Disruption of Membrane Integrity by the Bacterium-Derived Antifungal Jagaricin

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ABSTRACT Jagaricin is a lipopeptide produced by the bacterial mushroom pathogen Janthinobacterium agaricidamnosum, the causative agent of mushroom soft rot disease. Apart from causing lesions in mushrooms, jagaricin is a potent antifungal active against human-pathogenic fungi. We show that jagaricin acts by impairing membrane integrity, resulting in a rapid flux of ions, including Ca$^{2+}$, into susceptible target cells. Accordingly, the calcineurin pathway is required for jagaricin tolerance in the fungal pathogen Candida albicans. Transcriptional profiling of pathogenic yeasts further revealed that jagaricin triggers cell wall strengthening, general shutdown of membrane potential-driven transport, and the upregulation of lipid transporters, linking cell envelope integrity to jagaricin action and resistance. Whereas jagaricin shows hemo-lytic effects, it exhibited either no or low plant toxicity at concentrations at which the growth of prevalent phytopathogenic fungi is inhibited. Therefore, jagaricin may have potential for agricultural applications. The action of jagaricin as a membrane-disrupting antifungal is promising but would require modifications for use in humans.

KEYWORDS Candida albicans, jagaricin, mode of action, calcium influx, membrane integrity, pathogenic fungi, susceptibility testing

Historically, natural interactions between microbes and other organisms have proven to be the best source of antimicrobial compounds, and even human ingenuity cannot normally compete with billions of years of evolution (1). Antagonistic interactions between species often drive this evolution toward production of biologically highly active compounds. Recently, we described such an interaction by showing that the mushroom pathogen Janthinobacterium agaricidamnosum employs a novel cyclic lipopeptide, jagaricin, to cause soft rot disease of Agaricus bisporus. We further found that jagaricin is also active against common human-pathogenic fungi, such as Candida albicans and Aspergillus fumigatus, whereas bacteria are not affected (2). Identification of novel antifungal compounds—ideally with a novel mode of action—is desperately needed, because fungal diseases kill as many people as, e.g., tuberculosis, and invasive fungal infections are often associated with high mortality rates (3). The situation is further exacerbated by the emergence of drug-resistant fungal isolates in hospitals, exemplified recently by Candida auris (3, 4). Furthermore, fungal pathogens cause severe losses in agriculture (5–7), while the use of the same compound classes of antifungals in agriculture and medicine might lead to the emergence of resistant strains (8). This poses a significant challenge to the identification of new scaffolds for agricul-

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Lipopeptides consist of a linear or cyclic peptide moiety covalently linked to a lipid chain, resulting in typically amphiphilic characteristics and, depending on the ratio of hydrophobic to hydrophilic molecule parts, a tendency for self-aggregation and the formation of micelles, vesicles, or nanofibers (9). In general, biologically active lipopeptides can originate from natural sources, such as jagaricin, but especially membrane-active lipopeptides can also be artificially generated by linkage of a peptide with a lipid chain (10). Lipopeptides have been described to have (among others) surfactant, antibacterial, antifungal, antiviral and cytotoxic properties (see, for example, references 6, 9, and 10), and often have an effect on the integrity of membranes (e.g., fengycin, surfactin, and iturin; all from Bacillus spp. [11]) or by inhibiting cell wall biosynthetic enzymes of fungi (e.g., echinocandins [12]). Accordingly, some lipopeptides, such as polymyxins, caspofungin, micafungin, and daptomycin, are now in clinical use (13), and biologically derived lipopeptides continue to be a prolific source of inspiration for drug discovery. Here, we evaluate the lipopeptide jagaricin in regard to its mode of action, primary biological activities, and selectivity, and we suggest potential uses for this antifungal agent. We show that jagaricin most likely exerts its function by the formation of membrane pores, leading to nonreversible breakdown of the membrane potential as well as Ca\(^{2+}\) influx and consequently to cell death.

**RESULTS**

**Jagaricin is a fungicidal compound.** Fungal growth inactivation by antifungals can be roughly categorized into fungicidal and fungistatic, and this classification helps to guide the search for possible modes of action. We therefore determined killing of a very common human-pathogenic fungus, *C. albicans*, by jagaricin using propidium iodide (PI) staining, which allows detection of dead cells via fluorescence. After jagaricin application, we found strongly fluorescent yeast cells in defined growth medium (SD) (Fig. 1A), suggesting a general fungicidal mechanism. Accordingly, in control experiments, we found a corresponding reduction in CFU numbers after jagaricin exposure (data not shown). Notably, isolated killing of *C. albicans* cells was observed even at otherwise permissive jagaricin levels (Fig. 1A).
Membrane Disruption by Jagaricin

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Jagaricin targets determined via *C. albicans* and *C. glabrata* mutant and species comparison. In a first step to investigate the mechanism of action, we aimed to narrow down the potential fungal targets of jagaricin. Jagaricin inhibited growth of all tested fungal species (2), which makes fungus-specific cell wall structures or ergosterol-containing membranes potential targets of its action. We therefore tested *Dictyostelium discoideum*, an ergosterol-containing social amoeba lacking a cell wall, for its susceptibility. Since jagaricin was highly active against *D. discoideum* (Fig. 1B), we concluded that the cell wall is not the primary target.

To further identify the potential targets and mode of action of jagaricin, we performed a selected library screen with mutants of the common fungal pathogens *C. albicans* and *C. glabrata* as model organisms. The mutants were chosen based on their function in central signaling pathways or function in membrane or cell wall synthesis, allowing us to infer potential cellular target processes (Fig. 2). In accordance with the hypothesis that jagaricin does not target the fungal cell wall, *Candida* deletion mutants of the protein kinase C pathway (controlling cell wall integrity) showed no altered susceptibility toward jagaricin (*Cgrom2Δ, Cgslt2Δ, Cgslm1Δ, Carlm1Δ, and Camkk2Δ*) or only a slight increase in susceptibility (*Camkc1Δ*). Similarly, no effect was observed with mutants of cell wall-synthesizing enzymes (*Cgfks2Δ, Cgfks3Δ, and Casun41Δ*). In contrast, deletion of components of the calcineurin pathway (involved in general cell integrity) resulted in strongly increased jagaricin susceptibility in *C. albicans* (*Camrr1Δ, Camrr2Δ, and Camid1Δ*), but led to no effect in *C. glabrata* (*Cgmid1Δ*). The Hog1 mitogen-activated protein (MAP) kinase pathway is responsible, among others, for osmotic stress tolerance. Deletion of its core components (*Capbs2Δ, Cassk2Δ, and Cahog1Δ*) led to pronounced growth defects in the presence of jagaricin, although this was not the case for other sensors or effectors of the osmotic and/or oxidative stress response (*Cgskn7Δ, Caskn7Δ, Casko1Δ, and Cachk1Δ*). Interestingly, deletions of two of three tested genes encoding inositol polyphosphate phosphatases (*Cginp53Δ and Cginp51Δ*) led to higher resistance to jagaricin (no effect for *Cginp51Δ*), while a *Cgslm1Δ* strain (lacking a homolog of a phosphatidylinositol-4,5-bisphosphate binding protein) was hypersusceptible. These genes are linked to cell membrane organization via their role in regulation of actin cytoskeleton organization and endocytosis. In summary, these results strongly suggest that jagaricin might affect fungal cell membrane integrity, leading to osmotic stress.

