The Plasmodium falciparum Translationally Controlled Tumor Protein Homolog and Its Reaction with the Antimalarial Drug Artemisinin*

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Artemisinin and its derivatives are important new antimalarial drugs. When Plasmodium falciparum-infected erythrocytes are incubated with [10-3H]dihydroartemisinin, several malaria-specific proteins become labeled. One of these proteins is the P. falciparum translationally controlled tumor protein (TCTP) homolog. In vitro, dihydroartemisinin reacts covalently with recombinant TCTP in the presence of hemin. The association between drug and protein increases with increasing drug concentration, plateauing at approximately 1 drug/TCTP molecule. By Scatchard analysis, there appear to be 2 hemin binding sites on TCTP with dissociation constants of ~18 μM. When the single cysteine moiety is blocked by pretreatment with iodoacetamide, hemin binding is not affected, whereas drug binding is reduced by two-thirds. Thus, TCTP reacts with artemisinin in situ and in vitro in the presence of hemin and appears to bind to hemin. The function of the malarial TCTP and the role of this reaction in the mechanism of action of artemisinin await elucidation.

Endoperoxide-containing antimalarials are becoming increasingly important. Because these drugs are structurally unrelated to the classical quinoline and antifolate antimalarials, there is little or no cross-resistance. Endoperoxide antimalarials such as artemether and artesunate (see Fig. 1) are the first-line agents when multidrug-resistant strains of Plasmodium falciparum appear, such as in Southeast Asia (reviewed in Ref. 1).

Artemisinin (qinghaosu) (Fig. 1), the first drug of this class, was originally identified as a component of a Chinese herbal remedy. Artemisinin derivatives have been used to treat malaria in over 2 million people in Asia. Like quinoline antimalarials, artemisinin appears to be selectively toxic to malaria parasites by interacting with heme, a byproduct of hemoglobin digestion, which is present in the parasite in high amounts. It has been proposed that intraparasitic heme activates artemisinin into a carbon-centered free radical, which then reacts both with heme and specific intraparasitic proteins (reviewed in Refs. 1 and 2).

The alkylation of intraparasitic proteins by artemisinin derivatives has been shown to occur at physiological drug concentrations to be selective for nonabundant proteins and to be dependent on the presence of the endoperoxide bridge. Several different artemisinin derivatives were found to alkylate the same proteins, yet they did not alkylate proteins in uninfected erythrocytes (3). Thus, alkylation of proteins is clearly associated with the antimalarial activity of artemisinin, although proof that this process mediates the antimalarial activity of the drug has not been obtained.

To gain a better understanding of the mode of action of artemisinin, we have been isolating and identifying the most heavily labeled proteins. In this paper, we identify one of these target proteins as a translationally controlled tumor protein (TCTP) homolog.

EXPERIMENTAL PROCEDURES

Parasites P. falciparum-infected erythrocytes were cultured by the method of Trager and Jensen (4). Strain FCR3 was used for all experiments except pulse-field gels, for which strain 3D7 was employed. Parasites were synchronized with sorbitol lysis (5). For autoradiograms, cultures were grown in the presence of 0.5 μCi/ml [10-3H]dihydroartemisinin (1.4 Ci/mmol; Moravek Biochemical, Brea, CA) for 3 h at 37 °C. Parasites were isolated and lysed as described (6). Extracts were stored at −80 °C.

Chemicals and General Methods

All chemicals were from Sigma unless otherwise noted. Protein was measured by the method of Bradford (7). Bio-Rad.

Isolation, Identification, and Sequencing of Labeled Protein

Parasite extracts were mixed with sample buffer (0.57 gm/ml urea, 2% Nonidet P-40, 10 mm CHAPS, 5% Bio-Lyte 3/10 ampholytes (Bio-Rad), 5% mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 0.1 mg/ml phenylmethyisulfonyl fluoride) at room temperature and vortexed. After the undissolved material was removed by a short spin in a microcentrifuge, and the supernatant was loaded onto a 2 × 150-mm isoelectric focusing tube gel (8) and electrophoresed at 400 V for 10 h followed by 1000 V for 2 h. The gel was stained with Coomassie Blue.

