

THE ANTIMALARIAL DRUG QUININE DISRUPTS TAT2P-MEDIATED TRYPTOPHAN TRANSPORT AND CAUSES TRP STARVATION

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Quinine is a major drug of choice for the treatment of malaria. However, the primary mode of quinine action is unclear and its efficacy is marred by adverse reactions among patients. To help address these issues, a genome wide screen for quinine sensitivity was carried out using the yeast deletion strain collection. Quinine sensitive mutants identified in the screen included several that were defective for tryptophan biosynthesis (*trp* strains). This sensitivity was confirmed in independent assays and was suppressible with exogenous Trp, suggesting that quinine caused Trp starvation. Accordingly, quinine was found to inhibit [³H]-Trp uptake by cells and the quinine sensitivity of a *trp1Δ* mutant could be rescued by overexpression of Trp permeases, encoded by *TAT1* and *TAT2*. The site of quinine action was identified specifically as the high affinity Trp/Tyr permease, Tat2p, with which quinine associated in a Trp-suppressible manner. A resultant action also on Tyr levels was reflected by the Tyr-suppressible quinine hypersensitivity of an *aro7Δ* deletion strain, which is auxotrophic for Tyr (and Phe). The present genome-wide dataset provides an important resource for discovering modes of quinine toxicity. That potential was validated with our demonstration that Trp and Tyr uptake via Tat2p is a major target of cellular quinine toxicity. The results also suggest that dietary tryptophan supplements could help to avert the toxic effects of quinine.

Malaria is a global crisis that devastates poverty stricken regions of the world. In the absence of a vaccine, chemotherapy remains the most effective means of countering malaria. However, the value of anti-malarial drugs is limited by resistance among *Plasmodium* spp., the

causative organism, and by drug toxicity in patients. Such adverse drug reactions (ADRs) have been documented to all of the currently available anti-malarials; with effects ranging from relatively mild symptoms such as gastrointestinal disturbances, nausea and headache, to more serious complications including death (1,2).

Quinine remains the most commonly used anti-malarial for therapy, despite major toxicity concerns especially in children (1,3,4). A link between quinine toxicity and one well-studied genetic defect of humans (glucose-6-phosphate dehydrogenase deficiency) is known, but that example only accounts for a fraction of adverse responses to quinine (1). The basis for most adverse responses to quinine is unknown, and the principal mode of quinine action against the malaria parasite is also still largely unclear. These are critical shortcomings in our understanding of quinine action.

The yeast *Saccharomyces cerevisiae* provides an excellent model for mode of action discovery. The tools available with yeast include the complete sets of deletion mutants, which allow determination of the role of each open reading frame (ORF) in enabling (or disabling) growth under a condition of interest. This approach has led to genome-wide identification of genes determining resistance to toxic metals and other pro-oxidants (5-7), small chemicals and drugs (8-11) and antibiotics (12,13). Moreover, because of the evolutionary conservation of cellular functions between yeast and humans (14,15), results from such studies with yeast can be extrapolated to higher organisms where they have yielded key new insight to stress and disease states. Furthermore, recent studies have reinforced how yeast is providing critical insights into understanding the general biology of the malaria parasite (16-19).

The applicability of the yeast model also extends to adverse drug reactions as yeast, like humans, are resistant to many drugs that are used for targeting pathogens. Consequently, the yeast deletion strains have been used previously to identify genetic defects that in humans could be the basis for the sensitivities of certain individuals to antimicrobial drugs (12,20). In this study, we applied this approach to the problem of adverse reactions to a major antimalarial drug for the first time, noting that similar approaches have been exploited recently for mode of action discovery with certain other antimalarials (17,21). Our analysis led to the key finding that yeast *trp* mutants (which rely on an exogenous supply of Trp, like mammals) are quinine hypersensitive, and that quinine specifically obstructs Trp acquisition by cells. This finding raises the novel possibility that a simple tryptophan supplement could help to avert the adverse responses commonly associated with quinine therapy.

EXPERIMENTAL PROCEDURES

Strains and Plasmids — The full set of homozygous deletion strains used for screens, in the diploid BY4743 background (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*) was obtained from Euroscarf (Frankfurt, Germany). To construct a cassette giving *tet*-regulated *TAT1* or *TAT2* gene expression, a PCR fragment containing the relevant ORF was amplified from yeast genomic DNA with addition of 5' terminal *NotI* and 3' *PstI* or *NsiI* sites, and ligated between the *NotI* and *PstI* sites of pCM190 (22). Recombinant plasmids were transformed into *S. cerevisiae* with the lithium acetate method (23) and transformants maintained on yeast nitrogen base (YNB; Formedium), supplemented as required to give Ura⁺ selection (24). Restriction digests, DNA ligations, sequencing and PCR were carried out using standard protocols (24).

Deletion Strain Screen — The full set of homozygous deletion strains was routinely stored at -80°C in 96-well format, in YEPD medium [2% (w/v) glucose, 1% yeast extract, 2% bacto-peptone, 1.6% agar, all Oxoid]

supplemented with 15% (v/v) glycerol. For experimental purposes, deletion strains were replica inoculated from the frozen stocks in to YEPD broth with a 96-pin tool, and microplates incubated at 30°C with orbital shaking at 120 rev min⁻¹. After overnight incubation, cultures were diluted to a median OD₆₀₀~0.5 before further dilution to OD₆₀₀~0.05 in fresh YEPD broth (200 μl per well, final volume), supplemented as specified with quinine hydrochloride (MP Biochemicals) from a 300 mM stock solution in 100% ethanol. Control incubations received the same ethanol additions without the quinine. Growth readings (OD₆₀₀) were recorded after incubation for 7 h at 30°C, with orbital shaking at 120 rev min⁻¹. Growth ratios (25) for each strain were calculated by dividing OD₆₀₀ readings obtained under control conditions (minus quinine) by those for parallel quinine-supplemented incubations; the background OD₆₀₀ of the medium (0.083) was retained to eliminate zero denominators. Growth ratios were not calculated for slow growing strains (OD₆₀₀<0.133 in untreated plates after 7 h), as small OD₆₀₀ differences have a disproportionately large effect on their growth ratios (25). Each growth ratio was divided by the median growth ratio obtained for the 96-well plate holding that strain, in order to correct for any plate to plate variation (25). Strains with a mean growth ratio of ≥1.45 (n=2) from the initial screen were re-arrayed onto new 96-well plates and screened three further times in duplicate. Strains giving a median growth ratio across all screens ≥1.45 were deemed to be quinine hypersensitive. The value of 1.45 was arrived at empirically, by relating the growth ratio values obtained for selected strains to phenotypes observed in independent (spotting on agar ± quinine) assays.

