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In Vitro and *In Vivo* Antifungal Activities of 6-[(N-4-Bromophenyl) amino]-7-chloro-5,8-quinolinediones

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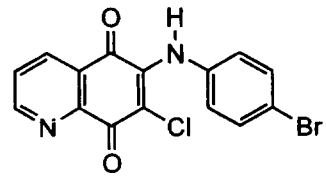
Antifungal activities of 6-[(N-4-bromophenyl)amino]-7-chloro-5,8-quinolinedione (RCK7) were tested. The MIC values of RCK7 were determined for antifungal suceptibility, *in vitro* against *Aspergillus niger, Cryptococcus neoformans* and *Trichophyton mentagrophyte* by standard agar streak method. *In vitro,* RCK7 showed more potent antifungal activity than fluconazole and ketoconazole. Also, RCK7 was tested for *in vivo* antifungal activitiy in the treatment of systemic infection with *Candida albicans* in normal mice. The therapeutic potential of RCK7 had been assessed by evaluating their survival rate against systemic infections compared with that of ketoconazole. ED_{50} of intraperitoneally administered RCK7 was 2.05 ± 0.30 mg/kg but that of ketoconazole was 8.00 ± 0.73 mg/kg, respectively. When RCK7 was administered intravenously at the ED_{50} (2.05 mg/kg), the colony counts of *Candida albicans* in the liver after 7 days and 14 days were reduced as likely as ketoconazole at the ED_{50} (8.00 mg/kg), and the better survival rates than ketoconazole's were achieved after 14 days. The results suggest that RCK7 may be a potent antifungal agent.

Key words : 6-[(N-4-Bromophenyl)amino]-7-chloro-5,8-quinolinedione, Minimum inhibitory concentration, *Candida albicans,* Candidiasis, *In vivo* antifungal activity

INTRODUCTION

The recent increase of fungal infections, especially among AIDS patients, has generated a renewed interest in antifungal drugs, including development of new antifungal agents (Clark *et al.*, 1992, Georgopapadakou *et al.*, 1994, Sternberg *et al.*, 1994).

In a program aimed at identifying novel antifungal agents, we focused on developing antifungal 5,8-quinolinediones that would be a potent fungicide and selective inhibitor of *de novo* pyrimidine biosynthesis in fungi due to blockade of mitochondrial electron transport in fungi (Roberts *et al.*, 1978, Hudson *et al.*, 1992). Following the observation that 6-substituted-7-chloro-5,8-quinolinediones (Jeschke *et al.*, 1993, Ryu *et al.*, 1994a, 1994b), had antifungal activities, newly prepared 6-[(N-4-bromophenyl)amino]-7-chloro-5,8-quinolinediones (RCK7, Fig. 1) were tested for *in vitro* antifungal activity against *Candida* spp.. We found that RCK7 showed more potent *in vitro* antifungal activities against *Candida* spp. than ketoconazole (Ryu *et al.*, 1994b). For the continuing evaluation on antifungal activities of RCK7, we tested its *in vitro* & *in vivo* activities. The *in vitro* activities were determined against other pathogenic fungi such as *Aspergillus niger, Cryptococcus neoformans* and *Trichophyton mentagrophyte.* The MIC values were determined by the standard agar streak method (Mcginnis *et al.*, 1991). The *in vivo* antifungal activities of RCK7 were tested in the treatment of systemic infection with *Candida albicans* in normal mice. The therapeutic potential of RCK7 had been assessed by evaluating their survival rate. We performed this study in an attempt to determine the ability of RCK7 to prolong the survival of mice and decrease colony counts of *Candida albicans* in the kidneys and liver in established models (Fisher *et al.*, 1989, Mcginnis *et al.*, 1991, Ryu *et al.*, 1995, Sugar *et al.*, 1994, Viscoli *et al.*, 1991) of murine disseminated candidiasis.



RCK7

Fig. 1. Chemical structure of RCK7.

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MATERIALS AND METHODS

Materials and apparatus

RCK7 (Fig. 1) prepared previously (Ryu *et al.*, 1994a) was used for *in vivo* antifungal activity tests.

Sabouraud agar and brain heart infusion (BHI) broth were purchased from Difco Lab (U.S.A.). Tween 20 was obtained from Aldrich Chemical Co. (U.S.A.), and ethanol from Shinyo Pure Chemicals Co. (Japan). Other chemicals such as ketoconazole and saline were reagent grade commercially available.

