Emodin Reduces the Activity of (1,3)-β-D-glucan Synthase from Candida albicans and Does Not Interact with Caspofungin

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Abstract

Candidiasis is the most common opportunistic yeast infection, with Candida albicans as a paramount causative species. (1,3)-β-D-glucan is one of the three main targets of clinically available antifungal agents used to treat Candida infections. It is one of the most abundant fungal cell wall components. Echinocandins represent the newest class of antifungals affecting cell wall biosynthesis through non-competitive inhibition of (1,3)-β-D-glucan synthase. Therefore, treatment with echinocandins causes defects in fungal cell integrity. In the present study, similar activity of emodin (6-methyl-1,3,8-trihydroxyanthraquinone) has been revealed. Many reports have already shown the antifungal potential of this pleiotropic molecule, including its activity against C. albicans. The aim of this report was to evaluate the activity of emodin towards a new molecular target, i.e. (1,3)-β-D-glucan synthase isolated from Candida cells. Moreover, given the identical mechanism of the activity of both molecules, interaction of emodin with caspofungin was determined. The study revealed that emodin reduced (1,3)-β-D-glucan synthase activity and increased cell wall damage, which was evidenced by both a sorbitol protection assay and an aniline blue staining assay. Furthermore, the synergy testing method showed mainly independence of the action of both tested antifungal agents, i.e. emodin and caspofungin used in combination.

Key words: Candida albicans, caspofungin, echinocandins, emodin, (1,3)-β-D-glucan synthase

Introduction

Candidiasis is one of the most prevalent superficial and deep-seated fungal infections in humans and, as such, a major global health problem, which is additionally associated with a high mortality rate. The most pervasive and problematic cause of infections of all Candida species is Candida albicans – a part of the commensal microbiota of more than half of the healthy population. It is a cause of both opportunistic and invasive fungal infections (Pfaller and Diekema 2007; Sardi et al. 2013). Yeast infections frequently develop in immunocompromised patients with AIDS, cancer, and neutropenia as well as those receiving immunosuppressive and antibiotic therapy (Canela et al. 2018). Recent reports indicate that Candida infections are often associated with bipolar disorder and schizophrenia (Sverance et al. 2016). The pathogenicity of Candida species is supported by a wide range of virulence factors and fitness attributes, such as biofilm formation, polymorphism, thigmotropism, phenotypic switching, secretion of hydrolytic enzymes, quick adaptation to fluctuations in environmental pH, metabolic flexibility, and strong stress response mechanisms (Mayer et al. 2013; Martins et al. 2014).

Due to the similarity of human and fungal cells, discovery of selective antifungal drugs is extremely difficult. Nevertheless, there are some elements differentiating both types of cells. One of them is the cell wall that does not exist in mammalian cells. (1,3)-β-D-glucan is the main polysaccharide in the fungal cell wall. It is synthesized in the fungal cell by glucan synthase located in the cell membrane. This enzyme is regarded as a molecular target in the search for compounds with potential antifungal activity. Echinocandins, the current antifungal drugs are the inhibitors of (1,3)-β-D-glucan synthase (Denning 2003).

Echinocandins represented by anidulafungin, caspofungin, and micafungin target the synthesis of (1,3)-β-D-glucan polymers through non-competitive...
inhibition of the glucan synthase enzyme. Inhibition of a fungal-specific target by these antibiotics leads to defects in fungal cell wall integrity (Pianalto and Alsophaugh 2016).

Despite the effectiveness of echinocandins in the control of many fungal infections, antifungal resistance and defensive mechanisms resulting from the use of these drugs have been observed in fungal cells. One of the beneficial alternatives for potentiating the antifungal drugs is combination therapy comprising an antibiotic with a natural product (Zacchino et al. 2017). A combination of several medicinal substances can significantly improve the therapeutic properties and reduce the effective concentration of antibiotics while eliminating their side effects (Martins et al. 2014; Singh and Yeh 2017).