Quinine Toxicity Assays — For quinine toxicity assays with specific deletion strains, organisms were cultured to mid/late exponential phase (OD₆₀₀~2.0) in either YEPD broth or YNB broth with appropriate selection (26). These cultures were diluted to OD₆₀₀~0.10–0.15 in fresh medium, supplemented where specified with quinine hydrochloride. Where indicated, L-tryptophan or L-tyrosine supplements were also added to the medium from 0.5 M stock solutions

prepared in 1 M NaOH. All controls were balanced for ethanol or NaOH additions. Aliquots (300 μ l) of the cultures were transferred to 48-well microtitre plates (Sigma, Greiner), which were sealed with parafilm. The plates were incubated at 30°C with shaking in a BioTek Powerwave XS microplate spectrophotometer, with OD₆₀₀ measured every 30 min. Cell division rate (the number of generations per hour) was derived from a minimum of at least four OD₆₀₀ determinations obtained during the linear portion of exponential growth.

Amino Acid and Quinine Uptake Assays — Mid/late exponential phase cells were harvested by centrifugation (6,000 g, 5 min) and washed with 10 mM sodium citrate, pH 4.5, before resuspension to OD₆₀₀~3.0 in 5 ml of buffer comprising 10 mM sodium citrate, 20 mM ammonium sulfate, 2 mM glucose, pH 4.5 adjusted with citric acid. Where specified, the buffer was supplemented as indicated with non-radiolabelled L-tryptophan, or quinine (pure molecule). Uptake assays were initiated by the addition of either L-[5-³H]-Tryptophan (GE Healthcare) to a final activity of 0.9 μ Ci ml⁻¹ [20-30 Ci (mmol Trp)⁻¹], or L-[³⁵S]-Methionine (GE Healthcare) to a final activity of 0.9 μ Ci ml⁻¹ [1000 Ci (mmol Met)⁻¹], or [9-³H]-quinine (American Radiolabelled Chemicals) to a final activity of 0.9 μ Ci ml⁻¹ [10-20 Ci (mmol quinine)⁻¹]. Aliquots (0.5 ml) of cell suspension were removed at time intervals and vacuum filtered through pre-soaked nitrocellulose filters (Whatman glass microfiber filter, 25mm diameter). Cells were washed with 10 ml of 10 mM sodium citrate, pH 4.5, before transfer to scintillation vials. Cell-associated radioactivity was quantified in a liquid scintillation counter (2250CA Packard Tri-carb) and the readings normalized to cell density (OD₆₀₀). For kinetic analysis of uptake data, Prism 5.0 software was used for weighted nonlinear regressions (Graphpad Software Inc., CA, USA).

RESULTS

Identification of Deletion Strains with Altered Quinine Resistances — The yeast homozygous

diploid deletion-strain collection was screened to identify genes that are required for quinine resistance. The effect of quinine on the growth of each deletion strain was assessed in broth culture in 96-well format (see Experimental Procedures). A concentration of 2 mM quinine was used for screens as preliminary experiments showed that this was just sufficient to exert a mild slowing on wild type growth. A total of 110 putative quinine-sensitive strains (growth ratio ≥ 1.45) were identified from the genome-wide screen, and 43 of these phenotypes were subsequently confirmed in specific tests of the 110 putative strains (Supplemental Table 1). With the FunCat analysis tool (27), the 43 deleted genes of the sensitive strains were grouped into functional categories and the resultant distributions analyzed for significant differences compared with genome-wide distributions for *S. cerevisiae*. ‘Tryptophan biosynthesis’ was the most highly over-represented category in the annotations of genes in the quinine sensitive dataset, owing to the sensitivity of *trp1 Δ* , *trp3 Δ* and *trp5 Δ* deletion strains (Table 1). This indicated that an intact Trp biosynthetic pathway is required for normal quinine resistance. Other over-represented categories were involved with metabolism of tryptophan or other aromatic amino acids (*aro2 Δ* and *aro7 Δ*), and phosphate metabolism.

Quinine Causes Tryptophan Starvation in Cells Lacking Trp Biosynthesis — We decided to subject the above indication of tryptophan-dependent quinine toxicity to further investigation. In yeast, the tryptophan branch of the aromatic amino acid biosynthesis pathway involves five genes, *TRP1* to *TRP5*, encoding seven enzyme activity domains. To substantiate the lead from the screening data and to test roles for the other genes of the Trp biosynthesis pathway, *TRP2* and *TRP4*, the *trp1 Δ* – *trp5 Δ* deletion strains were each tested individually for quinine sensitivity. This was according to continuous growth measurements in the absence and presence of quinine (Fig. 1A). The strains all had similar exponential growth rates (albeit after differing lag times) under control conditions. However, growth of all of the *trp* mutants was inhibited at a quinine concentration (4 mM) that had no discernible effect on wild type growth

(Fig. 1A). The results corroborated that a capacity for Trp biosynthesis is required for quinine resistance under these conditions. The fact that two of the five *trp* deletion strains were not detected in the original screen for quinine sensitivity reflects the stringency of the criteria that we set to avoid false positives, but also highlights how some genuinely sensitive strains may not be revealed. Like other genome-wide approaches, the screening data here are ideally suited to mining for broader trends and effects (e.g., quinine sensitivity of multiple Trp pathway mutants) rather than definitive assessment of each strain's individual behaviour.

