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Resistance mutations at the lipid substrate binding site of *Plasmodium falciparum* protein farnesyltransferase

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Abstract

The post-translational farnesylation of proteins serves to anchor a subset of intracellular proteins to membranes in eukaryotic organisms and also promotes protein-protein interactions. This enzymatic reaction is carried out by protein farnesyltransferase (PFT), which catalyzes the transfer of a 15-carbon isoprenoid lipid unit, a farnesyl group, from farnesyl pyrophosphate to the C-termini of proteins containing a CaaX motif. Inhibition of PFT is lethal to the pathogenic protozoa *Plasmodium falciparum*. Previously, we have shown that parasites resistant to a tetrahydroquinoline (THQ)-based PFT inhibitor BMS-388891 have mutations leading to amino acid substitutions in PFT that map to the peptide substrate binding domain. We now report the selection of parasites resistant to another THQ PFT inhibitor BMS-339941. In whole cell assays sensitivity to BMS-33941 was reduced by 33-fold in a resistant clone, and biochemical analysis demonstrated a corresponding 33-fold increase in the BMS-339941 K_i for the mutant PFT enzyme. More detailed kinetic analysis revealed that the mutant enzyme required higher concentration of peptide and farnesyl pyrophosphate substrates for optimum catalysis. Unlike previously characterized parasites resistant to BMS-388891, the resistant parasites have a mutation which is predicted to be in a distinct location of the enzymatic pocket, near the farnesyl pyrophosphate binding pocket. This is the first description of a mutation from any species affecting the farnesyl pyrophosphate binding pocket with reduced efficacy of PFT inhibitors. These data provide further support that PFT is the target of THQ inhibitors in *P. falciparum* and suggest that PFT inhibitors should be combined with other antimalarial agents to minimize the development of resistant parasites.

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#Note: Nucleotide sequence data reported in this paper have been submitted to GenBankTM data base with the accession number **DQ986323** (*P. falciparum* Dd2 PFT alpha subunit drug resistant phenotype), and **DQ986322** (*P. falciparum* Dd2 PFT beta subunit drug resistant phenotype).

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Keywords

Plasmodium; falciparum; farnesyltransferase; PFT; drug resistance; malaria

Introduction

Malaria causes about 300 million infections annually [1]. Approximately 90% of deaths occur in Africa, due to falciparum malaria. For decades, malaria chemotherapy has relied on a limited number of drugs. The emergence and spread of drug resistant *Plasmodium falciparum* is a cause for grave concern with respect to disease control. The acquisition and spread of resistance to available drugs is largely responsible for a recent increase in malaria-related mortality [2, 3], resulting in about 1–2 million deaths per year [1]. The increasing burden caused by drug resistant parasites has led investigators to seek out novel anti-malarial drug targets. Among these are enzymes necessary for cellular division and differentiation. Previous work has demonstrated that the enzyme protein farnesyltransferase (PFT) is a viable drug target for pathogenic protozoa, including the malaria parasite *P. falciparum* [4–8]. PFT inhibitors (PFTIs) have been developed by the pharmaceutical industry owing to their anti-cancer properties [9–11]. Utilizing this existing resource, we have been able to demonstrate that low nanomolar concentrations of tetrahydroquinoline (THQ)-based PFTIs inhibit *P. falciparum* PFT (P/PFT) and are cytotoxic to parasites both *in vitro* and *in vivo* [6].

Due to the enzymatic nature of the drug target, we have investigated the potential for *P. falciparum* to acquire resistance to PFTIs. Upon selection with THQ PFTI BMS-388891 we identified a Y837C mutation of the PFT beta subunit in BMS-388891 resistant parasites, predicted to be in the peptide binding pocket [12]. The corresponding residue in yeast had previously been demonstrated to alter peptide substrate binding and provide resistance to tricyclic inhibitors (Schering-Plough PFT inhibitors SCH44342 and SCH56582) [13].

