Published in final edited form as:

ACS Infect Dis. 2018 April 13; 4(4): 549–559. doi:10.1021/acsinfecdis.7b00159.

Biological Studies and Target Engagement of the 2-*C*-Methyl-D-Erythritol 4-Phosphate Cytidyltransferase (IspD)-Targeting Antimalarial Agent (1*R*,3*S*)-MMV008138 and Analogs

Maryam Ghavami[†], Emilio F. Merino^{‡,§}, Zhong-Ke Yao[†], Rubayet Elahi[‡], Morgan E. Simpson[‡], Maria L. Fernández-Murga[‡], Joshua H. Butler[§], Michael A. Casasanta[‡], Priscilla M. Krai[‡], Maxim M. Totrov[¶], Daniel J. Slade[‡], Paul R. Carlier^{†,*}, and Maria Belen Cassera^{‡,§,*}

[†]Department of Chemistry and Virginia Tech Center for Drug Discovery, Virginia Tech, Hahn Hall South, 800 West Campus Drive, Blacksburg, Virginia 24061, United States

[‡]Department of Biochemistry and Virginia Tech Center for Drug Discovery, Virginia Tech, Engel Hall, 340 West Campus Drive, Blacksburg, Virginia 24061, United States

[§]Department of Biochemistry and Molecular Biology and Center for Tropical and Emerging Global Diseases (CTEGD), University of Georgia, 120 Green Street, Athens, Georgia 30602, United States

[¶]Molsoft LLC, 11199 Sorrento Valley Road, San Diego, California 92121, United States

Abstract

Malaria continues to be one of the deadliest diseases worldwide, and the emergence of drug resistance parasites is a constant threat. *Plasmodium* parasites utilize the methylerythritol phosphate (MEP) pathway to synthesize isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are essential for parasite growth. Previously, we and others identified that the Malaria Box compound MMV008138 targets the apicoplast and that parasite growth inhibition by this compound can be reversed by supplementation of IPP. Further work has revealed that MMV008138 targets the enzyme 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase (IspD) in the MEP pathway, which converts MEP and cytidine triphosphate (CTP) to cytidinediphosphate methylerythritol (CDP-ME) and pyrophosphate. In this work, we

*Corresponding Authors: maria.cassera@uga.edu. Tel.: 706-542-5192. Fax: 706-542-1738 (M.B.C.). pcarlier@vt.edu. Tel.: 540-231-9219. Fax: 540-231-3255 (P.R.C.).

ORCID

Daniel J. Slade: 0000-0001-5634-7220

Paul R. Carlier: 0000-0002-6683-285X

Maria Belen Cassera: 0000-0001-6341-3169

Author Contributions

The study and experiments were designed by M.B.C., P.R.C., D.J.S., M.M.T., M.G., E.F.M., Z.-K.Y., R.E., M.E.S., M.L.F.-M., J.H.B., M.A.C., and P.M.K. Experiments were conducted by M.G., E.F.M., Z.-K.Y., R.E., M.E.S., M.L.F.-M., J.H.B., M.A.C., and P.M.K. All authors analyzed the data. M.B.C., P.R.C., M.G., M.M.T., and D.J.S. wrote the manuscript, and all authors have read and given approval to the final version.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfec-dis.7b00159.

Synthetic procedures and analytical characterization data for all the new compounds described in the paper. SDS-PAGE analysis of the *Pt*IspD used for enzyme inhibition studies (Figure S1), and determination of kinetic parameters for *Pt*IspD (Figure S2) (PDF)

sought to gain insight into the structure–activity relationships by probing the ability of MMV008138 analogs to inhibit *Pf*IspD recombinant enzyme. Here, we report *Pf*IspD inhibition data for fosmidomycin (FOS) and 19 previously disclosed analogs and report parasite growth and *Pf*IspD inhibition data for 27 new analogs of MMV008138. In addition, we show that MMV008138 does not target the recently characterized human IspD, reinforcing MMV008138 as a prototype of a new class of species-selective IspD-targeting antimalarial agents.

Graphical Abstract

Keywords

Plasmodium; malaria; MEP pathway; MMV008138; IspD; structure–activity studies

Human malaria is caused by five species of *Plasmodium*. Around 250 million cases of malaria occur every year with 95% of the infections caused by *Plasmodium falciparum* and *Plasmodium vivax*.¹ Malaria continues to be one of the deadliest diseases worldwide, and the continued emergence of drug resistance is a constant threat. Therefore, efforts to identify and characterize new leads for development of antimalarial drugs with different mechanisms of action are still needed. In order to catalyze the development of new antimalarials, the Medicines for Malaria Venture (MMV) and SCYNEXIS assembled the Malaria Box,² an open access library composed of 400 compounds originally identified by phenotypic screening of nearly 6 000 000 compounds from the research libraries of Saint Jude Children's Research Hospital, Novartis, and GlaxoSmithKline.³ Over 290 assays had been performed to screen the Malaria Box, and substantial information about these compounds is now available.⁴

Malaria parasites contain a vestigial plastid called the apicoplast, which performs vital functions such as the biosynthesis of isoprenoid precursors, fatty acids, and part of the heme.^{5,6} *Plasmodium* parasites utilize the methylerythritol phosphate (MEP) pathway to synthesize isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are essential for parasite growth.^{7,8} This pathway is absent in humans, who rely on the mevalonate pathway instead. Recently, it has been suggested that the MEP pathway and the biosynthesis of the isoprenoid precursors IPP and DMAPP represent the sole essential function of this organelle during asexual intraerythrocytic development of the parasites⁹ as well as during gametocytogenesis.¹⁰ The strongest support for this proposal stems from the observation that both loss of the apicoplast function and loss of the organelle can be chemically rescued by supplementing the growth medium with IPP. Therefore, the growth inhibitory effect of drugs that directly target biosynthesis of isoprenoid precursors or

indirectly disrupt their biosynthesis by interfering with processes essential for apicoplast biogenesis, such as apicoplast DNA replication, transcription, and protein translation, may be reverted by IPP supplementation.⁹ As a result, the reversal of growth inhibition by IPP supplementation can be used as a phenotypic screening diagnostic to identify compounds that target the apicoplast, thus identifying their mechanism of action and narrowing their potential molecular targets.⁹ Previously, we identified the drug-like Malaria Box compound MMV008138 (Figure 1) using this method and proposed that the molecular target may be within the MEP pathway, especially because we noted that MMV008138 did not present a delayed death phenotype and the apicoplast was not lost in the presence of IPP, similar to fosmidomycin (FOS).¹¹ Further analyses revealed that the active stereoisomer of this molecule is (1*R*,3*S*)-configured^{12,13} and that its MEP pathway target is the 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (IspD) (E.C.2.7.7.60) which converts methylerythritol phosphate (MEP) and cytidine triphosphate (CTP) to cytidine diphosphate methylerythritol (CDP-ME) and pyrophosphate.^{12,14}

One of the most striking features of (1*R*,3*S*)-MMV008138 (henceforth **1a**, Scheme 1) is its species selectivity toward the malarial IspD.^{12,14} Though **1a** was reported to inhibit *P. falciparum* IspD with K_i values in the 7–13 nM range, it did not inhibit *Arabidopsis thaliana*, *Mycobacterium tuberculosis*, or *Escherichia coli* IspDs at concentrations up to 10 μ M.^{12,14} This *E. coli* IspD (EclspD) insensitivity is especially important since the MEP pathway is present in the human microbiome.¹⁵ Therefore, unlike antibiotics such as doxycycline (DOX) that kill malaria parasites by interfering with the apicoplast's genome expression¹⁶ or FOS that inhibits 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in the MEP pathway also affecting the gut microbiome,^{8,17–19} compound **1a** may not affect beneficial gut bacteria. Moreover, doxycycline is used for prophylaxis in endemic areas with multidrug resistance and in combination therapies; however, it is not recommended for pregnant women and children under the age of 8.²⁰ Therefore, antimicrobial agents that are parasite-specific are ideally suited for the development of malaria chemopreventive agents that are not toxic to pregnant women and children and do not impact the human intestinal microbiome. Indeed, we previously confirmed that **1a** had no effect on *E. coli* growth at concentrations up to 500 μ M.¹³

Subsequent to our discovery of **1a** as a MEP-pathway inhibitor,¹¹ we prepared 34 close structural analogs designed to probe *P. falciparum* growth inhibition structure–activity relationships (SAR).¹³ We determined that, in addition to (1*R*,3*S*)-configuration, potent growth inhibition required 2',4'-disubstitution of the D-ring, featuring at least one electron-withdrawing substituent. For example, whereas **1a** inhibited *P. falciparum* (Dd2 strain) growth with a half-maximum inhibitory concentration (IC₅₀) value of 250 $\pm$ 50 nM, neither **1b** (bearing an unsubstituted phenyl ring) nor **1u** (bearing 2',4'-dimethyl substitution) inhibited *P. falciparum* growth at 10 000 nM (Figure 1). However, 2'-chloro substituted analog **1c** showed weak growth inhibition, and **1e** (2'-chloro-4'-methyl substituted) nearly recapitulated the potency of **1a**. The carboxy substituent at C3 of the C-ring also proved to be essential; replacement with CO₂Me (**3a**) or H (($\pm$)-**11a**) abrogated growth inhibition potency. However, weak growth inhibition was restored with the first amide analog **6a**, and methyl amide derivative **7a** proved equipotent to **1a**.

