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Jawsamycin exhibits in vivo antifungal properties by inhibiting Spt14/Gpi3-mediated biosynthesis of glycosylphosphatidylinositol

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Biosynthesis of glycosylphosphatidylinositol (GPI) is required for anchoring proteins to the plasma membrane, and is essential for the integrity of the fungal cell wall. Here, we use a reporter gene-based screen in Saccharomyces cerevisiae for the discovery of antifungal inhibitors of GPI-anchoring of proteins, and identify the oligocyclopropyl-containing natural product jawsamycin (FR-900848) as a potent hit. The compound targets the catalytic subunit Spt14 (also referred to as Gpi3) of the fungal UDP-glycosyltransferase, the first step in GPI biosynthesis, with good selectivity over the human functional homolog PIG-A. Jawsamycin displays antifungal activity in vitro against several pathogenic fungi including Mucorales, and in vivo in a mouse model of invasive pulmonary mucormycosis due to Rhyzopus delemar infection. Our results provide a starting point for the development of Spt14 inhibitors for treatment of invasive fungal infections.
Invasive human fungal infections are associated with high morbidity and mortality rates. The impact of these diseases is underappreciated but the annual death rate due to serious fungal infections exceeds that caused by tuberculosis or malaria. Despite increasing numbers of affected patients with life-threatening infections and the concomitant increased burden on public healthcare systems, there remains only three main classes of established antifungal agents to treat systemic infections. These are the polyenes, azoles, and echinocandins which act on the fungal plasma membrane, its biosynthesis pathway or cell wall components, respectively. These classes of anti-fungals suffer from restrictions in route of administration, spectrum of fungi. The screen identified jawsamycin as a novel antifungal lead compound.

Results
Identification of jawsamycin as a GPI pathway inhibitor. To identify novel inhibitors of the GPI pathway, we adapted a previously published reporter construct expressing the small Gaussia princeps luciferase gene fused to the GPI-anchoring signal of Candida albicans PGA59 (Fig. 1a). Expression of this construct from the galactose-inducible GALI/10 promoter in the non-pathogenic yeast Saccharomyces cerevisiae allowed to monitor cell surface-bound luciferase in relation to non-anchored, secreted luciferase signal in the medium in case of GPI pathway modulation. Testing the assay with two antifungal agents and one known GPI inhibitor revealed an increase of signal in the medium paralleled by a reduction of signal of the cell pellet with the GPI inhibitor only (Fig. 1b, Supplementary Fig. 1). This suggested a lack of anchoring of secreted reporter protein as expected. To benchmark if this reporter was specific for inhibition of the GPI pathway, we tested a panel of compounds with known, diverse mechanisms of action (Fig. 1c). Based on this set of compounds, it was confirmed that the signal of non-anchored luciferase in the medium increased only when GPI anchor biosynthesis was inhibited. Using this 1356-well supernatant signal assay, we screened a focused set of 12472 compounds selected from the Novartis natural product library at a fixed dose of 10 μM (Fig. 1d). The screen had acceptable quality with an overall z′ score of 0.42–0.54. Among the top hits were structural variants of known Gwt1 and Mcd4 inhibitors as well as two isolation batches of a natural product named jawsamycin. Jawsamycin (also known as FR-900848) is a structurally unique oligocyclopropyl-containing natural product and has been proposed to have potent antifungal activity (Fig. 1e). To support validation and further testing, we optimized fermentation conditions using Streptomyces luteoverticillatus to a yield of 10 mg/l in shake flasks and 5 mg/l in large scale fermenters (see Methods section) and restested the new purified batch in dose-response fashion in the reporter gene assay (Fig. 1f). Concentration-dependent decrease of luminescence in the cell-pellet fraction by jawsamycin was confirmed and a half maximal inhibitory concentration (IC50) of ~7 μM measured. This validated jawsamycin as a GPI pathway modulator.

Chemogenomic profiling points to first node in the GPI pathway. We next sought to discover the target of jawsamycin. Potent growth-inhibitory activity with an IC50 of ~800 nM (Fig. 1g) made it a suitable compound to investigate the mechanism of action by chemogenomic profiling using the S. cerevisiae heterozygous and homozygous deletion collections. Haploinsufficiency profiling (HIP) and homozygous profiling (HOP) are gene-dosage dependent methods that assesses the effect of compounds against potential targets encoded by the S. cerevisiae genome. HIP indicates pathways directly affected by compound. HOP (both gene copies deleted) indicates synthetic lethality and identifies compensating pathways to those directly affected by the compound.
Two independent HIP assays reproducibly identified, GPI1, GPI2, GPI15, and SPT14, as hits in essential genes with the best z-scores (Fig. 2a). These four components are 4 of 6 subunits which comprise a complex to transfer UDP-GlcNAc to phosphatidylinositol, the first intermediate in the synthesis of glycosylphosphatidylinositol (GPI) anchors 23.

