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Synthesis, Structure−Activity Relationship, and Antimalarial Activity of Ureas and Thioureas of 15-Membered Azalides

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Supporting Information

ABSTRACT:

n=3, R = cladinosyl, X = O, R2 = H, R' = alkyl or (alkyl)aryl
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n=2, R = cladinosyl, X = O, R2 = $CH_2CH_2CO\text{-}O\text{-}CH_2CH_3$, R' = (alkyl)aryl
n=2, R = cladinosyl, X = O, R2 = $CH_2CH_2CO\text{-}NH_2$, R' = (alkyl)aryl

Azithromycin, a first member of the azalide family of macrolides, while having substantial antimalarial activity, failed as a single agent for malaria prophylaxis. In this paper we present the first analogue campaign to identify more potent compounds from this class. Ureas and thioureas of 15-membered azalides, *N''*-substituted 9a-(*N'*-carbamoyl-*β*-aminoethyl), 9a-(*N'*-thiocarbamoyl-*β*-aminoethyl), 9a-[*N'*-(*β*-cyanoethyl)-*N'*-(carbamoyl-*β*-aminoethyl)], 9a-[*N'*-(*β*-cyanoethyl)-*N'*-(thiocarbamoyl-*β*-aminoethyl)], 9a-{*N'*-[*β*-(ethoxycarbonyl)ethyl]-*N'*(carbamoyl-*β*-aminoethyl)}, and 9a-[*N'*-(*β*-amidoethyl)-*N'*-(carbamoyl-*β*-aminoethyl)] of 9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A, were synthesized and their biological properties evaluated. The results obtained indicate a substantial improvement of the in vitro activity against *P. falciparum* (up to 88 times over azithromycin), particularly for compounds containing both sugars on the macrocyclic ring and aromatic moiety on 9a-position. The improved in vitro activity was not confirmed in the mouse model, likely due to an increase in lipophilicity of these analogues leading to a higher volume of distribution. Overall, with increased in vitro activity, promising PK properties, and modest in vivo efficacy, this series of molecules represents a good starting platform for the design of novel antimalarial azalides.

INTRODUCTION

Malaria is a serious disease with half of the world's population at risk of malaria, particularly those in sub-Saharan Africa and regions of Asia. An estimated 250 million cases led to nearly 1 million deaths in 2006, of which 91% were in Africa and 85% were of children under 5 years of age.[1] The disease is caused by a parasite, protozoa of the *Plasmodia* genus, which is transmitted by the female *Anopheles* mosquito. There are five species that infect humans, with *P. falciparum* causing the vast majority of the fatalities.

Drugs of diverse chemical classes such as chloroquine, mefloquine, halofantrine, artemisinin, atovaquone/proguanil, doxycycline, and primaquine have been developed for the treatment and/or prophylaxis of malaria. However, while successful against some strains of malaria, many strains of *Plasmodium* appear to have developed multiple drug resistance, and this remains an issue in antimalarial treatment and prevention.[2] This fact further underscores the urgent necessity to find new efficacious and safe compounds to maintain and improve the management and prevention of malaria in a new era of intensive malaria control, elimination, and eradication.[2]

Macrolides belong to the polyketide class of natural products. Macrolide antibiotics are an old and well-established class of antibacterial agents that have long played a significant role in the chemotherapy of infectious diseases.[3] The azalide azithromycin, a 15-membered macrolide, is characterized by an enhanced antibacterial profile and improved pharmacokinetic properties

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3 R = H, X = S, R' = alkyl or (alkyl)aryl
4 R = cladinosyl, X = O, R' = alkyl or (alkyl)aryl
5 R = cladinosyl, X = S, R' = alkyl or (alkyl)aryl

Figure 1. Ureas and thioureas of 15-membered azalides.[14]

in comparison to other 14-membered ring macrolides. Azithromycin is of particular interest because of its apparent safety in pregnancy.[4] Furthermore, since it is now available in generic forms, its cost has substantially decreased.

It has been known for almost 2 decades that azithromycin has the potential for therapeutic use in malaria.[5−7] The in vitro activity of azithromycin against *P. falciparum* is in the low micromolar range (IC_{50} values are between 0.5 and 10 μg/mL) in a 68 h assay.[8] Azithromycin is a slow acting antimalarial that targets the parasite apicoplast, and its prokariotic ribosomes and effects of its action increase with prolonged incubation time (from one to two generations), giving IC_{50} in the nanomolar range.[9] Azithromycin does not appear to be cross-resistant with standard antimalarial drugs. Although resistance can be induced in vitro,[9] the rapidity of loss to resistance in the field when used in combination with other antimalarial drugs is unknown (as is its ability to protect other antimalarial drugs from the emergence of resistance).

In the 1990s, azithromycin was shown to have prophylactic activity on malaria when administered daily and weekly by the Walter Reed Army Institute of Research.[6,10−12] Development was discontinued when the maximum tolerated daily dose only demonstrated an approximate efficacy of 65% in nonimmune adults (although efficacy was very high against *P. vivax*).[11,12] Despite the insufficient prophylactic activity, based on the activity observed with azithromycin, a three-pronged approach was encouraged for the treatment and/or prophylaxis of malaria including a better tolerated formulation,[13] drug combinations,[8] and the development of new analogues with increased potency and longer half-lives.

In this manuscript, we present the initial efforts made toward the discovery of novel antimalarial azalides with improved activity, efficacy, and safety while being affordable to the target patient population.[2]

As macrolide antibiotics exert their activity through the 50S ribosomal subunit, present both in bacteria and in *Plasmodium* apicoplast, the goal for the project was to identify new azalide derivatives with antibacterial activity comparable to that of azithromycin. Furthermore, the next generation antimalarial azalides needed to be produced by a low-step, high-yield synthetic route resulting in a low-cost final drug product. In our previous paper we have reported the synthesis of ureas and thioureas of 15-membered azalides and their comparable or improved antibacterial activity over that of azithromycin (Figure 1).[14]

Since these compounds were obtained by a straightforward and affordable chemical procedure, a set of compounds was selected for antimalarial profiling. Herein we report the in vitro and in vivo efficacy of these compounds against three *Plasmodium falciparum* strains. Their excellent antimalarial activity encouraged us to continue and extend our investigation to novel classes of ureas and thioureas of 15-membered azalides. In this paper we also report the synthesis and antimalarial activity of novel *N*″-substituted 9a-(*N*′-carbamoyl/thiocarbamoyl-*β*-aminoethyl) derivatives.

■ CHEMISTRY

We have previously developed an efficient synthetic methodology to access a wide range of *N*″-substituted 9a-(*N*′-carbamoyl/thiocarbamoyl-*γ*-aminopropyl) derivatives **1**−**5** (Figure 1).[14] This report will highlight the synthesis and in vitro antimalarial activity of a series of novel 9a-(*N*′-carbamoyl/thiocarbamoyl-*β*-aminoethyl) analogues **12**−**15** and **19**−**22**.