In case of an intracellular target, drug tolerance in fungi is often mediated by active drug efflux. We therefore tested a number of clinical isolates with known enhanced Cdr or Mdr efflux phenotypes, deletion mutants of known drug transporters and their regulators, as well as gain-of-function strains of drug transporter regulators (*CaTac1* and *CaMrr1*). Deletion of individual drug transporter genes did not increase jagaricin susceptibility (*Cgpd1Δ, Cgpd17Δ, Casnq2Δ, Cacdr1Δ, Cgcd1Δ, and Cgsnq2Δ*), and this was the same for both Cdr- and Mdr-based increased efflux (in clinical isolates EU0992, EU0989, EU0999, and EU0981). Similarly, *C. albicans* strains with synthetic gain-of-function mutations in the regulator genes _MRR1_ or _TAC1_, which cause upregulation of the transporters _Mdr1_ or _Cdr1/Cdr2_, respectively, had no effect on jagaricin susceptibility. Also, deletion of the same drug transporter regulator genes had no effect (*Camrr1Δ, Camrr2Δ, and Catac1Δ*). In summary, susceptibility toward jagaricin is not affected by the investigated drug transporters, indicating that jagaricin is either not transported outside or—more likely—does not act on a cytoplasmatic target but rather directly on the plasma membrane.

If jagaricin acts on the plasma membrane, the membrane composition should influence susceptibility. Since ergosterol and the ergosterol biosynthesis pathway are well-known targets of antifungal compounds, we continued our mutant analysis in this context (Fig. 2). Deletion of _ERG5_ did not significantly change susceptibility in *C. albicans* and *C. glabrata*, and a defect of the Erg6-mediated step (clinical *C. albicans* isolate EU0136) in ergosterol biosynthesis similarly did not result in a difference of jagaricin susceptibility compared to the *C. albicans* reference strain SC5314. In contrast, clinical isolates harboring disruptive mutations in the _ERG3_ gene (*C. albicans* EU0012...
FIG 2 Jagaricin susceptibility of selected C. albicans (black, orf19.nnn) and C. glabrata (light blue, CAGLOnnn) mutants. Jagaricin effects on selected C. albicans and C. glabrata mutants are shown. The growth of Candida strains at 3 μg/ml (growth-inhibiting), 2 μg/ml (Continued on next page)
and EU1008) showed strongly reduced jagaricin tolerance. Finally, a gain-of-function mutation in the ergosterol biosynthesis regulator UPC2 (showing increased Erg11 expression and plasma membrane ergosterol levels [14–16]) of C. albicans led to high susceptibility toward jagaricin, whereas a UPC2 deletion did not affect jagaricin susceptibility. Although these results do not necessarily indicate a highly specific binding of jagaricin to ergosterol, they imply a role of sterol levels and membrane composition for jagaricin susceptibility.

**C. albicans transcriptome changes in the presence of jagaricin.** We continued to assess the physiological effects of jagaricin on C. albicans by transcriptional profiling of yeasts exposed to the antifungal. To this end, we obtained transcriptomes from a short-term (30 min) exposure to nontoxic jagaricin levels (1 μg/ml, 5 × 10⁶ yeasts/ml) and from a culture growing in a jagaricin concentration still permitting growth (0.5 μg/ml, 2.5 × 10⁶ yeasts/ml), sampled at an optical density at 600 nm (OD₆₀₀) of 0.5.

**Short-term transcriptomic response.** The short-term transcriptional response of C. albicans toward jagaricin was analyzed via Gene Ontology term (GO-term) enrichment to determine global functional patterns. Our data show clear hallmarks of a general cell wall repair and strengthening response (Fig. 3; see also Fig. S1A and B and Table S1A in the supplemental material), suggesting that jagaricin affects the integrity of the cellular envelope. Furthermore, a loss of membrane chemiosmotic potential is indicated by these two transcriptional events: (i) a downregulation of secondary active transmembrane transporter genes (Fig. 4) and (ii) a downregulation of the gene for the major plasma membrane H⁺-ATPase, Pma1 (Fig. 4A; see Table S1A in the supplemental material). Against the general downregulation of ion transporter genes, certain Ca²⁺ transporter genes, such as PMC1, which codes for a Ca²⁺-detoxifying vacuolar ATPase, were strongly upregulated (Fig. 4B). We also observed an upregulation of the gene for the central transcription factor of the calcineurin pathway, CRZ1, and of Rcn1, a calcineurin regulator (17, 18) whose expression has been shown to be upregulated by calcineurin at least in *S. cerevisiae* (19) and humans (20). Together, this indicates an increased intracellular Ca²⁺ level and calcineurin activity. This is in agreement with our mutant-based finding that Crz1 is required for *C. albicans* jagaricin tolerance. In contrast to the general trend of downregulation of transporter-coding genes, gene set enrichment analysis (GSEA) revealed the enrichment of the set “aminophospholipid transporter activity” among jagaricin-associated genes (Fig. S1F and Table S1A) due to several upregulated transmembrane lipid transporter genes (RTA2, RTA3, RTA4, and a potential orthologue of DRS25). Furthermore, the transcriptional response includes downregulation of cell cycle processes, especially those associated with mitosis, and fatty acid biosynthesis (Fig. 3; see also Fig. S1B and Table S1A).

Amphotericin B exposure to *C. albicans* is known to elevate transcript levels of genes encoding potassium (HAK1), as well as sodium (ENA21) transporters (21). Both were also upregulated under jagaricin treatment. Furthermore, amphotericin B application to fungal cells has been shown to lead to oxidative stress (21, 22). Interestingly, jagaricin treatment led to transcriptional upregulation of a specific set of oxidative stress-related genes, namely, those which are mainly involved in cell envelope-associated oxidative stress responses (SOD5, PST3, and YCP4).

**Transcriptomes during growth under growth permissive jagaricin levels.** The transcriptomic response of exponentially growing cells under 0.5 μg/ml jagaricin was

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**FIG 2 Legend (Continued)**

(growth-permissive), and 0 μg/ml jagaricin was recorded in individual wells of a 96-well plate by measuring absorbance at 600 nm every 30 min over a period of 3 days under continuous incubation in an enzyme-linked immunosorbent assay reader. (A) The A (600 nm) % WT was calculated as the geometric mean of three biological replicates. The growth speed index is −log₂ of the maximum t₁/₂ ratio of the mutant and reference strain, where the maximum t₁/₂ is the time to half-maximal A (600 nm) value; negative values indicate slower and positive values faster growth than the wild type; the arithmetic means of three biological replicates are shown. The individual origins of the strains are listed in Table S2. Genes depicted in boldface showed “stable growth” (for the definition, see Materials and Methods) at the otherwise growth-inhibiting jagaricin concentration of 3 μg/ml. (B) Individual growth curves of these mutants (and the corresponding *C. glabrata* [ATCC 2001 hlt] and *C. albicans* [SN250] reference strains) are shown. Arithmetic means of A (600 nm) values of three biological replicates ± the standard deviations are shown.
less pronounced than the short-term response. In general, plasma membrane transport processes were upregulated (compare GO-term analyses, see Fig. S1C and S1D and Table S1B), in part contrasting their downregulation in the short-term experiment and possibly indicating a compensatory response. Notably, among downregulated genes, mitochondrion-associated GO-terms were partially enriched (e.g., GO:0005746: mitochondrial respiratory chain; Revigo summary log\(_{10}\) \(P = -1.8593\)), suggesting that low levels of jagaricin interfere either directly or indirectly with mitochondrial function. Analogous effects have been observed for the surfactants cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) (23).

