Sinefungin resistance of *Saccharomyces cerevisiae* arising from *sam3* mutations that inactivate the AdoMet transporter or from increased expression of AdoMet synthase plus mRNA cap guanine-N7 methyltransferase

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ABSTRACT

The *S*-adenosylmethionine (AdoMet) analog sinefungin is a natural product antibiotic that inhibits nucleic acid methyltransferases and arrests the growth of unicellular eukarya and eukaryal viruses. The basis for the particular sensitivity of fungi and protozoa to sinefungin is not known. Here we report the isolation and characterization of spontaneous sinefungin-resistant mutants of the budding yeast *Saccharomyces cerevisiae*. In all cases, sinefungin resistance was attributable to a loss-of-function mutation in Sam3, the yeast high-affinity AdoMet transporter. Overexpression of wild-type Sam3 increased the sensitivity of yeast to growth inhibition by sinefungin. Thus, Sam3 is a tunable determinant of sinefungin potency. The shared ability of protozoan parasites to import AdoMet might determine sinefungin's anti-infective spectrum. Insights to the intracellular action of sinefungin stem from the finding that increased gene dosage of yeast AdoMet synthase plus cap guanine-N7 methyltransferase afforded greater resistance to sinefungin than either enzyme alone. These results are consistent with the proposal that mRNA cap methylation is a principal target of sinefungin's bioactivity.

INTRODUCTION

Sinefungin is a natural product analog of *S*-adenosylmethionine (AdoMet) that has antifungal, antiprotozoal and antiviral activities (1–10). Sinefungin differs from AdoMet in that the S–CH$_3$ sulfonium moiety is replaced by a C–NH$_3$ amine. Sinefungin inhibits a wide spectrum of AdoMet-dependent nucleic acid methyltransferases *in vitro*, by competing with AdoMet for occupancy of the methyl donor site on the enzyme (9,11–14). Several studies suggest that sinefungin's antiviral and antifungal properties derive from selective inhibition of pathogen-encoded mRNA cap-methylating enzymes, specifically RNA (guanine-N7) methyltransferase, which adds a methyl group from AdoMet to GpppRNA to form the m^{7}GpppRNA cap (3,8–10). The cap promotes eukaryal translation initiation and protects RNAs from degradation by 5'-exoribonucleases.

The budding yeast *Saccharomyces cerevisiae* cap methyltransferase Abd1 is exquisitely sensitive to sinefungin *in vitro* (IC$_{50}$ of 24 nM sinefungin at 25 µM AdoMet); indeed, sinefungin is 900-fold more potent than the product AdoHcy in inhibiting cap methylation by Abd1 (9). Two recent studies implicated cap methylation as an antifungal target of sinefungin *in vivo*. First, isogenic *S. cerevisiae* strains containing fungal versus mammalian mRNA capping systems (15) displayed 5-fold differences in sensitivity to growth inhibition by sinefungin that correlated with the source of the cap (guanine-N7) methyltransferase component—the strain with the human cap methyltransferase being more resistant to sinefungin (8). Second, the susceptibility of budding yeast to growth inhibition by sinefungin was diminished when Abd1 was overexpressed by increased gene dosage (9).

To better understand sinefungin's antifungal mechanism, we attempted to identify missense mutations in the yeast cap methyltransferase that confer sinefungin resistance *in vivo*. The approach was to select for outgrowth of sinefungin-resistant (*sfr*) cells from a pool of yeasts containing mutagenized (but functional) *ABD1* genes on a single-copy plasmid. Although forced passage in liquid culture in the presence of 10 µM sinefungin yielded resistant strains, it quickly became apparent that the

resistance determinants did not reside on the *ABD1* plasmid. Genetic tests assigned sinefungin-resistance to *SAM3*, which encodes the yeast high-affinity AdoMet transporter (16,17). Spontaneous *sfr* Sam3 mutations resulted in loss of function in AdoMet uptake, as gauged by growth on medium containing exogenous AdoMet as the sulfur source. We found that over-expression of wild-type Sam3 increased the sensitivity of yeast to growth inhibition by sinefungin. Our results highlight sinefungin uptake by an AdoMet transporter as a critical determinant of its antifungal activity. The shared capacity of many unicellular parasites to import AdoMet (18–20) is likely to underlie sinefungin's broad anti-infective spectrum. The finding that overexpression of Abd1 plus AdoMet synthase confers sinefungin resistance in a wild-type *SAM3* background provides further support for the proposal that cap methylation is the principal intracellular target for sinefungin.

MATERIALS AND METHODS

Yeast strains and media

All *S. cerevisiae* strains used in this study are haploid derivatives of W303 with the following genotype: *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 can1-100*. The non-essential *SAM3* gene was replaced with a *sam3::kanMX* cassette in which the *SAM3* open reading frame was deleted and substituted by a bacterial gene that confers resistance to the antibiotic G418. Minimal B medium lacking exogenous sulfur was prepared as described in (21). B medium plates contained 1% agarose (from EMD Chemicals, Inc.) instead of agar. Sinefungin was purchased from Sigma.

Initial selection for sinefungin resistance

We generated a new library of PCR-mutated *ABD1* alleles (*ABD1**) in a *CEN TRP1* plasmid under the control of the *ABD1* promoter according to methods described previously (22), with minor modifications of the PCR reaction conditions. The p*ABD1** library was transformed into a *S. cerevisiae abd1Δ* p*ABD1* (*CEN URA3 ABD1*) strain and Trp$^+$ colonies were subjected to two rounds of replica-plating on medium containing 5-fluoroorotic acid (FOA) to select for loss of the *URA3*-marked pABD1 plasmid. Approximately 2500 FOA-resistant *abd1Δ* p*ABD1** colonies were harvested directly from agar plates and then divided into 15 separate pools that were stored in 17% glycerol at −80°C. Aliquots (50 μl) from each pool were inoculated into 1 ml of YPD medium containing 10 μM sinefungin. A control aliquot was inoculated into YPD medium with no drug. The cultures were incubated for 24 h at 30°C with constant shaking. Whereas the control culture became turbid after 24 h ($A_{600} > 4.0$), the sinefungin-containing cultures did not. Cells were collected by centrifugation from the sinefungin-containing cultures and resuspended in 1 ml of fresh YPD medium with 10 μM sinefungin. Incubation was continued for another 24 h, at which time the cells were again collected by centrifugation and resuspended in fresh medium with 10 μM sinefungin. After the third 24 h

incubation, several of the sinefungin-containing cultures had become moderately turbid. Aliquots of those cultures were added to 1 ml of fresh medium with 10 μM sinefungin to attain an A_{600} of 0.1. After incubation for another 24 h, these cultures were fully turbid. Single colonies were obtained from the cultures by plating on YPD agar. Sinefungin resistance was confirmed by inoculation of single colonies into YPD medium with 10 μM sinefungin and assessment of turbidity after overnight growth. Several of the cultures that had grown out in the presence of 10 μM sinefungin were subjected to dose escalation by serially inoculating them at 1-day intervals into YPD containing 20, 40, and 80 μM sinefungin. Single colonies were recovered at each step by plating on YPD agar.

