Sampangine Inhibits Heme Biosynthesis in both Yeast and Human

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Received 6 July 2011/Accepted 30 August 2011

The azaoxoaporphine alkaloid sampangine exhibits strong antiproliferation activity in various organisms. Previous studies suggested that it somehow affects heme metabolism and stimulates production of reactive oxygen species (ROS). In this study, we show that inhibition of heme biosynthesis is the primary mechanism of action by sampangine and that increases in the levels of reactive oxygen species are secondary to heme deficiency. We directly demonstrate that sampangine inhibits heme synthesis in the yeast Saccharomyces cerevisiae. It also causes accumulation of uroporphyrinogen and its decarboxylated derivatives, intermediate products of the heme biosynthesis pathway. Our results also suggest that sampangine likely works through an unusual mechanism—by hyperactivating uroporphyrinogen III synthase—to inhibit heme biosynthesis. We also show that the inhibitory effect of sampangine on heme synthesis is conserved in human cells. This study also reveals a surprising essential role for the interaction between the mitochondrial ATP synthase and the electron transport chain.

The plant-derived alkaloid sampangine has broad and potent antiproliferation activities against fungal pathogens, human cancer cell lines, malaria parasites, and mycobacteria (19, 20, 25, 31). In particular, its potency against various human fungal pathogens in vitro is comparable to the potencies of existing antifungal drugs (1). Other analogs of the same aporphine family of alkaloids have also been shown to exhibit antiproliferation activities against viruses, bacteria, fungi, parasites, and tumor cell lines (7, 8, 17, 24, 34, 36). However, the mechanism of action exhibited by sampangine and its analogs in both cancer cells and human pathogens remains unclear. Sampangine was shown to induce apoptosis in cancer cells by stimulating the generation of reactive oxygen species (ROS) (20). A closely related alkaloid, ascididemin, was shown to inhibit the growth of Mycobacterium tuberculosis through iron depletion (5). We have previously also shown that sampangine inhibits fungal growth, likely by interfering with heme metabolism (1). This was supported by the observations that multiple mutants with mutations affecting the Saccharomyces cerevisiae yeast heme biosynthesis pathway were comparatively more sensitive to sampangine than a wild-type strain and that exogenously supplied hemin partly suppressed the inhibitory activity of the drug (1). However, exogenous hemin failed to completely reverse the inhibitory effect of sampangine. Overexpressing genes in the heme biosynthesis pathway also failed to confer sampangine resistance in a wild-type strain background (unpublished results). These results raise the question of whether heme synthesis is a primary target of the drug and also whether there is any molecular relationship between the many different cellular effects of sampangine.

In this study, we took an unbiased functional genomic approach by systematically screening the yeast genome-wide deletion mutant libraries to identify mutants exhibiting hypersensitivity or resistance toward the drug. Although none of the mutants tested conferred obvious resistance, we identified 132 mutants that were hypersensitive. Among these, the most sensitive ones affected mitochondrial functions, especially subunits of the ATP synthase. We next performed genome-wide synthetic lethality analyses with strains with two representative mutations (atpΔ and ymeΔ) that conferred the highest drug sensitivity to identify common genes or pathways that, when disrupted, cause severe growth defects or lethality. This identified mutations that affect the electron transport chain (ETC) and heme synthesis (hem14Δ) to be among the most significant ones. Comparison of synthetic lethality profiles suggested that the hem14Δ mutation reproduced the effects of sampangine treatment much better than mutations in ETC (cox17Δ or cyc3Δ), suggesting that the heme biosynthesis pathway is a primary target of the drug. Consistent with this model, we found that sampangine inhibits heme production in both yeast and human cells. Moreover, our studies on porphyrin profiles and enzyme activity levels indicate that sampangine inhibits...
heme synthesis likely by hyperactivating Hem4, the fourth step within the pathway. We also found that the growth defect of a hem14Δ yeast mutant was partially suppressed by the antioxidant N-acetyl cysteine (NAC), indicating that heme deficiency likely accounts for the increased ROS levels observed in sampangine-treated cells (1, 20).