In the present work, we sought to gain insight into these structure–activity relationships by probing the ability of these compounds and new analogs to inhibit *Pf*IspD recombinant enzyme, the proposed parasite target of **1a**. In this paper, we report *Pf*IspD inhibition data for FOS and 19 previously disclosed analogs and report growth inhibition and *Pf*IspD inhibition data for 27 new analogs of **1a**. As we will show below, these analogs show an excellent correlation between *P. falciparum* Dd2 strain growth inhibition and *Pf*IspD inhibition, confirming the proposed antimalarial mode of action. In addition, we expanded the biological studies to assess cytotoxicity and stage specificity profile of **1a**.

RESULTS AND DISCUSSION

Structure–Activity Relationships

We first measured the kinetic parameters of recombinant IspD ($K_m^{\text{MEP}} = 12.0 \pm 2.5 \mu\text{M}$, $k_{\text{cat}}^{\text{MEP}} = 7.6 \pm 0.6 \text{ s}^{-1}$, $K_m^{\text{CTP}} = 9.3 \pm 2.5 \mu\text{M}$, $k_{\text{cat}}^{\text{CTP}} = 11.7 \pm 1.2 \text{ s}^{-1}$) using a PhosphoWorks Fluorimetric Pyrophosphate Assay Kit (AAT Bioquest, Inc.) which directly measures pyrophosphate (PP_i) released from the IspD-catalyzed reaction ($\text{MEP} + \text{CTP} \rightarrow \text{CDP-ME} + \text{PP}_i$) (see Figure S2). Previously reported kinetic parameters of the substrate CTP for this construct were $K_m^{\text{CTP}} = 59 \mu\text{M}$ and $k_{\text{cat}}^{\text{CTP}} = 0.43 \text{ s}^{-1}$.¹⁴ In addition, Wu et al. reported kinetic parameters of the substrate MEP of $K_m^{\text{MEP}} = 61 \mu\text{M}$ and $k_{\text{cat}}^{\text{MEP}} = 0.16 \text{ s}^{-1}$ using a histidine- and maltose binding protein-tagged *Pf*IspD protein.¹² However, in both cases, different detection assays (EnzChek Phosphate and Pyrophosphate Assay Kits) were used to indirectly measure release of pyrophosphate through its subsequent conversion to phosphate. After the kinetic parameters were determined, we first studied the inhibitory properties of **1a**, its three diastereomers **5a**, *ent*-**1a**, and *ent*-**5a**, and the antimalarial FOS (Table 1). As was first reported by Wu et al.,¹² we confirmed that, among the 4 stereoisomers of MMV008138, (1*R*,3*S*)-configured **1a** is the most potent inhibitor of *Pf* IspD ($\text{IC}_{50} = 44 \pm 15 \text{ nM}$). Thus, the stereochemical dependence of *P. falciparum* growth inhibition potency correlates very well with *Pf*IspD target engagement. Furthermore, as expected, the IspC (DXR)-targeting antimalarial FOS is a poor inhibitor of *Pf*IspD (~4% inhibition at $10 \mu\text{M}$). We would also note that the toxicological selectivity of **1a** is much higher than that of FOS: whereas **1a** has no effect on *E. coli* growth at 2000-fold of its *P. falciparum* growth inhibition IC_{50} value, FOS shows an *E. coli* MIC only 14-fold higher than its *P. falciparum* growth inhibition IC_{50} value. This toxicological selectivity is expected, since **1a** does not engage *Ec*IspD.^{12,14} Interestingly, while (1*S*,3*R*)-configured *ent*-**1a** and (1*S*,3*S*)-configured **5a** have no effect on *E. coli* growth at 250 and $125 \mu\text{M}$, (1*R*,3*R*)-configured *ent*-**5a** inhibited $18 \pm 6\%$ of *E. coli* growth at $250 \mu\text{M}$.

As alluded to above, previously, we observed a very tight D-ring SAR for analogs of **1a**; only analogs bearing at least one sterically small, electron-withdrawing substituent at 2' and/or 4' were potent *P. falciparum* growth inhibitors (e.g., **1e–g**).¹³ To further test this hypothesis, 17 new acid analogs of **1a** were prepared and compared to previously synthesized analogs (Table 2). As can be seen, *P. falciparum* growth inhibition potency is retained in compounds **1h–l** that feature single replacement of the Cl atoms of **1a** with F (**1h,i**), Br (**1j,k**), or both Cl atoms of **1a** with Br (**1l**). Within experimental error, compounds **1j** and **1k** are equipotent to **1a**, but compounds featuring 2'-I,4'-F, 2'-F,4'-I, or 2'-Br,4'-I

substitution (**1m–o**) were less potent growth inhibitors. Nevertheless, compounds **1e–n** all showed full rescue upon addition of 200 μ M IPP, confirming their action as MEP pathway inhibitors. However, replacement of one of the Cl atoms of **1a** with OMe, OH, or CO₂H (**1p–s**) significantly reduces growth inhibition potency. Compounds featuring very large electron-withdrawing groups (**1t**) or electron-releasing substituents at 2',4' (**1u**, **1v**) are also poor *P. falciparum* growth inhibitors. A few compounds featuring 3',4'-disubstitution (**1w**, **1x**), trisubstitution (**1y**, **1z**, **1aa**), and heterocyclic modification (**1ab**, **1ac**) were also explored and found to be poor *P. falciparum* growth inhibitors. Two modifications of the C–D-ring junction of **1a** were explored. Compound **1ad** features the insertion of a CH₂ unit between the C- and D-rings of **1a**, and this modification abrogated growth inhibition. Second, preparation of a derivative of **1a** that featured replacement of the C1–H with methyl was attempted via a ketone Pictet-Spengler reaction with 2',4'-dichlorophenyl methyl ketone (*vide infra*). Although the expected product of this reaction could not be isolated, compounds **1ae** and **1af** were successfully prepared by ketone Pictet-Spengler reactions with 4-chlorophenyl methyl ketone and acetophenone. However, these compounds also did not inhibit growth of the parasite below 5 μ M. Thus, the new compounds synthesized reinforce our earlier conclusion that potent growth inhibition by analogs of **1a** requires 2',4'-disubstitution of the D-ring, featuring at least one electron-withdrawing substituent.

