X-ray Structural Analysis of Plasmodium falciparum Enoyl Acyl Carrier Protein Reductase as a Pathway toward the Optimization of Triclosan Antimalarial Efficacy*

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The x-ray crystal structures of five triclosan analogs, in addition to that of the isoniazid-NAD adduct, are described in relation to their integral role in the design of potent inhibitors of the malarial enzyme Plasmodium falciparum enoyl acyl carrier protein reductase (PfENR). Many of the novel 5-substituted analogs exhibit low micromolar potency against in vitro cultures of drug-resistant and drug-sensitive strains of the P. falciparum parasite and inhibit purified PfENR enzyme with IC50 values of <200 nM. This study has significantly expanded the knowledge base with regard to the structure-activity relationship of triclosan while affording gains against cultured parasites and purified PfENR enzyme. In contrast to a recent report in the literature, these results demonstrate the ability to improve the in vitro potency of triclosan significantly by replacing the suboptimal 5-chloro group with larger hydrophobic moieties. The biochemical and x-ray crystallographic data thus demonstrate the flexibility of the active site and point to future rounds of optimization to improve compound potency against purified enzyme and intracellular Plasmodium parasites.

Malaria is a disease of worldwide significance, which is responsible for over one million deaths annually, mainly in children under the age of 5 years (1). Plasmodium falciparum infection is the most virulent form of malaria, accounting for the vast majority of deaths and >500 million infections yearly. The occurrence and spread of resistance to traditional drugs, such as chloroquine and sulfadoxine-pyrimethamine necessitate new, highly potent antimalarials that are orally bioavailable, affordable, safe, and unencumbered by existing resistance mechanisms (2).

Inhibition of type II fatty acid biosynthesis (FAS-II) appears to hold significant promise in devising novel antimalarials that meet these criteria (3–5). This dissociative FAS-II pathway, which occurs in the plastid-like apicoplast organelle (6), is composed of four separate enzymes. As such, it is fundamentally different from the associative FAS-I multifunctional polypeptide present in mammalian cells. Fatty acid synthesis by the parasite has been postulated to be crucial to membrane construction and energy production. The final reaction in the FAS-II pathway is catalyzed by the enoyl acyl carrier protein reductase enzyme (PfENR in P. falciparum, also known as PfFabI), which mediates the NADH-dependent reduction of trans-2-enoyl-ACP (acyl carrier protein) to acyl-ACP.

Triclosan (Fig. 1) is an uncompetitive inhibitor of purified PfENR (7, 8) and demonstrates potency against P. falciparum parasites cultured in vitro (7, 9, 10–12) as well as Plasmodium berghei in vivo infections in mice (7). A possible correlation among these biological activities is supported by the observation that triclosan produces a dose-dependent inhibition of [14C]acetate incorporation into fatty acids by cultured P. falciparum parasites and [14C]malonyl-CoA incorporation into parasite extracts freed from host red blood cells (7) and also inhibits a PfENR FAS-II in vitro reconstituted system (13). A requirement for fatty acids was demonstrated by the finding that P. falciparum in vitro growth was dependent on the presence of fatty acids in the medium, with a combination of C14, C16, and/or C18 fatty acids being sufficient to replace Albumax (14). Given that current antimalarial treatments do not feature a lipid synthesis inhibitor, we and others have explored triclosan analogs as potential therapeutics (10, 15–17). Of note, triclosan has displayed a satisfactory safety profile when administered either topically in humans or systemically in mice (7, 18). This is especially important in light of evidence suggesting that mammalian cells harbor a mitochondrial FAS-II system, producing a lipoic acid precursor, which could have raised concerns of host toxicity (19–22).

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S7.
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Key to our triclosan analog program has been the x-ray crystal structure of triclosan and its co-factor bound to PfENR (10). The phenol moiety has been clearly demonstrated to be crucial to triclosan binding to both the enzyme and co-factor. However, in vivo conjugation of the phenol is a metabolic liability (23). The crystal structure was utilized to suggest vectors off the triclosan diaryl ether scaffold where functionality could be appended to gain energetically favorable interactions with both the enzyme and co-factor. Sufficient increases in potency versus both PfENR and cultured parasites could facilitate eventual replacement or excision of the phenol. A campaign to explore SAR at the 4’-position resulted in minor gains in enzyme and anti-parasite potency, potentially due to enhanced hydrogen-bonding interactions with proximal active site residues (15). Examination of analogs at the 2’-position afforded very potent antiparasitic agents (EC₅₀ < 200 nM) that were only micromolar level inhibitors of PfENR activity (17).

Inspection of the PfENR-triclosan structure shows that the 5-chloro is in van der Waals contact with the side chains of Tyr-267, Pro-314, and Phe-368. We thus hypothesized that larger hydrophobic groups off the 5-position may better fill this enzyme pocket and confer greater potency against the purified enzyme and cultured parasites. More hydrophobic analogs of triclosan may also result in greater compound permeability to the four membranes surrounding the apicoplast (4, 24).

