thematic review

Thematic review series: Lipid Posttranslational Modifications

Fighting parasitic disease by blocking protein farnesylation

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Abstract Protein farnesylation is a form of posttranslational modification that occurs in most, if not all, eukaryotic cells. Inhibitors of protein farnesyltransferase (PFTIs) have been developed as anticancer chemotherapeutic agents. Using the knowledge gained from the development of PFTIs for the treatment of cancer, researchers are currently investigating the use of PFTIs for the treatment of eukaryotic pathogens. This "piggy-back" approach not only accelerates the development of a chemotherapeutic agent for protozoan pathogens but is also a means of mitigating the costs associated with de novo drug design. PFTIs have already been shown to be efficacious in the treatment of eukaryotic pathogens in animal models, including both *Trypanosoma brucei*, the causative agent of African sleeping sickness, and *Plasmodium falciparum*, one of the causative agents of malaria. Here, current evidence and progress are summarized that support the targeting of protein farnesyltransferase for the treatment of parasitic diseases.—Eastman, R. T., F. S. Buckner, K. Yokoyama, M. H. Gelb, and W. C. Van Voorhis. **Fighting parasitic disease by blocking protein farnesylation. *J. Lipid Res.* 2006.** 47: **233–240.**

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Parasitic diseases continue to have a major impact on morbidity and mortality in tropical and subtropical regions. Among these, malaria causes ~300 million infections annually, with 1–3 million deaths occurring in Africa (1). The emergence and spread of parasites resistant to existing antimalarial agents is largely responsible for the recent increase in malaria-related mortality. Another reemerging disease is African sleeping sickness (African trypanosomiasis), with an estimated 50,000 deaths in 2002 (1). The increasing burden of these diseases, along with the inadequacies of current drugs for African sleeping sickness in terms of safety, efficacy, and ease of administration, have led investigators to seek new chemotherapeutic agents (2, 3). Among the current drug targets under study are enzymes involved in protein prenylation, or the posttranslational modification of proteins by the covalent modification by isoprenyl lipids, C15 farnesyl and C20 geranylgeranyl (4–7). The isoprenyl lipid modification of proteins has been shown to be critical for various cellular activities in mammals and yeast, including proliferation and apoptosis (8, 9). Growth of the protozoan parasites has been shown to be severely impaired by the inhibition of protein farnesylation compared with mammalian cells, suggesting high potential of the enzyme protein farnesyltransferase (PFT) as an antiparasitic drug target (5, 10–13).

The isoprenoid synthesis pathway from mevalonic acid in many eukaryotes, including trypanosomatids (or deoxyxylulose in Apicomplexa, including *Plasmodium* and *Toxoplasma*, and plants) is essential for the production of sterols, dolichol, ubiquinone, and other isoprene derivatives in many eukaryotic cells. Indeed, these pathways have been the study of recent efforts to develop other antiparasitic chemotherapeutic agents, especially the targeting of isoprenoid pyrophosphate synthesis by nitrogen-containing bisphosphonates (14–16). Organisms belonging to the group Apicomplexa contain the nonmevalonate pathway of isoprenoid biosynthesis. One enzyme in this pathway is 2C-methyl-D-erythritol 4-phosphate synthase (IspC protein), which is inhibited by fosmidomycin (17). This has led to a clinical trial using fosmidomycin and clindamycin in combinational therapy for the treatment of malaria (18). This review will discuss the current efforts and progress in developing inhibitors of protein farnesyltransferase (PFTIs) as antiparasitic agents.

Abbreviations: ED_{50}, effective dose that inhibits 50% of parasite proliferation; PFT, protein farnesyltransferase; PFTI, protein farnesyltransferase inhibitor; PGGT-I, protein geranylgeranyltransferase type I; THQ, tetrahydroquinoline.

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PROTEIN PRENYLATION IN HIGHER EUKARYOTIC CELLS

Protein prenylation refers to the posttranslational modification of proteins by the covalent attachment of a 15 carbon farnesyl or a 20 carbon geranylgeranyl group. The structure of both the farnesyl and geranylgeranyl groups appended to proteins was determined in the early 1990s by Glomset, Gelb, and Farnsworth (19). This type of posttranslational modification creates a hydrophobic tail that facilitates membrane association as well as protein-protein interactions. Among known prenylated proteins are small GTPases, including Ras, Rac, Rho, and Rab, which play a role in cell signal transduction, vesicle trafficking, and cell cycle progression (20).

