CHAPTER 6

INHIBITOR SCREENING AGAINST
P. falciparum ENOYL REDUCTASE
6.1 Introduction

P. falciparum harbors a unique Fatty acid synthesis-II (FAS II) pathway compared to the human host. This pathway plays a pivotal role in the survival of the parasite through its involvement in the membrane construction and energy production (Freundlich et al., 2005). Moreover, its absence in humans makes it an attractive target for therapeutic intervention. FabI (P. falciparum enoyl acyl carrier protein reductase, PfENR), the enzyme catalyzing the elongation step in this pathway, has been shown to be indispensable in E. coli. It is a nuclear-encoded enzyme that functions in the apicoplast and as such should interact with PfHsp70-1 during its transport to the relict plastid. It was this context that initially caught our interest. The enzyme from various sources has been studied earlier and several classes of inhibitors have been tested against it. Triclosan has been identified as one of the most potent inhibitors of the enzyme from E. coli and P. falciparum. However, the poor oral bioavailability of Triclosan necessitates the need for the synthesis of other inhibitors with novel scaffolds. In a collaborative exercise involving the laboratories of Dr. Imran Siddiqui, Molecular and Structural Biology Division and Dr. Bijoy Kundu, Medicinal and Process Chemistry Division, Central Drug Research Institute, Lucknow, we set out to identify novel scaffolds with affinity and properties at least as good as triclosan to start with. The protein clone was a kind gift from Prof. Jim Sacchettini, Texas A&M University, USA, to our group. In this exercise Dr. Siddiqui’s group carried out the initial virtual screening and scaffold optimization while Dr. Kundu’s group synthesized the compounds. The details of the screening and synthesis are not mentioned here, as they are not a part of the thesis. Our group purified the protein and carried out the inhibition experiments. We also carried out co-crystallization experiments involving selected compounds. The results of the latter experiments are reported in this chapter.

6.2 Fatty acid synthases (FAS)

FAS are the enzymes that carry out fatty acid biosynthesis. There are mainly two types of FAS:

1. TypeI enzymes are a large multifunctional polypeptide complex, existing as a dimer, with distinct domains and are present in many eukaryotes. The newly
formed fatty acyl chain remains attached to this complex until the fatty acid synthesis reaction completes.

2. TypeII enzymes are separate proteins encoded by distinct genes catalyzing the various reactions of the fatty acid synthesis (FAS) pathway. These enzymes are generally present in prokaryotes.

The two pathways viz; FAS I and FAS II are therefore distinct as far as sequence homology is concerned but perform similar functions in the respective organisms (Kapoor et al., 2001).

6.2.1 Fatty acid synthesis pathway

The fatty acid synthesis pathway consists mainly of the following steps:

- Condensation reaction
- Reduction and dehydration reactions
- Elongation reaction

In the first step of the pathway, FabH (β-ketoacyl-ACP synthase III) condenses acetyl CoA with malonyl-ACP to form acetoacetyl-ACP. The reduction and hydration step is catalyzed involving several enzymes prominently being FabA or FabZ. The last elongation step however, is catalyzed by a single NADH-specific enoyl-ACP reductase (FabI/ PfENR). PfENR is a tetramer with a molecular weight of approximately 38kDa. Since the enzyme is involved in the rate-limiting step of the pathway it has garnered attention as an important anti-malarial target (Surolia & Surolia, 2001). The enzyme catalyses the following reaction:

\[
\text{ACP} + \text{NADH} \rightarrow \text{SAC} + \text{NAD}^+ + \text{H}^+
\]

In this final step of the fatty acid elongation cycle, PfENR reduces the α—β-unsaturated double bond of the fatty acids bound to ACP in NADH-dependent reaction. This results in the conversion of trans2-enoyl-ACP to saturated acyl ACP. The repeated cycles of this reaction leads to the formation of fatty acids of the required length (Kumar et al., 2007).