Now, using a different PFT inhibitor, we have selected resistant *P. falciparum* which possess a novel mutation in the PFT beta subunit. This mutation is predicted to be near the farnesyl pyrophosphate binding region of the enzyme catalytic site, implicating a unique mechanism of conferring resistance to PFTIs.

Materials and Methods

Parasites

Experiments described in this study were performed with a clone of *P. falciparum* Dd2 strain parasites [14]. Parasites were cultured asynchronously *in vitro* using standard conditions, and media [15]. Parasites from infected erythrocytes were isolated for PFT enzyme extraction by treatment with 0.1% (w/v) saponin.

Selection of BMS-339941-resistant Parasites

Selection of resistant parasites was conducted as described previously using a “one-step” selection procedure [14,16]. Briefly, triplicate 30 ml cultures with a 2% hematocrit were inoculated with 10^8 infected RBCs, of an isogenic, recently-cloned population of *P. falciparum* Dd2 (WT). These parasites were challenged with varying concentrations of THQ BMS-339941 (300 nM, 100 nM, 33.3 nM) in the culture media (Figure 1). In addition, triplicate flasks were inoculated with 10 parasites as a growth control, to gauge when limiting numbers of parasites would appear by microscopy. The medium was changed every other day, maintaining the initial drug concentration, and fresh RBCs were added once per week. Cultures were maintained under continuous drug pressure for 80 days. Giemsa stained blood smears of

cultures were used to detect parasite outgrowth, a 2% infected RBC level was considered positive. Upon selection parasites were cultured continually under drug pressure, clonal isolates from selection flasks were obtained by the limiting dilution method.

Proliferation Assays

In vitro responses to THQ PFTIs were calculated from 72-h [^3H]-hypoxanthine incorporation assays, as previously described [17,18]. Incorporation of [^3H]-hypoxanthine into nucleic acids was measured using a Chameleon liquid scintillation counter (Hidex, Finland). The ability of the PFTI to reduce parasite growth was reported as an ED_{50} value, the effective dose of PFTI that reduces the hypoxanthine incorporation by 50%, compared to the untreated positive control, after subtraction of a no parasite background control. These values were fitted using non-linear regression analysis using Prism 3.0 (Graphpad, San Diego, CA), drug concentrations are expressed as log values.

Genetic Analysis

Genomic DNA was isolated from parasites using the standard phenol/chloroform protocol. Duplicate independent PCR reactions for both the PFT alpha and beta subunits were amplified using Bio-X-Act Short Polymerase Mix (Bioline, Randolph, MA). These amplicons were directly sequenced using previously reported primers specific for the alpha and beta subunit [12]. Sequencing results were analyzed using Vector NTI Suite 9.0 (Informax, Invitrogen, Carlsbad, CA).

Protein Farnesyltransferase Assay and Enzyme Inhibition Assay

*Pf*PFT was partially purified from the isolated parasites by ammonium sulfate fractionation and Q Sepharose chromatography from parasite lysates as described by Chakrabarti *et al.* [5, 19]. Peak activity fractions were concentrated using a Vivaspinn 15R concentrator according to the manufacturer's recommendations (Vivascience, Hannover, Germany). Assays for *Pf*PFT activity were performed with a PFT-specific scintillation proximity assay (SPA) kit (Amersham Biosciences, Piscataway, NJ), slightly modified from previously described [6, 19]. One μM biotinylated lamin B peptide substrate (biotin-YRASNRSCAIM) was used. The concentration of [^3H]-farnesyl pyrophosphate (FPP) (3.7MBq) was increased to 1 μM to ensure that the enzyme substrate was in excess of its likely K_m value (the K_m of farnesyl diphosphate for human PFT is 9.3 ± 5.8 nM) [20]. Increasing the FPP concentration to 2 μM had no effect on the amount of product formed (data not shown). All reactions were performed in triplicate. Controls (no enzyme reaction values) were subtracted from experimental values before calculation of percent inhibition. The effectiveness of the PFTI is reported as an IC_{50} value, the concentration of inhibitor that reduces the activity of the enzyme by 50%, compared to the untreated positive control. These values were fitted using non-linear regression analysis using Prism 3.0 (Graphpad, San Diego, CA), drug concentrations are expressed as log values.