**MATERIALS AND METHODS**

**Yeasts and media.** The haploid selection synthetic complete (SC) medium without Leu, His, and Arg and with G418 and l-canavanine (Can) contained dextrose (20 g/liter), yeast nitrogen base without amino acids and ammonium sulfate (1.7 g/liter), SC medium Leu-His-Arg dropout mix (2 g/liter), sodium glutamate (1 g/liter), G418 (200 mg/ml), l-canavanine (60 mg/ml), and agar (2%). Sodium glutamate substituted for ammonium sulfate as the nitrogen source to make the G418 selection more reliable on the minimal medium. A version of this that lacked uracil was used to select for double mutants during synthetic lethality analyses. SC medium contained dextrose (20 g/liter), yeast nitrogen base without amino acids and ammonium sulfate (1.7 g/liter), SC medium mix (2 g/liter), and sodium glutamate (1 g/liter) with or without agar (2%). A similar medium that lacked uracil was also used. Solid sporulation medium contained potassium acetate (10 g/liter), zinc acetate (0.05 g/liter), and agar (2%).

ATP, N-acetyl cysteine, and antimycin were purchased from Sigma-Aldrich and dissolved in water as stock solutions of 1 M, 100 mg/ml, and 100 mg/ml, respectively. Sampangine was isolated as described previously (31) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 2 mg/ml.

**Yeasts strains and plasmids.** Yeast strains used in this study were haploid convertable heterozygous diploid yeast deletion mutants (mutants YSC4035 and YSC4428; Open Biosystems) and their haploid MATA convertants and were used after sporulation and haploid selection as previously described (29). The genotype of a typical haploid strain was MATA ura3Δ leu2Δ his3Δ met15Δ can1Δ LEU2-MFA1-pr his3Δ gorΔ:kanMX, where gorΔ stands for deletion of any gene of interest. A homoΔ:kanMX mutant was used as a surrogate wild-type control in most experiments because the HO gene is already mutated in the parent strain.

Wild-type genes HEM3, HEM4, and HEM12 were PCR amplified and cloned into the vector YEpplac195 (2μm, URA3) (13) to construct the overexpression plasmids.

**Screening for and validation of sampangine-hypersensitive haploid YKOs.** Screening for and validation of sampangine-hypersensitive haploid yeast knockout (YKOs) were carried out essentially as previously described (28, 29). For the screen, a pool of haploid-convertable heterozygote diploid yeast deletion mutants was sporulated. Pools of isogenic MATA haploid cells were derived by growth on a haploid selection medium (SC medium without Leu, His, and Arg and with G418 and canavanine) that either contained (experiment) or lacked (control) sampangine at 0.5 g/ml. Relative representation of each YKO in drug-treated samples was processed for high-performance liquid chromatography (HPLC) analysis at Frontier Scientific Inc. (Logan, UT) as described previously (4).

