In vitro Synergistic Effects of Anthracycline Antitumor Agents and Fluconazole Against Azole-Resistant Candida albicans Clinical Isolates

Matsumoto S, Kurakado S, Shiokama T, Ando Y, Aoki N, Cho O and Sugita T*

Department of Microbiology, Meiji Pharmaceutical University, Kiyose, Tokyo Japan

Abstract

The number of azole-resistant Candida albicans clinical isolates is increasing. This study searched for compounds that are functionally synergistic with fluconazole against azole-resistant C. albicans strains. Synergistic effects were evaluated using the checkerboard method in a time-kill study using anthracycline antitumor agents and azole-resistant C. albicans strains. Of the five anthracycline agents examined, aclarubicin by itself had antifungal effects, whereas daunorubicin, doxorubicin, epirubicin, and idarubicin did not show antifungal effects alone, but did exert dose- and time-dependent synergistic effects with fluconazole against the C. albicans strains. No antitumor agent other than anthracycline exhibited an anti-Candida effect. Anthracycline compounds may therefore be useful as seeds for development of new antifungal agents.

Keywords: Anthracycline; antitumor agent; azole-resistant Candida albicans; synergy

Introduction

Candida albicans is the most frequently observed opportunistic fungal pathogen and causes deep-seated fungal infection, mainly in immune compromised hosts such as cancer patients, transplant recipients, or patients with human immunodeficiency virus (HIV) infection [1]. Infection by this microorganism is often life threatening; however, antifungal therapy remains limited. Presently, only four chemical classes are available commercially for treating deep-seated fungal infection: polyenes, azoles, echinocandine, and nucleic acid analogs [2]. The first two inhibit the biosynthesis of cell membranes in fungal cells, whereas echinocandine inhibits synthesis of the cell wall. Worldwide, azole agents are the most widely used agents for treating deep-seated candidiasis; however, an increase in the number of azole-resistant C. albicans strains is causing problems in the treatment of candidiasis [3]. The number of available antifungal agents is very small compared with the numbers of antibacterial and antiviral agents. This is because it is difficult to identify unique targets not shared with the human host, as the fungal cell is eukaryotic, like human cells. Consequently, research on compounds that have synergistic effects with azole agents has flourished. Calcineurin inhibitors [4-7], HMG-CoA reductase inhibitors (statins) [8,9], and non-steroidal anti-inflammatory drugs (NSAIDs) are representative examples [10,11]

Low-molecular-weight antitumor agents are divided into six classes based on their mechanism of action: antimetabolites, alkylating agents, topoisomerase inhibitors, microtubule inhibitors, microtubule depolymerizing agents, and molecular target agents. Topoisomerase is an enzyme that plays roles in the processes of DNA cleavage and recombination and is divided into two classes, I and II, based on the mode of DNA cleavage. Topoisomerase I cleaves one strand of a duplex DNA molecule, whereas topoisomerase II cleaves both strands of the DNA molecule [12]. Topoisomerase inhibitors are further classified into four classes chemically: camptothecins, anthracyclines, epipodophyllotoxins, and quinolones. Of these, camptothecin targets topoisomerase I, whereas the others target topoisomerase II. Several studies suggested that topoisomerase might be a target for antifungal drugs [13-15]. As an example, aclarubicin, an anthracycline antitumour agent, inhibited the growth of C. albicans at low concentrations [16]. In this study, we found that anthracycline antitumor agents have synergistic effects with fluconazole against azole-resistant C. albicans strains.

Materials and Methods

Strains used

Twelve azole-resistant Candida albicans strains obtained from patients’ blood were examined in this study. All strains were cultured on Sabouraud dextrose agar (SDA) plates at 37°C.

Reagents

Fluconazole and five anthracycline antitumor agents (aclarubicin, daunorubicin, doxorubicin, epirubicin, and idarubicin) were examined. The chemical structures of the anthracycline antitumor agents are shown in (Figure 1). Additionally, the antitumor agents camptothecin, irinotecan, etoposide, paclitaxel, vincristine, and methotrexate were investigated for comparison. All reagents were purchased from Wako Pure Chemical (Osaka, Japan) and were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL. They were stored at -20°C in the dark until use.