The growth of \textit{C. albicans} in the presence of the ergosterol biosynthesis inhibitor ketoconazole is known to induce ergosterol biosynthesis genes, while amphotericin B treatment leads to the downregulation of \textit{ERG3} and \textit{ERG11} transcription (21). Similarly, jagaricin exposure affected ergosterol biosynthesis by upregulation of the gene for the main regulator of the ergosterol biosynthesis, \textit{UPC2}, in the short-term and downregulation in the growth-phase response (see Tables S1A and S1B in the supplemental material). As revealed by GSEA (Fig. S1F and Table S1B) and GO-term analysis (Fig. S1D...
Transcriptomic response: regulation of transporters. The regulation of transporter genes in response to short-term (i.e., after 30 min) jagaricin exposure or jagaricin exposure during growth phase (grown to an OD$_{600}$ of 0.5) was assessed. Genes

**FIG 4** Transcriptomic response: regulation of transporters. The regulation of transporter genes in response to short-term (i.e., after 30 min) jagaricin exposure or jagaricin exposure during growth phase (grown to an OD$_{600}$ of 0.5) was assessed. Genes

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and Table S1B), the latter was accompanied by the downregulation of several ergosterol biosynthesis genes (ERG1, ERG3, and ERG11; Table S1B).

**Jagaricin effects on plasma membrane resistance and Ca\(^{2+}\) influx.** These results indicated that jagaricin might interfere with membrane integrity. We thus tested artificial and natural membrane systems, where jagaricin showed no effect on the integrity of an artificial membrane based on soybean asolectin, even at 4 \(\mu\)g/ml (data not shown), but exhibited a significant lytic activity toward human erythrocytes with a half-maximal inhibitory concentration (IC\(_{50}\)) of 4.16 \(\mu\)g/ml (Fig. 5). This indicated that a membrane compound or property is required that is present in mammalian and fungal cells (and lacking in the investigated artificial membrane model). Our previous data hinted at an interference with the chemiosmotic potential, and we thus tested the ability of jagaricin to elicit ion fluxes through the plasma membrane of human HEK293T cells using patch-clamp assays (Fig. 6A). A few minutes after jagaricin application, we consistently observed a sudden and irreversible onset of a transmembrane current. Both the delay of the onset and the magnitude of the current were found to be concentration dependent. This would be consistent with an accumulation of jagaricin in the membrane, followed by breakdown of the plasma membrane chemiosmotic potential.

To test whether the possible cation influx into HEK293T cells included Ca\(^{2+}\) ions as indicated by our previous results, we measured intracellular Ca\(^{2+}\) levels using a fluorescent reporter assay (Fura-2-AM; Fig. 6B). At 5 \(\mu\)g/ml jagaricin, similar to the patch-clamp results, we observed a lag time, followed by a fast Ca\(^{2+}\) influx that plateaued within about 1 min and remained at this level for the duration of the experiment. At a lower concentration of 1 \(\mu\)g/ml, the lag time was considerably prolonged, and the intracellular Ca\(^{2+}\) levels increased more slowly. Drawing from these data from experimentally accessible mammalian cells and our previous data, we conclude that jagaricin likely also mediates Ca\(^{2+}\) influx in *C. albicans* in an analogous manner.

We were interested whether jagaricin causes this Ca\(^{2+}\) influx via the activation of membrane-spanning Ca\(^{2+}\) transporters or channels, as suggested recently for another bioactive lipopeptide, orfamide A (24). For this, we blocked Ca\(^{2+}\)-specific transport by addition of Cd\(^{2+}\), which, however, did not block the jagaricin-mediated Ca\(^{2+}\) influx into HEK293T cells (see Fig. S2 in the supplemental material). Altogether, our results are in
best agreement with a model where jagaricin creates membrane lesions large enough to allow diffusion even of strongly hydrated ions such as Ca\(^{2+}\). One possible scenario for the action of a lipopeptide against membranes is the “micellar mechanism,” the solubilization of host membrane parts into mixed micelles (25). We found that jagaricin forms micelles only at concentrations exceeding 176 \(\mu\)g/ml (Fig. S3), arguing for a different mode of action at biologically relevant concentrations.

Cooperative effects with commonly used antifungals. Based on the possible mechanisms of action, we continued testing for cooperative effects with other clinically used drugs. Synergistic interactions among drugs can help to reduce the side effects of a single drug at high doses and enables the investigation of further mechanistic relationships. We therefore tested the combined action of jagaricin with one representative each of the three major antimycotic compound classes that target cell wall and plasma membrane (the polyene amphotericin B, the echinocandin caspofungin, and the azole clotrimazole). We measured growth at 24 and 48h and estimated potential synergism or antagonism by calculating the fractional inhibitory concentration index (\(\text{FICI}\)). While this index indicated indifferent interactions (\(\text{FICI} > 0.5 \text{ but } \leq 4\)) of jagaricin with all three tested antymycotics at 24 h, the addition of clotrimazole restored Candida growth at the otherwise fungicidal jagaricin concentration of 4 \(\mu\)g/ml at 48 h (Fig. 7A). Time-resolved growth curves show that this growth in the presence of clotrimazole appears mainly after 24 h of incubation (Fig. 7B). Similarly, at a normally toxic amphotericin B level of 1 \(\mu\)g/ml, addition of low levels of jagaricin allowed for (barely detectable) growth, again visible at 48 h (Fig. 7A).

Potential application spectrum of jagaricin. So far, our investigations into the effectiveness and mode of action of jagaricin focused on human-pathogenic fungi. Phytopathogens are another major class of fungi which are responsible for large economic losses in crops. New and dedicated fungicides for agriculture could help to reduce these losses and limit the transfer of resistance against clinically used drugs to human pathogens. We therefore tested the susceptibility of a range of important phytopathogenic fungi (Alternaria alternata, Penicillium digitatum, Penicillium italicum, Colletotrichum gloeosporioides, Geotrichum candidum, Botrytis cinerea, and Fusarium graminearum) and found them all susceptible to jagaricin at an MIC range of 0.1 to 5 \(\mu\)g/ml, comparable to our data on human pathogens (Fig. 8). Importantly, when we...
tested the plant model hosts, *Sinapis alba* and *Lepidium sativum*, we detected no inhibition of germination even at 5 μg/ml jagaricin, the highest concentration in this range (Fig. 8). Only in the absence of light, there was an intermediate reduction of 40.4% in root growth observed for *S. alba*, but there was no reduction independent of light for *L. sativum*. In summary, these data seem very promising for future, more detailed investigations into the application of jagaricin against phytopathogenic fungi.

