ENHANCEMENT BY UBENIMEX (BESTATIN) OF HOST RESISTANCE
TO *Candida albicans* INFECTION

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Ubenimex is a low molecular weight microbial metabolite which has been demonstrated to have antitumor and immunomodulatory activities. In this study, the protective effect of ubenimex on *Candida albicans* infection was investigated in normal and immunosuppressed mice. In normal mice, treatment with ubenimex at 0.5, 5 and 25 mg/kg for 5 days prior to infection prolonged survival time in a dose-dependent manner. In immunosuppressed mice treated with a single dose of cyclophosphamide 4 days prior to infection, ubenimex treatment at 5 mg/kg for 5 days significantly increased the number of survivors. Ubenimex-treated mice had a significant increase in number of peritoneal exudate cells with neutrophils as well as enhanced functions, including phagocytosis and active oxygen production. These results suggest the potential usefulness of ubenimex as a prophylactic agent for the management of patients with opportunistic fungal infections.

Ubenimex was found by Umezawa *et al.* to be an inhibitor of aminopeptidases in culture filtrates of *Streptomyces olivoreticuli*[1] and it can be chemically synthesized. We have undertaken a variety of immunopharmacological studies on ubenimex and demonstrated that it stimulates macrophages, T cells and bone marrow cells and has antitumor activity[2,3]. It would be seem, therefore, that ubenimex enhances the production and/or induction of various cytokines from immunocompetent cells and modulates the function of antitumor effectors such as macrophages, cytotoxic T cells and natural killer (NK) cells. Since ubenimex is a substance with low molecular weight, its pharmacological behavior has been examined in detail[4]. An autoradiographic study of tissue distribution of [³H]-ubenimex demonstrated that it is efficiently distributed in macrophages in lymphoid tissues and solid tumors in mice[5]. Biochemical and histochemical studies proved that one of the binding sites for ubenimex is aminopeptidase N (EC 3.4.11.12) which is located on the surface of mammalian cells[6]. It is well absorbed by oral administration, extremely low in toxicity and can be safely administered over a long period to humans. The efficacy and toxicity of ubenimex in humans has been evaluated by many researchers in well-controlled clinical trials[3]. Ota *et al.* demonstrated that ubenimex prolongs the duration of remission and survival time in adult non-lymphocytic leukemia patients who achieved a complete remission by induction and consolidation chemotherapy[7]. It was also observed through these clinical studies that ubenimex might be effective in protecting immunodeficient patients from opportunistic infections.

There are several papers on preclinical studies of ubenimex with experimental infections. HARADA et al. reported that ubenimex enhanced host resistance to Listeria monocytogenes infection in mice[8]. DICKNITE et al. demonstrated that it reduced bacterial persistence in experimental chronic Salmonella typhimurium infection in mice[9], and TANAKA et al. observed that it has a prophylactic effect on experimental pyelonephritis induced by Pseudomonas aeruginosa[10]. In viral infections, ubenimex enhanced production of antibody to Herpes simplex in mice[11] and increased the number of CD4$^+$ lymphocytes in acquired immunodificiency syndrome (AIDS) and AIDS-related complex patients[12]. Considering these biological response modifier (BRM) characteristics and the beneficial effect on bacterial and viral infections, ubenimex was expected to be effective against fungal infections. We therefore examined its effects on experimental Candida albicans infection in mice and we report here prevention of this infection and activation of murine neutrophils in vivo.

Materials and Methods

Animals
Specific pathogen-free inbred male C3H/HeN mice 7 week-old and female CD1 mice 4 week-old were obtained from Charles River Japan, Inc., Atsugi, Kanagawa. Mice were housed at a temperature of $25 \pm 2°C$ and a humidity of $55 \pm 7\%$, and fed standard mouse diet (NMFR, Oriental Yeast Co., Ltd., Tokyo) and water ad libitum.

Fungi
C. albicans TIMM 0239 and 1768 were maintained at the Research Center for Medical Mycology, Teikyo University. Fungi were grown in Sabouraud Dextrose broth containing 0.5% yeast extract overnight at 37°C in a shaking water bath. Cultures were then centrifuged for 5 minutes at 1,500 rpm and resulting C. albicans cells washed five times with physiological saline. The number of C. albicans cells was counted with a hemocytometer.