Susceptibility testing

The minimum inhibitory concentrations (MICs) of fluconazole and the antitumor agents for the C. albicans strains were determined according to the method described in the Clinical and Laboratory Standards Institute (CLSI) guideline M27-A3 [17]. Briefly, a cell suspension of each strain was diluted in 3-[(N-morpholino) propane sulfonic (MOPS)-buffered RPMI 1640 medium to a final inoculum ranging between 0.5×10^7 and 2.5×10^7 cfu/mL. Serial two-fold dilutions were also made in the MOPS-buffered RPMI 1640 medium. A total volume of 200 μL of drug plus cell suspension was placed in each well of 96-well micro titration plates, and the plates were incubated at 35°C for

*Corresponding author: Takashi Sugita, Department of Microbiology, Meiji Pharmaceutical University, Kiyose, Tokyo Japan, Tel: +81-424-95-8762; E-mail: sugita@my-pharm.ac.jp

Received July 07, 2014; Accepted October 06, 2014; Published October 16, 2014

Citation: Matsumoto S, Kurakado S, Shiokama T, Ando Y, Aoki N, et al. (2014) In vitro Synergistic Effects of Anthracycline Antitumor Agents and Fluconazole Against Azole-Resistant Candida albicans Clinical Isolates. J Develop Drugs 3: 125. doi:10.4172/2329-6631.1000125

Copyright: © 2014 Matsumoto S, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
48 h. The MIC was defined as the lowest concentration of the agent that substantially inhibited the growth of Candida. A final concentration of 2% (v/v) DMSO was included in the wells; this concentration was found to not affect the viability of the Candida strains. The experiment was performed in triplicate.

**Checkerboard test**

The combinations antitumor agent and fluconazole were studied using the checkerboard method. Each antitumor agent was serially diluted two-fold in MOPS-buffered RPMI 1640. The final drug concentrations ranged from 32 to 0.03125 µg/mL for fluconazole and 50 to 0.0244 µg/mL for the antitumor agents. Each 50 µL dilution of fluconazole and antitumor agent was added to a well on a micro titer plate. A 100 µL suspension of the C. albicans strain was added to each well, and the plate was incubated at 35°C for 48 h. The MIC of both compounds in combination was determined in the same manner as the susceptibility testing described above. The fractional inhibitory concentration (FIC) and FIC index (FICI) were determined to assess the synergistic activity of the drug combinations. The FIC was calculated by dividing the MIC of the combination of fluconazole and antitumor agent by the MIC of fluconazole or the antitumor agent alone. The FICI was calculated by adding both FICs. Synergism and antagonism were defined as an FICI ≤ 0.5 and >4, respectively. Intermediate values were considered indifferent [18]. The number of colony-forming units (cfu) in each well was also determined. A 100-µL sample was removed from the well and serially diluted 10-fold in sterile saline solution, and the solution was subsequently plated onto SDA. The fungi static effect was considered indifferent [18]. The number of colony-forming units (cfu) in each well was also determined. A 100-µL sample was removed from the well and serially diluted 10-fold in sterile saline solution, and the solution was subsequently plated onto SDA. A 100 µL suspension of the C. albicans strain was added to each well.

**Time-kill curves analysis**

Time-kill curves were plotted for the combination of fluconazole and aclarubicin or daunorubicin against three C. albicans strains (strains 36, 40, 42). Each experiment was conducted for six concentration groups of culture tubes: control (no drug), 1/4×MIC, 1/2×MIC, 1×MIC, 2×MIC, and 4×MIC against fluconazole. Then, 1×MIC of aclarubicin or daunorubicin was added to each tube, except for the control group. Consequently, the fluconazole concentrations varied in the wells, while the concentrations of the antitumor agents were kept constant [19]. A 100 µL sample was removed from the tube and serially diluted 10-fold in sterile saline solution, and the solution was subsequently plated onto SDA. The experiment was performed in triplicate for each strain.

**Results**

**Checkerboard method**

Aclarubicin inhibited the growth of C. albicans strains at MICs of 6.25-25 µg/mL, whereas no growth inhibition was observed for the other 10 compounds at a concentration of 200 µg/mL (Supplementary Table 1). Of the 10 compounds, the MICs of aclarubicin, daunorubicin, doxorubicin, epirubicin, and idarubicin against the microorganisms were between 0.098 and 25 µg/mL in the presence of fluconazole, which was at concentrations of 0.125-4 µg/mL, with an FICI of 0.0032-0.0781. The remaining six compounds did not show any effect on the growth of C. albicans. The inhibitory effect of aclarubicin increased 100–200 times in combination with fluconazole. Aclarubicin and daunorubicin in combination with fluconazole showed fungicidal activity, whereas the other three anthracycline compounds were fungicidal [20].