**DISCUSSION**

Cell integrity disturbing lipopeptides generally act either by direct membrane disruption or by targeting cell wall-synthesizing enzymes (reviewed in reference 10). Here, we investigated the mode of action of the recently discovered lipopeptide, jagaricin, on fungal and mammalian cells and found that it disrupts membrane integrity, leading to a permanent depolarization of the plasma membrane. In mammalian and likely fungal cells, this is accompanied by an influx of Ca²⁺ and, most likely, other ions. We found that the drop in membrane resistance, as well as the influx of Ca²⁺, was rapid and nonreversible in a mammalian cell line. Furthermore, we demonstrated that Ca²⁺ influx was not inhibited in the presence of Cd²⁺, a potent blocker of Ca²⁺ channels, suggesting the formation of pores within the plasma membrane that are large enough to allow unspecific Ca²⁺ flow and do not seal spontaneously. This resembles the activity of other membrane-permeabilizing agents, such as fengycins, which also lead to the formation of large pores (26, 27). In contrast, e.g., amphotericin B creates smaller, defined pores allowing ions and smaller metabolites to pass, in particular K⁺ (28, 29), and the antibacterial lipopeptide daptomycin forms K⁺-selective pores (30; for a review, see reference 31). Importantly, we observed a strong effect on fungal and human cell membranes but no evident toxicity toward bacteria (2) and only
specific and limited toxicity toward plants (Fig. 8). Based on the data presented here, which mechanisms could therefore best explain the membrane disruption effect of jagaricin as well as its specificity?

The social amoeba, fungal, and mammalian cell death (including hemolysis) seems to be mediated by membrane disruption. Our transcriptional and electrophysiological data demonstrate this for fungi and a human cell line, and the influx of Ca\(^{2+}\) directly observed in human cells was reflected by the calcineurin-related transcriptional events in fungal cells. We therefore propose that the mechanisms are conceptually similar for fungal and mammalian cells.

Similar to other lipopeptides, jagaricin starts to form micelles at its critical micellar concentration (CMC) value of 176 \(\mu g/ml\) (Fig. S3), 2 to 3 orders of magnitude higher than the concentrations required for biological activity. We therefore conclude that the “micellar mechanism,” the solubilization of part of the host membrane into mixed micelles (as defined in reference 25), is unlikely to play a major role for jagaricin action. Based on the structure of jagaricin, we assume that rather the lipid tail is anchored into the membrane, while the peptide part is at least partially exposed to the outside. In this regard, it is interesting to note that all polar side chains of the jagaricin molecule (Fig. 9) that are able to participate in hydrogen bond interactions (hydroxyl group of the fatty acid, \(\alpha\)-allo-Thr-4, \(\alpha\)-allo-Thr-9, and \(\alpha\)-His-10) cluster around the hydrophobic ring-closing ester moiety (\(\alpha\)-Thr-3 and its neighbor Dhb-2), separating the hydrophobic tail from a second hydrophobic cluster (\(\alpha\)-Tyr-5, Dhb-6, Dhb-7, and Gly-8). This may result in a three-dimensional structure with the hydrophilic parts located within the phospholipid head layer and the hydrophobic peptide cluster and lipid chain located in the lipophilic part of the membrane. Notably, the lipid chain of the jagaricin molecule is rather short; such a conformation would therefore exert a strong bending stress toward the local membrane environment but might also trigger self-aggregation of jagaricin molecules. In general, aggregate formation within fungal membranes has been sug-

![FIG 8 Susceptibility tests for application (plants and phytopathogenic fungi). Phytotoxicity assays with jagaricin were performed. Lepidium sativum (left) or Sinapis alba (right) seedlings were grown in the presence (top) or absence (bottom) of light in substrate containing distilled water (control; A, C, E, and G) or jagaricin at 5 \(\mu g/ml\) (B, D, F, and H). In general, 5 \(\mu g/ml\) jagaricin caused no inhibition of seed germination; root length was only inhibited with Sinapis alba in the absence of light (root growth inhibition = 40.4%). (J) Jagaricin shows low MICs for different phytopathogenic fungi. The bars represent the arithmetic means of two biological replicates.](http://aac.asm.org/)
gested as crucial for the antifungal action of the *Bacillus* lipopeptide classes of fengycins and iturins (26, 27, 32–35). This phenomenon is explained by the combined effect of aggregates of, e.g., inverted-cone shaped lipopeptides on the local membrane bending, while monomers are typically insufficient to significantly alter local membrane structures. Fengycin aggregate formation was found to be triggered by immiscibility of the lipopeptide with the target membrane (27, 35, 36). This also explains the missing activity of fengycin against bacteria, since fengycin can readily interact with their large amounts of phosphatidylethanolamine, thus mixing with membrane components and inhibiting formation of biologically active aggregates (35). In fungal membranes, phosphatidylcholine and fengycin do not interact, and aggregate formation takes place. Thus, contrary to intuition, the presence of an avid binding partner in a membrane can actually diminish the effect of an intercalating agent. In the case of jagaricin, we can speculate whether aggregate formation in membranes is relevant to its biological activity and which possible role sterols may play in the biological activity of jagaricin.

Interestingly, organisms containing cholesterol (mammals) and ergosterol (fungi and social amoebae) were targeted, while bacteria (containing no sterols) and plants, whose membranes typically contain a diverse mixture of sterols (mainly phytosterols, with cholesterol typically accounting only for 1 to 2% of total sterols [37]), remained largely unaffected. When we investigated the ergosterol metabolism in yeasts, we observed both positive and negative effects on jagaricin susceptibility: strains defective at different steps of ergosterol biosynthesis (*Caerg5Δ, Cgerg5Δ*, and a clinical *C. albicans* isolate with deficiency at Erg6) showed no altered susceptibility toward jagaricin, while clinical isolates deficient for Erg3 showed reduced jagaricin tolerance. These strains are known to accumulate episterol, ergosta-7-enol, and ergosta-7,22-dienol instead of ergosterol under typical culture conditions (38, 39). Such compounds could potentially serve as a jagaricin interaction partner as well or better than ergosterol to explain this phenotype. Importantly, these compounds do not have the same detrimental effect as 14α-methylergosta-8,24(28)-dien-3β,6α-diol to the organism (40), which accumulates in membranes of azole-treated yeasts and increases their fluidity (40). Since we ob-

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**FIG 9** Polarity distribution within the jagaricin molecule. The structure and distribution of polar and apolar parts within the jagaricin molecule are depicted. Polar functional groups are highlighted in light yellow.
served a rescue of yeast growth when clotrimazole was added together with otherwise toxic jagaricin levels, it seems possible that such a more fluid membrane decreases the activity of jagaricin, for example via reduced aggregation or faster closing of lesions formed by jagaricin. Indeed, it is known that higher plasma membrane sterol levels (and concomitant decreased fluidity) prolong the lifetime of opened pore states for iturins (32, 33). We similarly observed that the UPC2 gain-of-function strain, with its increased ergosterol plasma membrane levels (14–16), was hypersusceptible to jagaricin.