UV spectrophotometer from Shimadzu UV-120-02 (Japan) was used. The microorganisms were incubated in an incubator bath from Vision Scientific Co. (Korea).

In vitro antifungal activities

In the determination of *in vitro* antifungal activities, the following fungal strains were used as target microorganisms: *Aspergillus niger* KCTC 1231, *Cryptococcus neoformans* KCTC 7224 and *Trichophyton mentagrophytes* KCTC 6085. Prior to determination of antifungal activity, the strains of fungi were cultured in Sabouraud agar at 30°C for 3~7 days. The number of cells was adjusted with sterile sline or the sterile BHI broth to 2×10^5 microorganisms and then used for the test. Test compounds (4 mg) were dissolved in 2 ml of DMSO and subjected to two-fold step dilution of the solution (0.05 ml). Then that was added to the melted Sabouraud agar for fungi over a final concentration range of 0.8 to 100 μg/ml. Each 3 μl of fungal inocula containing about 2×10^5 microorganisms was incubated by making a 2 cm long streak with loop on solidified agar plates. All the plates were incubated at 30°C for appropriate periods of time that sufficed to show clearly visible growth of colonies on drug-free control plates. The MIC was defined as the lowest concentration of a drug at which there was no visible colonial growth. Fluconazole and ketoconazole as antifungal standard substances were used (Table I).

Table I. *In vitro* antifungal activities of RCK7

Comp.	MIC (μg/ml)		
	A. niger	*C. neoformans*	*T. mentagrophytes*
RCK7	3.2	3.2	1.5
Fluconazole	25.0	12.5	25.0
Ketoconazole	25.0	12.5	12.5

a) Fungi tested; *Aspergillus niger* KCTC 1231, *Cryptococcus neoformans* KCTC 7224 and *Trichophyton mentagrophytes* KCTC 6085. The inoculum sizes contained approximately 2×10^5 CFU/ml.

b) Culture media tested was modified Sabouraud dextrose agar. MIC values were read after 3 days for *A. niger* and *C. neoformans*, and 7 days for *T. mentagrophytes* at 30°C.

Systemic infection and evaluation of *in vivo* antifungal activities

The evaluation of *in vivo* antifungal activities was conducted by the established methods (Fisher *et al.*, 1989, Ryu *et al.*, 1995b, Sugar *et al.*, 1994, Viscoli *et al.*, 1991).

The RCK7 and ketoconazole were suspended in saline with 0.25% Tween 20 and were administered by the intravenous injection of 0.1 ml.

Candida albicans used in this study was recent clinical isolates from Kyung Hee University Hospital, Seoul, Korea, which was maintained on Sabouraud dextrose agar slants at 4°C. A large loopful of yeast cells was suspended in fresh Sabouraud dextrose broth and incubated for 24 hrs at 37°C. Blastoconidia were harvested and washed twice in sterile buffered saline (pH 7.4) by centrifugation. Cells were counted in a hemacytometer and the concentration was adjusted to 1×10^5, 1×10^6 and 1×10^7 cells per ml. The number of yeast cells administered to the mice was determined by planting the same inoculum on Sabouraud dextrose agar plates. Colonies were counted 24 to 48 hrs later.

ICR male mice, 3 to 4 weeks old, were purchased from Daehan experimental animal center (Korea). At the start of each experiment, the mice were weighed the average being 20g. Six mice were housed per cage in filter-topped cages and were fed standard mouse chow and water *ad libitum*. Each group of survival experiments consisted of eight mice. Additional cohorts of mice were infected for studies of kidneys and liver colony counts.

Determination of injected size on toxicity of *Candida albicans*: Each group consisted of eight mice in-

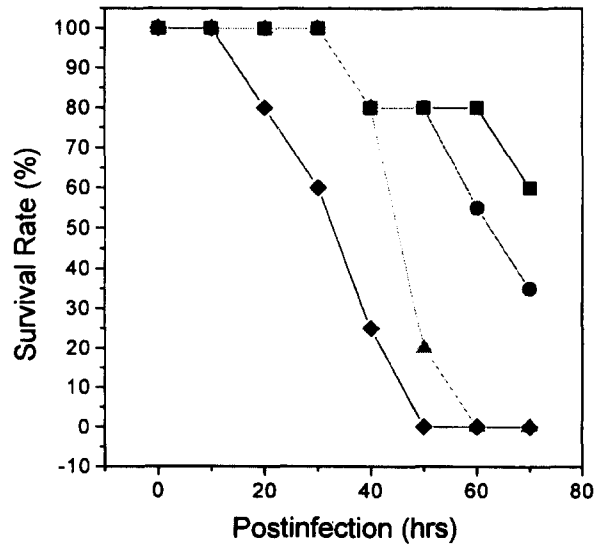

Fig. 2. Effect of injected size on toxicity of *C. albicans*. ■: 1×10^6, ●: 2×10^6, ▲: 5×10^6, ◆: 1×10^7/mouse.