In this work we have used 9-deoxo-9a-aza-9a-homoerythromycin A (**7**) as a convenient source of starting material for further chemical modification leading to novel classes of *N*″-substituted 9a-(*N*′-carbamoyl-*β*-aminoethyl) (**12**), 9a-(*N*′-thiocarbamoyl-*β*-aminoethyl) (**14**), 9a-[*N*′-(*β*-cyanoethyl)-*N*′-(carbamoyl-*β*-aminoethyl)] (**19**), 9a-[*N*′-(*β*-cyanoethyl)-*N*′-(thiocarbamoyl-*β*-aminoethyl)] (**20**), 9a-{*N*′-[*β*-(ethoxycarbonyl)ethyl]-*N*′-(carbamoyl-*β*-aminoethyl)} (**21**), and 9a-[*N*′-(*β*-amidoethyl)-*N*′-(carbamoyl-*β*-aminoethyl)] (**22**) derivatives (Scheme 1). The 9a-*N*-*β*-aminoethyl precursor **10** was synthesized in three steps starting from compound **7**. It is well-known that the simple 9a-*N*-alkyl derivatives of **7** can be prepared by a straightforward reductive amination of **7** with aldehydes, while other 9a-*N*-alkyl analogues require alkyl halide reaction with 3′-*N*-oxide **8**.[15] Thus, in order to prevent quaternization at the 3′-nitrogen, the basic desosamine sugar 3′-*N*-oxide **8** was prepared by oxidation with H_2O_2. The following reaction of **8** with bromacetonitrile and subsequent reduction of the resulting cyanomethyl compound **9** involved the simultaneous deoxygenation at the 3′-dimethylamino group and afforded the precursor **10** to ureas **12**, **19**, **21**, **22** and thioureas **14**, **20**. Intermediates **16** and **17** were obtained by Michael addition of ethyl acrylate followed by amidation. 9a-[*N*′-(*β*-Cyanoethyl)-*β*-aminoethyl] derivative **18** was prepared by Michael addition of acrylonitrile according to a procedure described previously.[14]

Scheme 1. Synthesis of Novel Ureas and Thioureas of 15-Membered Azalides[a]

6 R1 = CH_3, R = Cladinosyl
7 R1 = H, R = Cladinosyl

8 R = Cladinosyl

9 R = Cladinosyl

Cladinosyl =

10 R = Cladinosyl
11 R = H

12 R = Cladinosyl, X = O
13 R = H, X = O
14 R = Cladinosyl, X = S
15 R = H, X = S

17 R = Cladinosyl

16 R = Cladinosyl

18 R = Cladinosyl

22 R = Cladinosyl, X = O

21 R = Cladinosyl, X = O

19 R = Cladinosyl, X = O
20 R = Cladinosyl, X = S

Cmpd	R'	Cmpd	R'	Cmpd	R'	Cmpd	R'
a		d		g		i	
b		e		h		j (±)	
c		f					

[a] Reagents and conditions: (i) H_2O_2, MeOH, room temp; (ii) bromoacetonitrile, K_2CO_3, DCM, room temp; (iii) $LiB(Et)_3H$, THF, room temp; (iv) alkyl or aryl isocyanate/isothiocyanate, CH_2Cl_2, room temp, 1 h; (v) 0.25 M hydrochloric acid, room temp; (vi) ethyl acrylate, ethanol, room temp; (vii) acrylonitrile, methanol, reflux, 22 h; (viii) 25% ammonia solution, ethanol, room temp.

Table 1. Antimalarial Activity of Substituted 9a-(N'-Carbamoyl-β-aminoethyl) 12, 13, 19, 21, 22 and 9a-(N'-Thiocarbamoyl-β-aminoethyl) 14, 15, 20 Derivatives against Three *Plasmodium falciparum* Strains and Cytotoxicity against Two Immortal Human Cell Lines THP-1 (Monocytes) and Hep G2 (Hepatocytes)[a]

1 n=3, R = cladinosyl, X = O, R2 = H
2 n=3, R = cladinosyl, X = S, R2 = H
3 n=3, R = H, X = S, R2 = H
4 n=3, R = cladinosyl, X = O, R2 = CH_2CH_2CN
5 n=3, R = cladinosyl, X = S, R2 = CH_2CH_2CN
12 n=2, R = cladinosyl, X = O, R2 = H
13 n=2, R = H, X = O, R2 = H
14 n=2, R = cladinosyl, X = S, R2 = H
15 n=2, R = H, X = S, R2 = H
19 n=2, R = cladinosyl, X = O, R2 = CH_2CH_2CN
20 n=2, R = cladinosyl, X = S, R2 = CH_2CH_2CN
21 n=2, R = cladinosyl, X = O, R2 = $CH_2CH_2CO\text{-}O\text{-}CH_2CH_3$
22 n=2, R = cladinosyl, X = O, R2 = $CH_2CH_2CO\text{-}NH_2$

						IC_{50} (nM) *Plasmodium falciparum* strain			IC_{50} (μM)	
Cmpd	R	R2	X	R'	n	TM91C235	D6	W2	THP-1	Hep-G2
6	cladinosyl	-	-	-	-	2114	1039	2294	>50	>50
1b	cladinosyl	H	O		3	>5699	>1425	>5699	ND*	ND
1h	cladinosyl	H	O		3	131	215	173	>50	>50
1i	cladinosyl	H	O		3	81	138	127	40	48
1j	cladinosyl	H	O		3	107	292	106	46	>50
2d	cladinosyl	H	S		3	156	257	121	47	>50
2e	cladinosyl	H	S		3	200	215	167	23	47
2f	cladinosyl	H	S		3	82	107	85	15	22
2h	cladinosyl	H	S		3	131	116	155	40	48
3d	H	H	S		3	1543	2038	3632	>50	>50
4b	cladinosyl	NC	O		3	1274	>13426	>13426	ND	ND
4g	cladinosyl	NC	O	Cl Cl	3	123	229	199	15	22
4h	cladinosyl	NC	O		3	255	523	1382	ND	ND
4i	cladinosyl	NC	O		3	116	118	178	ND	ND
4j	cladinosyl	NC	O		3	97	200	136	40	48
5d	cladinosyl	NC	S		3	126	204	139	11	44
5e	cladinosyl	NC	S		3	48	88	51	6	13
5f	cladinosyl	NC	S		3	62	179	88	5	9
12a	cladinosyl	H	O		2	978	3623	4930	ND*	ND
12b	cladinosyl	H	O		2	717	1401	2059	ND	ND
12c	cladinosyl	H	O		2	103	223	67	27	48

Table 1. Continued

Cmpd	R	R2	X	R'	n	IC50 (nM) *Plasmodium falciparum* strain TM91C235	D6	W2	IC50 (μM) THP-1	Hep-G2
12d	cladinosyl	H	O		2	170	320	287	35	>50
12e	cladinosyl	H	O		2	243	546	105	45	>50
12h	cladinosyl	H	O		2	42	142	79	14	35
12i	cladinosyl	H	O		2	24	71	30	5	9
12j	cladinosyl	H	O		2	46	179	33	8	10
13i	H	H	O		2	106	318	99	8	16
14c	cladinosyl	H	S		2	113	183	443	16	48
14d	cladinosyl	H	S		2	106	109	72	9	14
14e	cladinosyl	H	S		2	128	203	102	4	9
14f	cladinosyl	H	S		2	82	182	52	5	8
14h	cladinosyl	H	S		2	130	225	99	9	20
15f	H	H	S		2	226	654	178	7	14
19i	cladinosyl	NC	O		2	224	525	125	13	11
20e	cladinosyl	NC	S		2	369	295	64	6	7
21i	cladinosyl	O, O	O		2	2154	773	207	20	23
22i	cladinosyl	NH_2, O	O		2	39	190	62	7	13

[a] (*) ND: not determined.

The addition of isocyanates or thioisocyanates to primary and secondary amines is an efficient method for the preparation of substituted ureas and thioureas, respectively.[16] N''-Aryl and alkyl substituted derivatives **12**−**15** and **19**−**22** were obtained by the reaction of the corresponding isocyanates and thioisocyanates with amines **10**, **16**, **17**, and **18** in high yield. The reaction proceeded smoothly in an aprotic solvent and at room temperature to form less polar products (Scheme 1). It is remarkable that no carbamate was formed in these reactions, since hydroxy groups are quite reactive with isocyanates and isothiocyanates. Also, site selective addition to secondary amino groups in the presence of primary amide in compound **17** was a noteworthy outcome.