Protein prenylation is mediated by three distinct enzymes: PFT, protein geranylgeranyltransferase type I (PGGT-I), and PGGT-II. PFT recognizes a CaaX motif at the C terminus of specific proteins and transfers a farnesyl from farnesyl pyrophosphate to the thiol group of the cysteine: the CaaX motif is a cysteine followed by two amino acids (typically aliphatic) and a terminal amino acid, X, which is typically a Ser, Met, Ala, or Gln (21). PFT is a zinc-dependent heterodimeric enzyme with an α- and a β-subunit. PGGT-I shares the same α-subunit as PFT but has a distinct β-subunit. PGGT-I catalyzes the attachment of geranylgeranyl to proteins with the CaaX motif, in which X is usually a Leu or Phe (22). For both PFT and PGGT-I, other residues may be tolerated in the X position (23). After the action of either PFT or PGGT-I, a prenyl protein-specific protease cleaves the terminal tripeptide from the prenylated protein (24). The final step is methylation of the terminal carboxylic acid by a prenyl protein-specific methyltransferase (25–27). Both of these subsequent enzymatic steps have been shown to be required for the proper localization of certain mammalian proteins and are currently being investigated as additional chemotherapeutic targets (28, 29). The third prenylation enzyme, PGGT-II, catalyzes the addition of two geranylgeranyl groups onto the terminal residues of proteins ending with CC, CCXX, or CXC motifs. To date, proteins modified by PGGT-II have been exclusively members of the Rab low molecular weight G protein family (21).

Because of the discoveries that the Ras oncogene is farnesylated and that this modification is required for the proper localization and function of Ras (30–32), protein prenylation has received significant attention as a potential anticancer chemotherapeutic target (33–35). Mutations in Ras are associated with 20–25% of human cancers and 90% of pancreatic carcinomas (34). Numerous pharmaceutical companies have initiated drug discovery programs to generate PFTIs for the treatment of cancer. The first PFTI, which was described in 1993, was found in a chemical library screen based on the ability to inhibit yeast PFT activity (36). There are currently >2,000 primary publications on PFT inhibitors and >300 patents worldwide. Four companies have entered clinical trials for the development of PFTIs as a cancer chemotherapeutic agent: Janssen/Johnson & Johnson, Schering-Plough, Merck, and Bristol-Myers Squibb (37–42). Janssen/Johnson & Johnson and Schering-Plough are advancing to late clinical trials for the use of PFTIs in the treatment of certain leukemias (43). To date, PFTIs have proven to be relatively nontoxic in clinical trials and effective when combined with other chemotherapeutic agents for the treatment of certain cancers in vivo (43, 44). Because of strong interest in the development of PFTIs for the treatment of cancer, there is a wealth of pharmacologic information about PFTIs. This pharmacologic information, the lack of toxicity, and a rich source of small-molecule PFTI libraries provide an excellent opportunity for the "piggy-back" investigation of PFTIs for the treatment of tropical diseases such as malaria and African sleeping sickness.

PFT IN PATHOGENIC PROTOZOA

Protein prenylation occurs in a wide variety of pathogenic protozoa, including *Trypanosoma brucei* (6, 45), *Trypanosoma cruzi* (46), *Leishmania* species (46), *Plasmodium falciparum* (4, 5), *Toxoplasma gondii* (47), *Giardia lamblia* (48), and *Entamoeba histolytica* (49). Cloning and characterization of the PFT enzyme from trypanosomatid parasites was originally described by our group (46, 50). PFT enzymatic activity was detected in cytosolic fractions of *T. brucei* using the yeast Ras1 protein containing the C-terminal CaaX sequence Cys-Val-Ile-Met as a substrate (6). *T. brucei* PFT was subsequently isolated and purified using affinity chromatography with the CaaX peptide Ser-Ser-Cys-Ala-Leu-Met (51). Similar to mammalian PFT, *T. brucei* PFT is a heterodimer. However, the subunits are larger, owing to numerous peptide segment insertions. These insertions are predicted, by molecular modeling using the known mammalian PFT structure, to be in loops on the surface of the protein and distant from the active site (46, 51). Insertions are also observed in *T. cruzi*, *Leishmania* species, and *P. falciparum* PFTs (5, 46), but their function is as yet unknown. The CaaX substrate specificity differs in *T. brucei* PFT compared with mammalian PFT, with a higher preference for substrates with a Met or Gln at the X position. Alteration of four amino acid residues in the putative X binding pocket in the active site of *T. brucei* could be responsible for the restricted peptide substrate specificity and suggests the potential for developing parasite-specific PFT inhibitors (50).

