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Discovery, biosynthesis and antifungal mechanism of the polyene-polyol meijiemycin

Zhen Jie Low,[†] Juan Xiong,[†] Ying Xie,[†] Guang-Lei Ma,[†] Howard Saw, Hoa Thi Tran, Wong Soo Lin, Li Mei Pang, July Fong, Peng Lu, Jin-Feng Hu, Liang Yang, Yansong Miao,^{*} Zhao-Xun Liang^{*}

Abstract: We report the genome-guided discovery of meijiemycin from a *Streptomyces* strain. Meijiemycin is a gigantic linear polyene that has structural features not seen in other members of the polyene-polyol family. We demonstrate that meijiemycin inhibits hyphal growth by inducing the aggregation of ergosterol and restructuring of fungal plasma membrane.

Invasive filamentous fungal infections remain a growing concern as the immunocompromised and aging population of the world increases. With an increasing number of resistant strains, it is vital to continuously supply the drug pipeline with novel antifungal compounds.^[1] Among the main classes of antifungal drugs, the polyene-polyol amphotericin B is the standard antifungal drug for treating deep-seated systemic fungal infections despite the debilitating nephrotoxicity and other side effects.^[2] One of the reasons for amphotericin B to remain indispensable in our limited antifungal arsenal today is the rare occurrence of drug resistance compared to other classes of antifungal drugs due to the unique ergosterol-binding mechanism.^[3]

Here we report the genome mining-guided discovery of meijiemycin, a unique polyol-polyene produced by the newly isolated *Streptomyces* sp. SD50 strain. SD50 has a large genome (11.6 Mb) that harbours several unique cryptic BGCs that are not found in other bacterial genomes from public databases. Among the unique BGCs found in SD50, there is a large cryptic BGC (~160 kb, referred to as *mjm*) that encodes a modular PKS system and other ancillary proteins (Fig. 1A, Table S2). Analysis of the PKS modules suggested that the *mjm* BGC is likely to produce a polyene-polyol with novel scaffold. Initial metabolite profiling revealed a fermentation product (**1**) with the characteristic UV-Vis spectrum of a hexaene-containing compound. Our initial effort to isolate **1** for structural elucidation was however unsuccessful due to low fermentation titer (< 10 µg/L).

Genome-mining and metabolite profiling suggested that SD50 contains constitutively active oxazolomycin (*ozm*) and nanchangmycin (*nan*) biosynthetic pathways.^[4] Reasoning that the disruption of these

two BGCs would boost the supply of malonyl-CoA and NADPH, and thus, the production of **1**, we employed the CRISPR/Cas9 method^[5] to disrupt the *ozm* and *nan* BGCs (Fig. S1). Disruption of *ozmH* and *nanA1* significantly increased the production of **1** (Fig. 1B), with the highest titer (8–12 mg/L) observed when the *ozmH/nanA1* double mutant was cultured in liquid media containing mannitol (Fig. 1C). To confirm that *mjm* BGC is responsible for the biosynthesis of **1**, the genes *mjmB* and *mjmE* were disrupted individually using the CRISPR/Cas9 method. The abolished production of **1** in the resultant mutants suggested that **1** is the product of the *mjm* BGC (Fig. 1D). Scaled-up fermentation led to the isolation of **1** that is subsequently named as meijiemycin. Meijiemycin has a molecular formula of C₆₆H₁₀₅NO₁₉ as suggested by the protonated molecular ion of *m/z* 1216.7357 [M+H]⁺ (calcd 1216.7359) (Fig. S2). As described in detail in the supporting material, the NMR data suggest that meijiemycin contains a linear carbon backbone and a perosamine sugar (Fig. 1E and Table S3). Comparing to the three currently used antifungal polyene-polyol drugs natamycin,^[6] amphotericin B,^[7] nystatin,^[8] meijiemycin has a larger and linear molecular architecture, a perosamine instead of a mycosamine sugar moiety (Fig. S5).

