In Vitro Antifungal Activity of CAN-296: A Naturally Occurring Complex Carbohydrate

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The *in vitro* activity of a naturally occurring complex carbohydrate, CAN-296, was evaluated by testing 132 clinical and ATCC isolates of yeast and *Aspergillus fumigatus*, many of which were azole-resistant. The *in vitro* susceptibility tests were performed by standardized broth micro- and macrodilution methods and results were compared with those obtained for amphotericin B, fluconazole, ketoconazole, flucytosine and the pneumocandin L-733,560. All tested *Candida* species showed highly uniform susceptibility to CAN-296 at concentrations of 0.078 to 0.312 μg/ml; non-*albicans Candida* were as susceptible to CAN-296 as the *Candida albicans* strains. Multi-azole-resistant *Candida* species were highly sensitive to CAN-296. Minimum inhibitory concentration measurements did not differ from minimum lethal concentrations by more than two-fold for all tested *Candida* species. *Aspergillus fumigatus*, on the other hand, showed only moderate susceptibility to CAN-296. The kinetics of the anti-*Candida* activity of CAN-296 was investigated by kill-curve experiments using *C. albicans* and *C. glabrata* and the results were compared with those obtain for amphotericin B. CAN-296 was found to be rapidly fungicidal in concentrations ranging from 4~16 fold the mean MIC value. The broad spectrum of anti-*Candida* activity together with the rapid fungicidal effect make this complex carbohydrate a promising agent for clinical use.

The incidence of fungal infections increased dramatically in the past decade. This is due, in part, to an expanding population of immunocompromised patients (from cytotoxic therapy, steroid therapy and AIDS) and technical advances that facilitate fungal invasion. The increased frequency, severity, and number of fungal species identified as pathogens has further created a critical need for new, safe anti-fungal drugs. Today, the most commonly used drugs for systemic and local fungal infections are amphotericin B (AMB) and azole agents[1]. AMB is still the drug of choice in many systemic mycoses due to its broad spectrum and fungicidal activity, however, AMB is nephrotoxic and is administered intravenously only[2]. The triazoles, fluconazole and itraconazole have broad spectrum of activity, can be administered orally, and are less toxic than AMB. However, azoles are fungistatic and resistance has become a significant problem[3~8]. Primary and secondary resistance to flucytosine is common and this drug is generally used only in combination with AMB.

In this study we report on the *in vitro* antifungal effects of CAN-296, a new, naturally occurring, complex carbohydrate isolated from the cell wall of the fungus *Mucor rouxii*. Although its molecular structure has not been completely elucidated yet, CAN-296 is known to consist of terminal and branched *N*-acetyl-D-glucosamine. It has a molecular mass of 4300 daltons.

Materials and Methods

We tested the activity of CAN-296 against 132 clinical and ATCC isolates of yeast and *Aspergillus fumigatus*, including azole-resistant strains. The results were compared with the antifungal activity of azoles, AMB, flucytosine and L-733,560 pneumocandin[6].

Organisms and Culture Conditions

Yeasts: A total of 112 strains from 14 yeast species were tested. Most of the strains (90%) were clinical isolates; the rest were obtained from the American Type Culture Collection (Rockville, MD). All isolates had been stored in Litmus Milk (Becton Dickinson Microbiology Systems, Cockeysville, MD), and kept frozen in -70°C. For use, approximately 10 μl of the stock culture was plated onto Sabouraud dextrose agar (SDA) and incubated for 48 hours, and then a colony was transferred to another SDA plate and incubated for 24 hours. The latter served as the source of the inoculum for most of the studies. About 30% of the tested isolates were azoles-resistant (fluconazole 48 hours MIC >32 μg/ml).

The distribution of species included 29 *Candida albicans* (14 azole-resistant), 15 *Candida glabrata* (5 azole-resistant), 10 *Candida parapsilosis* (2 azole-resistant), 10 *Candida lusitaniae*; 2 *Candida rugosa*; 10 *Candida tropicalis* (2 azole-resistant), 1 azole-resistant *Candida lambica*; 12 azole-resistant *Candida krusei*; 10 *Candida guillermondii*; 5 *Candida kefyr*; 4 *Candida stellatoidea*; 1 *Candida paratropicalis*; 1 *Candida lipolitica* and 2 *Saccharomyces cerevisiae*.