In the light of these facts, the medicinal potential of phytochemicals, their synergistic action with antifungal agents, and their interrelated mechanisms of action have been extensively studied (Kanafani and Perfect 2008; Sher 2009; Agarwal et al. 2010). Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) is one of the most promising natural compounds. Many reports regard emodin as a plant component with antioxidant, antibacterial, antiviral, antimutagenic, antitumor, and immunosuppressive properties (Shrimali et al. 2013; Dong et al. 2016). The latest research on the biological activity of this compound points to its anti- C. albicans activity (Kong et al. 2009; Janeczko et al. 2017).

The aim of the present study was to evaluate the inhibitory properties of emodin against (1,3)-β-D-glucan synthase from C. albicans cells. Moreover, the influence of the compound on C. albicans cell wall destruction was evidenced with the use of a sorbitol protection assay and aniline blue staining. A checkerboard microtiter plate assay was also used to determine the FICI (Fractional Inhibitory Concentration Index) in order to evaluate the combined activities of emodin and caspofungin against C. albicans strains by determining the FICI (Fractional Inhibitory Concentration Index).

**Experimental**

**Materials and Methods**

*C. albicans strains*. The experiments were performed on C. albicans reference strain ATCC 10231. Additionally, 20 clinical strains isolated from urinary tracts and 20 clinical strains isolated from reproductive systems of gynecological patients of Jan Boży Independent Public Provinicial Hospital in Lublin, Poland, were included in this study. The strains were identified using VITEK 2 YST IC CARDS (Biomerieux).

**Determination of Minimal Inhibitory Concentrations (MICs)**. The MICs of emodin (Sigma-Aldrich, USA), caspofungin (Sigma-Aldrich, USA), and amphotericin B (Sigma-Aldrich, USA) were determined with the broth dilution method as recommended by CLSI, with some modifications (CLSI 2017). Two-fold serial dilutions of emodin (0.8–400 μg/ml) or antibiotics (0.015–10 μg/ml) were prepared in 96-well microtiter plates using RPMI-1640 medium (with L-glutamine and phenol red, without bicarbonate) (Sigma-Aldrich, USA) buffered with 0.165 M 3-(N-morpholino)propane sulfonic acid (MOPS) (Sigma-Aldrich, USA). Adjacent wells of the microtiter plates contained 100 μl of each dilution. The inoculum was prepared by dilution of C. albicans cells with RPMI. The turbidity of this suspension was adjusted to 1–5 × 10^3 at a 530 nm wavelength. After addition of 20 μl of the inoculum to the wells, the plates were incubated at 37°C for 48 hours. 100 μl of uninoculated medium was included as a sterility control (blank). The MIC was taken as the lowest concentration of disinfectant that inhibits fungal growth. The experiments were performed in triplicate.

**Sorbitol protection assay**. The sorbitol protection assay was carried out to determine the effect of emodin on the destabilization of the fungal cell membrane. To this end, duplicate plates containing either emodin or amphotericin B and caspofungin were prepared as controls. One plate from each pair contained only the substance tested and the other plate contained an adequate antifungal and, additionally, 0.8 M sorbitol as an osmotic protectant (Frost et al. 1995). MICs for each trial were determined by the modified CLSI protocol as described above. Each assay was performed in triplicate.

**Preparation and quantification of (1,3)-β-D-glucan synthase.** (1,3)-β-D-glucan synthase from C. albicans cells (ATCC 10231) was prepared using the method proposed by Shedletzky et al. (1997) with some modifications described by Lee and Kim (2016). The enzyme was isolated from C. albicans cells cultivated in 11 of Sabouraud Dextrose Agar Broth (Biocorp, Poland) at 37°C for 16 h. The cells were homogenized in a Bead Beater (Minilys Homogenizer, Bertin Instruments) in 12 cycles of 1 min with 0.5-mm acid-washed glass beads. The protein concentration in the microsome and membrane fraction was measured using the Bradford method in accordance with the manufacturer’s instructions (Sigma-Aldrich, USA). The (1,3)-β-D-glucan synthase assay was performed according to the method developed by Frost et al. (1995) and modified by Lee and Kim (2016). The glucans stained specifically with aniline blue solution (0.1%) were a measure of the enzyme activity. Fluorescence was measured using a spectrofluorometer (Pharmacia Biotech) at 400-nm excitation and 460-nm emission wavelengths. The effect of emodin on the enzyme activity was determined at concentrations corresponding to the MIC/4, MIC/2, MIC, and 2 × MIC, and DMSO was used as a control.
The assays were performed in triplicate in three independent experiments.

