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Antifungal Mechanism of FK463 against *Candida albicans* and *Aspergillus fumigatus*

KAZUO HATANO*, YOSHIHIKO MORISHITA, TORU NAKAI and FUMIAKI IKEDA

Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd. 1-6, Kashima 2-chome, Yodogawa-ku, Osaka 532-8514, Japan

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Fungal infections, particularly deep-seated mycoses, have dramatically increased in frequency in recent decades due to a growing number of immunocompromised or neutropenic patients[1]. FK463 has potent anti-fungal activity against clinical important fungi[2~4]. In the present report, we studied antifungal mechanisms of action of FK463 using biochemical techniques.

Cell free 1,3-β-D-glucan synthesis by *Candida albicans* ATCC90028 (purchased from the American Type Culture Collection, VA, USA) and by *Aspergillus fumigatus* TIMM0063 (kindly gifted from the Teikyo University Institute of Medical Mycology, Tokyo, Japan) were studied by the methods described below. *C. albicans* was precultured in YPD broth-A [1% w/v of Bacto-peptone, 0.5% w/v yeast extract, 2% w/v glucose] at 35°C overnight. Preculture was inoculated into fresh same medium (inoculum=10% of total volume) and grown for 6 hours at 35°C with shaking. Cells were harvested by centrifugation at 4°C, washed once with ice-cold deionized water and then with buffer-A [50 mM Tris/HCl buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM 2-mercaptoethanol (2-ME), 1 M sucrose], and stored at −80°C until use. *A. fumigatus* was grown on potato-dextrose agar for 7 days at 35°C. Conidia suspension (approximately 10^8 cells/ml) was prepared with saline containing 0.2% Tween 80. Conidia were inoculated into YPD broth-A (inoculum=1% of total volume) and incubated for 24 hours at 35°C with shaking. Cells were harvested by filtration through Whatman filter paper, and washed with ice-cold deionized water, harvested by centrifugation at 4°C after resuspension with buffer-A, and then stored at −80°C until use. Both frozen cells were thawed by the tap water and suspended in buffer-A containing 25 μM guanosine 5′-triphosphate (GTP). Cell breakage was performed by sonication with 0.4 mm-diameter acid-washed glass beads at 4°C. Breakage was typically >80% as judged by light-microscopy. Cell debris, unbroken cells and glass beads were removed by low-speed centrifugation at 4°C, and supernatant was subjected to centrifugation at 35,000 rpm for 1 hour at 4°C. The pellet was resuspended in a small volume of buffer-B [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM 2-ME and 25 μM GTP] and centrifuged at 40,000 rpm for 1 hour at 4°C. The pellet was suspended in buffer-C [buffer-B : glycerol=2 : 1] and stored at −80°C until use as the membrane fraction. Protein content of the membrane fraction was measured by the Bradford method and adjusted to 4 mg/ml of protein, standardized with bovine serum albumin. 10 μl of membrane fraction was added to 10 μl of 5× reaction buffer [final concentration; 100 mM Tris/HCl (pH 7.0), 1 mM EDTA, 10 mM NaF, 4% glycerol, 0.1 mM GTP and 0.25% BSA]. A further 10 μl of FK463 (final concentration; for *C. albicans*: 0.4, 0.3, 0.2, 0.1 and 0 μM: for *A. fumigatus*: 0.016, 0.012, 0.008, 0.004 and 0 μM) was added. After 15 minutes incubation at room temperature (approximately 25°C), 20 μl of UDP-glucose (final concentration: 0.25, 0.5, or 1 mM) with 0.35 μCi/ml UDP-[^{14}C]-glucose was added and incubated for 60 minutes (*C. albicans*) or for 150 minutes (*A. fumigatus*) at room temperature. Reaction was stopped by addition of 100 μl of 5% of trichloroacetic acid (TCA), and the reaction mixture was filtered through a Whatman glass filter and washed with 5% TCA using a rapid filtration technique. Glass filters were air dried and radioactivities were measured with a liquid scintillation counter. Reaction velocity was defined as incorporation of UDP-glucose for 1 minute with 1 mg of membrane fraction and calculated by the formula described bellow.