Aspergillus: Twenty *Aspergillus fumigatus* [10 clinical isolates (Microbiology Laboratory, Detroit Medical Center, Detroit, Michigan), 5 laboratory isolates each resistant to itraconazole and amphotericin B] strains were used to study the effect of CAN-296 on growth of *Aspergillus* species. Stock cultures were prepared using conidia as inoculum on YPD (Sigma Chemical Company, St. Louis, MO) agar slants. The slants were incubated at 30°C for 4 days, or until the cultures conidiated, and fresh conidia from the subcultures were used as the source of inoculum for all the cultures in subsequent work. The subculturing enabled us to assess further the purity of the original cultures.

CAN-296

CAN-296 is a naturally occurring heat stable complex carbohydrate isolated from the cell wall of the fungus *Mucor rouxii*. Structurally, CAN-296 belongs to a novel class of antifungal agents. It consists mostly of 1,4-, 3,4- and 4,6-linked, and terminal *N*-acetyl-D-glucosamine residues. It has a molecular mass of 4300 daltons. The compound was obtained as a 0.1 mg/ml aqueous solution from IGG International Inc., Cambridge, MA.

Yeast Antifungal Susceptibility Assays

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of CAN-296 were determined using the broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS)[9] except Peptone Yeast Extract Glucose Medium (PYG) was used for the assay instead of RPMI 1640. Briefly, the microorganisms were grown in PYG medium to which CAN-296 was added to a final concentration of 10 to 0.019 μg/ml. The MIC was defined as the lowest concentration that inhibited growth completely. The MICs were recorded after 48 hours of incubation at 35°C. MLC was determined by subculturing 100 μl from the first well demonstrating complete growth inhibition and from all wells that had no visible growth onto Sabouraud dextrose agar plates that were incubated at 30°C for 24 hours. MLC was defined as the lowest concentration at which 99% of the initial inoculum was killed.

Aspergillus fumigatus Susceptibility Studies

The susceptibility of *A. fumigatus* to CAN-296 as well as to itraconazole and amphotericin B was determined by the broth macrodilution technique according to the procedure of ESPINEL-INGROFF *et al.*[10] as modified by MANAVATHU *et al.*[11] using fresh conidia as the source of inoculum. For the preparation of conidial suspensions, cultures of various isolates were grown on YPD agar plates for 6 days at 30°C until the whole plate was covered with fungal growth. The conidia were collected by flooding the agar surfaces with sterile growth medium (20 ml) followed by gentle scraping with a sterile rubber policeman. The resulting suspension, containing fragments of mycelium, small pieces of agar, and other cellular debris, was collected by aspiration. The aspirate was vortexed vigorously to release conidia from the conidiophores and was filtered through a sterile cotton plug fitted in a sterile Nalgene filtration funnel. In general, a single filtration step was sufficient to obtain a clarified condial suspension. The cell density was determined by hemocytometer count. Each sample was counted four times independently, and the mean value of the quadruplicate was used for the calculation of the cell density. Under these condition we routinely obtained approximately $1 \sim 3 \times 10^7$ conidia per ml when 20 ml of growth medium was used for resuspension. The relationship between cell density estimated by hemocytometer count and the number of colony forming units (CFU) was determined by plating appropriately diluted cell suspensions on YPD agar. The cell density estimation by CFU production was approximately 10 to 20 percent lower than the value obtained by hemocytometer count.

The broth macrodilution experiments were performed in sterile 6-ml polystyrene tubes (Falcon 2058) with a final volume of 1 ml. Two times the required final concentrations of CAN-296, itraconazole and AMB were prepared in 0.5 ml growth medium by two-fold serial dilutions. Each well was inoculated with an equal volume (0.5 ml) of conidial suspension (2× the required final CFU prepared in growth medium by two-fold serial dilution) to obtain a final CFU of 1×10^4 per ml. Each series of drug concentration was tested in triplicate and each MIC determination was repeated at least once. The AMB tubes were wrapped in aluminum foil to prevent light exposure, and all tubes were incubated at 35°C for 48 hours, and scored for visible growth after vortexing the tubes gently, or scraping the walls of the tube fol-

Table 1-1. *In vitro* susceptibilities of *C. albicans* and non-*albicans Candida* to CAN-296 in comparison to azoles, amphotericin B and L-733,560.