The structures of all synthesized compounds were determined by NMR spectroscopy and mass spectrometry. The assignments of proton and carbon chemical shifts were made by a combined use of one- (^{1}H and APT) and two-dimensional (COSY, HSQC, and HMBC) NMR spectra. Carbon and proton chemical shifts of all compounds are given in the experimental part.

IN VITRO ANTIMALARIAL AND CYTOTOXIC ACTIVITY

The preliminary library contained a total of 36 urea and thiourea derivatives of 15-membered azalides, more specifically, 17 N''-substituted 9a-(N'-carbamoyl/thiocarbamoyl-γ-aminopropyl)-derivatives (**1**-**5**) and 19 N''-substituted 9a-(N'-carbamoyl/thiocarbamoyl-β-aminoethyl)-derivatives (**12**−**15** and

19-22). The in vitro antimalarial activity was evaluated against three *Plasmodium falciparum* strains: TM91C235 (multidrug resistant), D6 (chloroquine sensitive), and W2 (chloroquine/pyrimethamine resistant) (Table 1) and compared to azithromycin **6** as standard. Among these, TM91C235 was considered the most important strain because of its multidrug resistance profile. Excellent in vitro activity of the tested library was confirmed by the total number of compounds more active than azithromycin against all three strains (30 compounds out of 36). An overall analysis of the data revealed an interesting property of these novel macrolides, mainly that higher in vitro potency was seen against one or both resistant strains than against the sensitive D6 strain.

By structure−activity relationship (SAR) analysis, the influence of the cladinose sugar, nature of the substituent R′, and type of the linker (variations of alkyl chains on 9a-*N* position on macrolide ring) on the in vitro activity was investigated for this set of compounds. Each feature was analyzed separately; however, it was also noted that the combination of key structural features contributed significantly to the enhancement of the antimalarial activity.

Initially, the significance of the cladinose sugar was explored. An evident trend was observed for a tested set of compounds where the absence of the cladinosyl moiety decreased antimalarial activity up to 10-fold (decladinosyl derivative **3d** in comparison to cladinosyl derivative **2d** with IC_{50} of 1543 and 156 μM, respectively). The removal of the cladinose sugar was shown to be the most influential structural feature that resulted in the reduction of in vitro antimalarial activity.

Further exploration of the SAR was focused on the nature of the substituents R′, R2, and the length of the linkers (Table 1): linker A, chain between 9a-*N* position on macrolide ring and *N*′ position on carbamoyl/thiocarbamoyl group; linker B, chain between *N*′′ position on carbamoyl/thiocarbamoyl group and alkyl/aromatic moiety R′. The observations on these SAR features include the following: (1) Introduction of aromatic groups significantly improved in vitro activity over azithromycin **6** (up to 88-fold) and over alkyl groups in the same position (**1b**, **4b**, **12a**, **12b**). (2) Reduction of activity was approximately 10-fold by the introduction of additional substituents R2 on urea and thiourea moieties: β-cyanoethyl, β-(ethoxycarbonyl)ethyl, and β-amidoethyl chains. For example, activities of **19i**, **21i**, and **22i** were significantly reduced in comparison to **12i**. (3) There was no clear-cut effect on in vitro activity for all derivatives with respect to the linker A length. The more active urea derivatives in the in vitro assay had short linker A (**1h**, **1i**, **1j** vs **12h**, **12i**, **12j**), while in thiourea series derivatives with longer linker A (propyl chain) showed similar or equal activity compared with the compounds with shorter and/or more rigid ethyl chain (**2d**, **2e**, **2f**, **2h** and **14d**, **14e**, **14f**, **14h**). (4) There was slightly improved antimalarial activity for compounds with a longer linker B (compound **14f** with propyl linker more active than compounds with shorter linkers **14c**, **14d**, and **14e**). (5) A combination of the structural features, urea/thiourea and naphthyl/phenyl R′ aromatic moieties, enhances antimalarial activity. The most active urea and thiourea analogues demonstrated that ureas with naphthyl moiety exhibit better activity than their thiourea analogues (e.g., **12h** vs **14h** with IC_{50} of 42 and 130 μM, respectively). However, thioureas with a phenyl substituent demonstrated slightly better activity than urea derivatives. For example, compounds **14e** and **14d** with thiourea moiety showed better activity than urea analogues **12e** and **12d** (IC_{50} of 128, 106, 243, 170 μM, respectively).

Table 2. Pharmacokinetic Parameters Estimated in Blood after Intravenous (iv) and Oral Gavage (po) Administration to Sprague−Dawley Rats (10 mg/kg iv and 30 mg/kg po)[a]

	CL ((mL/min)/kg)	V_d (L/kg)	$T_{1/2}$ (h)	oral *F* (%)
azithromycin	11.0	20.0	24.0	33.0
2d	4.0	10.4	30.0	3.4
2f[b]	2.3	2.6	13.4	1.3
4g	7.7	23.3	41.5	18.4
5f	24.5	31.7	15.2	21

[a] CL, blood clearance; V_d, apparent volume of distribution at the terminal phase based on drug concentration in blood, $T_{1/2}$, half-life; oral *F*, oral bioavailability, [b] The iv parameters were determined in one rat.

In vitro cytotoxicity measured for the selected set of compounds revealed that all compounds showed relatively low cytotoxicity in vitro ($IC_{50} \geq 4\ \mu$M). SAR analysis indicted that (1) thiourea derivatives showed slightly higher cytotoxicity than corresponding urea analogues (i.e., **14d** > **12d**, **14e** > **12e**, **14h** > **12h**) and (2) an ethyl chain between 9a-*N* and urea/thiourea functionality increases cytotoxicity in comparison to a propyl chain derivatives (i.e., **12i** > **1i**, **12h** > **1h**, **14d** > **2d**, **14f** > **2f**).

In general, the antimalarial activity of compounds tested was accompanied by a decrease in IC_{50} determined for Hep G2 and THP-1 cell lines. Despite this fact, most active compounds showed *P. falciparum* IC_{50} more than 2 log units above cellular inhibition IC_{50}, and this marked them as potent and selective compounds for further profiling.[17]

PRELIMINARY PHARMACOKINETICS AND IN VIVO ANTIMALARIAL ACTIVITY

On the basis of in vitro activity (IC_{50} against *P. falciparum* strain TM91C235 of <200 nM and HepG2 IC_{50} of >9 μM) and their structural similarity, several compounds were screened for metabolic stability in vitro (**1h**, **1i**, **1j**, **2d**, **2e**, **2f**, **2h**, **4g**, **4j**, **5d**, **5e**, **5f**) using human and mouse liver microsomes, and only a few were selected for in vivo rat pharmacokinetic studies in order to determine their pharmacokinetic profiles (Table 2). In vitro, all compounds demonstrated good metabolic stability with $t_{1/2}$ greater than 120 min ($t_{1/2}$ = 103 min for compound **5e** in human liver microsomes). As was observed with azithromycin and in line with the in vitro data, these analogues had a low systemic clearance, moderate to high volume of distribution, and a very long half-life; however, the oral bioavailability was low (**2d**, **2f**) to moderate (**4g**). Nevertheless, all 12 compounds (**1h**, **1i**, **1j**, **2d**, **2e**, **2f**, **2h**, **4g**, **4j**, **5d**, **5e**, **5f**) were progressed to be tested in vivo in the mouse *P. berghei* model, as the dosing regimen was subcutaneous. Compounds were administered subcutaneously at 160/40/10 mg/kg twice daily for 3 days (total doses of 960/240/60 mg/kg, high/medium/low dose), including azithromycin, which was used as a comparator. The results from the efficacy model were expressed as a curative dose 50 (CD_{50}), defined as the daily dose of compound that cures 50% of mice. Animals that have a negative blood smear on the last day of the experiment are considered cured. The results of the efficacy experiments are presented in Table 3.

