THE NOVEL IMMUNOSTIMULANT N-563, AN ANALOGUE OF DEOXYSPERGUALIN, PROMOTES RESISTANCE TO *Candida albicans* INFECTION IN MICE

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An analogue of deoxyspergualin, N-563 has an immunostimulating activity whereas the mother compound has been found to be a potent immunosuppressant. In this study, the protective effect of the analogue against *C. albicans* infection was investigated in normal and immunosuppressed mice. In normal mice, N-563 treatment at 10 mg/kg for 3 days prior to infection significantly prolonged the survival time. In immunosuppressed mice treated with a single dose of cyclophosphamide 4 days prior to infection, N-563 at 3 and 10 mg/kg for 3 days prior to infection also significantly prolonged the survival time of mice. In addition, it augmented the phagocytic activity of neutrophils and enhanced the delayed type hypersensitivity reaction against *C. albicans*. Coincidentally, N-563 appeared to protect against secondary infection with *C. albicans* in the delayed type hypersensitivity-positive mice.

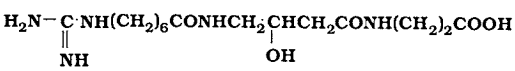
Spergualin is a *Bacillus* metabolite which possesses immunosuppressive activity in animals[1,2]. An analogue of spergualin, deoxyspergualin, shows strong immunosuppressive activity in various models of experimental organ transplantation[3], and has a therapeutic effect in rescuing kidney transplant patients with on-going graft rejection[4]. During the course of screening spergualin derivatives for more potent immunosuppressant, we surprisingly found several derivatives which acted as immunostimulants. These had a –COOH moiety in place of the spermidine $-NH_2$ moiety. Preliminary study showed that N-563, the most active analogue, restored the production of antibodies to sheep red blood cells in immunosuppressed mice. This reversal of effect of deoxyspergualin derivatives prompted us to investigate the antimicrobial activity of N-563. In this paper, we present evidence showing that N-563 protects mice from experimental infection with *Candida albicans*.

Materials and Methods

Animals

Specific pathogen-free inbred male C3H/HeN mice and female CD1 mice were obtained from Charles River Japan, Inc at 7 and 4 weeks of age, respectively. They were housed at a temperature of 25 ± 2°C and a humidity of 55 ± 7%, and fed standard mouse diet (NMFR, Oriental Yeast Co., Ltd.) and water *ad libitum*.

Fig. 1. Chemical structure of N-563.



Fungi

C. albicans TIMM 1623 and 1768 maintained at the Research Center for Medical Mycology, Teikyo University, were grown in Sabouraud dextrose broth (SDB) containing 0.5% yeast extract overnight at 37°C in a shaking water bath. *C. albicans* cells were precipitated by centrifugation for 5 minutes at 1,500 rpm and washed five times with physiological saline. The number of *C. albicans* cells was counted with a hemocytometer.

Agents

N-563 was prepared by Nippon Kayaku Co., Ltd. It was dissolved in physiological saline and filtered through a Millipore filter (0.22 μm). Cyclophosphamide was purchased from Shionogi Pharmaceuticals, Co., Ltd., and dissolved in distilled water before use. Levamizole and lentinan were purchased from Sigma Chemical Co. and Ajinomoto Co., Ltd., respectively. MDP-Lys (L-18), fluconazole, econazole, and ketoconazole were the kind gifts of Daiich pharmaceutical Co., Ltd., Pfizer Pharmaceutical Japan, Otsuka Pharmaceutical Co., Ltd., and Kyowa Hakko Co., Ltd., respectively.

Systemic Infection of Mice

CD1 mice (age 5 weeks) were used in these experiments as previously described[5]. Normal mice were infected intravenously (iv) with 1×10^6 cells of *C. albicans* TIMM 1768. Immunosuppression was induced by intraperitoneal treatment with a dose of 200 mg/kg of cyclophosphamide (CY) 4 days prior to infection. Mice were infected (iv) with 4×10^4 cells of *C. albicans*. N-563 was administered intraperitoneally (ip) for the 3 consecutive days prior to infection.

Detection of *C. albicans* in Kidneys

At the end of the experimental period, surviving mice were sacrificed, and the kidneys were excised and latitudinally sectioned under sterile conditions. The cut surface of each kidney was pressed against the surface of *Candida* GS agar plates containing 1% peptone, 0.5% yeast extract, 4% glucose, 2% agar and 0.05% 5-nitro-2-furfurylidine-aminoguanide hydrochloride. They were incubated for 48 hours at 37°C to observe the growth of *C. albicans* colonies.

