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Determination of the active stereoisomer of the MEP pathway-targeting antimalarial agent MMV008138, and initial structure-activity studies

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Abstract

Compounds that target isoprenoid biosynthesis in *Plasmodium falciparum* could be a welcome addition to malaria chemotherapy, since the methylerythritol phosphate (MEP) pathway used by the parasite is not present in humans. We previously reported that MMV008138 targets the apicoplast of *P. falciparum* and that its target in the MEP pathway differs from that of Fosmidomycin. In this article, we determine that the active stereoisomer of MMV008138 is **4a**, which is (1*R*,3*S*)-configured. 2',4'-Disubstitution of the D ring was also found to be crucial for inhibition of the parasite growth. Limited variation of the C3-carboxylic acid substituent was carried out, and methylamide derivative **8a** was found to be more potent than **4a**; other amides, acylhydrazines, and esters were less potent. Finally, lead compounds **4a**, **4e**, **4f**, **4h**, **8a**, and **8e** did not inhibit growth of *Escherichia coli*, suggesting that protozoan-selective inhibition of the MEP pathway of *P. falciparum* can be achieved.

Keywords

malaria; *Plasmodium*; apicoplast; MEP pathway; Non-mevalonate

Malaria continues to be one of the most deadly diseases worldwide, with an estimated 584,000 deaths in 2013.¹ The reductions seen in annual mortality over the last 10 years are greatly welcomed, but these advances are at risk due to several factors. Chief among these risk factors is emerging resistance to existing antimalarial drugs, such as chloroquine and

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Supplementary Material

Supplementary data (38 pages) includes synthetic procedures, analytical tabulations, and copies of the ¹H and ¹³C NMR spectra for all tested compounds; biological assay procedures and biological data can be found in the online version.

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artemether (Figure 1). Thus, there is a pressing need to develop antimalarial drugs that engage new targets. Since the discovery that the malaria parasite *Plasmodium falciparum* carried an apicoplast, there has been considerable interest in developing chemical agents that could specifically target this relict chloroplast, due to its unusual non-mammalian metabolism.² Of particular interest is the fact that isoprenoid precursor biosynthesis in *P. falciparum* occurs in the apicoplast not via the mevalonate pathway, but rather through the methylerythritol phosphate (MEP) pathway.³ Moreover, supply of isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) is the sole essential function of this organelle during disease-causing asexual intraerythrocytic stages of the parasite⁴ and during gametocyte development, the malaria stages transmitted to the mosquito.⁵ Since the MEP pathway is absent in humans, agents targeting it could be both safe to the human host and effective. However, the MEP pathway is present in the human microbiome,⁶ therefore, compounds that can selectively inhibit the *P. falciparum* enzymes are highly desirable to reduce their impact on the host microbiome. Fosmidomycin (FOS) was shown to inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR or IspC), the second enzyme in the MEP pathway, and it cured mice infected with *P. vinckei*.^{3a} In combination with clindamycin, FOS was evaluated in clinical trials.⁷ However, because of poor oral bioavailability, recent attention has focused on development of more hydrophobic FOS analogs.⁸

To identify chemotypes that might inhibit other enzymes in the MEP pathway, the compounds present in the publicly available Malaria Box⁹ were evaluated against asexual blood-stages of *P. falciparum* Dd2 strain. IPP supplementation⁴ was then employed to attempt chemical rescue of drug-treated parasites; in the case of FOS, 100% rescue was observed, consistent with its proposed mechanism of action.¹⁰ Of the 400 compounds in the Malaria Box that showed >95% growth inhibition at 5 μ M, only MMV008138 exhibited >60% rescue following the addition of 200 μ M IPP.¹⁰ Several antibiotics such as doxycycline kill malaria parasites by interfering with apicoplast genome replication, transcription, or protein translation.¹¹ Parasites treated with these antibiotics initially present normal morphology and continue to grow and release daughter cells (merozoites) that are capable of invading new host cells. However, this progeny will not complete the subsequent intraerythrocytic cycle since the apicoplast will fail to replicate and segregate; this phenomenon is known as the delayed death phenotype.¹² Like FOS, MMV008138 did not show a delayed death phenotype, and inhibition of apicoplast elongation was reversed by IPP supplementation; such rescue is not seen with antibiotics like doxycycline. However, the efficacy of MMV008138 observed against FOS-resistant parasites suggested that its target within the MEP pathway is not DXR.¹⁰

Since the stereochemical purity and identity of MMV008138 was not known, we synthesized all four possible stereoisomers by Pictet-Spengler reaction¹³ of (*S*)- and (*R*)-tryptophan methyl ester (Trp-OMe) with 2,4-dichlorobenzaldehyde **1a** (Scheme 1).

