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Effect of Biphenyl Dimethyl Dicarboxylate on the Immunosuppression of Ketokonazole

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비페닐 디메칠 디카르복실레이트가 케토코나졸의 면역억제에 미치는 영향

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Ketoconazole is an imidazole antifungal agent which inhibits the biosynthesis of fungal cell-membrane ergosterol and has immunosuppressive properties *in vitro*. Biphenyl dimethyl dicarboxylate (PMC) has been utilized for antioxidative action and for liver-protective purposes. Studies were undertaken to investigate effects of biphenyl dimethyl dicarboxylate (PMC) on the immunosuppression of ketoconazole in ICR mice. In the combination of PMC and ketoconazole, as compared with the treatment of ketoconazole alone, there were significant increases in activities of natural killer (NK) cells and phagocytes along with circulation leukocytes. The elevation of serum glutamic-pyruvic transaminase (S-GPT) and total protein levels caused by ketoconazole were reduced by the combination of PMC and ketoconazole. In addition, lower serum albumin and albumin/globulin (A/G) ratio were also increased to normal level.

Keywords—Biphenyl dimethyl dicarboxylate, Ketoconazole, Immunosuppression, Natural killer, Phagocytic activity

Ketoconazole is an imidazole antifungal agent which inhibits the biosynthesis of fungal cell-membrane ergosterol.[1] It is an orally active agent with a broadspectrum antifungal activity.[2] Nevertheless, it has been reported that adverse effects of this drug consist of anaphylaxis, gastrointestinal disturbances, thrombocytopenia, gynecomastia, and hepatitis.[3] Immunologically, ketoconazole has been shown to interfere with lymphocytic and phagocytic functions, neutrophil chemotaxis, random movement, deoxy glucose uptake, and hexomonophosphate shunt activity.[4] It has been further found that the systemic antifungal agents available for clinical use are usually immunotoxic.[5] Thus, it would be important to abrogate the deleterious effects of these antifungal agents using agents with a more physiological action as well as little adverse side-effects.

Biphenyl dimethyl dicarboxylate (PMC) is a substance derived from the synthesis of *Schizandra sp.* constituents which have been utilized for tonic and sedative purposes in traditional medicine for over 2,000 years, and recently for antioxidative action and for liver-protective purposes.[6] PMC has been shown to be effective in treating or preventing chronic hepatitis due to drug poisoning.[7] Clinical trials on chronic hepatitis virus of type B showed that PMC markedly improved impaired liver functions, as shown by elevated levels of glutamic-pyruvic transaminase (GPT), bilirubin, α-fetoprotein, and other symptoms of patients.[8]

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PMC was also found to protect mice against CCl_4-induced liver damage, and to increase hepatic microsomal cytochrome P-450 in rats.[9] It was found that PMC also was capable of stimulating serum antibody production after immunization with ovalbumin (OVA) in complete Freund's adjuvant in BALB/c mice. Further, it has been shown that PMC is a useful modulator of oral tolerance to OVA in C_3H/HeN and BALB/c mice.[10] Thus, it can be considered that PMC has the immunosuppressive effects of ketoconazole without adverse side-effects, suggesting that PMC modulates oral tolerance to OVA as well as enhances total serum immunoglobulin levels in experimental animals.

The present study was undertaken, therefore, to investigate the effects of PMC on the immunosuppression of oral ketoconazole in ICR mice.

Experimental

Experimental Animals

Male ICR mice, 6 weeks of age, weighing 20±2 g, were used. The experimental animals were housed individually in each cage and acclimatized for at least 7 days prior to use. The cages were maintained at 23±2°C and 50±5% of relative humidity throughout the whole experimental period. All experimental mice were fed with animal chows and tap water *ad libitum* but deprived of the animal chows for 16 hrs prior to sacrifice.