jected intraperitoneally with 0.1 ml of sterile saline calculated containing each 1×10^5, 1×10^6 and 1×10^7 blastospores of *Candida albicans* per ml. The injected size at 3 day's post-infection was determined by counting survival rate of those mice (Fig. 2).

Efficacy of RCK7 and ketoconazole: Acute infection in normal mice was produced by intraperitoneal injection (via the lateral tail vein) with 0.1 ml of sterile saline calculated containing 10^6 blastospores of *Candida albicans*, a dose uniformly lethal for placebo-treated animals within 48 hrs. Groups of six animals received one of the following dose range; RCK7 0.1, 0.5, 1.0, 2.0, 10.0 mg/kg; ketoconazole 0.2, 1.0, 2.0, 10.0, 40.0 mg/kg. Appropriate doses in 0.1 ml of diluent were administered intravenously at 1, 4 and 24 hrs post-infection. The ED_{50} value at 2 days post-infection was calculated by fitting survival data to logistic dose response model (Table II).

Survival experiments and study of colony counts:

Table II. Efficacy of RCK7 against systemic infection with *Candida albicans* in normal mice

Compound	Mean $ED_{50}\pm SD$ ($n\geq5$) in normal mice (mg/kg)
RCK7	2.05±0.30
Ketoconazole	8.00±0.73

1)Dose range; RCK7 0.1, 0.5, 1.0, 2.0, 10.0 mg/kg
; ketoconazole 0.2, 1.0, 2.0, 10.0, 40.0 mg/kg
2) Drugs were administered intraperitoneally at 1, 4 and 24 hrs post-infection.
3) ED_{50} at 2 day's pos-tinfection

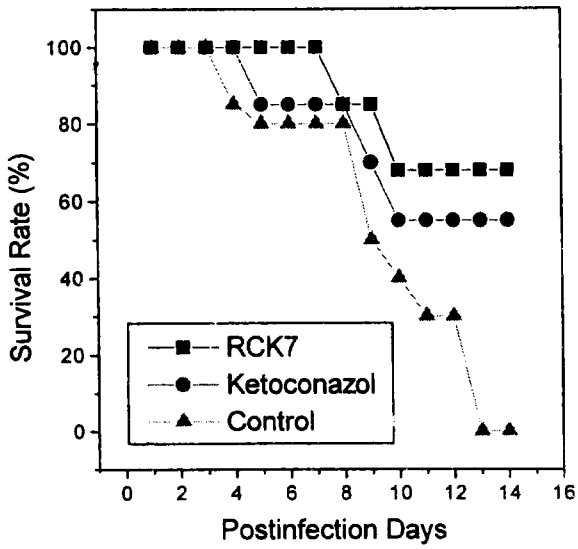

Fig. 3. Survival of *C. albicans* systemically infected mice treated with RCK7 and ketoconazole. Treatment was begun from 4 days after infection and continued for a total of 14 days. Mice (6 per group) received intravenous therapy once daily. Data for groups given RCK7 at the ED_{50} (2.05 mg/kg/day) and ketoconazole at the ED_{50} (8.0 mg/kg/day). ■: RCK7, ●: Ketoconazole, ▲: Control (saline with 0.25% Tween 20).