Diastereomers **2a** and **3a** derive from (*S*)-Trp-OMe and were easily separated by column chromatography; the relative configuration of the C1 substituent (*cis*- or *trans*-) in each compound was determined by ¹³C NMR spectroscopy, using the empirical rule of Cook.¹⁴ Similarly (*R*)-Trp-OMe provided *ent*-**2a** and *ent*-**3a**. Hydrolysis of the esters using an

Amberlyst resin catch and release protocol¹⁵ gave the desired stereoisomers of MMV008138, **4a**, **5a**, *ent*-**4a**, and *ent*-**5a**, free from inorganic salt contamination. ¹H NMR spectroscopic analysis of commercial MMV008138 indicated that it was >95% *trans*-configured, like **4a** or *ent*-**4a** (Supplementary Material, Figure S1). The four stereoisomers were then examined for growth inhibitory activity and reversal of growth inhibition by 200 μ M IPP (Table 1), using MMV008138 and FOS as controls. As can be seen, **4a** ($IC_{50} = 250 \pm 70$ nM) was by far the most potent stereoisomer of the four (cf. entries 3, 4, 15 and 16). Compounds **5a** and *ent*-**4a** showed no inhibition of growth at the highest concentration tested (10,000 nM), and *ent*-**5a** had an IC_{50} of 3,000 nM.

Furthermore, the *P. falciparum* toxicity of **4a** was reversed (100% at 2.5 μ M) by supplementation with IPP, similar to MMV008138. These observations and the similar measured IC_{50} values for MMV008138 and **4a** (210 ± 90 and 250 ± 70 nM, entries 2 and 3 respectively) confirm that MMV008138 is the pure (1*R*,3*S*)-stereoisomer and is thus properly depicted as **4a**.

To ascertain the structural determinants of the apicoplast-targeting growth inhibitory activity of **4a**, we explored the effect of variation of the D-ring substituents. With slight modification, the same synthetic methods were used to prepare (1*R*,3*S*)-configured acid derivatives **4b–k** from benzaldehydes **1b–k**, and a smaller number of (1*S*,3*S*)-configured acids **5b**, **e**, **g**, **h** were also investigated (Table 2).

Bioassays of **4b–k** revealed that potent growth inhibition requires 2',4'-disubstitution of the D-ring. Compound **4b** lacking D-ring substitution has no toxicity to *P. falciparum* at 10,000 nM (Table 1 entry 5). Growth inhibitory activity is regained with a 2'-Cl (**4c**) or a 4'-Cl (**4d**) substituent, though with substantially reduced potencies (Table 1 entries 6–7). Among the 2',4'-disubstitution patterns explored, the relatively conservative 2'-chloro-4'-methyl (**4e**, Table 1 entry 8) and 2'-methyl-4'-chloro (**4f**, Table 1 entry 9) substitutions came closest to recapitulating the activity of **4a**. Although **4e** ($IC_{50} = 410 \pm 40$ nM) is not as potent as **4a** (cf. Table 1 entries 8 and 3), it demonstrated full rescue by 200 μ M IPP supplementation. The size of the substituents at positions 2' and 4' of the D ring appears to be critical: the 2',4'-difluoro analog (**4h**, Table 1 entry 11) is more potent than the 2',4'-dimethyl analog (**4g**, Table 1 entry 10), and both the 2',4'-dimethoxy and 2',4'-bis(trifluoromethyl) analogs failed to show growth inhibitory activity at 10,000 or 20,000 nM (**4i** and **4j**, Table 1 entries 12–13). The sole case of 3',4'-disubstitution explored (**4k**, Table 1 entry 14) did not inhibit parasite growth at 20,000 nM. Thus the steric requirements for the D-ring substituents are very precise. It seems likely that the 2'- and 4'-substituents complement the shape of a rather tight binding pocket. In addition, the presence of the 2'-substituent may also constrain the D-ring to lie perpendicular to the C-ring. Finally, a limited number of (1*S*,3*S*)-configured analogs was explored (**5b**, **5e**, **5g**, **5h**). As expected based on the low potency of **5a**, none inhibited parasite growth at 10,000 or 20,000 nM (Table 1, entries 17–20).

Since the carboxylic acid group is often considered a liability in drug development, we investigated a limited series of analogs of **4a** that varied in the C3 substituent (Table 3). Compound **2a**, the methyl ester precursor of **4a** had only weak growth inhibitory activity ($IC_{50} = 6,800 \pm 1,400$ nM, Table 3 entry 1).