For biosynthesis, the polyene-polyol carbon skeleton is likely to be assembled by the Type 1 modular PKS system encoded by genes *mjmA-H* (Fig. 2). The biosynthesis appears to obey the collinearity rule,^[9] with the loading module and 26 extension modules of *mjm* PKS agreeing with the 54-carbon long scaffold. Detailed sequence analysis of the acyltransferase (AT), ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains lent further support to the methylation pattern and oxidation state of the β-groups deduced from the NMR data. Due to the presence of a YASH motif, the AT domains of modules 8, 17, 18, 19, 21 and 24 would prefer methylmalonyl-CoA, whereas the remaining HAFH motif-containing ATs prefer malonyl-CoA (Fig. S6).^[10] This translates into a polyketide chain consisting of twenty-six acetate units with five methyl groups (C55–C59) and one carboxylate group (C60) that is likely derived from a methyl group. Among the 26 KR domains, KR10 is likely to be inactive due to the absence of a catalytic tyrosine residue, consistent with the keto group at C35 that forms the hemiketal. The 25 active KRs were assigned as B-type or A-type KRs (Fig. S7), with KR8 and KR19 considered A2-type KRs that can epimerize the α-methyl group from *R* to an *S* configuration.^[11] The considerations led us to propose the absolute configuration of the β-carbons and the α-methyl groups at C12, C16, C18, C20, and C38. DH13 and DH18 lack key residues and are likely catalytically inactive (Fig. S8),^[12] consistent with the two hydroxyl groups at C19 and C29. During the extension of the polyketide chain, a six-membered ring is formed via the intramolecular nucleophilic attack of the hydroxyl group of C39 to the carbonyl group at C35.

The PKS product is likely to be released from MjmH via the thioesterase (TE) domain-mediated hydrolysis. The nascent polyketide product is subjected to further enzymatic tailoring to yield meijiemycin.

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Figure 1. Genome-mining guided discovery of meijiemycin. (A) Organization of the genes in the cryptic *mjm* BGC (also see Table S2 for gene annotation). (B) Comparison of metabolite profiles of the SD50 strain and three mutants grown on solid MS agar. The peak of the putative polyene-polyol product (1) is depicted by the red asterisk. (C) HPLC chromatograms of the $\Delta nan/\Delta ozmH$ double mutant fermented in YM media. The peak of 1 is depicted by the red asterisk. (D) HPLC chromatograms of SD50 mutants fermented using mannitol-supplemented YM liquid medium. The peak of 1 is depicted by the red asterisk. (E) Chemical structure of meijiemycin (1).

The methyl group at C38 is oxidized to yield a carboxyl group and is likely catalysed by the cytochrome P450 monooxygenase MjmOI. MjmOI is a close homologue of AmphN, the enzyme responsible for the oxidation of the C16 methyl group of amphotericin B.^[13] A perosamine moiety is installed onto the hydroxyl group at C41 by the glycosyltransferase MjmSIII. The perosamine originates from GDP-D-mannose via a process that involves MjmSI and MjmSII. The biosynthesis of perosamine and mycosamine share a similar pathway that involves the initial conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose. To yield perosamine, the keto group of GDP-4-keto-6-deoxy-D-mannose is converted by a perosamine synthase into an amino group,^[14] whereas mycosamine biosynthesis involves an

additional isomerization step.^[15] The fact that mannitol can be converted directly to mannose and GDP-D-mannose explains why mannitol increased the fermentation titer by several hundred-fold.

Meijiemycin was found to inhibit *C. albicans* DF2162R, a clinical strain isolated from a patient with urinary tract infection, with a minimal inhibitory concentration (MIC) of 12 ± 4 $\mu\text{g/mL}$. Little inhibitory effect was seen against the aspergillosis-causing *Aspergillus fumigatus*^[16] and the environmental strain *Aspergillus westerdijkiae* CBS112803^[17] up to 128 $\mu\text{g/mL}$. Hence, meijiemycin appeared to have a narrower spectrum-of-activity than the other antifungal polyene-polyols such as amphotericin B.^[18] Meijiemycin did not exhibit noticeable cytotoxicity

in the macrophage cells up to 128 $\mu\text{g/mL}$ (Fig. S9), suggesting that the compound is likely to act on a specific target in fungal cells.