Growth Inhibition of *C. albicans*

Growth inhibition assay was performed as described by FUKAZAWA *et al.*[6]. Briefly, *C. albicans* TIMM 1623 cells were suspended in SDB at a concentration of 2×10^4 cells/ml. N-563, fluconazole, econazole, and ketoconazole were diluted with SDB to a final concentration of 0.16 to 20 μg/ml. *C. albicans* cell suspension (100 μl) and each drug dilution (100 μl) were mixed in 96-well microtest flat plate, and absorption ($\lambda = 620$ nm) at the initial time was measured (OD_0). The *C. albicans* cell suspension was incubated for 24 hours at 37°C, and 100 μl of 0.1% triton X-100 was added with vigorous mixing. Fifty microliters of ethanol was added, and absorption was measured 24 hours later (OD_{24}). The difference between OD_{24} and OD_0 was plotted to the concentration of each drug. The inhibitory concentration (IC) was obtained from this standard curve.

White Blood Cell (WBC) Count in Peripheral Blood

CY-treated C3H/HeN mice were given N-563 at a dose of 10 mg/kg daily for 3 days prior to retroorbital blood sampling. The number of WBC was counted with a hemocytometer (Celltac, Nippon Koden Co., Ltd.).

Preparation of Murine Neutrophils

Purification of neutrophils was performed as described previously[5]. Briefly, C3H/HeN mice were injected (ip) with 2 ml of 8% casein sodium. Six hours later, peritoneal exudate cells (PEC) were recovered with an injection (ip) of 5 ml/mouse of Dulbecco's phosphate buffered saline (PBS), then resuspended in PBS after centrifugation for 5 minutes 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 confirmed to consist of more than 95% neutrophils by microscopical estimation using Diff-quik (Green Cross Co., Ltd.) staining, and then used as a neutrophil preparation.

Phagocytosis of *C. albicans* Cells by Neutrophils

C3H/HeN mice were treated (ip) with 10 mg/kg of N-563 daily for 3 days. Murine neutrophils were suspended in RPMI-1640 medium containing 10% fetal calf serum at a concentration of 4×10^6 cells/ml. Heat-killed *C. albicans* TIMM 1768 cells were also suspended in the same medium at 2×10^7 cells/ml. The neutrophil suspension (500 μl), heat-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. Phagocytic activity was determined by measuring under a microscope the total number of phagocytosing neutrophils per 200 neutrophiis and the average number of phagocytozed *C. albicans* per neutrophil.

Cell-Mediated Immunity

Mice were immunized by intravenous injection of 1×10^4 cells of viable *C. albicans* TIMM 1768 at 4 days after CY-treatment at 200 mg/kg. N-563 at 10 mg/kg was administered (ip) daily for the 3 days prior to immunization, and 10 mg/kg of fluconazole was administered (ip) on the day of immunization and the next day. At 13 days after immunization, footpad sensitivity to *C. albicans* antigen was performed as described by KAGAYA *et al.*[7]. Twenty microliters of sonicated dead *C. albicans* antigen (5 mg/ml protein) or saline was injected into the hind footpad of the immunized mice. Twenty-four hours after injection, the thickness of the footpad was measured with calipers. At 14 days after immunization, all mice, including non-treated mice as a control, were challenged (iv) with 1×10^6 cells of viable *C. albicans* to detect protective activity against *C. albicans* infection.

Statistical Analysis

Statistical analysis was carried out by Mantel-Cox test, Student's *t*-test, and χ^2-test.

Results

Effect of N-563 on *C. albicans* Infection in Immunosuppressed Mice

We examined the protective effect of N-563 against *C. albicans* infection in CY-induced immunosuppressed mice. Mice were given (ip) 200 mg/kg of CY 4 days before infection, and administered saline or N-563 at 3,10, 30, and 90 mg/kg daily for 3 days prior to infection. As shown in Table1, treatment

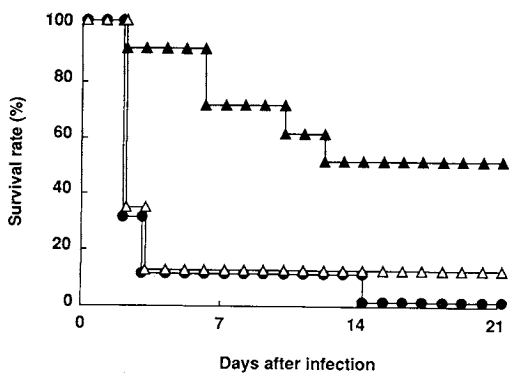
Table 1. Protective effect of N-563 against *Candida albicans* in cyclophosphamide-induced immunosuppression in mice[a].