P. falciparum PFT was first characterized by Chakrabarti et al. (4, 5). After partial purification by $(NH_4)_2SO_4$ precipitation and anion-exchange chromatography, it was shown that the CaaX substrate specificity was similar to that of *T. brucei* PFT, favoring a Met or Gln in the terminal position. Metabolic radiolabeling of prenylated cellular proteins with [^{3}H]farnesol demonstrated the incorporation of ^{3}H into 50 kDa proteins and some lower molecular mass proteins. The 50 kDa proteins were analyzed and found to be modified by a farnesyl group; the lower molecular mass proteins, however, were found to be geranylgeranylated, presumably after the conversion of farnesol into both farnesyl pyrophosphate and geranylger-

anyl pyrophosphate. Our group later showed that the 50 kDa farnesylated proteins, but not the lower molecular mass geranylgeranylated proteins, were specifically inhibited by PFTIs (11). Using synchronized *P. falciparum*, Chakrabarti et al. (5) demonstrated the stage-specific incorporation of prenylation precursors, the highest amount of incorporation occurring in the trophozoite (mid erythrocytic stage) to schizont (cell division stage) and schizont to ring (early erythrocytic stage) transition states in the erythrocytic life cycle of the parasite.

Using a polyclonal antibody raised against rat PFT, Ibrahim et al. (47) immunoprecipitated and identified the *T. gondii* PFT enzyme. PFT enzyme activity was confirmed using a CaaX-containing lamin substrate. Incubating tachyzoites (intracellular replicative form) with radiolabeled farnesol or geranylgeraniol demonstrated the in vivo prenylation of proteins, with the geranylgeranylation of proteins of 29 kDa and the farnesylation of proteins of 47 kDa (47). Inhibition of the *T. gondii* PFT enzyme occurred using hydrophobic the peptidomimetic inhibitors

