

Sphingolipid Analogues Inhibit Development of Malaria Parasites

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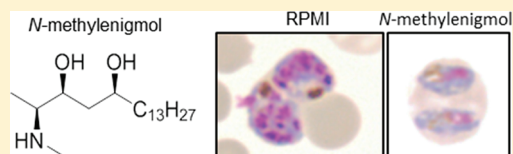
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Supporting Information

ABSTRACT: *Plasmodium*-infected erythrocytes have been shown to employ sphingolipids from both endogenous metabolism as well as existing host pools. Therapeutic agents that limit these supplies have thus emerged as intriguing, mechanistically distinct putative targets for the treatment of malaria infections. In an initial screen of our library of sphingolipid pathway modulators for efficacy against two strains of the predominant human malaria species *Plasmodium falciparum* and *Plasmodium knowlesi*, a series of orally available, 1-deoxysphingoid bases were found to possess promising in vitro antimalarial activity. To better understand the structural requirements that are necessary for this observed activity, a second series of modified analogues were prepared and evaluated. Initial pharmacokinetic assessments of key analogues were investigated to evaluate plasma and red blood cell concentrations in vivo.

KEYWORDS: Enigmols, sphingolipids, malaria, drugs, antimalarials, *Plasmodium*



Novel antimalarials are especially needed in the face of widespread drug resistance against *Plasmodium falciparum* and to provide alternative compounds for inclusion in future combination therapies.¹ Data have also emerged demonstrating that the sphingolipid pool is an important factor in modulating vital eukaryotic cellular functions, including those of the protozoan genus, *Plasmodium*. In malaria infections, membrane formation is critical for the growth and development of the *Plasmodium* parasite inside host erythrocytes. New membraneous structures are formed as the parasite invades these host red blood cells (RBCs) with the formation of a parasitophorous vacuole and then throughout the RBC cytoplasm to create trafficking networks, critical for the import and export of nutrients and waste products as the parasite grows and multiplies through its development stages.² The *Plasmodium* sphingolipid metabolic pathway is known to have activity of several enzymes (e.g., sphingomyelin synthase, glucosylceramide synthase, and two sphingomyelinases),^{3–6} and compounds such as fumonisins B⁷ or 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP)^{5,8} have been shown to interfere with the sphingolipid metabolism of *P. falciparum* (see the Supporting Information for the structures of these and other compounds that have been investigated in the context of their effect on *P. falciparum*). Here, we show that selected sphingoid base analogues inhibit the normal growth and development of both *P. falciparum*- and *Plasmodium knowlesi*-infected erythrocytes, and morphological studies

suggest that the inhibitory effect begins in the ring stage of the parasite's development.

Since the mid-1990s, we have been designing sphingolipid analogues to modulate the biosynthesis of sphingolipids, such as sphingosine, to suppress cellular proliferation for use as anticancer agents with a higher safety margin than those that are currently available. Toward this end, we have compiled a rationally designed library of synthetic analogues and, in doing so, developed several synthetic routes toward this compound class.^{9–12} Recently, other groups have also shown interest in this area and have published additional synthetic routes toward this class of compounds.^{13–18} Of particular interest to us are the 1-deoxy-5-hydroxy-sphingoid base analogues, since they retain many of the physical characteristics of the natural sphingolipids but lack a C-1 primary hydroxyl group. We hypothesized that by trans-locating the C-1 hydroxyl group to the C-5 position, we would maintain similar compound hydrophobicity and enzymatic recognition while eliminating the possibility for phosphorylation of the C-1 hydroxyl group by sphingosine kinase (SK). This is desirable since the phosphate intermediates formed by SK are subject to catabolic degradation and also possess undesirable pro-mitogenic and antiapoptotic properties.