Furthermore, transcription of the regulator of ergosterol biosynthesis in C. albicans, UPC2, itself was upregulated upon short-term exposure to jagaricin, possibly indicating membrane stress. However, it was downregulated, together with some ergosterol biosynthesis genes (ERG1, ERG3, and ERG11), under longer-term growth permissive jagaricin treatment. This would lower the ergosterol levels and thus jagaricin susceptibility. Whether or not this happened as a direct response to the action of jagaricin will be interesting to study in the future, for example, to determine whether this mechanism could be inhibited to enhance the antifungal action of jagaricin. In summary, we conclude that composition and levels of membrane sterols can influence jagaricin susceptibility, and a reduction of ergosterol biosynthesis rates might enable yeasts to grow in the presence of otherwise toxic jagaricin levels—interestingly the opposite of a classical azole resistance mechanism.

So far, we have discussed the direct interaction of jagaricin with the plasma membrane. But what are the consequences of this interaction for susceptible target cells? Lesions caused by jagaricin are likely large, since they allow the transit of large hydrated ions like Ca2+ and nonselective, since the influx of Ca2+ was not inhibited by Cd2+ in a mammalian cell line. As a result, these ion fluxes will lead to the breakdown of the plasma membrane potential. This would lead to increased osmosensitivity, and in fact deletion of essentially any of the MAP kinases of the high osmolarity glycerol pathway resulted in a decreased jagaricin resistance. This is similar to iturins which have been shown to provoke osmotic stress in the fungal phytopathogen Verticillium dahliae, and fungal resistance toward iturins depends on Hog1 activation (41). In conclusion, we hypothesize that the breakdown of plasma membrane potential, together with the intracellular accumulation of Ca2+, aided by osmotic stress and possibly ROS generation, will in sum lead to fungal cell death.

Our data using growth permissive jagaricin concentrations revealed important hints regarding the molecular events triggered by jagaricin in fungi: during exposure, we found transcriptional indications of a shutdown of mitochondria function, which could hint toward a disruption of mitochondrial membrane potential by sublethal jagaricin levels, similar to the surfactants SDS and CTAB (23) or feasibly apoptotic processes under disrupted Ca2+ homeostasis (42). Are mitochondria thus a relevant target for jagaricin toxicity? Loss of mitochondrial membrane potential will disrupt ATP generation, and we would expect a pronounced reduction of energy-consuming cellular processes such as transcription, translation, amino acid biosynthesis, and fatty acid synthesis. Except for the transcriptional downregulation of fatty acid biosynthesis in the short-term transcriptomic response, we did not observe such reductions neither in the transcriptional short-term nor in the growth response. We therefore propose that the observed transcriptional shutdown of plasma membrane transport processes, as well as the downregulation of the gene for the main electrogenic proton pump, Pma1 (43), in C. albicans is caused by the loss of the plasma membrane chemiosmotic potential and Ca2+ influx rather than by ATP level reduction. In support of this view, Ca2+-dependent calcineurin signaling is known to control Pma1 activity in S. cerevisiae (44, 45). Furthermore, a subinhibitory jagaricin concentration elicited limited cell death in a subpopulation of C. albicans cells as shown by PI staining, although the majority continued to grow (Fig. 1A).

Interestingly, during our screening we found two deletion mutants with increased resistance toward jagaricin, both lacking genes for inositol polyphosphate phosphatases (cginp53Δ and cainp51Δ but not cginp51Δ). Both enzymes dephosphorylate inositol phosphates, including PIP2, which is located in the inner membrane leaflet and
known to play a pivotal role in actin cytoskeleton organization, endocytosis, and integrity of the cell envelope as a membrane anchor for proteins (46, 47). This complements the observed hypersusceptibility of Cgslm1Δ, a C. glabrata mutant lacking the homolog of the S. cerevisiae Pip2-binding protein Slim1. The latter is known to regulate actin cytoskeleton organization in response to stress (48). In summary, this membrane-bound regulator of endocytosis and actin polymerization is proposed to play an important role in withstanding jagaricin, again linking membrane composition to jagaricin susceptibility.

Permanently elevated intracellular Ca2+ levels, breakdown of plasma membrane potential and membrane pores all together disrupt the cell’s highly ordered state. Elevated cytosolic Ca2+ levels alter the localization of cationic proteins away from the cytoplasm-facing membrane leaflet (49), and Ca2+ influx through membrane lesions also triggers the onset of local membrane repair processes (50). Therefore, the transcriptional upregulation of phospholipid translocases could counteract jagaricin action in two ways: first, directly via promotion of (e.g., endosome-driven) membrane repair processes (51, 52) and, second, by potentially increasing negatively charged phospholipids in the cytoplasmic leaflet of the plasma membrane to restore protein organization. This could provide an additional explanation for the resistance phenotype of the inositol polyphosphate phosphatases, since an elevated level of highly phosphorylated inositol phosphates would have the same effect. Interestingly, of the three most strongly upregulated jagaricin phospholipid translocases, Rta2 and Rta4 are targets of calcineurin regulation and upregulated under extracellular Ca2+ stress (53, 54), and Rta3 has been shown to regulate membrane asymmetry in C. albicans (55). Therefore, membrane asymmetry regulation in response to elevated cytosolic Ca2+ levels might (partially) restore cell homeostasis under the severe stress induced by jagaricin.

Overall, these proposed mechanisms render jagaricin an interesting candidate for a fungicide directly targeting the membrane. However, its lytic effect on erythrocytes seems to prohibit its use in mammals in its unmodified form. Potentially, chemical modification could be used to increase its specificity. One possible aim of further optimization should be increased self-aggregation within ergosterol-containing membranes over cholesterol-containing membranes, if aggregates in fact represent the active forms of jagaricin. Jagaricin derivatives with decreased overall toxicity could potentially be combined with non-azole ergosterol biosynthesis inhibitors which increase sterol by-products to enhance its efficacy, similar to what we observed for the Erg3-deficient C. albicans strains with jagaricin.

As an interesting feature, we observed broad activity against phytopathogenic fungi, but no or very specific and limited detrimental effects on their host plants: all tested phytopathogens were susceptible in a comparable concentration range, while plant root growth was either unaffected (L. sativum) or only affected under one experimental condition (S. alba). Despite modern crop management, phytopathogenic fungi still cause devastating yield losses (5–7, 56), and agricultural use of substance classes also in use for human disease treatment is controversial (8, 57). As biotechnological production of jagaricin appears feasible, further optimization of fermentation conditions of Janthinobacterium agaridamnosum and future process development will enable the production of ample amounts for agricultural uses.

Interestingly, Bacillus species producing various amounts of surfactins, iturins, and fengycins are used as biocontrol species in agriculture instead of the purified compounds (58), which have been reported to stimulate the plant defense against pathogens in addition to their direct antimicrobial action (58). Janthinobacteria are commonly found in soil (59, 60) raising the question whether jagaricin-related compounds that have coevolved in symbiotic bacterium-plant interactions exist. Therefore, Janthinobacteria might merit further investigations to elucidate their potential as biocontrol species.

