Detection of novel Lipid II-targeting antibiotics from fungi

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ABSTRACT

Bacterial resistance to antibiotics is one of the biggest threats to global health today. Therefore, there is a pressing need for new antibiotics. In this study, we examined four fungal strains for novel Lipid II-targeting antibiotic compounds. To do this, we analyzed fungus-extracted fractions by antibacterial activity assays in which Lipid II was included to detect antagonism, TLC and LC-MS. We identified eighteen Lipid II-targeting active fractions. Further purification and characterization of one active compound suggested a hydrophobic nature and a corresponding m/z value of 805 or 1300. Further research should elucidate the structure of the compound.

Keywords

Antibiotics, Lipid II, fungi, resistance

In this paper, details on fungal species and strains have been removed for confidentiality.

INTRODUCTION

The widespread and inappropriate use of antibiotics has caused the emergence of multidrug resistant bacteria. As a result, patient morbidity and mortality rates have increased[1]. According to the World Health Organization, these resistant bacteria are one of the biggest threats to global health today. However, despite the pressing need for new antibiotics, the introduction of new antibiotics has decreased significantly over the past decades[2].

For a potential new antibiotic it is of importance that it acts on a specific bacterial target to avoid toxicity for mammalian cells. A target that has proven to be successful is Lipid II, a bacteria-specific membrane component that is involved in peptidoglycan synthesis[3]. Peptidoglycan, the main component of the cell wall, is a crosslinked polymer that provides bacteria with the structural rigidity needed to cope with osmotic pressure differences[4,5]. In peptidoglycan synthesis, Lipid II carries the peptidoglycan building blocks from the cytoplasm to the periplasm. Therefore, Lipid II is essential for bacterial survival.

The importance of Lipid II, in combination with its specificity for bacteria and its accessibility as a membrane compound, makes Lipid II an effective target for antibiotics. This is illustrated by the multitude of existing Lipid II-targeting antibiotics (e.g. several glycopeptides such as vancomycin and ramoplanin, several lantibiotics such as nisin, etc.). It is striking that these antibiotics differ from each other in many aspects, such as interacting site, affinity and mechanism[6]. This indicates that there are many opportunities for antibiotic attack involving Lipid II.

Moreover, the effectiveness of Lipid II as target is illustrated by the reliability of Lipid II-targeting antibiotics in treating bacterial infections. In fact, vancomycin has long been used as a drug of last resort for treating *Methicillin-resistant Staphylococcus aureus* (MRSA)[7]. This can be explained by the fact that Lipid II is a non-proteinaceous target and can therefore not be altered directly through mutations. Unfortunately, bacterial strains resistant to vancomycin have arisen in the last decades. In the recent past, MRSA strains highly resistant to vancomycin have been described in clinical literature[8]. Therefore, there is a need for novel classes of antibiotics. In this study, we focused on finding new Lipid II-targeting antibiotics. As fungi have been valuable sources of antibiotic compounds in the past[9], we opted to use fungi as our starting point. We approached our search by extracting compounds from four fungal strains, fractionating the compounds and screening for activity against *Staphylococcus simulans,* a bacterium from the same genus as MRSA that is safe to work with in the lab. Active fractions were tested for Lipid II activity by antagonism experiments, followed by further identification and characterization of the Lipid II-active compounds.

EXPERIMENTAL PROCEDURES

Chemicals

Fungi were kindly provided by Jan Dijksterhuis (Westerdijk Institute). UDP-N-Acetylmuramyl-pentapeptide (lysine form) was derived from *Staphylococcus simulans*. UDP-N-Acetylglucosamine was purchased from Sigma Aldrich. All other chemicals used were of analytical grade or better.

Lipid II synthesis and purification

Lipid II (lysine form) was synthesized and purified as described by Breukink *et al.*[10], except that solely undecaprenyl was used instead of varying prenylchain lengths.