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Given the significant role(s) that sphingolipids may play in the formation of membrane structures of the malaria parasite growing in RBCs, we hypothesized that our existing lead enigmol¹² **1** and its analogues may also have unique potential as novel antimalarial agents. Enigmol was shown to specifically interrupt the sphingolipid pathway in a pleiotropic manner¹⁹ and thus had the potential to impede development of the parasite in the infected RBC. We selected enigmol and an initial set of existing novel sphingolipid analogs to study the potential of such compounds as anti-malarial therapies. The syntheses of enigmol, and its analogs appearing in Table 1 were performed

Table 1. First Generation Sphingolipid Analogue SAR Data^a

Compound	Structure	IC ₅₀ (μM)		
		PfW2	PfD6	Pk
Enigmol 1		7.4 ±1.5	12.6 ±2.4	10.5 ±0.4
2		9.2 ±1.6	17.8 ±1.1	20.3 ±2.0
3		8.1 ±0.2	14.6 ±0.6	NT
4		8.2 ±1.8	12.7 ±1.2	17.6 ±1.8
N-methyl-enigmol 5		1.6 ±0.1	4.7 ±0.1	1.7 ±0.1
6		4.4 ±1.1	7.0 ±0.6	6.8 ±0.1
7		4.4 ±1.5	9.7 ±1.0	6.3 ±0.3
8		5.6 ±0.1	12.7 ±0.3	NT
9		2.4 ±0.3	7.3 ±0.1	NT
10		13.1± 3.7	11.1± 0.5	NE
11		NE	NE	NE
Chloroquine	See Supp. Info.	0.18	0.02	0.01
Mefloquine	See Supp. Info.	0.02	0.08	0.04

^aPf, *P. falciparum*; Pk, *P. knowlesi*; NE, no effect; and NT, not tested. The error is represented as the standard deviation. Bold number, average of two experiments.

as previously described (see Supporting Information for specific approaches and experimentals).^{11,12,20,21} The highly reliable

[³H]-labeled hypoxanthine uptake method was utilized to assay our analogues for their ability to inhibit the in vitro growth and replication of malaria parasites in RBCs (see the Supporting Information).²² Preliminary dose–response assays were conducted using the W2 and D6 strains of *P. falciparum*, with known relative chloroquine (CQ) resistance and sensitivity values, respectively, in such assays.²³ The simian malaria parasite *P. knowlesi* has also been recognized as a human pathogen of public health concern in South East Asia,²⁴ and it can be cultured effectively in vitro.²⁵ This species was therefore included to probe the potential of multispecies efficacy within this compound class.

Table 1 shows comparative inhibitory concentration (IC₅₀) values for *P. falciparum* W2 and D6 strains and *P. knowlesi* when tested for their sensitivity to enigmol and 10 selected analogues. Our initial results showed that most analogues tested exhibited antimalarial activity on both W2 and D6 strains at less than 13 μM. There was an observable structure–activity relationship (SAR) between the compounds, ranging over roughly 1 order of magnitude. This SAR was quantitatively similar for both *P. falciparum* strains. Enigmol was found to have an IC₅₀ between 7 and 12 μM, which is similar to the level of antiproliferative activity that it displayed in various cancer cell lines. This is in turn sufficient to result in in vivo efficacy in human cancer xenograft cancer models. N-Methylation tended to increase potency as compared to the parent compounds. N-Methyl-enigmol (**5**) was particularly potent against all three malaria parasites with potencies in the low micromolar range, and this is within an order of magnitude in potency relative to the known positive control, chloroquine, in the W2 strain. N-Methyl analogues **3** and **9** also displayed increased potency, albeit modest, as compared to the parent compounds; however, this potency advantage was lost with the addition of a second N-methyl group as observed with N,N-dimethylenigmol analogue **6**. Pyrrolidine analogue **7** constrains C1 and the C2 amine functionality into a ring and demonstrates that this relatively severe modification is well tolerated. Fluorination of the side chain, such as with analogue **8** and its N-methyl equivalent, **9**, is also well tolerated. N-Acylation of enigmol (**10**) or the N-palmitoyl analogue (**11**), with ceramide-like functionality, displayed no activity. Interestingly, in all of these cases, the analogues were most effective against *P. knowlesi* and the *P. falciparum* W2 strain (PfW2) (known for its relative resistance to chloroquine), supporting our supposition that the sphingolipid analogues may be functioning through mechanisms different from those that impart chloroquine resistance. Taken together, these data also support our hypothesis that sphingolipid analogues may function by distinctive inhibitory mechanism(s) providing potentially multiple drug targets for future consideration. Finally, as an initial assessment of the viability of these compounds as human therapies, we tested the cytotoxicity of enigmol in human foreskin fibroblasts (HFF-1) cells. We used the standard sulforhodamine B assay with a density of 20,000 cells per plate and found that it was noncytotoxic after 72 h at 10 μM incubation, but cytotoxic at 50 μM, thus providing a narrow but sufficient safety index for an acute therapy such as the one we are targeting.