p*ABD1** DNA was recovered from individual *sfr* strains and amplified clonally by transformation in *Escherichia coli*. p*ABD1** DNAs isolated from bacteria were transformed into *S. cerevisiae abd1Δ* p*ABD1* (*CEN URA3 ABD1*) and exchanged for the wild-type p*ABD1* plasmid by selection on medium containing FOA. In no instance was the resulting p*ABD1** yeast strain capable of growing during a 24 h incubation in YPD medium with 10 μM sinefungin. Thus, the sinefungin-resistant phenotype was apparently not linked to the *ABD1** plasmid. This conclusion was verified by transforming several of the *sfr* strains with wild-type p*ABD1* and then screening for spontaneous loss of the p*ABD1** (*TRP1*) plasmid by tryptophan auxotrophy. The reintroduction of wild-type *ABD1* did not ameliorate the sinefungin-resistance, implying that the putative resistance-conferring mutation(s) reside in one or more yeast chromosomal genes.

The *sfr abd1Δ* p*ABD1* strains were backcrossed to a sinefungin-sensitive *ABD1* strain and the diploids were subjected to sporulation and tetrad dissection. The haploid progeny of four-spore tetrads were screened for sensitivity to sinefungin by spotting aliquots of the drug on a freshly plated lawn of cells and assessing the size of any resulting zone of growth inhibition (9). Sensitivity and resistance segregated in a 2:2 pattern (Figure 1), suggesting that resistance was conferred by mutation of a single genetic locus. Haploid *sfr Mat*a progeny that contained a wild-type chromosomal *ABD1* locus were selected for further study. These *sfr* strains were provisionally designated *sfr-1*, *sfr-2*, *sfr-3*, etc.

RESULTS

Sinefungin-resistant yeast mutants

We isolated *sfr* strains by forced passage in liquid cultures of YPD medium containing 10 μM sinefungin. Although our selection strategy was designed to identify *sfr* versions of the yeast cap methyltransferase Abd1, initial genetic linkage tests showed that none of the yeast strains that grew in the presence of sinefungin did so because of resistance-conferring changes in the plasmid-borne *ABD1* gene. By backcrossing the *sfr* strains to a sinefungin-sensitive yeast strain, and then subjecting the diploids to sporulation and tetrad analysis, we obtained evidence that drug-resistance was caused by a mutation that segregated 2:2 with drug sensitivity. Figure 1 illustrates the use of a
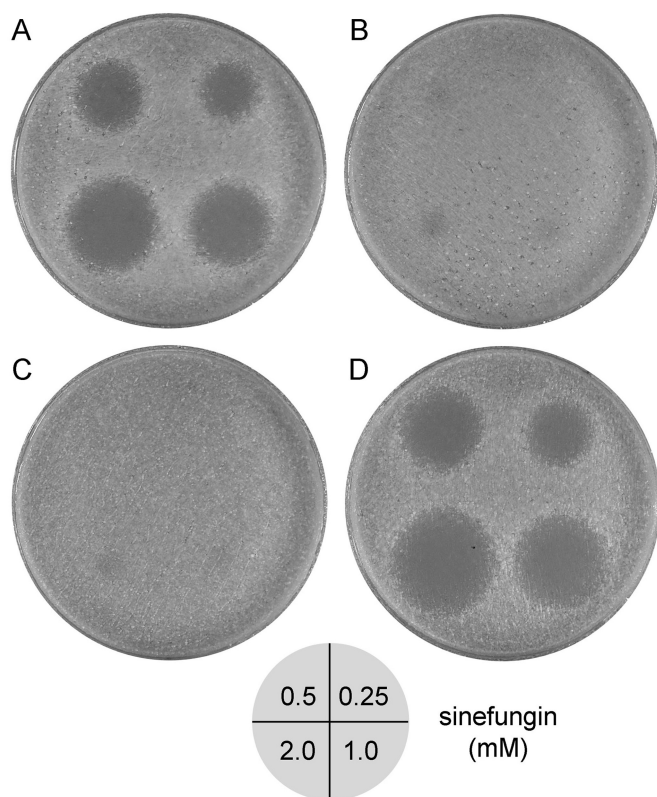
Figure 1. Meiotic segregation of sinefungin resistance. The four haploid progeny (**A**, **B**, **C** and **D**) were grown in YPD medium to mid-log phase and aliquots (10^6 cells) were spread on YPD agar plates (15-cm diameter). After incubation of the plates for 1 h at 30°C to allow the cell suspension to dry, 2 μl aliquots of aqueous solutions of sinefungin (0.25, 0.5, 1.0 or 2.0 mM as diagrammed at the bottom) were spotted on the agar plates. The plates were incubated for 2 days at 30°C and then photographed.

simple spot test to gauge sinefungin's effect on the growth of the four haploid progeny of one such backcross. Aliquots (2 μl) of an aqueous solution of sinefungin (either 0.25, 0.5, 1.0, or 2.0 mM) were spotted on a freshly plated lawn of yeast cells on YPD agar medium. In the case of two of the haploid progeny (Figure 1A and D), diffusion of the drug from the site of application resulted in a circular zone of growth inhibition with a sharp demarcation at the circumference. The diameter of the zone of inhibition increased with the increasing sinefungin concentration, as expected. The two other haploid progeny (Figure 1B and C) showed hardly any zone of growth inhibition at even the highest sinefungin dose. Three other tetrads from the same diploid also yielded two sinefungin-sensitive and two *sfr* haploid progeny (data not shown). This pattern of meiotic segregation is consistent with sinefungin resistance being caused by a single-gene mutation.