PIG19, encoding one additional, essential subunit displayed moderate hypersensitivity in the HIP assay, the sixth subunit encoded by ERI1 was not resolved as this strain is not part of the standard yeast deletion collection. Hypersensitivity of these hits was unique to jawsamycin and has never been observed in chemogenomic profiling experiments of more than 4000 diverse other compounds22. Not only does this underline the biological relevance of the hits, but also suggests that the used reporter assay successfully enriches for compounds modulating the GPI pathway. Re-testing by dose-response experiments of the individual strains confirmed the finding from the genome-wide profiling experiment (Supplementary Fig. 2a). GPI-anchored proteins receive the GPI anchor as a conserved posttranslational modification in the lumen of the endoplasmic reticulum (ER). After anchor attachment, the GPI anchor is structurally remodeled to function as a transport signal that actively triggers the delivery of GPI-anchored proteins from the ER to the plasma membrane8 where many have essential functions in cell-wall synthesis7. Inhibition of GPI biosynthesis has been shown to lead to disrupted proteostasis in the ER triggering the activation of the unfolded protein response (UPR)5. In agreement with inhibition of GPI biosynthesis by jawsamycin, the two key components for activation of the UPR, HAC1 and

Fig. 1 Identification of jawsamycin in a screen for GPI biosynthesis inhibitors. a Schematic representation of reporter gene construct and assay principle. b Characterization of signal distribution in medium and pellet upon growth inhibition. Approximate IC50 compound concentrations determined in pre-experiments were used in the test (1 μM for voriconazole and E1210, 10 μM amphotericin B). c Testing of assay specificity using inhibitors against different known targets and pathways. d Primary assay data of 12472 natural products screened at 10 μM. e Structure of jawsamycin, a key hit in the screen. f Dose-response validation of jawsamycin in the reporter gene assay. g Dose-response validation of jawsamycin against growth of S. cerevisiae. Bars in panel b depict the mean of 3, in panel c the mean of 6 measured wells, dots in panel f and g depict the mean of 2 replicates; in all panels error bars depict +/− one standard deviation. All raw data is available in the Source Data file.
IRE1 scored as prominent hits in the HOP experiment (Fig. 2b). As for the hits from the HIP experiment, hypersensitivity of hac1 and ire1 deletion strains was confirmed in single strain experiments (Supplementary Fig. 2b). Therefore, unbiased chemogenomic profiling experiments identified heterozygous and homozygous deletion strains in agreement with inhibition of GPI anchor biosynthesis.

**Mutations in SPT14/GPI3 confer jawsamycin resistance.** Chemogenomic profiling allowed us to postulate that jawsamycin
Fig. 2 Identification of the target of jawsamycin. a Two independent haploinsufficiency profiling (HIP) experiments identify strains compromised in genes encoding the phosphatidylinositol N-acetylgalactosamine transferase complex (GPI, GPI15, GPI2, and SPT14) as selectively and reproducibly hypersensitive against Jawsamycin. GPI15, encoding an additional subunit of this complex scored some hypersensitivity, response of ER11 also associated with this complex could not be recorded as this strain is not part of the standard yeast deletion collection. b Two independent homozygous profiling (HOP) experiments with jawsamycin reproducibly identify synthetic genetic nodes in the unfolded protein response (HAC1, IRE1), cell wall integrity (MID2, JIP3, RLM1) and the pre-mRNA splicing component gene MUD2. The profiles plot sensitivity (y-axis) vs. z-score (x-axis) and the entire dataset is available in the Source Data file. The z-score represents a measure for uniqueness of a hit in relation to >3000 tested, diverse chemical compounds (see materials and methods section).
Although some mutations in PIG-A are reported to be embryonic lethal in mice\(^2^7\), human cells with PIG-A lead to loss of extracellular concentration of GPI-anchored proteins in HCT116 cells as assessed by staining with the fluorescently labeled GPI-binding compound aerolysin (FLAER) and flow-cytometry analysis. In line with jawsamycin inhibiting GPI biosynthesis and consequently compromising cell viability and demonstrating a fungicidal effect.