Fungal extractions

Fungus A, Fungus B, Fungus C and Fungus D* were grown for one and two weeks on different solid media: Malt Extract Broth (MEB), Yeast Extract with Supplements (YES) and/or Czapek Yeast Agar (CYA). The agar plates were cut in pieces and extracted two times with 40 mL ethyl acetate under sonication. In the extraction, the agar of the one and two weeks old plates of the same fungus and medium were combined. The extracts were filtered through cellulose filters (Whatman) to purify the extracts from possible spores. Ethyl acetate was removed by rotary evaporation under vacuum. The dried fungal extracts were dissolved in methanol (*Table 1*).

Table 1. Amount of dry matter obtained for each fungus and volume of methanol it was dissolved in.

Fungus	Medium	Total dry matter (mg)	Dissolved in mL methanol
A	MEB	21.6	2.0
A	YES	136.3	3.0
B	MEB	33.6	2.0
C	MEB	15.6	1.0
C	YES	63.3	3.0
D	MEB	9.4	1.0
D	CYA	12.4	1.0

Fractionation of fungal extracts

A volume of fungal extract containing 8 mg dry matter was applied to a C_{18} reversed phase column (2×2.5 cm). Elution was performed stepwise with 10%, 30%, 50%, 70% and 90% methanol in water. The collected fractions were dried and resuspended in 200 µL methanol.

Activity screens

The fungal extract fractions were tested by spotting 2 and 4 µL on 1.6% tryptic soy broth (TSB) agar plates containing *Staphylococcus simulans*. Active fractions were selected and screened for Lipid II-specific activity. 0, 1, 2 and 5 nmol Lipid II, dissolved in 50 mM Tris-HCl 0.1% Triton X-100 buffer pH 7.5, were spotted on new bacterial plates at a distance corresponding to the halo size of the initial activity screen. The bacterial plates were placed in a 30°C stove for ~16 hours before analysis.

Thin layer chromatography (TLC)

All fractions were filtered over a 4 mm syringe driven filter unit (Millex). 5 µL of each fraction was put on a reversed phase TLC plate (Silica gel 60 RP-18 F_{254}s; Merck) using the Linomat 5 (Camag) and developed in 70% methanol in water at T=20°C and rH=57% using the Automatic Developing Chamber 2 (Camag). The bands were visualized under UV at 254, 302 and 365 nm. For further analysis, some fractions were again put on reversed phase TLC and developed in 80% methanol in water. The silica of interesting bands was scratched off and extracted twice in 100 µL methanol/2-propanol (1:1). The extracts were filtered over a 4 mm syringe driven filter unit (Millex), dried and resuspended in 5 µL methanol. The extract were then tested using activity screens as described above.

Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS analysis was performed using a LCQ Deca XP mass spectrometer (Thermo Finnigan), coupled to a Surveyor autosampler and Surveyor pump (Thermo Finnigan). Compounds were first trapped on a Gemini µm NX-C18 110 Å LC column (150×2 mm; Phenomenex) using 0.1% TFA in water and 2-propanol (19:1). Elution was performed with a flow rate of 200 µL/min and an increasing concentration of 2-propanol as follows: 5% 2-propanol for 10 min, from 5% to 100% 2-propanol in 30 min (linear gradient) and finally 100% 2-propanol for 10 min. The column effluent was introduced into the ESI-source of the mass spectrometer, which was set to positive polarity. Mass spectra were acquired and processed with Xcalibur software (Thermo Fischer Scientific).

RESULTS

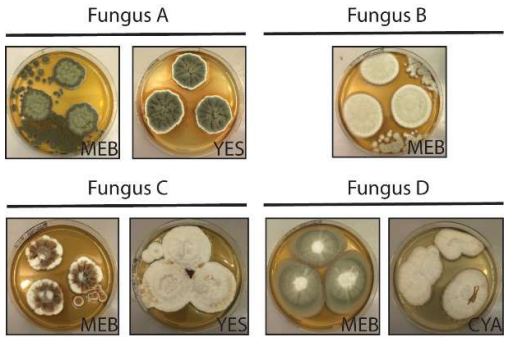

Figure 1. **Pictures of the investigated fungi.** All pictures were taken after growing fungus for 2 weeks. MEB = Malt Extract Broth, YES = Yeast Extract with Supplements, CYA = Czapek Yeast Agar.