It was noted that Trp catabolic genes (*BNA1* to *BNA6*) were not among the quinine sensitive group from the screen, suggesting that specific downstream products of Trp such as kynurenine and nicotinamide adenine dinucleotide (NAD) are not involved in quinine resistance. We corroborated this by testing the *bnal1* – *bnal6* deletion strains individually, as described above for the *trp* mutants. None of the *bnal* mutants were quinine sensitive compared with the wild type (data not shown).

In the absence of *de novo* Trp biosynthesis, *trp* mutants rely on Trp taken up from the growth medium (28). It was reasoned that the Trp available in the medium alone may not be sufficient to meet the cellular Trp demand during quinine exposure. To test this, quinine exposure was performed in the presence of added L-tryptophan in the medium. For clarity, the data are presented as effects on cell division rate, which were determined from full growth curves (see Methods). The marked growth defect of quinine-exposed *trp1Δ* cells was rescued by exogenous Trp (Fig. 1B). Thus, growth in the presence of quinine was ameliorated by the addition of L-Trp to 0.05 or 0.1 mM and, at ≥ 0.5 mM L-Trp, the growth of *trp1Δ* cells was not affected by quinine [the natural Trp concentration of YEPD is reportedly 0.47 mM, but that is decreased by glucose addition and autoclaving (29)]. The Trp additions had no effect on growth in the absence of quinine (Fig. 1B), or on quinine resistance of the wild type (not shown). The data indicated that quinine provokes Trp starvation in cells that cannot synthesize their own tryptophan.

Quinine Targets the High Affinity Trp/Tyr Permease, Tat2p — We hypothesized that quinine interferes with tryptophan uptake, which would affect the phenotype only of biosynthesis-defective *trp* cells (by Trp limitation). To test this hypothesis, [3 H]-Trp uptake was compared in the absence and presence of quinine. The amount of [3 H]-Trp accumulated by cells after 60 min was decreased by $\sim 25\%$ and $\sim 70\%$ at 2 mM and 4 mM quinine, respectively (Fig. 2A). The uptake of [35 S]-methionine was also tested to help distinguish Trp-specific effects from any generic effects on (amino acid) transport. Quinine did not affect Met uptake by cells (Fig. 2B). This supported the screening data (Table 1, Supplemental Table 1), which did not suggest that quinine had general effects on amino acid transport.

To give further insight to the nature of quinine-mediated inhibition of Trp uptake, the kinetics of [3 H]-Trp transport (over 20 min) were examined in the absence and presence of quinine. Experiments were designed according to the principles described elsewhere (30,31), to facilitate reliable determination of kinetic transport parameters. The data were linearized by plotting reciprocal values for transport rate (V) versus Trp concentration (S) (Fig. 2C); the resultant plots indicated that quinine is a competitive-type inhibitor of L-Trp uptake, i.e., the K_m (negative reciprocal of the x axis intercept) but not the V_{max} (reciprocal of the y axis intercept) for L-Trp transport was altered by quinine. This suggested that Trp and quinine compete reversibly for the same binding site(s) on the relevant Trp transporter. The apparent K_m for Trp transport obtained in the absence of quinine (45 ± 4 μ M) was consistent with the range (15–50 μ M) reported previously in yeast (32).

Tryptophan uptake in yeast is mediated principally by the high-affinity (Tat2p) and low-affinity (Tat1p) Trp permeases. To reinforce the above data and to give an initial indication as to whether quinine may act at these permeases, *TAT1* and *TAT2* were tested as suppressors of the quinine-sensitive phenotype of *trp1Δ* cells. The *TAT1* and *TAT2* ORFs were cloned into plasmid pCM190 behind a *tet*-regulatable promoter module, which is induced >1000 -fold

in tetracycline-free media (33). High level expression of Tat1p or Tat2p in this way caused some slowing of growth in cells incubated without quinine (Fig. 3). Moreover, overexpression of either of these permeases conferred a marked advantage to *trp1Δ* cells incubated with quinine, rescuing the quinine sensitivity of this mutant.

The suggestion that Tat1p and/or Tat2p may be involved in the action of quinine was resolved more rigorously by comparing [³H]-Trp uptake in wild type, *tat1Δ* and *tat2Δ* strains, in the absence and presence of quinine (Fig. 4). Consistent with the greater contribution that Tat2p makes to cellular Trp uptake (34), *TAT2* deletion caused a larger decrease in Trp uptake in the absence of quinine than *TAT1* deletion. Moreover, the addition of quinine had no further effect on Trp uptake by *tat2Δ* cells. In contrast, the quinine-dependent inhibition of Trp uptake evident in wild type cells was accentuated in cells defective for the alternative Trp permease, Tat1p (Fig. 4). Therefore, quinine inhibits Trp uptake only in cells expressing the Tat2p permease, pointing to Tat2p as the site of this quinine action.

The evidence indicated that quinine competitively inhibits Trp uptake via Tat2p. Therefore, it was reasoned that Tat2p may also mediate quinine uptake by cells. Quinine uptake was measured with radiolabelled [³H]-quinine. This was available as purum quinine rather than the quinine hydrochloride salt used elsewhere in this study, but we confirmed that the *trp* mutants exhibited the same sensitivity to both purum and HCl-salt quinine preparations (data not shown). Initial time course studies showed that quinine uptake by cells was complete within 2 min (Fig. 5, inset). The absence of any further uptake up to 2 h was consistent with quinine uptake comprising a rapid association with the cell surface but without subsequent intracellular accumulation, unlike tryptophan uptake (Fig. 2). Quinine uptake was decreased by >80% in *tat2Δ* cells compared with the wild type (Fig. 5), indicating that Tat2p was primarily responsible for quinine association with cells. A similar decrease in quinine uptake by wild type cells was achieved by incubation in the presence of 1 mM L-tryptophan. In contrast, Trp supplementation had no effect on (the low level

of) quinine uptake by the *tat2Δ* mutant. These data substantiated that Tat2p is the site of quinine competition with Trp for uptake.