Determination of K_m values for the Peptide Substrate and for the Farnesyl Pyrophosphate Substrate

Determination of the K_m value for the peptide substrate of the PFT enzyme was conducted using the SPA, as described above. The [^3H]-FPP substrate concentration was kept constant at 1 μM while the peptide substrate concentrations used were 0, 0.25, 0.5, 1, 2, 3, 4, and 5 μM . Each experiment was repeated in duplicate for both the WT and mutant enzymes. Control values obtained from reactions with no peptide substrate were subtracted from experimental values. Experimental values were graphed as velocity, determined by counts per minute, as a function of peptide substrate concentration. These values were fitted to the Michaelis-Menten equation using Prism 3.0 (Graphpad, San Diego, CA). For determination of the K_m value for the FPP, assays were conducted using the SPA, as described above. The peptide substrate

concentration was 5 μ M while the [3 H]-FPP substrate concentrations used were 0, 0.9, 1.875, 3.75, 7.5, 15, 30, 60, 120 nM.

Determination of the K_i Values for BMS-339941

K_i values of BMS-339941 for both the WT and mutant native PFT enzyme were obtained by non-linear regression fit of percent inhibition as a function of inhibitor concentration at fixed peptide substrate concentration (1 μ M) and fixed FPP concentration (600 nM). The K_m values for the WT and G612A enzyme were used to obtain the value of K_i for the PFTI by fitting the observed rate data, from the IC₅₀ experiments, as a function of inhibitor concentration at constant peptide substrate concentration using Equation 1, by non-linear regression. V_i/V_o is the ratio of enzyme activity in the presence of inhibitor over the activity without inhibitor, [S] is the peptide substrate concentration, K_m is the value determined for each enzyme (see Table 1), and [I] is the concentration of BMS-339941. For comparison purposes the K_i of BMS-339941 was also obtained for the Y837C PFT mutant enzyme, and the K_i of BMS-388891 was obtained for the G612A mutant enzyme

$$V_i/V_o = ([S] + K_m) / ([S] + K_m(1 + [I]/K_i)) \quad (\text{Eq. 1})$$

Homology Modeling of PfPFT

The homology model of PfPFT obtained earlier was used for docking BMS-339941 into the binding site of the enzyme [12]. Several Monte Carlo searches were carried out with the program FLO (version 6.02) [21]. The initial placements of BMS-339941 were obtained by modifying the structures of two analogous THQs. They were PB-93 as observed in the x-ray structure with rat PFT (Corey Strickland, personal communication), or STN-48, as observed in the x-ray structure with human PFT (PDB 2IEJ), after superpositioning these structures on the PfPFT homology model. 2000 cycles of Metropolis Monte Carlo conformational searches were performed for each starting pose of BMS-339941 in the PfPFT binding site employing the MCDOCK module of the FLO program. A distance restraint was introduced for the bond between the imidazole nitrogen and the zinc ion.

Results

BMS-339941 Resistant *P. falciparum* Clones can be Selected

Earlier studies demonstrated that *P. falciparum* 3D7 was highly sensitive to growth inhibition by the THQ PFTI, BMS-388891 and BMS-339941, with an ED₅₀ of 7.0 nM and 5.0 nM, respectively [6] (Figure 1). Previously, we found that using a one-step selection protocol, it was possible to isolate *P. falciparum* Dd2 parasites that displayed a drug resistant phenotype to BMS-388891. Using the same selection protocol, we wanted to determine if drug resistance parasites could be isolated when selected with another THQ, BMS-339941 (Figure 1). We employed the same recently cloned Dd2 strain, as previously [12], which is prone to the acquisition of drug resistance [14]. In cultures treated with 100 nM BMS-339941, two of three flasks developed parasite growth by day 30. Flasks with inhibitor concentration of 33.3 nM had detectable parasitemia by day 25, and the control flask inoculated with 10 parasites, without drug, all had demonstrable parasites by day 15. In contrast, after 80 days of continuous culturing, the remaining flask challenged with 100 nM BMS-388891, and all of the flasks challenged with 300 nM BMS-388891 remained negative for parasites. Clonal parasites were derived from the populations of the two 100 nM flasks in which parasite outgrowth occurred, and these clones were used for further experimentation. Growth rate of the drug resistant clones with or without drug pressure did not differ significantly from the WT population without drug pressure (data not shown).