The *Pf*IspD inhibition potencies of these compounds largely follow the same trend (Tables 1 and 2). Unsubstituted or monosubstituted D-ring derivatives (**1b–d**, IC₅₀ = 510 to >5000 nM) are weaker inhibitors than **1a** (IC₅₀ = 44 $\pm$ 15 nM). Close analogs of **1a** featuring 2',4'-disubstitution with at least one small electron-withdrawing group (**1e–l**) feature *Pf*IspD IC₅₀ values ranging from 31 to 260 nM, consistent with their potent growth inhibition (IC₅₀ = 320–860 nM). Note that several potent compounds (**1a**, **1e**, **1i–l**) have *Pf*IspD IC₅₀ values close to the nominal enzyme concentration in the assay (60 nM), and thus, these values represent our current best estimates. This nominal enzyme concentration represents a practical current lower limit, on the basis of the sensitivity of the assay (0.1 μ M of PPI); if assay at lower nominal enzyme concentration could be performed, it is possible that the measured IC₅₀ values of these compounds would be lower. Therefore, differences in the measured *Pf*IspD IC₅₀ values of **1a**, **1e**, and **1i–l** (31–100 nM) may not be reflective of actual differences in target-site affinity of these compounds. Finally, as was seen for growth inhibition, replacement of the C2' and C4' Cl groups of **1a** with CF₃, Me, or OMe (**1t–v**) or 3',4'-Cl₂ substitution (**1w**) significantly reduced *Pf*IspD inhibition. Thus, the antimalarial activity of D-ring analogs of **1a** appears very well correlated to the extent of their engagement with *Pf*IspD. This tight SAR suggests that the D-ring projects into a very well-defined cavity of *Pf*IspD and is not solvent-exposed. The requirement that at least one of the substituents at C2' or C4' must be a halogen (cf. **1a** vs **1e/1f** vs **1u**) is curious and could be a consequence of a number of phenomena. It is possible that halogen-bonding²¹ to one or more *Pf*IspD backbone carbonyl groups contributes to affinity, as was characterized crystallographically for the 1,3-dihalobenzene moieties present in the pseudillin inhibitors of *A. thaliana* IspD.²²

We then examined several of the carboxylic acid replacement analogs of **1a** disclosed in our earlier publication (**3a**, **6a**, **7a**, **7e**, **11a**), and six new ones (**7i–k**, **8a**, **9a**, **10a**, Table 3). As

mentioned earlier, methyl ester (**3a**) was a poor *P. falciparum* growth inhibitor, and as expected, this compound does not measurably inhibit *Pf*IspD at 1000 nM. The 1° amide **6a** does inhibit growth of *P. falciparum* in the 1 μ M range, and its potency to inhibit *Pf*IspD is similar. Methyl amides (**7a**, **7e**, **7i–k**) proved to be excellent replacements for the carboxylic acid group, both for growth inhibition (IC_{50} = 190–506 nM) and for *Pf*IspD inhibition (IC_{50} = 21–360 nM). With respect to growth inhibition, **7j** and **7k** are equipotent to **1a** (within error), and **7a**, **7e**, and **7i–k** all show 100% rescue upon supplementation with 200 μ M IPP. The methyl amides also show very weak inhibition of *E. coli* growth at 250–500 μ M. Finally, the ethyl amide (**8a**), *N,N*-dimethyl amide (**9a**), 1° alcohol analog (**10a**), and racemic des-carboxy derivative **11a** showed poor growth and *Pf*IspD inhibition. Thus, within this limited series, antimalarial activity is also well-correlated to *Pf*IspD engagement. These data and those in Table 1 show that the carboxylate group of **1a** plays a critical role in interacting with *Pf*IspD; however, the nature of this interaction is not clear. A purely electrostatic interaction of the carboxylate of **1a** with *Pf*IspD is ruled out by the potency of methyl amide **7a**. Yet, the carboxylate cannot simply serve as a hydrogen-bond acceptor, since the isosteric 1° amide **6a** does not potently engage *Pf*IspD. In any event, the carboxylate and methylamide groups of **1a** and **7a** must project into a rather constricted pocket, since ethyl amide **8a** and *N,N*-dimethyl amide **9a** dramatically lose potency relative to methyl amide **7a**.

***Pf* IspD Inhibitor 1a Is Cytocidal in Late Trophozoite/Early Schizont Stages but Not in Ring Stage**

In our previous study, we demonstrated that **1a** (MMV008138) targets the apicoplast, where it inhibits elongation and disturbs the mitochondrial membrane; these effects are reversed upon IPP supplementation.¹¹ However, the parasite's development was arrested at the early schizont stage, since DNA replication (assessed by Hoechst staining) was still observed after 40 h of treatment with 2.5 μ M of **1a**.¹¹ Therefore, to better assess if **1a** is cytocidal and parasite stage specific, we determined the half-maximum lethal concentration (LC_{50}) at 72 h using highly synchronous cultures starting at ring stage and bolus incubation times ranging from 6 to 24 h. At each indicated time, **1a** was washed out and parasites were returned to culture to complete 72 h of growth, at which point growth was measured by the SYBR Green assay (Figure 2). In parallel, cultures were smeared and stained with Giemsa for stage assessment of **1a**-treated and control parasites at the time that **1a** was washed out. The SYBR Green assay at 72 h confirmed that parasites were able to grow similarly to untreated controls following 6 and 12 h incubation with **1a**; therefore, the LC_{50} values could not be determined. However, when incubation with **1a** was extended to the late trophozoite/early schizont stage (18 h), the LC_{50} value was 1.20 ± 0.08 μ M. When the drug incubation was extended to 24 h, the measured LC_{50} value further decreased to 0.46 ± 0.02 μ M, which was very close to the measured IC_{50} value at 72 h performed simultaneously with these assays (0.35 ± 0.03 μ M). The cytocidal action of **1a**, therefore, manifests at 18–24 h, which is the time needed for parasites to progress from ring to late trophozoite/early schizont stage. This transition marks the period of highest metabolic activity of the MEP pathway, as discussed below (Figure 3). The absence of growth inhibition when cultures were treated for only 6 or 12 h is interesting (Figure 2A), given that the MEP pathway is known to be active through the entire asexual intraerythrocytic cycle.^{18,23} Analysis of MEP pathway intermediates at

ring (12 h postinvasion, hpi), trophozoite (19 hpi), and schizont (33 hpi) showed that IPP concentrations increase markedly between 19 and 33 hpi (Figure 3), which is in agreement with our cytotoxic analysis showing that the measured LC_{50} value in cultures exposed for only 24 h was very close to the IC_{50} value measured at 72 h. Cassera et al. have previously shown that transcript abundance of genes involved in the MEP pathway and the isoprenoid products dolichol and ubiquinone all peak at the schizont stage.²³ Therefore, one potential explanation for the lack of growth inhibition observed at 72 h when **1a** was present for only 6 or 12 h is that isoprenoid products were present at a sufficient level at the time that **1a** treatment started to allow parasites to progress through ring and early trophozoite stages until **1a** was washed out.

Methylamide Derivative **7a** Inhibits CDP-ME Formation *in Vivo* Better than the Carboxylic Acid **1a**

In 2015, we reported that the methylamide derivative **7a** was equipotent to **1a** for *P. falciparum* growth inhibition,¹³ and in this study, we determined that its *Pf*IspD inhibition values are identical within experimental error (44 ± 15 and 57 ± 10 nM, respectively, Tables 1 and 3). As a further means to compare these compounds, we assessed *in vivo* IspD inhibition in *P. falciparum* at schizont stage by quantitative liquid chromatography–mass spectrometry (LC-MS/MS, Figure 4) in highly synchronous schizont stage parasites treated for 2 and 4 h with 1 μ M **1a** or **7a** (~4 times the IC_{50} value). FOS was used as positive control at 10 μ M (~10 times the IC_{50} value). After 2 h of drug exposure, **7a** caused $93 \pm 5\%$ decrease in the cellular levels of the *Pf*IspD product CDP-ME, while **1a** caused $67 \pm 2\%$ decrease. The 27% greater reduction caused by **7a** was statistically significant by the Benjamini-Hochberg significance test. After 4 h of treatments, **1a** caused $84 \pm 8\%$ decrease in the levels of CDP-ME while the reduction caused by **7a** remained the same. Interestingly, FOS treatment did not cause significant reduction of the CDP-ME levels after 2 or 4 h of drug exposure. As mentioned above, FOS inhibits MEP synthesis and does not inhibit the enzymatic activity of recombinant *Pf*IspD (Table 1). Previously, Zhang et al. reported that parasites treated with 5 μ M FOS for 10 h starting at ring stage presented increased levels of MEP and an unknown metabolite, presumptively identified as 2-*C*-methylerythrose 4-phosphate, and reduced levels of CDP-ME.²⁴ Accumulation of MEP and the potential 2-*C*-methylerythrose 4-phosphate was unexpected, and the authors hypothesized that FOS may be indirectly inhibiting *Pf*IspD through the intermediate 2-*C*-methylerythrose 4-phosphate that accumulates following FOS treatment or through other control mechanisms that remain to be identified. Because our experiments involved shorter treatments at a different *P. falciparum* stage, the results cannot be directly compared; however, in both cases, FOS induced unexpected levels of metabolites downstream of its enzyme target DXR, suggesting that FOS is triggering other control mechanisms that regulate the MEP pathway in the malaria parasite.