Treatment[b]	Dose (mg/kg)	Survived mice/Treated mice[c]			
		Exp. 1	Exp. 2	Exp. 3	Total
Saline		0/10	1/10	2/16	3/36
N-563	3		5/9		5/9[e]
	10	5/10[f]	4/8[d]	4/10	13/28[f]
	30	1/9	4/9	3/10	8/28
	90			2/10	2/10

[a] Female CD1 mice were given cyclophosphamide (200 mg/kg, ip) on day-4, and infected (iv) with 4×10^4 *C. albicans* on day 0.
[b] Saline or N-563 were administered (ip) on day-3 to day-1.
[c] Number of surviving mice was determined on day 21.
[d] $P<0.05$ by the Mantel-Cox test (*vs.* Saline).
[e] $P<0.01$ by the Mantel-Cox test (*vs.* Saline).
[f] $P<0.001$ by the Mantel-Cox test (*vs.* Saline).

Fig. 2. Protective effect of N-563 against *Candida albicans* in immunosuppressed mice.

Female CD1 mice (9 or 10 animals/group) were infected (iv) with 4×10^4 *C. albicans* on day 0. Cyclophosphamide (200 mg/kg) was injected (ip) on day-4. N-563 10 mg/kg (▲), 30 mg/kg (△) or saline (●) was administered (ip) on day-3 to day -1.



with N-563 at 3 and 10 mg/kg significantly prolonged the survival time of mice, while partial protection was observed at 30 mg/kg. In case of Exp. 2 mean survival time of saline group, that of 3 mg/kg, 10 mg/kg, and 30 mg/kg of N-563-treated groups were 8.3, 4.2, 4.9 and 15.0 days, respectively. Optimal dose of N-563 was seemingly from 3 mg/kg to 30 mg/kg, but not clear as cytocidal antifungal agents.

Fig. 2 shows survival curves of a representative experiment. While all saline-treated mice died within 14 days after infection and median survival time was 2.3 days, N-563 (10 mg/kg)-treated mice showed the significantly prolonged median survival time of 20.6 days. Half the N-563-treated mice were still alive at 21 days after infection. At the end of the observation period, surviving mice were sacrificed to determine whether viable *C. albicans* were still present in the kidneys. These surviving mice showed no detectable *C. albicans* in either kidney, except one with detectable *C. albicans* in one kidney. This finding indicates that N-563 enhanced the elimination of in CY-immunosuppressed mice.

Comparison of Antifungal Activity of N-563 with That of Various Biological Response Modifiers (BRMs) on *C. albicans* Infection

Various BRMs have been reported to have a prophylactic effect on experimental *C. albicans* infection in mice. To evaluate the relative antifungal activity of N-563 in comparison with the BRMs levamisole, lentinan[8] and MDP-Lys (L18)[9], normal mice were infected (iv) with 1×10^6 cells of *C. albicans* TIMM 1768, and treated with each BRM at the dosages and schedules reported by CHEN[8] and OTANI *et al.*[9]. As shown in Table 2, all saline-treated mice died within 18 days after infection with a median survival time of 11.5 days. When N-563 at 10 mg/kg was administered (ip) 3 days prior to infection, survival time was significantly prolonged to 18.0 days, and 4 of 10 mice survived. Lentinan and MDP-Lys (L18) induced a non-significant prolongation of survival time, while levamisole had no effect.

We also examined the therapeutic effect of N-563 administered after *C. albicans* infection in normal and CY-immunosuppressed mice. N-563 was given (ip) by single daily administration at 1, 3, 10, or 30 mg/kg for 3 days after infection. However, no prolongation of survival time was observed (data not shown). These results indicate that prior treatment was necessary to prevent mice from fatal infection, and suggest that the time of N-563 administration was a critical factor in this drug's efficacy under these experimental conditions.

In Vitro Antifungal Activity of N-563 against *C. albicans*

In vitro antifungal activity of N-563 against *C. albicans* was examined. No direct inhibitory effect of

Table 2. Protective effect of N-563 against *Candida albicans* in normal mice[a].