In this study, we examined four fungal strains for Lipid II-specific antibiotic activity. Fungi and growth media were chosen based on previous research in our lab, which showed that different strains, and even the same strain grown on a different medium, produced different compounds[11]. In line with this research, we observed clear differences between the appearances of the fungi depending on strain and medium (*Figure 1*). To obtain fungal compounds, we extracted cut-up pieces of agar with ethyl acetate. In this way, we obtained seven fungal extracts. These extracts were separated into fractions of different hydrophobicity using a reversed phase C_{18} column, which was eluted stepwise with increasing concentrations of methanol. The obtained fractions were then concentrated and used for further analysis.

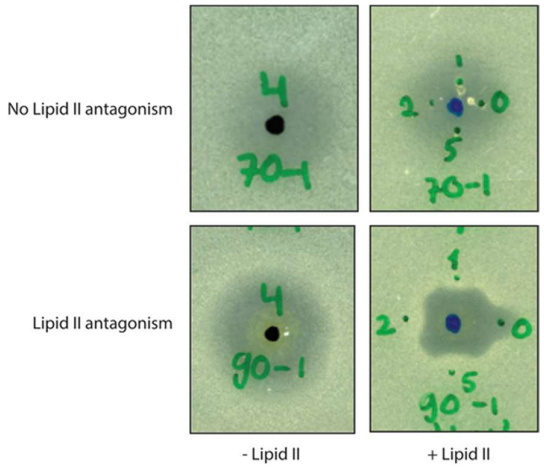

Figure 2. **Representative images of fractions showing no Lipid II antagonism and fractions showing Lipid II antagonism.** 0, 1, 2 and 5 indicate the amount of Lipid II spotted (nmol). Images were taken from fraction 70-1 of Fungus B on Malt Extract Broth (top) and from fraction 90-1 of Fungus B on Malt Extract Broth (bottom).

First, we investigated which fractions contained antibiotic activity. To do this, we spotted 2 and 4 µL of the fraction on agar plates containing *Staphylococcus simulans*. If a fractions contained antibiotic activity this could be seen because a halo, where no bacteria grew, arose after putting the agar plate in the stove overnight. Representative images of fractions showing activity are shown in *Figure 2* (left column). In total, we found activity in 35 of the 74 tested fractions.

We then analyzed for which fractions this activity was Lipid II-specific. To do this, 0, 1, 2 and 5 nmol of Lipid II were spotted on the predicted edge of the halo before

spotting the active fraction. This way, the antibiotic activity of the fungal extract was antagonized by Lipid II if the activity was Lipid II-specific. As a result, the round halo form was disturbed. This could be seen on the agar plates after growing the bacteria overnight (*Figure 2*, bottom right). Of the 35 fractions tested, eighteen showed clear Lipid II-antagonism, implying that the activity of these fractions was Lipid II-targeting. The amount and hydrophobicity of Lipid II active fractions differed for different fungi and media, confirming the importance of growth medium and fungal strain for the production of antibiotic compounds.

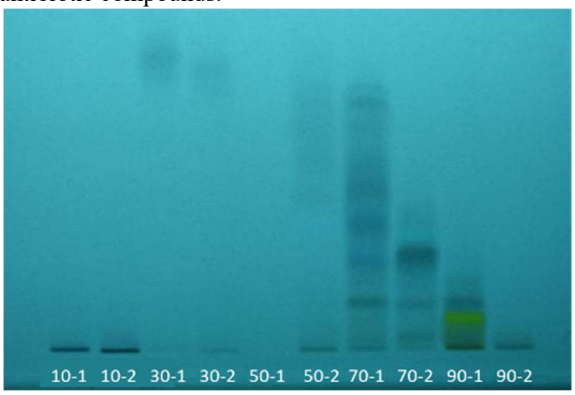

Figure 3. **Thin layer chromatography (TLC) plate obtained from fractions of Fungus B on Malt Extract Broth.** 10, 30, 50, 70 and 90 indicate the concentration of methanol (%) in water of the fraction. 1 and 2 indicate the first and second collected fraction. Picture of TLC was taken under UV light (λ = 302 nm).