The structure based virtual screening, performed by our collaborators, resulted in the identification of a new class of compounds with inhibitory potential and was
distinct compared to the earlier known class of inhibitors based on Triclosan (Chhibber et al., 2006; Freundlich et al., 2007; Kumar et al., 2007). The predicted compounds (~17) selected after virtual screening were synthesized and screened initially against the *P. falciparum* in whole cell assays. We checked the *in vitro* inhibitory efficacy of only those compounds that exhibited good MIC values in whole cell assays.

**6.3 Materials and Methods**

**6.3.1 Protein expression and purification**

The *P. falciparum* enoyl acyl carrier protein reductase (PfENR) clone was a kind gift from Dr. James C. Sacchetini, Department of Biochemistry and Biophysics, Texas A & M University, USA. The PfENR protein was expressed and purified as described previously (Nicola et al., 2007). Briefly, the PfENR clone was transformed and protein was overexpressed in BL21 (DE3) Codon+ -RIL cells (Novagen). Culture was grown in Terrific Broth till A600 reached 0.8; the cells were induced with 1mM isopropyl-1-thio-β-D-galactopyranoside for 6h at 37°C. Cell pellets were resuspended in buffer A (20mM Tris-HCl, pH: 8.0, 500mM NaCl, and 50mM Imidazole) and sonicated. The soluble protein was then purified using Ni-NTA chelating affinity chromatography. The column was pre-equilibrated with buffer A and supernatant was loaded. Subsequently column was washed with buffer A followed by buffer B (20mM Tris-HCl pH: 8.0, 500mM NaCl, 150mM Imidazole). Protein was eluted with buffer C (20mM Tris-HCl pH: 8.0, 500mM NaCl, and 400mM Imidazole). The protein was concentrated using a 10kd cutoff Centricon (Millipore) and subsequently applied onto a Superdex 200 size-exclusion column equilibrated with buffer D (20mM Tris-HCl pH: 7.5, 150mM NaCl, 10mM EDTA).

**6.3.2 In vitro enzyme inhibition assay**

The *in vitro* enzyme inhibition assays involved monitoring of the NADH oxidation at 340nm as described previously (Kumar et al., 2007). All experiments were carried out in 96 well ELISA plates. Since the compounds were not water soluble we tried other organic solvents like DMSO and acetonitrile for dissolving them. Finally, the compounds were dissolved in DMSO and subsequently diluted in buffer containing 20mM Tris-HCl pH: 7.5, 150mM NaCl, 1% v/v DMSO, 0.2mM NADH, and 0.02mM NAD^+ and used in the experiments. Spectrophotometric
readings were taken on BioTek Powerwave XS Elisa plate reader (version Gen5) at 25°C, immediately after addition of Crotonoyl CoA. Absorbance was determined every 30s interval for 5min at 340nm spectrophotometrically by following the oxidation of NADH to NAD⁺ at 340nm (ε = 6.3mM⁻¹ cm⁻¹). Initial enzyme velocity in terms of change in absorbance per unit time (ΔOD/min) was calculated from slope of time versus absorbance curve over zero to three minutes. Correlation coefficient values greater than 0.92 indicated linearity of the curve. IC₅₀ values for the inhibitors were calculated by nonlinear curve fitting of ΔOD/min versus log of inhibitor concentration using Graph pad Prism 5.0 (Swift, 1997).

Inhibition constants for the best compound were determined with respect to both the substrate Crotonoyl CoA and the cofactor NADH. The substrate concentration (200µM) was maintained at saturation. At various fixed cofactor NADH concentrations viz; 50µM, 75µM, 100 µM and 200µM, the inhibitor concentration was varied (10nM-1.5µM) to provide Kᵢ values for the cofactor. Similarly, the inhibition constant against the substrate was determined keeping the saturating concentration of NADH (100µM) taking different concentration of Crotonoyl CoA (100µM, 150µM, 200µM) and variable inhibitor concentration (10nM-1.5µM). Values for Kᵢ were determined from the x-intercept of a Dixon plot (Dixon, 1953) using mean values of three independent experiments.