Agents
Ubenimex was prepared by Nippon Kayaku Co., Ltd., Tokyo. It was dissolved in physiological saline and filtered through a Millipore filter (0.22 μm). Limulus hemocyto lysate assay (E-toxate, Sigma Chemical Co., St. Louis, MO) detected no endotoxin. Cyclophosphamide was purchased from Shionogi Co., Ltd., Osaka, and dissolved in distilled water before use. OK-432 was a gift from Chugai Co., Ltd., Tokyo. Luminol solution was purchased from Nichi-on Co., Ltd., Chiba.

Systemic Infection of Mice
Normal mice were intravenously (iv) infected with 1×10^6 cells of C. albicans TIMM 1768. Immunosuppressed mice produced by intraperitoneal treatment with a single dose of 200 mg/kg of cyclophosphamide 4 days prior to infection were iv infected with 5×10^4 cells of C. albicans. Ubenimex was intraperitoneally (ip) administered daily for the 5 consecutive days preceding the infection.

Detection of C. albicans in Kidneys
At the end of the experimental period surviving mice were sacrificed, and kidneys dissected under sterile conditions. The vertically cut face of each kidney was pressed against the surface of Candida GS agar (1% peptone, 0.5% yeast extract, 4% glucose, 2% agar and 0.05% 5-nitro-2-furfurylidine-aminoguanide hydrochloride) plates that were then incubated for 48 hours at 37°C to observe colonial growth of C. albicans.

Induction of Peritoneal exudate cells (PEC)
C3H/HeN mice were ip treated with ubenimex daily for 4 days. Eighteen hours after the last

administration, each mouse ip received an injection of 2 ml of 8% casein sodium. Six hours later the PEC were obtained with 5 ml/mouse of DULBECCO's phosphate buffered saline (PBS).

Preparation of Murine Neutrophils
Purification of neutrophils was performed as described by MORIKAWA et al.[13]. Briefly, PEC were obtained as described above and resuspended in PBS after 5 minutes centrifugation at 1,500 rpm. The suspension was layered gently on 90% Ficoll-paque, and centrifuged for 30 minutes at 1,700 rpm. The sedimented cells in the tube were recovered and confirmed to consist of more than 95% neutrophils by microscopical estimation using Diff-quik (Green Cross Co., Ltd., Osaka) staining.

Phagocytosis of C. albicans Cells by Neutrophils
Murine neutrophils in PEC following ubenimex treatment were suspended in RPMI-1640 medium containing 10% fetal calf serum at a concentration of 4×10^6 cells/ml. Formalin-killed C. albicans TIMM 0239 cells were also suspended in the same medium at 2×10^7 cells/ml. The neutrophil suspension (500 μl), formalin-killed C. albicans cell suspension (500 μl), and normal mouse serum (50 μl) were mixed together in a centrifuge tube, and incubated for 30 minutes at 37°C. Aliquots of the incubation mixture (50 μl) were then stained with an equal volume of 0.4% trypan blue and 0.2% eosin Y in PBS[14]. Phagocytic activity was determined by measuring the total number of phagocytized C. albicans cells in 200 neutrophils.

Chemiluminescence Response
The chemiluminescence response of the neutrophils was measured as described by ALLEN et al.[15]. Briefly, neutrophils were suspended in 10 mM HEPES buffer (pH 7.4) containing 5 mM KCl, 145 mM NaCl, 5.5 mM glucose and 1 mM $CaCl_2$ at a concentration of 3×10^6 cells/ml just prior to measurement. Twenty-five μl of 10 KE/ml of OK432 was added to 100 μl of the neutrophil suspension, and the mixture preincubated for one minute at 37°C. Then 100 μl of 2×10^{-4} M luminol solution was added with a microsyringe, and chemiluminescence was measured by Picolite (Model 6100 PICO-LITE, Packard, Tokyo).

Statistics
Statistical analysis was carried out by Fisher exact test or STUDENT's t-test.