Sinefungin-resistant mutants are defective in utilization of exogenous AdoMet

sfr haploids that contained a wild-type chromosomal *ABD1* locus after backcrossing were selected for further study. These *sfr* strains were provisionally designated *sfr-1*, *sfr-2*, *sfr-3*, *sfr-4*, etc. We considered several possible mechanisms for drug resistance including: (i) defective uptake of sinefungin from the medium; (ii) enhanced export of sinefungin out of the cell; (iii) accelerated metabolism of sinefungin to an inactive derivative; and (iv) resistance-conferring changes in an AdoMet-dependent methyltransferase other than Abd1. Our first suspicions focused on drug export in light of the well-studied phenomenon of yeast pleiotrophic drug resistance (PDR), which is caused by gain-of-function mutations in transcription factors that increase the level of expression of the ATP-dependent plasma membrane pumps that expel various xenobiotics from the cell (23). However, two preliminary results obtained by spot-testing for drug inhibition hinted that sinefungin resistance might not be mediated via PDR. First, the yeast *sfr* strains were no less sensitive than the parental wild-type to cycloheximide, a substrate for export by the PDR system. Second, a yeast *pdr1Δ* strain, which lacks a master transcriptional regulator of expression of the export pumps, was not more sensitive than a *PDR1* strain to sinefungin.
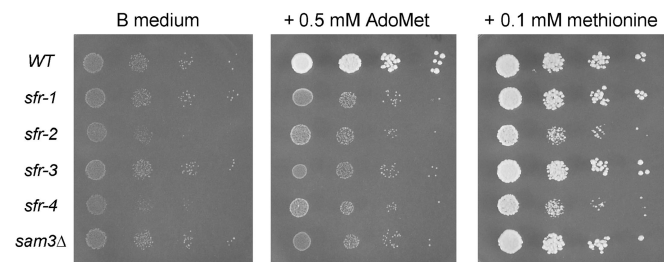
The prospect that sinefungin-sensitivity might rely on the same pathway used to assimilate exogenous AdoMet was suggested by an initial finding that the zone of inhibition of the wild-type yeast strain in the sinefungin spot test was diminished progressively by including increasing concentrations of AdoMet in the spotted solution along with a fixed concentration of sinefungin (data not shown). One explanation for this effect (though by no means the only one) is that sinefungin and AdoMet compete for a common yeast transport system. *Saccharomyces cerevisiae* can take up AdoMet from the medium; this capacity is not essential for growth under normal conditions, but becomes critical when the paralogous yeast *SAM1* and *SAM2* genes encoding AdoMet synthase are inactivated simultaneously, resulting in AdoMet auxotrophy (24). The uptake of AdoMet by *S. cerevisiae* requires Sam3, a 587-aa integral membrane protein that belongs to the amino acid permease superfamily (16). Although a yeast *sam3Δ* mutant is viable, the strain is defective for growth on B medium (a minimal medium lacking sulfur) when exogenous AdoMet is included as the lone sulfur source (16).

To test whether the four *sfr* strains were defective in utilizing exogenous AdoMet, we spotted serial dilutions of wild-type, *sfr*, and *sam3Δ* cells on unsupplemented B medium and on B medium containing either AdoMet or methionine as the sulfur source (Figure 2). All of the strains were impaired for growth on B medium. The *sam3Δ* mutant was unable to grow using AdoMet as the sulfur source, yet it was able to grow in the presence of methionine, as reported previously (16). The wild-type strain was the only one for which growth was restored by AdoMet. Each of the *sfr* strains phenocopied *sam3Δ* with respect to their ability to utilize methionine but not AdoMet (Figure 2). These results underscore a correlation between impaired AdoMet uptake and sinefungin-resistance in yeast. Yet, they do not reveal whether the *sfr* mutations elicit a *sam3*-like phenotype directly, via mutations in Sam3, or indirectly, through mutations in genes that regulate Sam3 expression or function.

sfr mutants are allelic to *SAM3*

To better quantify sinefungin resistance and simultaneously compare the sensitivities of various mutants strains, we applied 150 μl aliquots of 0.5, 1, 2 and 20 mM sinefungin stock solutions to the surfaces of YPD agar plates (containing 25 ml of medium) and allowed the drug to adsorb into the agar before spotting serial dilutions of wild-type, *sfr*, and *sam3Δ* cells. (If one assumes that the applied drug is evenly distributed through the z-plane of the agar plate, then these doses correspond to net sinefungin concentrations of 3, 6, 12 and 120 μM, respectively.) Wild-type yeast cells failed to grow on plates that had received ≥0.5 mM sinefungin (Figure 3). Back titration of the applied dose revealed that 150 μl of a 0.2 mM sinefungin solution sufficed to prevent growth of the wild-type yeast strain on a YPD agar plate (data not shown). The *sam3Δ* strain was impervious to the 20 mM dose of sinefungin, which translates into at least 100-fold resistance compared to the wild-type strain (Figure 3). This result signifies that sinefungin is transported into yeast cells by the same permease that transports AdoMet. The *sfr-1* and *sfr-3* strains phenocopied *sam3Δ* with respect to resistance to the highest level of sinefungin (Figure 3). However,

growth of the *sfr-2* and *sfr-4* strains, though resistant to the 0.5 mM sinefungin dose, was slowed at the 2 mM dose and inhibited fully by the 20 mM dose (Figure 3). These results highlight heterogeneity of the resistance phenotypes of different *sfr* strains.

To test whether the *sfr* mutations were allelic to *SAM3*, the *sfr* and *sam3Δ* strains were transformed with a *CEN TRP1* plasmid bearing the wild-type *SAM3* gene under the control of its native promoter. The strains were transformed in parallel with the empty *CEN TRP1* plasmid vector. The *SAM3* plasmid restored sinefungin sensitivity to the *sam3Δ* strain (Figure 4). The *sfr-1*, *sfr-2*, *sfr-3*, and *sfr-4* strains were also rendered sinefungin-sensitive by the plasmid-borne *SAM3* gene (Figure 4), implying that the *sfr* mutations are recessive and allelic to *SAM3*. The results are not consistent with the *sfr* mutations affecting a regulator of *SAM3* expression or function, insofar as a single copy of the *SAM3* gene on a plasmid would not be expected to restore Sam3 activity in such a scenario.