Organism	Antifungal agent	MIC (μg/ml) Range	MIC (μg/ml) Mean	MLC (μg/ml) Range
C. albicans (29)	CAN-296	0.156～0.312	0.268	0.156～0.312
	Amphotericin B	0.02～0.1	0.050	
	Flucytosine	0.04～1.25	0.330	
	Ketoconazole	0.01～6.25	0.282	
	Fluconazole	0.08～80	23.95	
	Itraconazole	0.01～12.5	0.660	
	L-733,560	0.05～0.78	0.203	
C. parapsilosis (10)	CAN-296	0.078～0.312	0.165	0.078～0.312
	Amphotericin B	0.05～0.2	0.185	
	Flucytosine	0.08～1.25	0.321	
	Ketoconazole	0.02～0.2	0.030	
	Fluconazole	0.16～20	3.730	
	Itraconazole	0.02～0.2	0.072	
	L-733,560	0.02～1.56	0.157	
C. glabrata (15)	CAN-296	0.078～10.00	0.812	0.078～10.00
	Amphotericin B	0.05～0.2	0.115	
	Flucytosine	0.04～0.3	0.070	
	Ketoconazole	0.01～6.3	0.440	
	Fluconazole	1.25～40	16.53	
	Itraconazole	0.02～6.3	1.613	
	L-733,560	0.1～0.39	0.300	
C. tropicalis (10)	CAN-296	0.039～0.312	0.195	0.039～0.312
	Amphotericin B	0.02～0.39	0.176	
	Flucytosine	0.08～0.63	0.215	
	Ketoconazole	0.02～3.12	0.460	
	Fluconazole	0.63～80	27.56	
	Itraconazole	0.02～6.25	0.885	
	L-733,560	0.1～0.78	0.308	
C. lusitaniae (10)	CAN-296	0.156～0.312	0.265	0.156～0.312
	Amphotericin B	0.05	0.05	
	Flucytosine	0.08	0.08	
	Ketoconazole	0.02～0.2	0.11	
	Fluconazole	0.31～20	7.186	
	Itraconazole	0.02～0.1	0.17	
	L-733,560	0.39～0.78	0.624	
C. krusei (12)	CAN-296	0.039～0.312	0.263	0.039～0.312
	Amphotericin B	0.1～0.39	0.222	
	Flucytosine	2.5～20	11.56	
	Ketoconazole	0.01～1.56	0.611	
	Fluconazole	0.16～80	51.27	
	Itraconazole	0.01～0.39	0.227	
	L-733,560	0.78	0.78	
C. guillermondii (10)	CAN-296	0.078～0.312	0.171	0.078～0.312
	Amphotericin B	0.2～0.78	0.392	
	Flucytosine	0.08～0.16	0.115	
	Ketoconazole	0.02～0.1	0.035	
	Fluconazole	5～10	5.70	
	Itraconazole	0.2～0.78	0.364	
	L-733,560	0.78～1.56	1.248	
C. kefyr (5)	CAN-296	0.156～0.312	0.187	0.156～0.312
	Amphotericin B	0.05～0.39	0.210	
	Flucytosine	0.08～0.16	0.1	
	Ketoconazole	0.02	0.02	
	Fluconazole	0.31～2.5	1.64	
	Itraconazole	0.2	0.2	
	L-733,560	0.02～0.78	0.295	
C. stellatoidea (4)	CAN-296	0.312	0.312	0.312
	Flucytosine	0.625～1.25	0.937	
	Ketoconazole	0.01	0.01	
	Fluconazole	0.16～0.31	0.235	
	Itraconazole	0.01	0.01	

Table 1-2. *In vitro* susceptibilities of non-*albicans Candida* to CAN-296 in comparison to azoles, amphotericin B and L-733,560.

