SHORT COMMUNICATION

Effects of combination of phenazine-1-carboxylic acid from Streptomyces kebangsaanensis with amphotericin B on clinical Fusarium solani species

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ABSTRACT

Aim: A novel endophyte, Streptomyces kebangsaanensis was isolated from the stem of a Malaysian ethnomedicinal plant, Portulaca oleracea in 2013. Studies on S. kebangsaanensis crude extract showed that it had antifungal activities and further work led to isolation of a novel compound, phenazine-1-carboxylic acid (PCA). This study investigated the combinatorial effect of PCA isolated from S. kebangsaanensis with amphotericin B on the growth of four clinical Fusarium solani isolates.

Methodology and results: Disk diffusion assay showed that the crude extract of S. kebangsaanensis inhibited growth of all four F. solani isolates. Whereas, the compound PCA from this extract inhibited two of the tested F. solani isolates, UZ541/12, and UZ667/13 at minimum inhibitory concentration of 18.00 µg/mL. Combinations of this compound with amphotericin B, reduced the minimum inhibitory concentration of amphotericin B for these two isolates from 8 to 0.13 µg/mL and 4 to 0.03 µg/mL respectively. Analysis of fractional inhibitory concentration index showed that a borderline synergism is present between the compound and amphotericin B.

Conclusion, significance and impact of the study: These results indicate PCA may be useful in improving actions of available drugs against antimicrobial resistant microorganisms.

Keywords: Streptomyces kebangsaanensis, phenazine-1-carboxylic acid, Fusarium solani, synergism

INTRODUCTION

Discovery of Streptomyces kebangsaanensis, a novel endophyte, has led to the study of the compounds produced by this bacterial species. S. kebangsaanensis produces phenazine-1-carboxylic acid (PCA) while its crude extract exhibited antifungal activities in in vitro assays (Sarmin, 2012; Sarmin et al., 2013; Zin et al., 2015). Apart from this discovery, genomic characterization of this species revealed the presence of gene clusters involved in polyketide, nonribosomal peptide, terpene, bacteriocin, and siderophore biosynthesis (Remali et al., 2017a; Remali et al., 2017b; Zin et al., 2017). PCA is a heterocyclic, nitrogen-containing secondary metabolite compound, predominantly produced by Pseudomonas species (Price-Whelan et al., 2006). It is also known as tubercymycin B, an antibiotic (Sarmin et al., 2013). Previous research has shown that the compound has antifungal activity against Fusarium spp., Phytophthora spp. and Rhizoctonia spp. As such, PCA has been regarded as having a high potential for use as an antifungal agent (Mavrodi et al., 2006). Therefore, it is worthwhile to investigate if PCA can be used together with existing drugs in order to combat drug-resistant fungal species. There is evidence indicating that combination of compounds or drugs used in clinical settings is particularly beneficial in the prevention of drug resistance emergence (Borisy et al., 2003).

In relation to drug resistance, an epidemiological survey on fusariosis conducted in Europe showed that Fusarium solani isolates were resistant to azoles and it was the most frequent etiology of disseminated and localized infections. The same survey also reported highest crude mortality rate for F. solani infections
compared to other *Fusarium* species (Tortorano et al., 2014). An older review whereas reported that *F. solani* isolates commonly exhibited higher minimum inhibitory concentration (MIC) for amphotericin B compared to other *Fusarium* species (Nucci and Anaissie, 2007). In Malaysia, reports on *Fusarium* infections are limited, but they have been mostly associated with keratitis where *Fusarium* was the most common fungal causative agent (Singh et al., 1981; Ratnalingam et al., 2017; Velayuthan et al., 2018; Yap et al., 2019). Antifungal susceptibility testing of several Malaysian *Fusarium* isolates have indicated resistance to amphotericin B and azoles (Santhanam et al., 2008; Tzar et al., 2016). Following the initial antifungal activities of PCA, in this study, we investigated the combination of PCA with amphotericin B with the aim of inducing an interaction which reduces the MIC of amphotericin B for four clinical *F. solani* isolates.

