**Abstract**

The shikimate pathway for aromatic biosynthesis presents a target for antimalarial drug development as this pathway is absent from animals. This study extends previous work on inhibitors of the shikimate pathway, by examining their interaction with the antimalarial drugs pyrimethamine and atovaquone. Combinations of atovaquone with several shikimate analogues exhibited synergistic effects. These findings highlight potential use of shikimate pathway inhibitors in combination therapy.

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**Index Descriptors and Abbreviations:** Shikimate; Aromatic; Antimalarial; Antiparasitic; Chemotherapy; Drug target; Atovaquone; *Plasmodium falciparum*; Malaria; Ubiquinone; Apicomplexa; *P. falciparum*, *Plasmodium falciparum*; EPSP, 5-enolpyruvyl shikimate 3-phosphate; F-shikimate, Fluoro-shikimate, pABA, para-Aminobenzoic acid

1. **Introduction**

With the continuing incidence of malaria worldwide (500 million cases per year) and associated mortality (1–2 million per annum) and the spread of resistance there is an urgent need for novel therapies (WHO Report 2002). One novel target for development of antimalarial chemotherapy is the shikimate pathway for aromatic biosynthesis. This pathway consists of seven conserved enzymatic steps in plants, bacteria, and fungi for production of chorismate that is metabolized into aromatic amino acids, folate, ubiquinone, vitamin K, and enterochelin (bacteria) (reviewed in Pittard, 1987). Recently, the shikimate pathway has been found to exist in Apicomplexa but folate synthesized via *para*-aminobenzoic acid (pABA) is the only end product that has been demonstrated (McConkey et al., 2004). Detection of three enzymes of the shikimate pathway was described in early studies of *Plasmodium falciparum* lysates (Dieckmann and Jung, 1986). Although genes encoding homologues of the seven enzymes were identified in the related apicomplexan parasite *Toxoplasma gondii*, only the gene encoding chorismate synthase, catalyzing the terminal step, has been cloned and expressed from *P. falciparum* (Campbell et al., 2004; Fitzpatrick et al., 2001). Sensitive bioinformatic searches have identified homologues of other enzymes in the shikimate pathway with low homology in *Plasmodium* and other related apicomplexan parasites (McConkey et al., 2004).

*Plasmodium falciparum* requires a functioning shikimate biosynthetic pathway for growth. Both analogues of shikimate and the well-characterized herbicide glyphosate (*N*-phosphonomethylglycine), an inhibitor of 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase in the shikimate
pathway, inhibit *P. falciparum* growth (McConkey, 1999; Roberts et al., 1998). The shikimate analogue inhibition was abrogated by pABA demonstrating that the shikimate pathway was specifically targeted. The requirement for a functional shikimate pathway in *P. falciparum* was also shown by RNA interference studies in which dsRNA encoding chorismate synthase induced inhibition of parasite growth (McRobert and McConkey, 2002).

Treatment of *P. falciparum* was performed with an expanded set of shikimate analogues from the original study (Fig. 1). This set consists of C-6 fluorinated analogues including a stereoisomer of those previously tested, 6-R-F-4-epi-shikimate, (kindly, provided by Zeneca Pharmaceuticals, Alderley Park, UK) and C-3 fluorinated analogues (Jiang et al., 1999). Both the C-6 and C-3 of shikimate are involved in chorismate synthesis. In *Escherichia coli*, 6-R-fluoroshikimate is metabolized in the shikimate pathway but inhibits the terminal enzyme chorismate synthase due to an inability to remove the fluorine at C-6 (Davies et al., 1994). In contrast, 6-S-fluoroshikimate is metabolized to fluorochorismic acid (E. coli) which inhibits metabolism of chorismate to the folate precursor pABA.

*Plasmodium falciparum* cultures were treated as previously described in media deficient in aromatic compounds and containing serum substitute Albumax I (Invitrogen, Paisley) (McConkey, 1999). Growth was monitored by incorporation of radiolabeled hypoxanthine and 50% inhibitory concentration calculated by non-linear regression (Prism 4, GraphPad Software). Of the compounds tested, 6-R-fluoroshikimate remains the most active inhibitor of *P. falciparum* growth (IC$_{50}$ value 30 ± 10 μM) (McConkey, 1999). 6-R-F-4-epi-shikimate, an epimer of the highly active 6-R-F-shikimate, with the C-4 OH group occupying the opposite plane, was tested to define structural requirements. It exhibited a striking decrease in activity from 6-R-F-shikimate with a 12-fold higher IC$_{50}$ value (0.36 ± 0.04 μM).