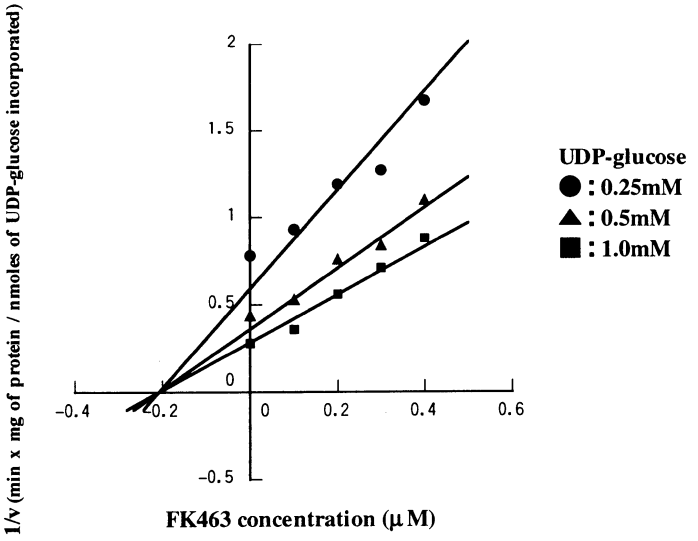
V=(concentration of UDP-glucose)×(reaction volume)×(dpm of incorporated UDP-[^{14}C]-glucose in reaction mixture)/(dpm of total amount of UDP-[^{14}C]-glucose in reaction mixture)/(incubation time: minutes)/(protein content of reaction mixture: mg)

Dixon plots of FK463 inhibition of 1,3-β-D-glucan synthesis in membrane fraction revealed that FK463 acts as a noncompetitive inhibitor, with an apparent *Ki* of 0.208 μM for *C. albicans* ATCC90028 and of 0.0158 μM for *A. fumigatus* TIMM0063 (Fig. 1 and 2).

Inhibition of chitin and mannan synthesis by FK463 was performed by the methods described below. 4 ml of preculture of *C. albicans* ATCC90028 was inoculated into 2 liters of YPD broth-B [1% yeast extract, 2% Bacto peptone

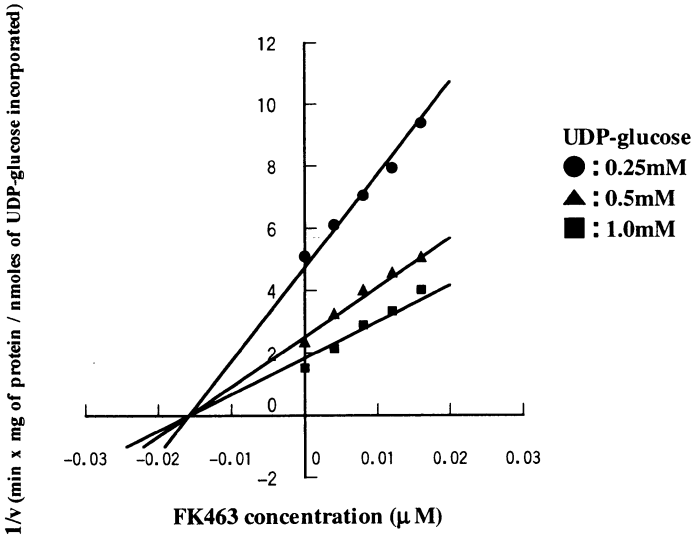
* Corresponding author: kazuo_hatano@po.fujisawa.co.jp

Fig. 1. Kinetics of FK463 inhibition against 1,3-β-D-glucan synthase in membrane fraction of *C. albicans* ATCC90028.



Glucan syntheses were run with different concentrations of UDP-glucose (ranging from 0.25 mM to 1 mM), with FK463 added at a final concentration of 0, 0.1, 0.2, 0.3 and 0.4 μM.

Fig. 2. Kinetics of FK463 inhibition against 1.3-β-D-glucan synthase in membrane fraction of *A. fumigatus* TIMM0063.