Jawsamycin does not inhibit PIG-A the human Spt14 homolog.

The Spt14 enzyme is also conserved in mammalian cells, named PIG-A, with an overall identity \(~40\%\) compared to the fungal homologs. To assess if jawsamycin also inhibits the mammalian enzyme, we tested cytotoxicity on human HCT116 cells. No effect on PGI-anchored proteins as observed by FLAER staining and peak heights normalized. Unstained and untreated control samples were used to set the gating strategy (gating strategy images and additional data are provided in the Source Data file).

Genetic validation of the FLAER assay: genetic editing of the PIG-A gene leads to loss of extracellular concentration of GPI-anchored proteins in HCT116 cells as assessed by staining with the fluorescein-labeled proaerolysin (FLAER) test that is used to diagnose PNH (paroxysmal nocturnal hemoglobinuria) caused by exposure of blood cells to the complement system\(^2^8\). A standard cytotoxicity assay might thus not capture to jawsamycin. Specifically, genetic inactivation of PIG-A using the CRISPR-Cas9 system lead to a strong increase in FLAER negative cells, whereas no such effect was observed with the jawsamycin-treated cells over a period of 6 days (Fig. 3b, c). Thus, we conclude that jawsamycin is a potent antifungal lead compound with good selectivity for the fungal enzymes.

A rapid and versatile chemical derivatization protocol. Jawsamycin is a unique and highly complex natural product containing five cyclopropyl moieties in the fatty acid tail. Its biosynthesis has been explored\(^2^0\) and there have been numerous approaches to chemically synthesize the various parts of the molecule (reviewed by Pietruszka et al\(^3^0\)). The current routes described for the total synthesis of jawsamycin are lengthy and tedious and the limited synthetic scope for derivatizing jawsamycin hampered the accessibility to a diverse set of testable compounds. As an alternate approach we started with the natural compound isolated by Pietruszka et al\(^3^0\). The current routes described for the total synthesis of jawsamycin are lengthy and tedious and the limited synthetic scope for derivatizing jawsamycin hampered the accessibility to a diverse set of testable compounds. As an alternate approach we started with the natural compound isolated by the producer strain and developed protocols to make it amenable to rapid and versatile chemical derivatization.

To allow for variations of the 5′-amino-5′′-deoxy-5,6-dihydrouridine nucleoside moiety we optimized the hydrolysis of jawsamycin to obtain the intact cyclopropanated carboxylic acid (compound 1, see Supplementary Methods). This part of the molecule subsequently served as starting material for the coupling of a limited series of nucleosides in order to explore antifungal structure-activity relationships (Fig. 4). In series a, only minor modifications of the dihydrouracil moiety were tolerated leading to reduced potency and antifungal activity against the four selected strains. The uridine analog JD-4 already loses activity on two strains, replacement of a ketone by a primary amine or introduction of a methyl group abolishes antifungal activity, as shown for compounds JD-2 and JD-3. In line with the identification that the compound inhibits the UDP-glycosyltransferase Spt14, exchange of the headgroup by purine

**Fig. 3** Jawsamycin does not display activity against the human SPT14 homolog PIG-A. **a** Cytotoxicity assessment by dose-response testing of jawsamycin and the control compound benzalkonium chloride against human HCT116 cells. **b** Genetic validation of the FLAER assay: genetic editing of the PIG-A gene leads to loss of extracellular concentration of GPI-anchored proteins in HCT116 cells as assessed by staining with the fluorescein-labeled GPI-binding compound aerolysin (FLAER) and flow-cytometry analysis. **c** Incubation of HCT116 cells with different doses of jawsamycin has no apparent effect on GPI-anchored proteins as observed by FLAER staining and flow-cytometry analysis. For each histogram in panel a and b, 10,000 events were recorded and peak heights normalized. Unstained and untreated control samples were used to set the gating strategy (gating strategy images and additional data are provided in the Source Data file).
rendered compound JD-1 inactive. The N-methyl analog JD-6 retained activity for three fungal strains but not for A. fumigatus. Any elimination or substitution of the two hydroxy ribose moieties resulted in a significant drop of potency as exemplified with compounds JD-7 - JD 14 of series b. Although not comprehensive, this structure-activity analysis suggests that any deviation from the natural uridine substrate is not well tolerated for inhibition of the UDP-glycosyltransferase.