Agent	Administration			Median Survival Time (range) (days)	Survival Ratio[b]
	Route	Dose (mg/kg)	Schedule		
Saline	ip	—	day-3, -2, -1	11.5 (4~18)	0/13
N-563	ip	10	day-3, -2, -1	18.0 (9~18)[c]	4/10
Levamisole	ip	10	day-1	11.0 (6~17)	1/10
Lentinan	ip	10	day-3, -2, -1	14.0 (7~16)	2/10
MDP-Lys (L18)	sc	5	day-1	12.0 (9~16)	2/10

[a] Female CD1 mice were infected (iv) with 1×10^6 *C. albicans* on day 0.
[b] Survival ratio was number of mice surviving on day 21 per number of mice treated.
[c] $P<0.05$ by the Mantel-Cox test (*vs.* Saline).

N-563 on growth of *C. albicans* could be detected at dose range between 0.16 and 20 μg/ml (data not shown), in contrast to fluconazole, econazole, and ketoconazole for which the 75% inhibitory concentration (IC_{75}) was determined to be 23 μg/ml, 0.3 μg/ml, and 0.16 μg/ml, respectively.

Augmentation of the Phagocytic Activity of Neutrophils by N-563

To understand the mechanism of the protective effect of N-563 against lethal *C. albicans* infection, we examined whether N-563 stimulated the production and function of neutrophils.

We first examined the number of WBC and PEC in CY-induced leukopenic mice. N-563 was administered (ip) to mice once a day for 3 days starting on the day after 200 mg/kg of CY injection, and the WBC numbers determined at various time points. WBC number in CY-treated mice rapidly reduced and reached nadir 4 days after CY-treatment ($8.2 \pm 1.6 \times 10^2$ cells/μl in CY-treatment mice *vs* $100.0 \pm 32.5 \times 10^2$ cells/μl in normal mice). N-563 did not show apparent inhibition or faster recovery from the reduction of WBC by CY-treatment ($12.6 \pm 4.0 \times 10^2$ cells/μl at 4 days after CY-treatment). Similarly N-563 did not significantly increase the number of PEC in CY-incduced leukopenic mice.

We next examined the effect of N-563 on neutrophil function, in particular on phagocytic activity. As shown in Table 3, N-563 significantly increased the total number of phagocytosing neutrophils per 200 neutrophils counted, but also the average number of phagocytozed *C. albicans* per neutrophil. These results indicate that N-563 augmented the phagocytic activity of these neutrophils.

Effect of N-563 on Cell-mediated Immunity

As a final means to elucidate the mechanism of N-563, we examined whether N-563 stimulates the footpad response in the delayed type hypersensitivity (DTH) reaction to *C. albicans* antigen in mice. N-563 enhanced the DTH reaction to *C. albicans* antigen in *C. albicans*-immunized mice (left column in Table 4). Further, we also examined the effect of N-563 on both the resistance of mice to secondary infection with *C. albicans* and on the DTH reaction to *C. albicans* antigen. Mice treated with N-563 prior to immunization with *C. albicans* antigen were completely protected to challenge with *C. albicans*

Table 3. Enhancement by N-563 of phagocytic activity of neutrophils against *Candida albicans in vitro*[a].

Treatment[b]	% of *C. albicans*-phagocytosing neutrophils (Mean ± SD)	No. of phagocytozed *C. albicans* per neutrophil (Mean ± SD)
Saline	42.8 ± 2.6	1.4 ± 0.2
N-563	63.3 ± 4.5[c]	2.1 ± 0.2[c]

[a] Heat-killed *C. albicans* (2×10^7 cells) and neutrophils (4×10^6 cells) were incubated, and phagocytosis was determined by trypan blue and eosin Y staining.
[b] Neutrophils were collected from C3H/HeN mice (n = 5) treated (ip) with saline or N-563 at 10 mg/kg daily for 3 days before collection.
[c] $P < 0.01$ by Student's *t*-test (*vs.* Saline).

Table 4. Effect of N-563 on delayed type hypersensitivity reaction against *Candida albicans* antigen and secondary infection of *C. albicans* in immunosuppressed mice[a].