Thirty-two regions of the isoelectric focusing gel were excised. For unlabeled extracts, corresponding regions from 10 different gels were pooled. Protein was then eluted in 5% Triton X-100, 3% SDS, and 5% mercaptoethanol to which solid Trizma (Tris base) was added until the pH was approximately 8. The suspensions were heated at 55 °C for 3 h and then transferred to a Centricon 3 and subjected to 100 V for 5 h in a Centrulitor Microelectroeluter (Millipore-Amicon, Bedford, MA). The eluate was then concentrated by centrifugation at 8,000 × g for 2 h, with the abbreviations used are: TCTP, translationally controlled tumor protein; IA, iodoacetamide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CAPS, 3-cyclohexylamino)propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
dissolved in sample buffer (10% SDS, 10% mercaptoethanol, 10% glycerol in 0.5 M Tris buffer, pH 6.8), and subjected to 15% SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue. For autoradiography, gels were treated with En3Hance (NEN Life Science Products), dried, and exposed to x-ray film for 30 days at ~80 °C.

A single band with a molecular mass of 25 kDa was found on the autoradiogram for the SDS gel lane run on the eluate from region 7 of the isoelectric focusing gel. This corresponded to the darkest band present in this lane as judged by Coomassie Blue staining (Fig. 2). This band was then excised from a series of gels run on unlabeled parasite extracts and sent to the University of Michigan Protein and Carbohydrate Structure Facility. The gel pieces were washed 4 times with 300 µl of 50% CH₃CN, 200 mM NH₄HCO₃, pH 8.9. 500 µl of cyanogen bromide (10 mg/ml) in 70% formic acid was added and incubated overnight at room temperature. Gel pieces were extracted 4 times with 300 µl of CH₃CN, 0.1% trifluoroacetic acid. Supernatants were pooled, lyophilized, run on a 10–20% Tricine gel (Novex, San Diego CA), electroblotted onto polyvinylidene fluoride membranes (Millipore) using 10 mM CAPS, pH 11, 10% methanol and then Coomassie-stained. The bands were excised and sequenced on an Applied Biosystems 473A protein sequencer.

**Preparation of Polyclonal Antipeptide**

An oligopeptide was synthesized containing the N-terminal 20 amino acids and linked to polylysine as a multiple antigenic peptide at the University of Michigan Protein and Carbohydrate Structure Facility. This was used to immunize two New Zealand White rabbits (Hazleton University of Michigan Protein and Carbohydrate Structure Facility). The isoelectric focusing gel. This corresponded to the darkest band present in this lane as judged by Coomassie Blue staining (Fig. 2). This band was then excised from a series of gels run on unlabeled parasite extracts and sent to the University of Michigan Protein and Carbohydrate Structure Facility. The gel pieces were washed 4 times with 300 µl of 50% CH₃CN, 200 mM NH₄HCO₃, pH 8.9. 500 µl of cyanogen bromide (10 mg/ml) in 70% formic acid was added and incubated overnight at room temperature. Gel pieces were extracted 4 times with 300 µl of CH₃CN, 0.1% trifluoroacetic acid. Supernatants were pooled, lyophilized, run on a 10–20% Tricine gel (Novex, San Diego CA), electroblotted onto polyvinylidene fluoride membranes (Millipore) using 10 mM CAPS, pH 11, 10% methanol and then Coomassie-stained. The bands were excised and sequenced on an Applied Biosystems 473A protein sequencer.

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**Autoradiography**

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**Molecular Cloning and Sequencing**

Clone Pf0946M was obtained and sequenced from a library of mung bean nuclease-digested genomic DNA fragments as part of the malaria genome project (10). The portion of the clone encoding the TCTP homolog has been fully sequenced in both strands. The DNA sequence upstream of the initiator methionine codon was obtained by inverse polymerase chain reaction (11) and sequenced. The molecular mass isoelectric point were predicted using ProtParam.²

**Chromosomal Localization of the TCTP Homolog**

Pulse-field gel blocks were made following the protocol of Kemp et al. (12) and incubated in lysis buffer (0.5 mM EDTA, 0.01 mM Tris-HCl, pH 9.5, protease K (2 mg/ml)) for 48 h with one change of solution. Blocks were stored at 4 °C in 0.05 mM EDTA. *P. falciparum* chromosomes were resolved on a 1% chromosomal grade agarose (Bio-Rad) gel run in 0.5× Tris-buffered EDTA at 7 °C using a CHEF-DRII (contour-clamped homogeneous electric field) apparatus (Bio-Rad) run at 80 V, 180–900 s (ramped) for 165 h. Gels were stained with ethidium bromide and photographed. They were then blotted by alkaline transfer onto Hybond N+ (Amersham) nylon membrane following manufacturer’s guidelines. The TCTP probe was radiolabeled by random priming (13) and hybridized under standard conditions at 65 °C. Blots were washed at high stringency (65 °C, 0.2× SSC (1× SSC, 0.15 mM NaCl and 0.015 mM sodium citrate), 0.1% SDS) for 2 h with one change of solution before exposure to film.