Modifications of the cyclopropanated fatty acid moiety were undertaken with the acetonide protected jawsamycin JD-14. This allowed modifications at positions C14 and C16 in the fatty acid tail (series c, Fig. 4), introducing functional groups such as hydroxy, butylamine, ethinyl and vinyl methylester (JD-17–20). JD-16 bearing a double bond in position 16/1731 was one of the few analogs isolated from the fermentation broth. It showed decent potency against the four fungal strains. As for modifications of the headgroup, also derivatization of the fatty acid tail resulted in dramatic drop in activity, except for the methylester analog JD-20 which revealed an antifungal profile similar to jawsamycin (Fig. 4).

Despite the observation that none of the tested modifications displayed improved potency or spectrum compared to the parent compound, we provide a robust and rapid synthetic protocol for the generation of extended libraries of jawsamycin analogs which allows rapid screening for antifungal activity.

Jawsamycin is efficacious in a pulmonary mucormycosis mouse model. As the limited chemical derivatization described above did not identify a compound with improved potency, we tested the antifungal potential of the original jawsamycin compound in vivo. The observed in vitro potency against Mucorales fungi led us to examine the efficacy of the compound in an in vivo model of invasive pulmonary mucormycosis due to intratracheal instillation of R. delemar32 (Fig. 5a). As depicted in Fig. 5b, c, oral dosing of jawsamycin at 100 mg/kg delivered for 6 consecutive days starting 24 h post infection, led to improved overall survival rate versus placebo-treated mice (45% and 10% overall survival for jawsamycin- and placebo-treated mice, respectively, \( P = 0.001 \)). A lower dose of 30 mg/kg of jawsamycin or a dose of the clinically used posaconazole tended to improve survival compared to placebo-treated mice with \( P = 0.08 \) for both drugs. Administration of jawsamycin for 11 days did not improve overall survival over the 7-day treatment regimen. The enhanced survival in the jawsamycin-treated mice was corroborated by a significant \(-1 \) log reduction in the fungal burden in both lung and brain when compared to placebo mice (Fig. 5d, e). Importantly, jawsamycin

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**Fig. 4 Chemical derivatization and bioactivity profiling of jawsamycin.** Chemical variation of the dihydro uracil moiety (a-series) the dihydro uridine moiety (b-series) and the cyclopropylated fatty acid tail moiety (c-series) was undertaken with the natural compound as starting material. The obtained jawsamycin derivatives (numbered JD-X) were purified, structure verified and tested against the indicated four different fungal pathogens to obtain minimal effective concentrations (MEC).
appeared to be well tolerated by mice with no signs of toxicity at the tested doses.

In summary, this is the first evidence that targeting fungal Spt14/Gpi3 by jawsamycin results in a beneficial effect against pulmonary *Rhizopus* infections. The demonstration of in vitro efficacy, the broad-spectrum activity against fungi in vitro, and its novel mechanism of action, warrants further investigations of jawsamycin as a novel antifungal lead.

**Discussion**

The GPI pathway has previously been proposed as a promising clinical intervention point to treat microbial infections. However, therapeutic exploitation has been hampered by the availability of few chemotypes targeting only two nodes. Here, we introduce a high-throughput screen which aimed at the availability of few chemotypes targeting only two nodes. The demonstration of in vitro efficacy, the broad-spectrum activity against fungi in vitro, and its novel mechanism of action, warrants further investigations of jawsamycin as a novel antifungal lead.

**Fig. 5 Testing of jawsamycin in a murine pulmonary mucormycosis model.** a Outline of experiment. On day 0, mice were infected with 2.5 × 10^5 spores of *R. delemar* by intratracheal instillation. CP cyclophosphamide, CA cortisone acetate, Cff cefazidime. Mice were treated for either 7 days (b) or 11 days (c) with the indicated compound treatments. b, c Kaplan–Meier plots depicting survival of mice under the different treatment regimens over 21 days. n = 20 mice/group from two independent experiments with similar results (confirmed average fungal spores delivered to lungs of 2.4 × 10^3). n = 10 mice/group from one experiment (confirmed fungal spores delivered to the lungs of 3.7 × 10^3). *p = 0.0014, and **p = 0.045 vs. placebo in b, c, respectively.

d, e Tissue fungal burden of lungs and brain harvested from 12 mice/group and processed on day 7 post infection. Tissue fungal burden was determined by real-time qPCR and expressed as Log10 CFU (colony forming units) equivalent/gram of tissue. P values < 0.05 were considered significant (details on data analysis and statistical significance are described in the Methods section).