Azithromycin cured all treated mice at the high and medium doses with a CD_{50} of 50 mg/kg, while in the low dose only three of five mice were cured. The most active of the compounds tested belonged to the 9a-(*N*′-carbamoyl-γ-aminopropyl) (**1**) and 9a-(*N*′-thiocarbamoyl-γ-aminopropyl) (**2**) groups. Compound

Table 3. Efficacy of Novel Macrolides in *Plasmodium berghei* Infected Mice after 3 Days of Subcutaneous Dosing[a]

cmpd	*P. berghei* in vivo CD_{50} (mg/kg)	(no. of mice without parasitemia on day 31)/(total no. of mice) at doses 960, 240, 60 mg/kg
1h	150	3/5, 3/5, 2/5
1i	>960	2/5, 0/5, 0/5
1j	397.5	4/4, 0/5, 1/5
2d	285	4/5, 2/5, 2/5
2e	780	3/5, 1/5, 0/5
2f	780	3/5, 1/5, 0/5
2h	195	4/5, 3/5, 1/5
4g	420	5/5, 2/5, 1/5
4j	510	4/5, 0/5, 1/5
5d	>960	1/5, 1/5, 2/5
5e	>960	0/4, 1/5, 0/5
5f	800	3/5, 0/5, 0/5
azithromycin	50	5/5, 5/5, 3/5
control[b]		0/5

[a] Groups of four to five *P. berghei* infected mice were treated subcutaneously on days +3, +4, +5 postinfection with macrolides suspended in palm oil at doses 160, 40, and 10 mg/kg twice daily for 3 days. Antimalarial activity was measured by curative dose 50, the daily dose that cures 50% of the mice. Animals that have a negative blood smear on the last day of the experiment are considered cured. [b] Mice that received only palm oil vehicle.

1h cured three mice at the high and medium doses and two mice at the low dose, while its thiourea derivative **2h** cured all four mice at the high dose (this was the only group that contained only four mice), three mice at the medium dose, and one mouse at the low dose. The CD_{50} values were determined to be 150 and 195 mg/kg, respectively. Compound **2d** had a CD_{50} of 285 mg/kg (four mice at the high dose and two mice at both the medium and low doses were cured), while its 9a-*N*′-(β-cyanoethyl) analogue **5d** was found to be much less active, curing only one mouse at the high and medium doses and two mice at the low dose with CD_{50} being larger than 960 mg/kg. In contrast to this analogue example, compound **1j** and its 9a-*N*′-(β-cyanoethyl) analogue **4j** did not exhibit such difference in their in vivo efficacy, as both had a similar pattern of efficacy (four mice cured at the high dose, zero mice in the middle dose, and one mouse at low dose) and their CD_{50} values were 398 and 510 mg/kg, respectively. Analogues **2f** and **5f** had no curative effect at the low dose, while at the high dose three mice were cured. At the medium dose one mouse was cured by **2f** and no mice by **5f**, so their respective calculated CD_{50} values were 780 and 800 mg/kg. The least active pair of analogues in this set of compounds was **2e** and **5e**, where **2e** exhibited a CD_{50} of 780 mg/kg whereas **5e** had CD_{50} of >960 mg/kg. Compound **2e** cured three mice in high dose and one mouse at the medium dose, while **5e** cured only one mouse at the medium dose. Compound **4g** without an analogue in this efficacy experiment exhibited a CD_{50} of 420 mg/kg and cured five mice at the high dose, two mice at the medium dose, and one mouse at the lower dose. Interestingly, the only 2-naphthyl analogue tested, **1i**, cured only two mice at the high dose and as such had a CD_{50} of >960 mg/kg.

DISCUSSION

The development of new antimalarial drugs is an urgent clinical need, as many drugs on the market are losing efficacy as multiresistant malaria parasites emerge.[2,18] This paper reports the in vitro and in vivo results for a novel class of antimalarial compounds: the 9a-*N*-substituted azalides. Azithromycin, an azalide antibiotic currently tested in clinical settings for the treatment and prophylaxis of malaria, has low in vitro activity against *P. falciparum* strains[8] as well as low clinical efficacy when used as monotherapy.[11,12,19] Therefore, a novel macrolide derivative with an equivalent safety profile and more potent antimalarial activity is of interest to all involved in malaria eradication and control. The most prominent feature in antimalarial activity of novel *N*″-substituted urea and thiourea derivatives was a substantial improvement of their in vitro activity against *P. falciparum* strains when compared to azithromycin. The most outstanding increase in activity was observed against resistant *P. falciparum* strains TM91C235 and W2, where the IC_{50} values of the most active compound **12i** were 88 and 76 times lower when compared to azithromycin, respectively. The increases in in vitro potency were not that pronounced for the drug sensitive *P. falciparum* strain D6, where the improvement over azithromycin was no more than 14 times.

On the basis of such encouraging in vitro antimalarial activity over azithromycin, structural pairing, and good in vitro metabolic stability, a total of 12 compounds were tested for their in vivo efficacy in the *P. berghei* mouse model. The curative effects in vivo were in line with previously observed in vitro SAR trends, with naphthyl substituted urea and thiourea derivatives (**1h**, **1j**, **2h**) and the thiourea phenyl derivative (**2d**) being the most active, while compounds with a β-cyanoethyl substituent on urea and thiourea moieties had lower curative outcomes than their analogues lacking this side chain. Nevertheless, the substantially improved in vitro activity in comparison to azithromycin was not fully translated in vivo, leading to at least 3-fold higher CD_{50} in the mouse model. Preliminary in vitro microsomal stability data indicated that these compounds had good metabolic stability, as was confirmed by low clearances in vivo for the compounds tested. In comparison to azithromycin, known for its extensive tissue distribution,[3] these derivatives had a tendency toward higher volumes of distribution, in line with their increased lipophilic character (approximately 2−3 log units higher than azithromycin, according to calculated log *P* values, data not shown) because of the presence of strong lipophilic aromatic phenyl and naphthyl rings in the 9a-*N* substituent. More extensive tissue penetration could lead to lower concentrations at the site of action (blood), resulting in lower efficacy in vivo. Finally, the in vitro activity against human malaria parasite *P. falciparum* is considered to be the guiding tool for the progression of compounds to one of the standard in vivo mouse models in malaria drug discovery; however, the data gathered in this research suggest that the *P. berghei* mouse model used may not be appropriate for the evaluation of this class of compounds, as previously observed for some other compound classes.[20] Overall, with increased in vitro activity, promising PK properties, and modest in vivo efficacy, this series of molecules represents a good starting platform for the design of novel antimalarial azalides. On the basis of the data reported here, other new series were proposed and synthesized.

CONCLUSIONS

Given the enormous burden malaria is causing to the affected population and the limited resources for their healthcare, the usual requirements for efficacy, safety, compliance, and

affordability are particularly stringent for novel antimalarials. Our research on the antimalarial macrolides has contributed substantially to the area of antimalarial drug discovery. A novel class of antimalarial agents, 15-membered azalide urea and thiourea derivatives, with substantially improved in vitro antimalarial activity over azithromycin and comparable pharmacokinetic properties was invented. Straightforward and affordable synthetic procedures for the preparation of novel ureas and thioureas of 15-membered azalides (N''-substituted 9a-(N'-carbamoyl/thiocarbamoyl-γ-aminopropyl) derivatives and N''-substituted 9a-(N'-carbamoyl/thiocarbamoyl-β-aminoethyl) derivatives) were developed.

In our research some compounds showed promising efficacy in the mouse model, although the model used may not be fully appropriate for the evaluation of this class of compounds.

This research established a good starting platform for the exploration of a wider chemical space around this novel series that will direct medicinal chemistry toward further optimization of the 9a-*N* substituent.