Glucan syntheses were run with different concentrations of DUP-glucose (ranging from 0.25 mM to 1 mM), with FK463 added at a final concentration of 0, 0.004, 0.008, 0.012 and 0.016 μM.

and 2% glucose], then grown at 30°C overnight. Cells were harvested by centrifugation (5,000 rpm for 5 minutes at 4°C) and washed with 1 mM EDTA. Cells were suspended in breaking buffer [0.5 M NaCl, 1 mM EDTA, 100 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin and 0.5 μg/ml leupeptin], and disrupted by sonication with 0.4 mm-diameter acid-washed glass beads at 4°C. Breakage was typically >80% as judged by light-microscopy. Cell debris, unbroken cells and glass beads were removed by filtration with Miracloth (Calbiochem, CA, USA) and then by low-speed centrifugation (3,000 rpm for 5 minutes at 4°C). The supernatant was centrifuged at 40,000 rpm for 30 minutes at 4°C. Precipitate was suspended in membrane buffer [50 mM Tris/HCl (pH 7.5), 10 mM EDTA, 1 mM 2-ME, 33% glycerol] and stored at −80°C until use as a membrane fraction. For the chitin synthesis activity and FK463 inhibition assay, a 1/100 volume of trypsin solution [10 mg/ml in 0.1 M Tris/HCl buffer (pH 7.5)] was added into membrane fraction and incubated at room temperature for 10 minutes, after which a 1/50 volume of trypsin inhibitor solution [10 mg/ml in 0.1 M Tris/HCl buffer (pH 7.5)] was added and incubated for 5 minutes on ice. 2.5 μl of FK463 solution (20, 200 or 2,000 μg/ml), 10 μl of trypsin-digested membrane fraction and 12.5 μl of reaction buffer [100 mM MES (pH 6.5), 20 mM $MgCl_2$] were transferred into wells of 96 well microplate and incubated for 10 minutes at room temperature. After that 25 μl of substrate solution [52.5 μl/ml UDP-[U-^{14}C]-*N*-acetylglucosamine, 0.6 mg/ml UDP-*N*-acetylglucosamine and 177 mg/ml *N*-acetylglucosamine] was added and incubated for 60 minutes at room temperature. Final concentrations of FK463 were 1, 10 or 100 μg/ml, respectively. Reaction was stopped by addition of 100 μl of 10% cold-TCA and reaction products were harvested using a 96 well cell harvester. Radioactivities were measured using a liquid scintillation counter. Nikkomycin X (made by Fujisawa Pharmaceutical Co., Ltd.) was used as a positive control in place of FK463. Nikkomycin X was shown to have an IC_{50} value of less than 0.1 μg/ml. Under the same conditions, FK463 was shown to have an IC_{50} value of over 100 μg/ml. For the mannan synthesis activity and FK463 inhibition assay, 2.5 μl of FK463 solution (20, 200 and 2,000 μg/ml), 10 μl of trypsin-digested membrane fraction and 12.5 μl of reaction buffer [0.1% Chaps, 0.05% Tween 80, 20 mM sodium cacodylate-HCl pH 6.5, 10 mM $MnCl_2$ and 1 mM dithiothreitol] were aliquoted into wells of a 96 well microplate and incubated for 15 minutes at room temperature. After that, 25 μl of substrate solution [GDP-[U-^{14}C]-mannose] was added and incubated for 60 minutes at room temperature. Final concentrations of FK463 were 1, 10 or 100 μg/ml, respectively. Reaction was stopped by addition of 100 μl of 10% cold-TCA and reaction products were harvested using a 96 well cell harvester. Radioactivities were measured using a liquid scintillation counter. The IC_{50} value of FK463 for mannan inhibition was over 100 μg/ml.