**Mycobacterium smegmatis** (4N9W). However Spt14 homology is low (~20% identity) and does not allow for calculation of homology models. Despite this, it is obvious that the headgroup of jawsamycin is a close analog of uridine thus it is plausible to hypothesize that jawsamycin engages at the active site and occupies the UDP binding pocket. This notion was supported by our chemistry efforts, in which the headgroup tolerated only minimal changes without complete loss of activity and thus only tolerated alterations still resembling the dihydro-UDP pharmacophore. One caveat of direct analog testing on fungi was that it did not allow differentiation between altered activity due to compound efflux or activity on the target. Although total synthesis of jawsamycin has been described, our optimized protocols for fermentation/isolation and chemical derivatization of the natural compound provide a valuable starting point to expand medicinal chemistry programs to further optimize the lead compound jawsamycin.

Our chemogenomic HOP profiling has identified the UPR key node Hac1/Ire1 to be synthetic lethal with inhibition by jawsamycin. This is in agreement with the notion that non-glypated proteins are retained in the ER and consequently disrupt proteostasis. Thus, it appears that inhibition at the level of Spt14 not only inhibits an essential function but furthermore exerts a dominant negative function. It has been demonstrated that compromising GPI biosynthesis result in cell-wall defects. Again, this is reflected in the high quality HIP HOP profiles where the two genes JIP3/MID2 score prominently in HOP. JIP3 encodes a dubious ORF and almost entirely overlaps the verified MID2 gene. Thus, it is reasonable to consider this as a second mid2 deletion strain. Mid2 acts as a sensor for cell wall integrity and activates the integrity signaling pathway. Thus, the
chremogenic profiling studies have identified the key relevant aspects of GPI biosynthesis: the targeted UDP-GlcNAC phosphatididylinositol transfer complex in HIP and the downstream consequences of target inhibition, UPR, and cell wall defects, in HOP.

Interestingly, the MUD2 gene coding for a protein involved in early pre-mRNA splicing significantly scored in the HOP profile of jaswamycin but not in those of the Gwt1 inhibitor CMB2683 a close El210 derivative, nor the Mcd4 inhibitor M743/CMB1539. As SPT14 is one of few genes in S. cerevisiae that contains an intron affecting SPT14 mRNA maturation in addition to inhibition of the protein by jaswamycin exerts synthetic lethality. This can be considered yet another line of support that SPT14 encodes the primary target of jaswamycin.

The mechanism of action was also apparent when performing microscopic analysis of Rhizopus spores upon incubation with jaswamycin. The compound treatment led to profound spore swelling, with the diameter range 15–20 μm, frequent leakage of cellular content and consequent cell death. This is consistent with defect in the integrity of fungal cell wall, whose biosynthesis requires many GPI-anchored proteins.

Recent positive data with the clinical Gwt1 inhibitors of the APX001 class in models of pulmonary aspergillosis, coccidioidomycosis, and against Candida auris validate the GPI pathway as a valuable druggable biological intervention point to tackle fungal diseases. With the availability of only a few chemogenomic profiles of jaswamycin, the studies were performed using a Multidrop combi dispenser (Thermo Inc.). In all, 3 μl of galactose induction media (100 g/l peptone; 20 g/l yeast extract; and CSM-Ura 0.8 g/l) was placed within lanes 45 and 46. Afterwards 3 μl of a luciferase reaction mixture and incubated for 20 min at room temperature. For luciferase reading a Pherastar FS reader (BMG Labtech, measurement time 10 s; focus height 0.8 mm; gain 4000) was used.

The data was analyzed with the internally developed Helios software tool. Normalized to the controls (DMSO negative control, a Gwt1 inhibitor at a final concentration of 10 μM corresponding to its IC50 value was used as active control, using the following calculation:"

\[ NC_{50} = \frac{x}{x} = \frac{100(x - NC)}{AC - NC} \]

\[ \text{efficacy parameter; } NC \text{ and } AC \text{ are averages (median) over the corresponding neutral control (NC) and active control (AC) values.} \]

Growth inhibition testing. Substances were first assayed for their potency using wild-type S. cerevisiae BY4743 by recording growth inhibition dose-response curves. OD50 values of exponentially growing S. cerevisiae cultures in rich medium with a robotic system. Twelve point serial dilutions, with a dilution factor of 3.16, were assayed in 96-well plates with a reaction volume of 150 μl. Cell growth was recorded as optical density at 600 nm using a Beckman DTX880 plate reader with a neutral detection software v3.3.9. Solutions containing dimethyl sulfoxide (DMSO) were normalized to 2%. IC50 values were calculated using logistic regression curve fits generated by GraphPad Prism (GraphPad Software, San Diego, California USA).