Results

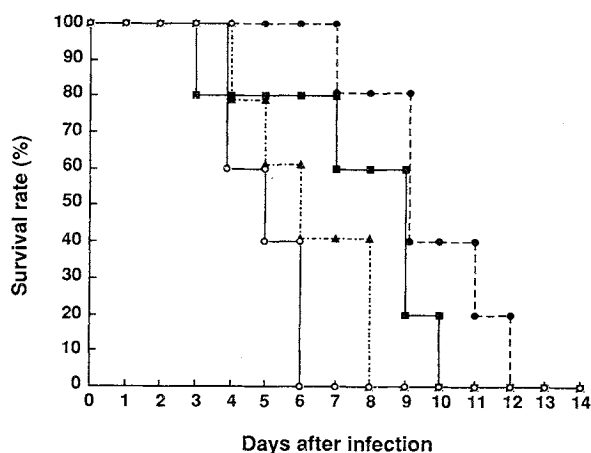### Prophylactic Effect of Ubenimex on C. albicans Infection in Normal Mice

The prophylactic effect on C. albicans infection was first examined in normal CD1 mice which were iv infected with 1×10^6 cells of C. albicans. All saline-treated mice died within 6 days after being infected. Treatment with 5 daily doses of ubenimex at 0.5, 5 or 25 mg/kg, however, prolonged the survival time in a dose-dependent manner as shown in Fig. 1, the most effective dose being 25 mg/kg.

Prophylactic Effect of Ubenimex on C. albicans Infection in Immunosuppressed Mice

Table 1 shows the results of experiments in which the prophylactic effect on C. albicans was examined in cyclophosphamide-induced immunosuppressed mice. The immunosuppressed mice died within 14 days following infection with 5×10^4 cells of C. albicans. Other mice were given ubenimex daily for 5 days prior to the infection and this treatment prolonged survival time; the number of animals surviving more than 14 days was: 4 out of 7 mice at 0.5 mg/kg, 4 out of 6 at 5 mg/kg and 1 out of 7 at 25 mg/kg. The surviving animals were then sacrificed and examined to determine whether C. albicans cells were still present in the kidneys. Two out of 4 mice at 0.5 mg/kg and 1 out of 4 at 5 mg/kg of ubenimex showed no detectable C. albicans in some kidney-stamps when examined 14 days after infection. This suggested that ubenimex was more effective against C. albicans infection in immunosuppressed mice than in normal mice at 5 mg/kg, but did not completely eliminate C. albicans.

Fig. 1.   Prophylactic effect of ubenimex on *Candida albicans* infection in normal mice.

Ubenimex 0.5 mg/kg (▲), 5 mg/kg (■), 25 mg/kg (●) or saline (○) was administered on day −5 to day −1.

Female CD1 mice (4 weeks, 5 animals/dose) were infected with 1×10^6 *C. albicans*/mouse on day 0.

Table 1.   Effect of ubenimex on *Candida albicans* infection in immunosuppressed mice.[a]

Treated with[b]		Survival on day 14 post-infection	Culture study with surviving mice[c] recovery of *C. albicans* from;	
			Both kidneys	One kidney
Saline		0/7	—	—
Ubenimex	0.5 mg/kg	4/7[d]	2/4	2/4
	5 mg/kg	4/6[d]	3/4	1/4
	25 mg/kg	1/7	1/1	0/1

[a]   Female CD1 mice (4 weeks, 7 animals/dose, except for 6 animals 5 mg/kg of ubenimex) were iv infected with 5×10^4 *C. albicans*/mouse on day 0. Cyclophosphamide (200 mg/kg) was injected ip on day −4.

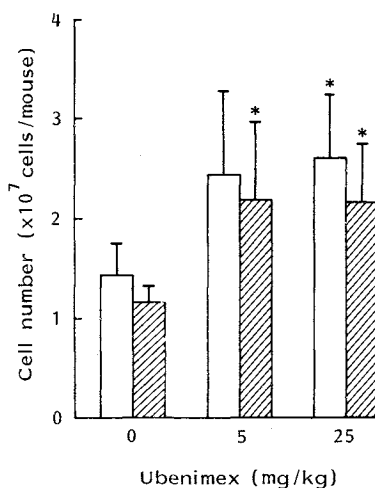[b]   Saline or ubenimex was ip administered on day −5 to −1.