**In vitro assays of porphobilinogen deaminase (PBGD), uroporphyrinogen III synthase (UIII), and uroporphyrinogen III decarboxylase (UroD).** (i) **PBGD assay.** A 100-μl mixture contained 330 μM porphobilinogen (PBG), 70 μM Tris (pH 7.65), 10 mM dithiothreitol (DTT), 6 μg purified recombinant human porphobilinogen deaminase (pPBGD), and 6 μl of stock DMSO that contained various amounts of sampangine. The mixture was then incubated at 37°C for 30 min in the dark. The reaction was terminated by adding 100 μl of 3 M HCl. The uroporphyrinogen I produced in the assay mixture was then oxidized to uroporphyrin I using 6% sulfuric acid. The supernatant was then centrifuged in a microcentrifuge. The supernatant was analyzed by ultra-performance liquid chromatography (UPLC) as described below. (ii) **UIII assay.** The UIII assay is performed as described above with the addition of 2 μg of purified recombinant UIII (rUIII). The porphyrin isomers were resolved by a method derived from those described previously (35, 39). An HPLC system that consisted of a Waters 2795 separations module (Alliance HT), a Waters 474 scanning fluorescence detector, a Waters 2996 photodiode array detector and a Phenomenex Gemini C18 column (250 mm by 4.6 mm by 5 μm) was used. The solvents were 1 M NH4OH in water adjusted to pH 5.16 with glacial acetic acid (solvent A), methanol (solvent B), acetonitrile (solvent C), and 10% (vol/vol) methanol in water (solvent D). All gradients were linear, and the flow rate was always 1 ml/min for the duration of the 40-min run. Solvent C was set at 10% throughout the run. The gradient conditions were set at 75% solvent A and 15% solvent B at 0 min, 60% solvent A and 30% solvent B at 8 min, 40% solvent A and 50% solvent B at 13 min, 28% solvent A and 62% solvent B at 17 min, and 70% solvent B and 20% solvent D at 20 min and 25 min and then went back to initial conditions at 28 min. (iii) **UroD assay.** The UroD assay was carried out essentially as previously described (32). Uroporphyrinogen I (30 to 35 nmol) for a single assay was enzymatically produced in a 100-μl mixture from PBG using 6 μg pPBGD and 2 μg rUIII as described above.

We note that sampangine at this concentration did not obviously reduce growth of the wild-type strain during this treatment period. Similar numbers of cells from each culture were harvested, washed with ice-cold water, and homogenized in 500 μl of lysis buffer as previously described (27). Two microliters of each cell lysate was used to measure the amount of heme with a hemin assay kit (Bio-Vision, CA) according to the manufacturer’s instructions. The protein concentration of each lysate was also measured and used to calculate the heme concentration as fmol/μg protein. Three independent experiments were performed for each culture condition, and the results were averaged. The average heme level in the wild-type strain containing the empty vector that was grown in the absence of sampangine was set at 100%.

**Measuring heme levels in human cancer cell lines.** Exponentially growing cultures (2 × 10^6) of the acute T cell leukemia Jurkat and non-small cell lung cancer NCI-H1299 cell lines were seeded into 6-well plates containing RPMI 1640 supplemented with fetal bovine serum (10%; Sigma-Aldrich), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and incubated at 37°C in humidified air with 5% CO2. After an overnight incubation, cells were treated with sampangine (0.01 μg/ml or 0.1 μg/ml) or DMSO as the vehicle control and incubated for another 24 h, harvested, and washed softly with gelfree phosphate-buffered saline. About 5,000 cells from each culture were used to measure the amount of heme present using a hemin assay kit (Bio-Vision, CA) according to the manufacturer’s instructions. At least three independent experiments were performed for each culture condition, and the results were averaged. The average heme level for each cell line grown in the absence of sampangine was set at 100%.

**Determination of porphyrin profile in yeast.** An overnight culture of wild-type yeast grown in liquid SC medium was used to inoculate 100 ml fresh medium at 0 OD600. After one day, either sampangine (0.01 μg/ml) or DMSO (0.25%) was added to the cultures. The cells were allowed to grow for 14 h after treatment, and equal numbers of cells from the corresponding treated and untreated cultures were harvested by centrifugation. The cells were washed once with sterile distilled water, and the cell pellets were flash frozen in liquid nitrogen. The experimental conditions, including the medium, temperature, aeration, and concentration of sampangine, were identical to the conditions used in a previously reported study (4). Three independent experiments were performed on independently grown cultures. The samples were processed for high-performance liquid chromatography (HPLC) analysis at Frontier Scientific Inc. (Logan, UT) as described previously (4).