Organism	Antifungal agent	MIC (μg/ml) Range	MIC (μg/ml) Mean	MLC (μg/ml) Range
C. rugosa (2)	CAN-296	0.312	0.312	0.312
	Flucytosine	0.08	0.08	
	Ketoconazole	0.01	0.01	
	Fluconazole	5	5	
	Itraconazole	0.02	0.02	
C. lambica (1)	CAN-296	0.078	0.078	0.078
	Amphotericin B	0.01	0.01	
	Flucytosine	0.31	0.31	
	Ketoconazole	0.01	0.01	
	Fluconazole	20	20	
	Itraconazole	0.01	0.01	
C. paratropicalis (1)	CAN-296	0.312	0.312	0.312
C. lipolitica (1)	CAN-296	0.312	0.312	0.312
Saccharomyces cerevisiae (2)	CAN-296	0.156	0.156	0.156

lowed by vortexing. The MIC was defined as the lowest concentration of the drug in which no visible growth occurred. The medium recommended by NCCLS was RPMI 1640. However this media was unsuitable for studying the susceptibility of yeast to CAN-296 because this complex carbohydrate consistently precipitated in RPMI 1640, even at low concentration, whereas no precipitation occurred in PYG medium.

Time-kill Study

The anti-*Candida* activity of CAN-296 was examined by kill-curve experiments using *Candida albicans* isolate (B311), and *Candida glabrata* (32554). Briefly, test organisms were grown in YPG broth for 24 hours at 30°C on a gyratory shaker (160 rpm). The fresh 24 hours culture was diluted approximately 1000-fold to obtain a cell density of 1×10^6 CFU per ml. Five ml aliquots of the diluted cultures were incubated at 30°C with various concentrations ($0 \sim 5$ μg/ml) of CAN-296. For comparison with AMB, 5 ml aliquots of the diluted cultures were incubated at 30°C in the presence of 5 and 10 μg/ml of CAN-296 and 4 and 8 μg/ml of AMB. At various time intervals ($0 \sim 24$ hours) 0.1 ml aliquots of cell suspension were removed, serially diluted (10^2 to 10^6 fold) and 0.1 ml aliquots were spread on YPD agar plates in replicates. After incubation at 30°C for 48 hours, the number of CFU per ml of cultures were calculated and plotted against the time of exposure to CAN-296 to construct a kill-curve.

Results

Susceptibility of Yeasts to CAN-296

Table 1 provides a summary of the *in vitro* susceptibility studies. The vast majority (99%) of the tested species showed highly uniform susceptibility to CAN-296 at concentration of 0.078 to 0.312 μg/ml. Azole-resistant and azole-susceptible *Candida* species were equally sensitive, and when compared to L-733560, CAN-296 had a narrower and more consistent therapeutic range. The MICs of CAN-296 were comparable with those of AMB. One strain of *Candida glabrata* was relatively insensitive to CAN-296 (MIC = 10 μg/ml). For all azole-susceptible and resistant *Candida* species, the MIC and MLC did not differ by more than two fold.

Susceptibility of *A. fumigatus* to CAN-296

The MIC values of CAN-296 for clinical and laboratory isolates of *A. fumigatus* were compared with those obtained for AMB and ITZ. Nineteen of the 20 isolates tested showed no inhibition of growth (MIC > 5 μg/ml). The only exception was the clinical isolate W73355 which yielded an MIC value of 0.625 μg/ml. Both itraconazole-resistant and amphotericin B-resistant aspergillus isolates, were as susceptible to CAN-296 as were the itraconazole- and amphotericin B-susceptible strains.

Fungicidal Activity

The time-dependent fungicidal activity of CAN-296 for *Candida albicans* and *Candida glabrata* was studied by kill curve experiments. The concentrations of CAN-296 used for the time-kill study ranged from approximately $2 \sim 16$ fold the mean MIC value for most *Candida*

species. As shown in Fig. 1-A, the fungicidal activity of CAN-296 was both concentration and time dependent. The fungicidal action was rapid and greater than 99% of the cells were killed within 15 minutes of exposure to the compound when CAN-296 concentration used was greater than 16 fold the MIC (5 μg/ml). Greater than 99% killing was achieved within 45 minutes at a concentration of 2.5 μg/ml and within 120 minutes at a concentration of 1.25 μg/ml (Fig. 1-A). *Candida glabrata* was shown to be slightly less susceptible than *Candida albicans*, however 95% killing was achieved within 120 minutes at a concentration of 5 μg/ml (Fig. 1-B).