Quinine Causes Tyrosine Starvation — Tat2p is a key cellular permease for tyrosine, as well as tryptophan. As the Tat2p-quinine interaction appeared to be responsible for the quinine hypersensitivity of Trp auxotrophs (above), it was hypothesized that quinine may also cause tyrosine limitation in cells that are defective for tyrosine biosynthesis. The only gene required exclusively for Tyr biosynthesis, *TYR1*, is not encompassed in the yeast deletion strain collection. However, it was noted that both an *aro2Δ* mutant (Tyr- Trp- Phe-) and, moreover, an *aro7Δ* mutant (Tyr- Phe-) were quinine hypersensitive according to the screening experiments (Table 1). We confirmed this phenotype in a specific assay with *aro7Δ* cells cultured in the presence of quinine (Fig. 6). To assign this phenotype specifically to Tyr limitation, as we did above with Trp, cultures were supplemented with varying concentrations of L-tyrosine. Growth of the *aro7Δ* mutant was rescued at 1.0 mM L-tyrosine (Fig. 6). The results collectively indicated that the cellular supply of exogenous Trp and Tyr (both key functions of Tat2p) are debilitated by quinine exposure.

DISCUSSION

The ability to perform genome-wide screens with the yeast deletion strain collection has found valuable applications in recent years. Here, this approach was used to gain insight to the mode of toxicity of quinine, a major antimalarial drug that is commonly associated with adverse reactions in humans (1-4). The deletion strain screen revealed a key role for Trp biosynthesis in quinine resistance. Through further experiments, this novel aspect of quinine activity was attributed to the drug's capacity to obstruct Trp (and Tyr) uptake, via the main high-affinity Trp transporter of yeast, Tat2p. This action and associated growth inhibition was observable only in strains defective for *de novo* Trp biosynthesis and, thus, which are wholly dependent on transporter function for Trp acquisition. The latter is an important point, as

like the quinine-hypersensitive yeast *trp* mutants, mammals and the malaria parasite also rely entirely on a dietary supply of Trp.

We have recorded sensitivity in the *trp* yeast strains at quinine concentrations of the same order as those measured in plasma (~45 μ M) and liver (~100 μ M) of humans receiving quinine therapy (35,36). It is accepted that absolute inhibitory concentrations in lab studies with yeast will not necessarily predict those *in vivo* with humans. However, a primary determinant of quinine toxicity revealed here is the quinine:tryptophan (or tyrosine) ratio. Given the tryptophan (and tyrosine) concentrations found in human plasma (~50 μ M) and liver (~40 μ M) (37,38), the quinine:tryptophan ratios in humans are comparable to those used in our experiments, i.e., with which we demonstrated physiological quinine-tryptophan interactions. Furthermore, the comparison holds whether considering either free or bound molecules, as a similar proportion of total quinine and total tryptophan in humans is bound (70-85%) versus unbound (38,39). These considerations reinforce the broader physiological relevance of our findings.

The respective roles of Tat1p and Tat2p permeases in Trp transport are well described in yeast. However, in the case of Tyr, both low- and high- affinity Tyr transport have been ascribed to Tat2p (34,40). In the present study, the observed quinine hypersensitivity of Tyr auxotrophs (*aro2 Δ* and *aro7 Δ*) and its suppression by exogenous Tyr, was analogous to the effects observed with Trp. We did not test inhibition of Tyr uptake by quinine specifically, but infer that the underlying basis of quinine sensitivity in Tyr auxotrophs is similar to that determined for Trp auxotrophs, i.e., perturbation of Tat2p-dependent Trp/Tyr transport. This inference is supported by the identification of Tat2p as the major site of quinine binding and of Trp-transport inhibition in the present study.

Several salient features of the quinine action revealed by the genomic approach here are shared by an emerging class of chemical and physical stressors; most prominently, the hypersensitive phenotype of Trp auxotrophs. However, the mechanisms underlying these phenotypes appear to be heterogeneous (41). In the case of quinine, our evidence for competitive

inhibition of Trp uptake was in keeping with toxicity suppression by supplementation with exogenous Trp. Tryptophan auxotrophy is also associated with hypersensitivity to the immunosuppressant FTY720, but Trp supplementation does not suppress FTY720 toxicity (42). Rather, FTY720 provokes ubiquitin-dependent Tat1p degradation and a resultant broader inhibitory action on transport, encompassing Leu as well as Trp. This scenario is similar to that for other stressors that induce Tat1p or Tat2p degradation, such as rapamycin and the immunosuppressant FK506 (43). In the present screen, the quinine-sensitive strains did not include auxotrophs for any amino acid other than those transported by Tat2p, consistent with quinine exerting a specific inhibitory effect at Tat2p. Nonetheless, in common with the other studies, overexpression of either *TAT1* or *TAT2* could enhance quinine resistance.

The major source of commercial quinine remains the bark of the *Cinchona ledgeriana* tree, in which the drug constitutes ~13% of the dry weight. Tryptophan is thought to be a precursor for quinine biosynthesis in the tree, and these two molecules have marked structural similarity (44,45) (Fig. 7). This similarity probably accounts for the observed competitive inhibition of Trp transport by quinine at Tat2p. Although quinine itself does not appear to be actively transported into the cell by Tat2p (Fig. 5), owing to their similar structures we propose that quinine binds reversibly in place of Trp at the Trp binding site of Tat2p. This model is wholly consistent with the experimental observations made here.

More broadly, the reasons that *S. cerevisiae* appears especially vulnerable to inhibition of Trp uptake specifically, in response to a wide multiple stressors, may relate to the relative costs of biosynthesis of different amino acids. According to several measures, Trp emerges as the most energetically costly of the amino acids (46), and recent studies with *S. cerevisiae* have substantiated this (47). If amino acid import is inhibited, the magnitude of the metabolic burden experienced by the cell should reflect the relative biosynthetic cost of de novo synthesis of the affected amino acid(s). This would be greatest in the case of Trp, and may help to explain why Trp limitation specifically

arises frequently as a factor in stressor resistance.