**Expression and Purification of the TCTP**

The TCTP coding sequence was cloned into the BamHI site of pET3a containing a 12-amino acid T7 fusion tag (Novagen, Madison WI). *Escherichia coli* BL21(DE3)pLysS host cells transformed with the recombinant plasmid were induced with isopropyl-D-thiogalactopyranoside at 37 °C and grown with vigorous aeration for 3 h. Cell pellets were obtained by centrifugation, lysed by freeze-thawing, and resuspended in buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂) containing 50 nM RNase. Cellular debris and unlysed cells were removed by centrifugation for 30 min at 11,500 × g over a layer of 27% sucrose in the same buffer. The recombinant protein, which remained in the supernatant, was then diluted 10:1 with loading buffer (50 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride), applied onto a DEAE-cellulose column (0.75 × 7.5 cm), and eluted with a step gradient of this buffer containing 50 mM, then 100 mM, then 150 mM, and then 188 mM NaCl.

² http://expasy.hcuge.ch/sprot/protparam.html.
Reaction of Protein with Radiolabeled Dihydroartemisinin

Specificity of Reaction—Immediately before the experiment, hemin was dissolved in 0.1 N NaOH, neutralized with 0.1 N HCl to pH 8, and then diluted with water to a final stock concentration of 1 mM. Unlabeled artemisinin was prepared as a 0.5 mM stock in ethanol. For each experiment, a series of 100-μl incubation mixtures were then prepared containing various combinations of purified recombinant TCTP (10 μg), hemin, [3H]dihydroartemisinin (5 nCi), and/or cold artemisinin and incubated in the dark at 37 °C for 24 h.

RESULTS

Equilibrium Dialysis Studies of Hemin

Recombinant P. falciparum TCTP (125 μg/ml, 5 μM) was dissolved in Tris-HCl buffer (50 mM, pH 7.5). Aliquots of hemin, prepared as above, were added to a quartz microcuvette containing 400 μl of the TCTP solution as well as to another cuvette containing 400 μl of buffer. UV-visible scans were performed on a Hewlett Packard 8452A diode array spectrophotometer. The scans were saved and imported into Microsoft Excel, version 4.0 for Macintosh.

Time Course—10 μg of TCTP, bovine serum albumin, or bovine erythrocyte superoxide dismutase (Sigma) were dissolved in 100 μl of Tris-HCl buffer (50 mM, pH 7.5) containing 5000 cpm of [3H]dihydroartemisinin (5 nCi). In separate incubations, unlabeled hemin was added to obtain 1 μM hemin (previously prepared as in the previous section). The mixtures were incubated for 2, 8, or 24 h in the dark at 37 °C and then dialyzed for 24 h in Slide-A-Lyzer cassettes (10,000 molecular weight cut-off, Pierce) in Tris-HCl buffer to remove free drug. The retentate was then measured as above.

Spectroscopic Studies on the Ligand Binding Properties of Recombinant TCTP Homolog

Recombinant P. falciparum TCTP (125 μg/ml, 5 μM) was dissolved in Tris-HCl buffer (50 mM, pH 7.5). Aliquots of hemin, prepared as above, were added to a quartz microcuvette containing 400 μl of the TCTP solution as well as to another cuvette containing 400 μl of buffer. UV-visible scans were performed on a Hewlett Packard 8452A diode array spectrophotometer. The scans were saved and imported into Microsoft Excel, version 4.0 for Macintosh, where difference spectra were calculated. The binding of the buffer plus hemin scan from the TCTP plus hemin scan. To better understand the nature of the binding, a mixture of 5 μM TCTP and 25 μM hemin was incubated in Tris-HCl buffer (50 mM, pH 7.5) for 1 h at room temperature in the dark, after which the free hemin was removed by the addition of charcoal-coated dextran. The charcoal was separated by centrifugation, and the supernatant was measured before and after the addition of a few milligrams of sodium dithionite.