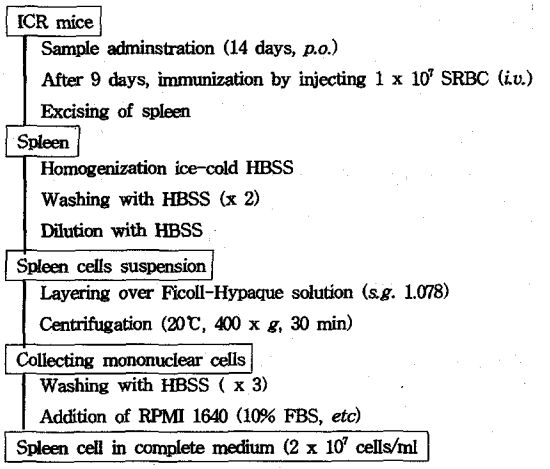
Materials and Treatments

PMC (Bimethyl-4,4′-dimethoxy-5,6,5,6′-dimethylene-dioxybiphenyl-2.2′-dicarboxylate) was supplied by Dong Kwang Pharmaceutical Co. (Korea) and suspended in 2% starch solution. PMC (6 mg/kg) was administered orally to mice daily for 14 consecutive days. Ketoconazole (Dong Kwang Pharmaceutical Co., Korea) was suspended in RPMI 1640 medium (Hybri-Max®) and also administered at 160 mg/kg orally, 2 hrs after the administration of PMC, daily for 14 consecutive days. Control animals received the appropriate vehicle only and were treated at the same time as the corresponding experimental animals.

Natural Killer (NK) Cell Activity

For preparation of effector cells, 5 mice from every experimental group were sacrificed, and 5 spleens were pooled in each petri-dish containing 20 m*l* cold Hanks' balanced salts (HBSS, Hybri-Max®). After washout twice with HBSS, spleen cell suspensions were prepared in cold HBSS by the gentle teasing of the organ with forceps, and passing it through nylon mesh to remove major tissue aggregates. After allowing the tissue debris to sediment for 5 minutes in an ice-bath, the cell suspensions in HBSS were layered on Ficoll-Hypaque solution (specific gravity 1.078) and centrifuged at 400×*g* for 30 minutes at 18-20°C. The mononuclear cell band was harvested and washed 3 times with HBSS. All cells were resuspended in complete medium to the desired concentration (2×10^7 cells/m*l*). Complete medium was RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum 100 unit penicillin/m*l*, 100 μg streptomycin/m*l*, and 2 mM glutamine (Scheme I).

For preparation of target cells, Yac-1 cell line,



Scheme I—Preparation of effector cells from ICR mice.

YAC-1 cell
Labelling with $Na_2{}^{51}CrO_4$ (1 mCi/ml) of viable cells
Incubation for 1 hr at 37℃ in 5% CO_2 incubator
Cell suspension
Washing with HBSS (10% FBS)
Labelled cell suspension in 10% FBS--HBSS (2 x10^5 cells/ml)

Scheme II—Preparation of target cell from YAC-I cell.

Effector cells/ target cells
(x 100)
Incubation 4 hrs at 37℃ in 5% CO_2 incubator
Centrifugation at 4℃, 500 x g, for 10 min.
Collecting the supernatant (100 μl)
Gamma counter

Scheme III—Procedure for natural killer cell activity assay.

a cell line of Moloney virus induced lymphoma of A/Sn origin, was used as target cells.

The target cells were labelled by incubating 2×10^5 cells in 1 m*l* medium with 100 μ Ci of $Na_2{}^{51}CrO_4$ (specific activity 283.58 m*l* Ci/m*l*, 1 mCi/m*l*: New England Nuclear) for 1 hr at 37℃ in CO_2 incubator. The labelled cells were washed three times with HBSS supplemented with 10% fetal bovine serum (10% FBS-HBSS) and adjusted to the desired concentration (2×10^5 cells/m*l*) (Scheme II).

NK cell assay was determined by the modified method of Kiesseling et al.,[11] as shown in Scheme III.

Desired concentrations of effector cells were mixed with labelled target cells in 1 m*l* per 96-well flat bottomed microplates in triplicate, and incubated for 4 hrs at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Most experiments were performed with effector to target ratios of 100 : 1. The tubes were centrifuged for 10 minutes $500\times g$ at 4℃ and 100 μ*l* of the supernatants were harvested from each well and were counted in an automatic gamma counter (Beckman, U.S.A.).