The new insight to quinine action described here may have important implications for our understanding of quinine toxicity in humans and the parasitocidal activity of quinine. Neither humans nor *Plasmodium* spp. synthesize Trp de novo and both depend on dietary Trp, analogous to the quinine sensitive yeast *trp* mutants. We have performed sequence comparison analyses, which revealed that neither humans nor *Plasmodium* spp. express Trp transporters that are orthologous to yeast Tat2p. Nevertheless, convergently evolved (homoplastic) Trp transporters are expressed (48,49). The probability that competitive inhibition of Trp uptake by quinine stems from these molecules' structural similarities (see above), suggests that such inhibition is likely to occur also in homoplastic Trp transporters. As Trp transporter function is essential to humans and *Plasmodium*, it follows that any quinine-dependent inhibition of Trp uptake activity could be highly debilitating in these organisms.

In the case of humans, one prominent example of the importance of Trp transport is at the blood-brain barrier, where Trp is a direct precursor for synthesis of the key neurotransmitter 5-hydroxytryptamine (serotonin). The role of Trp availability in determining neurological conditions and glucose homeostasis defects is well described. Interestingly, the symptoms of Trp limitation in humans are similar to those of adverse-reactions to quinine, e.g., quinine causes cardiac arrhythmias and tryptophan deficiency is associated with heart rate variability and related cardiomyopathies (1,2,50-52). Furthermore, cognitive effects of tryptophan depletion such as tinnitus (35,53) overlap with common quinine side effects: nausea, tinnitus, dysphoria, headache and blurred vision (1,2). This supports the possibility that a large fraction of adverse reactions to quinine in humans might simply arise from Trp transport inhibition and Trp starvation. It follows that people with pre-existing genetic or dietary tryptophan defects, the latter being common in poor and undernourished populations, would be especially susceptible. Moreover, the present findings raise the possibility that administration of a dietary

tryptophan supplement might prove a cheap and simple solution for suppressing adverse responses associated with quinine therapy. This strategy would also fit well with another reported benefit of tryptophan supplementation during infection, that is, a potential stimulation of host responses to the malaria parasite (54).

In the case of *Plasmodium* spp., the major route of amino acid acquisition is via protease-mediated degradation of host-derived proteins in the parasite's food vacuole (FV) (55). Liberated amino acids are subsequently transported out from this organelle. Quinine is known to accumulate in the FV during the parasite's intra-erythrocytic phase, which has led to the inference that the FV may be the site of quinine action; resultant models of quinine activity include degradation of proteins in the FV and/or inhibition of the sequestration of toxic metabolites arising from this degradation (56). We suggest an additional possibility: that the accumulation of quinine in the parasite's FV could result in Trp starvation due to inhibition of Trp transport. If this is borne out by future studies, it should open new possibilities for rational drug design to improve the parasitocidal action of quinine.

This study has highlighted the applicability of the yeast mutant collection for gaining novel insight to the activity of antimalarial drugs, which are commonly undermined by adverse responses in humans. Moreover, the data led us to the discovery that quinine targets tryptophan transport and causes tryptophan starvation. The future potential for personalized therapy against malaria (57) relies, at least in part, on such advances and this aim may now be closer at hand.

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TABLE 1**Over-representation of specific functional categories in the annotations of genes from the quinine-sensitive dataset**

The dataset comprises the defective gene of each deletion strain that scored quinine sensitive.

Functional category ^a	<i>P</i> value ^b	Genes
Biosynthesis of tryptophan	4.09 x10 ⁻⁵	<i>TRP1, TRP3, TRP5</i>
Metabolism of tryptophan	1.13 x10 ⁻³	<i>ARO2, ARO7</i>
Metabolism of phenylalanine	3.09 x10 ⁻³	<i>ARO2, ARO7</i>
Metabolism of tyrosine	3.59 x10 ⁻³	<i>ARO2, ARO7</i>
Regulation of phosphate metabolism	5.93 x10 ⁻³	<i>SIS2, LSP1</i>

^aAll functional categories scoring $P \leq 0.01$ are shown

^bAccording to <http://funspec.med.utoronto.ca/>

LEGENDS TO FIGURES

FIGURE 1. The importance of tryptophan availability for quinine resistance. A, *S. cerevisiae* BY4743 (○), *trp1Δ* (●), *trp2Δ* (□), *trp3Δ* (■), *trp4Δ* (△) or *trp5Δ* (▲) strains were cultured in either the absence (top) or presence (bottom) of 4 mM quinine hydrochloride in 300 μl YEPD medium in a 48-well microplate. Growth was monitored with a BioTek Powerwave plate reader with shaking at 30°C. Typical curves from one of three independent experiments are shown. B, The *S. cerevisiae trp1Δ* deletion strain was cultured as in (A) but in YEPD medium supplemented with varying concentrations of L-tryptophan as well as 0 mM (□) or 4 mM (■) quinine. Division rates (the number of generations per hour) were derived from OD₆₀₀ measurements obtained during the exponential phase of growth. The values are means ± SEM from three replicate experiments. Control analyses (not shown for clarity) confirmed that the wild type division rate was not affected by quinine or L-tryptophan at the concentrations used.

FIGURE 2. Quinine inhibits cellular tryptophan uptake. Exponential phase cells (wild type) were incubated in YEPD medium supplemented with 0 mM (○), 2 mM (●) or 4 mM (□) quinine and with either 0.9 μCi ml⁻¹ [³H]-Trp (A) or 0.9 μCi ml⁻¹ [³⁵S]-Met. (B). Cellular uptake of radiolabel was determined at intervals and the derived scintillation counts normalized to cell density. Typical results from one of three independent experiments are shown. SEM values from replicate samples were smaller than the dimensions of the symbols. C, Tryptophan uptake rates were determined during 20 min after the addition of [³H]-Trp to cells in buffer supplemented with 0.025 mM, 0.05 mM, 0.1 mM or 0.25 mM L-tryptophan, and either 0 mM (○), 2 mM (●) or 4 mM (□) quinine. Reciprocal values of Trp concentration (S; mM) and Trp uptake rate [V; pmol Trp (10⁹ cells)⁻¹ min⁻¹] were derived for the Lineweaver-Burk analysis presented. The points are means from three replicate determinations and typical results from one of three independent experiments are shown. SEM values are smaller than the dimensions of the symbols.