Sinefungin resistance is caused by a variety of mutations in the *SAM3* gene

Convincing evidence that mutations in the *SAM3* gene were the cause of spontaneous sinefungin-resistance was obtained by PCR-amplifying, cloning, and sequencing the *SAM3* locus from the wild-type, *sfr-1*, *sfr-2*, *sfr-3*, and *sfr-4* strains. Four plasmid clones were sequenced from each strain to avoid specious attribution of phenotypes to PCR-generated changes. The sequence of the wild-type *SAM3* open reading frame encoded a 587-aa polypeptide identical to the wild-type Sam3 reported previously (16). The *sfr-1* strain had a single nucleotide coding change in the *SAM3* gene that resulted in a stop codon in lieu of the codon for Ser444 (Table 1). The *sfr-3* strain had a 2-nucleotide deletion in the codons for Val371 and Ser372; the −2 frameshift created a mutant peptide sequence from Leu373 to the truncated C-terminus at Gly404 (Table 1). Note that the protein truncations caused by *sfr-1* and *sfr-3* mutations mimicked the null phenotype of the *sam3Δ* deletion with respect to extreme sinefungin-resistance (Figure 3). By contrast, the *sfr-2* and *sfr-4* strains, which were less resistant to sinefungin than
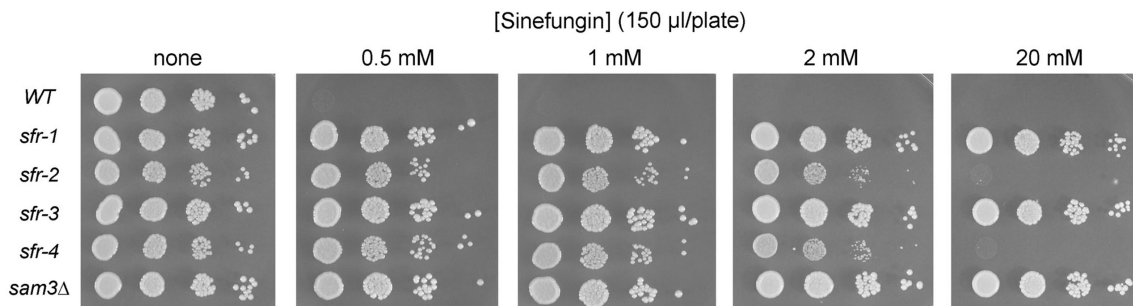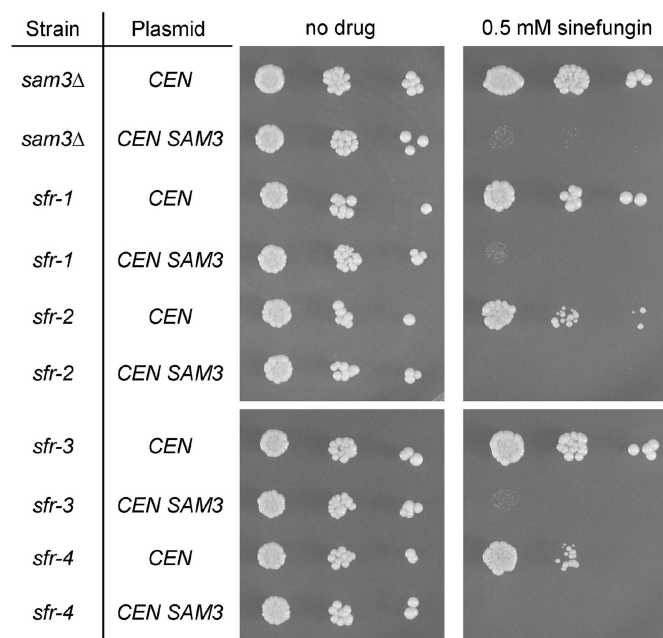

Figure 2. The *sfr* strains are defective in utilization of exogenous AdoMet. The indicated yeast strains were grown in YPD medium at 30°C until A_{600} reached ~0.7. The cells were harvested by centrifugation, suspended in water, recentrifuged and resuspended in water. Serial 10-fold dilutions (in water) of the washed cells were prepared and aliquots (2 μl) were spotted on B medium agarose plates or B medium supplemented with 0.5 mM AdoMet or 0.1 mM methionine. The plates were photographed after incubation for 2 days at 30°C.

Figure 3. Sinefungin resistance of *sfr* and *sam3Δ* strains. The indicated yeast strains were grown in YPD medium at 30°C until A_{600} reached ~0.7. The cells were harvested by centrifugation and suspended in water. Serial 10-fold dilutions were prepared and aliquots (2 μl) were spotted on an unsupplemented agar plate ('none') and on YPD agar plates onto which 150 μl aliquots of a sinefungin solution (0.5, 1, 2 or 20 mM) had been applied and spread. If one assumes that the applied drug is evenly distributed through the depth of the agar plate, then these doses correspond to net sinefungin concentrations of 3, 6, 12 and 120 μM, respectively. The plates were photographed after incubation for 2 days at 30°C.

sam3Δ (Figure 3), had single-nucleotide missense mutations in the *SAM3* gene that resulted in E213K and S476F changes in the Sam3 protein, respectively. We surmise that the defective Sam3 E213K and S476F proteins retained some low level of transport function *in vivo* that allowed uptake of sinefungin when the drug was present at very high concentration in the medium.

To check that the *SAM3* alleles obtained from these four *sfr* strains were indeed functionally compromised, we cloned them into *CEN TRP1* plasmids and then used these vectors, and a wild-type *SAM3* control, to transform the *sam3Δ* strain. The transformants were tested for growth on Trp⁻ plates that had been overlaid with 0.5 or 2 mM sinefungin. The wild-type *SAM3* gene restored sinefungin-sensitivity, but the empty vector control did not, nor did any of the four mutated *sam3* genes from the *sfr* strains (Figure 5).

To better survey the spectrum of spontaneous Sam3 lesions that cause sinefungin-resistance, we sequenced the *SAM3* locus from eight additional *sfr* strains isolated in the screen. The *sfr-5, sfr-10 and sfr-11* strains had single nucleotide changes that resulted in translation stops at the codons for amino acids 180, 400 and 60, respectively (Table 1). The *sfr-6, sfr-7* and *sfr-9* strains had single nucleotide mutations that resulted in Q126P, S171R and S171R missense changes in Sam3, respectively. Note that the identical S171R protein mutations in the *sfr-7* and *sfr-9* strains were the result of independent spontaneous mutations of the Ser171 AGC codon to AGA and AGG arginine codons, respectively (Table 1). The *sfr-8* and

sfr-12 strains had single nucleotide deletions in the codons for Pro262 and Thr90, respectively; the −1 frameshifts created mutant C-terminal Sam3 peptides that stopped after Ser282 and Val98, respectively (Table 1). These results underscore that complete inactivation of Sam3 by a protein-truncating stop codon is the predominant route to sinefungin resistance.