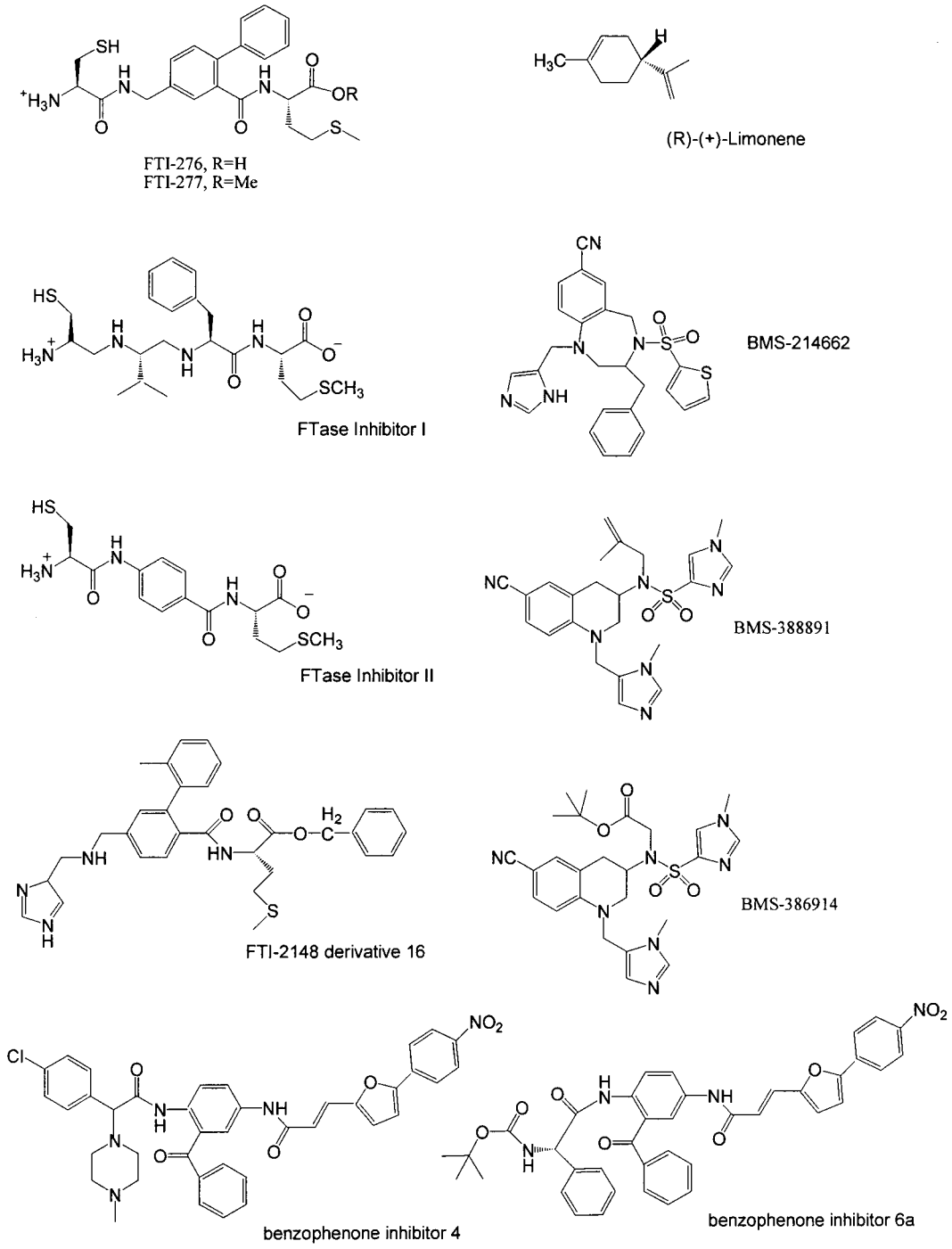

Fig. 1. The structures of selected protein farnesyltransferase inhibitors (PFTIs).

FTase Inhibitor I and II (**Fig. 1**). However, these inhibitors had no effect against the inhibition of PFT enzyme activity in intact parasites, presumably because of poor cellular penetration, as indicated by normal radiolabeling of proteins with [^{3}H]farnesol in inhibitor-treated cultures (47).

Although the PFT enzyme of *G. lamblia* has not been isolated, prenylation of proteins has been demonstrated in this primitive eukaryote (48). [^{3}H]mevalonic acid was specifically incorporated into cellular proteins of 50 and 20–30 kDa. After cleavage with methyl iodide, the isoprenoid substituents of these proteins were subjected to HPLC analysis and found to run with the same retention time as farnesol and geranylgeraniol (48). Inhibitors of PFT, such as limonene (Fig. 1), perillic acid, and perillyl alcohol, showed a dose-dependent effect on trophozoite (the replicative form of *Giardia*) growth in vitro (48); however, these agents are not very potent inhibitors of PFTs, and it is difficult to judge whether inhibition of *Giardia* PFT is the reason for the growth arrest.

Based on a genome search, Kumagai et al. (49) have identified and characterized PFT in *E. histolytica*. Interestingly, it was found that *E. histolytica* PFT does not preferentially modify proteins with a terminal Met residue. Instead, *E. histolytica* PFT favors CaaX substrates with smaller terminal amino acids, Ala and Ser, which suggests an altered binding cleft for the CaaX substrate. In further support of the altered substrate specificity of *E. histolytica* PFT, this PFT has a higher resistance to the CaaM peptidomimetic FTI-276 (Fig. 1) compared with other PFT enzymes (49).