FIGURE 3. Overexpression of tryptophan permeases rescues the quinine hypersensitivity of the *trp1Δ* mutant. *Saccharomyces cerevisiae* BY4743 (wild type) or *trp1Δ* strains were transformed or not with pCM190 ('empty' vector; ev), pCM190-*TAT1* (+*TAT1*) or pCM190-*TAT2* (+*TAT2*). Strains were cultured in the absence (□) or presence (■) of 4 mM quinine hydrochloride in 300 μl YNB medium in a 48-well microplate, with appropriate selection and supplemented with 0.02mM L-Trp. (The use of minimal YNB medium for plasmid selection accounts for the slower division rates in this experiment versus Fig. 1). Expression of the plasmid borne *TAT* genes was fully de-repressed by the absence of tetracycline in the medium. Growth was monitored with a BioTek Powerwave plate reader with shaking at 30°C. Division rates were derived from OD₆₀₀ measurements obtained during the exponential phase of growth. The values are means ± SEM from three replicate determinations.

FIGURE 4. Quinine inhibits Tat2p-dependent tryptophan uptake. Exponential phase *S. cerevisiae* BY4743 (wild type), *tat1Δ* or *tat2Δ* cells were incubated in buffer supplemented with 0 mM (○) or 5 mM (●) quinine and with 0.025 mM L-tryptophan supplemented with 0.9 μCi ml⁻¹ [³H]-Trp. Cellular uptake of radiolabel was determined at intervals and the derived scintillation counts normalized to cell density. The points are means from three replicate determinations and typical results from one of three independent experiments are shown. SEM values are smaller than the dimensions of the symbols.

FIGURE 5. Quinine association with cells depends on Tat2p. Exponential phase *S. cerevisiae* BY4743 (□) or *tat2Δ* (■) cells were incubated in buffer in the presence of 0.5 mM quinine supplemented with 0.9 μCi ml⁻¹ [³H]-quinine. Where indicated, 1.0 mM L-tryptophan was also included in the buffer. Cellular radiolabel uptake was determined after 10 min and the derived scintillation counts normalized to cell density. Inset: time course of quinine uptake by *S. cerevisiae* B4743. All the values are means ± SEM

from five replicate determinations, and typical results from one of two independent experiments are shown.

FIGURE 6. Tyrosine supplementation suppresses quinine hypersensitivity of a Tyr- auxotroph. *S. cerevisiae aro7*Δ cells (Tyr- Phe-) were cultured in either the absence (left) or presence (right) of 4 mM quinine hydrochloride in YEPD medium supplemented with either no additional L-tyrosine (○), or with 0.1 mM (●), 0.5 mM (□) or 1.0 mM (■) L-tyrosine. Growth was in 300 μl volumes of medium in 48-well microplates, monitored with a BioTek Powerwave plate reader with shaking at 30°C. Typical curves from triplicate independent experiments are shown.

FIGURE 7. Structures of the quinine and tryptophan molecules. Imported from BioDraw Ultra 11.0 software.