EXPERIMENTAL SECTION

Chemistry. TLC was performed on Merck 60 F_{254} plates using DCM/MeOH/NH_4OH = 90:9:0.5 as eluents. Column chromatography was performed on Merck silica gel 60 (0.043−0.060 mm).

NMR spectra were recorded on a Bruker Avance DRX500 and Bruker Avance DPX 300 spectrometer operating at 500 and 300 MHz for ^1H. The chemical shifts were reported as δ ppm relative to TMS, using the residual solvent peak as the reference unless otherwise noted. The following abbreviations were used to express the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet. The solvents used were pyridine-d_5 and DMSO-d_6. All spectra were recorded at ambient temperature.

Mass spectra were obtained on a Waters Micromass ZQmass spectrometer for ES^+ MS. Electrospray positive ion mass spectra were acquired using a Micromass Q-Tof2 hybrid quadrupole time-of-flight mass spectrometer, equipped with a Z-spray interface, over a mass range of 100−2000 Da, with a scan time of 1.5 s and an interscan delay of 0.1 s in continuum mode. Reserpine was used as the external mass calibrant lock mass ($[M + H]^+$ = 609.2812 Da). The elemental composition was calculated using a MassLynx, version 4.1, for the $[M + H]^+$, and the mass error quoted was within the $\pm$5 ppm range.

The purity of each compound was determined on an Agilent 1100 series LC/MSD trap. The analysis was carried out using MS and UV detection at 240 nm on a YMC Pack Pro C_{18} column, 150 mm × 4.6 mm i.d., 3 μm particle size. Gradient elution at a flow rate of 1.0 mL/min started with 10% of acetonitrile/90% of 40 mM ammonium acetate buffer solution and ended after 15 min with 90% of acetonitrile/10% of 40 mM ammonium acetate buffer solution. The ESI source was operated in the positive ion mode. Nitrogen was used as nebulizing and drying gas at 350 °C. The m/z range scanned in the MS measurement was 200−1200. Complete system control, data acquisition, and processing were done using the ChemStation for LC/MSD, version 4.2, from Agilent. By use of the described method, the determined purity for each compound was $\geq$95%.

9-Deoxo-9-dihydro-3′-*N*-oxide-9a-aza-9a-homoerythromycin A (8). To a solution of 9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (7) (20 g, 27.21 mmol) in MeOH (80 mL) at 0 °C, a 30% water solution of H_2O_2 (30 mL) was added dropwise over 30 min. The reaction mixture was stirred for an additional 1.5 h at room temperature. After detection of complete transformation the reaction mixture was poured into ice−water (400 mL) and DCM (200 mL). A saturated water solution of Na_2SO_3 (150 mL) was added to remove excess H_2O_2. The layers were separated, and the water layer was extracted with DCM (2 × 200 mL). The combined organic layers were evaporated under reduced pressure, and the residue was precipitated from DCM and diisopropyl ether, yielding **8** (21.5 g, 94.3% yield). MS (ES^+) m/z: 751.6.

9-Deoxo-9-dihydro-3′-*N*-oxide-9a-cyanomethyl-9a-aza-9a-homoerythromycin A (9). To a DCM (200 mL) solution of **8** (20 g, 26.63 mmol) K_2CO_3 (7.35 g, 53.26 mmol) was added, and the reaction mixture was stirred for 10 min at room temperature. Then bromoacetonitrile (3.71 mL, 53.26 mmol) was added, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was washed with brine, yielding after evaporation 20 g of the crude product. Precipitation from water yielded intermediate **9** (7.1 g, 31.3% yield). MS (ES^+) m/z: 790.6.

9-Deoxo-9-dihydro-9a-(β-aminoethyl)-9a-aza-9a-homoerythromycin A (10). To the solution of **9** (3 g, 3.80 mmol) in THF (25 mL), $LiB(Et)_3H$ (10 mL, 1 M THF solution) was added dropwise over 20 min at −20 °C. The mixture was stirred for 10 min at −20 °C to complete conversion. To the reaction mixture water (50 mL) and DCM (50 mL) were added, and gradient extraction was performed at pH 4.5 and 10. Evaporation of the combined organic extracts at pH 10 yielded 1.6 g of the crude product. Column chromatography using elution system DCM/MeOH/NH_4OH = 90:9:0.5 yielded **10** (0.87 g, 29.5% yield). MS (ES^+) m/z: 778.5.

3-*O*-Decladinosyl-9-deoxo-9-dihydro-9a-(β-aminoethyl)-9a-aza-9a-homoerythromycin A (11). A solution of intermediate **10** (1.5 g, 1.93 mol) in 0.25 N HC1 (50 mL) was stirred for 20 h at room temperature. To the reaction mixture DCM (50 mL) was added, and gradient extraction was performed at pH 1.1 and 9.5. Evaporation of the combined organic extracts at pH 9.5 yielded 0.98 g of crude product. Column chromatography using elution system DCM/MeOH/NH_4OH = 90:9:1.5 yielded compound **11** (0.76 g, 62.7% yield). ^1H NMR (500 MHz, DMSO-d_6) δ: 5.08 (d, 1H, H-13), 4.98 (d, 1H, 3 OH), 4.53 (d, 1H, H-1′), 3.51 (s, 1H, H-11), 3.45 (d, 1H, H-5), 3.43 (br s, 1H, H-5′), 3.32−3.35 (m, 1H, H-3), 3.08 (dd, 1H, H-2′), 2.70−2.80 (m, 1H, β-CH_2), 2.65 (d, 1H, H-10), 2.52−2.53 (m, 2H, H-9, α-CH_2), 2.45−2.48 (m, 2H, H-3′, H-2), 2.40−2.45 (m, 1H, β-CH_2), 2.24−2.27 (m, 2H, H-9, α-CH_2), 2.22 (s, 6H, 3′NMe_2), 2.07−2.12 (m, 1H, H-4), 1.76−1.85 (m, 1H, H-14), 1.65−1.72 (m, 1H, H-8), 1.56−1.64 (m, 2H, H-7, H-4′), 1.37 (ddd, 1H, H-14), 1.15 (s, 3H, 6 Me), 1.13 (d, 3H, 5′ Me), 1.12 (d, 3H, 2 Me), 1.10−1.11 (m, 1H, H-4′), 1.08−1.10 (m, 1H, H-7), 0.97 (s, 3H, 12 Me), 0.97 (d, 3H, 10 Me), 0.89 (d, 3H, 4 Me), 0.83 (d, 3H, 8 Me), 0.75 (t, 3H, 15 Me). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 174.8, 102.5, 86.9, 76.4, 76.1, 75.3, 73.9, 73.5, 70.5, 68.1, 64.6, 61.1, 43.8, 40.6, 40.2, 37.6, 36.1, 30.5, 29.6, 25.9, 21.3, 21.1, 20.9, 16.7, 16.1, 10.5, 8.5, 8.4. MS (ES^+) m/z: 620.6.