The nucleic acid synthesis activity and FK463 inhibition were measured by the method described below. Preculture of *C. albicans* ATCC90028 was incubated in GYS broth [0.4% glucose, 0.2% yeast extract and 0.8 M sorbitol] at 35°C overnight. Medium was changed by centrifugation and incubated for more 3 hours at 37°C with shaking. For determination of total nucleic acid (DNA plus RNA) synthesis and FK463 inhibition, 20 μl of FK463 solution (0.25, 2.5, 25, 250 or 2500 μg/ml) were added to 460 μl of prepared culture described above (approximately 4×10^7 cells/ml), and pre-incubated at 37°C for 5 minutes. 20 μl of [^{3}H]-adenine (2.5 μCi/ml) was added to the pre-incubated mixture and incubated at 37°C for 30 minutes with shaking. The reaction was stopped by addition of an equal volume of 10% TCA, and reaction products were harvested through a glass filters. Filters were washed with 5% TCA, air dried and radioactivities were measured in a liquid scintillation counter. For DNA synthesis and FK463 inhibition, the same manner of incorporation protocol was used. After 30 minutes incubation, an equal volume of 1 M KOH was added and incubated at 60°C for 2 hours, then 900 μl of 20% TCA was added and stored at 4°C overnight. Reaction products were harvested using glass filters. Filters were washed with 5% TCA, air dried and radioactivities were measured with a liquid scintillation counter. RNA synthesis was calculated by subtracting DNA synthesis from total nucleic acid synthesis. 5-Fluorocytosine (5-FC, Wako-junyaku, Osaka, Japan) was used as a positive control of DNA and RNA synthesis inhibition. FK463 was shown to have over 100 μg/ml IC_{50} values for DNA and RNA synthesis, while 5-FC, a DNA and RNA synthesis inhibitor, was shown to have IC_{50} values of 0.44 and 0.7 μg/ml, respectively.

The protein synthesis activity and FK463 inhibition were measured by the method described below. Preculture of *C. albicans* ATCC90028 was incubated in GYS broth at 35°C overnight. Medium was changed by centrifugation and incubated for more 3 hours at 37°C with shaking. 20 μl of FK463 solution (2.5, 25, 250 or 2500 μg/ml) was added to 460 μl of prepared culture described above (approximately 4×10^7 cells/ml), and pre-incubated at 37°C for 5 minutes. 20 μl of [^{3}H]-leucine (25 μCi/ml) was added into the pre-incubated mixture and incubated at 37°C for 30 minutes with shaking. Reaction was stopped by addition of an equal volume of preheated 10% TCA (approximately 60°C),

incubated at 90°C for 15 minutes, and then chilled on ice. Reaction products were harvested using glass filters. The filters were washed with 5% TCA, air dried and radioactivities were measured using a liquid scintillation counter. Blasticidin S (purchased from Kakenseiyaku, Tokyo, Japan) was used as a positive control of protein synthesis inhibition. FK463 was shown to have an IC_{50} value for protein synthesis over 100 μg/ml, while blasticidin-S, a protein synthesis inhibitor, was shown to have an IC_{50} value of 0.02 μg/ml.

Antifungal agents commercially available or on development can be classified into the following categories: 1) membrane-active compounds (*e.g.*, polyenes); 2) ergosterol biosynthesis inhibitors (*e.g.*, azoles); 3) nucleic acid biosynthesis inhibitors (*e.g.*, 5-FC); 4) protein synthesis inhibitors (*e.g.*, sordarin derivatives); and 5) cell wall-active compounds including β-glucan biosynthesis inhibitors (*e.g.*, echinocandins), chitin biosynthesis inhibitors (*e.g.*, nikkomycins), and mannan biosynthesis inhibitors (*e.g.*, benanomycin A). Results from the present study suggest that the antifungal action of FK463 against *C. albicans* is not due to inhibition of nucleic acid biosynthesis nor protein biosynthesis, nor chitin or mannan biosynthesis. FK463 inhibits UDP-glucose incorporation into glucan polymers in a concentration-dependent manner. Its enzymological kinetics of inhibition indicates a non-competitive inhibition pattern from Dixon plots using several concentrations of substrate (0.25 mM, 0.5 mM and 1.0 mM of UDP-glucose). BEAULIEU[5], also using Dixon plot analysis, reported that inhibition kinetics of cilofungin, an echinocandin derivative, against 1,3-β-D-glucan synthase derived from *A. fumigatus* works in a non-competitive manner. Furthermore, other analysis methods such as Lineweaver-Burk plot and Hanes-Wolff plots, of pneumocandin derivatives and echinocandin derivatives on 1,3-β-D-glucan synthesis indicate non-competitive patterns of inhibition[6~8]. 1,3-β-D-Glucan synthase has been reported to be an enzyme complex composed of at least 2 components, the catalytic subunit Fks1p/Fks2p[9~11] (genome code: *FKS1*/*FKS2*) and soluble regulatory subunit Rho1p GTPase[12,13] (genome code: *RHO1*). The detailed antifungal mechanism of FK463 has not yet been completely elucidated. To determine its mechanism of action it will be necessary in the future to define the exact antifungal activity of FK463 on a molecular basis.