To learn more about the content of the Lipid II-active fractions, we analyzed all fractions using reversed phase thin layer chromatography (TLC). The TLC plates were analyzed under UV light. The colour, height and number of bands for each fraction differed greatly for each fungus/medium (results not shown), again showing the role of strain and medium for antibiotic production. One fraction (Fungus B, 90-1) was of particular interest to us, as it contained strong antibiotic activity (*Figure 2,* bottom row) and the TLC plate showed one bright yellow band that was absent in surrounding fractions (*Figure 3*, 90-1). To investigate whether this band contained the antibiotic activity, we purified the band from TLC. For this, a new TLC plate of the Fungus B 90-1 fraction was made and developed in 80% methanol instead of 70%. Now, a red band appeared that was not visible in the previous TLC plate (*Figure 4A)*.

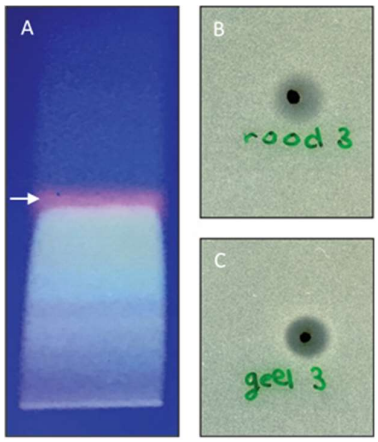

Figure 4. **Analysis of Fungus B fraction 90-1.** A) TLC plate of the 90-1 fraction of Fungus B developed in 80% methanol. λ = 365 nm. Arrow indicates red band that was not visible after development in 70% methanol. B) Activity assay done with extract of red band. C) Activity assay done with extract of yellow band.

To find out whether the red band, yellow band or neither contained the antibiotic activity, we scratched both compounds off and extracted the silica using methanol/2-propanol (1:1). To prevent contamination of the red band in the yellow and vice versa, the silica was scratched off in such a way that the yellow band was pure and the red contained yellow contaminations. The extracts were then tested for antibiotic activity by spotting the extract on agar plates containing *S. simulans* as described above. Both extracts showed activity, although the halo for the yellow extract was clearer (*Figure 4B and 4C)*. From this experiment we concluded that the yellow band contained the antibiotic activity. The weak activity seen for the red band can be explained by the yellow contaminations that were present.

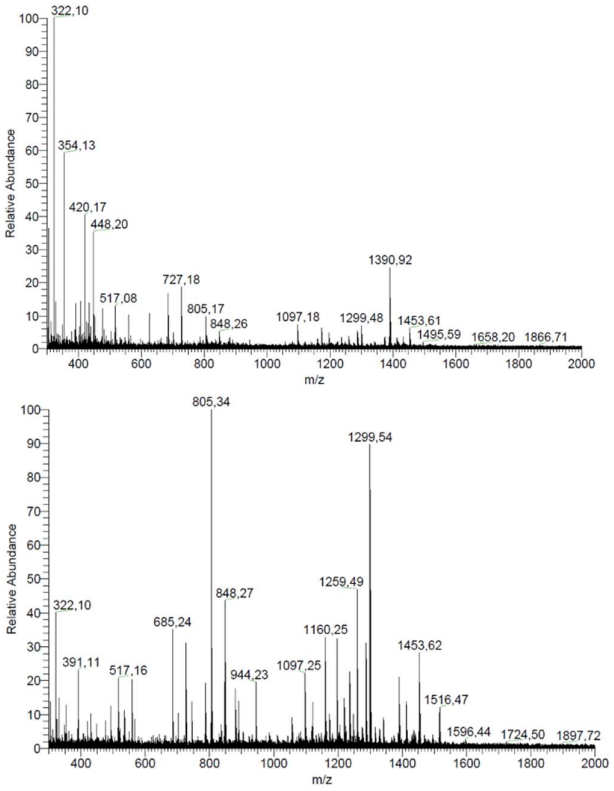

Figure 5. **Averaged mass spectra of liquid chromatography eluents.** Averaged mass spectra of eluents that eluted from 36 min until 46 min from a blanc (top) and the 90-1 fraction of Fungus B (bottom).