Overexpression of Sam3 sensitizes yeast to sinefungin

We reasoned that if loss of Sam3 transporter function results in sinefungin resistance, then overexpression of Sam3 might enhance the sinefungin sensitivity of *S. cerevisiae*. Previous studies had established that introducing *SAM3* on a high copy plasmid into a wild-type *SAM3* strain elicited a 3.5-fold increase in AdoMet uptake (16). We expected that *SAM3* overexpression would also increase sinefungin bioavailability. Thus, we cloned the wild-type *SAM3* gene into a high-copy 2 μ *HIS3* plasmid and then introduced the 2 μ *SAM3* plasmid into wild-type yeast, in parallel with the empty 2 μ vector. The transformants were tested for growth on His⁻ plates with no added drug and His⁻ plates that had been overlaid with sinefungin. Preliminary experiments established that growth of wild-type yeast cells was suppressed by applying 150 μl of a 25–50 μM solution of sinefungin to the minimal agar medium. Thus, we tested for the effects of Sam3 overexpression at lower doses of sinefungin (150 μl of a 1.6 μM or 3.1 μM sinefungin solution) and observed that increasing the copy number of the *SAM3* gene sensitized the cells to inhibition of growth by a sinefungin dose that had little impact on cells that had only a single copy of *SAM3* (Figure 6). Thus, Sam3 is a tunable determinant of sinefungin sensitivity and resistance.

Overexpression of AdoMet synthase plus cap methyltransferase confers sinefungin resistance

The genetic studies presented above highlight Sam3 as the major determinant of sinefungin susceptibility of budding

Figure 4. *SAM3* restores sinefungin sensitivity to yeast *sfr* strains. The indicated yeast strains were transformed with either the control *CEN TRP1* plasmid or a derivative bearing wild-type *SAM3*. Cells were grown in Trp⁻ medium at 30°C until A_{600} reached ~0.7, then harvested by centrifugation and suspended in water. Serial 10-fold dilutions were prepared and aliquots (2 μl) were spotted on an unsupplemented Trp⁻ agar plate ('no drug') and on a Trp⁻ plate onto which 150 μl of a 0.5 mM sinefungin solution had been applied and spread. The plates were photographed after incubation for 3 days at 30°C.
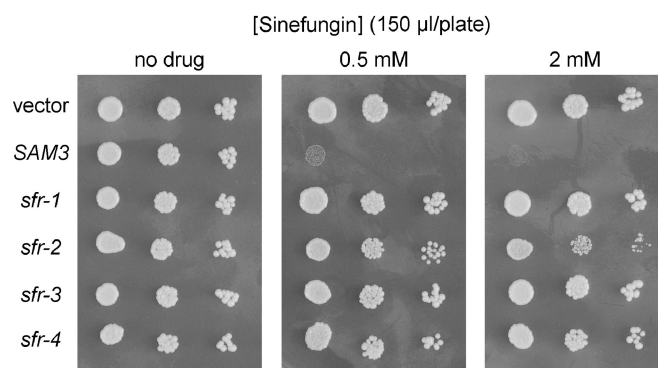

Figure 5. Sinefungin phenotype of *sfr sam3Δ* strains. *sam3Δ* cells transformed with a *CEN TRP1* vector containing the genes specified at left were grown in Trp⁻ medium at 30°C until A_{600} reached ~0.7. The cells were harvested by centrifugation and suspended in water. Serial 10-fold dilutions were prepared and aliquots (2 μl) were spotted on an unsupplemented Trp⁻ agar plate ('no drug') and on Trp⁻ plates onto which 150 μl of a 0.5 mM or 2 mM sinefungin solution had been applied and spread. The plates were photographed after incubation for 3 days at 30°C.

Table 1. Spontaneous *SAM3* mutations that confer sinefungin resistance

sfr allele	Sam3 protein mutations	*SAM3* DNA mutations
sfr-1	Ser444 → ter	TCA → TAA
sfr-2	Glu213 → Lys	GAG → AAG
sfr-3	[371]VSVCNSCVYASSRLIQALGASGQLPSVCSYMDRK... → [371]VSLQFLRLCFFKTNSSFRCIWPTSFGMFLHGQKG[404](ter)	−2 frameshift GT*GT*CAGTT → GTCAGTTTG
sfr-4	Ser476 → Phe	TCC → TTC
sfr-5	Ser180 → ter	TCA → TAA
sfr-6	Gln126 → Pro	CAG → CCG
sfr-7	Ser171 → Arg	AGC → AGA
sfr-8	[262]PVFKNLCNTFVSAAFSFGGSEL... → [262]LSSRICVTHSFLLFPLVVS[282](ter)	−1 frameshift C*C*TGTC → CTGTCT
sfr-9	Ser171 → Arg	AGC → AGG
sfr-10	Tyr400 → ter	TAC → TAA
sfr-11	Glu60 → ter	GAG → TAG
sfr-12	[90]TLGTGLFIGL... → [90]PWGRDCSLV[98](ter)	−1 frameshift and T → C *A*CTTTG → CCTTGG

The *sfr* strains were selected for growth by passage in YPD medium with 10 μM sinefungin, except for *sfr-1* and *sfr-5*, which were subjected to dose escalation and selection for growth in 80 μM sinefungin. The nucleotide changes in the *SAM3* genes of each strain are shown in the right column. Single missense or nonsense nucleotide changes in the *sfr* strains are underlined. The nucleotides of the wild-type *SAM3* gene that were deleted in the *sfr-3*, *sfr-8*, and *sfr-12* strains are shown in italics. The resulting mutations in the Sam3 protein are indicated in the middle column.
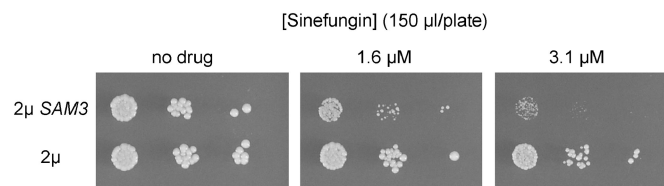
Figure 6. Sam3 overexpression sensitizes yeast to sinefungin inhibition. Wild-type *SAM3* cells were transformed with 2 μ *SAM3* or 2 μ plasmids and grown in His⁻ medium at 30°C until A_{600} reached ~0.7. The cells were harvested by centrifugation and suspended in water. Serial 10-fold dilutions were prepared and aliquots (2 μl) were spotted on an unsupplemented His⁻ agar plate ('no drug') and on His⁻ plates onto which 150 μl of a 1.6 μM or 3.1 μM sinefungin solution had been applied and spread. If the applied drug is evenly distributed through the depth of the agar plate, these doses correspond to net sinefungin concentrations of about 10 and 19 nM, respectively. The plates were photographed after incubation for 3 days at 30°C.