**MATERIALS AND METHODS**

**Microorganisms**

*S. kebangsaanensis* was obtained from Novel Antibiotic Research Laboratory, Universiti Kebangsaan Malaysia and was maintained in International Streptomyces Project medium 2 (ISP-2) at 28 °C (Shirling and Gottlieb, 1966). The *F. solani* strains; UZ541/12, UZ667/13, M2309, and UZ 994/12 were clinical isolates maintained in the same research laboratory, on potato dextrose agar (PDA) at 27 °C.

**Isolation of phenazine-1-carboxylic acid (PCA)**

*S. kebangsaanensis* was grown on the ISP-2 agar plate for 14 days at 28 °C in order for the culture to reach a stage where it produces aerial spore-mass and substrate mycelia. Ten agar blocks (1 × 1 cm) from the growth were inoculated into 600 mL nutrient broth and were incubated at 28 °C on orbital shaker (150 rpm) for another 14 days. The supernatant was collected and extracted with ethyl acetate (1/2 ratio) three times and the resulting extract was subjected to dryness using a rotary evaporator which yielded the crude extract. An aliquot of the crude extract (40 µL of 10 mg/mL) was analyzed with an analytical C18-reversed phase HPLC using the following gradient solvent system: 1 min at 5% methanol (MeOH)/ milliQ water (H2O); a linear gradient to 100% MeOH over 20 min; isocratic at 100% MeOH for 4 min; a linear gradient to 5% MeOH/ H2O over 3 min; isocratic at 5% MeOH/ H2O over 2 min. HPLC was performed on Agilent 1200 HPLC system (Santa Clara, CA, USA) using a reversed phase analytical column (C18 Waters ® 4.6 x 250 mm, 5 µm) with UV detector at 250 nm. Fraction containing PCA eluting at 17.54 min (Sarmin, 2012; Remali et al., 2017b) was collected and the process was repeated to collect enough sample for testing.

**Bioassays**

The disk diffusion assay was done based on recommendations by M02-A12 Clinical Laboratory and Standard Institute document (The Clinical and Laboratory Standards Institute, 2015). In brief, the disks were impregnated with different amounts (100 µg, 80 µg, 60 µg and 40 µg) of *S. kebangsaanensis* crude extract and were air-dried before being placed on Mueller-Hinton agar (MHA) which was swabbed with *F. solani* strains suspension. Test plates were incubated at 37 °C for 48 h and results were scored as diameter of inhibition (mm). The positive control used in this assay was disk with amphotericin B (Nacalai Tesque, Japan) at 0.25 µg and negative control was disk impregnated with the diluent used to dissolve the crude extract, ethyl acetate. Determination of MIC of PCA and amphotericin B for *F. solani* strains was carried out via broth dilution assay as described in CLSI M38-A2 document (The Clinical and Laboratory Standards Institute, 2017).

Evaluation of combinatorial effect of PCA with amphotericin B on the growth of four clinical *F. solani* isolates was studied using methods described by Dawis et al. (2003). Briefly, PCA at three sub-MIC concentrations (1/2 × MIC, 1/4 × MIC and 1/8 × MIC) was combined with amphotericin B with concentrations ranging from 1/2 × MIC to 1/256 × MIC. Media used was RPMI-1640 and *F. solani* inoculum size in the assay was approximately 5 × 10⁴ CFU/mL. The results were expressed in fractional inhibitory concentration (FIC) index, calculated using a previously published formula: 

\[
\text{FIC} = (\frac{\text{MIC B tested alone}}{\text{MIC B in combination}} + \frac{\text{MIC A tested alone}}{\text{MIC A tested in combination}})
\]

where MIC is the minimum inhibitory concentration (FIC) index,

The combination was defined as synergy if the FIC index was ≤ 0.5, partial synergy was defined > 0.5 but ≤ 1, additivity was defined = 1, indifference was defined >1 but < 4, and antagonism was defined ≥ 4 (Dawis et al., 2003).