Thus the methyl ester group cannot recapitulate the binding of the carboxylic acid/carboxylate of **4a**, though it might serve well in a prodrug. A number of other esters were examined and found to be inactive; in particular *ent*-**2a** and **2b** (the methyl ester precursors of *ent*-**4a** and **4b**) had little growth inhibitory activity (Table 3, entries 2–3). Tryptamine was condensed with **1a** to yield ($\pm$)-**6a** in 42 % yield, which lacks a C3-substituent (Scheme 2). As can be seen in Table 3, ($\pm$)-**6a** is only a weak inhibitor of parasite growth (entry 4). More success was obtained with amide and hydrazide derivatives, that were simply prepared by treatment of the requisite methyl ester with the desired amine or hydrazine (Scheme 2).

The primary amide **7a** was weakly potent, but methylamide **8a** proved slightly more potent than **4a** ($IC_{50} = 190 \pm 30$ nM, Table 3 entry 6, $p < 0.04$ ANOVA test) and exhibited 100% rescue upon IPP supplementation. X-ray crystallography of **8a** confirmed (1*R*,3*S*)-configuration (Figure 2).

Methylamide **8e** featuring a 2'-chloro-4'-methylphenyl group in place of 2',4'-dichlorophenyl also showed good potency ($IC_{50} = 340 \pm 50$ nM, Table 3 entry 7); methylamides **8f–h** proved to be less potent. Methylamide **8j** had no activity, consistent with the low activity of the corresponding acid **4j**. Amides featuring larger alkyl groups (**9a–11a**, Table 3 entries 12–14) were also less potent than methylamide **8a**. One acylhydrazine **12a** was explored, and proved to be less potent than **8a** as well.

Finally, we noted that the amide derivatives bore a resemblance to the erectile dysfunction drug tadalafil. As can be seen in Table 3, this compound showed no growth inhibition at 20,000 nM. This low potency can be attributed to at least three factors: i) non-optimum (*R,R*)-configuration; ii) 3',4'-disubstitution on the D-ring; and iii) the as yet unknown effect of rendering N2 non-basic.

To assess possible off-target effects of potent inhibitors **4a** and **8a** we explored their parasite growth inhibition activity in the presence of 200 μ M IPP (Figure 3). As can be seen, the presence of 200 μ M IPP raises the IC_{50} values of these compounds to roughly 10,000 nM. Similar results were seen for **4e**, **4f**, and **4h** (Supplementary Material, Figure S2). Thus the off-target *P. falciparum* growth inhibition activity of these compounds occurs at concentrations roughly 40-fold higher than those that target the MEP pathway. In addition we note that when tested at 10–20 μ M, many of the weakly potent (micromolar IC_{50} values) analogs in Tables 1 and 3 do not show full rescue upon IPP supplementation (e.g. **4c,d**, *ent*-**5a**, **2a**, *ent*-**2a**, ($\pm$)-**6a**, **7a**, **8f**, **11a**, **12a**). It is thus likely that the same off-target toxic mechanisms are also operative for these compounds. As mentioned above, the MEP pathway is present in the human microbiome and specificity of these inhibitors to the malaria parasite is highly desirable. Therefore, compounds **4a**, **4e**, **4f**, **4h**, **8a**, **8e**, and **8h**, were tested for antibacterial activity against *Escherichia coli*. None of these seven compounds inhibited *E. coli* growth at 500 μ M (Supplementary Material, Figure S3).

In conclusion, we have demonstrated that the active stereoisomer of MMV008138 is structure **4a**. In addition our survey of 33 analogs of **4a** demonstrated that potent MEP pathway-targeting antiparasitic activity within this scaffold requires a) (1*R*,3*S*)-configuration; b) a carboxylate/carboxylic acid or methylamide functionality at C3; and c)

and sterically modest 2',4'-disubstitution of the D-ring. Lead compounds **4a**, **4e**, **4f**, **4h**, **8a**, and **8e** have *P. falciparum* growth inhibition potencies ranging from 190 to 780 nM, and do not inhibit the growth of *E. coli* at 500 μ M. Thus it is possible to selectively inhibit the MEP pathway of *P. falciparum* without affecting that of helpful gut bacteria; these tetrahydro- β -carboline derivatives therefore represent promising new antimalarial leads. Recently, other researchers have identified the molecular target of MMV008138 as IspD, the third enzyme of the MEP pathway; these authors also concluded that the active stereoisomer of MMV008138 was (1*R*,3*S*)-configured.¹⁷ Our characterization of the aforementioned lead compounds against that and related enzymes, and further structural optimization studies will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Antimalarial agents used clinically (chloroquine, artemether) and MEP pathway-targeting drug candidates (FOS, MMV008138).

Figure 2. Anisotropic displacement ellipsoid drawings (50%) of the X-ray structure of compound **8a**. This structure was deposited at the Cambridge Structural Database (CCDC-1042325) and was visualized using OLEX2.¹⁶

Figure 3.

Effects of **4a** and **8a** on *P. falciparum* Dd2 strain growth in the absence and presence of 200 μ M IPP (72 h incubation). Data represent the average and standard error of five independent experiments.