References

1) WADE, J. C. : Treatment of fungal and other opportunistic infections in immunocompromised patients. Leukemia 11 (Suppl. 4): S38~39, 1997

2) TAWARA, S.; F. IKEDA, K. MAKI, Y. MORISHITA, K. OTOMO, N. TERATANI, T. GOTO, M. TOMISHIMA, H. OHKI, A. YAMADA, K. KAWABATA, H. TAKASUGI, K. SAKANE, H. TANAKA, F. MATSUMOTO & S. KUWAHARA: *In vitro* activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. Antimicrob. Agents Chemother. 44: 57~62, 2000

3) IKEDA, F.; Y. WAKAI, S. MATSUMOTO, K. MAKI, E. WATABE, S. TAWARA, T. GOTO, Y. WATANABE, F. MATSUMOTO & S. KUWAHARA: Efficacy of FK463, a new lipopeptide antifungal agent, in mouse models of disseminated candidiasis and aspergillosis. Antimicrob. Agents Chemother. 44: 614~618, 2000

4) MATSUMOTO, S.; Y. WAKAI, T. NAKAI, K. HATANO, T. USHITANI, F. IKEDA, S. TAWARA, T. GOTO, F. MATSUMOTO & S. KUWAHARA: Efficacy of FK463, a new lipopeptide antifungal agent, in mouse models of pulmonary aspergillosis. Antimicrob. Agents Chemother. 44: 619~621, 2000

5) BEAULIEU, D.; J. TANG, S. B. YAN, J. M. VESSELS, J. A. RADDING & T. R. PARR, Jr.: Characterization and cilofungin inhibition of solubilized *Aspergillus fumigatus* (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. 38: 937~944, 1994

6) TAFT, C. S.; T. STARK & C. P. SELITRENNIKOFF: Cilofungin (LY121019) inhibits *Candida albicans* (1,3)-β-D-glucan synthase activity. Antimicrob. Agents Chemother. 32: 1901~1903, 1988

7) TANG, J. & T. R. PARR, Jr. :W-1 solubilization and kinetics of inhibition by cilofungin of *Candida albicans* (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. 35: 99~103, 1991

8) KURTZ, M. B.; I. B. HEATH, J. MARRINAN, S. DREIKORN, J. ONISHI & C. DOUGLAS: Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. 38: 1480~1489, 1994

9) DOUGLAS, C. M.; J. A. D'IPPOLITO, G. J. SHEI, M. MEINZ, J. A. MARRINAN, W. LI, G. K. ABRUZZO, A. FLATTERY, K. BARTIZAL, A. MITCHELL & M. B. KURTZ: Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-β-D-glucan synthase inhibitors. Antimicrob. Agents Chemother. 41: 2471~2479, 1997

10) MIO, T.; M. ADACHI-SHIMIZU, Y. TACHIBANA, H. TABUCHI, S. B. INOUE, T. YABE, T. YAMADA-OKABE, M. ARISAWA, T. WATANABE & H. YAMADA-OKABE: Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae GSC1*/*FKS1* and its involvement in β-1,3-glucan synthesis. J. Bacteriol. 179: 4096~4105, 1997

11) DOUGLAS, C. M.; F. FOOR, J. A. MARRINAN, N. MORIN, J. B. NIELSEN, A. M. DAHL, P. MAZUR, W. BAGINSKY, W. LI, M. EL-SHERBEINI, J. A. CLEMAS, S. M. MANDALA, B. R. FROMMER & M. B. KURTZ: The *Saccharomyces cerevisiae FKS1*(*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA 91: 12907~12911, 1994

12) KONDOH, O.; Y. TACHIBANA, Y. OHYA, M. ARISAWA & T. WATANABE: Cloning of the *RHO1* gene from *Candida albicans* and its regulation of β-1,3-glucan synthesis. J. Bacteriol. 179: 7734~7741, 1997

13) MAZUR, P. & W. BAGINSKY: *In vitro* activity of 1,3-β-D-glucan synthase requires the GTP-binding protein Rho1. J. Biol. Chem. 271: 14604~14609, 1996