Two groups have recently published work describing 5-substituted triclosan analogs (16, 25). Sullivan et al. (25) reported 5-alkyl-substituted triclosan derivatives assayed against Mycobacterium tuberculosis enoyl acyl ACP reductase (InhA) that did not feature chlorine substitution on the B-ring. Chhibber et al. (16) described the synthesis and activity, versus both purified PfENR and cultured parasites, of triclosan analogs with primarily hydrophilic 5-substituents. These compounds were significantly less potent than triclosan in both assay systems. This led the investigators to propose that chlorine is the ideal 5-moiety given its small size. Our studies, described below, do not support this finding and lead us to identify alternative 5-substituted analogs with enhanced potency.

**EXPERIMENTAL PROCEDURES**

Synthesis of 5-Substituted Triclosan Analogs—The compounds depicted in Table 1 were prepared via a route reliant on the coupling of A-ring and B-ring precursors through a nucleophilic aromatic substitution reaction, following protocols similar to those published previously (15, 17). In brief, A-ring phenol precursors were either purchased or prepared via the Suzuki-Miyaura reaction of 4-bromo-2-methoxyphenol with an arylboronic acid (26). Alternatively, the diaryl ether scaffold was constructed by the reaction of a 4-substituted-2-methoxyaryl fluoride with 2,4-dichlorophenol.

With the exception of 2, benzaldehyde A was a key intermediate on the way to the 5-substituted alkyl derivatives shown in Table 2 (Scheme 1). Reaction of the aldehyde with an excess of the appropriate Grignard reagent afforded a benzyl alcohol that underwent “ionic hydrogenation” by stirring with triethylsilane in the presence of trifluoroacetic acid (27). Deprotection of the methyl ether afforded 5-alkyl derivatives. This Grignard method was also harnessed in the synthesis of derivatives 20–22 in Table 3, whereas use of the appropriate lithiated pyridines (28) afforded 26–28. Synthesis of the aryl and heteroaryl analogs of 3 shown in Table 3 relied on the transformation of 4-bromo-2-methoxyphenol or 5-bromotriclosan via variants of the Suzuki-Miyaura reaction (26, 29, 30).

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**Activation of Isoniazid (INH) and Synthesis of INH-NAD—**Purified InhA (2 μM) was incubated with INH (500 μM), NAD⁺ (500 μM), and Mn(III) pyrophosphate (500 μM) in 2 ml of sodium phosphate buffer for 1 h. After InhA inhibition, the enzyme solution was concentrated to 0.3 mM and purified by a Superdex™ 200 column preequilibrated with 50 mM phos-
X-ray Structural Analysis of PfENR

TABLE 3
Inhibitory properties of selected 5-aryl and heteroaryl derivatives

| Compound | R<sub>1</sub> | R<sub>2</sub> | EC<sub>50</sub> | IC<sub>50</sub> |
|----------|-------------|-------------|-------------|-------------|
| 3        | Ph          | Cl          | 23.2 ± 0.3 | 1.1 ± 0.5  |
| 15       | o-ch<sub>2</sub>Ph | Cl          | 1.6 ± 0.3 | 0.5 ± 0.6  |
| 16       | o-ch<sub>2</sub>Ph | CN          | 2.6 ± 0.3 | 0.7 ± 0.3  |
| 17       | m-ch<sub>2</sub>Ph | CN          | 3.5 ± 0.3 | 0.6 ± 0.5  |
| 18       | o-ch<sub>3</sub>Ph | CN          | 2.9 ± 0.6 | 1.4 ± 0.3  |
| 19       | p-F-Pb      | Cl          | 2.1 ± 0.5 | 0.3 ± 0.3  |
| 20       | ChCl        | Cl          | 11.1 ± 2  | 9.2 ± 1    |
| 21       | Ch<sub>2</sub>Cl | Cl          | 5.6 ± 0.2 | 9.1 ± 1    |
| 22       | Ch<sub>2</sub>Cl | CN          | 6.4 ± 0.1 | 0.3 ± 0.1  |
| 23       | pyridyl     | Cl          | 8.1 ± 1  | 8.0 ± 0    |
| 24       | pyridyl     | Cl          | 23.4 ± 0.9| 0.1 ± 0.0  |
| 25       | pyridyl     | Cl          | 11.4 ± 1.2| 0.2 ± 0.9  |
| 26       | CH<sub>2</sub>pyridyl | Cl       | 7.3 ± 0.6 | 0.3 ± 0.1  |
| 27       | CH<sub>2</sub>pyridyl | CN       | 6.9 ± 0.3 | 0.3 ± 0.1  |
| 28       | CH<sub>2</sub>pyridyl | CN       | 7.4 ± 0.3 | 0.3 ± 0.2  |

<sup>a</sup> Values are reported as mean ± S.E.

<sup>β</sup> 1

Phosphate buffer (pH 7.5). InhA in complex with inhibitor was then concentrated and heated for 40 s at 100 °C. After the heat treatment, the adduct was separated from denatured enzyme by filtration (Amicon cut-off size 30 kDa). The concentration of the inhibitor was determined by its absorbances at 260 and 326 nm, according to the procedure described by Nguyen (31).