The time-dependent fungicidal activity of CAN-296 against *Candida albicans* and *Candida glabrata* was notably expeditious when compared with that of AMB (Figs. 2 and 3). CAN-296 at 5 μg/ml and 10 μg/ml killed greater than 95% of the cells within 60 and 30 minutes of exposure to the drug, respectively. No significant difference in the rate of killing was observed between the two *Candida* species used. The fungicidal activity of AMB was not as rapid, with 95% kill achieved within 120 minutes at a concentration of 8 μg/ml and within 4 hours at a concentration of 4 μg/ml. Moreover, *C. glabrata* was less susceptible to killing by AMB than *C. albicans*, at a concentration of 4 μg/ml considerably more than 4 hours of exposure was required to obtain >95% killing (Fig. 3).

Fig. 1. Kill-curve study of CAN-296 at concentrations 2~16 fold MIC value against *Candida albicans* B311 (A) and *Candida glabrata* 32554 (B).

● 0 μg/ml, ■ 1.25 μg/ml, ▲ 2.5 μg/ml, ▼ 5 μg/ml.

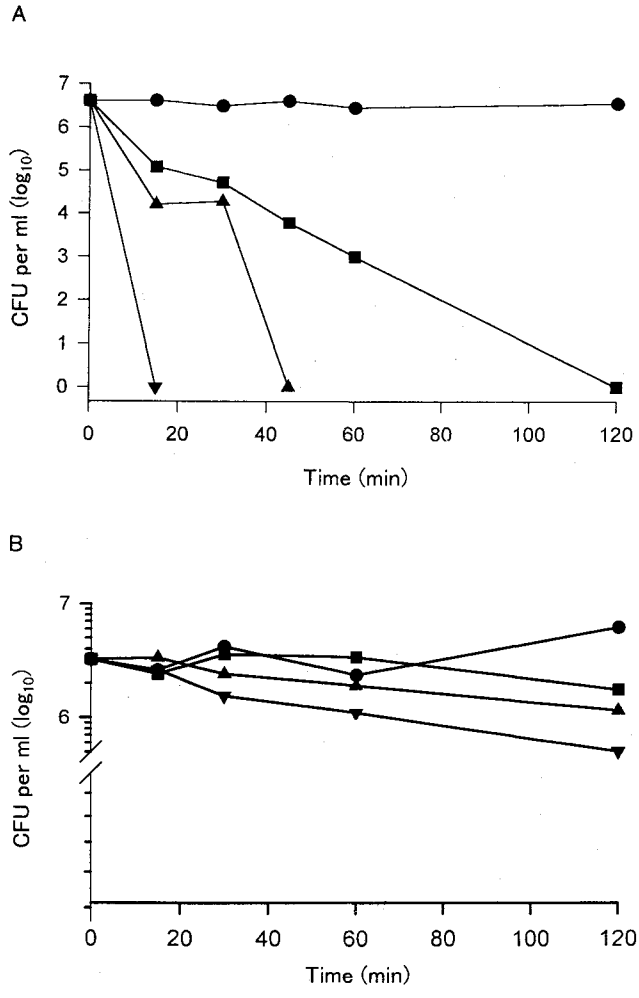


Fig. 2. Comparison of fungicidal activity of CAN-296 and amphotericin B—for *C. albicans*.

○ CAN-296; 5 μg/ml, □ CAN-296; 10 μg/ml, ◇ AMB; 4 μg/ml, △ AMB; 8 μg/ml.