Aniline blue staining of (1,3)-β-glucan in the C. albicans cell wall. The aniline blue staining method and fluorescence microscopy were used to visualize the effect of emodin and caspofungin on (1,3)-β-D-glucans in the C. albicans (ATCC 10231) cell walls. The yeast cells at the exponential phase were harvested by centrifugation at 4500 \( \times g \) at 4°C for 5 min. Next, the cells were washed twice and resuspended in 0.85% NaCl. Emodin at concentrations corresponding to MIC/2 and MIC/4, caspofungin at MIC/2, and 1% DMSO as control were added to the cell suspensions and incubated at 37°C for 10 h. The cells were harvested and washed in 0.85% NaCl; next, the cell density of each experimental group was adjusted to 1 x 10^6 cells/ml and the cells were resuspended in an aniline blue solution (0.1%). The samples were stained at 50°C for 30 min. A drop of each suspension was squashed between the microscope slide and the cover glass. The preparation was sealed and examined in a fluorescence microscope under UV illumination (Nicon). Images were taken with a cooled monochrome camera.

Caspofungin – emodin combination assay (a checkerboard method). Interactions between caspofungin and emodin were measured by calculation of the fractional inhibitory concentration index (FICI). A total of 100 µl of RPMI-1640 medium was distributed into each well of the microdilution plates. The first antibiotic of the combination – caspofungin was serially diluted along the abscissa at a concentration range of 0–100 µg/ml while the other drug – emodin was diluted along the ordinate at a concentration range of 0–1.2 µg/ml. The inoculum was prepared from C. albicans in RPMI-1640 medium as described in the MIC assay. Each microtiter well was inoculated with 20 µl of the yeast inoculum and the plates were incubated at 37°C for 48 h. The MIC values were detected with the naked eye. The FICI values were calculated for each well with the equation FICI = FICA + FICB = (MICA+B/MICA) + (MICB + A/MICB), where MICA and MICB are the MICs of drugs A and B alone, respectively, and MICA + B and MICB + A are the concentrations of the drugs applied in combination, respectively, in all the wells corresponding to the MIC. A combination of two drugs is considered synergistic when the FICI is ≤ 0.5, indifferent when the FICI is > 0.5 to ≤ 4, and antagonistic when the FICI is > 4 (Odss 2003; Petersen et al. 2006).

Results and Discussion

Emodin is a natural anthraquinone derivative found mainly in the roots and rhizomes of numerous plants. Pharmacological studies have demonstrated that emodin with its various biological functions has been used in the treatment of cancers and inflammatory diseases (Wei et al. 2013; Dong et al. 2016; Monisha et al. 2016). The unique therapeutic potential of emodin results from its ability to interact with many molecular targets, e.g. protein kinases, NADH-oxidase, topoisomerase II, survivin, XIAP, STAT3, p53, and p21 (Shrimali et al. 2013). Furthermore, emodin was found to have antimicrobial activity (Alves et al. 2004; Kong et al. 2009; Liu et al. 2013; Cao et al. 2015; Liu et al. 2015; Janeczko et al. 2017).