**PfENR Enzymatic Inhibition Assay**—PfENR inhibition assays were carried out on a Cary 100 Bio Spectrophotometer or POLARstar Optima at 25 °C by monitoring oxidation of NADH at 340 nm as described previously (10, 15). Briefly, reactions were initiated by adding crotonoyl-CoA (300 mM) to assay mixtures containing buffer (150 mM NaCl and 20 mM Tris at pH 7.9), PfENR (50 nM), NADH (400 μM), and inhibitor at concentrations listed below. This mixture was added to a place containing 300 μM crotonoyl-CoA. The concentration of the well solution consisting of 2.5 M (NH₄)₂SO₄, 100 mM NaOAc, and 200 mM Tris, pH 7.7, and equilibrated at 17 °C to afford the binary complex. The triclosan analog was dissolved in acetonitrile and directly added to this crystallization drop containing crystals of binary complex with a space group P4<sub>2</sub>2<sub>1</sub>2. Data were collected after 10 days of soaking at 120 K using a Raxis IV++

Coefficient. These plots are shown in the supplemental data. Inhibitor concentrations were as follows: [2] = 200 nM, [4] = 94 nM, [15] = 440 nM, [19] = 54 nM, [20] = 78 nM, [21] = 87 nM, [22] = 660 nM, [23] = 1.5 μM, [26] = 640 nM, and [27] = 530 nM.

**Crystallization Details for PfENR-INH-NAD**—Purified PfENR (2 μM) was incubated with INH (500 μM), NAD<sup>+</sup> (500 μM), and Mn(III) pyrophosphate (500 μM) in 2 ml of sodium phosphate buffer for 1 h. After PfENR inhibition, the enzyme solution was concentrated to 0.3 mM and used for crystallization. PfENR-INH-NAD was crystallized in hanging drop containing 2 μl of protein solution at 10 ml/mg and 2 μl of buffer (2.4 M (NH₄)₂SO₄, 0.1 M MES, pH 5.6) at 16 °C in Linbro plates against 1 ml of the same buffer. Protein crystals formed after ~4 days with a space group of P4<sub>2</sub>2<sub>1</sub>2. Data were collected at 121 K using paratone as cryoprotectant. Crystals of PfENR-INH-NAD diffracted X-rays to 2.5 Å with a Bruker Proteum CCD Detector system (Proteum 6000) coupled with a Rigaku generator (MM007 HF). The data were integrated and reduced using Proteum software. Crystals produced from PfENR in complex with INH-NAD were isomorphous to those of the native enzyme. Initial phases were obtained by molecular replacement using the apo-PfENR structure (Protein Data Bank 1VRW) and refined with CNS (32). F<sub>o</sub> − F<sub>c</sub> and 2F<sub>o</sub> − F<sub>c</sub> electron density maps were calculated, and additional density resembling the INH-NAD was found. The ligand was incorporated into the additional density, and the whole model was rebuilt using XtalView (33). After the final cycles of the refinement, water molecules were added into peaks above 3-σ of the F<sub>o</sub> − F<sub>c</sub> electron density maps that were within hydrogen-bonding distances from the appropriate protein atoms. The final model was refined to an R<sub>free</sub> of 21% and an R<sub>free</sub> of 27%. At the end of the refinement and model building, a final simulated annealing was conducted to eliminate overrefinement, for this and all other models reported in this study.

**Crystallization Details for 15**—Since crystals of the PfENR-NAD<sup>+</sup>15 complex were isomorphous to those of the native enzyme, the protein coordinates of the PfENR-NADH complex (Protein Data Bank code 1VRW) were used to calculate 2F<sub>o</sub> − F<sub>c</sub> and F<sub>o</sub> − F<sub>c</sub> maps. The F<sub>o</sub> − F<sub>c</sub> map calculated at 2.5 Å resolution identified additional density consistent with the structure of 15. The inhibitor was built into the additional density with XtalView (33), and subsequent refinement with REFMAC (34) gave rise to an R<sub>work</sub> of 20% and an R<sub>free</sub> of 26% after placement of ordered water molecules.

**Crystallization Details for 21–23 and 26**—The crystallization conditions utilized the hanging drop and vapor diffusion methods. Typically, 24 mg/ml PfENR was incubated with 4 mM NADH in 20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, and 1 mM dithiothreitol. 2 μl of this mixture was mixed with 2 μl of the well solution consisting of 2.5 M (NH₄)₂SO₄, 100 mM NaOAc, and 200 mM Tris, pH 7.7, and equilibrated at 17 °C to afford the binary complex. The triclosan analog was dissolved in acetonitrile and directly added to this crystallization drop containing crystals of binary complex with a space group P4<sub>2</sub>2<sub>1</sub>2 to a final concentration of 2.1 mM. Diffraction data were collected after 10 days of soaking at 120 K using a Raxis IV++