[c]   Recovery of *C. albicans* from each kidney of the survivors was examined on day 14.

[d] $P < 0.05$, by Fisher exact test.

Fig. 2.   Increment by ubenimex of number of casein-induced neutrophils in the peritoneal cavity of normal mice.

C3H/HeN mice were administered ubenimex on day −4 to day −1 ($n = 5$). The number of casein-induced PEC (open) and the number of neutrophils in PEC (closed) were determined on day 0.

* $P < 0.05$, against saline-treated group by STUDENT's t-test.

Effect on Intraperitoneal Exudation of Neutrophils and Other Cells and Their Functions

The total number of PEC and the number of neutrophils in PEC were examined following ubenimex administration in mice with or without casein induction. In the absence of casein induction, the total number of PEC was about 5×10^6 cells/mouse and the proportion of neutrophils to whole PEC was about 17% in the saline-treated group. Ubenimex treatment of mice did not change these values.

Table 2. Phagocytic activity of neutrophils from ube-
nimex-pretreated normal mice.

Neutrophils from mice treated with[a]	% of C. albicans-phagocytizing neutrophils[b]	
	Mean ± SD	Index[c]
Saline	18.9 ± 3.2	100
Ubenimex 25 mg/kg	28.0 ± 7.5[d]	148

[a] C3H/HeN mice were administered saline or ubenimex
     daily for 4 days (n = 6).
[b] Formaline-killed C. albicans (2 × 10⁷ cells) and
     neutrophils (4 × 10⁶ cells) were incubated, and
     phagocytosis was determined by trypan blue and
     eosin Y staining.
[c] Relative value of C. albicans-phagocytizing neutro-
     phils to control.
[d] $P < 0.05$, by STUDENT's t-test.

Table 3. Enhancement by ubenimex of chemilumine-
scence response of PEC from cyclophosphamide-
treated mice.[a]

Neutrophils from mice treated with[b]	Counts per minute[c]	
	Mean ± SD	Index[d]
Saline	2,053 ± 223	100
Ubenimex 0.5 mg/kg	2,544 ± 476	124
5 mg/kg	3,026 ± 328[e]	147
25 mg/kg	3,059 ± 77[f]	149

[a] C3H/HeN mice were given cyclophosphamide
     (200 mg/kg, ip) on day −4.
[b] Mice were administered saline or ubenimex ip on day
     −4 to day −1 (n = 3).
[c] Chemiluminescence was measured as described in
     Materials and Methods.
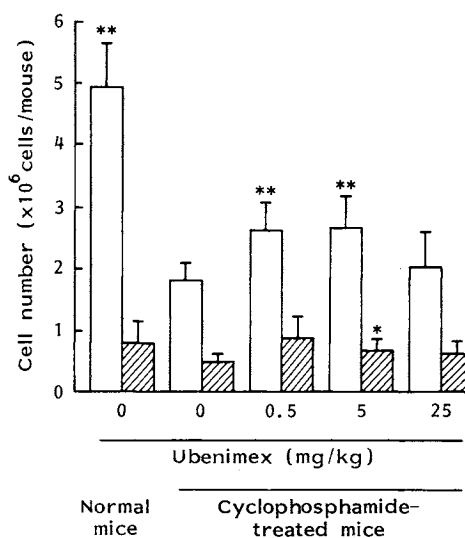[d] Relative value of chemiluminescence response to
     control.
[e] $P < 0.05$, by STUDENT's t-test.
[f] $P < 0.01$, by STUDENT's t-test.

Fig. 3. Effects of ubenimex on the number of
neutrophils in the peritoneal cavity of cyclophos-
phamide-treated mice.

Ubenimex (mg/kg)

Normal mice	Cyclophosphamide-treated mice

C3H/HeN mice were given cyclophosphamide
(200 mg/kg, ip) on day −4 and ubenimex was
administered on day −4 to day −1 (n = 9 for saline
group and 5 mg/kg, and n = 4 for group of 0.5 mg/kg
and 25 mg/kg). The number of PEC (open) and the
number of neutrophils in PEC (closed) were
determined on day 0.
    * $P < 0.05$, ** $P < 0.01$ against cyclophosphamide-
saline-treated group by STUDENT's t-test.