Genetic and Phenotypic analysis of Parasites

The parent clone has been previously sequenced [12], (GenBank/EBI Data Bank accession number [AY880032](#) PFT alpha and [AY880033](#) PFT beta subunit). Based on previous selection results, the PFT coding region of drug resistant clones was sequenced to determine if any genotypic changes had occurred in the drug selected parasites. Independent PCRs were used for sequencing to rule out PCR-induced errors. A single nucleotide difference in the PFT beta gene was found in clone 1a, and a single nucleotide difference in the PFT alpha gene was identified in clone 3a. Additional clones isolated from the each flask had the same mutations, suggesting only a single resistant genotype had arisen in each flask. These mutations altered the predicted amino acid coding of the beta subunit of clone 1a at position 612 from Glycine to Alanine, and in the alpha subunit of clone 3a at position 315 from Asparagine to Tyrosine. Phenotypic analysis, using a THQ inhibitor BMS-339941 dose response assay, demonstrated altered drug susceptibility for both clones, compared to the parent clone. Clone 1a had an ED₅₀ value of 193 ± 19.6 nM (Figure 2A) and clone 3a a value of 46 ± 5.2 nM (Data not shown), representing a 33-fold and 6-fold increase, respectively, in resistance to the inhibitor when compared to the parental ED₅₀ value of 5.7 ± 1.3 nM. Dose response experiments were conducted multiple times (WT n=3, 3a n=3, 1a n=3). Student's t-test demonstrated significance between the mean ED₅₀ values of the WT and clone 1a (*p*-value of 0.0044). The difference between WT and clone 3a, however, failed to yield a significant *p*-value.

G612A PfPFT has a Decreased Affinity for BMS-339941 and has an Decreased Affinity for Enzyme Substrates

The IC₅₀ value for PFT enzyme extracted from the *P. falciparum* Dd2 WT clone was 0.96 ± 0.34 nM against BMS-339941. This is similar to the value that was found previously for *P. falciparum* 3D7 (0.6 nM) [6]. However, the mutant PFT enzyme, extracted from resistant clone 1a, had an IC₅₀ value of 19.8 ± 0.06 nM against BMS-339941, 20-fold higher than the WT enzyme, as determined by an enzyme inhibition assay (Figure 2B). We were unable to extract functional PFT enzyme from clone 3a after multiple attempts. Therefore, further biochemical work concentrated on clone 1a. Enzyme inhibition experiments for both the WT enzyme and the G612A mutant enzyme were conducted 3 times, with a Student's t-test demonstrating a *p*-value of 0.02.

The K_m values of the WT and G612A mutant PFT enzyme for both the peptide (YRASNRSCAIM) and FPP substrate are listed in Table 1. For comparison, values for Y837C PFT are also included. BMS-388891 and BMS-339941 K_i values for WT, G612A, and Y837C enzyme are shown in Table 1. The G612A mutated enzyme has a decreased affinity for both the peptide substrate and FPP substrate (higher K_m), and also a 33-fold reduced binding affinity of BMS-388891 (higher K_i). Together it is hypothesized that these alterations allow the mutant PFT enzyme to function in the presence of higher concentrations of PFTI compared with WT PFT enzyme.