**RESULTS**

Disk diffusion assay done on two separate occasions showed that the crude extract of *S. kebangsaanensis* inhibited strain UZ541/12 and UZ667/13 consistently in both experiments (except at 100 µg for which no inhibition was observed for strain UZ667/13 in one experiment) as shown in Table 1. For the other two strains (M13002309 and UZ994/13) inhibition was observed only at 100 µg crude extract, which was the highest tested amount, for strain UZ994/13, while inhibition of strain M13002309 was inconsistent at ≥ 80 µg. Hence, these two strains were excluded in further testing and strains UZ541/12 and UZ667/13 were chosen for combinatorial effect assays. Besides that, the results also showed that zones of inhibition with diameter ranging from 8-23 mm were recorded for all the tested strains when they were exposed to amphotericin B at 0.25 µg.
Table 1: Antifungal effect of S. kebangsaanensis crude extract (SK-CE) and amphotericin B on F. solani clinical strains. Values shown were obtained from two independent experiments (N=1 and N=2).

| F. solani strains | Amphotericin B 0.25 µg | SK-CE 100 µg | SK-CE 80 µg | SK-CE 60 µg | SK-CE 40 µg |
|-------------------|------------------------|-------------|-------------|-------------|-------------|
|                   | N=1                    | N=2         | N=1         | N=2         | N=1         | N=2         | N=1         | N=2         | N=1         | N=2         |
| UZ541/12          | 11.0                   | 10.0        | 10.0        | 9.0         | 10.0        | 7.0         | 8.0         | 8.0         | 6.5         | 7.0         |
| M13002309         | 8.5                    | 8.0         | 7.5         | -           | 7.0         | -           | -           | -           | -           | -           |
| UZ994/13          | 8.0                    | 9.0         | 7.0         | 7.5         | -           | -           | -           | -           | -           | -           |
| UZ667/13          | 21.0                   | 23.0        | 8.0         | -           | 8.0         | 8.0         | 7.0         | 8.0         | 7.0         | 8.0         |

Evaluation of the combinatorial effects of PCA with amphotericin B is shown in Table 2. The MIC value of PCA alone was 18 µg/mL for both tested strains (UZ541/12 and UZ667/13). Whereas, MIC of amphotericin B alone for strain UZ541/12 was 8 µg/mL and 4 µg/mL for strain UZ667/13.

When PCA was combined with amphotericin B, two combinations (1/64×MIC amp B + 1/2×MIC PCA and 1/256×MIC amp B + 1/2×MIC PCA) resulted in inhibition of the tested fungal strains whereby the FIC indices of these combinations suggested presence of partial synergism between the combined test agents (Table 2). Presence of PCA was noted to improve the activity of amphotericin B markedly whereby its MIC was reduced to 1/64 and 1/256 of its initial MIC for strains UZ541/12 and UZ667/13 respectively. These combination treatments were also observed to decrease MIC of PCA by half, from 18 µg/mL to 9 µg/mL. Although the other tested combinations also resulted in inhibition of fungal growth, the FIC index indicated the interaction of the agents (amphotericin B and PCA) were indifferent (results not shown). PCA isolation was done using HPLC system and Figure 1 shows peak at approximately 17.54 min when the compound PCA elutes.

Table 2: Combinatorial effects of phenazine-1-carboxylic (PCA) with amphotericin B (amp B) on F. solani strains. Values shown represent triplicates of each combination treatment (n=3).

| F. solani strains | MIC amp B alone (µg/mL) | MIC PCA alone (µg/mL) | MIC amp B in combination (µg/mL) | MIC PCA in combination (µg/mL) | FIC indexa (interaction) |
|-------------------|-------------------------|-----------------------|----------------------------------|--------------------------------|--------------------------|
| UZ541/12          | 8.00                    | 18.00                 | 0.13b                             | 9.00b                          | 0.516 (partial synergy)  |
| UZ667/13          | 4.00                    | 18.00                 | 0.02c                             | 9.00c                          | 0.505 (partial synergy)  |

a1/64 × MIC amp B
b1/2 × MIC PCA
c1/256 × MIC amp B
d1/2 × MIC PCA
FIC= (MIC amp B in combination/MIC amp tested alone) + (MIC PCA in combination/ MIC PCA tested alone)