9-Deoxo-9-dihydro-9a-{*N*′-[β-(ethoxycarbonyl)ethyl]-β-aminoethyl}-9a-aza-9a-homoerythromycin A (16). To the EtOH (10 mL) solution of **10** (1.22 g, 1.57 mmol), ethyl acrylate (0.08 mL, 1.22 mmol) was added. The reaction mixture was stirred for 8 days at room temperature. After completing the reaction, the solvent was evaporated and the residue was extracted with DCM (3 × 30 mL). The crude product was purified by the solid phase extraction technique (SPE 10 g), yielding the title compound (0.69 g, 62.9% yield). ^1H NMR (500 MHz, DMSO-d_6) δ: 5.52 (br s, 1H, 6 OH), 4.95 (dd, 1H, H-13), 4.83 (d, 1H, H-1″), 4.43 (d, 1H, H-1′), 4.26 (d, 1H, 4″OH), 4.04−4.11 (m, 1H, H-5″), 4.05 (q, 2H, CH_2/Et), 3.97−4.00 (m, 1H, H-3), 3.97 (br s, 1H, 2′ OH), 3.94 (s, 1H, 12 OH), 3.63−3.71 (m, 1H, H-5′), 3.52 (d, 1H, H-5), 3.46 (s, 1H, H-11), 3.22 (s, 3H, 3″OMe), 3.06−3.11 (m, 1H, α-CH_2), 3.00−3.06 (m, 1H, H-2′), 2.87−2.93 (m, 1H, H-4″), 2.76−2.80 (m, 1H, α'-CH_2), 2.72−2.76 (m, 3H, β-CH_2), 2.67−2.71 (m, 1H, H-10), 2.51−2.54 (m, 1H, H-9), 2.41−2.48 (m, 2H, β'-CH_2), 2.39−2.41 (m, 1H, H-3′), 2.31−2.37 (m, 1H, α-CH_2), 2.29 (d, 1H, H-2″), 2.22 (s, 6H, 3′NMe_2), 2.15−2.20 (m, 1H, H-9), 1.92−1.99 (m, 1H, H-4), 1.80−1.83 (m, 1H, H-8), 1.73−1.79 (m, 1H, H-14), 1.59 (dd,

1H, H-4′), 1.50−1.55 (m, 1H, H-7), 1.51 (dd, 1H, H-2″), 1.37 (ddd, 1H, H-14), 1.28 (dd, 1H, H-7), 1.20 (s, 3H, 6 Me), 1.18 (t, 3H, CH_3/Et), 1.16 (d, 3H, 5″ Me), 1.14 (s, 3H, 3″ Me), 1.11 (d, 3H, 2 Me), 1.08 (d, 3H, 5′ Me), 1.06−1.06 (m, 1H, H-4′), 1.00 (d, 3H, 4 Me), 0.96−0.98 (m, 6H, 10 me, 12 Me), 0.85 (d, 3H, 8 Me), 0.78 (t, 3H15 Me). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 175.7, 172.0, 101.9, 95.2, 82.6, 78.4, 77.3, 76.4, 74.9, 74.0, 73.6, 72.6, 70.6, 67.0, 66.0, 64.8, 64.5, 60.3, 59.6, 51.4, 48.7, 48.0, 44.8, 44.2, 40.3, 40.3, 40.2, 34.8, 34.2, 29.9, 28.7, 26.5, 22.0, 21.4, 20.9, 20.9, 18.4, 17.3, 15.3, 14.0, 10.8, 9.5, 8.8. MS (ES$^+$) m/z: 878.5.

9-Deoxo-9-dihydro-9a-[*N*′-(*β*-amidoethyl)-*β*-aminoethyl]-9a-aza-9a-homoerythromycin A (17). To the EtOH (10 mL) solution of **16** (0.44 g, 0.50 mol), 25% ammonia solution (8 mL) was added. The reaction mixture was stirred at room temperature for 3 days. After evaporation of the solvent the crude residue was dissolved in DCM (30 mL). Water (30 mL) was added, and the pH was adjusted to 9.6. After extraction with DCM (2 × 30 mL) the combined organic layers were dried over K_2CO_3 and the solvent was evaporated, yielding the title compound **17** (0.30 g, 72.2% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.91 (dd, 1H, H-13), 4.82 (d, 1H, H-1″), 4.43 (d, 1H, H-1′), 4.03−4.11 (m, 1H, H-5″), 3.95 (d, 1H, H-3), 3.62−3.70 (m, 1H, H-5′), 3.53 (d, 1H, H-5), 3.48 (s, 1H, H-11), 3.24−3.27 (m, 1H, α-CH_2), 3.22 (s, 3H, 3″OMe), 3.03 (dd, 1H, H-1′), 2.88−2.95 (m, 2H, α′-CH_2), 2.83−2.88 (m, 1H, H-4″), 2.70−2.79 (m, 4H, H-2, H-10, *β*-CH_2), 2.44−2.49 (m, 2H, α-CH_2, H-9), 2.38−2.44 (m, 1H, H-3′), 2.28 (d, 1H, H-2″), 2.23−2.26 (m, 2H, *β*′-CH_2), 2.22 (s, 6H, 3′NMe_2), 2.16−2.21 (m, 1H, H-9), 1.90−1.99 (m, 1H, H-4), 1.74−1.82 (m, 1H, H-14), 1.68−1.73 (m, 1H, H-8), 1.59 (dd, 1H, H-4′), 1.49−1.55 (m, 2H, H-7, H-2″), 1.35−1.44 (m, 1H, H-14), 1.26 (br s, 1H, H-7), 1.23 (s, 3H, 6 Me), 1.18 (d, 3H, 5″ Me), 1.13 (s, 3H, 3″ Me), 1.12 (d, 3H, 2 Me), 1.08 (s, 1H, H-4′), 1.07 (s, 3H, 5′Me), 1.02 (d, 3H, 4 Me), 0.97−1.00 (m, 6H, 10 Me, 12 Me), 0.85 (d, 3H, 8 Me), 0.79 (t, 3H, 15 Me). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 175.5, 173.7, 101.9, 95.6, 82.4, 79.1, 77.3, 76.8, 76.0, 74.0, 72.6, 70.6, 67.0, 65.2, 64.8, 64.5, 60.1, 48.9, 48.7, 45.6, 44.4, 44.0, 40.3, 39.9, 38.6, 35.0, 32.3, 29.8, 28.9, 26.4, 21.6, 21.4, 21.0, 20.8, 18.6, 17.7, 15.6, 10.7, 9.6, 8.7. MS (ES$^+$) m/z: 850.6.

9-Deoxo-9-dihydro-9a-[*N*′-(*β*-cyanoethyl)-*β*-aminoethyl]-9a-aza-9a-homoerythromycin A (18). To the MeOH (15 mL) solution of intermediate **10** (0.92 g, 1.18 mol), a solution of acrylonitrile (0.08 mL, 1.221 mmol) in MeOH (2 mL) was added dropwise. The reaction mixture was refluxed for 22 h. After the reaction was completed, the solvent was evaporated and the residue extracted with DCM (3 × 30 mL). The crude product was purified by the solid phase extraction technique (SPE 10 g), yielding the title compound (0.63 g, 89.0% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.98 (dd, 1H, H-13), 4.81 (d, 1H, H-1″), 4.43 (d, 1H, H-1′), 4.26 (d, 1H, 4″ OH), 4.08 (dq, 1H, H-5″), 3.97 (s, 1H, 12 OH), 3.92−3.96 (m, 2H, 2′OH), 3.63−3.71 (m, 1H, H-5′), 3.52 (d, 1H, H-5), 3.46 (br s, 1H, H-11), 3.22 (s, 3H, 3″OMe), 3.10−3.16 (m, 1H, α-CH_2), 3.03 (ddd, 1H, H-2′), 2.91 (dd, 1H, H-4″), 2.78−2.83 (m, 1H, α′-CH_2), 2.74−2.77 (m, 1H, H-2), 2.71−2.74 (m, 1H, H-10), 2.69−2.71 (m, 1H, α′-CH_2), 2.67−2.68 (m, 1H, *β*-CH_2), 2.57 (t, 2H, *β*′-CH_2), 2.52−2.53 (m, 1H, H-9), 2.46−2.48 (m, 1H, *β*-CH_2), 2.39−2.45 (m, 1H, H-3′), 2.34−2.38 (m, 1H, α-CH_2), 2.28 (d, 1H, H-2″), 2.21 (s, 6H, 3′NMe_2), 2.16−2.20 (m, 1H, H-9), 1.92−2.01 (m, 1H, H-4), 1.75−1.83 (m, 1H, H-14), 1.72−1.75 (m, 1H, H-8), 1.59 (dt, 1H, H-4′), 1.49−1.55 (m, 1H, H-7), 1.52 (dd, 1H, H-2″), 1.32−1.42 (m, 1H, H-14), 1.24−1.29 (m, 1H, H-7), 1.22 (s, 3H, 6 Me), 1.18 (d, 3H, 5″Me), 1.14 (s, 3H, 3″Me), 1.12 (d, 3H, 2 Me), 1.08 (d, 3H, 5′Me), 1.06 (br s, 1H, H-4′), 1.01 (d, 3H, 4 Me), 0.96−0.99 (m, 6H, 10 Me, 12 Me), 0.84 (d, 3H, 8 Me), 0.78 (t, 3H, 15 Me). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 175.6, 120.0, 101.8, 95.4, 82.5, 78.8, 77.3, 76.5, 75.2, 74.0, 73.7, 72.6, 70.6, 67.0, 65.9, 64.8, 64.5, 60.3, 51.5, 48.7, 47.7, 44.8, 44.3, 40.2, 38.9, 34.9, 29.8, 29.0, 26.6, 21.8, 21.4, 20.9, 20.8, 18.5, 17.6, 17.4, 15.5, 10.7, 9.6, 9.0. MS (ES$^+$) m/z: 831.4.

