SD ($n = 3$). $^*p < 0.005$, when compared with the control.

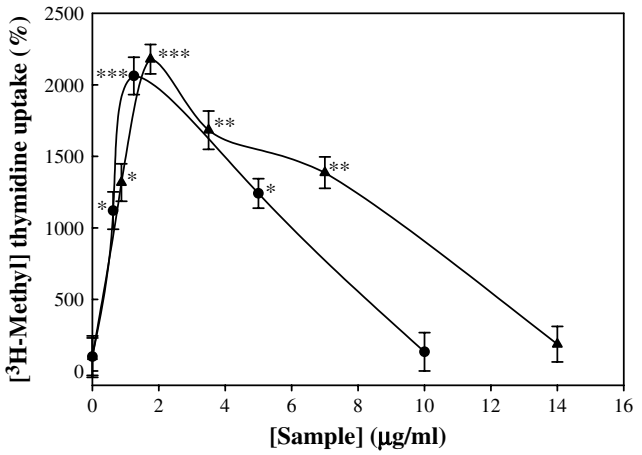


**Figure 3** Effects of MCoCI and Con A on proliferation of bone marrow cells. BALB/c bone marrow cells ($2 \times 10^5$ cells/well) were incubated with various concentrations of MCoCI (●, 0–10 μg/ml) or Con A (▲, 0–14 μg/ml) at 37 °C for 48 h. Cultures were then pulsed with 0.5 μCi of $^3$H-TdR for 8 h before harvest. Each value represents mean ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, when compared with the control.

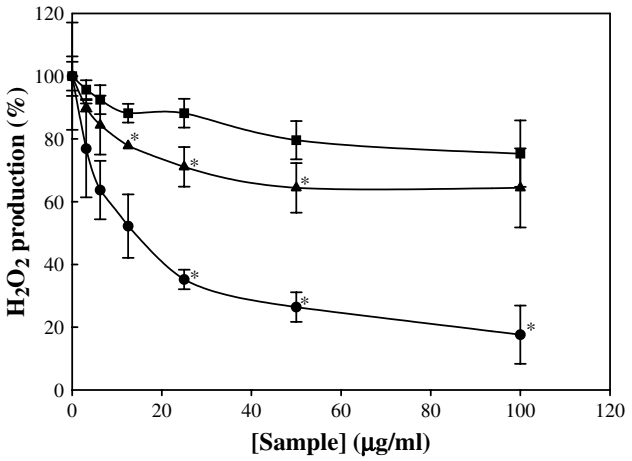


**Figure 4** Effects of protease inhibitors on $H_2O_2$ production in neutrophils. Suspensions of neutrophils ($4 \times 10^4$ cells) were seeded to a 96-well plate and were preincubated with 10 μg/ml LPS for 6 h. After the preincubation, cells were washed twice with PBS. Different concentrations (0–100 μg/ml) of MCoCI (●), aprotinin (▲) and SBTI (■) were added to the cells and incubated with 25 nM phenol red and 50 μg/ml horseradish peroxidase at 37 °C for 2 h. The absorbance was determined at 590 nm. Each value represents mean ± SD ($n = 3$). $^*p < 0.05$, when compared with the control.

was more potent than those of the protease inhibitors, aprotinin and SBTI, which caused a maximal inhibition of only 36% and 25%, respectively (Figure 4).

### Effect of MCoCI on Macrophages

MCoCI stimulated the growth of macrophages by about 39% at 12.5 μg/ml of MCoCI (Figure 5(A)). Similar to the case of neutrophils, MCoCI inhibited LPS-induced $H_2O_2$ production in macrophages in a dose-dependent manner. It showed about 58% inhibition on $H_2O_2$ production at a concentration of 100 μg/ml (Figure 5(B)).

## DISCUSSION

In the search for new immunomodulatory agents over the past few years, many traditional herbal medicines

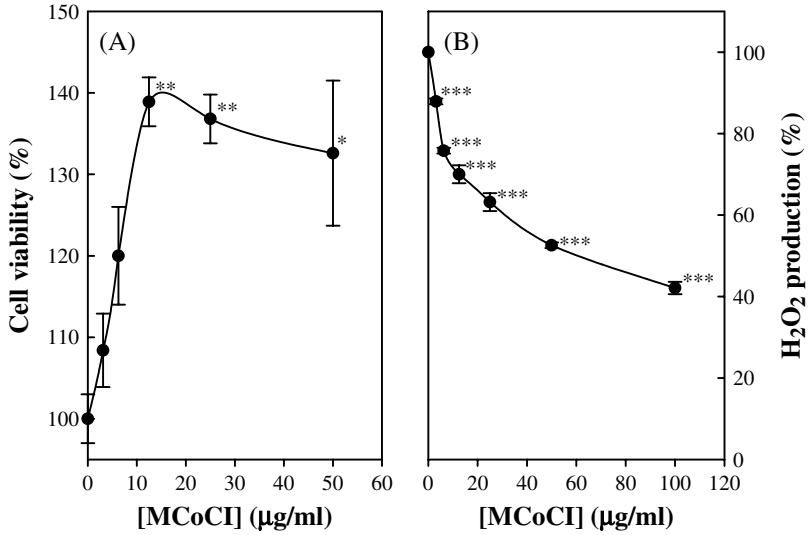


**Figure 5** Effect of MCoCI on macrophages. (A) Cell viability. Mouse resident peritoneal macrophages ($4 \times 10^4$ cells) were seeded to a 96-well plate and incubated for 48 h with different concentrations (0–50 μg/ml) of MCoCI. After the incubation, cell viability was evaluated by the MTT assay. (B) $H_2O_2$ production. Mouse resident peritoneal macrophages ($4 \times 10^4$ cells) were seeded to a 96-well plate and were preincubated with 10 μg/ml LPS for 6 h. Following the preincubation, cells were washed twice with PBS. Different concentrations (0–100 μg/ml) of MCoCI were added to the cells and incubated with phenol red and horseradish peroxidase at 37 °C for 2 h. The absorbance was determined at 590 nm. Each value represents mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, when compared with the control.