6.3.3 Co-crystallization trials

The compounds that exhibited good in vitro efficacy were used further for co-crystallization trials. Purified PfENR (2µM) was incubated with different concentrations of inhibitors (ranging from 100µM- 600µM), both in the presence (500µM) and absence of NAD⁺. Crystallization was tried using hanging drop method containing 2µl of the protein solution and 2µl of buffer (2.4 M (NH₄)₂SO₄, 0.1 M MES, pH: 5.6). We also tried the other strategy of co-crystallization where, the crystals of the native protein obtained in the conditions specified above were soaked in different concentrations of inhibitors (100µM-1mM).

6.3.4 Molecular docking

The best compound (no.685) was docked into the active site of the PfENR. The three-dimensional structure of the compound was built and optimized using the Builder module of the Insight II package (M/s Accelrys Inc.). The compound was
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submitted to the web-based docking server IS-IT: In Silico Inhibitor Identification Tool (http://myc.cdri.res.in/new2/login_homepage.html) developed by our group that uses the AUTODOCK 4.0 (Morris G.M. et al., 1998) docking engine for the calculations. The docking was carried out using the available crystal structure of PfENR (PDB id: 1UH5). The docking grid for the calculations was centered on the triclosan (inhibitor) with a grid size of 50 X 50 X 50. Interaction energies between compound and the protein were calculated using the scoring function implemented in the AUTODOCK4.0 program.

6.4 Results and Discussion

The availability of the co-crystal structure of PfENR in complex with Triclosan (Surolia & Surolia, 2001) and numerous other diaryl ether inhibitors (Freundlich et al., 2005; Freundlich et al., 2007) have led to directed efforts using the structure-based drug design strategy to identify new inhibitors. However, Triclosan has problems with oral bioavailability and the efficacy of other inhibitors still requires validation using in vivo assays. It is therefore important to identify new lead compounds against PfENR to exploit its potential in the development of novel therapeutics.

The structure based virtual screening resulted in the discovery of a new scaffold bearing biaryl amide moiety possessing ENR agonistic characteristics that were distinct from the previously known compounds based upon Triclosan, having the biaryl ether functionality. There were a total of 17 compounds that were screened for the activity. We examined all those compounds that showed good docking score against PfENR as well as good MIC values in whole cell assays, for their antimalarial activity against the target protein. Amongst the compounds that were screened for the in vitro activity, those with the hydrophobic amide unit flanked by two aromatic rings exhibited considerable activity. Although, the basic functional unit is different in both the Triclosan and the new class of compounds, small substituent groups viz; chloro and hydroxy remains unchanged, imparting some similarity to the two classes of compounds. It was also observed that presence of one or two chloro groups in one aromatic ring as well as one hydroxy substituent in the other like Triclosan led to an enhanced potency. Compounds tested for in vitro activity along with the respective ICso values are listed in Table 6.1. Some compounds exhibited inhibition at more than 100μM concentration in the in vitro inhibition assays. Compound 685 depicted higher
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order of activity than Triclosan with an IC$_{50}$ value of $34.67 \pm 1.56$ (nM). Thus, we subsequently studied its inhibition in greater detail. The compound 687 (IC$_{50} = 10.30 \pm 1.56$ (µM) and compound 927 (IC$_{50} = 0.42 \pm 1.39$ (µM) also showed considerable inhibition, although it was not better than Triclosan.

We carried out inhibition assays in the presence of the substrate and the cofactor. The K$_i$ value with respect to Crotonoyl CoA is 874nM and with respect to NADH is 39.56nM. The Dixon plot indicates that compound 685 exhibits competitive inhibition with respect to Crotonoyl CoA (Fig.6.1) and is non-competitive for the cofactor NADH (Fig.6.2). This compound therefore shows a mode of inhibition that is mechanistically similar to the rhodanine class of inhibitors that were shown to be 13 times more potent than Triclosan and possessed inhibition mechanism distinct from the latter (Kumar et al., 2007). The earlier studies where triclosan analogs with primarily 5-substituents had been screened against PfENR did not result in enhanced potency (Chhibber et al., 2006). However, in vitro potency of Triclosan analogs that involved substitution of a 5-chloro group with large hydrophobic moieties improved the inhibition potency (Freundlich et al., 2007). Earlier virtual screening techniques had been successfully applied to screen inhibitors against PfENR that exhibited in vitro potency against the target similar to Triclosan (Nicola et al., 2007).