TABLE 1. Comparative enzyme and cellular efficacy of protein farnesyltransferase inhibitors against protozoa

Compound	Protein Farnesyltransferase Inhibition	Cell Growth Inhibition	Reference
	nM		
BMS-214662	8 (*Pf*), 1.9 (*Tb*)	200 (*Pf*), 200 (*Tb*)	11
BMS-386914	0.7 (*Pf*), 50 (*Tb*)	5 (*Pf*), 500 (*Tb*)	11
FTI-276	1 (*Pf*), 1.7 (*Tb*)	ND	5, 51
FTI-277	ND	60,000 (*Pf*), 700 (*Tb*)	5, 51
FTI-2148 derivative 16	1,000 (*Pf*)	150 (*Pf*)	10
FTase Inhibitor I	270 (*Tg*)	ND	48
FTase Inhibitor II	0.97 (*Tg*)	ND	48
Limonene	ND	1,220 (*Tg*)	49
Benzophenone 6a	8 (*Pf*)	150 (*Pf*)	69

ND, not determined; *Pf*, *P. falciparum*; *Tb*, *T. brucei* bloodstream forms (strain BF427); *Tg*, *T. gondii*.

PFTIs

As a result of the interest in the development of PFTIs as a cancer chemotherapeutic agent, there are a large number of structurally diverse PFTIs reported in the primary and patent literature. Therefore, this resource can be used for the development of compounds for the treatment of pathogenic protozoa, a resource-poor area of drug development. Interestingly, even though almost all PFTIs were developed with the aim of inhibiting human PFT, compounds with similar ability to inhibit both the mammalian and parasite PFT display greater cytotoxicity against parasite cultures than mammalian cells (**Table 1**). Although the reason that PFTIs are more toxic to cancer cells and parasites than normal mammalian cells remains unclear, it is clear from inhibition studies that the selectivity is based on a biological difference and not on selective enzyme inhibition. Two potential explanations for the biological difference have emerged. One is that pathogenic protozoa may lack PGGT-I, which may act in a redundant manner when PFT is inhibited in mammalian cells. It has been shown in mammalian cells that when PFT is inhibited, proteins that are normally farnesylated can be geranylgeranylated by PGGT-I (52–56). Another explanation is that the farnesylation of proteins that are essential for parasite viability are blocked at lower concentrations of PFTI than the farnesylation of essential proteins in mammalian cells. Because very little is known about the identities of prenylated proteins in pathogenic protozoa, the mechanism of cytotoxicity to protozoa remains unclear.

Recently, a few prenylated proteins have been identified in protozoa. These include, for example, a small GTPase and a protein tyrosine phosphatase from *T. cruzi* (57, 58) and two small GTPases from *T. brucei* (59, 60). A bioinformatic approach, a search of potential prenylated proteins (those containing a cysteine at the −4 position from the C terminus) from *T. brucei*, yields 190 open reading frames of >50 amino acids. Some proteins from this search are strongly predicted to be prenylated based on data regarding the ability of *T. brucei* PFT to farnesylate various CaaX peptides in vitro (50), although not all permutations of CaaX have been tested in this manner. This bioinformatics approach is not as fruitful when applied to predicting potentially prenylated proteins in *P. falciparum*, in which inaccuracy in the identification of introns leads to inaccurate prediction of the C terminus. A new technique, the use of a farnesyl analog, anilinogeranyl alcohol, to specifically label farnesylated proteins, may provide a means to label and identify native farnesylated proteins (61–63).

The presence of a functional PGGT-I enzyme is still unclear in many of the parasites mentioned above. Only recently have the presence and biochemical activity of PGGT-I been demonstrated in *E. histolytica*, with clear demonstration of the incorporation of geranylgeranyl pyrophosphate into Ras-like proteins with a Leu in the terminal CaaX position (64). In *P. falciparum*, Chakrabarti and others (4–7) demonstrated possible PGGT-I activity from fractionated lysates. However, a BLAST search of the genome reveals three orthologs of protein prenyltransferase β-subunits, one of which is the PFT β-subunit and two show low but significant homology with both PGGT-I and II β-subunits. These two genes have not been cloned or biochemically characterized, so it is not possible to assign specific functions to them. In *T. cruzi*, a BLAST search has revealed a possible PGGT-I, which is currently under

investigation by our group. Both the *T. brucei* and *Leishmania major* genomes have been fully sequenced, and an ortholog for the PGGT-I β-subunit cannot be identified by BLAST search. In contrast, the *T. brucei* and *L. major* orthologs to the PFT and PGGT-II β-subunits are readily identifiable by BLAST searches.