In this study, the antifungal activity of emodin against the reference and clinical strains of C. albicans has been confirmed by the CLSI method in RPMI medium. The minimal inhibitory concentration against the standard strain was 12.5 µg/ml. The control antibiotics, caspofungin and amphotericin B, inhibited yeast growth at concentrations of 0.15 µg/ml and 1 µg/ml, respectively (Table I). Moreover, emodin suppressed the growth of all clinical strains isolated from the urinary tracts or the vaginas of the gynecological patients. The activity against these species has been shown at values of MICs between 6.25 and 50 µg/ml. Also, all isolates were susceptible to caspofungin. The MICs ranged from 0.03 to 0.6 µg/ml. The MIC values of the antifungal agents tested individually are summarized in Table II and Table III. These results were comparable to MICs obtained in our previous work (Janeczko et al. 2017). As demonstrated in the previous study, emodin suppressed the growth of C. albicans and other reference strains, such as C. krusei, C. parapsilosis, and C. tropicalis, as well as clinical Candida strains. In addition, fungicidal activity against these species has been shown at values of MICs and MFCs (Minimal Fungicidal Concentrations) between 12.5 and 200 µg/ml. Moreover, we have proved that this compound has anti-virulent potential by reducing hyphal formation, suppressing adhesion, which is the first and critical phase of fungal infection, and destabilizing fully established biofilm. In terms of the high pleiotropic nature of emodin, it has been confirmed that this compound is an effective inhibitor of protein kinase 2 (CK2) isolated from C. albicans cells (Janeczko et al. 2017).

In order to verify the influence of emodin on C. albicans cells, the previous research on the molecular

| MIC (µg/ml) | Without sorbitol | With sorbitol |
|------------|------------------|---------------|
| Emodin     | 12.5             | 25            |
| Caspofungin| 0.15             | 0.6           |
| Amphotericin B | 1              | 1             |

Table I

Effect of sorbitol on the MICs of emodin and antibiotics against C. albicans ATCC 10231.
The fungal cell wall is a unique structure built of α- and β-linked glucans, chitin, polysaccharides, and mucopolysaccharides. Many of these biopolymers are essential for proper functioning of fungal cells. Enzymes synthesizing these biopolymers could be desirable antifungal targets (Wiederhold 2018). Since emodin is a highly pleiotropic molecule capable of interacting with several major molecular targets and damaging C. albicans cell walls, the influence of the compound on the activity of (1,3)-β-D-glucan synthase (GS) has been analyzed. This enzyme is a glucosyltransferase involved in synthesis of 1,3-β-D-glucan in fungi – one of the main molecular targets used in clinically available antifungals and also a pharmacological target for echinocandins (Denning 2003). Inhibition of GS activity and the following depletion of β-glucans from the fungal cell wall result in cell lysis under osmotic stress (Frost et al. 1995).

An aniline blue assay was used to determine the effect of emodin on the activity of (1,3)-β-D-glucan synthase obtained from a microsomal membrane fraction from C. albicans. The decrease in the GS activity after the treatment with the anthraquinone tested was shown as a percentage of the DMSO control. As shown in Fig. 1, emodin reduced the GS activity approxi-
Effect of emodin on glucan synthase activity

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...approximately to 67.6% ± 3.4%, 76% ± 4%, 87.4% ± 5.2%, and 91.2% ± 3.8% at concentrations equal to 2×MIC, MIC, MIC/2, and MIC/4, respectively, when compared to the DMSO control cells. The reduced activity of GS proved that emodin prevented the synthesis of the C. albicans cell wall, and therefore, the effect of its activity corresponds to that of echinocandins.