In our preliminary examinations, morphological changes were detected in the PfW2 strain after incubation of the cultures with N-methylenigmol (**5**). The effect was evident between 28 and 48 h of incubation, with a quantitatively high percentage of various aberrant or stunted forms of ring-stage and trophozoite-stage parasites found on Giemsa-stained thin

blood smears (Figure 1). These findings can be contrasted with the normal growth, development, and maturation of the

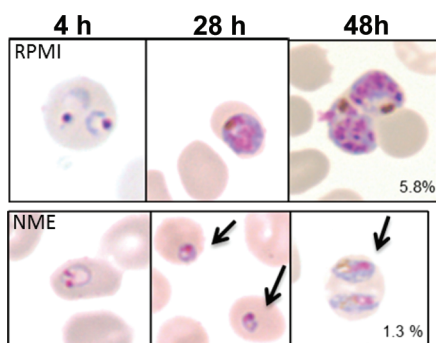
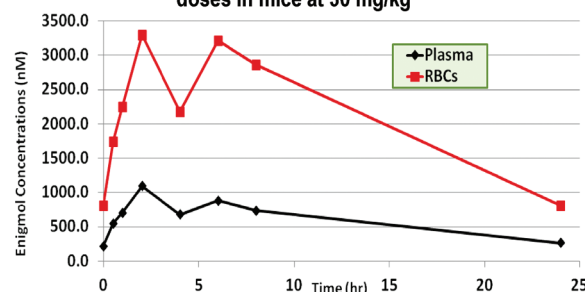


Figure 1. Compound 5 (NME = *N*-methylenigmol) inhibits the normal development of *P. falciparum* (W2 strain). IRBC samples taken 28 and 48 h postinoculation with 5 were compared to untreated parasites (RPMI). The arrows point to stunted ring-stage parasites at 28 h and to an iRBC-containing two trophozoites with an abnormal vacuolated appearance at 48 h.

parasites without the drug. By the 48 h time point, the parasitemia increased from 1 to $5.8 \pm 0.5\%$ with healthy appearing parasites in the control samples, as expected, while it remained at $1.3 \pm 0.3\%$ and with aberrant parasite forms in the sample incubated with *N*-methylenigmol (Figure 1). The relative percentages of rings, trophozoites, and schizonts in the cultures with and without the 5 compound were 41.9, 39.2, and 18.9% versus 87.9, 4.2, and 8.05, respectively. These data reflect the fact that the parasites that were incubated with *N*-methylenigmol did not progress with normal development, while the untreated parasites progressed through schizogony with a high percentage of newly invaded RBCs and normal development of new young ring-stage parasites.