In summary, we showed that jagaricin is an example for a virulence factor developed by a pathogenic bacterium against its mushroom host that exerts its function by disrupting cell membranes. We found a certain degree of species specificity and suggest that this is at least partly based on membrane composition. Interestingly, the
target and the mode of action of jagaricin are superficially similar to toxins of other mushroom-infecting bacteria such as tolaasin from *Pseudomonas tolaasii* (61, 62) and WLIP from *Pseudomonas reactans* (61). This kind of interorganism interaction could thus be an interesting source to identify new antifungals for human medicine. Jagaricin and its future derivatives are therefore candidates with great potential as antifungal agents.

**MATERIALS AND METHODS**

**Yeast growth conditions.** Yeasts were routinely streaked on YPD (1% yeast extract, 2% peptone, 2% glucose; pH 7) agar and incubated 1 to 2 days at 30°C. Yeast cultures were stored at 4°C for up to 1 month. Yeast cultures were routinely grown overnight in YPD (1% yeast extract, 1% peptone, 2% glucose; pH 6) at 30°C with shaking (180 rpm).

**Yeast strains.** For the PI assay, the transcriptome analysis and the combinatorial drug tests, the *C. albicans* reference strain SC5314 was used. For *C. albicans* and *C. glabrata* strains used in the mutant test screening, see Table S1B in the supplemental material.

**Chemicals and media.** A total of 500 ml of 2× SD (pH 6) (+ CSM) (+ uridine) liquid medium, 6.7 g of YNB without amino acids (Difco, catalog no. 291940, 100 g), and optionally 0.395 g of complete supplement mixture (CSM, Formedium, catalog no. DCS0019) were dissolved in 400 ml of double-distilled water, adjusted to pH 6 with NaOH, and autoclaved. Then, 100 ml of autoclaved 20% (wt/vol) glucose solution was added. When required, 5 ml of water was replaced by an autoclaved uridine (5 mg/ml; Carl Roth, catalog no. 07143) solution added after autoclaving to reach 50 μg/ml.

Jagaricin powder was dissolved at a concentration of 1 mg/ml in 10% ethanol by sonication, aliquoted, and stored at −80°C until usage. Aliquots were thawed immediately before use. Amphotericin B (Sigma, catalog no. A8880) was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 10 mg/ml, aliquoted, and stored at 4°C in the dark for a maximum of 6 months. Aliquots were thawed immediately before use. Caspofungin diacetate (Sigma, catalog no. 32343, 10 mg) was dissolved in sterile phosphate-buffered saline (PBS) to give a final concentration of 20 mg/ml, aliquoted, and stored at −20°C. Aliquots were thawed immediately before use. Clotrimazole powder (Bayer) was dissolved at 1 mg/ml in 10% ethanol to yield a cloudy stock solution, which cleared upon further dilutions. Aliquots were stored at −80°C and thawed immediately before use.

**Jagaricin production and purification.** A cryostock of *Janthibacterium agaricidamnosum* DSM 9628 (~0.5 ml) was used to inoculate 50 ml of sterile nutrient broth (1 g/liter beef extract, 2 g/liter yeast extract, 5 g/liter peptone, 5 g/liter NaCl), and the culture was grown at 25°C for 20 h at 150 rpm. Five Erlenmeyer flasks containing 200 ml of a suitable sterile growth medium (5 g/liter glycerol, 10 g/liter glucose, 10 g/liter yeast extract, 5 g/liter sodium glutamate, 3 g/liter CaCO₃ after sterilization, 2 ml/liter trace element solution, containing 4 g/liter CaCl₂-2H₂O, 1 g/liter ironIII citrate-H₂O, 0.2 g/liter MnSO₄, 0.1 g/liter ZnCl₂, 0.03 g/liter CoCl₂·6H₂O, 0.04 g/liter CuSO₄·5H₂O, 0.03 g/liter Na₂MoO₄·2H₂O, and 0.06 g/liter Na₂B₄O₇·10H₂O was added, and the pH was adjusted to 6.8 using NaOH) were inoculated with 1 ml of the preculture. The cultures were grown at 25°C for 20 h at 150 rpm. Fermentation was performed at a 30-liter scale using the same medium and 0.167 g/liter antifoaming agent SAG471. The fermentor was inoculated with 800 ml of the preculture. The pH was regulated in a range of 6.2 to 7.8, and the temperature was set to 23°C. The culture was aerated with a sterile airflow of 5 to 10 liters/min and a stirrer speed of 200 to 500 rpm to reach a saturation of >20% O₂. The fermentation was stopped after 51 h. Then, 15 liters of the fermentation broth was twice extracted with ethyl acetate (27 and 15 liters), and the collected extracts were concentrated to dryness. The residue was taken up in methanol (20 ml), and ice-cold ethyl acetate (40 ml) was added. The mixture was sonicated and stirred until a homogeneous slurry was obtained. The solid was removed by filtration and washed with small portions of ice-cold ethyl acetate and dried under reduced pressure. A portion of the solid (50 mg) was treated with methanol (~1 ml), centrifuged to remove undissolved material, and the supernatant was subjected to preparative high-pressure liquid chromatography (Macherey & Nagel Nucleodur C₁₈ column [5 μm, 40 by 250 mm]; gradient, 25 to 75% acetonitrile with 0.1% trifluoroacetic acid; flow, 30 ml/min) to obtain pure jagaricin (28 mg).

**Candida mutant susceptibility testing.** Yeast overnight cultures were harvested by centrifugation for 1 min at 5,000 × g, washed twice with distilled water, and resuspended in 1 volume of distilled water. Yeast cells were then diluted 1:100 in 2× SD plus CSM medium and adjusted to 5 × 10⁶ yeast cells/ml. All dilutions were stored on ice to prevent further yeast growth. Jagaricin stock solutions were diluted to 6 or 4 μg/ml in 1% ethanol. For the assay, 100 μl of yeast cells was mixed with 100 μl of jagaricin or control solution in a 96-well plate (TPP, catalog no. 92696), resulting in 5 × 10⁶ cells per well in 1× SD plus CSM plus 0.5% ethanol (pH 6). Each strain was tested at 3, 2, and 0 μg/ml jagaricin in technical duplicates for each of three biological replicates. For growth curve measurements, the plate was covered with sealing foil (Excel Scientific, STR-SEAL-PLT), transferred to a microplate reader (Tecan Infinite M200; i-control software), and incubated for 3 days at 30°C, with absorbance measurements at 600 nm (λ₅₅₀) every 30 min after 10 s of orbital shaking.

Occasional, late-onset residual growth occurred even at the usually toxic dose of 3 μg/ml jagaricin. If all or the majority of strains showed unexpected fast growth at 3 μg/ml jagaricin, the assays were excluded from further analysis, and new biological replicates were performed with a fresh jagaricin aliquot (in a total of two cases). Stable growth of a mutant at 3 μg/ml jagaricin was defined as detectable growth in at least two of three biological replicates and a maximum change in A of ΔA = max (A − A_背景) of ±0.15 (the mean of three biological replicates). We used the growth curve data to calculate...
the relative $A_{\text{max}}$ as follows: relative $A_{\text{max}}$ (%) = ($A_4$ [mutant]$/A_4$ [wild type]) × 100. The relative half-maximal time ($t_{1/2}$) was defined as the $-\log$ value of the ratio of the wild type and mutant $t_{1/2}$ values.