On the other hand, the reduction of the cellular levels of the methylerythritol cyclodiphosphate (MEcPP) and the final MEP pathway product, IPP, did not show significant differences among the three treatments after 2 and 4 h of exposure to **1a**, **7a**, and FOS as assessed by the Benjamini-Hochberg significance test. However, the overall reduction of the MEcPP and IPP levels was greater after 4 h of treatment with the three

inhibitors where >90% reduction of the IPP levels was achieved (Figure 4). Overall, these results are in agreement with the results described above showing that **1a** is cytotoxic in late trophozoite/early schizont stages where there is a higher demand of isoprenoid products such as dolichol and ubiquinone.²³

Human IspD Is Not Targeted by **1a**

Isoprenoid synthase domain-containing (IspD-like) proteins belong to a large family of glycosyltransferases conserved from bacteria to mammals, and defective function of a IspD-like protein disrupts glycosylation of α -dystroglycan causing Walker–Warburg syndrome, a congenital muscular dystrophy accompanied by a variety of brain and eye malformations.^{25,26} Recently, functional studies revealed that human IspD is a ribitol-5-phosphate cytidyltransferase.^{27–29} Because of the possibility that drugs directed against IspD enzymes from pathogenic bacteria and parasites could also inhibit the human IspD enzyme causing undesired side effects, the validity of IspD as drug target has been questioned.^{29,30} In order to assess if **1a** inhibits human IspD activity, its enzymatic activity was measured by LC-MS/MS to monitor CDP-ribitol formation in the presence of 20 and 200 μ M of **1a**. The CDP-ribitol formation was not inhibited by the presence of **1a** at either concentration (Figure 5). This result was expected since **1a** has been shown to be active against *P. falciparum* and *P. vivax* but not bacterial or plant IspD homologues.^{12,14} Therefore, our results support the validity of *Pf*IspD as drug target and the further development of this chemotype for malaria prophylaxis and pharmacotherapy. As other *Pf*IspD inhibitor chemotypes emerge, testing against hIspD will help elucidate the structural basis of this important selectivity.

METHODS

Synthesis of Analogs of **1a**

Compound **1a** and its D-ring variants **1b–ae** were prepared by Pictet-Spengler reaction of the requisite aldehydes with (*S*)-Trp-OMe as described in Scheme 1. The *trans*-configured methyl ester intermediates **3a–ac** were separated from their *cis*-isomers by column chromatography on silica gel (using dichloromethane–ethyl acetate–hexanes mixtures to elute, as described in the Supporting Information). The relative configuration of each isomer was determined by ¹³C NMR according to the method of Cook and co-workers,³¹ and in every case examined thus far, the *cis*-isomer eluted before the *trans*-isomer. Compounds **1h–s**, **1w**, and **1y–af** have not been previously described. C1-methyl analogs **1ae** and **1af** were prepared by ketone Pictet-Spengler reaction³² with (*S*)-Trp-OMe (Scheme 2). Note that application of this protocol to 2,4-dichlorophenyl methyl ketone did not give the expected C1-methyl analog of **1a**. Methyl ester **3a**, 1° amide **6a**, and methyl amides **7a**, **7e**, and ($\pm$)-**11a** were prepared as described previously.¹³ New methyl amides **7i–k** were prepared by treating the corresponding methyl esters **3i–k** with methylamine in ethanol. Ethyl amide **8a** and *N,N*-dimethyl amide **9a** were prepared by HATU coupling of **1a** with ethylamine and dimethylamine, respectively. The 1° alcohol analog **10a** was prepared by LiAlH₄ reduction of **1a**.

***P. falciparum* Culture**

Parasite Dd2 (MRA-150) strain was maintained in O⁺ human erythrocytes at 4% hematocrit in RPMI 1640 media supplemented with 2 g/L glucose (Sigma-Aldrich), 2.3 g/L sodium bicarbonate (Sigma-Aldrich), 50 mg/L hypoxanthine (Sigma-Aldrich), 5.94 g/L HEPES, 20 mg/L gentamycin (GIBCO Life Technologies), and 5 g/L Albumax I (GIBCO Life Technologies). Parasites were kept at 37 °C under reduced oxygen conditions (5% CO₂, 5% O₂, and 90% N₂). Synchronous cultures in ring stage (>95%) were obtained by two cycles of 5% sorbitol treatment.

***P. falciparum* Growth Inhibition (IC₅₀) and IPP Reversal of Growth Assays**

The effects of **1a** and analogs were evaluated against *P. falciparum*, Dd2 strain, by the SYBR Green assay as described previously in a 72 h assay since we previously reported that this class of apicoplast-targeting compounds does not present a delayed death phenotype.¹¹ Studies were performed with the Dd2 strain since it is chloroquine resistant and we have previously showed that there is no significant differences between resistant and sensitive *P. falciparum* strains.¹¹ Briefly, ring stage parasite cultures (100 μ L per well, with 1% hematocrit and 1% parasitemia) were grown for 72 h in the presence of increasing concentrations of the inhibitor under reduced oxygen conditions (5% CO₂, 5% O₂, and 90% N₂) at 37 °C. After 72 h in culture, parasite's growth inhibition was determined by DNA quantitation using SYBR Green I as described previously.³³ The half-maximum inhibitory concentration (IC₅₀) values were calculated with GraphPad Prism (GraphPad Software, Inc.) using nonlinear regression curve fitting. The reported values represent averages of at least three independent experiments performed in triplicate, using 10-point serial dilutions, with standard errors of the mean (S.E.M.). The range for serial dilutions was adjusted accordingly for each analog after the first screening to set the IC₅₀ value in the middle of the concentration range.

To establish reversal of growth inhibition by IPP, ring stage parasite cultures were grown for 72 h in the presence of increasing concentrations of drug and in the presence or absence of 200 μ M IPP. The reported values represent averages of at least two independent experiments. SEM values are only indicated for mean values below 100%. The parasite's growth inhibition and recovery was assessed by SYBR Green as described previously.¹¹

Cytocidal (LC₅₀) Assay

In order to determine the concentration of a bolus dose of the resynthesized 1*R*,3*S*-MMV008138 (**1a**) that kills 50% of parasites (LC₅₀), *P. falciparum* (Dd2 strain) cultures were exposed to increasing concentrations of **1a** and then the drug was washed away at 6, 12, 18, and 24 h to also probe stage specificity for the activity of **1a** as described previously.³⁴ Briefly, following the bolus dose incubation, plates were centrifuged at 700*g* for 3 min and **1a**-containing medium was removed. Cell pellets were washed three times with 100 μ L of medium using the same centrifuge settings and then resuspended in 100 μ L of media without the inhibitor. Washed plates were then incubated at 37 °C to complete a total of 72 h after setting the assays, and growth of surviving parasites was assessed by SYBR Green. The half-maximum lethal concentration (LC₅₀) values were calculated with GraphPad Prism software (GraphPad Software, Inc.) using nonlinear regression curve fitting, and the reported

values represent averages and SEM of at least three independent assays, with each assay performed in triplicate. Parasites untreated or treated with **1a** were smeared and stained with Giemsa before washing the inhibitor in order to assess stage development at the time that **1a** was removed.

***E. coli* Growth Inhibition Assays**

In order to investigate the effect of selected compounds against *E. coli*, strain BL21(DE3), an overnight culture of *E. coli* cultivated at 37 °C at 200 rpm agitation was diluted 100-fold into LB broth medium and incubated to an OD₆₀₀ of ~0.6. The culture was then diluted 10 000-fold into LB broth medium. Then, 750 μ L of this *E. coli* inoculum was inoculated into a culture tube containing 750 μ L of the test compounds previously diluted in LB broth medium at three final concentrations (500, 250, and 125 μ M). The final DMSO concentration was 5%. Cultures were incubated for 18 h at 37 °C and 200 rpm agitation. The following controls were performed: 100 μ M fosmidomycin (FOS) treatment, which targets the MEP pathway in *E. coli*, media without inoculum, 5% DMSO (vehicle), and control with inoculum alone (untreated). After 18 h of incubation, bacteria growth was measured using a cell density meter. The percentage of growth was normalized to that of untreated control bacteria and potential inhibition of growth of **1a** analogs was determined by comparison to the 5% DMSO control, which does not affect *E. coli* growth, as we described previously.¹³ The data represents the mean and SEM of two independent experiments.