General Procedure for the Preparation of Ureas 12, 13, 19, 21, 22 and Thioureas 14, 15, 20. To the solution of **10**, **11**, **16**, or **18** in dichloromethane, 20% excess isocyanate or isothiocyanate was added, and the reaction mixture was stirred about 1 h at 0−5 °C. The crude product was evaporated, wherefrom by column chromatography on silica gel using solvent system $CH_2Cl_2/CH_3OH/NH_3(25\%)$ = 90:9:1.5 the pure compound was obtained.

*9-Deoxo-9-dihydro-9a-N-(N′-ethylcarbamoyl-β-aminoethyl)-9a-aza-9a-homoerythromycin A (**12a**).* Yield: 0.30 g, 61.7%. ^{1}H NMR (500 MHz, pyridine-d_5) δ: 6.57 (t, 1H, NH), 5.43 (dd, 1H, H-13), 5.25 (d, 1H, H-1″), 5.00 (d, 1H, H-1′), 4.69 (d, 1H, H-3), 4.64 (m, 1H, H-5″), 4.25 (d, 1H, H-11), 4.17 (d, 1H, H-5), 4.02 (m, 1H, H-5′), 3.86 (m, 1H, *β*-CH_2), 3.71 (dd, 1H, H-2′), 3.63 (m, 1H, α-CH_2), 3.54 (s, 3H, 3″OMe), 3.50 (m, 1H, *β*-CH_2), 3.36 (m, 2H, CH_2/Et), 3.31 (m, 1H, H-4″), 3.19 (m, 1H, H-2), 3.15 (m, 1H, H-10), 2.84 (m, 1H, H-3′), 2.76 (m, 1H, α-CH_2), 2.76 (d, 1H, H-9), 2.54 (m, 1H, H-4), 2.48 (d, 1H, H-2″), 2.39 (m, 1H, H-9), 2.32 (s, 6H, 3′NMe_2), 2.30 (m, 1H, H-8), 2.19 (m, 1H, H-14), 1.99, (m, 1H, H-7), 1.86 (m, 1H, H-7), 1.82 (s, 3H, 6 Me), 1.73 (m, 1H, H-14), 1.65 (s, 3H, 3″ Me), 1.64 (d, 3H, 4 Me), 1.60 (dd, 1H, H-2″), 1.56 (d, 1H, H-4′), 1.42 (d, 3H, 10 Me), 1.37 (d, 3H, 2 Me), 1.35 (s, 3H, 12 Me), 1.34 (d, 3H, 5″ Me), 1.33 (d, 3H, 5′ Me), 1.25 (m, 1H, H-4′), 1.12 (t, 3H, CH_3/Et), 0.97 (d, 3H, 8 Me), 0.94 (t, 3H, 15 Me). ^{13}C NMR (125 MHz, pyridine-d_5) δ: 178.0, 159.9, 103.8, 97.2, 84.4, 80.8, 79.9, 79.2, 76.9, 75.7, 74.1, 72.0, 68.5, 66.7, 66.4, 66.0, 62.1, 53.4, 50.2, 46.2, 41.4, 40.9, 40.1, 36.2, 35.9, 31.2, 30.4, 28.2, 22.8, 22.6, 22.3, 22.0, 19.9, 18.7, 16.6, 16.4, 11.9, 11.0, 9.7. MS (ES$^+$) m/z: 849.1.

*9-Deoxo-9-dihydro-9a-[N′-(β-cyanoethyl)-N′-(2-naphthyl)carbamoyl-β-aminoethyl]-9a-aza-9a-homoerythromycin A (**19i**).* Yield: 0.22 g, 81.4%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.62 (s, 1H, NH), 8.04 (d, 1H, naphthyl, a), 7.77−7.81 (m, 2H, naphthyl, d, e), 7.74 (d, 1H, naphthyl, h), 7.66 (dd, 1H, naphthyl, c), 7.40−7.44 (m, 1H, naphthyl, f), 7.32−7.36 (m, 1H, naphthyl, g), 5.33 (br s, 1H, 6OH), 4.98 (dd, 1H, H-13), 4.79 (d, 1H, H-1″), 4.46 (br s, 1H, 12OH), 4.44 (d, 1H, H-1′), 4.41 (d, 1H, 11OH), 4.25 (d, 1H, 4″OH), 4.03−4.11 (m, 1H, H-5″), 4.02 (dd, 1H, H-3), 3.96 (br s, 1H, 2′OH), 3.66−3.70 (m, 1H, H-5′), 3.62−3.66 (m, 2H, H-5′), 3.51−3.56 (m, 2H, H-11, H-5), 3.44−3.51 (m, 2H, *β*-CH_2), 3.22 (s, 3H, 3″OMe), 3.02−3.08 (m, 1H, H-2′), 2.97−3.02 (m, 1H, α-CH_2), 2.89 (dd, 1H, H-4″), 2.83 (d, 1H, H-10), 2.79 (t, 2H, *β*′-CH_2), 2.75 (d, 1H, H-2), 2.67 (dd, 1H, H-9), 2.59−2.65 (m, 1H, α-CH_2), 2.39−2.46 (m, 1H, H-3′), 2.26 (d, 1H, H-2″), 2.21 (s, 6H, 3′NMe_2), 2.16−2.19 (m, 1H, H-9), 1.92−1.97 (m, 1H, H-4), 1.88−1.92 (m, 1H, H-8), 1.76−1.85 (m, 1H, H-14), 1.55−1.62 (m, 1H, H-4′), 1.53−1.56 (m, 1H, H-7), 1.48 (dd, 1H, H-2″), 1.41 (ddd, 1H, H-14), 1.32 (d, 1H, H-7), 1.24 (s, 3H, 6Me), 1.15 (d, 3H, 5″Me), 1.10−1.13 (m, 6H, 2Me, 3″Me), 1.08−1.09 (m, 1H, H-4′), 1.07 (d, 3H, 5′Me), 1.04 (s, 3H, 12Me), 1.01−1.04 (m, 3H, 10Me), 1.01 (s, 3H, 4Me), 0.89 (d, 3H, 8Me), 0.82 (t, 3H, H-15). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 175.9, 154.6, 137.9, 133.4, 129.1, 127.5, 127.2, 126.8, 125.9, 123.8, 121.3, 119.2, 115.3, 101.9, 95.3, 82.9, 78.4, 77.2, 76.6, 76.2, 74.1, 73.6, 72.6, 70.6, 67.0, 64.8, 64.5, 63.9, 59.4, 50.1, 48.7, 46.5, 44.2, 43.1, 40.2, 39.9, 39.6, 34.8, 29.8, 28.1, 26.8, 22.1, 21.3, 21.2, 20.8, 18.4, 18.1, 16.7, 15.2, 10.9, 9.4, 8.8. MS (ESI) m/z calcd for $C_{53}H_{86}N_5O_{13}$ $[M + H]^+$ 1000.6222; found 1000.6255.