We note that sampangine at this concentration did not obviously reduce growth of the wild-type strain during this treatment period. Similar numbers of cells from each culture were harvested, washed with ice-cold water, and homogenized in 500 μl of lysis buffer as previously described (27). Two microliters of each cell lysate was used to measure the amount of heme with a hemin assay kit (Bio-Vision, CA) according to the manufacturer’s instructions. The protein concentration of each lysate was also measured and used to calculate the heme concentration as fmol/μg protein. Three independent experiments were performed for each culture condition, and the results were averaged. The average heme level in the wild-type strain containing the empty vector that was grown in the absence of sampangine was set at 100%.
The flow rate was set at 0.7 ml per min. Two solvents were used: solvent A consisted of 0.1% formic acid in water, while solvent B was pure acetonitrile. The gradient conditions were set at 60% solvent A at 0.0 min, 35% solvent A at 2.0 min, 10% solvent A at 2.1 min, and 1% solvent A at 2.6 min and then went back to initial conditions at 2.7 min. Except at 2.0 min, where the gradient was set at convex 5, all the rest were set at linear 6. The millivolt signals detected for the various porphyrins were individually compared with those in a standard solution that contained 5 pmol each of 8-, 7-, 6-, 5-, 4-, and 2-carboxylporphyrin per 10/μl injection. The chromatograms were processed using Waters Empower Pro software.

RESULTS

Mutants of mitochondrial ATP synthase are hypersensitive to sampangine. To identify the primary target of sampangine in a caykaryote, we first systematically screened the yeast genome-wide heterozygous diploid deletion mutants (11) and found that the mutant with the TOM40/tom40 mutation, which affects mitochondrial protein import (37), was the only one exhibiting significant hypersensitivity. However, this hypersensitivity was likely due to the intrinsic growth defects exhibited by this mutant (data not shown). We also screened a genome-wide open reading frame overexpression library that we constructed (Z. Huang and X. Pan, unpublished data) and found that overexpressing the multidrug ABC transporter SNQ2 (33) confers sampangine resistance (data not shown), possibly due to decreased drug accumulation inside yeast cells. We next screened the genome-wide haploid deletion mutants with complete loss of gene functions for increased drug sensitivity, reasoning that such mutants could define cellular functions closely related to the drug’s target, if not the target itself. Upon individual validation, we identified 132 haploid deletion mutants that were significantly more sensitive to sampangine at 0.5 μg/ml than a wild-type strain (see Table S1 in the supplemental material). Highly enriched among these were mutants with mutations affecting the mitochondrial ATP synthase, histone modification and chromatin remodeling, peroxisome biogenesis, and endoplasmic reticulum-Golgi functions (Fig. 1A; see Table S1 in the supplemental material). Mutations affecting oxidative stress response (e.g., lys7Δ) and DNA damage repair (e.g., rad50Δ) were also identified (Fig. 1B; see Table S1 in the supplemental material), consistent with previous observations that the drug causes oxidative damage (1, 20). A hem14Δ mutant lacking the only nonessential gene of the heme biosynthesis pathway in yeast was also sensitive to the drug (Fig. 1B; see Table S1 in the supplemental material). Among all these mutants, those with mutations affecting multiple subunits of the mitochondrial ATP synthase (e.g., Atp1p) and several other mitochondrial proteins, such as Yme1p, the catalytic subunit of the mitochondrial inner membrane i-AAA protease involved in the turnover of unfolded or misfolded mitochondrial membrane proteins (22, 26, 30, 38), exhibited the highest sensitivity (Fig. 1B and C; see Table S1 in the supplemental material). The sampangine-hypersensitive phenotype of the atp1Δ mutant was partly suppressed both by exogenously supplied ATP (1 mM) and/or NAC (0.1 μg/ml) in atp1Δ and yme1Δ mutants.
ATP but was barely suppressed by NAC under the same conditions (Fig. 1C), suggesting that the increase in ROS levels may not represent the most fundamental challenge faced by cells treated with sampangine.