***P. falciparum* IspD Protein Purification and IspD Enzymatic Assay Conditions**

Plasmid containing wild-type *PfIspD* (pBG1869 *PfIspD*), as described in Imlay et al., was a gift from Dr. Audrey Odom.¹⁴ pBG1869 *PfIspD* was transformed into Arctic Express (DE3) RIL *E. coli* cells (Stratagene), followed by protein expression and purification as described previously (see Figure S1).¹⁴ The effect of **1a** analogs on inhibiting *PfIspD* enzymatic activity was measured using a PhosphoWorks Fluorimetric Pyrophosphate Assay Kit (AAT Bioquest, Inc.) following the manufacturer's instructions.^{35–37} This fluorimetric assay directly measures pyrophosphate (PP_i) with a linear range of 0.3 to 30 μ M (detection limit of 0.1 μ M) as described by the manufacturer. We measured PP_i released in the enzymatic reaction catalyzed by *PfIspD* (MEP + CTP $\rightarrow$ CDP-ME + PP_i). The reactions were performed in a solid black 96-well microplate in a final volume of 50 μ L (25 μ L of test samples and 25 μ L of assay solution) according to the manufacturer's protocol. Briefly, various concentrations of **1a** analogs were incubated for 10 min with 60 μ M CTP, 60 μ M MEP, 100 mM Tris-HCl (pH 7.4), and 1.6 mM MgCl₂. Reactions were initiated by adding 60 nM *PfIspD*. In assays with variable MEP, 100 μ M CTP was used, and in assays with variable CTP, 100 μ M MEP was used. Fluorescence signal was continuously monitored at 316 nm (Ex) and 456 nm (Em) using a Cytation-5 multimode plate reader (BioTek). The half-maximum inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism software (GraphPad Software, Inc.) from a 7-data points curve from at least three independent experimental replicates. The data represents the mean and SEM. A reaction mix without *PfIspD* was performed in all assays and used for background subtraction. The range for serial dilutions was adjusted accordingly for each analog after the first screening to set the IC₅₀ value in the middle of the concentration range.

Human IspD Protein Enzymatic Assay

Human IspD (hIspD) recombinant protein and its substrate ribitol-5-phosphate were a generous gift from Dr. Lance Wells and Dr. Osman Sheikh (University of Georgia).^{28,29} The effect of **1a** on inhibiting hIspD enzymatic activity was measured by LC-MS/MS to monitor CDP-ribitol formation as described below for the detection of the MEP intermediates.¹⁰ Briefly, the enzymatic assay was performed using 2 μ M hIspD, 50 mM Tris-HCl (pH 7.2), 2 mM MgCl₂, 2 mM DTT, 1 mM CTP, and 1 mM ribitol-5-phosphate in the absence or presence of **1a** at 20 and 200 μ M. Reactions were carried out at 37 °C for 16 h. The reported mean and SEM values were calculated from two independent assays.

MEP Pathway Metabolite Profiling

Mycoplasma-free parasite cultures were tightly synchronized and grown to recover ring, trophozoite, and schizont stages for MEP pathway intermediates level assessment or early schizont stage (~27 h postinvasion) for drug treatments and MEP pathway intermediates level assessment. Cultures in early schizont stage were treated for 2 and 4 h with 0.1% DMSO (control), 1 μ M **1a**, 1 μ M **7a**, or 10 μ M FOS. In all cases, parasites from infected erythrocytes were released from the host cell by lysis with 0.03% saponin in cold PBS containing 2 g/L glucose and washed three times with PBS/glucose by centrifuging 7 min at 10 000g and 4 °C. Metabolite extraction was performed by adding 250 μ L per sample of ice-cold extraction solvent consisting of chloroform/methanol/acetonitrile (2:1:1, v/v/v) containing 1 μ M isopentenyl *S*-thiolodiphosphate (ISPP) as internal standard. Samples were sonicated for 10 min in a water bath sonicator with ice. Then, 500 μ L of ice-cold MS water was added, and the solution was mixed by vortexing and centrifuged at 12 000g and 4 °C for 4 min. The polar upper phase was transferred to a 10 kDa centricom tube and centrifuged at 12 000g and 4 °C for 10 min. The flow through was lyophilized, resuspended in 120 μ L of ice-cold MS water, and centrifuged at 12 000g and 4 °C for 10 min. Samples were transferred to a total recovery vial (Waters), and two injections of 40 μ L of each sample underwent LC-MS/MS analysis as previously described using ion-pair reversed phase ultraperformance liquid chromatography in tandem with mass spectrometry (IP-RP-UPLC-MS/MS).³⁸ Data were acquired using MassLynx Software, v. 4.1, and processed using TargetLynx Application Manager (Waters). Relative quantification of the level of each metabolite was performed by determining the analyte-to-internal standard ratio (response) calculated by dividing the area of the analyte peak by the area of the internal standard (ISPP) peak. The response of each detected metabolite is expressed as the mean and SEM of two biological replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health (AI128362 (PRC), AI082581 (PRC), and AI108819 (MBC)), the Fralin Life Science Institute at Virginia Tech, and the Virginia Tech Center for Drug Discovery for financial support. The following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium falciparum* Dd2 strain, MRA-150, deposited by D. Walliker. We thank Dr. Audrey Odom (Washington

University) for the gift of the plasmid containing *PfIspD* for protein expression. We thank Dr. Lance Wells and Dr. M. Osman Sheikh (University of Georgia) for the gift of the human IspD protein and ribitol-5-phosphate.

ABBREVIATIONS

MEP	methylethanol phosphate
IPP	isopentenyl pyrophosphate
DMAPP	dimethylallyl pyrophosphate
CTP	cytidine triphosphate
CDP-ME	cytidine diphosphate methylethanol
MEcPP	methylethanol cyclodiphosphate
FOS	fosmidomycin
SAR	structure–activity relationships
hpi	hours post-invasion
IC₅₀	half-maximum inhibitory concentration
LC₅₀	half-maximum lethal concentration

References

1. WHO. World Malaria Report 2015. World Health Organization; Geneva: 2016.
2. Spangenberg T, Burrows JN, Kowalczyk P, McDonald S, Wells TNC, Willis P. The Open Access Malaria Box: A Drug Discovery Catalyst for Neglected Diseases. *PLoS One*. 2013; 8(6):e62906. [PubMed: 23798988]
3. Guiguemde WA, Shelat AA, Garcia-Bustos JF, Diagana TT, Gamo FJ, Guy RK. Global phenotypic screening for antimalarials. *Chem Biol*. 2012; 19(1):116–29. [PubMed: 22284359]
4. Van Voorhis WC, Adams JH, Adelfio R, Ah Yong V, Akabas MH, Alano P, Alday A, Aleman Resto Y, Alsibae A, Alzualde A, Andrews KT, Avery SV, Avery VM, Ayong L, Baker M, Baker S, Ben Mamoun C, Bhatia S, Bickle Q, Bounaadia L, Bowling T, Bosch J, Boucher LE, Boyom FF, Brea J, Brennan M, Burton A, Caffrey CR, Camarda G, Carrasquilla M, Carter D, Belen Cassera M, Chih-Chien Cheng K, Chindaoudomsate W, Chubb A, Colon BL, Colon-Lopez DD, Corbett Y, Crowther GJ, Cowan N, D'Alessandro S, Le Dang N, Delves M, DeRisi JL, Du AY, Duffy S, Abd El-Salam El-Sayed S, Ferdig MT, Fernandez Robledo JA, Fidock DA, Florent I, Fokou PV, Galstian A, Gamo FJ, Gokool S, Gold B, Golub T, Goldgof GM, Guha R, Guiguemde WA, Gural N, Guy RK, Hansen MA, Hanson KK, Hemphill A, Hooft van Huijsduijnen R, Horii T, Horrocks P, Hughes TB, Huston C, Igarashi I, Ingram-Sieber K, Itoe MA, Jadhav A, Naranuntarat Jensen A, Jensen LT, Jiang RH, Kaiser A, Keiser J, Ketas T, Kicka S, Kim S, Kirk K, Kumar VP, Kyle DE, Lafuente MJ, Landfear S, Lee N, Lee S, Lehane AM, Li F, Little D, Liu L, Llinas M, Loza MI, Lubar A, Lucantoni L, Lucet I, Maes L, Mancama D, Mansour NR, March S, McGowan S, Medina Vera I, Meister S, Mercer L, Mestres J, Mfopa AN, Misra RN, Moon S, Moore JP, Morais Rodrigues da Costa F, Muller J, Muriana A, Nakazawa Hewitt S, Nare B, Nathan C, Narraido N, Nawaratna S, Ojo KK, Ortiz D, Panic G, Papadatos G, Parapini S, Patra K, Pham N, Prats S, Plouffe DM, Poulsen SA, Pradhan A, Quevedo C, Quinn RJ, Rice CA, Abdo Rizk M, Ruecker A, StOnge R, Salgado Ferreira R, Samra J, Robinett NG, Schlecht U, Schmitt M, Silva Villela F, Silvestrini F, Sinden R, Smith DA, Soldati T, Spitzmuller A, Stamm SM, Sullivan DJ, Sullivan W, Suresh S, Suzuki BM, Suzuki Y, Swamidass SJ, Taramelli D, Tchokouaha LR, Theron A, Thomas D, Tonissen KF, Townson S, Tripathi AK, Trofimov V, Udenze KO, Ullah I, Vallieres C, Vigil E, Vinetz JM, Voong Vinh P, Vu