25438 JOURNAL OF BIOLOGICAL CHEMISTRY

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Expression of katG in P. falciparum—This gene was expressed using the Bxb1 integrase system, recently developed to enable rapid, site-specific transgene integration in P. falciparum (41). Here, the transgene expression plasmid pLN-ENR-V5, which is identical to pLN-ENR-GFP (41) except that the GFP sequence was replaced by a V5 epitope tag, was adapted to drive katG expression from the ring stage hrp3 promoter. This 1.9-kb promoter was amplified using the primers 5′-taaGCATGCCCGAGTCACACGTCTGGTTAAAGCG (SpfI site underlined) and 5′-taaCCTAGGTTTTTTTTAATAACTGTATTATATAAAA (AvrII site underlined), cloned into pGEM-T (Promega), and then subcloned as an SpfI-AvrII fragment into pLN-ENR-V5 digested with SpfI and AvrII to remove the calmodulin promoter. katG was amplified from M. tuberculosis genomic DNA using the primers 5′-taaCCTAGGATGTCCGAGCTAAACACTG-3′ and 5′-taaGGTATGCTCGGACAACCTCACCC (AvrII site underlined) and 5′-taaGGTGTCACACGTCTGGTTAAAGCG (SpfI site underlined) and cloned into pGEM-T. An error-free insert was identified following double-stranded sequencing and subcloned as a 2.2-kb AvrII-BsiWI fragment into the hrp3 construct digested with AvrII and BsiWI to remove the pfenr gene without disrupting the V5 tag. The resulting 9.5-kb vector, denoted pLN-hrp3-katG-V5, was electroporated into Dd2_attB parasites along with the pINT plasmid that expresses Bxb1 integrase. Integrase-mediated insertion of pLN-hrp3-katG-V5 into the chromosomal attB site was demonstrated by PCR as described (41). Western blot analysis with antibodies to the V5 epitope tag was performed with parasite extracts prepared from the katG-expressing line or the parental control Dd2_attB line, as described (41).

RESULTS AND DISCUSSION

Our design efforts at the triclosan 5-position were initially aided by concurrent investigations in our laboratories to study the inhibition of PfENR by INH (Fig. 1). INH, a front-line antitubercular drug used since 1952 (42, 43), inhibits InhA via the formation of a covalent adduct between activated INH and NADH (44–47). Consistent with the 30% sequence identity between InhA and PfENR (10), the INH-NAD adduct was determined to inhibit the PfENR enzyme with an IC_{50} of 320 ± 40 mM. INH was reported to have modest activity against P. falciparum parasites cultured in vitro (48) and, in our hands, exhibited EC_{50} values of 1.4 ± 0.2 and 1.9 ± 0.1 mM versus the P. falciparum 3D7 and Dd2 strains, respectively.

Given the requirement for activation of the INH prodrug in M. tuberculosis by the catalase peroxidase KatG (49), we tested whether expression of katG in P. falciparum would result in activation of the INH prodrug and an increase in potency against the cultured malarial parasites. Using site-specific integration techniques (41), recombinant parasites were obtained in which M. tuberculosis katG was integrated into the genome, as confirmed by PCR and Southern blot analyses. Expression of katG was also confirmed by Western blot using a monoclonal antibody to a V5 epitope tag that had been appended to the C-terminal end of KatG. This study was designed to express KatG in the relatively voluminous parasite cytoplasm, where it could activate INH prior to the drug attaining the apicoplast, and did not include a parallel experiment to direct KatG to the apicoplast using an apicoplast targeting sequence (3). Drug assays with INH yielded IC_{50} values of 1.8–2.0 mM for Dd2 parasites expressing katG as well as for the control lines Dd2 and Dd2_attB (41). A similar result was obtained for lines created with the 3D7 strain, with IC_{50} values of 1.2–1.7 mM for the katG-expressing and control lines. The very weak antiparasitic activity of INH is inconsistent with its significant inhibition of purified PfENR and may be due to a lack of proper activation of INH within the malarial parasite or possibly because INH activity against cultured parasites is not caused by inhibition of PfENR.

Despite the lack of whole-cell activity, we were able to generate the INH-NAD adduct bound to PfENR. PfENR was crystallized in the presence of NAD^+ and, in INH was activated through oxidation by manganese(III) pyrophosphate (50). The overall structure (Fig. 2A), including the nucleotide binding site and the proposed fatty acyl substrate binding pocket of PfENR-INH-NAD, is similar to that observed in the structure of PfENR-NAD (10). The INH-NAD binding region includes the same nucleotide binding site observed in PfENR-NAD and an extended small pocket housing the isonicotinoyl moiety. This small pocket, partially overlapping with the purported fatty acyl substrate-binding site, is lined predominantly by side chains of hydrophobic residues Tyr-267, Tyr-277, Gly-313, Pro-314, Phe-368, Ile-369, Ala-372, and Ile-373. This pocket also serves as a small portal to the solvent at one side of the active site,
where the isonicotinoyl group of the adduct points toward the entrance. Comparison of the structures of PfENR-NADH and PfENR-INH-NAD reveals that the side chain of Tyr-267 has rotated \( \sim 15^\circ \) to form an aromatic ring-stacking interaction with the pyridine ring of the isonicotinoyl group. The isonicotinoyl nitrogen, oxygen of the \( \text{Tyr}-267 \) hydroxyl group, and an ordered water molecule interact in a triangle hydrogen-bonding network. A similar conformational change is observed with \( \text{Tyr}-277 \), whose side chain has also twisted \( \sim 15^\circ \) to form a hydrogen bond with a highly conserved water molecule located 2.8 Å away. This water molecule is in the center of a triangle hydrogen-bonding network formed by the isonicotinoyl carbonyl oxygen, \( 2' \)-hydroxyl oxygen of the co-factor ribose, and oxygen of the \( \text{Tyr}-277 \) hydroxyl group.