The ATP synthase and Yme1p become essential when the ETC or heme synthesis is defective. To identify the primary function(s) affected by sampangine, we next performed genome-wide synthetic lethality analyses with the \( \text{atp1} \Delta \) and \( \text{yme1} \Delta \) mutations using a high-throughput technology that we previously described (28, 29). Given that both mutations conferred hypersensitivity to sampangine yet the corresponding proteins seem to have distinct biological functions, we expected that genome-wide synthetic lethality analyses with these two mutations would identify common as well as distinct genetic interactions. Some of the common synthetically lethal interactions would likely define the pathway(s) targeted by sampangine. Upon individual validation, we identified 172 and 190 synthetically lethal or sick interactions for \( \text{atp1} \Delta \) and \( \text{yme1} \Delta \), respectively, with the majority of them being surprisingly common (Fig. 2A). Most of these common interactions affected mitochondrial functions, particularly the mitochondrial ribosome and the ETC (Fig. 2B and C; see Table S2 in the supplemental material). While mutations of the mitochondrial ribosome typically cause severe growth defects on their own and tend to exhibit synthetic lethality interactions with other mutations, single mutations affecting mitochondrial ETC cause only modest growth defects (data not shown), and their synthetic lethality interactions with \( \text{atp1} \Delta \) and \( \text{yme1} \Delta \) were thus deemed more specific and significant. In addition, mutations of mitochondrial ribosome could affect expression of some of the ETC components. These results suggested that the \( \text{atp1} \Delta \) and \( \text{yme1} \Delta \) mutants both need a functional ETC to survive, and this was further corroborated by the observation that they were both highly sensitive to the ETC inhibitor antimycin (Fig. 2D). However, the ETC itself is unlikely a primary target of sampangine because it is dispensable for yeast cell survival, whereas the drug completely inhibits cell growth at >2 \( \mu \)g/ml. Instead, sampangine likely targets a process whose inhibition severely compromises the function of ETC.

We next considered the essential pathway(s) critical for the function(s) of ETC as a potential sampangine target(s) and focused on heme biosynthesis for three main reasons. First, our previous studies have implicated heme in the antifungal activity of sampangine. Second, heme is a physical component of ETC as a cofactor of the cytochromes. Third, synthetic lethality interactions were observed between heme deficiency (caused...
shown), and a synthetic lethality interaction was not observed when cytochrome b6f was deleted. The mutations of the ETC components were no more sensitive to sampangine treatment.

**Conclusion**

The heme biosynthesis pathway is a primary target of sampangine. The genetic interactions between a hem14Δ mutant and mutations affecting DNA repair, peroxisome biogenesis, and oxidative stress response were examined. The results indicated that exogenously supplied hemin suppressed the lethality caused by deleting heme biosynthesis genes.

**Further Experiments**

Experiments were conducted to determine the mechanism of sampangine treatment. The expression of genes involved in heme biosynthesis was measured to determine the effect of sampangine treatment. The results showed that sampangine treatment suppressed the expression of heme biosynthesis genes.

**Discussion**

The heme biosynthesis pathway is a primary target of sampangine. The genetic interactions between a hem14Δ mutant and mutations affecting DNA repair, peroxisome biogenesis, and oxidative stress response were examined. The results indicated that exogenously supplied hemin suppressed the lethality caused by deleting heme biosynthesis genes.
of the heme biosynthesis pathway even in the absence of sampangine (Fig. 3D). It is also possible that sampangine inhibits cell proliferation by targeting heme biosynthesis as well as other yet-to-be identified pathways. In this case, exogenously supplying hemin will unlikely restore all functions inhibited by sampangine.

**Sampangine inhibits heme biosynthesis.** Given the phenotypic similarity caused by sampangine treatment and a hem14Δ mutation, as discussed above, we further tested the model in which sampangine inhibits heme biosynthesis. As expected, the hem14Δ mutant showed greatly reduced heme levels compared to a wild-type strain (Fig. 4A). A similar effect was observed when a wild-type yeast strain was treated with sampangine at the IC₅₀ after one round of cell division (Fig. 4A). Such an inhibitory effect of sampangine on heme levels was also observed in human cells. Treatment with sampangine significantly reduced the intracellular levels of heme in two different human cancer cell lines tested (Fig. 4B). These results together indicate that sampangine inhibits heme synthesis in both yeast and human cells.