DISCUSSION

Results of disk diffusion assay confirms the earlier findings of antifungal activities exhibited by S. kebangsaanensis (Sarmin, 2012). However, in general, the zones of inhibition produced by crude extract of S. kebangsaanensis against growth of F. solani isolates were smaller when compared to the antifungal agent, amphotericin B. This indicates the greater antifungal activity of amphotericin B at much lower concentration (0.25 µg) compared to the crude extract; the exposure to amphotericin B managed to produce similar inhibition as the crude extract at 100 µg for strain UZ541/12 (both treatments produced zone of diameter ~10 mm). However, strain UZ667/13 was far more susceptible to amphotericin B with an inhibition zone of 23 mm compared to the crude extract at 100 µg which produced 8 mm inhibition zone. The disk diffusion assay also showed that the crude extract had low activity against the F. solani strains M13002309 and UZ994/13 as the antimicrobial activity was observed at only the higher crude extract concentrations (Table 1). From these results, it is evident that the crude extract of S. kebangsaanensis is able to suppress growth of F. solani isolates, however, not as effectively as amphotericin B. In fact, in crude extract form, the activity is relatively poor. A plausible explanation...
for the weak activity of the crude extract is due to composition of the extract which is a mixture of various metabolites. As such, the activity of the active compound may not be prominent when tested in crude form. Evidently, when PCA was isolated and tested against the strain UZ541/12 and UZ667/13; the antifungal activity was remarkably improved as observed in determination of MIC (Table 2).

MIC determination for amphotericin B for the selected strains (UZ541/12 and UZ667/13) indicated that the obtained values were within the CLSI epidemiological cut-off value for susceptibility to this drug, which is 8 μg/mL for F. solani (Espinel-Ingroff et al., 2004). Nevertheless, it important to note that MIC of amphotericin B for strain UZ541/12 is 8 μg/mL leading to the notion that any increase in this value denotes resistance to amphotericin B. Preceding research has identified that clinical fungi of Fusarium genus tend to be multi-resistant in nature (Alistruey-Izquierdo et al., 2008) and reveal high resistance patterns to antifungal drugs (Taj-Aldeen et al., 2016). The FIC index bioassay in this study showed that presence of PCA in the combination treatments lowered MIC of amphotericin B for both of the strains as much as 1/64 and 1/256 of the original MIC and partial synergism was present. This is a preliminary result supporting the hypotheses that addition of PCA to amphotericin B may be useful in improving the sensitivity of F. solani strains to the drug and this could be beneficial in treatment of drug-resistant strains.

PCA belonging to phenazine group, is commonly found occurring as natural products of Pseudomonas and Streptomyces species. In addition, phenazines are also found in other bacterial genera from marine and soil. A review in 2004, reported that phenazines possess many biological activities such as antibiotic, antitumor and antiparasitic activities (Laursen and Nielsen, 2004). This group of compounds is also known to inhibit plant pathogens. The mode of action of phenazine is associated with their ability to interact with polynucleotides, inhibition of topoisomerase and radical scavenging activity (Xu et al., 2015). In relation to antifungal activities, PCA was identified as important for bio-control of the phytopathogens, F. oxysporum and F. solani (Upadhyay and Srivastava, 2011; Jain and Pandey, 2016). Studies on synergism of PCA with antifungal drugs revealed that combinations of PCA with azoles produced synergistic effects in inhibiting growth of Candida species. The same research also suggested that this synergism implies that PCA may be useful in delaying development of resistance. Furthermore, PCA at 200 μg/mL showed no cytotoxicity to normal human cell lines (Nishanth Kumar et al., 2014).

CONCLUSION

In conclusion, presence of PCA in combination with amphotericin B was observed to reduce the MIC of amphotericin B for two clinical F. solani strains used in this study. Following this, the use of PCA with other antifungal agents may be further explored especially against drug-resistant fungal strains.
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