Homology Modeling of PfPFT

DNA sequence analysis of the PFT coding regions demonstrated two different single point mutations each of which are predicted to alter the amino acid sequence. Clone 3a possessed a mutation in the alpha subunit, N315Y, and clone 1a a mutation in the beta subunit, G612A. Both of these mutations are predicted to be near the binding pocket of the FPP substrate. Shown in figure 3 is a model of the predicted PFT structure of the G612A mutated *P. falciparum* enzyme. The mutation at residue 612 to an alanine introduces a methyl group, which is predicted to sterically interfere with the native binding mode of the FPP substrate. Specifically, the methyl group is predicted to contact the central isoprene unit of the FPP substrate (closest distance is 2.0 Å). In turn, movement of the FPP would alter the contact between the FPP and

inhibitor, and likely reduce the size of the inhibitor binding pocket; thus weakening the affinity of the BMS-339941 THQ inhibitor for the mutated enzyme.

Discussion

In this paper we report the selection of *P. falciparum* parasites resistant to PFTIs, specifically a THQ BMS-339941. The resulting parasites demonstrated an increased resistance to BMS-339941, which was used to select for resistance, and cross-resistance to other THQs (ED₅₀ values of BMS-388891 listed in Table 1). Similar to previous findings, resistance was associated with single point mutations in the target enzyme, protein farnesyltransferase. Since resistance was generated by continuous growth of parasites in the presence of inhibitor, without the addition of a mutagenic agent or process, these results raise the possibility that PFTIs may succumb to resistance if not protected through drug combinations. However, it remains to be seen if such mutants would emerge *in vivo* under therapeutically relevant dosing.

The predicted PFT structure, Figure 3, illustrates the proximity and orientation of the identified mutation in relation to the FPP binding site. It is predicted that introduction of a methyl group on amino acid 612 would introduce steric interference with the FPP binding pocket, which would in turn alter the binding of BMS-339941. Our *Pj*PFT model is consistent with the observed enzymological studies, predicting altered binding for both the FPP substrate and BMS-339941 in the G612A mutated enzyme. Experimental results using PFT enzyme purified from the G612A mutant demonstrated that the K_m value for both substrates were elevated compared to the WT enzyme. In addition, the G612A mutated enzyme demonstrates reduced binding affinity for BMS-339941, as demonstrated by a 130-fold increase in the K_i of BMS-339941 for the G612A versus WT enzyme. The WT and mutant parasite clones have similar growth rates (data not shown). This implies that although the mutation causes a weakening in the affinities for the substrates this decrease in enzyme efficiency does not have an observable effect on the parasites growth rate.

An alternative reason may explain why the G612A mutation confers PFTI resistance. It has been established for PFTs from yeast and mammals that FPP binds to free enzyme before the peptide substrate binds (ordered binding) [22,23]. The lower concentration of *Pj*PFT-FPP complex due to the lower affinity of FPP for the mutant *Pj*PFT would lead to an apparent increase in the value of the K_m for the peptide substrate and the K_i for the THQ PFT because there is a smaller fraction of enzyme in the form that can bind these molecules. In this context, it should be noted that the K_m for the peptide substrate and the K_i for the THQ PFTI are apparent values, dependent on the concentration of FPP and thus the concentration of *Pj*PFT-FPP complex in the assay mixture.

Previous studies in yeast and *P. falciparum* demonstrated that alteration of residues near the catalytic zinc ion altered substrate and inhibitor binding [12,13,24,25]. Del Villar *et al.* demonstrated that alteration of Tyr β 361, in the CAAX substrate binding pocket altered susceptibility to tricyclic PFT inhibitors SCH44342 and SCH56582 [13]. The Del Villar *et al.* study demonstrated that alteration of the tyrosine at position 361 to a hydrophobic residue, leucine, altered the affinity for certain peptide substrates, implicating its involvement in binding the peptide substrate. Previous findings from our group, found alteration of the homologous residue in the *P. falciparum* PFT enzyme, Y837C, also altered substrate affinity and conferred resistance to THQ inhibitors [12]. However, these earlier studies did not identify any mutations near the farnesyl pyrophosphate binding site. Also, as shown in Table 1, the G612A mutation has a greater effect on substrate binding and inhibitor binding than the previously described Y837C mutation. To our knowledge, this is the first report of an amino acid mutation near the FPP substrate binding pocket altering PFTI efficacy. This finding may have general significance to PFTI therapy and resistance for diseases beyond malaria.