The percentage of released isotope was calculated by the following formula :

$$\%\ \text{specific}^{51}\text{Cr release} = \frac{\text{c.p.m. Experimental} - \text{c.p.m. SR}}{\text{c.p.m. MR} - \text{c.p.m. SR}} \times 100$$

Where spontaneous release (SR) was defined as the counts per minute (c.p.m.) released ^{51}Cr from targets incubated with medium alone, and maximal release (MR) was determined as the c.p.m. in the supernatants after lysis of target with 1% Triton X-100. MR was higher than 95% of total isotope uptake, and SR was less than 10%.

Assay of Phagocytic Activity

Phagocytic assay was determined by the modified method of Biozzi *et al.*[12] In brief, for the preparation of colloidal carbon solution, rotring ink was diluted 1/6 with 1% gelatin and kept in a stoppered tube at 37℃ during the experiment. In order to measure the phagocytic activity, separate groups of mice were challenged *via* the lateral tail vein by using a 1 m*l* syringe with 26 gauge needle at the dose of 0.01 m*l* of colloidal carbon solution per gram of mouse. At the interval of 10, 20 and 30 min, 20 μ*l* of blood sample was obtained from the retro-orbital venous plexus. The collected blood samples were expelled into each vial containing 2 m*l* of 0.1% sodium carbonate, and the contents were well mixed for the lysis of erythrocytes. The absorbance of the colloidal carbon contained in blood was measured with spectrophotometer (Varian, Cary 219) at 600 nm using water as blank. Ten times of density readings were converted into logarithmic scale and plotted against time. The slope of the line was called phagocytic coefficient K. The mice were killed and the weights of spleen and liver were measured. Corrected phagocytic index is a measure of phagocytic activity per unit weight of tissue.

$$\text{Corrected phagocytic index} = \frac{\text{body}_{wt.}}{\text{spleen}_{wt.} + \text{liver}_{wt.}} \times \sqrt[3]{K}$$

Count of Circulating Leukocyte

Blood was collected from the retro-orbital plexus of mice. Türk's solution (glacial acetic acid 1 m*l*, 1%-gentian violet 1 m*l* and distilled water to 100 m*l*) was used for the staining of leukocytes and lysis of unnucleated cells. The number of nucleated cells was counted in a hemacytometer chamber under a microscope. Triple counting per sample was carried out and the mean value of the results was calculated.

Serum Chemistries

Glutamic-pyruvic transaminase (S-GPT) levels were determined according to the method described by Reitman and Frankel.[13] Protein was ditermined by the method of Lowry *et al.*[14]

Statistical Analysis

The values were expressed as means±standard error (S. E.). All data were examined for their statistical significance of difference with Student's t-test.

Results

Effects of PMC on Natural Killer Cell Activity in Ketoconazole-treated Mice

The effects of PMC on natural killer (NK) cell activity in ketoconazole-treated mice are summarized in Table I. NK cell activity was significantly decreased in ketoconazole-treated mice, compared with those in controls (21.8±1.9). NK cell activity of PMC together with ketoconazole showed 19.8±1.7 ($p<0.05$) and the value was significantly increased as compared with those in ketoconazole-treated mice (13.16±2.0).

Effects of PMC on Phagocytic activity in Ketoconazole-treated Mice

The effects of PMC on phagocytic activity in ketoconazole-treated mice are summarized in Table I. Phagocytic activity was significantly decreased in ketoconazole-treated mice (4.14±0.19, $p<0.05$), compared with those in controls (5.28±0.46), but the decrease in phagocytic activity was restored by the combined administration of ketoconazole and PMC (5.01±0.25, $p<0.05$).