FTI-276 and FTI-277 were the first mammalian PFT inhibitors shown to be effective against pathogenic protozoa, inhibiting *T. brucei* PFT and effective at inhibiting *T. brucei* growth in culture (51, 65). These compounds are peptidomimetics, with FTI-276 the active free acid form and FTI-277 the methyl ester prodrug form. It is reasoned that cellular enzymes convert the ester prodrug to the active free acid form in the cell, which is then able to bind to PFT with high affinity. However, this class of compounds has a shortcoming, in that in vivo, serum esterases rapidly metabolize the methyl ester prodrug into the free acid form, which is not sufficiently hydrophobic to enter parasites. Recent work using this prodrug approach produced FTI-2148 derivative 16 (Fig. 1), which contains a benzyl ester. This compound demonstrated significant anti-malarial activity [effective dose that inhibits 50% of parasite proliferation (ED_{50}) = 150 nM] and was able to suppress parasitemia by 46% (dosed at 50 mg/kg/day) in the *P. berghei* mouse model (10).

Chemists at Bristol-Myers Squibb developed the tetrahydrobenzodiazepine-based PFTI series, eventually evolving into the anticancer clinical candidate BMS-214662 (Fig. 1) (66). Our group tested BMS-214662 against *T. brucei* and *P. falciparum* PFT enzymes and found that it inhibited at low nanomolar concentrations and had inhibitory effects on parasite growth. A diverse collection of ~150 compounds related to BMS-214662 were screened against both *T. brucei* and *P. falciparum* PFTs to identify potent inhibitors of these enzymes. Compounds containing the tetrahydroquinoline (THQ) core possessed increased potency at inhibiting both *T. brucei* and *P. falciparum* PFTs, and these have become the focus of our efforts for drug development supported by the Medicines for Malaria Venture and the Drugs for Neglected Diseases Initiative. We have also tested a number of other series of PFTIs, including R115777 (Janssen/Johnson & Johnson) and SCH-66336 (Schering-Plough), which are in clinical development as anticancer agents, but none was as potent as the THQ series at blocking parasite growth (11).

We were able to identify a number of molecules with the THQ core that inhibited *P. falciparum* PFT at low nanomolar concentrations. One such compound, BMS-386914 (Fig. 1), displays an ED_{50} against *P. falciparum* 3D7 at 5 nM, which is approximately half the observed ED_{50} of chloroquine using chloroquine-sensitive strains of *P. falciparum* (11). In addition, BMS-386914 was shown to have an effect on both the morphological development of *P. falciparum* and the incorporation of radiolabeled farnesol into proteins (11). Twenty-one other THQs, including BMS-388891 and BMS-339941, display 3D7 ED_{50} values of <25 nM (Fig. 1). The ED_{50} values of five of our most potent THQs against four *P. falciparum* strains (W2, K1, HB3, and Dd2) were within 3-fold of the *P. falciparum* 3D7 ED_{50} values. These five strains represent a variety of drug resistance patterns and geographic distribution, demonstrating that cross-resistance of PFTIs and existing antimalarial agents probably does not occur and that strains from all over the world are susceptible to PFTI growth inhibition.

The pharmacodymanic properties of PFTIs for the treatment of infection by many of the organisms discussed here remain unknown. For *P. falciparum*, however, we have begun to determine these properties to develop an effective clinical treatment for malaria. To produce complete killing in vitro, *P. falciparum* requires exposure to 30-fold the ED_{50} of BMS-386914 for greater than 72 h (**Fig. 2**). This suggests that parasites are able to progress through one complete replication cycle, which is 48 h for *P. falciparum*, until the cytotoxic effects of the PFTI are irreversible upon removal of the drug. This is not unsurprising, because the proposed mechanism of action of PFTIs is the inhibition of protein modification of one or more essential proteins; thus, it is likely that protein turnover or replication may be required to decrease the abundance of previously farnesylated proteins to a cytotoxic level.