Superimposition of the PfENR-INH-NAD and PfENR-triclosan structures revealed that this ordered water molecule is in the same location as the hydroxyl group of triclosan. Although the structure of PfENR bound with its substrate has yet to be defined, it is very likely that this water occupies the binding site of the carbonyl group of the fatty acyl substrate, based on analysis of the complex structure of InhA bound with fatty acyl substrate (51). Although the isonicotinoyl group and the A-ring of triclosan both fit inside the hydrophobic pocket, the former extends \( \sim 3 \) Å deeper into the pocket along a vector most easily reached from the 5-position of triclosan. The introduction of a bulky 5-substituent could block the binding of the fatty acyl substrate, fill the enzyme hydrophobic pocket, and displace the bound water molecule. These observations support our hypothesis that hydrophobic 5-position substituents may be highly desirable.

To test our 5-position hypothesis further, a range of differentially substituted 5-position analogs was synthesized and examined for activity versus PfENR and cultured parasites (Table 1). All final compounds (except for 23) were tested on at least three separate occasions to determine their inhibition of purified PfENR enzymatic activity and parasite whole-cell growth via a \([3H]\)hypoxanthine uptake assay (39, 40).

A clear preference for hydrophobic groups was established at the outset. Methyl 2 is slightly less potent than triclosan against purified enzyme, whereas it is \( \sim 4 \)-fold less potent versus the cultured parasites. Phenyl analog 3, although marginally less active against the enzyme, is essentially equipotent to triclosan in the parasite assay. In contrast, analogs with polar functionality, such as tetrazole (5), carboxamide (6), and carboxylic acid (7), are much less efficacious in both assay systems than tri-

![FIGURE 2. X-ray crystal structures of ligands bound to PfENR. A, INH-NAD (stick drawing) bound to PfENR (ribbon and tube). The INH isonicotinamide pyridyl nitrogen and the Tyr-267 side chain phenol are hydrogen-bonded to a water molecule (WAT30). The side chain phenol of Tyr-277, the carbonyl of the INH isonicotinamide moiety, and NADH ribose hydroxyl are all hydrogen-bonding to a water molecule (WAT64). Distances shown are in angstroms. B, 15 (stick drawing; gold coloring) bound to PfENR (ribbon and tube) in the presence of NAD (space fill), with an overlay of INH-NAD (stick drawing) bound to PfENR. The enzyme active site accommodates the 5-substituent most evident by rotation of Phe-368 and movement of Tyr-267. C, 23 (stick drawing) bound to PfENR (ribbon and tube) and NAD (stick drawing), with an overlay of 15 (stick drawing; turquoise blue coloring) bound to PfENR. Phe-368 is colored according to the ligand bound to PfENR-NAD (atom color 23 bound) or turquoise blue (15 bound). The 2-pyridyl moiety of 23 is engaged in hydrophobic interactions with Pro-314, Phe-368, Ile-369, and Ala-372 and a face-to-face \( \pi \)-stacking interaction with Tyr-267. For all PfENR structures in Figs. 2 and 3, key residues are noted in stick format, and parts of the structure are removed for clarity. Both figures were made using the program SwissPDB viewer (63).](image-url)
closan. Carboxylic acid 7 was prepared and tested by Chhibber et al. (16) and demonstrated a similar lack of antiparasitic activity. Intriguingly, in our hands, 7 is inactive against PfENR, having an IC_{50} of >100 μM (n = 4 independent trials) compared with their reported value of 560 nM. The potent enzyme inhibition of 4 is consistent with our design hypothesis, since the 5-nitrile moiety should extend deeper into the proximal binding pocket than the triclosan 5-chloro group, with molecular modeling suggesting the potential for an energetically favorable hydrogen-bonding interaction between the nitrile nitrogen and the hydroxyl group of Tyr-267. In addition, 4 was estimated to be only slightly less hydrophobic (< 1 log unit) than triclosan, based on AlogP and MlogP values (calculated using Accord for Excel, Version 6.1, Accelrys Software Inc.; data not shown). The reduced hydrophobicity may explain why the antiparasitic activity of 4 is diminished compared with that of triclosan. For our compound series (Tables 1–3), a modest correlation (r^2 = 0.55) was observed between antiparasitic activity and the calculated hydrophobicity of triclosan and its 5-substituted analogs, as predicted by MlogP and AlogP values.

