Journal of Developing Drugs
ISSN: 2329-6631

Matsumoto et al., J Develop Drugs 2014, 3:2
DOI: 10.4172/2329-6631.1000125

Research Article Open Access

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

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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 anthrax cycline antitumor 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 0.5×10^3 and 2.5×10^3 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

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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

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 by 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 defined as a reduction in cfu/mL from the starting inoculum of <99.9%. The experiment was performed in triplicate.

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 fungi static [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^{-3}, respectively, for aclarubicin and by 9×10^{-2}, 7×10^{-2}, and 4×10^{-2} 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 is a potential target of antifungal agents [12-15,16] found that the 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 trisaccharide, whereas the other anthracyclines are monosaccharides (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

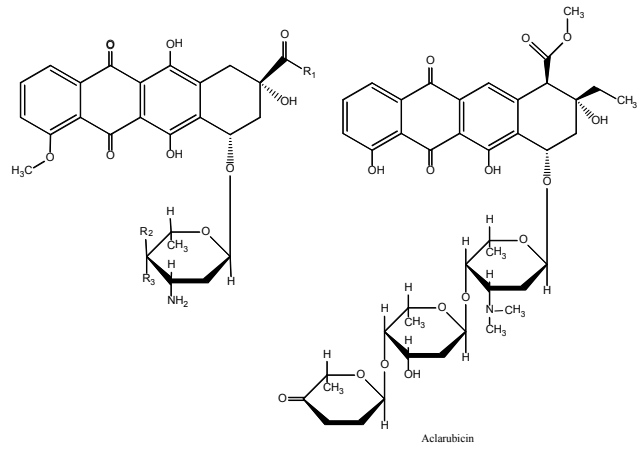

Figure 1: Chemical structures of the anthracycline antitumor agents.

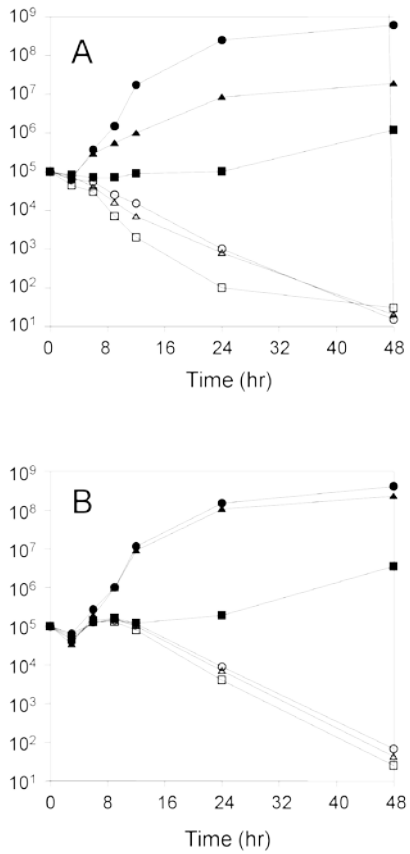

Figure 2: Representative time-kill curves of A) aclarubicin or B) daunorubicin in combination with fluconazole against azole-resistant *Candida albicans* isolate 36. Closed circles, control; solid triangles, 1/4×MIC; solid squares, 1/2×MIC; open circles, 1×MIC; open triangles, 2×MIC; open squares, 4×MIC.

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).

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