Transcriptome analysis. For RNA isolation, C. albicans SCS314 was grown in YPD overnight, 1 ml of this culture harvested by centrifugation (10,000 × g, 1 min), washed twice with distilled water, and used to inoculate a second YPD culture at OD$_{600}$ of 0.1 in 5 to 10 ml of YPD, which was grown at 30°C and 180 rpm until reaching a OD$_{600}$ of 1 to 4. Cells were pelleted (10,000 × g, 1 min), washed twice with distilled water, and adjusted to either 10$^7$ ml$^{-1}$ or 5 × 10$^8$ ml$^{-1}$ in 2 × SD (pH 6) for short-term exposure to and growth in jagaricin, respectively.

For short-term exposure, yeasts were mixed 1:1 with jagaricin or control solution to reach 5 × 10$^6$ yeast cells/ml in SD (pH 6) plus 0.05% ethanol with (1 μg/ml) or without jagaricin in 5 ml and then incubated at 30°C and 180 rpm. Yeast cells were harvested for RNA isolation after 30 min, and continued yeast viability was ascertained by CFU plating. Higher concentrations of jagaricin (5 μg/ml) consistently led to cell death (data not shown).

For the transcriptome response of C. albicans cells growing in the presence of jagaricin, yeasts were mixed with jagaricin, respectively, control solution to reach 2.5 × 10$^6$ yeast cells/ml in SD (pH 6) plus 0.05% ethanol with (0.5 μg/ml) or without jagaricin in 15 ml, followed by incubation at 30°C and 180 rpm. We found that 0.5 μg/ml jagaricin in this assay increased the lag time by several hours but did not influence the final growth rate. Samples were taken at the early growth phase at an OD$_{600}$ of 0.5. Three biological replicates were performed for each condition.

RNA was then isolated using an RNasey minikit (Qiagen) and Cy5-labeled cRNA (Cy5 CTP; GE Healthcare) generated using a QuickAmp labeling kit (Agilent). Samples were cohybridized with a common Cy3-labeled reference (RNA from mid-log-phase-grown C. albicans SCS314 [63]) on Agilent arrays (AMADID 026869), scanned in a GenePix 4200AL with GenePix Pro 6.1 (Auto PMT; pixel size, 5 μm), and scanned in AEGP (AEGP). Data analysis was performed with GSEA v2.2.0 (Broad Institute) [64, 65], and Revigo (66).

Hemolysis assay. Blood for hemolysis assays was taken from healthy volunteers with written informed consent according to the principles expressed in the Declaration of Helsinki. The blood donation protocol and use of blood were approved by the institutional ethics committee of the University Hospital Jena (permission number 2207-01/08). Blood was collected using EDTA-containing 5-Monovette tubes (Sarstedt). Portions (1 ml) were centrifuged for 5 min and 1,000 × g down to 0.1ng/ml; the positive growth control

C. albicans PI assay. Yeast overnight cultures of C. albicans SCS314 were harvested for 1 min at 5,000 × g, washed twice with distilled water, resuspended in either 2 × SD or 2 × PBS (pH 7.4) at 5 × 10$^5$ ml$^{-1}$, and kept on ice until the start of the assay. Portions (100 μl/well) were then pipetted into a 96-well plate (TPP) with 1 μl of propidium iodide (PI) solution (1 mg/ml), and the cells were allowed to settle for 15 min. Then, 100 μl of jagaricin solution was added to a final concentration of 0 to 8 μg/ml jagaricin (containing 0.1% [vol/vol] ethanol). Immediately after jagaricin addition, the plate was imaged hourly with a CellDiscoverer 7 microscope and ZEN 2.3 software (Zeiss) at 30°C for 20 h; three biological replicates were performed. The images were analyzed by automatic detection in ImageJ (68–70), with yeasts and PI-positive signals defined as signal intensities of greater than or equal to the means plus 3 × standard deviation in the contrast enhanced bright-field or fluorescence channel, respectively. A fraction of yeasts lost the PI signal over time. The fraction size and decay time of PI signal were thus separately determined with 150 yeasts in 3, 4, and 8 μg/ml jagaricin and used to correct the loss of PI signal of the respective jagaricin concentrations during longer measurements.

Cooperative drug tests. Overnight cultures of C. albicans SCS314 were harvested for 1 min at 5,000 g, washed twice and resuspended in distilled water, and adjusted to 5 × 10$^6$ ml$^{-1}$ and kept on ice until start of the assay. Jagaricin and other drugs (clotrimazole, caspofungin or amphotericin B) were diluted from stocks with double-distilled water to appropriate concentrations. Assays were performed as follows: 100 μl of yeasts were mixed with 50 μl each of jagaricin and combinatorial drug in a microplate well (TPP, Ref. no. 92696) to a final 2.5 × 10$^4$ cells per well in 1 × SD (pH 6) plus 0.05% (vol/vol) ethanol (amphotericin B, caspofungin) or 0.15% (vol/vol) ethanol (clotrimazole), and 0.05% (vol/vol) DMSO (amphotericin B). The plate was covered with a gas-permeable sealing foil (Attitude; product code 4ti-0516/96) and absorbance at 600 nm determined every 30 min in a Tecan infinite M200 microplate reader (software: i-control) set to 30°C for a total duration of 48 h. Three biological replicates were performed for each combination scenario. For FICI calculations, MIC95 was used for jagaricin, AMB and CAS, while MIC75 was applied for CLT.

Growth inhibition assay D. discoideum. A total of 3,000 D. discoideum cells (AX2) were cultured at 22°C as triplicates in 96-well plates (Sarstedt) in 200 μl of H5S medium containing 1% DMSO (Carl Roth) and a 2-fold serial dilution of jagaricin from 1 μg/ml down to 0.1 ng/ml; the positive growth control

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lacked jagaricin. After 72 h, the cell concentration was determined at a size range of 7.6 to 17.6 μm with a CASYCell Counter and an Analyser system (model TT [Roche Innovatis AG]; equipped with a 60-μm capillary). The viable cell concentration was plotted against the logarithmic concentration of the compound to determine the IC50 value using Prism. The assay was repeated three times to obtain the weighted mean and error of the logIC50, IC50, as described previously (67).

[Ca2+]i and patch-clamp measurements. HEK293T cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained in DMEM/F-12 (Dulbecco modified Eagle medium: nutrient mixture F-12; Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) at 37°C in a 95% air–5% CO2 atmosphere and saturated humidity. Measurements of intracellular free Ca2+ levels ([Ca2+]i) were performed as described previously (71) with slight modifications: HEK293T cells were loaded with 1 μM Fura-2-AM (Life Technologies) in complete culture medium for about 20 min at 37°C. The cells were washed, and recording was performed in a HEPES-buffered solution containing 150 mM NaCl, 2 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES (pH 7.4) at room temperature. In control experiments, the specific Ca2+ transport was blocked by the addition of 0.1 mM CdCl2 to the recording buffer. Fluorescence was measured using a microscopy-based system after alternating the illumination at 340 or 380 nm with a sample rate of 0.1 Hz. The data were analyzed using TillvisION software (TILL Photonics, Gräfelfing, Germany). The 340-nm/380-nm ratio of the background-corrected fluorescence signal was calculated on a single-cell level and plotted as a function of time for 60 to 100 cells per experiment in at least five independent experiments for each condition.