Identification of 25-kDa Protein That Binds to [3H]Dihydroartemisinin—Sequential isoelectric focusing and SDS gels were performed on both labeled and unlabeled P. falciparum homogenates. One band was found on the autoradiogram with a pl of ~5 and molecular mass 25 kDa (Fig. 1). The corresponding band was excised and eluted from the focusing gel, and the eluate was re-run on an SDS-PAGE gel to confirm that the labeled protein was indeed recombinant TCTP. The protein was separated from bound drug by centrifugation through Centricon-3 as described above. To assess the effect of IA on the binding between recombinant TCTP and hemin, IA was added to the incubation mixtures. The retentate was then analyzed as above.

Effect of Sulphydryl Group Modification on Ligand Binding

Recombinant TCTP (50 μg) was dissolved in 100 μl of 50 mM Tris-HCl, pH 7.5, in the presence of 30 mM iodoacetamide (IA) or 0.1 mM β-mercaptoethanol and incubated for 20 min at room temperature. Free IA and β-mercaptoethanol were eliminated by centrifuging through a Centricon-3 (with two washes) or by dialysis against 4 liters of 50 mM Tris-HCl buffer at 4 °C with two changes of buffer. To measure the effects of these treatments on drug binding, aliquots (10 μg) of recombinant TCTP were incubated with hemin (1 μM) and [3H]dihydroartemisinin (10,000 cpm) at 37 °C for 24 h in a total volume of 100 μl. After incubation, free drug was separated from bound drug using the Centricon-3 as described previously. The radioactivity in the retentate and filtrate was assayed as above.
demonstrated that a single copy of the gene was present on chromosome 4.

Cloning and Expression of TCTP—The TCTP was expressed with a 12-amino acid fusion peptide on pET3a. The protein was purified to >95% purity as judged by SDS-polyacrylamide gels (Fig. 4A). The recombinant protein has an apparent molecular mass of 25 kDa and a pI of 4.9 (Fig. 4B).

Stage Specificity of TCTP Expression—Polyclonal antibodies prepared against the N-terminal 20-amino acid peptide react with a single band at 25 kDa (Fig. 5), which is presumably the TCTP. A much stronger band is present in trophozoites than in rings. The higher molecular mass bands seen on this immunoblot were also seen when prebleed antisera was used instead of anti-TCTP (not shown).

Binding of Recombinant TCTP with Dihydroartemisinin—When [3H]dihydroartemisinin and TCTP were incubated in vitro in the presence of hemin, 2.1 ± 0.46% of the total radioactivity used (n = 4) remained in the retentate with the protein. In contrast, when drug + TCTP alone or drug + hemin alone were incubated, less than one-third as much radioactivity was retained (0 ± 0.23, n = 3, and 0.67 ± 0.34, n = 3, respectively). This difference is statistically significant (p < 0.01), indicating that a hemin-dependent reaction between drug and TCTP occurred. The binding between drug and TCTP is specific, because much less radioactivity was retained in the presence of an excess of cold artemisinin (0.61 ± 0.33, n = 3, p < 0.01). No reaction between TCTP and drug was seen when 1 μM Fe-EDTA was added instead of hemin.

A covalent bond formed between drug and protein in a time-dependent and specific manner. [3H]Dihydroartemisinin and hemin were incubated in the presence of TCTP, bovine serum albumin, superoxide dismutase, or no protein for 2, 8, and 24 h. Between 2.5 and 5 times as much radioactivity associated with TCTP than with the other proteins. After 24 h, retentates from the TCTP, albumin, superoxide dismutase, and no protein incubations contained 1.26, 0.48, 0.23, and 0.26% total radioactivity used, respectively. The drug reacted with the TCTP protein, not with hemin, since a radiolabeled protein band was observed by autoradiography (Fig. 4C).

Stoichiometry of Dihydroartemisinin Binding—The binding of dihydroartemisinin to TCTP was measured at various hemin concentrations (Fig. 6A). In the presence of TCTP (10 μg), the amount of radioactivity retained was proportional to the concentration of hemin for the range of concentrations tested (R² = 0.98). Radioactivity was also retained in the absence of TCTP, although the slope of this line was only one-third of that found in the presence of TCTP (R² = 0.92). This is probably due to the formation of hemin-dihydroartemisinin adducts and hemin aggregates.

To determine the number of dihydroartemisinin binding sites and whether the binding is saturable, dihydroartemisinin-TCTP binding was measured in the presence of varying concentrations of drug (Fig. 6B). The quantity of drug retained increased until the drug reached a concentration of 200 μM, at which point the quantity appears to plateau. Because 10 μg of TCTP are present in each incubation (~0.4 nmol), the magnitude of the plateau (0.4–0.5 nmol) suggests that a maximum of one drug molecule binds/molecule of protein.