Additionally, aniline blue was used to verify the in vivo influence of emodin on changes in the content of (1,3)-β-D-glucans in C. albicans by inhibition of their biosynthesis. This fluorescent dye binds to (1,3)-β-D-glucans in the cell wall. The biosynthesis of C. albicans cell wall components was shut down by inhibition of GS. The fungi were grown in the presence of emodin at the concentrations of 6.25 µg/ml and 3.125 µg/ml corresponding to MIC/2 and MIC/4, respectively or in the presence of caspofungin at 0.07 µg/ml (MIC/2). The control contained DMSO at the same concentration as in the samples with emodin. As shown in Fig. 2, the intensity of fluorescence of fungal cells stained with blue aniline was lower in cells treated with emodin and caspofungin than in the controls. The apparent significant loss of fluorescence of the fungal cell walls and, above all, the disintegration of cells under the pressure of caspofungin at MIC/2 correlated with the sensitivity of this species to echinocandin, i.e. a 1,3-β-D-glucan synthesis inhibitor (Fig. 2B). The treatment of C. albicans cells with emodin at concentrations MIC/2 and MIC/4 resulted in reduction of the number of cells, but the reduction of the glucan content in the cell walls was almost imperceptible (Fig. 2C and Fig. 2D).

Recently, natural product screening has also been a source of a number of distinct GS inhibitors. Antifungal activity was shown by natural lipopeptides and triterpenes containing a polar (acidic) moiety (Vicente

Table III

**In vitro** activity of emodin and caspofungin alone and in combination assessed by the broth dilution method against clinical strains of C. albicans isolated from vaginas.

| Strain No. | MIC of the drug used alone (µg/ml) | MIC of the drug used in combination (µg/ml) | FICI | Interaction |
|------------|-----------------------------------|--------------------------------------------|------|------------|
|            | Emodin | Caspofungin | Emodin + Caspofungin |      |            |
| 1          | 50     | 0.3        | 50 + 0.3               | 2    | Indifferent |
| 2          | 50     | 0.6        | 50 + 0.6               | 2    | Indifferent |
| 3          | 25     | 0.15       | 25 + 0.3               | 3    | Indifferent |
| 4          | 50     | 0.3        | 50 + 0.3               | 2    | Indifferent |
| 5          | 25     | 0.15       | 25 + 0.3               | 3    | Indifferent |
| 6          | 50     | 0.07       | 25 + 0.15              | 2.64 | Indifferent |
| 7          | 50     | 0.3        | 50 + 0.6               | 3    | Indifferent |
| 8          | 50     | 0.6        | 25 + 0.3               | 1    | Indifferent |
| 9          | 12.5   | 0.3        | 25 + 0.3               | 3    | Indifferent |
| 10         | 50     | 0.15       | 50 + 0.6               | 5    | Antagonism  |
| 11         | 50     | 0.07       | 50 + 0.15              | 3    | Indifferent |
| 12         | 50     | 0.6        | 50 + 0.3               | 1.5  | Indifferent |
| 13         | 25     | 0.3        | 50 + 0.3               | 3    | Indifferent |
| 14         | 25     | 0.6        | 50 + 0.15              | 2.25 | Indifferent |
| 15         | 25     | 0.3        | 50 + 0.15              | 2.5  | Indifferent |
| 16         | 12.5   | 0.03       | 25 + 0.15              | 5    | Antagonism  |
| 17         | 12.5   | 0.6        | 12.5 + 0.6             | 2    | Indifferent |
| 18         | 50     | 0.3        | 50 + 0.6               | 3    | Indifferent |
| 19         | 50     | 0.15       | 25 + 0.07              | 1    | Indifferent |
| 20         | 50     | 0.6        | 50 + 0.3               | 1.5  | Indifferent |

Fig. 1. Effect of emodin on (1,3)-β-D-glucan synthase activity.
In addition, several new investigational agents are currently under development. Among these, there are semi-synthetic enfumafungins modified by replacement with amino ethers (Apgar et al. 2015), SCY-078, which derives from enfumafungin, as well as a cyclic hexapeptide rezafungin (CD101, biafungin, previously SP3025) (Wiederhold 2018).