Currents were recorded from HEK293T cells in the whole-cell configuration of the patch-clamp method using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany), operated with PatchMaster software (HEKA; for further details, see reference 72). Cells were clamped to −40 mV, while jagaricin was applied by complete bath exchange. Solutions were as follows (in mM): internal (pipette), 140 KCl, 10 EGTA, and 10 HEPES (pH 7.4 with KOH); and external (bath), 146 NaCl, 4 KCl, 2 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.4 with NaOH).

Black lipid membranes. Planar lipid bilayers of asolectin from soybean (Sigma, catalog no. 11145-50G), dissolved in o-decane (10 mg of lipid per ml of solvent), were formed on a 1-mm-diameter Teflon septum with both bath chambers containing 100 mM KCl and 10 mM Tris-HCl (pH 8.0). The membrane current was measured with a Turbo TEC-10CD amplifier (NPI Electronic GmbH, Tamm, Germany), combined with repetitive stimulation with voltage ramps from −50 to 50 mV (see reference 73). Jagaricin was applied on both sides of the membrane up to a final concentration of 4 μg/ml amphotericin B (2 μg/ml) and gramicidin A (1 μM) served as positive controls.

Plant susceptibility testing. Jagaricin phytotoxicity was investigated at 5 μg/ml using PhytoTest-Kit (MicroBioTests, Inc.) with two plants according to the manufacturer’s instructions: Lepidium sativum and Sinapis alba were incubated at 25°C in the presence or absence of light for 72 h in three or two biological replicates, respectively, and evaluated for inhibition of seed germination and root length.

Susceptibility test of phytopathogenic fungi. Antifungal activity was determined in broth dilution according to CLSI reference method M38-A2, with slight modifications, for the major agricultural phytopathogens Alternaria alternata, Penicillium digitatum, Penicillium italicum, Geotrichum candidum, Colletotrichum gloeosporioides, Fusarium graminearum, and Botrytis cinerea. The tests were performed in 96-well plates in duplicate, with 195 μl of RPMI 1640 medium per well supplemented from a jagaricin stock solution (1.28 mg/ml dissolved in 5% DMSO) to reach a final concentration range of 20 to 0.08 μg/ml. Then, 5 μl of each fungal species was added to reach 5 × 104 CFU/ml, followed by incubation for 48 h at 30°C. As a positive control, ketoconazole (50 μg/ml) was used, and 5% DMSO in RPMI 1640 medium served as the negative control.

Critical micellar concentration (CMC) determination. Measurements were performed as described previously (74). Fluorescence measurements were performed on a Jasco FP6200 instrument using 10-μm fused silica cuvettes. Excitation was carried out at 331.5 nm, and emission spectra were recorded at 400 to 450 nm. l/l0 ratios were calculated from the intensities at 374 nm (1) and 386 nm (3). The matrix solution was prepared from ultrapure water (Thermo GenPure system) containing approximately 2 μM pyrene. Pyrene (≈0.4 mg) was taken up in 20% ethanol using an ultrasonic bath (80°C) and diluted with water to give a final ethanol content of 0.5%. To this solution, defined volumes of a suitable stock solution of jagaricin (1, 10, or 100 mg/ml in methanol) were added to give final volumes of 4 ml and total methanol contents of <2%, preferably <0.5%. Individual samples for each data point were prepared, gently mixed, and incubated for at least 30 min at room temperature prior to fluorescence measurement.

Statistical analysis. Experiments were performed in biological replicates from independent samples (n ≥ 3) unless stated otherwise (see “Plant susceptibility testing”). All experiments were performed unblinded. Data points in graphs represent either (weighted) arithmetic or geometric means (see individual graphs) ± the standard deviations. Data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, ImageX (68–70), Excel 2010, GeneSpring GX (v14.8; Agilent Technologies, Inc., Santa Clara, CA), GSEA v2.2.0 (Broad Institute) (64, 65), and Revigo (66). Where applicable, samples were tested for significance (P < 0.05) using a two-sided t test, and GO-term enrichment was determined by using the Fisher exact test.

Data availability. All relevant data are available by request from the authors, with the restriction of data that would compromise the confidentiality of blood donors. The microarray data are available in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7718.
SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00707-19.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 1.8 MB.
SUPPLEMENTAL FILE 3, XLSX file, 1.7 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank Vito Valiante and Jakob Weber for sharing unpublished results and helpful discussions, Dominique Sanglard and Oliver Bader for kindly providing some strains for the mutant analysis, Karin Martin for performing and optimizing the jagaricin preparation, Nadja Jablonowski, Daniela Schulz, and Dorothee Eckhardt for technical help with the Candida mutant tests, and Ankido Gustin for technical help with the C. albicans killing assay.

D.F., B.H., and S.B. have been supported by the Cluster of Excellence “Balance of the Microverse” of the Friedrich Schiller University Jena and by the Leibniz Science Campus “InfectoOptics” and Collaborative Research Centre/Transregio 124—“FungiNet” (project C1) of the Deutsche Forschungsgemeinschaft. S.H.H. has been supported by the German Federal Ministry of Research and Education (Competence Cluster for Nutrition and Cardiovascular Health—nutriCARD, grant 01E1411A). R.B. was a member of the Jena School for Microbial Communication. F.K. has been supported by InfectControl 2020 (FKZ 03ZZO803A). The work of C.H., K.S., K.D., and T.P.F. has been supported by the SFB 1127 ChemBioSys. T.P.F. has been supported by a Capes-Humboldt research fellowship. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

D.F. performed experiments (C. albicans killing, Candida mutant screen, C. albicans transcriptome analysis, hemolysis assay, and the cooperative drug test), analyzed the data, wrote the manuscript, and prepared the figures. G.G. and K.T. performed patch clamp assays and measurements of intracellular free Ca^{2+} levels, analyzed the data, and edited the manuscript. T.P.F. performed susceptibility tests of phytopathogenic fungi and plant susceptibility testing, analyzed the data, and edited the manuscript. R.B. and P.S. performed the D. discoideum susceptibility test, analyzed the data, and edited the manuscript. F.K. and K.S. isolated and provided jagaricin. F.K. performed the CMC determination, analyzed the data, and edited the manuscript. K.D., B.H., and C.H. discussed and interpreted the data and edited the manuscript. S.H.H. discussed the data, edited the manuscript, designed parts of the study (patch clamp assays and intracellular calcium measurements), and prepared the corresponding figures. K.S. and S.B. conceived and designed the study, K.S. edited the manuscript, and S.B. cowrote the manuscript.

We declare no competing interests.

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Membrane Disruption by Jagaricin

Antimicrobial Agents and Chemotherapy

September 2019 Volume 63 Issue 9 e00707-19 aac.asm.org