**Figure 4.** Recombinant TCTP. A, 10% SDS-polyacrylamide gel (Nu-PAGE, Novex) of E. coli extract after sucrose gradient (lane 1) and after DEAE-cellulose chromatography (lane 2). B, isoelectric focusing gel of recombinant TCTP after a 24-h incubation in the presence of [3H]dihydroartemisinin and hemin.

**Figure 5.** Immunoblots of 10% polyacrylamide gels of ring-stage (lane 1) and trophozoite-stage (lane 2) parasites using polyclonal antibodies to N-terminal peptide.
When TCTP and heme were incubated for 1 h and then treated with charcoal-coated dextran, much of the heme spectra was retained (not shown). Interestingly, however, no peak was seen at 415 nm, suggesting that the binding in this case was occurring to \( \mu \)-oxo-dimers (17). When the latter was reduced, no change in spectra was seen, suggesting that the heme-TCTP complex is not a redox-active enzyme.

**DISCUSSION**

In this paper, we have purified, sequenced, cloned, and expressed the *P. falciparum* TCTP, which appears to be one of the principal malaria proteins that react with dihydroartemisinin when infected erythrocytes are incubated with radiolabeled drug (3). Evidence that dihydroartemisinin reacts specifically with recombinant TCTP includes 1) the slow reactions of dihydroartemisinin with control proteins, 2) the binding of dihydroartemisinin to TCTP increases with increasing drug concentration, plateauing at approximately 1 drug bound/protein molecule, and 3) the inhibition of protein binding by a thiol-blocking reagent. Both the *in vitro* and *in vivo* reactions between dihydroartemisinin and TCTP may partly be the result of the presence of bound heme. The recombinant TCTP was found to have two hemin binding sites/molecule with modest affinity (\( K_d = 18 \ \mu M \)).

The 25-kDa artemisinin target protein is unlikely to be anything other than TCTP, based on comparisons of gels and

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**FIG. 6. Hemin and Dihydroartemisinin concentration-dependence of drug-TCTP binding.** A, varying concentrations of heme were incubated with \( [3H] \) dihydroartemisinin (5 nCi) plus 10 \( \mu \)M cold dihydroartemisinin in the presence (■) or absence (▲) of TCTP (10 \( \mu \)g/100 \( \mu l \)). Ordinate shows nmols of total dihydroartemisinin retained in Centricon, based on measured radioactivity. B, varying concentrations of cold dihydroartemisinin were incubated with \( [3H] \) dihydroartemisinin (5 nCi) plus 50 \( \mu \)M heme in the presence (■) or absence (▲) of TCTP (10 \( \mu \)g/100 \( \mu l \)).
autoradiograms. Antisera prepared against synthetic peptide reacts with one band in parasite extracts at $M_r$ 25 kDa (Fig. 5). This is the same $M_r$ as the band seen on autoradiograms (Fig. 2) and as that determined for the recombinant TCTP (Fig. 4). 25 kDa is higher than the predicted $M_r$ for both native and recombinant TCTP (20 and 21 kDa, respectively), but the differences could be due to secondary structure. On isoelectric focusing gels, the antibody reacts with several bands, including one at pI 4.8 (not shown). The recombinant TCTP has a slightly higher pI (4.9, Fig. 4). This difference is probably due to contributions from the 12-amino acid tag, since the predicted pI for the recombinant TCTP is slightly higher than that predicted for native TCTP (4.63 versus 4.58). Autoradiograms of isoelectric focusing gels run on [$^3$H]dihydroartemisinin-treated parasites show a band at pI 4.85 (not shown), which is consistent with the possibility that the reaction of an amino acid residue with dihydroartemisinin leads to a slight shift in pI.

Two other observations confirm that TCTP is the 25-kDa protein that binds to dihydroartemisinin in vivo. First, because artesinin may alter the pI of the target protein, it is theoretically possible that the autoradiogram band was aligned to a protein band with a pI different from that of the target protein. However, no other discrete 25-kDa protein band was seen at either higher or lower pIs (Fig. 2). Second, because bands were excised from more than 100 gels, pooled, and submitted for three separate sequencing runs, one would have expected some ambiguities if two proteins were co-migrating. In contrast, unambiguous and consistent sequence patterns were obtained (data not shown). Third, as predicted, artesinin appears to react selectively with the recombinant protein in vitro.