yeast, but they do not illuminate the intracellular actions of the drug once it is taken up from the medium. If sinefungin is blocking growth by acting as a competitive inhibitor of AdoMet binding to an essential yeast methyltransferase, then we reasoned that increasing the intracellular level of AdoMet might ameliorate the effects of sinefungin on its methyltransferase target. *S. cerevisiae* encodes two AdoMet synthases: Sam1 and Sam2 (24). Previous findings that increased *SAM1* or *SAM2* gene dosage can suppress temperature-sensitive *abd1* mutations (22) implied that Abd1 function is responsive to intracellular AdoMet levels. Here we introduced into yeast a multicopy 2 μ *URA3* plasmid containing *SAM1* or *SAM2* under the control of the constitutive yeast *TPI1* promoter (22). Compared to an empty 2 μ *URA3* vector control, overexpression of either isozyme of AdoMet synthase enhanced cell growth on minimal medium overlaid with 150 μl of 25 μM or 50 μM sinefungin, but did not allow for growth at a higher drug dosage (Figure 7A). Transformation of yeast with a 2 μ *TRP1* plasmid containing wild-type *ABD1* driven by the *TPI1* promoter also enabled

growth on minimal medium dosed with 150 μl of 25 μM sinefungin (Figure 7B), but not with ≥50 μM sinefungin (not shown). Cotransformation with 2 μ *ABD1* plus either 2 μ *SAM1* or 2 μ *SAM2* plasmids had an additive effect on sinefungin-resistance, whereby the strains overexpressing AdoMet synthase and cap methyltransferase grew on minimal medium overlaid with 150 μl of 100 μM sinefungin, a condition in which neither wild-type yeast nor yeast overexpressing Sam1 or Sam2 alone were able to form colonies (Figure 7A).

DISCUSSION

A classical genetic approach to identifying the target of a drug is to screen for a drug-resistant mutant and then identify the gene responsible for the resistance phenotype. There have been several earlier reports of the isolation of *sfr* mutants or resistant strain variants of protozoan organisms that are normally sensitive to the drug (25–30). An *sfr* isolate of *Leishmania* displayed impaired uptake of sinefungin compared with the wild-type strain (30). The present study shows that impaired sinefungin uptake is the predominant route to spontaneous sinefungin-resistance of *S. cerevisiae*, which is caused by a variety of loss-of-function mutations in the yeast high-affinity AdoMet transporter Sam3. To our knowledge, sinefungin resistance-conferring genetic changes in a free-living organism had not been assigned previously to specific genes.

SAM3 was identified initially by Rouillon *et al.* (16) as a gene required for growth of budding yeast on sulfur-free medium containing AdoMet as the sole sulfur source. The Sam3 protein consists of 12 putative membrane-spanning segments and is classified as a member of the amino acid permease superfamily. Sam3 is capable of discriminating structurally related sulfonium derivatives of methionine, insofar as Sam3 suffices to transport AdoMet, but not *S*-methylmethionine (16). The genetic evidence presented here indicates that Sam3 is the principle (if not the only) yeast transporter of sinefungin. Although sinefungin has a
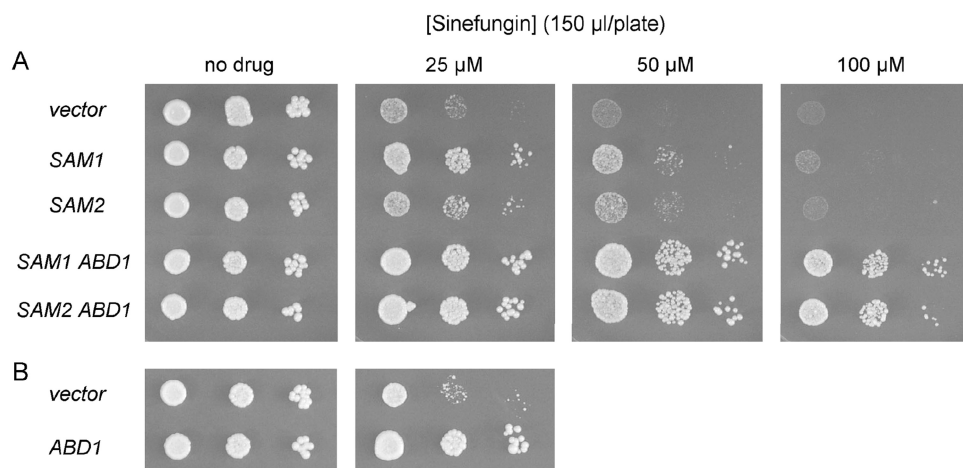
Figure 7. Increased gene dosage of *SAM1* or *SAM2* plus *ABD1* confers sinefungin-resistance. (**A**) Yeast *abd1Δ* cells containing either a *CEN TRP1 ABD1* plasmid (with *ABD1* expression controlled by its native promoter) or a 2μ *TRP1 ABD1* plasmid (with *ABD1* driven by the strong *TPI1* promoter) were transformed with a 2μ *URA3* plasmid bearing either *SAM1* or *SAM2* or no insert. Transformants were grown in Ura⁻ medium at 30°C until A_{600} reached ∼0.7. The cells were harvested by centrifugation and suspended in water. Serial 10-fold dilutions were prepared and aliquots (2μl) were spotted on an unsupplemented Ura⁻ agar plate ('no drug') and on Ura⁻ plates onto which 150μl of a 25, 50 or 100μM sinefungin solution had been applied. If the applied drug is evenly distributed through the depth of the agar plate, these doses correspond to net sinefungin concentrations of 0.15, 0.3 and 0.6μM, respectively. The plates were photographed after incubation for 3 days at 30°C. (**B**) Aliquots (2μl) of serial 10-fold dilutions of wild-type yeast cells carrying either an empty *CEN TRP1* vector or a 2μ *TRP1 ABD1* plasmid were spotted on an unsupplemented Trp⁻ agar plate ('no drug') and on Trp⁻ plates onto which 150μl of a 25μM sinefungin solution had been applied. The no drug and 25μM sinefungin plates were photographed after incubation at 30°C for 3 days and 5 days, respectively.

primary amine instead of the sulfonium center found in AdoMet, the two compounds are virtually isosteric and both have a positive charge on the amine/sulfonium groups. Thus, it is sensible that Sam3 would be able to transport sinefungin. A recent study suggests that Sam3 is also able to transport other positively charged compounds such as putrescine and spermidine (17).