Chemogenic profiling. The growth-inhibitory potency of test substances was determined using wild-type S. cerevisiae BY4743 by recording growth inhibition dose-response curves. OD50 values of exponentially growing S. cerevisiae cultures in rich medium with a robotic system. Twelve point serial dilutions, with a dilution factor of 3.16, were assayed in 96-well plates with a reaction volume of 150 μl. Cell growth was recorded as optical density at 600 nm using a Beckman DTX880 plate reader with a neutral detection software v3.3.9. Solutions containing dimethyl sulfoxide (DMSO) were normalized to 2%. IC50 values were calculated using logistic regression curve fits generated by TIBCO Spotfire Spotfire 7.9–10.3 (TIBCO Software Inc.).

The Saccharomyces haploinsufficiency profiling (HIP) and homogygous profiling (HOP) and microarray analysis was performed as published previously. The basic concept behind this assay is that HIP identifies genes where one functional copy, compared to two, confers hypersensitivity to inhibition by the compound. This indicates pathways directly affected by the compound. HOP (both gene copies deleted) indicates synthetic lethality and identifies compensating pathways to those directly affected by the compound. Thus genome-wide heterozygous and homozygous deletion libraries of S. cerevisiae strains were purchased, (OpenBiosystems, Cat #YSC1056 and YSC1055) and pools were constructed as published previously. Each HIP strain is heterozygous and each HOP strain compensating for 10 yeast generations (generation 10–12). The HIP strain is heterozygous and each HOP strain completely null for one gene (and each strain being identified by a unique DNA sequence, called “bar-code” or “tag” inserted into the deleted gene). For each HIP and HOP experiment, each test substance was assayed in duplicate (2 wells) at its IC50 concentration, in 24-well plates (Greiner 662102), with 1600 μl/well YPD. DMSO was normalized to 2%. At the onset of the HIP experiment ~250 yeast cells/strain (100 μl of a 1.5 OD50/mL culture) from an overnight log phase pre-culture were plated onto a concentration of 10 μM corresponding to its IC50 value was used as active control, using the following calculation:"

\[ NC_{50} = \frac{x}{x} = \frac{100(x - NC)}{AC - NC} \]

\[ \text{efficacy parameter; } NC \text{ and } AC \text{ are averages (median) over the corresponding neutral control (NC) and active control (AC) values.} \]
(110 µl of a 1.5 OD<sub>600</sub>/ml culture) were transferred into each well. The cell material from the final HPI and HOP plates was harvested, the gDNA extracted and the tags amplified by PCR reaction22. Then relative abundance of each strain in the compound treated wells was compared to eight no-drug control samples that were produced in the same experiment.

For the experimental analysis we used the same computation of normalized tag intensities, outlier masking, and saturation correction as published previously22. Sensitivity was defined as the median absolute deviation (MAD) score for each compound/concentration combination, then gene-wise z-scores based on a robust parametric estimation of gene variability from >3000 different profiles allowing for up to 15% outliers were computed as described in detail22. Profiles were visualized using TIBCO Spotfire 7.9–10.3.

Selection of drug-resistant S. cerevisiae. Strain BY4743Δλ, derived from BY4741 but deleted for eight genes involved in drug resistance (eflux pumps: SNQ2, PDR5, and YOR1; transcription factors: PDR1, PDR2, PDR3, YAPI, and YRM1) was incubated in 2.5% ethylmethane sulfonate until only 50% of the cells formed colonies. A total of 2 × 10<sup>6</sup> mutated cells were plated on two 14-cm dishes with synthetic complete medium (0.7 g/l Difco yeast nitrogen base w/o amino acids, 0.79 g/l MPbio CSM amino acid mixture, and 2% Glucose) containing 500 mM jaysamycin. After 4 days resistant colonies could be isolated and resistance confirmed by restreaking on selective medium. Genomic DNA extraction, whole-genome sequencing and focused sequencing to confirm findings were performed as published22. Mutations were cloned into non-mutagenized wild-type cells and fold resistance compared to wild-type assessed by dose-response testing.