We next investigated whether sampangine inhibits a specific step in the heme biosynthesis pathway (Fig. 4C) (14). We compared the porphyrin intermediate metabolite profiles of yeast cells grown in the presence and absence of the drug. In particular, we measured the levels of uroporphyrin III, coproporphyrin III, and protoporphyrin IX, the oxidized intermediates of the fourth, fifth, and sixth steps of this pathway, respectively, because sampangine treatment leads to accumulation of a red pigmentmentation, which we thought to be porphyrin (1). Similar to what was reported previously (21), wild-type yeast cells not treated with the drug mostly accumulated coproporphyrin III and protoporphyrin IX, whereas the level of uroporphyrin III was extremely low and beyond detection (Fig. 4D). Treatment with the drug increased the total amount of porphyrins by 5- to 20-fold in three independent experiments (data not shown). More importantly, the porphyrin profile in the drug-treated cells was drastically altered, with the relative level of uroporphyrin greatly increased and the levels of co- and protoporphyrins by 5- to 20-fold in three independent experiments. These results together indicate that sampangine inhibits heme synthesis in both yeast and human cells.

We next tested the possibility that sampangine inhibits UroD. Using a well-characterized in vitro assay with a recombinant human enzyme (32), we surprisingly found that the activity of purified UroD was not significantly affected by excess sampangine at three concentrations tested (Fig. 5A). On the contrary, sampangine treatment significantly increased the

**FIG. 4.** Sampangine inhibits heme biosynthesis in vivo. (A) Sampangine treatment reduces heme levels in wild-type yeast. The heme concentration in each cell line not treated with the drug, calculated as fmol per μg of total protein, was set at 100%. Values shown are means ± standard deviations from assays performed in triplicate. The absolute levels of heme in the hem14Δ mutant were close to the background levels and the detection limit of the assay. (B) Sampangine treatments (0.01 μg/ml and 0.1 μg/ml) reduce heme levels in two human cancer cell lines, as indicated. The heme concentration in each cell line not treated with the drug, calculated as fmol per μg of total protein, was set at 100%. Values shown are means ± standard deviations from assays performed in triplicate. (C) Heme biosynthesis pathway in yeast. CoA, coenzyme A. (D) Effects of sampangine treatment (1 μg/ml) on protein levels of each of the eight enzymes of the yeast heme biosynthesis pathway analyzed by Western blotting. Enzymes were expressed as tandem affinity purification (TAP) tag fusion proteins from the endogenous loci. The fusion proteins are indicated with black arrows. Tub2 was used as a loading control.
activities of both porphobilinogen deaminase (PBGD) ($P = 0.0009$) and uroporphyrinogen III synthase (UIIIS) ($P = 0.01$), enzymes preceding UroD in the heme biosynthesis pathway (Fig. 5A). That sampangine stimulates the activities of these two enzymes could explain the increased levels of uroporphyrinogen III observed in drug-treated yeast cells (Fig. 4D). However, it was not clear how this might be related to the blockade of heme synthesis by sampangine treatment observed in vivo (Fig. 4A). It is possible that hyperactivation of PBGD and/or UIIIS disrupts the balance within the entire heme synthesis pathway and leads to decreased heme levels. To test this hypothesis, we measured cellular levels of hemin in yeast strains that overexpress HEM3, HEM4, and HEM12, genes encoding PBGD, UIIIS, and UroD, respectively (2, 10, 18), from high-copy-number plasmids. Overexpressing both HEM3 and HEM12 increased heme levels in yeast cells, and these effects were blocked by sampangine treatment (Fig. 5B). On the contrary, HEM4 overexpression reproducibly caused a reduction in heme levels even in the absence of sampangine ($P = 0.0005$) (Fig. 5B). Consistent with this observation, overexpression of HEM4 caused a growth defect in both a wild-type strain and an atp1Δ mutant (Fig. 5C). This inhibitory effect was further exacerbated by sampangine treatment (Fig. 5C). These results together suggested that a higher level of activity of HEM4 in a diploid yeast strain by half did not confer sampangine resistance (data not shown). An attempt with targeted random mutagenesis also failed to produce sampangine-resistant mutants, despite screening about 50,000 distinct alleles of the HEM4 gene (data not shown), possibly because potential resistance alleles are also nonfunctional.