Our finding that a single amino acid substitution can shift the sensitivity of *Pf*PFT by 20-fold reaffirms some of the liabilities of enzyme inhibitors for malaria therapy. This, however, does not negate this or other enzyme inhibitors for potential use as chemotherapeutic agents for malaria. It is likely that THQ PFTIs, as well as other antimalarials, will need to be combined with other agents, to achieve cures and to decrease the occurrence of drug resistance.

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Figure 1.
Structures of the tetrahydroquinolines BMS-339941 and BMS-388891.

Figure 2.

Demonstration of resistance to BMS-339941. (A) Growth inhibition by BMS-339941 of *P. falciparum* WT and resistant clone 1a. Shown is an *in vitro* growth inhibition assay, using [³H]-hypoxanthine uptake as a measurement of parasite proliferation. Results are shown as percent inhibition as a function of BMS-339941 inhibitor concentration. *P. falciparum* Dd2 WT (■), *P. falciparum* Dd2 G612A clone 1a (▲). (B) BMS-339941 inhibition of PFT enzyme from the parent clone and resistant clone 1a. Using native PFT enzyme isolated from both *P. falciparum* Dd2 WT (■) and G612A mutant clone 1a (▲), the percent enzyme inhibition as a function of BMS-339941 inhibitor concentration was determined. Both graphs are the mean of multiple replicates and standard deviation. Values were graphed using non-linear regression analysis, X-axes values are log of BMS-339941 drug concentration [nM].

Figure 3. Modeled location of G612A mutation site in *Pf* PFT. The extra methyl of Ala 612, shown as a white surface mesh, is predicted to push FPP more into the binding site of the inhibitor BMS-339941, thereby perturbing the good fit and reducing the affinity. (The zinc ion bound by PFT and the inhibitor is shown as a small white sphere).

Table 1

Difference in affinity of the G612A mutant PFT enzyme for substrates and THQs. Determination of the K_m for the YRASNRSCAIM peptide substrate, for both *P. falciparum* Dd2 WT and G612A mutant enzyme isolated from clone 1a, was determined using the Michaelis-Menten plot of activity as a function of peptide substrate concentration. Also shown is the K_m value of *P. falciparum* Y837C mutant enzyme, for comparison purposes [12]. The farnesyl pyrophosphate substrate K_m values for both the *P. falciparum* Dd2 WT and G612A mutant enzyme isolated from clone 1a as determined using the Michaelis-Menten plot of activity as a function of substrate concentration. Also shown is the K_m value of *P. falciparum* Y837C mutant enzyme, K_i of BMS-388891 and ED_{50} values against BMS-388891, for comparison purposes. The K_i values of WT, G612A, and Y837C *P. falciparum* PFT enzyme was determined by fitting the IC_{50} data as a function of inhibitor concentration at constant peptide substrate concentration [1 μ M] by non-linear regression. The ED_{50} value for BMS-388891, shown in parentheses, of WT, G612A, and Y837C *P. falciparum* for were determined by non-linear regression analysis of [3 H]-hypoxanthine incorporation data. All results are given as the mean $\pm$ S.D. of three experimental replicates.

Clone	K_m		K_i		ED_{50}	
	Peptide [μ M]	FPP [nM]	BMS-339941	BMS-388891	BMS-388891	BMS-388891
<i>P. falciparum</i> Dd2 WT	0.64 $\pm$ 0.12 ^a	23.83 $\pm$ 3.5	0.9 $\pm$ 0.2	0.22 $\pm$ 0.01 ^a	10 $\pm$ 2.1 ^a	
<i>P. falciparum</i> Dd2 G612A	3.03 $\pm$ 0.8	247.9 $\pm$ 30.3	30.08 $\pm$ 5.85	11.2 $\pm$ 1.6	132 $\pm$ 33.2	
<i>P. falciparum</i> Dd2 Y837C	0.32 $\pm$ 0.1 ^a	127.5 $\pm$ 34.7	2.8 $\pm$ 0.3	1.2 $\pm$ 0.2 ^a	120 $\pm$ 23.4 ^a	