- H, Watanabe NA, Weatherby K, White PM, Wilks AF, Winzeler EA, Wojcik E, Wree M, Wu W, Yokoyama N, Zollo PH, Abula N, Blasco B, Burrows J, Laleu B, Leroy D, Spangenberg T, Wells T, Willis PA. Open Source Drug Discovery with the Malaria Box Compound Collection for Neglected Diseases and Beyond. *PLoS Pathog.* 2016; 12(7):e1005763. [PubMed: 27467575]
5. Goodman CD, McFadden GI. Targeting apicoplasts in malaria parasites. *Expert Opin Ther Targets.* 2013; 17(2):167–77. [PubMed: 23231425]
 6. van Dooren GG, Striepen B. The algal past and parasite present of the apicoplast. *Annu Rev Microbiol.* 2013; 67:271–89. [PubMed: 23808340]
 7. Odom AR, Van Voorhis WC. Functional genetic analysis of the *Plasmodium falciparum* deoxyxylulose 5-phosphate reductoisomerase gene. *Mol Biochem Parasitol.* 2010; 170(2):108–11. [PubMed: 20018214]
 8. Nair SC, Brooks CF, Goodman CD, Strum A, McFadden GI, Sundriyal S, Anglin JL, Song Y, Moreno SN, Striepen B. Apicoplast isoprenoid precursor synthesis and the molecular basis of fosmidomycin resistance in *Toxoplasma gondii*. *J Exp Med.* 2011; 208(7):1547–59. [PubMed: 21690250]
 9. Yeh E, DeRisi JL. Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. *PLoS Biol.* 2011; 9(8):e1001138. [PubMed: 21912516]
 10. Wiley JD, Merino EF, Krai PM, McLean KJ, Tripathi AK, Vega-Rodriguez J, Jacobs-Lorena M, Klemba M, Cassera MB. Isoprenoid precursor biosynthesis is the essential metabolic role of the apicoplast during gametocytogenesis in *Plasmodium falciparum*. *Eukaryotic Cell.* 2015; 14(2):128–39. [PubMed: 25446055]
 11. Bowman JD, Merino EF, Brooks CF, Striepen B, Carlier PR, Cassera MB. Antiapicoplast and gametocytocidal screening to identify the mechanisms of action of compounds within the malaria box. *Antimicrob Agents Chemother.* 2014; 58(2):811–9. [PubMed: 24247137]
 12. Wu W, Herrera Z, Ebert D, Baska K, Cho SH, DeRisi JL, Yeh E. A chemical rescue screen identifies a *Plasmodium falciparum* apicoplast inhibitor targeting MEP isoprenoid precursor biosynthesis. *Antimicrob Agents Chemother.* 2015; 59(1):356–64. [PubMed: 25367906]
 13. Yao ZK, Krai PM, Merino EF, Simpson ME, Slebodnick C, Cassera MB, Carlier PR. Determination of the active stereoisomer of the MEP pathway-targeting antimalarial agent MMV008138, and initial structure-activity studies. *Bioorg Med Chem Lett.* 2015; 25(7):1515–9. [PubMed: 25754494]
 14. Imlay LS, Armstrong CM, Masters MC, Li T, Price KE, Edwards RL, Mann KM, Li LX, Stallings CL, Berry NG, O'Neill PM, Odom AR. *Plasmodium* IspD (2-C-Methyl-D-erythritol 4-Phosphate Cytidyltransferase), an Essential and Druggable Antimalarial Target. *ACS Infect Dis.* 2015; 1(4):157–167. [PubMed: 26783558]
 15. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. *Science.* 2006; 312(5778):1355–9. [PubMed: 16741115]
 16. Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ. Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. *Antimicrob Agents Chemother.* 2006; 50(9):3124–31. [PubMed: 16940111]
 17. Dahl EL, Rosenthal PJ. Apicoplast translation, transcription and genome replication: targets for antimalarial antibiotics. *Trends Parasitol.* 2008; 24(6):279–84. [PubMed: 18450512]
 18. Cassera MB, Gozzo FC, D'Alexandri FL, Merino EF, del Portillo HA, Peres VJ, Almeida IC, Eberlin MN, Wunderlich G, Wiesner J, Jomaa H, Kimura EA, Katzin AM. The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem.* 2004; 279(50):51749–59. [PubMed: 15452112]
 19. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D, Beck E. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science.* 1999; 285(5433):1573–1576. [PubMed: 10477522]