Overall, the results in Table 1 are consistent with our observations concerning the 5-position binding pocket derived from the x-ray crystal structure of triclosan bound to the PfENR-NAD^+ complex. Analogs 2 and 3 represent reasonable origins from which to test our hypothesis that suitable hydrophobic groups should better fill the 5-position pocket than the triclosan 5-chloro group.

While attempts were undertaken to determine the x-ray structures of bound 2 and/or 3 to aid design efforts, we prepared a number of alkyl-substituted triclosan derivatives (Table 2) to expand the SAR at the 5-position. Compounds 8–14 all achieve an improvement in antiparasitic activity over methyl 2. It is interesting to note that the introduction of a cyclic constraint (11/12 → 14) does not consistently improve potency in the parasite and enzyme assays. Although 8 and 13, in addition, improve on the enzymatic potency of methyl 2, they are slightly less efficacious than triclosan. These 5-alkyl derivatives do not demonstrate a correlation between enzyme inhibition and antiparasitic efficacy. This may be due to a number of factors, including differences in their pharmacokinetic profiles and potential off-target activities.

The initial examination of analogs of 5-phenyl 3 began with the tolyl compounds 15–18 (Table 3). The o-tolyl analog 15 is the most potent 5-substituted analog versus cultured parasites prepared to date, being 2-fold more active than triclosan. In contrast, the placement of a methyl group on the phenyl ring of 3 has a detrimental effect on enzyme activity. This structural perturbation affects parasite growth inhibition in a manner dependent on the methyl group location. It is interesting to note that p-F-phenyl derivative 19 is the most potent triclosan analog we have prepared to date versus purified enzyme, being 2-fold more active than triclosan. 19, also slightly more active than triclosan against both strains of parasite, is proposed through molecular modeling studies to attain its enhanced binding affinity to PfENR via a hydrogen bond between the fluoro group and the proximal hydroxyl moiety of Tyr-267.

Comparison of the structure of 15 (Fig. 2b) with that of bound triclosan clearly shows that the 5-o-tolyl moiety better fills the enzyme hydrophobic pocket into which the triclosan 5-chloro group projects. This results in an increase in surface area of interaction of ~50 Å^2 between 15 and the enzyme active site, compared with the binding of triclosan. The o-tolyl group has enhanced van der Waals interactions with Pro-314 and Phe-368. Phe-368 has rotated ~60° from its conformation in the triclosan structure to engage the o-tolyl moiety in an edge-to-face interaction. Tyr-267 is now participating in a face-to-face interaction (d-centroid-centroid = 3.7 Å) with the o-tolyl group and has been displaced from C6 of the triclosan A-ring by ~0.5 Å. Due to its o-tolyl moiety, 15 is also engaged in van der Waals interactions with Ile-323 of the putative substrate-binding loop and Ala-372. The chloro group of triclosan is incapable of making these interactions. Comparison of the x-ray structure of bound 15 with that of PfENR-NAD^+ (Fig. 1A) is aided by an overlay of the two structures. The two ligands clearly both occupy the hydrophobic binding pocket while extending into the proposed substrate-binding pocket.

Despite the presence of these novel and presumably energetically favorable contacts between 15 and PfENR, we note that 15 is six times less potent than triclosan against purified enzyme. The primary interaction of triclosan with PfENR is that of the phenol with the 2’-hydroxyl of the ribose unit and the hydroxyl of Tyr-277 (10). The metrics describing these interactions, and those pertinent to the face stacking of the cofactor nicotinamide ring with the A-ring of the diaryl ether scaffold, are essentially the same for 15 and triclosan. It is plausible that the binding of 15 to the PfENR-NAD^+ complex results in unfavorable interactions not observed in the analysis of the x-ray structure.

In an attempt to better mimic the INH pyridyl moiety, analogs 23–25 were prepared and assayed. It is interesting to note that replacement of the o-tolyl group in 15 with a 2-, 3-, or 4-pyridyl increases neither enzyme binding affinity nor antiparasitic activity. These compounds are also significantly less active than INH versus PfENR. Noticeably, 3-pyridyl analog 24 is the least active of the trio.

The complex structure of PfENR-NAD^+·23 (Fig. 2C) readily aligns with that of the corresponding o-tolyl analog 15. It should be noted that the 4’-substituent (Cl versus CN) does not significantly perturb the overall structures. The A-ring and B-ring atoms of the triclosan scaffolds nearly overlay, whereas the conformations of the 5-substituents are clearly different. The 2-pyridyl moiety of 23 has flipped 40° from the position of the o-tolyl group of 15. Correspondingly, Phe-368 returns close to its position in the structure with bound triclosan by rotating 45° from its position in the o-tolyl structure, obviating the possibility of an edge-to-face interaction with the 5-substituent. The 2-pyridyl group still maintains van der Waals contact with Phe-368 in addition to Pro-314, Ile-369, and Ala-372. Similar to the structure of bound 15, Tyr-267 has moved 0.4 Å away from C6 of the triclosan A-ring to facilitate a face-to-face interaction with the 5-(2-pyridyl) moiety.