**DISCUSSION**

By using a combination of genomic, genetic, and biochemical approaches, we have demonstrated that the plant alkaloid sampangine antagonizes cellular proliferation mainly by inhibiting heme biosynthesis, a function essential for cell survival. This mechanism of action is apparently conserved in yeast and human cells, further proving yeast to be a valuable system for investigating the mechanisms of action exhibited by bioactive small molecules. In order to discover the pathway(s) most significantly affected by sampangine treatment, we used a combination of genome-wide deletion mutant fitness profiling and synthetic lethality analysis. We first identified mutations that confer the highest sensitivity toward the drug. Cells with representative mutations were then used in genome-wide synthetic lethality analyses to reveal the cellular functions or pathways most likely affected by the drug. This approach will likely be useful in revealing the mechanisms of action exhibited by other drugs. Apparently, this was not the most straightforward approach for identifying drug targets in yeast cells. However, it was necessary with sampangine because both screening the genome-wide heterozygous diploid deletion mutants for drug-induced haploinsufficiency and analyzing a genome-wide gene overexpression library for drug-resistant clones, which could presumably directly reveal drug targets (6, 12, 23), had failed. We note that some heterozygous diploid mutants of the heme synthesis pathway were comparatively more sensitive to the drug than a wild-type strain (1) (data not shown). However, their defects were too subtle to be detected by the genome-wide screen that we performed.

Our results suggest that heme biosynthesis is a primary target of sampangine in yeast cells and likely also in human cells. This was supported by the evidence that heme deficiency due to a hem14Δ mutation largely mimicked the effects of sampangine treatment and that sampangine treatment inhibited heme synthesis in vivo. We also showed that heme deficiency is at least partly responsible for the increased levels of ROS observed in sampangine-treated cells (1, 20), possibly due to a defect in the ETC. In particular, we observed synthetic sick interactions between heme deficiency (due to the hem14Δ mutation) and oxidative stress response defects (due to the lys7Δ mutation) (Fig. 3C). In addition, the growth defect of a hem14Δ mutant was partly suppressed by the antioxidant NAC (Fig. 3C). Both sampangine treatment and heme deficiency were also shown to downregulate expression of multiple genes involved in iron uptake in yeast (1, 9). This could deprive cells of iron and further contribute to heme deficiency. This also points to the possibility that heme deficiency might also be responsible for the iron depletion observed in mycobacteria treated with the structurally related ascididemin (5). Taken together, inhibition of heme biosynthesis is likely the primary cause of the various biological effects of sampangine and its analogs.
Our results have also provided a glimpse into how sampangine might inhibit heme biosynthesis. It most likely involves hyperactivation of UIIIS (encoded by HEM4), which is by far the most active enzyme within the heme biosynthesis pathway (14). This model is based on the observations that sampangine stimulates the activity of human UIIIS in vitro and that overexpressing Hem4 inhibits heme synthesis and causes growth defects in yeast cells (Fig. 5). In addition, yeast cells treated with sampangine also accumulate higher levels of uroporphyrinogen III, the product of UIIIS, and its decarboxylation derivatives (Fig. 4A). However, it still remains to be investigated how sampangine treatment and hyperactivation of UIIIS might inhibit heme synthesis. It certainly does not involve reduced levels of expression of any of the downstream enzymes of the pathway (Fig. 4E). Overexpressing none of the downstream enzymes suppressed the detrimental effects of HEM4 overexpression and sampangine treatment (data not shown). We speculate that Hem4 hyperactivation might somehow cause irreversible damage to the pathway. This might help to explain the puzzling observation that heterozygous diploid mutants with mutations of Hem1 and Hem2, which lie upstream to Hem4 in the heme synthesis pathway, are hypersensitive to sampangine (1). Possibly, reducing the gene dosage of the upstream components in these mutants has little effect on Heme4 activation by the drug. However, once Hem4 is hyperactivated and the pathway is damaged, reducing the flux of the pathway further exacerbates the defects in heme synthesis.