FIGURE 1

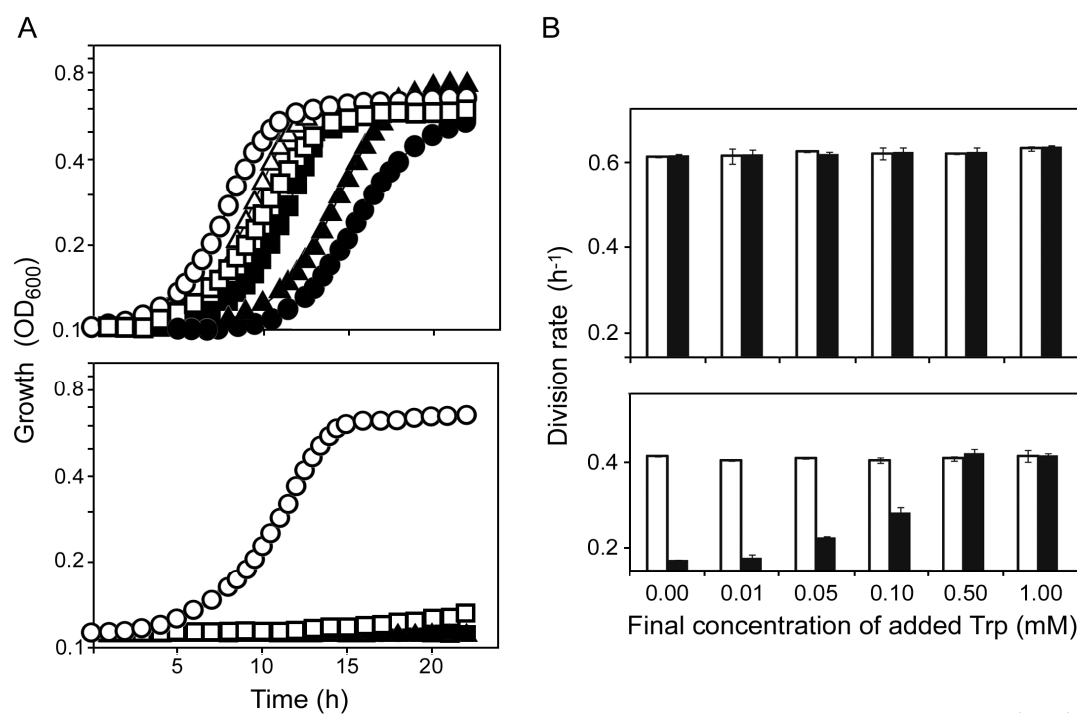


FIGURE 2

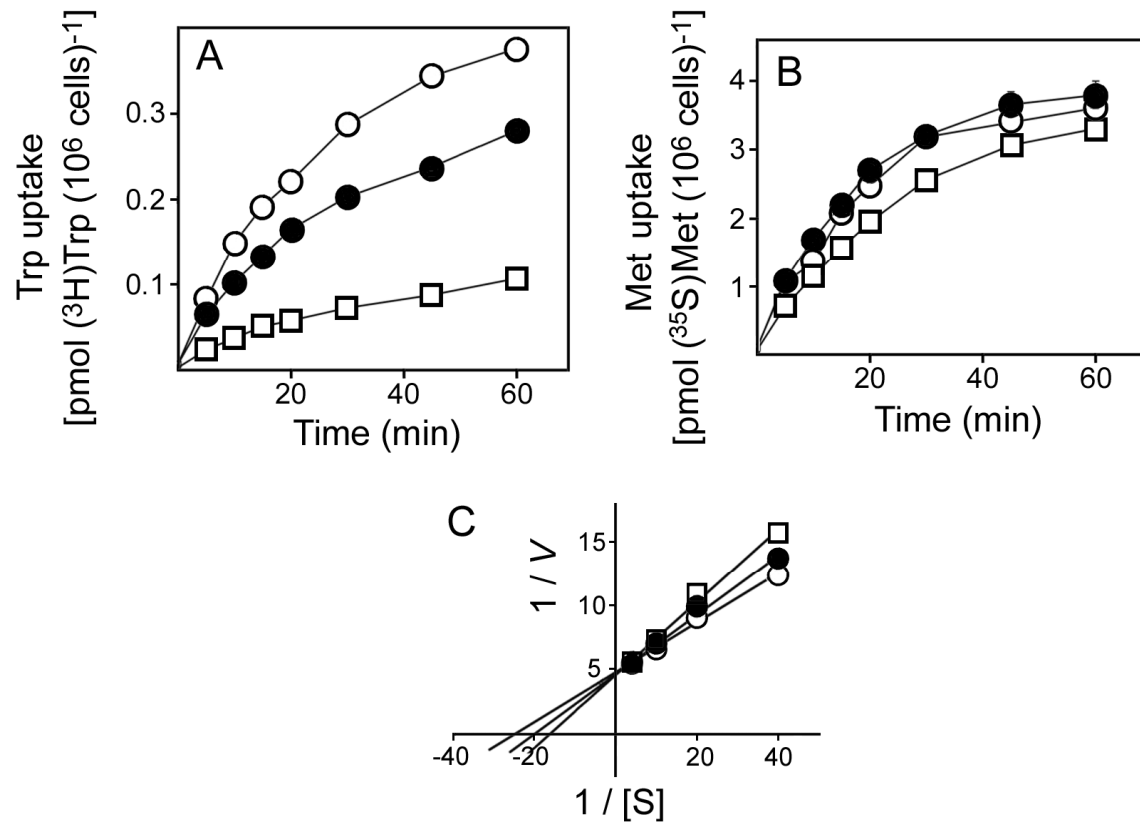


FIGURE 3

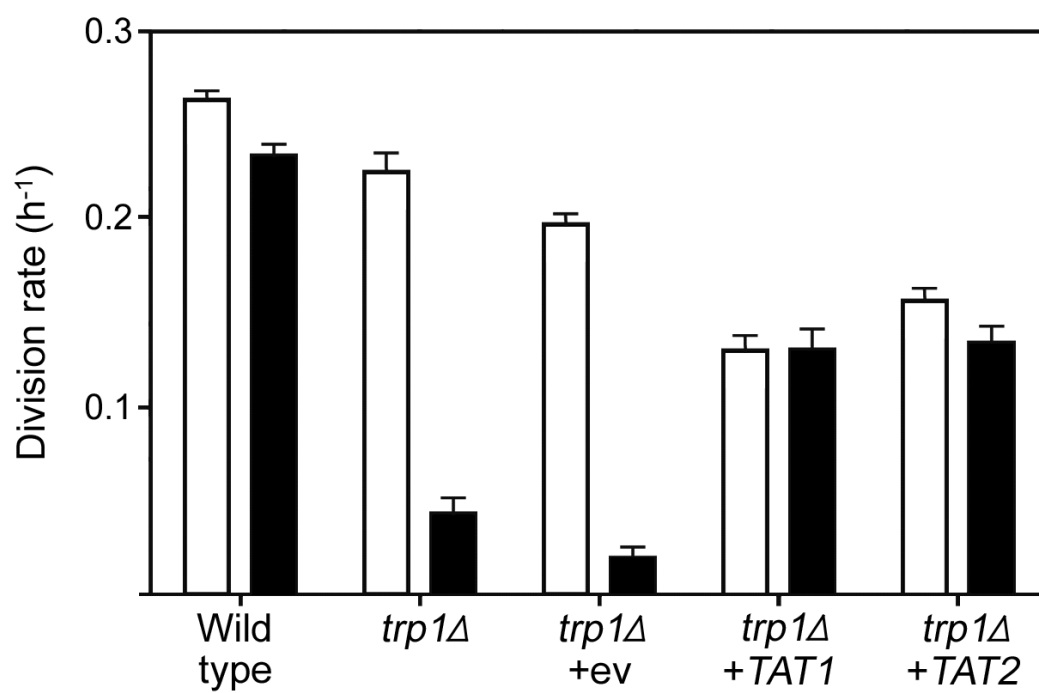


FIGURE 4

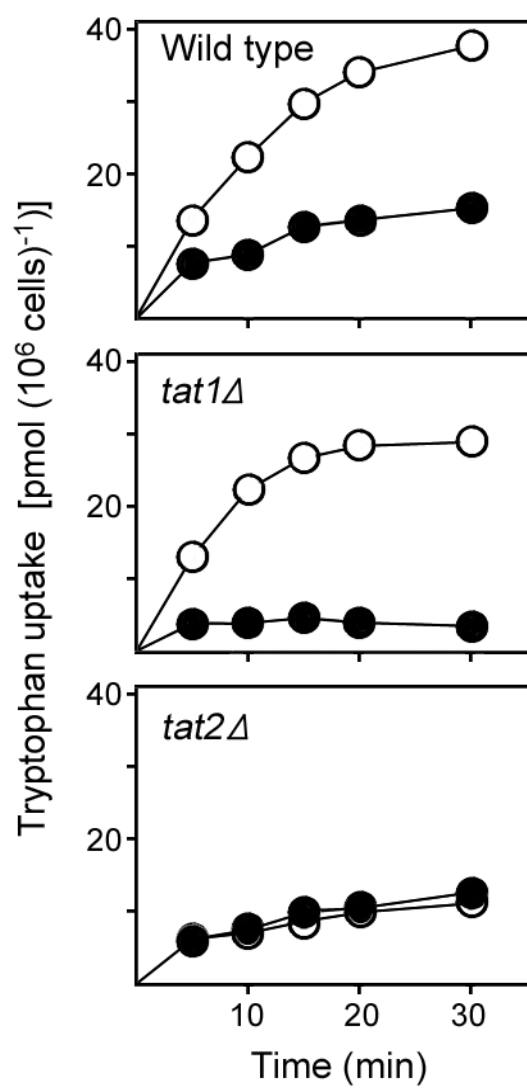


FIGURE 5

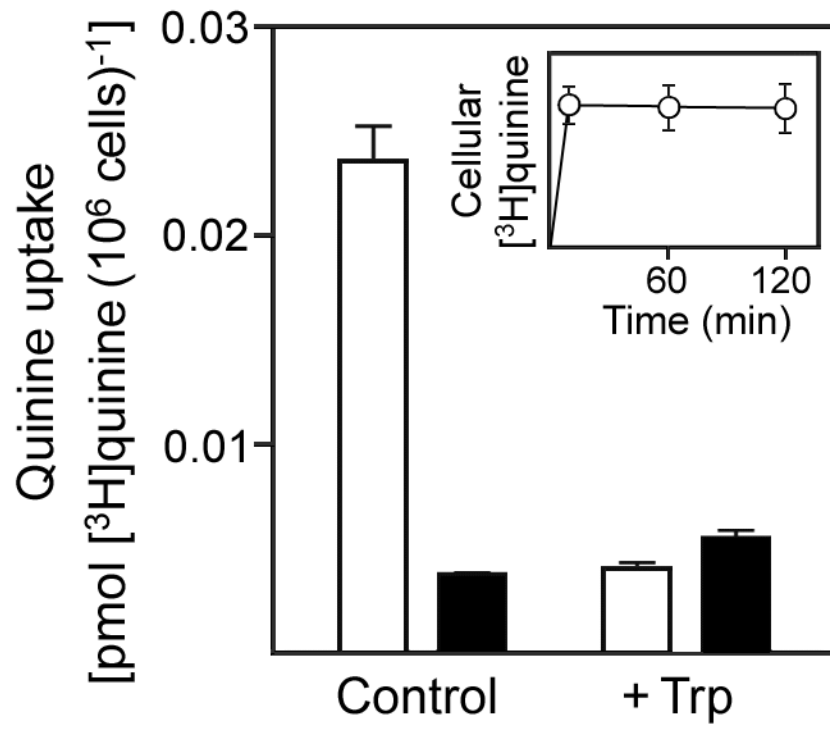