A combination of two prior strategies was then explored: use of an alkyl linker in conjunction with an aryl or pyridyl group. It was hoped that the PfENR active site would exhibit further flexibility and that enhanced interactions with the 5-substituent would confer considerable improvements in compound
X-ray Structural Analysis of PfENR

FIGURE 3. X-ray crystal structures of ligands bound to PfENR. A, 26 (stick drawing) bound to PfENR (ribbon and tube) and NAD⁺ (stick drawing), with an overlay of triclosan (stick drawing; gold coloring) bound to PfENR. Phe-368 is colored according to the ligand bound to PfENR-NAD⁺ atom color (26 bound) or gold (triclosan bound). The 2-pyridyl moiety of 26 is engaged in hydrophobic interactions with Pro-314, Ile-323, Phe-368, Ile-369, and Ala-372 and a hydrogen bond (d\textsubscript{N-O} = 2.6 Å) with the hydroxyl moiety of Tyr-267. B, 21 (stick drawing) bound to PfENR (ribbon and tube) and NAD⁺ (stick drawing), with an overlay of triclosan (stick drawing; gold coloring) bound to PfENR. Phe-368 is colored according to the ligand bound to PfENR-NAD⁺ atom color (26 bound) and gold (triclosan bound). The 5-pyridylmethyl moiety interacts via van der Waals contacts with Val-274, Tyr-277, Pro-314, Ala-320, Ile-323, Phe-368, Ile-369, and Ala-372. C, 22 (stick drawing; gold coloring) bound to PfENR (ribbon and tube) and NAD⁺ (stick drawing; magenta coloring), with an overlay of triclosan (stick drawing) bound to PfENR. Phe-368 is colored according to the ligand bound to PfENR-NAD⁺ atom color (triclosan bound) or gold (22 bound). The 5-phenylpropyl moiety interacts via van der Waals contacts with Val-274, Gly-276, Tyr-277, Pro-314, Ala-320, Ile-323, Phe-368, Ile-369, and Ala-372.

potency. Examination of analogs of the type (CH\textsubscript{2})\textsubscript{n}Ph (n = 1–3) demonstrated that benzyl 20 and phenethyl 21 are essentially equipotent to triclosan against PfENR. This increase in enzyme inhibition upon one carbon homologation is also observed in the pyridyl series. Interestingly, further homologation of 21 to 22 affords a much weaker PfENR inhibitor while not significantly altering activity against cultured parasites.

Analysis of the x-ray crystal structure of 26 (Fig. 3A) demonstrates how the enzyme active site similarly accommodates a group at the 5-position larger than chloride. Phe-368 has moved in deference to the 2-pyridylmethyl group, interacting instead with the 5-substituent aromatic ring in an edge-to-face mode. By virtue of its formal one-carbon homologation from 23, 26 is able to utilize the pyridyl group nitrogen to form a hydrogen bond with the hydroxyl group of Tyr-267 instead of participating in a π-stacking interaction. The 5-pyridylmethyl group additionally participates in hydrophobic interactions with Pro-314, and the purported substrate binding loop residue Ile-323, Ile-369, and Ala-372.

The bound structure of 21 (Fig. 3B) is instructive in demonstrating the ability of the active site to house the even larger 5-phenethyl moiety. The A- and B-ring skeletons of 21 and triclosan overlap well, whereas the 5-phenethyl moiety extends deep into the proximal hydrophobic pocket lined by Val-274, Tyr-277, Pro-314, Ala-320, Ile-323 of the proposed substrate binding loop, Phe-368, Ile-369, and Ala-372. Phe-368 rotates more than 90° from its position in the respective triclosan structure such that only its side chain C\textsubscript{α} is interacting with the 5-position pendant phenyl group.

One-carbon homologation of the alkyl tether in 21 affords phenylpropyl 22, where the x-ray structure (Fig. 3C) demonstrates further flexibility in the PfENR active site. The structure is quite similar to that of bound 21, with the main deviation occurring due to extension of the phenylpropyl side chain further into the hydrophobic pocket. The result is an additional displacement of Phe-368 by 0.5 Å to accommodate the larger 5-substituent and an interaction with Gly-276.

Overall, these x-ray structures allow a detailed examination of the structural effects of increasing the size of the triclosan 5-substituent from that of a chloride to an aryl/pyridyl group spaced between zero and three carbons from the A-ring. This comparison illustrates that the most obvious way the enzyme accommodates the larger 5-substituent is by movement of the Phe-368 side chain. The effect on Phe-368 becomes more pro-
nounced as the size of the 5-substituent is increased. We do not, however, observe significant changes in the key metrics describing the \( \pi \)-stacking of the A-ring with the nicotinamide ring and the hydrogen bonding of the phenol with the ribose 2'-hydroxyl and Tyr-277 hydroxyl.