We next set out to learn more about the active compound. To do this, we performed liquid chromatography-mass spectrometry (LC-MS). In the LC-MS experiment, elution form the reversed phase column was performed with an increasing concentration of 2-propanol. Comparing the mass spectrum of the 90-1 fraction to a blanc resulted in several m/z values that were more abundant in the 90-1 fraction (*Figure 5*). Especially the peaks at m/z 805 and 1300 stood out, suggesting these peaks belonged to the active compound. The 805 and 1300 peaks started eluting from ~36 min and ~37 min respectively, when the 2-propanol concentration was almost 100%. This indicated a hydrophobic nature of the active compound. This is in line with the fact that the active compound was located in the 90% methanol fraction of the initial fractionation.

DISCUSSION AND IMPLICATIONS

In this paper, we have found fungal compounds with potential of being developed into novel antibiotics using antibacterial activity assays. Fungi have proven useful for the production of antibiotic compounds in the past[9] and

this was also the case in this study, as 35 of the 74 tested fractions contained antibiotic activity. The activity of eighteen of these fractions was also Lipid II-targeting, as became clear from antagonism experiments.

One active compound, located in fraction 90-1 of Fungus B, has been further characterized using thin layer chromatography and liquid chromatography-mass spectrometry (LC-MS). Our results suggest potential masses and a hydrophobic active compound. In the LC-MS experiment, the Fungus B 90-1 fraction was compared to a blanc. It was found that m/z values of 805 and 1300 may correspond to the active compound. However, these peaks can also be seen in the spectrum recorded for the blanc (although with a lower relative abundance). This observation can be explained by the fact the column was used several times prior to recording the blanc to record Fungus B 90-1 fractions. Since the active compound is so hydrophobic, some of the active compound could have still been present on the column when recording the blanc spectrum. Although more research is required to determine the mass with certainty and to elucidate the structure of this compound, it may eventually be developed further into an antibiotic that can be used in the clinic.

Next to having detected promising compounds with potential to be used as antibiotics, this study provides a simple yet functional approach in the search for novel antibiotics. There is still a large variety of fungi that can be investigated in this way, thus providing many opportunities for finding new antibacterial compounds. This is important to be able to meet the pressing need for new antibiotics that is a major health problem today and will likely remain so in the future.

In spite of the promise that fungal derived compounds hold, there is one challenge that needs to be solved. As can be seen from the TLC plates and the differences in activity, the medium on which a fungus is grown has an influence on the compounds the fungus produces. Moreover, we have experienced in our lab that there can even be differences between two fungi of the same strain that are grown on the same medium. More research needs to be conducted to find stable growth conditions in which more or less the same compounds are reproducibly produced by the fungus. Because of this, it is important that our experiments are repeated to see if the same results are obtained.

In addition to finding stable growth conditions, finding growth conditions in which the natural competitors of fungi (e.g. bacteria) are present may provide a way to make the fungus produce even more substances that have potential as antibiotic. This would be an interesting concept for future research.

CONCLUSION

In this study, we set out to find novel Lipid II-specific antibiotic compounds from fungi. From four fungal strains, we identified eighteen fractions that contained Lipid II-specific antibiotic activity. One of these fractions, 90-1 from Fungus B, was further characterized. We found some important properties of the active molecule: the band corresponding to the molecule on TLC, its hydrophobic nature and possible molecular weights. From these results, we conclude that we have successfully detected Lipid II-targeting antibiotic compounds. Further research should elucidate what the structure of these compounds is and whether these compounds could be developed into antibiotics for clinical use. Moreover, the method used in the study provides a model for analysis of other fungi.

ROLE OF THE STUDENT

Irene Zaalberg was an undergraduate student working under the supervision of Eefjan Breukink when this research was performed. The topic and the experimental procedures were proposed by the supervisor. The experiments were performed by the student in collaboration with Paul Schürmann, another undergraduate student. The processing of the data as well as the writing of the research paper were performed by the student independently.

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