The Sam3 mutations that confer sinefungin-resistance also result in loss-of-function in AdoMet uptake, as gauged by impaired growth on AdoMet-containing B medium. Most of the *sfr* mutants isolated in the screen are functionally null for Sam3 because of premature translation stops that remove three or more of the 12 predicted membrane-spanning segments. The Sam3 missense mutations that confer sinefungin-resistance are Q126P, S171R, E213K, and S476F. Gln126, Ser171 and Ser476 are located in predicted membrane spanning segments 2, 3 and 11, respectively. Glu213 is situated between the third and fourth membrane-spanning segments. All four of these Sam3 side chains are conserved in the yeast Mmp1 permease that transports *S*-methylmethionine (16).

Our findings that sinefungin's antifungal activity is contingent on its import by the yeast AdoMet permease resonate strongly with previous studies of AdoMet and sinefungin in protozoan parasites. Sinefungin-sensitive kinetoplastid parasites such as *Leishmania* and *Trypanosoma* can import exogenous AdoMet. Inhibition of AdoMet uptake by sinefungin and direct assays of intracellular sinefungin accumulation suggest that kinetoplastids import sinefungin *via* the AdoMet uptake pathway (19,20,30,31). Resistance of *Leishmania* to sinefungin is correlated with decreased uptake of both sinefungin and AdoMet (30,31). To our knowledge, there has been no published report of the identification of the gene encoding the putative kinetoplastid AdoMet/sinefungin transporter.

To a first approximation, it appears that the anti-infective spectrum of sinefungin correlates with the presence in the susceptible organisms of an AdoMet transport system that accepts sinefungin as cargo. The acquisition of spontaneous sinefungin-resistance by inactivating mutations of the fungal AdoMet permease is a potential obstacle to sustained clinical efficacy of a sinefungin-based drug. However, this problem is less daunting if the parasite relies on AdoMet uptake for growth or persistence in the animal host, in which case mutations in the AdoMet transporter that affect sinefungin susceptibility would simultaneously diminish the virulence of the parasite. This is a plausible scenario for the fungal pathogen *Pneumocystis carinii*, which has no detectable AdoMet synthase activity and is naturally auxotrophic for exogenous AdoMet taken up by a high-affinity transporter (18). Sinefungin inhibits *Pneumocystis* growth in culture (32).

The fact that the *sfr* phenotype in yeast is dominated by *sam3* mutations confounded our initial efforts to identify an intracellular methyltransferase target of sinefungin by screening haploid strains for drug-resistance. Because the sinefungin-sensitive trait of wild-type *SAM3* is dominant in the presence of a *sfr sam3* allele, it might be feasible to screen in diploid strains for non-Sam3 resistance-conferring changes. However, we observed the rapid emergence of *sfr* colonies in the middle of the zone of inhibition when drug was spotted on a lawn of the *SAM3 sam3(sfr-1)* diploid strain (data not shown). We surmise that gene conversion between homologous chromosomes resulted in transfer of the resistance mutation to the previously wild-type *SAM3* locus. A potentially better

way to seek resistance mutations in intracellular targets is to exploit the observation that a 2 μ *SAM3* plasmid sensitizes yeast to AdoMet. The high copy number of the 2 μ plasmid makes it unlikely that an inactivating mutation in the chromosomal *SAM3* gene, or any one of the plasmid-borne genes, will result in resistance in the presence of an excess of wild-type *SAM3* alleles. In this background, non-Sam3 resistance-conferring mutations might be identified.

The observation that wild-type *SAM3* cells become *sfr* upon overexpression of AdoMet synthase plus Abd1 reinforces the earlier suggestion that RNA cap methylation is a principal intracellular event targeted by the drug (8,9). It is sensible that simultaneously increasing the concentration of the enzyme responsible for AdoMet production and the level of the relevant methyltransferase enzyme target should generate greater resistance than either maneuver alone.