Two compounds with fluorine replacements at the C-3 position, 3-deoxy-3,3-difluoro-shikimate, and 3-deoxy-3,3-difluoro-4-epi-shikimate (compounds 5 and 6 in Fig. 1) were also tested (Jiang et al., 1999). The C-3 position is involved in phosphorylation of shikimate by shikimate kinase. Both compounds exhibited low activity in growth assays (IC$_{50}$ values of 0.81 ± 0.08 and 0.75 ± 0.07 mM, respectively) although 2-fluoroshikimate remains the least active analogue tested; probably because the 2-C of shikimate is not involved in catalysis. From these compounds the priority in importance of fluorinated substitutions is: 6-R > 6-S > 6-R-4-epi > 3,3-difluoro > 2-fluoro-shikimate.

As predicted from the conserved synthetic pathway for chorismate, alterations of the C-6 position of shikimate affect growth whereas fluorination at the C-2 position has no effect on growth. The required conformation of shikimate is very strict as altering the stereoisotype of the fluorine or a hydroxyl on C-4 greatly affects activity. Similar results were observed with a decrease in activity of fluoro-shikimates by alterations of the C-4 hydroxyl in bacteria (G. Davies, personal communication). The poor inhibitory activity of analogues with fluorine substitutions on C-3 carbon was unexpected as phosphorylation of the C-3 position is essential in the shikimate pathway in other organisms. It may be that the di-fluoro compounds are poor competitors for shikimate kinase.

The shikimate pathway is poorly characterized in *Plasmodium*. Gene search and pathway reconstruction software (http://bioinformatics.leeds.ac.uk/shark/) has identified putative genes encoding four of the seven shikimate pathway enzymes (3-dehydroquinase, shikimate kinase, 5-enolpyruvylshikimate 3-phosphate synthase, and chorismate synthase) in the *P. falciparum* genome (Pinney et al., 2005). Biochemical characterization of the putative enzymes of the shikimate pathway identified in the *P. falciparum* and other *Plasmodia* genomes (e.g., rodent malarials *P. yoelii*, *P. chabaudi*, and *P. berghei* and simian malaria *P. knowlesi*) is needed.

Combination therapy has become the basis of antimalarial chemotherapy in recent years to reduce the potential of drug-resistance arising (Ridley, 2002). This may extend the useful lifespan of antimalarial drugs. In this study, combinations of the fluoroshikimates with pyrimethamine and atovaquone were tested. Mixtures containing atovaquone and pyrimethamine at a set concentration and several concentrations of a shikimate analogue were tested to determine the concentration of fluoro-shikimate at which 50% of parasite growth is

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**Fig. 1.** Chemical structures of shikimate and shikimate analogues, pyrimethamine, and atovaquone.
Bacteria and yeast synthesize ubiquinone via the shikimate pathway. The synthesis of ubiquinone in *Plasmodium* is not described. Enzymes catalyzing ubiquinone synthesis from shikimate have not been detected in the genome, but the later steps in which the isoprenoid units are added has been found to occur in the apicoplast of *P. falciparum* (Gardner et al., 2002; Jomaa et al., 1999). Additionally, the protozoa *Tetrahymena pyriformis* and *Leishmania major* incorporate radiolabeled shikimate into ubiquinone demonstrating that these protozoa can catabolize shikimate to ubiquinone (Ranganathan and Mukkada, 1995). Experimental evidence is needed to assess whether the synergism of atovaquone and the fluoro-shikimates is due to inhibition of different steps in ubiquinone metabolism.

Combinations of pyrimethamine with fluoro-shikimates were tested (Table 1). 6-R-F-shikimate increased the inhibition by pyrimethamine in an additive fashion (i.e., ∑FIC value = 1). The other two shikimate analogues tested, 6-S-F-shikimate and 6-R-F-4-epi-shikimate, did not potentiate pyrimethamine inhibition. One may have predicted synergy with these inhibitors as pyrimethamine inhibits dihydrofolate reductase, the enzyme responsible for recycling folate (Yuvaniyama et al., 2003) and folate has been demonstrated to be a product of the shikimate pathway in *Plasmodium* based on auxotrophic pABA-requiring mutants, reversal of inhibition of shikimate inhibitors by folate, and identification of genes encoding the enzymes for folate synthesis (i.e., pABA synthase) (Hyde, 2005; McConkey et al., 1994; McConkey, 1999). Although synergy indicates an interaction of biochemical pathways inhibited, the lack of synergy does not indicate that the inhibitors are blocking independent pathways. The observations may be explained by the complex biochemical relationship: the product of the shikimate pathway, chorismate, may be a common precursor to more than one product and there is a non-stoichiometric relationship of chorismate to dihydrofolate recycling.