Effects of PMC on the Number of Circulating Leukocyte in Ketoconazole-treated Mice

The effects of PMC on the number of circulating leukocytes in ketoconazole-treated mice are summarized in Table I. The number of circulating leukocytes was significantly decreased in ketoconazole-treated mice (4,780±350, $p<0.05$), compared with those in controls (6,170±530). But the decrease in the number of circulating leukocytes administration of ketoconazole and PMC (7,600±650, $p<0.01$), as compared with those in ketoconazole-treated mice.

Effects of PMC on Serum Chemistries in

Table I— *The Effects of Biphenyl Dimethyl Dicarboxylate in Ketoconazole-treated Mice*

Group	% Specific lysis of ^{51}Cr-labelled targe cell[b] (Effector : Target Cell (100 : 1))	Corrected phagocytic index[c]	Number of circulating leukocyte[d] (/mm^3)
Control	21.8±1.9	5.28±0.46	6,170±530
Ketoconazole	13.1±2.0*[e]	4.14±0.19*	4,780±350*
Ketoconazole+PMC	19.8±1.7§[f]	5.01±0.25§	7,600±650§§
PMC[a]	22.3±2.8	5.07±0.32	7,720±800

Each value represents the mean±S. E. of 5 to 7 mice. a) Biphenyl dimethyl dicarboxylate, PMC, b) The % lysis was determined by a standard 4 hrs ^{51}Cr release assay and effector to target ratio was 100 : 1, c) Corrected phagocytic index is a constant obtained from a formula relating the cube root K to the ratio of body weight to the weights of the liver and spleen, d) Blood samples for measuring leukocytes in mice were collected from the retro-orbital plexus immediately before assay, e) Asterisks denote a significant difference (*; $p<0.05$) compared with the values in control mice, f) Section marks denote a significant difference between ketoconazole and ketoconazole plus PMC groups (§; $p<0.05$, §§; $p<0.01$).

Table II— *The Effects of Biphenyl Dimethyl Dicarboxylate on Serum Chemistry in Ketoconazole-treated Mice*

Group	GPT (IU/ml)	Albumin (g/dl)	Protein (g/dl)	A/G ratio
Control	38.2±3.3	4.18±0.33	6.98±0.13	1.49±0.08
Ketoconazole	64.8±5.9**[b)]	3.38±0.16*	8.04±0.36*	0.73±0.14**
Ketoconazole+PMC	38.0±5.7§§[c)]	4.02±0.17§	6.82±0.18§§	1.44±0.11§§
PMC[a)]	34.3±3.3	4.08±0.18	6.73±0.17	1.54±0.13

Each value represents the mean ± S. E. of 6 to 7 mice. a) Biphenyl dimethyl dicarboxylate, PMC, b) Asterisks denote a significant difference (*; $p<0.05$, **; $p<0.01$) compared with the values in control mice. c) Section marks denote a significant difference between ketoconazole and ketoconazole plus PMC groups (§; $p<0.05$, §§; $p<0.01$).

Ketoconazole-treated Mice

The effects of PMC on serum chemistries are shown in Table II. The elevated S-GPT and total protein levels were significantly reduced to normal limit by the combined administration of ketoconazole and PMC (*i.e.*, 38.0±5.7 IU/ml, $p<0.01$ as compared with 64.8±5.9 IU/ml in ketoconazole alone mice and 6.82±0.18 g/dl, $p<0.01$ as compared with 8.04±0.36 g/dl in ketoconazole alone, respectively). In addition, lower serum albumin and A/G ratio were also restored to normal limit.

Thus, PMC is thought to inhibit the induction of hepatitis due to other antifungal agents by blocking the release of hepatotoxic factors.