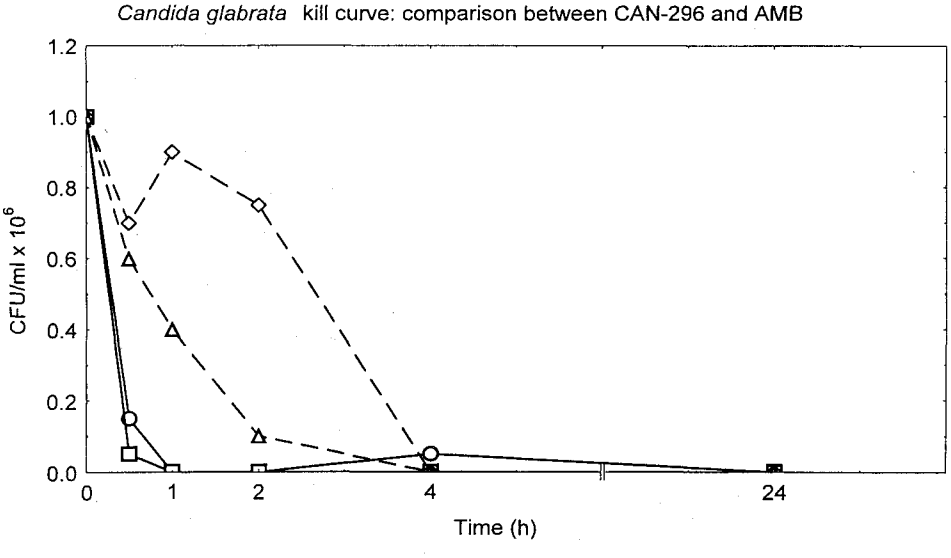
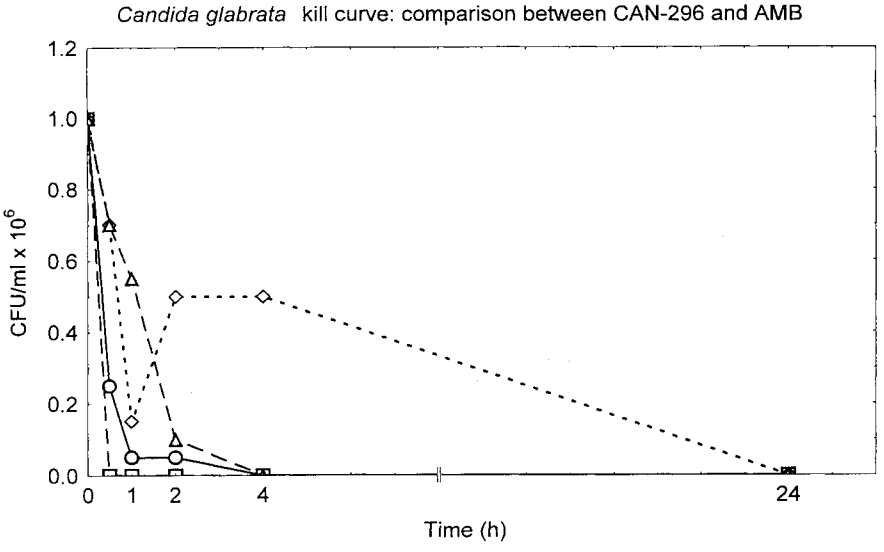


Fig. 3. Comparison of fungicidal activity of CAN-296 and amphotericin B—for *C. glabrata*.
○ CAN-296; 5 μg/ml, □ CAN-296; 10 μg/ml, ◇ AMB; 4 μg/ml, △ AMB; 8 μg/ml.



Discussion

The results of the present study demonstrate that CAN-296 is a highly active antifungal agent *in vitro*. Its broad anti-*Candida* activity is comparable to that of AMB. It possesses a very narrow therapeutic range (0.078～0.312 μg/ml) in all *Candida* species tested. The non-*albicans Candida* species were as susceptible as the *Candida albicans* strains. MICs did not differ between the azole-susceptible and the azole-resistant *Candida* species. When compared with L-733,560, CAN-296 was found to possess uniform activity against all the tested *Candida* species; in contrast L-733,560 demonstrated a much wider therapeutic range 0.02～1.56 μg/ml with the same species tested (Table 1).

Use of CAN-296 at concentrations of 8 and 16 fold greater than the MIC resulted in the killing of 99.6% and 99.9% of *Candida albicans* within 15 minutes of exposure, suggesting that CAN-296 is a rapidly acting fungicidal agent. The fungicidal activity of CAN-296 was more rapid than that of AMB in both *Candida* species.

The lack of marked activity of CAN-296 against *A. fumigatus* was in contrast to its impressive fungicidal activity demonstrated against *Candida* species. The reason for the relative resistance of *A. fumigatus* to CAN-296 is not clear. However, it is not unusual for an antibiotic that is highly active against one group of fungi to possess little activity against others[12].

In summary, CAN-296 demonstrates an excellent *in vitro* inhibitory antifungal and fungicidal spectrum. CAN-296 was active against azole-resistant *Candida*. Coupled with its *in vitro* stability and rapidity of action, this complex carbohydrate makes an excellent candidate for a next generation therapeutic agent. Further investigations of its *in vivo* activity and toxicity are warranted.

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