Immunization	Agent[b]	DTH reaction[c] Mean ± SD ($\times 10^2$ mm)	No. of surviving mice at 10 days after challenge[d]
None	Saline	−0.5 ± 0.87[f]	1/8
Immunized[e]	Saline	7.71 ± 4.27	5/7[g]
Immunized	N-563	17.86 ± 5.17[f]	7/7[h]
Immunized	Fluconazole	6.29 ± 6.90	5/7[g]

[a] Female CD1 mice were given cyclophosphamide (200 mg/kg, ip) on day-4.
[b] Saline or N-563 at 10 mg/kg was administered (ip) on day-3 to day-1. Fluconazole at 10 mg/kg was administered (ip) on day 0 and day 1.
[c] *C. albicans* antigen was injected into the footpad on day 13. Twenty-four hours later, DTH reaction was determined.
[d] All mice were challenged (iv) with 1×10^6 cells of viable *C. albicans* on day 14.
[e] Mice were immunized (iv) with 1×10^4 cells of viable *C. albicans* on day 0.
[f] $P < 0.01$ by Student's *t*-test (*vs.* Saline-immunized).
[g] $P < 0.05$ by the χ^2-test (*vs.* Non-immunized).
[h] $P < 0.01$ by the χ^2-test (*vs.* Non-immunized).

infection (right column in Table 4), indicating that N-563 enhanced not only neutrophil fungicidal activities but also cell-mediated immunity.

Discussion

Our preliminary study showed that N-563 had stimulant activity on the production of antibodies to sheep red blood cells in immunosuppressed mice. Further study has been undertaken to elucidate the immunomodulatory profile of N-563. Patients with underlying diseases such as leukemia or cancer often suffer recurrent opportunistic infections, including *Candida albicans* infection. We investigated the protective effect of N-563 against experimental systemic infection with *C. albicans*.

N-563 had a prophylactic effect against *C. albicans* infection in CY-treated and normal mice. In CY-treated mice, treatment with N-563 at the dose of 10 mg/kg markedly prolonged survival time after infection, and 50% of N-563-treated mice survived the infection. Under these experimental conditions, N-563 enhanced the elimination of *C. albicans* from the kidney, a target organ of *C. albicans* infection.

In normal mice, N-563 treatment at the dose of 10 mg/kg for 3 days prior to infection also prolonged survival time, and 40% of N-563-treated mice survived. CHEN *et al.*[8] reported that levamisol and lentinan had a protective effect against *C. albicans* in normal mice, while OTANI *et al.*[9] reported that MDP-Lys (L18) had also protective effect against this pathogen in normal mice. We were unfortunately unable to confirm the efficacy of these compounds under the limited conditions in our experiment. We have demonstrated, however, that N-563 had a prophylactic effect equal to or greater than those of these BRMs when given prior to *Candida* challenge. The findings that N-563 showed no therapeutic activity when given after infection and no direct fungicidal activity *in vitro* suggests that the protective effect of N-563 on *C. albicans* infection involved a host-mediated action. Host-mediated antimicrobial agents such as ubenimex were known to show the maximal protective activity to *Candida* infection only when given at its optimal dose range[5]. Therefore, it is not curious that N-563 given at the doses of 30~90 mg/kg was less effectively protective from *Candida* infection than that 10 mg/kg as shown in Table 1.

N-563 clearly augmented the phagocytic activity of neutrophils without increasing the number of WBC in peripheral blood and the number of PEC. This suggests that neutrophil activation by N-563 may have an important role in the mechanism of protections against *C. albicans* infection. Some BRMs, including granulocyte-colony stimulating factor[10], the enterococcal preparation FK-23[11] and ubenimex[5] are reported to confer resistance to experimental mice against *C. albicans* infection. They were all reported to clearly increase the number of neutrophils in peripheral blood or the peritoneal cavity, and their function included phagocytic and killing activities[5,10,11].

N-563 augmented the phagocytic activity of neutrophils without increasing the number of neutrophils. Thus, we speculate that the protective effect of N-563 against *C. albicans* infection may involve some other activities, such as cellular immunity.

KAGAYA *et al.*[7] reported that cell-mediated immunity plays an important role in mechanisms of defence against *C. albicans*; mean survival time following secondary infection of immunized mice correlated with the degree of DTH response to *C. albicans* antigen. N-563 enhanced the DTH reaction against *C. albicans* when DTH was elicited by sonicated *C. albicans* antigen. Coincidentally, N-563 appeared to protect against secondary infection with *C. albicans* in the DTH-positive mice. Thus, part of the protective effect of N-563 against candidiasis may be due to augmented cellular immunity, as detected by enhanced DTH response to *C. albicans* antigen. This assumption may be supported by the survival curve of the N-563-treated infected mice (Fig. 2), which shows that N-563 clearly protected the mice against fatal infection in later periods, especially later than day 12 after infection.

These results suggest that N-563, an immunomodulator, is potentially useful as a prophylactic agent in the management of patients with severe opportunistic fungal infection.

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