Scheme 1.

Synthesis of the possible stereoisomers of MMV008138 (**4a**, **5a**) and methyl ester precursors (**2a**, **3a**). i) 4 Å molecular sieves, CH₂Cl₂, 20 h; TFA (2 equiv, 44 h). ii) Amberlyst hydroxide, THF/MeOH/H₂O; AcOH, H₂O.

Scheme 2.

Synthesis of C3 variants of **4a**/MMV008138. i) 2,4-dichlorobenzaldehyde (**1a**), 4 Å molecular sieves, CH₂Cl₂, 24 h. ii) Requisite amine or hydrazine, 25 °C or 50 °C (**9a**, **11a**), 24 h.

Table 1

P. falciparum growth inhibitory activity and effect of IPP supplementation of MMV08138 stereoisomers and D-ring variants.

Entry	Compound	X	<i>P. falciparum</i> Dd2 strain ^a IC ₅₀ (nM)	% Rescue (+ 200 μM IPP) ^b
1	FOS		880 ± 70	100 @ 10 μM
2	MMV008138	2',4'-Cl ₂	210 ± 90	100 @ 2.5 μM
3	4a	2',4'-Cl ₂	250 ± 70	100 @ 2.5 μM
4	ent-4a	2',4'-Cl ₂	>10,000	ND
5	4b	H	>10,000	ND
6	4c	2'-Cl	3,280 ± 990	60 @ 10 μM
7	4d	4'-Cl	1,170 ± 60	50 @ 10 μM
8	4e	2'-Cl, 4'-Me	410 ± 40	100 @ 2.5 μM
9	4f	2'-Me, 4'-Cl	700 ± 90	100 @ 2.5 μM
10	4g	2',4'-Me ₂	70% inhibition @ 10,000	ND
11	4h	2',4'-F ₂	780 ± 175	100 @ 5 μM
12	4i	2',4'-(OMe) ₂	>20,000	ND
13	4j	2',4'-(CF ₃) ₂	>10,000	ND
14	4k	3',4'-(OMe) ₂	>20,000	ND
15	5a	2',4'-Cl ₂	>10,000	ND
16	ent-5a	2',4'-Cl ₂	3,000 ± 200	60 @ 10 μM
17	5b	H	>10,000	ND
18	5e	2'-Cl, 4'-Me	>10,000	ND
19	5g	2',4'-Me ₂	>10,000	ND
20	5h	2',4'-F ₂	>20,000	ND

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

^b Drug at indicated concentration; nd signifies "not determined."

Table 2

Synthesis and isolated yields for D-ring variants of **2a** and **3a**, and those of their acid derivatives (**4** and **5**).

	%Yield	%Yield	%Yield	%Yield			
2b	14	3b	67	4b	99	5b	95
2c	18	3c	59	4c	95		
2d	20	3d	61	4d	95		
2e	40	3e	60	4e	78	5e	69
2f	21	3f	37	4f	56		
2g	40	3g	52	4g	85	5g	83
2h	30	3h	68	4h	74	5h	68
2i	27	3i	19	4i	90		
2j	39	3j	61	4j	84		
2k	41	3k	36	4k	86		

Table 3

The effect of C3-substitution on the *P. falciparum* growth inhibitory activity of **4a** and D-ring variants.

Entry	Compound	R	<i>P. falciparum</i> Dd2 strain ^a IC ₅₀ (nM)	% Rescue (+ 200 μM IPP) ^b
1	2a	CO ₂ Me	6,800 ± 1,400	~ 20 @ 20 μM
2	<i>ent</i> - 2a	CO ₂ Me	7,700 ± 1,400	0 @ 20 μM
3	2b	CO ₂ Me	~20% inhibition @ 20,000	ND
4	(±)- 6a	H	10,000 ± 1,600	0 @ 20 μM
5	7a	CONH ₂	1,200 ± 100	50 @ 10 μM
6	8a	CONHMe	190 ± 30	100 @ 2.5 μM
7	8e	CONHMe	340 ± 50	100 @ 2.5 μM
8	8f	CONHMe	1,850 ± 600	50 @ 10 μM
9	8g	CONHMe	70% inhibition @ 10,000	ND
10	8h	CONHMe	1,200 ± 100	100 @ 2.5 μM
11	8j	CONHMe	>10,000	ND
12	9a	CONHi-Pr	50% inhibition @ 10,000	ND
13	10a	CONHBu	80% inhibition @ 10,000	ND
14	11a	CONH <i>c</i> -C ₆ H ₁₁	2,830 ± 500	0 @ 10 μM
15	12a	CONHNHMe	1,940 ± 200	80 @ 10 μM
16	tadalafil	NA ^c	>20,000	ND

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

^bDrug at indicated concentration; ND signifies “not determined.”

^cNot applicable.