The clinical success of the echinocandins is associated with their fewer toxic side effects in comparison to polyenes and their fewer drug-drug interactions compared to azoles. These drugs are primarily used for the treatment of invasive candidiasis and as an alternative therapy for aspergillosis treatment (Odds et al. 2003; Denning and Hope 2010). Unfortunately, the effectiveness of these antibiotics is compromised due to a critical increase in the emergence of drug-resistant Candida strains. In the face of this problem, another strategy has been developed to overcome the treatment failures by combining different antifungals. Many reviews indicate that the combination of antibiotics, phytochemicals, or both natural plant products and well-known antibiotics offers significant potential for the development of novel antimicrobial therapies and treatment of several diseases caused by microorganisms. The advantages of the synergistic action of antibiotics and plant extracts include reduction of

Fig. 2. Aniline blue staining of C. albicans cell walls. A) treatment with DMSO at 1% (control); B) caspofungin at 0.07 µg/ml; C) emodin at 6.25 µg/ml; D) emodin at 3.12 µg/ml.
undesirable effects and increased efficiency. It is also important to increase the stability and bioavailability of free agents and achieve an adequate therapeutic effect with relatively small doses compared to any synthetic medication (Hemaiswarya et al. 2008).

Since emodin affects the activity of (1,3)-β-D-glucan synthase in the same range as caspofungin, the interactions between this anthraquinone and the antibiotic were investigated. In this case, two possibilities of interaction could be expected – synergism or antagonism in the action against C. albicans cells. The third option was indifferent interaction between the two tested substances. Synergism is defined as a positive interaction occurring when two agents combined together exert an inhibitory effect that is greater than the sum of their individual effects. In turn, the term antagonism is used when the effect of both drugs together is worse than the effect of either alone. Then, indifference means that no effect is exhibited. The caspofungin- emodin combination effect was measured using the checkerboard microtitre plate method and calculation of the fractional inhibitory concentration index (FICI). The course and result of the experiment for the reference C. albicans strain is schematically shown in Fig. 3. The combination of both antifungals showed a tendency towards indifference between the tested compounds at most concentrations and ratios. Thus, emodin at 0.19–3.12 μg/ml did not affect the caspofungin activity against C. albicans. Similarly, caspofungin at 0.007–0.6 μg/ml did not change the MIC values for emodin. Only in one case, the MIC of caspofungin increased from 0.15 μg/ml to 0.3 μg/ml in the presence of 6.25 μg/ml emodin. Based on the MIC values in various concentration combinations of both antifungal compounds, the FICI was 3 and did not show any interactions between the compounds.

The checkerboard assays evaluated against 40 clinical isolates of C. albicans showed that the combination of emodin with caspofungin changed mainly the MIC values with respect to caspofungin. MICs increased for 18 strains, decreased for nine strains, and remained unchanged for 13 isolates. The composite emodin/ caspofungin caused a change in the MICs with respect to emodin to a lesser extent. The MIC values decreased for four strains and increased for five strains. They were ca. 2–4-fold lower or higher than the values for the compounds applied alone. The FICI values of the combinations of the antifungal drugs ranged from 1 to 6. This combination showed predominantly indifferent interactions between emodin and caspofungin (87.5% isolates) with the FICI in the range from 1 to 4. An antagonistic effect was proved only against five strains tested (12.5%) with FICI > 4. Otherwise, no synergism was observed (FICI < 0.5). These results were comparable to the FICI of the C. albicans reference strain. The MIC and FICI values of the antifungal agents tested in combination are summarized in Table II and Table III.

In conclusion, as shown above, the antifungal activity of emodin against C. albicans may be related to the inhibition of (1,3)-β-D-glucan synthase activity, leading to disruption of (1,3)-β-D-glucans in the fungal cell wall. This completely new molecular target for emodin is highly desirable due to the high specificity of this type of antifungals in relation to host cells. The novel mechanism of emodin action could hypothetically amplify the activity of echinocandins; however, in combination with caspofungin, this anthraquinone shows indifferent or antagonistic interactions. The data from the studies of the interactions between emodin/caspofungin suggest that these combinations could not be an effective strategy against C. albicans infections.

Conflict of interest
Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.
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