Initial pharmacokinetic studies previously reported by our group¹² suggest that enigmol and *N*-methylenigmol possess attractive pharmacologic properties that provide sustained plasma and tissue levels in the micromolar range. We are therefore encouraged that compounds from this class could potentially provide antimalarial efficacy in vivo, even given the observed micromolar potency levels. Both compounds displayed very large volumes of distribution, suggesting substantial tissue accumulation. We believe that this might offer a possible explanation for the fact that we observe consistent pharmacological efficacy in vivo in mouse human tumor xenograft studies with enigmol despite the fact that the observed plasma levels from oral dosing are lower than the cellular IC_{50} .¹² As an initial test of this hypothesis, mice were dosed with enigmol once per day orally at 30 mg/kg, and drug levels were followed in both plasma and RBCs on day 1 (data not shown) and day 5 of dosing (Figure 2). We found that plasma levels increased by about 30% from accumulation and that drug concentrations in the RBCs were roughly 3-fold higher than plasma levels at any given time, thus supporting the notion that enigmol partitions into membranous tissues. At the high dose (30 mg/kg), drug levels in RBCs approached the IC_{50} of enigmol and were sustained in the micromolar range for nearly 24 h, while plasma levels remained mostly in the nanomolar range. Accordingly, we were encouraged that a rational drug design effort could deliver a second generation of agents with improved potency and/or higher distribution properties into RBCs that would potentially provide effective,

Mean levels of enigmol in plasma and RBCs on day 5 after 5 daily doses in mice at 30 mg/kg



Mean pharmacokinetic parameters from study B

Drug	Matrices	t_{max} (hr)	C_{max} (nM)	$t_{1/2}$ (hr)	Vz_F (L/kg)	AUC (0- ∞) (hr*nM)
Enigmol	Plasma	2	1,095	10.7	4.4	14,411
Enigmol	RBC's	2	3,300	8.9	25	50,732

Figure 2. Enigmol shows plasma accumulation after five daily doses and 3–4-fold higher levels in RBCs.

acute antimalarial treatments. Importantly, no signs or symptoms of toxicity have so far been observed in any of our in vivo studies.

As we were preparing a second generation of analogues, it was necessary to conjugate the compounds to bovine serum albumin (BSA) to increase their solubility as described previously²⁶ for testing in the [³H]-labeled hypoxanthine uptake assay. The results for enigmol and *N*-methylenigmol shown in Table 2 indicate that the formation of BSA complexes with our sphingolipid analogues provides for slightly greater efficacy in the assay, presumably due to the resolution of solubility challenges or aggregation barriers.

The design of the second generation of analogues was driven in large part by the hypothesis that the primary amine at C2 plays a key role in shaping the metabolism and tissue distribution properties of the analogues in vivo. Additional steric bulk at C2 could potentially reduce in vivo *N*-acylation by ceramide synthase and thus increase plasma concentrations of the free amine. Analogues were therefore prepared that were modified at C2 with a set of dimethyl and cyclopropyl analogues that would eliminate the chirality of the center. Analogues of this type were accessed via method B (Supporting Information), and a specific example for analogues 16, 17, and 18 is presented in Scheme 1. The oxime analogue 22 was available via reaction of 16 with $HONH_2 \cdot HCl$. The related cyclopropyl analogues were prepared similarly using method B and starting with cyclopropyl methylketone. Unfortunately, elimination of chirality at C2 had a detrimental effect on the stereoselectivity of the aldol reaction in both cases. However, because our initial results (Table 1) seemed to show that the absolute stereochemistry played a minimal role, we decided to test the racemic mixtures. Optimization of the synthesis to yield enantiopure materials is currently in progress.