Finally, having noted the structural perturbation of the 5-substituent on the PfENR active site, we wished to determine if a correlation exists between the nature of this moiety and its compound's binding kinetics. The time course of the PfENR reduction of crotonoyl-CoA was followed spectrophotometrically in the presence and absence of inhibitor (see “Experimental Procedures” and supplemental data). A subset of compounds (IC\textsubscript{50} values versus PfENR of 38–840 nM) was selected with a variety of 5-substituents: cyano 4, alkyl (methyl 2), aryl (o-toly 15; p-F-phenyl 19; benzyl 20; phenethyl 21; phenylpropyl 22), and heteroaryl (2-pyridyl 23; 2-pyridylmethyl 26; 3-pyridylmethyl 27). The compounds demonstrate what may be described as a range of behaviors varying between slow binding and classical reversible kinetics (52). 2-Pyridyl 23 and 2-pyridylmethyl 26 most closely exemplify classical reversible inhibition. It should be noted that careful inspection of the respective x-ray crystal structures of 23 and 26 bound to the PfENR-NAD\(^+\) complex did not uncover significant differences as compared with the previously reported structures of PfENR-NAD\(^+\)-triclosan and PfENR-NADH (10). Methyl 2, o-toly 15, benzyl 20, phenylpropyl 22, and 3-pyridylmethyl 27, on the contrary, clearly exhibit slow binding kinetics. With these slow binders, the preincubation of enzyme with compound, NAD\(^+\), and NADH for 30 min led to a significant decrease in catalytic activity as compared with the use of no preincubation period. Triclosan also has been reported to behave similarly (8, 53, 54). Clearly, the placement of a 5-substituent on triclosan has an effect on the binding kinetics with respect to PfENR that, at this moment, does not appear to follow a trend that is structure- or activity-based.

In this study, a series of 5-substituted triclosan derivatives has been prepared and assayed for inhibition of PfENR and parasite growth. The efforts described herein significantly expand the knowledge of triclosan analog SAR at the 5-position while resulting in 2-fold improvements in the antiparasitic and/or enzyme potencies of triclosan. Further profiling of these compounds will require an analysis of whether they also inhibit mammalian enzymes, especially in light of recent reports documenting the existence of FAS-II pathway in the mitochondria in mammalian cells (19–22). We note that changes in enzyme and parasite whole-cell inhibition data do not always correlate, suggesting that properties other than inhibition of PfENR under cell-free \textit{in vitro} conditions can influence potency in whole-cell assays. These could include differences between compounds in their levels of drug accumulation and access to target or off-target effects in cultured parasites.

A comparison of our whole-cell and enzyme inhibition, performed for inhibitors whose enzyme IC\textsubscript{50} values were submicromolar, revealed that IC\textsubscript{50} values were 4–400-fold lower than whole-cell EC\textsubscript{50} values (with the geometric mean of the -fold difference being 20). Similar discrepancies, attributed to inefficient uptake of compounds into the parasite, have been reported with inhibitors of the \textit{P. falciparum} cysteine proteases (55) or dihydroorotate dehydrogenase (56). Limited cell permeability could equally be a cause for the discrepancy between whole-cell and enzyme inhibition values observed with triclosan and its analogs, particularly in view of the requirement for apicoplast inhibitors to traverse four membranes surrounding this organelle (4, 24) in addition to the host cell and parasite membranes.

The 5-substituted triclosan derivatives presented herein offer an expansion of the general diarylether phenol chemotype as previously explored by other researchers and ourselves (10, 15–17). It is interesting to compare these triclosan analogs versus other known inhibitors of PfENR. Most closely related to the triclosan series is a single less potent diphenylamine, prepared in a directed attempt to replace the diaryl ether oxygen of triclosan (10). A high throughput screen of small molecules for activity versus InhA uncovered an amide-substituted indole and a disubstituted pyrazole that also afforded modest inhibition of purified PfENR and cultured parasites (11). In the case of the amide-substituted indole, the central amide moiety is most probably a surrogate for the phenol group of triclosan. An independent study subsequently reported modest PfENR inhibition with a remarkably similar set of pyrazoles (57). These researchers also reported the discovery of a rhodanine class of small molecule binders to PfENR, with the most efficacious member of this class approximating the activity of triclosan (58). Building on initial investigations into plant polyphenols as antibacterial FAS-II inhibitors (59), other researchers have independently examined natural product inhibitors of PfENR where a common structural theme is the presence of one or more phenolic moieties (60, 61). Interestingly, select members of the green tea catechin family were high nanomolar inhibitors of PfENR alone while also potentiating the efficacy of triclosan inhibition supposedly through the formation of a PfENR-catechin-triclosan complex (62).

Our experimental results stand in sharp contrast to a recent proposal that a 5-chloro group is optimal for enzyme inhibition due to its small size (16). Against PfENR, 5-p-fluorophenyl 19 is 2 times more potent than triclosan, and the corresponding 5-benzyl 20 and 5-phenethyl 21 analogs are equipotent to triclosan. These groups are all larger than chloride, and x-ray crystallographic studies demonstrate that the enzyme can undergo conformational changes to support moieties larger than a chloride. Future efforts will seek to utilize the insights from this structural study to design more potent inhibitors of PfENR and parasite growth. This will be done in conjunction with probing other positions along the diaryl ether scaffold of triclosan.

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