Although BMS-386914 is not well absorbed when administered orally, when administered by subcutaneous infusion pump in the mouse *P. berghei* malaria model, BMS-386914 was able to suppress parasitemia in 100% of the mice infected (n = 5) and to eliminate parasites in 60% of the mice (11). A new compound, based on the THQ structure, is able to suppress parasitemia in the *P. berghei* model when administered orally. We are currently working on improving the oral absorption and serum half-lives, both of which are necessary for effective malaria chemotherapeutic agents. Using a structural model of the *P. falciparum* PFT enzyme (based on the rat PFT-BMS-214662 and rat PFT-THQ crystal structures), we are modifying the substituents attached to the core THQ structure to improve the pharmacokinetic properties of the inhibitors while retaining or improving potency.

A major difficulty with current antimalarial agents is the emergence and spread of drug-resistant parasites. In an

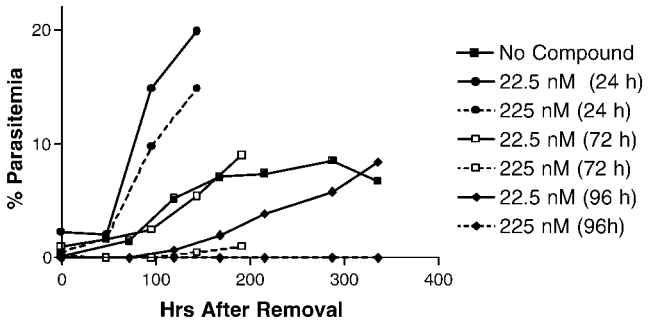

Fig. 2. Varying the length of exposure of PFTI demonstrates that 96 h of exposure kills *P. falciparum*. *P. falciparum* 3D7 in red blood cell cultures was exposed to 22.5 or 225 nM BMS-388891 for varying lengths of time before washing the parasites free of drug and reculturing to test whether the parasites are viable. A no-compound control was included.

effort to understand the potential for the generation of resistance to PFTIs, we have selected in vitro *P. falciparum* parasites resistant to a THQ PFTI (67). These parasites all possessed the same point mutation in the PFT β-subunit at amino acid position 837, which correlated with a 12-fold increase in the ED_{50} and a 13-fold increase in the inhibitor concentration that inhibits 50% of enzymatic activity of the native PFT enzyme. These data are consistent with PFT as the target of THQ PFTIs. Of note, it was found recently that BMS-214662 has some inhibitory activity against PGGT-II (68); the data described above, however, provide support for PFT being the major target for THQ PFTIs in *P. falciparum*. Importantly, we have been unable to select for parasites resistant to higher concentrations of THQ inhibitors, and it remains unclear whether parasites are able to acquire resistance to PFTIs in vivo.

In parallel with developing THQ inhibitors for the treatment of malaria, we are developing THQ-containing compounds for the treatment of African sleeping sickness. Our most potent inhibitor possesses an ED_{50} of 60 nM against *T. brucei* blood-stage parasites. Our current focus is to improve potency and pharmacokinetic properties. This, similar to our malaria project, is being guided by a structural model of the *T. brucei* PFT enzyme.

PFTIs based on a benzophenone scaffold are currently being developed for the treatment of malaria (12, 69). Earlier studies demonstrated an active compound class that possessed a nitrophenyl-furylacryloyl residue at the 5 amino group, benzophenone inhibitor 4 (Fig. 1), which was designed to use an aryl binding site of PFT. Although the compounds have no oral activity, and some have poor solubility in aqueous solution, it was shown that one had activity in the *P. vinckei* model of malaria in mice with an ED_{50} at 21 mg/kg when dosed intraperitoneally for 3 days (12). Recent work has been focused on increasing aqueous solubility, with the introduction of an α-amino group into the phenylacetic acid substructure (Fig. 1), which had the desired effect of increasing solubility (69). Indeed, this modification preserved activity against the PFT enzyme of *P. falciparum*; however, activity against parasite culture was detrimentally affected, suggesting a lack of cellular penetration.

CONCLUSIONS

The piggy-back approach to drug development for protozoan parasitic diseases is a logical way around the perennial problems associated with meeting the costs of de novo drug design. These methods are clearly starting to pay off in the case of the PFT inhibitors, with effective, novel pharmacophores being developed. The challenge is not the development of potent PFT inhibitors that are toxic to parasites but the development of compounds with suitable pharmacokinetic properties to be effective chemotherapy for the treatment of pathogenic protozoa.

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