FIGURE 6

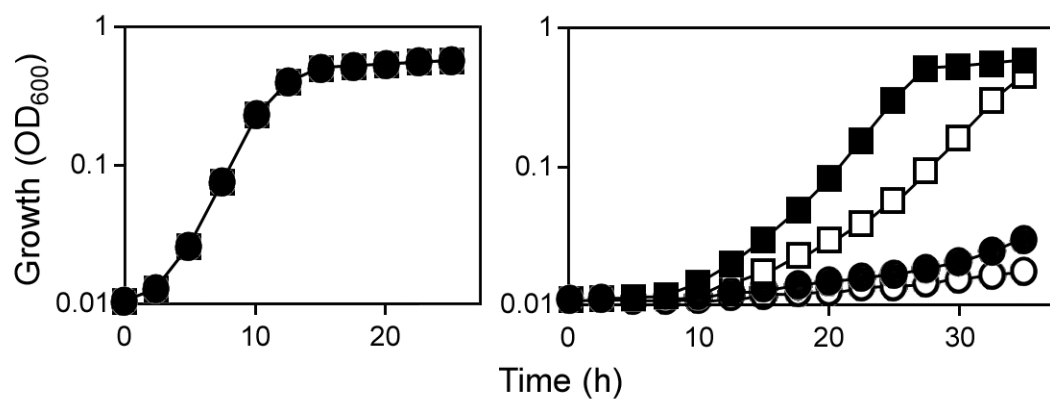
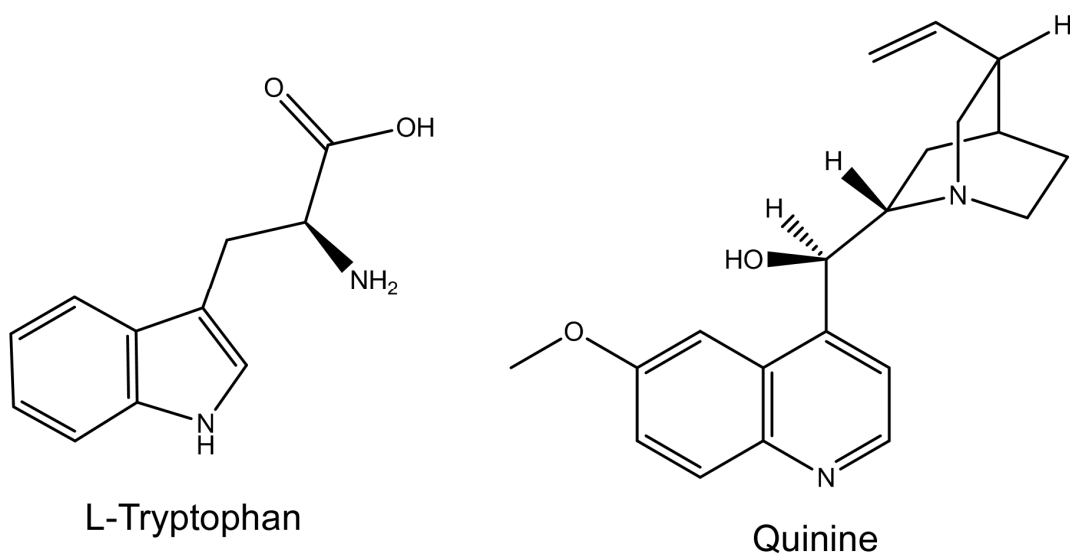


FIGURE 7



The antimalarial drug quinine disrupts Tat2p-mediated tryptophan transport and causes Trp starvation

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