have been evaluated. Traditional medicines that are used as tonics are thought to nourish and strengthen the defense mechanisms of the host. Although many reports show that herbal extracts can enhance various types of immune responses [13,14], it is not clear how they work, particularly the physiological effects of the individual components in the crude extract.

The seeds of *M. cochinchinensis* (family Cucurbitaceae) are used as a crude extract for their anti-inflammatory effects in traditional medicine [8]. The seeds of *M. cochinchinensis*, like those from other Cucurbitaceous species, are rich in serine protease inhibitors, e.g. trypsin inhibitors and elastase inhibitors [15–17]. Moreover, a strong chymotrypsin-inhibitory activity has also been detected, which is virtually unknown in other plants of the same family [9]. As the interaction between proteases and protease inhibitors plays an important role in the proper functioning of the immune system [18,19], it is of interest to determine whether the chymotrypsin-specific inhibitor MCoCI purified from the seeds of *M. cochinchinensis* is responsible for at least a part of the antiinflammatory effects of the herbal medicine.

MCoCI exerted a mitogenic effect on splenocytes, albeit with a lower potency than that of the bifunctional trypsin-chymotrypsin inhibitor isolated from broad bean by Ye *et al.* [20]. Mitogenic activity on splenocytes is generally accompanied by the induction of cytokines, e.g. IL-2 [21]. MCoCI also induced the formation of IL-2 in splenocytes. However, it is obvious that the proliferation of splenocytes was not entirely due to the effect of IL-2 as the increase in the proliferation of splenocytes by MCoCI was much lower than that of Con A, though the increase in the production of IL-2 by MCoCI was higher.

Both MCoCI and Con A exhibited a biphasic effect on the growth of lymphocytes. The dose-response curve showed that while the maximal stimulation was similar, MCoCI was less potent and a higher concentration was needed to obtain maximal stimulation. Little information is available in the literature about the effect of protease inhibitors on lymphocyte proliferation and cytokine production. IL-2 is one of the cytokines for lymphocyte activation [22]. The present results showed that MCoCI could stimulate the production of IL-2 in lymphocytes, similar to that in splenocytes, in a dose-dependent manner, suggesting that MCoCI plays a role in the signaling pathway.

MCoCI stimulated the proliferation of bone marrow cells and the pattern of stimulation was similar to that in lymphocytes, i.e. growth stimulation occurred at low concentrations of MCoCI while the stimulating effect gradually disappeared at higher concentrations. Several protease inhibitors have been shown to act on bone marrow cells. For example, specific cysteine protease inhibitors could prevent bone metastasis [23]. Different metalloproteinases were found in murine cartilages and the tissue metalloproteinase inhibitors could be used to prevent the degradation of cartilages [19]. On the other hand, there have been no reports on the effect of serine protease inhibitors on bone marrow cells.

The activation of neutrophils is accompanied by the secretion of neutrophil enzymes like proteases [24] and the production of $H_2O_2$, which may exert an adverse

effect on cells. The interaction between the proteases and their inhibitors may result in the fine modulation of the physiological functions of neutrophils, namely, the cytotoxic function against external invasion. MCoCI was found to inhibit the production of $H_2O_2$ in neutrophils. Its action was more potent than that of aprotinin and SBTI, consistent with the findings of Kitagawa *et al.* [25] that chymotrypsin-specific inhibitors purified from legumes were more effective than the trypsin inhibitors.

MCoCI increased the proliferation of macrophages. Such results agreed with those of Abate and Schroder [26] who found that the serine protease inhibitors TLCK and *N*-tosylphenylalanine chloromethyl ketone (TPCK) could protect against the growth suppression effect induced by the cytotoxic LPS in macrophages. Similarly, Zhu *et al.* [27] have also shown that the response to LPS could be blocked by transfection of SLPI into macrophages. Like the case in neutrophils, MCoCI also inhibited $H_2O_2$ production in macrophages, though the effectiveness was slightly lower.

In conclusion, MCoCI showed an immunostimulatory effect on the proliferation of splenocytes, lymphocytes and bone marrow cells. It was involved in the differentiation of splenocytes and lymphocytes as indicated by the increase in the level of IL-2. MCoCI also exhibited an antiinflammatory activity and removed the reactive species in cells. It inhibited the production of $H_2O_2$ in both neutrophils and macrophages. Neutrophils generally adhere to the vascular endothelium and migrate to the site of infection, leading to ingestion and killing of the pathogen while macrophages play critical, accessory, inflammatory and effector roles in the nonseptic T-cell-mediated inflammatory response [28–30]. These immunostimulatory and antiinflammatory effects of MCoCI may explain some of the therapeutic actions of Mubiezhi, the herbal medicine from which it was isolated.

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