Given that sampangine inhibits heme biosynthesis in both yeast and human cells and possibly other organisms, it might lack the needed specificity as a therapeutic agent against human pathogens and cancer cells. However, high specificity could still be achieved by exploiting unique genetic interaction networks within particular pathogens or cancer cell types. The list of sampangine-hypersensitive mutations identified in this study could aid in identifying pathogens or human cancer types that might be particularly sensitive to this drug and be a guide for selecting drug combinations that exhibit higher therapeutic potency and/or broader therapeutic indexes. Regardless of these, sampangine could serve as a chemical tool for investigating the physiological functions of heme metabolism in various biological systems and for studying the molecular mechanisms of human diseases related to heme deficiency, because its effect in yeast was largely mimicked by that of heme deficiency (Fig. 3A; see Table S1 in the supplemental material). However, for such applications, one should take into consideration possible off-target effects of sampangine because heme deficiency is probably not the only biological effect of sampangine treatment in yeast. This is because exogenous hemin partly rescued the growth inhibition caused by deleting heme biosynthesis genes yet completely failed to suppress sampangine’s lethal effects at high concentrations (Fig. 3D). Possibly, inhibition of the heme biosynthesis pathway by sampangine confers additional cellular defects that cannot be overcome simply by supplying cells with heme. For example, the accumulation of uroporphyrinogen III and potentially increased levels of other metabolic products derived from uroporphyrinogen III might also contribute to sampangine’s cytotoxic effect. It is also possible that, in addition to heme biosynthesis, sampangine directly inhibits an additional yet-to-be-discovered pathway. Our study also revealed a surprising synthetic lethality interaction relationship between mutations in the mitochondrial ATP synthase and those in the mitochondrial ATP synthase and ETC are together required for producing ATP via respiration, which is thought to be dispensable for yeast cell survival under normal growth conditions because this organism gets enough energy from glycolysis alone. We also found that the lethality of an atpLΔ cyc3A double mutant was not suppressed by exogenously supplied ATP (data not shown), even though the same amount of ATP partly suppressed the sampangine hypersensitivity of an atpLΔ mutant (Fig. 1D). NAC, when used alone or together with ATP, also failed to restore growth in the atpLΔ cyc3A double mutant (data not shown), suggesting that the lethality of this mutant simply due to decreased ATP production and increased ROS levels is unlikely. Therefore, in addition to ATP production, mitochondrial ATP synthase and the ETC together likely play an additional undefined role that is essential for yeast cell survival. Understanding the molecular mechanism of the lethality observed in the atpLΔ cyc3A double mutant could provide insights into such a novel function of mitochondrial ETC and ATP synthase.

ACKNOWLEDGMENTS

We thank Ping Shi for providing human cancer cell lines and advice for measuring heme in human cells. We also thank Hector Bergonia and Isaiah Davies for their assistance in porphyrin separation and method development.

This work was supported by NIH grants R01 HG004840 (to X.P.), R01 DK020503 and U54 DK083907 (to J.D.P.), and R01 AI27094 (to A.M.C.) and by USDA-ARS Specific Cooperative Agreement 58-6408-2-0009 (to the University of Mississippi).

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