Discussion

Several investigators have commented on the potentially harmful effects of systemic antifungal agents with immunosuppressive properties, especially when they are used for long-term treatment of patients who are already immunocompromised by their medical condition.[15] In this regard, ketoconazole has been widely studied for its side-effects associated with immunological studies as well as clinical trials on antifungal activity.[16] On the contrary, PMC was found to protect against CCl_4-induced immunotoxicity in mice,[17] CCl_4, D-galactosamine and thioacetamide-induced liver injuries in mice or rat,[18] and to be effective in treating or preventing chronic hepatitis due to drug poisoning.[7] Thus, the objective of this study was to investigate the protective effects of PMC against the immunosuppression of ketoconazole. We selected the toxic dose of 160 mg/kg from the toxicological studies of ketoconazole, which had been previously reported by Lavrijsen *et al.*[19] On the other hand, PMC at 6 mg/kg is known to be nontoxic and quite effective in enhancing antibody production, without causing any adverse side-effects.[10]

It has been suggested that the enhancement of natural killer (NK) cell activity might play a role in providing resistance against tumor cell and viral activity *in vitro* and *in vivo*.[20] In addition, NK cells produce and secrete lymphokines, which may serve as a feedback mechanism to turn off antibody production of B lymphocytes.[21] In view of these reports and the data shown in Table I, it is thought that PMC may restore the reduction of NK cell activity in ketoconazole-treated mice by blocking the suppression of lymphokine production by ketoconazole.

Phagocytes, such as macropghaes and polymorphonuclear leukocytes, play a significant part in immunological function, imflammation, infection, autoimmune diseases, tumor necrosis, and other important biological responses. They are known to release many cytokines that play important roles in maintaining homeostasis. It was shown by Gergely *et al.*[22] that ketoconazole depressed host resistance mechanism *in vitro*. Our previous study of itraconazole, which is known to have even fewer side effects than ketoconazole, has found that it also significantly suppressed phagocytic activity and leukocyte counts in mice.[10]

In the present study, we have further shown that PMC combined with ketoconazole significantly enhanced phagocytic activity and circulating leukocyte counts it ketoconazole-immunosuppressed mice (Table I). Therefore, oral PMC is also likely to more strongly enhance the reticuloendothelial system (RES), including macrophages. The mechanism by which PMC enhances phagocytic activity is not yet known, but may be due in part to recovery from the inhibition of RES by ketoconazole.

In vivo studies were performed to determine whether PMC also would result in a significant recovery of the hepatotoxicity produced by ketoconazole. The basis of selecting PMC for these studies was previously published in a report by Xie *et al*,[18] in which they demonstrated that pretreatment of mice with PMC resulted in the abrogation of CCl_4-induced liver damage. The present study further shows that PMC combined with ketoconazole significantly decreased serum GPT levels and total protein in the serum elevated by ketoconazole alone. Serum albumin levels and A/G ratio as well as liver weight were also found to be significantly increased (Table II). Then, it is thought that PMC may prevent hepatic damage such as the degenerations of endoplasmic reticulum of liver cells by ketoconazole, as discussed the enhancement of RES associated with immune function through the inhibition of liver damage induced by CCl_4 in mice.[10]

The above findings suggest that the enhancing effect of PMC on the immune responses suppressed by ketoconazole may give some practical benefits for the prevention of immunobiological diseases by other antifungal agents. Therefore, it is desirable to prepare mixed medicine with ketoconazole and PMC. It is necessary, however, to study further the mechanism of action of PMC and also the differences according to animal species.

In conclusion, PMC showed significant restoration from the immunotoxic status induced by ketoconazole.

Conclusion

The present study was undertaken to investigate the effect of biphenyl dimethyl dicarboxylate (PMC) on the immunosuppression of ketoconazole in ICR mice.

PMC at dose of 6 mg/kg was administered orally to the mice daily for 14 consecutive days. Ketoconazole was suspended in RPMI 1640 medium and also orally administered at 160 mg/kg/day 2 hrs after the administration of PMC. Mice were immunized and challenged with sheep red blood cells (SRBC).

The results of the present study are summarized as follows:

In the combination of PMC and ketoconazole, as compared with the treatment of ketoconazole alone, there were significant increases in activities of natural killer (NK) cells and phagocytes along with circulation leukocytes.

The elevation of serum glutamic-pyruvic transaminase (S-GPT) and total protein levels caused by ketoconazole were reduced by the combination of PMC and ketoconazole. In addition, lower serum albumin and A/G ratio were also increased to normal level.

These findings indicate that PMC decreased against ketoconazole-induced immunotoxic status.

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