Figure 2. A proposed biosynthetic mechanism for meijiemycin. (ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; KS, ketosynthase; TE, thioesterase).

We found that the Alexa Fluor™ 488 dye-labelled meijiemycin can be readily incorporated into the glycerophospholipid liposome (Fig. 3A), and that the dye-labelled meijiemycin was localized in or near the plasma membrane of *C. albicans* during yeast or hyphal growth (Fig. 3B), which suggests that meijiemycin prefers the lipid bilayer environment. *C. albicans* cells showed decreased hyphal formation when treated with meijiemycin, with less than 40% of the cells exhibiting filamentous growth upon hyphae induction (Figs. 3C and 3D),

or with reduced hyphae length (Fig. 3E). The growth of fungal hyphae relies on the temporal compartmentalization of lipid rafts.^[19] By using the fluorescent probe filipin,^[20] we found that the ergosterol on the surface of the polarized hyphae tip clustered to form compartmentalized aggregates upon the treatment of meijiemycin (Fig. 3F). The observations suggest that the inhibition of hyphal growth by meijiemycin could be caused by altered distribution of ergosterol on the plasma membrane.

Temporal compartmentalization of different lipid species is critical for the correct assembly of actin filament as well as the actin-powered endocytosis required for filamentous growth.^[21] Yeast cells rely on the sterol-enriched eisosomes to maintain temporal lipid compartmentalization. Eisosomes form 200–300 nm furrow-like structure in the plasma membrane,^[22] with the protein Pil1 bound to the lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and playing a key role during endocytosis and filamentous fungal growth.^[23] We tracked the fluorescent protein-tagged eisosome maker Pil1 and Lsp1 to examine how meijiemyacin affects lipid compartmentalization. Treatment with meijiemyacin inhibited the growth of budding yeast and changed the morphology of the eisosomes from punctate structures to more elongated furrow structures (Figs. 3G and 3H), reflecting a redistribution of sterols as well as PI(4,5)P₂.^[24] The cells treated by meijiemyacin also showed a redistribution of actin patches from bud-tip to mother cells to indicate the occurrence of cell depolarization and impairment of polarized cell elongation (Fig. 3I). Consistently, longer lifetimes were also observed for the protein markers Abp1 (~1.5 folds increase) and Sla1 (~2.9 folds increase)^[25] in meijiemyacin-treated cells (Figs. 3J–L), suggesting a delay in endocytosis and polarized cell growth.

In summary, the genome-guided approach led us to the discovery of meijiemyacin, an antifungal polyene-polyol produced by a largest BGC. With structural features not seen in the well-documented antifungal macrocyclic polyene-polyols, meijiemyacin inhibits fungal hyphal growth by inducing the aggregation of ergosterol and remodelling of the lipid microdomains on the plasma membrane. One of the defining characteristics of *C. albicans* is its ability to switch between the yeast and hyphal forms of growth. Targeting hyphal growth by inhibiting the yeast-to-hypha transition is emerging as an attractive antifungal strategy.^[26] Elucidation of the biosynthetic mechanism opens up the opportunities to produce meijiemyacin derivatives hyphal-inhibiting with improved physicochemical and pharmacological properties by genetic engineering, as demonstrated by recent studies on other polyene-polyols.^[27]

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RESEARCH ARTICLE

Genome-guided discovery of an unusual antifungal polyene-polyol that induces ergosterol aggregation and disrupts endocytosis on the fungal plasma membrane.

Zhen Jie Low,[†] Juan Xiong,[†] Ying Xie,[†]
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