**Time-kill curves analysis**

As aclarubicin and daunorubicin had high synergistic effects in combination with fluconazole, time-kill-curve analysis was performed for the two compounds. Representative time-kill curves are shown in (Figure 2) using strain 36. At concentrations of 1×MIC, 2×MIC, and 4×MIC, both compounds were fungicidal against azole-resistant strains after 48 h in a concentration and time-dependent manner. After 24 h, the viable count at 1×MIC, 2×MIC, and 4×MIC was reduced by 1×10^2, 8×10^3, and 1×10^4, respectively, for aclarubicin and by 9×10^2, 7×10^4, and 4×10^5 for daunorubicin. Aclarubicin inhibited the growth of the microorganism more rapidly than did daunorubicin. Among the three strains analyzed in this study, no remarkable differences were found.

**Discussion**

We found that anthracycline antitumor agents have a synergistic effect with fluconazole against azole-resistant C. albicans strains. Several studies have suggested that DNA topoisomerase inhibitor aclarubicin at 0.8-7.3 µg/mL inhibited the growth of C. albicans in vitro, whereas other inhibitors, including daunorubicin, doxorubicin, idarubicin, beta-lapachone, camptothecin, irinotecan, topotecan, etoposide, and mitoxantrone, did not inhibit the growth. Nevertheless, the first four of these compounds affected the morphology of C. albicans. In the present study, only the DNA topoisomerase inhibitor anthracycline was found to have inhibitory or synergistic effects against azole-resistant C. albicans strains. Chemical structure may play a significant role in the uptake of compounds into fungal cells. Of the anthracycline compounds, only aclarubicin had anti-Candida activity when used alone, with MICs of 6.25-25 µg/mL. Anthracyclines are glycosides. Aclarubicin is a topoisomerase inhibitor that is more rapidly absorbed into fungal cells than the other anthracyclines are (Figure 1). This structural difference may also influence the uptake of these compounds into fungal cells. However, the synergistic effects of aclarubicin and daunorubicin with fluconazole were similar, i.e., the FICIs of aclarubicin and daunorubicin were 0.0083-0.0162 and 0.0083-0.0162, respectively, for aclarubicin and daunorubicin.
0.0032-0.0256, respectively. Fluconazole is known to have synergistic effects with several compounds, including calcineurin inhibitors [4-7], HMG-CoA reductase inhibitors (statins) [8,9], and non-steroidal anti-inflammatory drugs [10, 11]. In addition to their synergistic effect with fluconazole, anthracyclines have a unique function. Phospholipase B and secretory aspartyl protease are major virulence factors. Of these, anthracycline compounds inhibit the activity of phospholipase B in a dose-dependent manner [21]. Doxorubicin also inhibits the replication of HIV [22], herpes simplex virus [23], dengue virus, and yellow fever virus [24]. Novel antimicrobial agents might be developed using anthracycline as the lead compound.

Acknowledgement

This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan (H26-shinkoujitsuyoka-ippan-010).

References

1. Pfaller MA, Diekema DJ (2007) Epidemiology of Invasive Candidiasis: a Persistent Public Health Problem. Clin Microbiol Rev 20: 133-163.
2. Thompson GR III, Cadena J, Patterson TF (2009) Overview of antifungal agents. Clin Chest Med 0: 203-215.
3. Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, et al. (2009) Efflux-mediated antifungal drug resistance. Clin Microbiol Rev 2: 291-321.
4. Marchetti O, Moreillon P, Glaser M, Bille J, Sanglard D (2000) Potent synergism of the combination of fluconazole and cyclosporine in Candida albicans. Antimicrob Agents Chemother 44: 2373-2381.