REFERENCES

1. Hamill,R.L. and Hoehn,M.M. (1973) A9145, a new adenine containing antifungal antibiotic: discovery and isolation. *J. Antibiot.*, **26**, 463–465.
2. Gordee,R.S. and Butler,T.F. (1973) A9145, a new adenine containing antifungal antibiotic: biological activity. *J. Antibiot.*, **26**, 466–470.
3. Pugh,C.S.G., Borchardt,R.T. and Stone,H.O. (1978) Sinefungin, a potent inhibitor of virion mRNA(guanine-7-)-methyltransferase, mRNA(nucleoside-2′-)-methyltransferase, and viral multiplication. *J. Biol. Chem.*, **253**, 4075–4077.
4. Dube,D.K., Mpimbaza,G., Allison,A.C., Lederer,E. and Rovis,L. (1983) Antitrypanosomal activity of sinefungin. *Am. J. Trop. Med. Hyg.*, **32**, 31–33.
5. Ferrante,A., Ljungstron,I., Huldt,G. and Lederer,E. (1984) Amoebicidal activity of the antifungal antibiotic sinefungin against *Entamoeba histolytica*. *Trans. R. Soc. Trop. Med. Hyg.*, **78**, 837–838.
6. Messika,E., Golenser,J., Abu-Elheiga,L., Robert-Gero,M., Lederer,E. and Bachrach,U. (1990) Effect of sinefungin on macromolecular biosynthesis and cell cycle of *Plasmodium falciparum*. *Trop. Med. Parasitol.*, **41**, 273–278.
7. Brassuer,P., Lemetheil,D. and Ballet,J.J. (1993) Curative and preventive anticryptosporidium activities of sinefungin in an immunosuppressed adult rat model. *Antimicrob. Agents Chemother.*, **37**, 889–892.
8. Chrebet,G.L., Wisniewski,D., Perkins,A.L., Deng,Q., Kurtz,M.B., Marcy,A. and Parent,S.A. (2005) Cell-based assays to detect inhibitors of fungal mRNA capping enzymes and characterization of sinefungin as a cap methyltransferase inhibitor. *J. Biomol. Screen.*, **10**, 355–364.
9. Zheng,S., Hausmann,S., Liu,Q., Ghosh,A., Schwer,B., Lima,C.D. and Shuman,S. (2006) Mutational analysis of *Encephalitozoon cuniculi* mRNA cap (guanine-N7) methyltransferase, structure of the enzyme bound to sinefungin, and evidence that cap methyltransferase is the target of sinefungin. *J. Biol. Chem.*, **281**, 35904–35913.
10. Li,J., Chorba,J.S. and Whelan,S.P.J. (2007) Vesicular stomatitis viruses resistant to the methylase inhibitor sinefungin upregulate RNA synthesis and reveal mutations that affect mRNA cap methylation. *J. Virol.*, **81**, 4104–4115.
11. Schluckebier,G., Kozak,M., Bleimling,N., Weinhold,E. and Saenger,W. (1997) Differential bind of *S*-adenosylmethionine, *S*-adenosylhomocysteine and sinefungin to the adenine-specific DNA methyltransferase M.TaqI. *J. Mol. Biol.*, **265**, 56–67.
12. Thomas,C.B., Scavettta,R.D., Gumport,R.I. and Churchill,M.E.A. (2003) Structures of liganded and unliganded RsrI N6-adenine DNA methyltransferase. *J. Biol. Chem.*, **278**, 26094–26101.
13. Schluckebier,G., Zhong,P., Stewart,K.D., Kavanaugh,T.J. and Abad-Zapatero,C. (1999) The 2.2 Å structure of the rRNA methyltransferase ErmC′ and its complexes with cofactor and cofactor analogs: implications for the reaction mechanism. *J. Mol. Biol.*, **289**, 277–291.
14. Horton,J.R., Liebert,K., Hattman,S., Jeltsch,A. and Cheng,X. (2005) Transition from nonspecific to specific DNA interactions along the substrate-recognition pathway of Dam methyltranserase. *Cell*, **121**, 349–361.
15. Saha,N., Schwer,B. and Shuman,S. (1999) Characterization of human, *Schizosaccharomyces pombe* and *Candida albicans* mRNA cap methyltransferases and complete replacement of the yeast capping apparatus by mammalian enzymes. *J. Biol. Chem.*, **274**, 16553–16562.
16. Rouillon,A., Surdin-Kerjan,Y. and Thomas,D. (1999) Transport of sulfonium compounds: characterization of the *S*-adenolysmethionine and *S*-methylmethionine permeases from the yeast Saccharomyces cerevisiae. *J. Biol. Chem.*, **274**, 28096–28105.
17. Uemura,T., Kashiwagi,K. and Igarashi,K. (2007) Polyamine uptake by DUR3 and SAM3 in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **282**, 7733–7741.
18. Merali,S., Vargas,D., Franklin,M. and Clarkson,A.B. (2000) S-adenosylmethionine and Pneumocystis carinii. *J. Biol. Chem.*, **275**, 14958–14963.
19. Goldberg,B., Yarlett,N., Sufrin,J., Lloyd,D. and Bacchi,C.J. (1997) A unique transporter of *S*-adenosylmethionine in African trypanosomes. *FASEB J.*, **11**, 256–260.
20. Goldberg,B., Yarlett,N., Sufrin,J., Lloyd,D. and Bacchi,C.J. (1998) Effects of intermediates of methionine metabolism and nucleoside analogs on *S*-adenosylmethionine transport by Trypanosoma brucei brucei and a drug-resistant Trypanosoma brucei rhodesiense. *FASEB J.*, **56**, 95–103.
21. Cherest,H. and Surdiin-Kurjan,Y. (1992) Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating the sulfur metabolism pathway. *Genetics*, **130**, 51–58.
22. Schwer,B., Saha,N., Mao,X., Chen,H.W. and Shuman,S. (2000) Structure-function analysis of yeast mRNA cap methyltransferase and high-copy suppression of conditional mutants by AdoMet synthase and the ubiquitin conjugating enzyme Cdc34p. *Genetics*, **155**, 1561–1576.
23. Bauer,B.E., Wolfger,H. and Kuchler,K. (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim. Biophys. Acta*, **1461**, 217–236.
24. Thomas,D., Rothstein,R., Rosenberg,N. and Surdin-Kerjan,Y. (1988) *SAM2* encodes the second methionine S-adenosyl transferase in Saccharomyces cerevisiae: physiology and regulation of both enzymes. *Mol. Cell. Biol.*, **8**, 5132–5139.
25. Inselburg,J. (1984) Induction and selection of drug resistant mutants of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.*, **10**, 89–98.
26. Kaminsky,R. and Zweygarth,E. (1989) Feeder layer-free in vitro assay for screening antitrypanosomal compounds against *Trypanosoma brucei brucei* and *T.b. evansii*. *Antimicrob. Agents Chemother.*, **33**, 881–885.
27. Sibley,L.D., LeBlanc,A.J., Pfefferkorn,E.R. and Boothroyd,J.C. (1992) Generation of a restriction fragment length polymorphism linkage map for *Toxoplasma gondii*. *Genetics*, **132**, 1003–1015.
28. Phelouzat,M.A., Lawrence,F. and Robert-Gero,M. (1993) Characterization of sinefungin-resistant *Leishmania donovani* promastigotes. *Parasitol. Res.*, **79**, 683–689.

29. Haughan,P.A., Chance,M.L. and Goad,L.J. (1993) Effects of sinefungin on growth and sterol composition of *Leishmania* promastigates. *Exp. Parasitol.*, **77**, 147–154.

30. Lawrence,F., Derbecourt,T. and Robert-Gero,M. (1998) Proton-ATPase activities involved in the uptake of an *S*-adenosylmethionine analogue. *Mol. Biochem. Parasitol.*, **92**, 99–109.

31. Phelouzat,M.A., Basselin,M., Lawrence,F. and Robert-Gero,M. (1995) Sinefungin shares AdoMet-uptake system to enter *Leishmania donovani* promastigotes. *Biochem. J.*, **305**, 133–137.

32. Merali,S. and Clarkson,A.B. (2004) S-adenosylmethionine and pneumocystis. *FEMS Microbiol. Lett.*, **237**, 179–186.