Mammalian cell cytotoxicity testing. Effects on mammalian HCT116 cells. (CCL-247, ATCC) were assayed by testing serially diluted compounds in 384-well plates seeded with 750 cells/well in DMEM high glucose, Glutamax, pyruvate medium (3196047, Life Technologies). DMSO was normalized to 0.2%. Viability (CCL-247, ATCC) were assayed by testing serially diluted compounds in 384-well plates seeded with 10,000 cells/well. The cell material (110 µl of a 1.5 OD<sub>600</sub>/ml culture) were transferred into each well. The cell material was incubated the following day ±jaysamycin. After 4 days resistant colonies could be isolated and resistance confirmed by restreaking on selective medium. Genomic DNA extraction, whole-genome sequencing and focused sequencing to confirm findings were performed as published22. Mutations were cloned into non-mutagenized wild-type cells and fold resistance compared to wild-type assessed by dose-response testing.

Media optimization and fermentation. The producer strain was cultivated at 28 °C for 7 days under agitation in shake flasks and the titers of jaysamycin were quantified by UPLC-UV. Medium 300–3000, consisting of agar (sucrose), 1 g/l; malt extract 5 g/l, glycerol 1 g/l, NaCl 0.05 g/l, CaCO<sub>3</sub> 0.05 g/l, KH<sub>2</sub>PO<sub>4</sub> 0.25 g/l, H<sub>2</sub>PO<sub>4</sub> 0.5 g/l, MgSO<sub>4</sub> 0.1 g/l, yeast extract 1.35 g/l, NZ-amines 2.5 g/l, malt extract 5.85 g/l, L-asparagine monohydrate 1 g/l, soy protein (Unicorn 75) 2.5 g/l, potato starch (Norex A150) 7.5 g/l, cecereus 7.5 g/l, HEPEX 6 g/l, trace element stock solution 1 ml/l and 100 mM ferrous ion (Fe<sub>2</sub>SO<sub>4</sub> 1 g/l), was used as starting point for media optimization. For the optimization of the production medium a Plackett-Burman experimental design was applied as first measure in order to identify the media components that are most crucial for production of jaysamycin. The highest positive media component for potato starch, malt extract, yeast extract, and KH<sub>2</sub>PO<sub>4</sub>. These components were selected for further optimization in a central composite design experiment. In total, 25 different variations were prepared and used for cultivation followed by quantification of jaysamycin. The optimized medium 300–3000, consisting of agar (Bacto) 1 g/l, glycerol 7.5 g/l, NaCl 0.05 g/l, CaCO<sub>3</sub> 0.05 g/l, KH<sub>2</sub>PO<sub>4</sub> 0.5 g/l, K<sub>2</sub>HPO<sub>4</sub> 1 g/l, MgSO<sub>4</sub> 0.1 g/l, yeast extract 15 g/l, NZ-amines 2.5 g/l, malt extract 15 g/l, L-asparagine monohydrate 1 g/l, soy protein (Unicorn 75) 2.5 g/l, potato starch (Norex A150) 20 g/l, and cecereus 7.5 g/l, HEPEX 6 g/l, and trace element stock solution 1 ml/l resulted in a titer of 0.7 mgjl jaysamycin in shake flasks. The same medium, but without agar, was used for fermentations at the 3700 liter scale. In all, 3500 liters of medium were inoculated with 200 liters of seed culture and cultivated for 5 days at 28 °C. The pH was controlled at pH 7 ± 0.2 with H<sub>2</sub>SO<sub>4</sub> and NaOH, and the pO<sub>2</sub> was controlled at 40% of saturation by means of the stirrer speed. After 5 days the fermentations were harvested with an average titer of 4.5–4.5 mg/l.