With the rapid development of resistance, there is a great need for antimalarials that target novel sites and can be used in combination therapy. These studies suggest that shikimate pathway inhibitors can fulfill both these requirements. In this study, the required doses of atovaquone were reduced 250-fold by using in combination with micromolar concentrations of 6-R-F-shikimate. This suggests that atovaquone might be used in combination with fluoro-shikimate lowering the required concentrations of both compounds. Shikimate pathway inhibitors are likely to exhibit low toxicity as this metabolic pathway is absent from humans. Indeed, the shikimate pathway inhibitor glyphosate (Roundup) is one of the herbicides with the lowest of toxicity (Hoo-geem, 1989). Due to their high specificity and absence of this pathway in the host, shikimate pathway inhibitors should be considered for combination therapy.

### Table 1

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>∑FICa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atovaquone + 6-R-F-shikimate</td>
<td>0.7 ± 0.04</td>
</tr>
<tr>
<td>Atovaquone + 6-R-F-4-epi-shikimate</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>Atovaquone + 3-deoxy-3,3-difluoro-shikimate</td>
<td>0.5 ± 0.08</td>
</tr>
<tr>
<td>Atovaquone + 3-deoxy-3,3-difluoro-4-epi-shikimate</td>
<td>0.7 ± 0.04</td>
</tr>
<tr>
<td>Pyramethamine + 6-R-F-shikimate</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Pyramethamine + 6-R-F-4-epi-shikimate</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>Atovaquone + cycloguanin</td>
<td>0.5a</td>
</tr>
</tbody>
</table>

a Values are expressed as the sums of the fractional inhibitory concentrations (FIC) calculated as: ∑FIC = IC50 of A in mixture/IC50 of A + IC50 of B in mixture/IC50 of B.

1. From Canfield et al. (1995).

Inhibited using the radiolabel assay described above. The assays were repeated with set concentrations of fluoro-shikimate and varying the antimalarial drug (‘chequerboard assay’). The results are expressed as the sums of the fractional inhibitory concentrations (FIC) which is defined as follows: ∑FIC = IC50 of A in mixture/IC50 of A + IC50 of B in mixture/IC50 of B.

Combinations of atovaquone with four shikimate analogues were tested: 6-R-F-shikimate, 6-R-F-4-epi-shikimate, 3-deoxy-3,3-difluoro-shikimate, and 3-deoxy-3,3-difluoro-4-epi-shikimate. Values for the sum of the fractional inhibitory concentrations ∑FIC indicate synergy (Canfield et al., 1995). Atovaquone in combination with any of the fluorinated shikimates is <1 indicating synergy between fluoro-shikimates and atovaquone (Table 1). Indeed, the IC50 for atovaquone in the presence of micromolar concentrations of 6-R-F-shikimate is reduced 250-fold to 8 × 10⁻¹² M. Several antimalarial compounds including primaquine, tetracycline, and cycloguanil (active metabolite of proguanil) were found to act in synergy with atovaquone with atovaquone + cycloguanil the most active combination (Canfield et al., 1995). The ∑FIC value for atovaquone + cycloguanil is shown in Table 1 for comparison.

Compounds that act in synergy may exert their effect on related biochemical pathways (as found with dihydrofolate reductase inhibitors and sulfonamides). As the fluoro-shikimates potentiate the inhibition of parasite growth by atovaquone (i.e., their inhibition atovaquone in combination is greater than the sums of their individual contributions), their may be a biochemical relationship between the targeted enzymes. The antimalarial drug atovaquone (prescribed in combination with proguanil as Malarone) is an analogue of reduced ubiquinone that inhibits electron transport within the *P. falciparum* mitochondria; its primary site of action is the cytochrome bcl complex (Fry and Pudney, 1992). Atovaquone acts in synergy with proguanil by lowering the effective concentration at which atovaquone uncouples electron transport (Srivastava and Vaidya, 1999).
References