5. Maesaki S, Marichal P, Hossain MA, Sangiadi D, Vanden Bossche H, et al. (1998) Synergetic effects of tacrolimus and azole antifungal agents against azole-resistant Candida albicans strains. J Antimicrob Chemother 42: 747-753.
6. Uppuluri P, Nett J, Heitman J, Andes D (2008) Synergistic effect of calcineurin inhibitors and fluconazole against Candida albicans biofilms. Antimicrob Agents Chemother 52: 1127-1132.
7. Del Poeta M, Cruz MC, Cardenas ME, Perfect JR, Heitman J (2000) Synergistic antifungal activities of bafilomycin A(1), fluconazole, and the pneumocandin MK-0991/caspofungin acetate (L-743,873) with calcineurin inhibitors FK506 and L-685,818 against Cryptococcus neoformans. Antimicrob Agents Chemother 44: 739-746.
8. Nash JD, Burgess DS, Talbert RL (2002) Effect of fluvastatin and pravastatin, HMG-CoA reductase inhibitors, on fluconazole activity against Candida albicans. J Med Microbiol 51: 105-109.
9. Song JL, Lyons CN, Holleman S, Oliver BG, White TC (2003) Antifungal activity of fluconazole in combination with lovastatin and their effects on gene expression in the ergosterol and prenylation pathways in Candida albicans. Med Mycol 41: 417-425.
10. Pina-Vaz C, Sansonetto F, Rodrigues AG, Martinez-De-Oliveira J, Fonseca AF, et al. (2000) Antifungal activity of ibuprofen alone and in combination with fluconazole against Candida species. J Med Microbiol 49: 831-840.
11. Aral R, Sugita T, Nishikawa A (2005) Reassessment of the in vitro synergistic effect of fluconazole with the non-steroidal anti-inflammatory agent ibuprofen against Candida albicans. Mycoses 48: 38-41.
12. Shen LL, Baranowski J, Fostel J, Montgomery DA, Larney PA (1992) DNA topoisomerases from pathogenic fungi: targets for the discovery of antifungal drugs. Antimicrob Agents Chemother 36: 2775-2784.
13. Fostel JM, Montgomery DA, Shen LL (1992) Characterization of DNA topoisomerase I from Candida albicans as a target for drug discovery. Antimicrob Agents Chemother 36: 2131-2138.
14. Del Poeta M, Toffaletti DL, Rude TH, Dykstra CC, Heitman J, et al. (1999) Topoisomerase I is essential in Cryptococcus neoformans: role in pathobiology and as an antifungal target. Genetics 152: 167-178.
15. Goto T, Wang JG (1985) Cloning of yeast TOP1, the gene encoding DNA topoisomerase I, and construction of mutants defective in both DNA topoisomerase I and DNA topoisomerase II. Proc Natl Acad Sci USA 62: 7178-7182.
16. Kwon SC, Schelenz S, Wang X, Steverding D (2010) In vitro effect of DNA topoisomerase inhibitors on Candida albicans. Med Mycol 48: 155-160.
17. National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard M27-A3. Wayne, PA, National Committee for Clinical Laboratory Standard 2002.
18. Odds FC (2003) Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemotherapy 52: 1.
19. Kofla G, Turner V, Schulz B, Storch U, Froehlich D, et al. (2011) Doxorubicin induces drug efflux pumps in Candida albicans. J Antimicrob Chemother 69: 134-142.
20. Cruz MC, Goldstein AL, Blankenship JR, Del Poeta M, Davis D, et al. (2002) Calcineurin is essential for survival during membrane stress in Candida albicans. EMBO J 21: 546-559.
21. Aral R, Sugita T, Nishikawa A (2004) The anthracycline antitumor agents doxorubicin and daunorubicin reduce the activity of Candida albicans phospholipase B. Microbiol Immunol 48: 695-697.
22. Jeyeselam R, Kuraboyashi M, Kedes L (1996) Doxorubicin inhibits Tat-dependent translaction of HIV type 1 LTR. AIDS Res Hum Retroviruses 2: 569-576.
23. Ash RJ, Diekema KA (1987) Inhibition of herpes simplex virus replication by anthracycline compounds. Antiviral Res 8: 71-83.
24. Kaptein SJ, De Burghgraeve T, Froeyen M, Pastoorino B, Alten MM, et al. (2010) A derivate of the antibiotic doxorubicin is a selective inhibitor of dengue and yellow fever virus replication in vitro. Antimicrob Agents Chemotherapy 54: 5269-5280.