Extraction and isolation. The fermentation broths were adjusted to pH 5.5 at time of harvest and extracted with equal volumes of ethyl acetate. Phases were separated with a Westphalia SB21 separator. The organic layer was evaporated and the viscous oil obtained thereby was suspended in a mixture of methanol/water 9:1 and extracted with cyclohexane to remove lipids. The aqueous phase was discarded and the methanol/water phase was extracted two more times with cyclohexane. Methanol was evaporated and the aqueous layer extracted with ethyl acetate. The amounts of extracts obtained by this procedure from the large scale fermentations were in the range of 900 g–1 kg. The crude extracts were separated by successive chromatographic steps. Initial separation was carried out on large scale preparative HPLC (RP18 YMC ODSA SP, 5–15 µm, 200 × 700 mm). The mobile phases used were water and methanol, both supplemented with 0.1% formic acid. The flowrate was 500 ml/min and the gradient started at 50% methanol kept constant for 10 min, followed by a gradient step to 65% methanol and isocratic elution for 50 min. This was followed by another gradient step to 75% methanol and further isocratic elution until the separation was finished and the column cleaned with 100% methanol. During the preparation of the first sample for injection in 50% methanol and 50% water, a precipitate was observed, which turned out to consist mostly of jaysamycin. Therefore the crude extract was dissolved in 50% methanol and 50% water, concentrated by evaporation and stored at 4 °C overnight. The resulting precipitate was obtained by filtration and resulted in a significant enrichment of jaysamycin which was submitted to separation as described above. Final purification was performed by preparative HPLC on a Sunfire-C18 column (5 µm, 30 × 150 mm, Waters). The mobile phases used were water and acetonitrile, both supplemented with 0.1% formic acid. The flowrate was 60 ml/min and the gradient started at 50% acetonitrile kept constant for 1.5 min, followed by a linear gradient from 1.5 to 17 min from 50 to 70% acetonitrile, followed by flushing and reequilibration of the column. Fractions containing the compound were concentrated under reduced pressure, followed by freezing and lyophilization. Depending on the intended purpose of the material (starting material for semi-synthesis, in vitro studies or in vivo studies) jaysamycin was purified to 85–95% purity as assessed by UPLC-UV-CAD-MS and NMR.

In total several fermentations at volumes from 20 to 1001 and 3 fermentations at 37001 scale were carried out and a combined 33.2 g of jaysamycin were purified.

Chemical derivatization of jaysamycin. For detailed protocols on the derivatization of jaysamycin, see the Supplementary Methods section in the Supplementary Data document provided as an online supplement.

Mucormycosis mouse model and antifungal efficacy testing. The mucormycosis model was conducted as previously described32. Male ICR mice (20–25 g) were purchased from Taconic (Germantown, NY) and were housed in groups of five mice each. They were given irradiated feed and sterile water containing 50 mg/l oxacin (Baytril; Bayer, Leverkusen, Germany) was added to the drinking water from day 3 to day 0. Ceftazidine (Cfa, 5 µg/dose/0.2 ml) replaced 10.3. 

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enrolfoxacin treatment on day 0 and was administered daily by subcutaneous injection from day 0 until day 6 (tissue fungal burden) or day 13 (survival). Intratracheal instillation of R. deltorum spores was carried out by a modification of the method of Luo and coworkers. Briefly, while pulling the tongue of isolofrane gas-anesthetized mice anteriorly to the side with forceps, 25 μl of fungal spores (2.5 × 10^6 cells) in PBS was injected through the vocal cords into the trachea with a Fisherbrand gel-loading tip (catalog number 02-707-138). To confirm the inoculation, the红旗 were delaid to the lungs, two or three mice were killed immediately after the infection, and lungs were dissected, homogenized, and quantitatively cultured on PDA plates plus 0.1% Triton. Colony forming units (CFU) were enumerated after incubation at 37 °C for 24 h. After infection, the mice were randomly sorted into different treatment groups. For uninfected control mice, 25 μl of PBS alone was intratracheally injected.

For testing jawsamycin antifungal efficacy, the compound was formulated as nanoparticle suspension in 1% hydroxypropyl cellulose (HPC)/0.1% Tween 80, and the chemical derivatives presented in Fig. 4 are provided in the Supplementary Lab. Invest. 79, 293–299 (1999).

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Author contributions
D.E., C.S., T.A., and R.R. performed the target identification experiments; Y.F., F.F., and F.G. designed and executed the GPI screen; Y.F. conducted fungicidal characterizations; D.P., K.M., E.W., E.R., and F.P. were involved in isolation and purification of the natural compound; synthesis of compound derivatives was performed by M.M., S.R., and J.H.; antifungal testing was conducted by Y.P. and A.S.I.; bioinformatic analysis was done by S.S. The project was conceived, figures designed and the manuscript written by J.T. and D.H. with feedback from all authors.

Competing interests
The authors declare no competing interests.

Additional information
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