20. Tan KR, Magill AJ, Parise ME, Arguin PM. Centers for Disease, C.; Prevention, Doxycycline for malaria chemoprophylaxis and treatment: report from the CDC expert meeting on malaria chemoprophylaxis. *Am J Trop Med Hyg.* 2011; 84(4):517–531. [PubMed: 21460003]
21. Scholfield MR, Zanden CMV, Carter M, Ho PS. Halogen bonding (X-bonding): A biological perspective. *Protein Sci.* 2013; 22(2):139–152. [PubMed: 23225628]
22. Kunfermann A, Witschel M, Illarionov B, Martin R, Rottmann M, Höffken HW, Seet M, Eisenreich W, Knölker H-J, Fischer M, Bacher A, Groll M, Diederich F. Pseudilins: Halogenated, Allosteric Inhibitors of the Non-Mevalonate Pathway Enzyme IspD. *Angew Chem, Int Ed.* 2014; 53(8):2235–2239.
23. Cassera MB, Merino EF, Peres VJ, Kimura EA, Wunderlich G, Katzin AM. Effect of fosmidomycin on metabolic and transcript profiles of the methylerythritol phosphate pathway in *Plasmodium falciparum*. *Mem Inst Oswaldo Cruz.* 2007; 102(3):377–83. [PubMed: 17568945]
24. Zhang B, Watts KM, Hodge D, Kemp LM, Hunstad DA, Hicks LM, Odom AR. A second target of the antimalarial and antibacterial agent fosmidomycin revealed by cellular metabolic profiling. *Biochemistry.* 2011; 50(17):3570–7. [PubMed: 21438569]
25. Roscioli T, Kamsteeg EJ, Buysse K, Maystadt I, van Reeuwijk J, van den Elzen C, van Beusekom E, Riemersma M, Pfundt R, Vissers LE, Schraders M, Altunoglu U, Buckley MF, Brunner HG, Grisart B, Zhou H, Veltman JA, Gilissen C, Mancini GM, Delree P, Willemsen MA, Ramadza DP, Chitayat D, Bennett C, Sheridan E, Peeters EA, Tan-Sindhunata GM, de Die-Smulders CE, Devriendt K, Kayserili H, El-Hashash OA, Stemple DL, Lefeber DJ, Lin YY, van Bokhoven H. Mutations in ISPD cause Walker-Warburg syndrome and defective glycosylation of alpha-dystroglycan. *Nat Genet.* 2012; 44(5):581–5. [PubMed: 22522421]
26. Willer T, Lee H, Lommel M, Yoshida-Moriguchi T, de Bernabe DB, Venzke D, Cirak S, Schachter H, Vajsar J, Voit T, Muntoni F, Loder AS, Dobyns WB, Winder TL, Strahl S, Mathews KD, Nelson SF, Moore SA, Campbell KP. ISPD loss-of-function mutations disrupt dystroglycan O-mannosylation and cause Walker-Warburg syndrome. *Nat Genet.* 2012; 44(5):575–80. [PubMed: 22522420]
27. Gerin I, Ury B, Breloy I, Bouchet-Seraphin C, Bolsee J, Halbout M, Graff J, Vertommen D, Muccioli GG, Seta N, Cuisset JM, Dabaj I, Quijano-Roy S, Grahn A, Van Schaftingen E, Bommer GT. ISPD produces CDP-ribitol used by FKTN and FKRP to transfer ribitol phosphate onto alpha-dystroglycan. *Nat Commun.* 2016; 7:11534. [PubMed: 27194101]
28. Praissman JL, Willer T, Sheikh MO, Toi A, Chitayat D, Lin YY, Lee H, Stalnaker SH, Wang S, Prabhakar PK, Nelson SF, Stemple DL, Moore SA, Moremen KW, Campbell KP, Wells L. The functional O-mannose glycan on alpha-dystroglycan contains a phospho-ribitol primed for matriglycan addition. *eLife.* 2016; 5:e14473. [PubMed: 27130732]
29. Riemersma M, Froese DS, van Tol W, Engelke UF, Kopec J, van Scherpenzeel M, Ashikov A, Krojer T, von Delft F, Tessari M, Buczkowska A, Swiezewska E, Jae LT, Brummelkamp TR, Many H, Endo T, van Bokhoven H, Yue WW, Lefeber DJ. Human ISPD Is a Cytidyltransferase Required for Dystroglycan O-Mannosylation. *Chem Biol.* 2015; 22(12):1643–52. [PubMed: 26687144]
30. Perez-Gil J, Rodriguez-Concepcion M. Metabolic plasticity for isoprenoid biosynthesis in bacteria. *Biochem J.* 2013; 452(1):19–25. [PubMed: 23614721]
31. Ungemach F, Soerens D, Weber R, DiPierro M, Campos O, Mokry P, Cook JM, Silverton JV. General method for the assignment of stereochemistry of 1,3-disubstituted 1,2,3,4-tetrahydro- β -carboline by carbon-13 spectroscopy. *J Am Chem Soc.* 1980; 102(23):6976–6984.
32. Horiguchi Y, Nakamura M, Saitoh T, Sano T. A Synthesis of Chiral 1,1,3-Trisubstituted 1,2,3,4-Tetrahydro- β -carboline by the Pictet-Spengler Reaction of Tryptophan and Ketones: Conversion of (1*R*,3*S*)-Diastereomers into their (1*S*,3*S*)-Counterparts by Scission of the C(1)-N(2) bond. *Chem Pharm Bull.* 2003; 51(12):1368–1373. [PubMed: 14646311]
33. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother.* 2004; 48(5):1803–6. [PubMed: 15105138]
34. Paguio MF, Bogle KL, Roepe PD. *Plasmodium falciparum* resistance to cytotoxic versus cytostatic effects of chloroquine. *Mol Biochem Parasitol.* 2011; 178(1–2):1–6. [PubMed: 21470564]

35. King DT, Wasney GA, Nosella M, Fong A, Strynadka NC. Structural Insights into Inhibition of *Escherichia coli* Penicillin-binding Protein 1B. *J Biol Chem*. 2017; 292(3):979–993. [PubMed: 27899450]
36. Sabra A, Bessoule JJ, Atanasova-Penichon V, Noel T, Dementhon K. Host-pathogen interaction and signaling molecule secretion are modified in the dpp3 knockout mutant of *Candida lusitanae*. *Infect Immun*. 2014; 82(1):413–22. [PubMed: 24191303]
37. Wang M, Yang X, Ren L, Li S, He X, Wu X, Liu T, Lin L, Li Y, Sun C. Biomarkers identified by urinary metabonomics for noninvasive diagnosis of nutritional rickets. *J Proteome Res*. 2014; 13(9):4131–42. [PubMed: 25051233]
38. Laouridakis CD, Merino EF, Neilson AP, Cassera MB. Comprehensive quantitative analysis of purines and pyrimidines in the human malaria parasite using ion-pairing ultra-performance liquid chromatography-mass spectrometry. *J Chromatogr B: Anal Technol Biomed Life Sci*. 2014; 967:127–33.

Figure 1. MMV008138 as originally disclosed, its active stereoisomer **1a** ((1*R*,3*S*)-MMV008138), and selected analogs.

Figure 2.

LC₅₀ value of **1a** decreases with increasing drug exposure time. (A) Parasite growth was measured at 72 h by the SYBR Green assay in cultures where **1a** was washed out at 6 or 12 h, and parasites were returned to culture to complete 72 h. A representative growth inhibition curve at 72 h from parasites continuously exposed to **1a** is also shown. (B) **1a** becomes cytotoxic at 18 h drug bolus time when parasites reach late trophozoite/early schizont stages. The reported values represent averages and SEM of at least three independent assays.

Figure 3.

Highly synchronous parasites were recovered at different stages of *P. falciparum* intraerythrocytic cycle (12 hpi: rings; 19 hpi: trophozoites; 33 hpi: schizonts), and the presence of the MEP pathway intermediates was assessed by quantitative LC-MS/MS where only CDP-ME, MEcPP, and IPP were detected. The levels of all three detected intermediates increased markedly between trophozoite (19 hpi) and schizont (33 hpi) stages. The response of each detected metabolite is expressed as the mean and SEM of two biological replicates.

1: 1-Deoxy-D-xylulose-5-phosphate (DOXP) reductoisomerase; 2: IspD; 3: CDP-ME kinase; 4: 2C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) synthase; 5: 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) synthase; 6: HMBPP reductase.

Figure 4.

In vivo IspD inhibition assessed by quantitative LC-MS/MS. Highly synchronous schizont stage parasites were treated for 2 and 4 h with 1 μ M **1a** or **7a**. FOS was used as positive control at 10 μ M. After 2 h of drug exposure, **7a** caused a greater reduction on the cellular levels of the *Pf*IspD product CDP-ME than **1a**. The detected difference was statistically significant by the Benjamini-Hochberg significance test. Reduction of the cellular levels of the MEcPP and the final MEP pathway product, IPP, did not show significant differences among the three treatments after 2 and 4 h of exposure to **1a**, **7a**, and FOS as assessed by the Benjamini-Hochberg significance test. Results are expressed as a percentage of untreated control $\pm$ SEM of two biological replicates.

Figure 5.

Human IspD is not targeted by **1a**. Human IspD enzymatic activity in the absence or presence of 20 and 200 μ M of **1a** was measured by LC-MS/MS to monitor CDP-ribitol formation. The CDP-ribitol formation was not inhibited by the presence of **1a** at either concentration. The small reductions observed were not statistically significant by the Benjamini-Hochberg significance test. Mean $\pm$ SEM values were calculated from two independent assays.

Scheme 1.
Synthesis of 1a and Its D-Ring Analogs

Scheme 2.

Synthesis of C1-Methyl Analogs of 1a

Table 1

*Pf*IspD Inhibition Potency of MMV008138 Stereoisomers and FOS

compound	configuration	<i>P. falciparum</i> growth inhibition IC ₅₀ (nM) ^a	% recovery (200 μ M IPP) ^c	<i>Pf</i> IspD IC ₅₀ (nM) ^d	% <i>E. coli</i> growth inhibition (18 h) ^e
1a	(1 <i>R</i> ,3 <i>S</i>)	250 $\pm$ 70 ^b	100 @ 2.5 μ M ^b	44 $\pm$ 15	NI @ 500 μ M ^b
5a	(1 <i>S</i> ,3 <i>S</i>)	>10 000 ^b	ND ^b	NI @ 1 μ M	NI @ 125 μ M
<i>ent</i> - 1a	(1 <i>S</i> ,3 <i>R</i>)	>10 000 ^b	ND ^b	NI @ 1 μ M	NI @ 250 μ M
<i>ent</i> - 5a	(1 <i>R</i> ,3 <i>R</i>)	3000 $\pm$ 200 ^b	60 $\pm$ 5 @ 10 μ M ^b	27 $\pm$ 5% inh. @ 1 μ M	18 $\pm$ 6 @ 250 μ M
FOS	NA	880 $\pm$ 70 ^b	100 ^b	4 $\pm$ 2% inh. @ 10 μ M	11.99 $\pm$ 0.02 μ M (MIC)

^aMRA-150, chloroquine-resistant (intermediate), pyrimethamine-resistant, mefloquine-resistant.