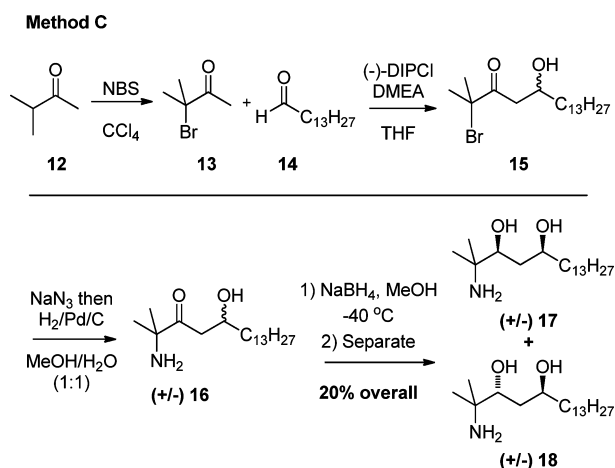
The second generation of compounds was tested after being complexed with BSA, and the observed in vivo potencies were generally within the range that was observed with enigmol and *N*-methylenigmol using the same method. In general, the incorporation of an additional substituent at C2 was well tolerated and exerted a minimal effect on potency. Interestingly, conversion of C3 to an SP2-hybridized ketone moiety (16) was also well tolerated and provided a slight potency advantage over the SP3 analogues 17 and 18; however, conversion of this

Table 2. Second Generation Sphingolipid Analogue SAR Data*

Compound	Structure	IC ₅₀ (μM)	
		W2	D6
enigmol 1	See Table 1	9.2 ± 2.1	5.3 ± 1.0
<i>N</i> -methyl-enigmol 5	See Table 1	0.9 ± 0.3	1.7 ± 1.1
19		7.2 ± 3.8	11.2 ^a
20		9.9 ± 7.1	5.5 ^a
17		4.7 ± 1.4	5.2 ± 3.2
18		7.4 ^a	4.1 ± 0.7
21		0.25 ^a	0.7 ± 0.5
16		2.6 ± 1.5	2.5 ± 0.1
22		10.0 ± 2.7	7.44 ^a
Chloroquine	Supp. Info.	0.093	0.01
Mefloquine	Supp. Info.	0.004	0.03

*All samples except chloroquine and mefloquine were tested as 1:1 molar complexes with BSA. The error is represented as the standard deviation. ^aSingle experiment in triplicate wells.

Scheme 1. Synthetic Methods for the Preparation of Enigmol and Its Analogues



center to the more metabolically stable oxime **22** reversed this potency advantage. As with enigmol, *N*-methylation in this series of compounds tends to boost the potency of the analogues by roughly 1 order of magnitude. In the case of compound **33**, we observed an IC₅₀ of 250 nM, which provides

evidence that further drug design and SAR investigations may lead to increasingly potent compounds in this series.

In this report, we have demonstrated that sphingolipid pathway modulators of the type represented by enigmol and its analogues can inhibit the growth and replication of multiple species of *Plasmodium* in RBCs in standardized in vitro assays. While further research in this area is required, data to date suggest that the mechanism(s) of action may be unique from any of the commonly employed antimalarials. Thus, these compounds may represent a potential new class of therapies that may prove invaluable for drug resistant malaria strains either as single agents or in combination with others. Importantly, the impressive pharmacokinetic and tissue distribution profile of enigmol and *N*-methylenigmol gives us confidence that we will be able to identify a compelling compound from this family of molecules that will prove to be safe, if in fact it is shown to be an effective treatment for malaria. As a next step, additional research is critical to test these compounds in vivo, to gain a deeper understanding of their mechanism(s) of action, and to develop new generation structures with increased potency.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic experimental details, analytical data of compounds, and biological assay protocols. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of E.M., J.J.H., D.S.M., R.B.H., M.R.G., and M.G.N. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

RBC, red blood cell; IC, inhibitory concentration; BSA, bovine serum albumin; PPMP, phenyl-2-palmitoylamino-3-morpholino-1-propanol; SK, sphingosine kinase; PfW2, *P. falciparum* W2 strain; PfD6, *P. falciparum* D6 strain; DIP-Cl, (-)-*B*-Chlorodiisopinocampheylborane; DMEA, Dimethylethyl amine; NaN₃, sodium azide; NaBH₄, sodium borohydride; NBS, *N*-bromosuccinimide

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