In casein-induced mice, both the number of PEC
and the proportion of neutrophils significantly
increased compared with the values for non-induced
mice. Ubenimex treatment enhanced the effect of
casein induction (Fig. 2). To study whether or not
ubenimex augments the function of neutrophils, the
effect on phagocytosis of C. albicans by neutrophils was also investigated. As shown in Table 2,
ubenimex significantly increased the number of phagocytizing neutrophils. With some other func-
tions of neutrophils, such as production of active oxygen and adhesion to a plastic surface, no
statistically significant difference was observed between the ubenimex-untreated and -treated groups of
normal mice (data not shown).

    Fig. 3 shows the result of experiments on the effect of ubenimex on neutrophil recovery following
treatment with an immunosuppressive dose of cyclophosphamide. The total number of PEC and the number
of neutrophils were determined without casein induction in ubenimex-treated mice. Cyclophosphamide
reduced the total number of PEC to $1.80 × 10^6$ cells/mouse. Under this experimental condition, ubenimex
treatment significantly diminished the reduction ($2.65 × 10^6$ cells/mouse). The number of neutrophils in the
PEC of ubenimex (5 mg/kg)-treated mice was also slightly but significantly larger than cyclophos-

phamide-treated control. Investigations of the ability of active oxygen production by PEC following cyclophosphamide treatment (Table 3) revealed that ubenimex treatment at doses of 5 and 25 mg/kg significantly enhanced active oxygen production by PEC obtained from cyclophosphamide-treated mice. These results indicate that the administration of ubenimex is effective not only in increasing the number of PEC but also in activating their functions.

Discussion

We demonstrated that ubenimex enhanced host resistance against *C. albicans* infection in both normal and immunosuppressed mice. To our knowledge this is the first report that ubenimex has protective activity against fungal infection *in vivo*. In normal mice ubenimex showed dose-dependent efficacy, namely, 25 mg/kg was more beneficial than 5 mg/kg. However, in immunosuppressed mice ubenimex was more effective at the lower dose of 5 mg/kg. These results correspond to the effects of ubenimex on the number of neutrophils in the peritoneal cavity (Figs. 2 and 3). It is well known that neutrophils, along with macrophages, are one of the major defensive factors against *Candida* infection. Preclinical data has revealed that ubenimex is a potent macrophage-augmenting agent causing increased release of interleukin-1 and lysosomal enzymes[16]. Ubenimex augments macrophage tumoricidal activity and has the ability to increase mixed lymphocyte response. However, its effect on neutrophil functions had not been previously clarified in mice. The results of the present study showed that ubenimex induced an enhancement of phagocytosis and production of active oxygen by neutrophils. In addition, it caused a significant increase in the number of neutrophils exudated into the peritoneal cavity in mice. The protective action of this microbial metabolite against candidasis is thus probably due to its stimulating effects on neutrophils. We have recently reported that treatment of immunosuppressed mice with ubenimex increases the frequency and absolute numbers of colony forming unit granulocyte macrophages (CFU-GM), resulting in an increase in the number of nucleated bone marrow cells and white blood cells[17]. In human, JARSTRAND *et al.* demonstrated that oral administration of ubenimex potentiated phagocytic activity of granulocytes[18]. SUSS *et al.* reported that ubenimex *in vitro* stimulated the phagocytic activity of human leucocytes against *Saccharomyces cervisiae*[19]. These results strongly support that ubenimex stimulates granulocytes both in mice and humans.

Ubenimex is a potent inhibitor of leucine aminopeptidase and aminopeptidase B. LOOK *et al.* reported that aminopeptidase N is identical to human myeloid plasma membrane glycoprotein CD13 (gp 150)[20]. The possible interaction of ubenimex with CD13 might be involved in neutrophil activation.

Recently CHEN *et al.* have reported that BRMs including lipopolysaccharide (LPS), lentinan and levamisole can prolong survival time of mice experimentally infected with a lethal dose of *C. albicans*[21]. Ubenimex might have a greater advantage to control fungal infections in immunosuppressed hosts including those with chronic leukemia or receiving intensive cancer chemotherapy because of its lower toxicity.

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