The specific amino acid residue that is modified by the drug

| Pretreatment   | Ligand              | Percent control ± S.D. (No. experiments) |
|----------------|---------------------|------------------------------------------|
| $\beta$-Mercaptoethanol | dihydroartemisinin | 96 ± 5.8 (3)                           |
| Iodoacetamide  | dihydroartemisinin | 33 ± 11.2 (4)                           |
| Iodoacetamide  | hemin               | 92 ± 8.5 (3)                            |

**Fig. 7.** Scatchard plot of recombinant TCTP-hemin binding in 50 mM Tris-HCl buffer, pH 7.5. The ordinate (bound/free) is micromolar hemin bound/micromolar hemin free. The abscissa (bound) is the micromolar hemin bound. The data shown are from the equilibrium dialysis experiment using 50 mM Tris-HCl, pH 7.5, at 4 °C. The recombinant TCTP concentration was 10 µM and those of hemin were 2, 5, 15, 20, 30, 40, 60 µM.

**Fig. 8.** Difference spectra, TCTP minus buffer. Hemin at 5 µM (---), 15 µM (-- -- ), 25 µM (— — ), and 40 µM (----) was added either to a solution of TCTP (5 µM) in Tris-HCl buffer, pH 7.5, or to Tris-HCl buffer alone. For each hemin concentration, the spectra for the latter was then subtracted from the spectra for the former.
is still unknown. The observed reaction between artemisinin and recombinant TCTP does not occur on the T7 fusion peptide tag, because no reaction was seen when \(^{\text{[3]H}}\)dihydroartemisinin was incubated with hemin and recombinant plasmepsin I containing the same fusion tag (data not shown). On the other hand, the single cysteine appears to be necessary for the reaction between dihydroartemisinin and TCTP. It is possible that this cysteine is the actual amino acid modified by the drug. Alternatively, it might serve as a source of electrons for the activation of the drug into a free radical.

The results presented here offer new leads to understanding the mechanism of action of artemisinin and its derivatives. There is general agreement that free radical generation is involved in the mechanism of action of artemisinin, and that free radicals form as a result of an interaction with heme (reviewed in Refs. 1 and 2). What isn’t clear is how the resulting free radicals lead to parasite death. It has been suggested that artemisinin might kill parasites by the generation of activated oxygen, but there are many observations that are inconsistent with this mechanism (18). A second possibility is that formation of artemisinin-hemin adducts might lead to parasite death (19, 20). However, the preformed adduct is not toxic to parasites (21), and artemisinin treatment neither inhibits hemozoin formation nor causes its degradation (22). Finally, it has been suggested that artemisinin might kill parasites by inactivating specific malaria proteins (3).

Currently, there is no direct evidence demonstrating that alkylation of TCTP or any other malaria protein by the drug is the proximate cause of parasite killing. The observation reported here that trophozoite-stage parasites, which are most sensitive to artemisinin derivatives (23), produce higher levels of TCTP than ring stages is consistent with its role as a drug target but does not prove it. Even if TCTP is alkylated as an “innocent bystander,” the fact that this reaction occurs both in vitro and in vivo suggests that TCTP might somehow be associated with the true drug target.

It is difficult to understand how TCTP alkylation could lead to parasite death, because relatively little is known about the physiological roles of TCTPs in general. TCTPs in other organisms appear to be cytosolic proteins that bind calcium and microtubules (24–26). In this paper, we present the first evidence that they bind heme and react with an endoperoxide. Thus, the observations presented here suggest new approaches to understanding the functions of this class of proteins.

Recombinant TCTP binds to heme with modest affinity (18 \(\mu\text{M}\)). Thus, it has a binding affinity for hemin that is similar to that of human serum albumin (50 \(\mu\text{M}\) (17)). Concentrations of heme in the parasite food vacuole are extremely high; if TCTP were to enter the food vacuole, it is likely that both sites would become saturated. Thus, the observation of a modest heme binding could be physiologically relevant.

Interestingly, 26 of the amino acids present in the \(P.\ falciparum\) TCTP (15.2%) are phenylalanines and tyrosines. 10 of these residues in the \(P.\ falciparum\) TCTP are conserved in at least 5 of the 6 other species (Fig. 3). Thus, some of the protein-heme binding may be based on pi–pi interactions between aromatic amino acids and the pyrrole rings of the heme.

Although it is possible that TCTP is the artemisinin drug target, the observed reaction may also be unrelated to the killing effect of the drug. Nevertheless, the observed interactions between TCTP, hemin, and artemisinin might provide clues to the true target as well as insights into the physiological role of TCTP.

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