^bData reported previously.¹³

^cDrug at indicated concentration.

^dRecombinant *P. falciparum* IspD IC₅₀ values measured at 60 nM *Pf*IspD.

^ePercent growth inhibition at the indicated concentration or the minimum inhibitory concentration (MIC). Values represent averages of at least two independent assays $\pm$ SEM. NA signifies “not applicable”. ND signifies “not determined”. NI signifies “no inhibition”.

Table 2

Growth Inhibition (*P. falciparum*, *E. coli*) and *Pf*IspD Inhibition of D-Ring Variants of 1a

compound ^a	X	<i>P. falciparum</i> growth inhibition IC ₅₀ (nM) ^f	% recovery (200 μM IPP) ^e	<i>Pf</i> IspD IC ₅₀ (nM) ^f	% <i>E. coli</i> growth inhibition (18 h) ^g
1b	H	>10 000 ^d	ND	>5000 ^g	NI @ 250 μM
1c	2'-Cl	3280 ± 990 ^d	60 ± 5 @ 10 μM	~1000	NI @ 250 μM
1d	4'-Cl	1170 ± 60 ^d	50 ± 7 @ 10 μM	510 ± 90	NI @ 250 μM
1e	2'-Cl,4'-Me	410 ± 40 ^d	100 @ 2.5 μM	82 ± 10	NI @ 500 μM ^c
1f	2'-Me,4'-Cl	700 ± 90 ^d	100 @ 2.5 μM	260 ± 50	NI @ 500 μM ^c
1g	2',4'-F ₂	780 ± 175 ^d	100 @ 5 μM	230 ± 10	NI @ 500 μM ^c
1h	2'-F,4'-Cl	860 ± 80	100 @ 5 μM	140 ± 30	NI @ 250 μM
1i	2'-Cl,4'-F	433 ± 55	100 @ 10 μM	100 ± 10	NI @ 250 μM
1j	2'-Cl,4'-Br	320 ± 60	100 @ 5 μM	34 ± 11	NI @ 250 μM
1k	2'-Br,4'-Cl	360 ± 40	100 @ 5 μM	31 ± 4	NI @ 125 μM
1l	2',4'-Br ₂	590 ± 20	100 @ 10 μM	84 ± 14	NI @ 125 μM
1m	2'-I,4'-F	970 ± 180	100 @ 10 μM	140 ± 70	NI @ 250 μM
1n	2'-F,4'-I	3343 ± 496	100 @ 10 μM	130 ± 20	NI @ 125 μM
1o	2'-Br,4'-I	1500 ± 200	80 ± 5 @ 10 μM	ND	ND
1p	2'-Cl,4'-OMe	>5000	ND	ND	ND
1q	2'-OMe,4'-Cl	2500 ± 600	ND	ND	ND

compound ^a	X	<i>P. falciparum</i> growth inhibition IC ₅₀ (nM) ^c	% recovery (200 μ M IPP) ^e	<i>Pf</i> /spD IC ₅₀ (nM) ^f	% <i>E. coli</i> growth inhibition (18 h) ^h
1r^b	2'-Cl,4'-OH	>5000	ND	ND	ND
1s	2'-Cl,4'-CO ₂ H	NI @ 10 000	ND	ND	ND
1t	2',4'-(CF ₃) ₂	>10 000 ^d	ND	>5000 ^g	NI @ 250 μ M
1u	2',4'-Me ₂	70% inh. @ 10 000 ^d	ND	~1000	NI @ 250 μ M
1v	2',4'-(OMe) ₂	>20 000 ^d	ND	>5000 ^g	NI @ 250 μ M
1w	3',4'-Cl ₂	>10 000	ND	NI @ 500 nM	NI @ 250 μ M
1x	3',4'-(OMe) ₂	>20 000 ^d	ND	ND	ND
1y	2',6'-F ₂ ,4'-Cl	1800 $\pm$ 150	80 $\pm$ 8 @ 10 μ M	ND	ND
1z	2',3',4'-F ₃	~20 000	ND	ND	ND
1aa	2'-Br,4'-F,5'-OMe	>10 000	ND	ND	ND
1ab	NA	>10 000	ND	ND	ND
1ac	NA	>5000	ND	ND	ND
1ad	NA	>10 000	ND	ND	ND
1ae	NA	>10 000	ND	ND	ND
1af	NA	>5000	ND	ND	ND

^a All compounds are *trans*-configured and derived from (*S*)-Trp-OMe; all are (1*R*,3*S*) except for **1ac**, which is (1*S*,3*S*) due to a Cahn-Ingold-Prelog priority switch.

^b This compound was tested as an approximate 1:1 mixture of the (1*R*,3*S*) and (1*S*,3*S*) stereoisomers.

^c MRA-150, chloroquine-resistant (intermediate), pyrimethamine-resistant, mefloquine-resistant.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

^dData reported previously.¹³

^eDrug at indicated concentration.

^fRecombinant *P. falciparum* IspD IC₅₀ values measured at 60 nM *PI*IspD.

^gApproximately 10% inhibition at 1000 nM.

^hNA signifies “not applicable”. NI signifies “no inhibition”. ND signifies “not determined”. Values represent averages of at least two independent assays ± SEM.

Table 3
Growth Inhibition (*P. falciparum*, *E. coli*) and *Pf*IspD Inhibition of C3-Carboxy Replacement Variants of 1a

Compound	X	<i>P. falciparum</i> growth inhibition IC ₅₀ (nM) ^d	% recovery (200 μ M IPP) ^d	<i>Pf</i> IspD IC ₅₀ (nM) ^e	% <i>E. coli</i> growth inhibition (18 h) ^f
3a	2',4'-Cl ₂	6800 $\pm$ 1400 ^c	20 $\pm$ 10 @ 20 μ M	NI @ 1000	NI @ 250 μ M
6a	2',4'-Cl ₂	1200 $\pm$ 100 ^c	50 $\pm$ 7 @ 10 μ M	~1000	NI @ 250 μ M
7a	2',4'-Cl ₂	190 $\pm$ 30 ^c	100 @ 2.5 μ M	57 $\pm$ 10	NI @ 500 μ M ^c
7e	2'-Cl,4'-Me	340 $\pm$ 50 ^c	100 @ 2.5 μ M	360 $\pm$ 40	NI @ 500 μ M ^c
7i	2'-Cl,4'-F	506 $\pm$ 45	100 @ 2.5 μ M	278 $\pm$ 27	21 $\pm$ 3 @ 250 μ M
7j	2'-Cl,4'-Br	300 $\pm$ 40	100 @ 5 μ M	21 $\pm$ 6	NI @ 250 μ M
7k	2'-Br,4'-Cl	340 $\pm$ 60	100 @ 5 μ M	31 $\pm$ 4	NI @ 250 μ M
8a	2',4'-Cl ₂	~5000	ND	~1000	NI @ 250 μ M
9a	2',4'-Cl ₂	>20 000	ND	~5% inh. @ 1000	NI @ 250 μ M
10a	2',4'-Cl ₂	>10 000	ND	ND	ND
11a	2',4'-Cl ₂	10 000 $\pm$ 1600 ^c	NI @ 20 μ M	7% inh. @ 1000	45 $\pm$ 10 @ 250 μ M

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

^gExcept for (±)-**11a**, all compounds are *trans*- and are (1*R*,3*S*)-configured.

^hMRA-150, chloroquine-resistant (intermediate), pyrimethamine-resistant, mefloquine-resistant.

^cData reported previously.¹³

^dDrug at indicated concentration.

^eRecombinant *P. falciparum* IspD IC₅₀ values measured at 60 nM *PT*IspD.

^fPercent growth inhibition at the indicated concentration. NI signifies “no inhibition”; ND signifies “not determined”. Values represent averages of at least two independent assays ± SEM.