Table III. Colony counts of *Candida albicans* recovered from the kidneys and liver of systemically infected mice

Organ	Agent & Dosage (ED_{50}, mg/kg)	Mean $\log_{10}$ CFU/g of tissue±S.E.	
		7-Day Rx[a]	14-Day Rx
Liver	Control[b]	4.90±0.59	4.95±0.78
	Ketoconazole (8.00)	3.37±0.70*	4.00±0.77*
	RCK7 (2.05)	3.08±0.60**	4.06±0.14*
Kidney[c]	Control	≥5.8	≥6.0
	Ketoconazole (8.00)	≥5.8	≥6.0
	RCK7 (2.05)	≥5.8	6.0

[a]Rx: Drug treatment, intravenously administered
[b]Control: saline with 0.25% Tween 20
[c]Mean for right and left kidneys
*$p<0.05$, **$p<0.01$

The groups of seven mice were infected by injection with 0.1 ml of sterile saline containing 2×10^4 blastoconidia in a lateral tail vein. Each group received the following treatment. The fresh suspensions of RCK 7 and ketoconazole were prepared daily in sterile saline with 0.25% Tween 20 and administered intravenously by the injection of 0.1 ml. Therapy was begun from 4 days after infection with *C. albicans* and was continued for a total of 14 days. The control group was injected with the sterile saline with 0.25% Tween 20 daily. Cages were observed twice daily for deaths (Fig. 3).

Randomly selected mice in each group were sacrificed at designated intervals. After the kidneys and liver were removed aseptically, they were homogenized in small volume of saline, and 10-fold dilutions were plated onto Sabouraud dextrose agar. The plates were incubated for 24 to 48 hrs at 37°C, and then the colonies were counted (Table III). Student's t test was used to compare the means of colony counts in the kidneys and liver. Significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

MICs of RCK7 are given in Table I by comparison with MICs of ketoconazole. The control cultures showed no antifungal activities against all the strain of fungi. As indicated in Table I, RCK7 showed generally potent antifungal activities with widely expanded spectra. RCK7 had not only antifungal activities against the five pathogenic *Candida* species (Ryu *et al.*, 1994) but also *Aspergillus niger, Cryptococcus neoformans* and *Trichophyton mentagrophytes*. RCK7 had more potent antifungal activities than ketoconazole. RCK7 completely inhibited the fungal growth at 3.2 μg/ml against all *Candida* species. On the other hand, ketoconazole inhibited the growth at 25.0 μg/ml respectively.

RCK7 was tested for determination of *in vivo* antifungal activities against pathogenic *Candida albicans* by the modified known techniques (Fisher *et al.*, 1989,

Ryu *et al.*, 1995, Sugar *et al.*, 1994, Viscoli *et al.*, 1991). The results are given in Fig. 3, Table II and Table III, compared with ketoconazole. The control groups showed no antifungal activities against the pathogenic *Candida albicans.*

The injected size at 3 day's post-infection, that of eight mice injected intraperitoneally with 0.1 ml of sterile saline containing each 1×10^5, 1×10^6 and 1×10^7 blastospores of *Candida albicans* per ml, was determined by counting survival rate of those mice. (Fig. 2). The injected size containing 10^6 blastospores of *Candida albicans*, made uniformly lethal for eight animals within 48 hrs. ED_{50} values of RCK7 and ketoconazole when administered intraperitoneally to mice infected with *Candida albicans* are summarized in Table II. RCK7 was approximately 80 times more potent than ketoconazole against the infection in normal mice. Placebo-treated mice were dead by 2 day's post-infection.

Analysis of a typical experiment revealed that any control (untreated) mice did not survive after receiving an inoculum of 2×10^4 *C. albicans* cells per mouse (Fig. 2). Intravenously administered RCK7 at the ED_{50} (0.10 mg/kg of body weight) per day prolonged their survival compared with the control. In spite of lower ED_{50} of RCK7 (2.05 mg/kg), better results were observed than higher ED_{50} of ketoconazole (8.00 mg/kg), including the prolonged survival length and increase of survival ratio after 7 day treatment, but at the end of the 14 day experiment the level of mortality was the same, as seen in Fig. 3. Liver colony counts obtained at the end of therapy for 7 days and 14 days showed substantial decreases in numbers of *Candida* organisms in the mice treated with RCK7 at ED_{50} as well as ketoconazole. And yet, both RCK7 and ketoconazole did not reduce significantly *Candida albicans* colony counts in the liver (Table III).

The results indicate that RCK7 possesses superior activity to that of ketoconazole in a range of animal models of systemic fungal infection. Continuing interest is to demonstrate that other 6-[N-(halophenyl)-amino]-7-chloro-5,8-quinolinediones (RCKs, RCK3, 4 11, 20) were also more protective than ketoconazole in the same murine cadidiasis model (Ryu *et al.*, 1995, 1996, Park *et al.*, 1996). The results suggest that RCKs may be potent antifungal agents. Further studies to continuously explore the *in vivo* antifungal activities and safeties of various new RCKs are required.

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