We tried to co-crystallize these compounds with PfENR using both the incubation and soaking strategies to identify the structural basis for the observed inhibition. Unfortunately no density for the compounds was seen. This may be due to either the compounds not interacting with the protein under the crystallization conditions or exhibiting conformational heterogeneity in the binding site. Therefore, we resorted to molecular docking as a tool to garner insights into inhibitor-protein interactions. The compound (no.685) that exhibits the best inhibitory efficacy was docked into the active site of the PfENR using the IS-IT? web server. The predicted interactions are summarized in Figure 6.3. Amongst other interactions, the compound 685 exhibits Π-stacking interactions with Tyr267 and Phe368.

Ongoing and future work in the lab involves the synthesis of inhibitors with improved efficacy based on the insights garnered during the course of the reported work. Some of the tested compounds (Table 6.1) however did not exhibit any inhibition and these results will also form part of a future Structure-Activity relationship analysis study based on more data involving more compounds to help the design of better inhibitors.
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Table 6.1 IC\textsubscript{50} values of various compounds tested for their respective anti PfENR inhibitory activity.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triclosan</td>
<td><img src="image" alt="Triclosan Structure" /></td>
<td>125.32 ± 1.36 (nM)</td>
</tr>
<tr>
<td>685</td>
<td><img src="image" alt="685 Structure" /></td>
<td>34.67 ± 1.56 (nM)</td>
</tr>
<tr>
<td>687</td>
<td><img src="image" alt="687 Structure" /></td>
<td>10.30 ± 1.56 (µM)</td>
</tr>
<tr>
<td>927</td>
<td><img src="image" alt="927 Structure" /></td>
<td>0.42 ± 1.39 (µM)</td>
</tr>
<tr>
<td>558</td>
<td><img src="image" alt="558 Structure" /></td>
<td>No apparent inhibition up to 100 µM</td>
</tr>
<tr>
<td>557</td>
<td><img src="image" alt="557 Structure" /></td>
<td>No apparent inhibition up to 100 µM</td>
</tr>
<tr>
<td>559</td>
<td><img src="image" alt="559 Structure" /></td>
<td>No inhibition.</td>
</tr>
</tbody>
</table>
374

No inhibition.

371

No inhibition.
Fig. 6.1 Dixon plot showing competitive inhibition of compound 685 w.r.t. CrCoA
■ -50nM, ▲ -100nM, ○ - 200nM. Line passing through individual data ranges is linear fit for that data.

Fig. 6.2 Dixon plot showing non-competitive inhibition of compound 685 w.r.t. NADH
■ -50nM, ▲ -100nM, ○ - 200nM. Line passing through individual data ranges is linear fit for that data.
Fig. 6.3 PfENR- compound 685 interactions
The inhibitor is shown in green while the cofactor NAD is wheat coloured. Phe368 and Tyr267, residues to which the compound makes π-stacking interactions, are depicted in yellow.
6.5 Summary

We have been successful in identifying inhibitors with a new scaffold bearing biaryl amide moiety that exhibited anti PfENR activity. One of the compounds (no.685) showed good *in vitro* inhibition activity, at least as good as the standard inhibitor Triclosan, against the malarial target PfENR. The compound has been demonstrated to act competitively with respect to Crotonyl CoA and noncompetitive with respect to the NAD cofactor. The basic scaffold in the compound was different from the biaryl ether moiety present in Triclosan. The synthesized inhibitors contained a different basic functional unit. However, small substituents like chloro and hydroxy were kept same, as in Triclosan (Table 6.1). The $K_i$ value of 685 was also comparable to that of Triclosan. The compounds with a biaryl ring did not exhibit much *in vitro* inhibition and the underlying reason needs to be probed further. Nevertheless, the reported work is a good starting point in the design of PfENR inhibitors with novel scaffolds to improve the chances of identifying a useful therapeutic.