*9-Deoxo-9-dihydro-9a-{N′-[β-(ethoxycarbonyl)ethyl]-N′-(2-naphthyl)carbamoyl-β-aminoethyl}-9a-aza-9a-homoerythromycin A (**21i**).* Yield: 0.18 g, 82.3%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.52 (s, 1H, NH), 8.02 (d, 1H, naphthyl, a), 7.79 (d, 1H, naphthyl, e), 7.77 (d, 1H, naphthyl, d), 7.73 (d, 1H, naphthyl, h), 7.63 (dd, 1H, naphthyl, c), 7.39−7.44 (m, 1H, naphthyl, g), 7.31−7.36 (m, 1H, naphthyl, f), 5.45 (br s, 1H, 6OH), 4.97 (dd, 1H, H-13), 4.79 (d, 1H, H-1″), 4.43−4.46 (m, 2H, H-1′), 4.34 (br s, 1H, 11OH), 4.25 (d, 1H, 4″OH), 4.09 (q, 2H, ethyl, CH_2), 4.04−4.07 (m, 1H, H-5″), 4.01−4.04 (m, 1H, H-3), 3.98 (br s, 1H, 2′OH), 3.63−3.71 (m, 1H, H-5′), 3.56−3.62 (m, 2H, α″-CH_2), 3.54 (d, 1H, H-5), 3.53 (br s, 1H, H-11), 3.43 (t, 2H, *β*-CH_2), 3.22 (s, 3H,

3″OMe), 3.02−3.07 (m, 1H, H-2′), 2.96−3.02 (m, 1H, α-CH_2), 2.89 (dd, 1H, H-4″), 2.79−2.85 (m, 1H, H-10), 2.75 (quin, 1H, H-2), 2.68 (dd, 1H, H-9), 2.62 (t, 2H, β″-CH_2), 2.58 (br s, 1H, α-CH_2), 2.39−2.46 (m, 1H, H-3′), 2.26 (d, 1H, H-2″), 2.21 (s, 6H, 3′NMe_2), 2.18−2.20 (m, 1H, H-9), 1.92−1.97 (m, 1H, H-4), 1.87−1.92 (m, 1H, H-8), 1.75−1.84 (m, 1H, H-14), 1.57−1.61 (m, 1H, H-4′), 1.51−1.55 (m, 1H, H-7), 1.43−1.50 (m, 1H, H-2″), 1.37−1.43 (m, 1H, H-14), 1.31−1.37 (m, 1H, H-7), 1.23 (s, 3H, 6Me), 1.20 (t, 3H, ethyl, CH_3), 1.15 (d, 3H, 5″Me), 1.10−1.13 (m, 6H, 2Me, 3″Me), 1.08−1.10 (m, 1H, H-4′), 1.07 (d, 3H, 5′Me), 1.04 (s, 3H, 12Me), 1.03 (d, 3H, 10Me), 1.00 (d, 3H, 4Me), 0.90 (d, 3H, 8Me), 0.82 (t, 3H, H-15). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 176.1, 171.6, 154.8, 138.2, 133.5, 129.0, 127.6, 127.3, 126.8, 126.0, 123.8, 121.2, 114.9, 101.9, 95.2, 82.8, 78.2, 77.3, 76.7, 76.0, 74.1, 73.6, 72.6, 70.6, 67.1, 64.9, 64.5, 64.0, 60.0, 59.4, 49.9, 48.8, 46.3, 44.2, 43.2, 40.3, 39.9, 39.8, 34.8, 33.3, 29.8, 28.2, 26.5, 22.1, 21.4, 21.2, 20.9, 18.4, 18.0, 15.2, 14.0, 10.9, 9.5, 8.8. MS (ESI) m/z calcd for $C_{55}H_{91}N_4O_{15}$ $[M + H]^+$ 1047.6481; found 1047.6488.

*9-Deoxo-9-dihydro-9a-[N′-(β-amidoethyl)-N′-(2-naphthyl)carbamoyl-β-aminoethyl]-9a-aza-9a-homoerythromycin A (**22i**).* Yield: 0.23 g, 73.2%. ^1H NMR (500 MHz, DMSO-d_6) δ: 8.52 (s, 1H, NH), 8.04 (d, 1H, naphthyl, a), 7.77 (d, 1H, naphthyl, d), 7.75 (d, 1H, naphthyl, e), 7.73 (d, 1H, naphthyl, h), 7.61 (dd, 1H, naphthyl, c), 7.38−7.42 (m, 1H, naphthyl, f), 7.32−7.36 (m, 1H, naphthyl, g), 5.42 (br s, 1H, 6OH), 4.98 (dd, 1H, H-13), 4.80 (d, 1H, H-1″), 4.44−4.48 (m, 2H, H-1′, 11OH), 4.32 (br s, 1H, 12OH), 4.24 (d, 1H, 4″OH), 4.04−4.07 (m, 1H, H-5″), 4.01−4.04 (m, 1H, H-3), 3.97 (br s, 1H, 2′OH), 3.64−3.72 (m, 1H, H-5′), 3.56−3.62 (m, 2H, H-11, H-5), 3.56 (d, 1H, β-CH_2), 3.20 (s, 3H, 3″OMe), 3.00−3.06 (m, 1H, α-CH_2), 2.96−3.02 (m, 1H, H-2′), 2.90 (dd, 1H, H-4″), 2.79−2.83 (m, 1H, β-CH_2), 2.75 (quin, 1H, H-2), 2.66 (dd, 1H, H-9), 2.60 (t, 2H, CH_2), 2.54 (br s, 1H, α-CH_2), 2.39−2.46 (m, 1H, H-3′), 2.23 (d, 1H, H-2″), 2.19 (s, 6H, 3′NMe_2), 2.15−2.18 (m, 1H, H-9), 1.92−1.97 (m, 1H, H-4), 1.85−1.90 (m, 1H, H-8), 1.75−1.84 (m, 1H, H-14), 1.58−1.62 (m, 1H, H-4′), 1.50−1.54 (m, 1H, H-7), 1.44−1.50 (m, 1H, H-2″), 1.38−1.43 (m, 1H, H-14), 1.31−1.36 (m, 1H, H-7), 1.20 (s, 3H, 6Me), 1.13 (d, 3H, 5″Me), 1.10−1.12 (m, 6H, 2Me, 3″Me), 1.06−1.08 (m, 1H, 5′Me), 1.07 (d, 3H, 12Me), 1.03 (s, 3H), 1.02 (d, 3H, 10Me), 1.00 (d, 3H, 4Me), 0.94 (d, 3H, 8Me), 0.85 (t, 3H, H-15). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 175.9, 173.6, 154.6, 138.0, 132.8, 129.4, 128.2, 127.4, 126.8, 126.1, 123.6, 121.4, 115.3, 101.4, 95.7, 82.1, 78.4, 77.3, 76.4, 74.0, 73.6, 72.6, 70.6, 67.0, 65.9, 64.5, 60.0, 59.4, 51.9, 48.8, 48.3, 44.6, 44.2, 40.3, 40.0, 39.8, 34.8, 34.3, 29.9, 28.6, 26.5, 22.1, 21.4, 21.0, 20.9, 18.4, 17.7, 15.2, 14.0, 10.9, 9.5, 8.8. MS (ESI) m/z calcd. for $C_{55}H_{91}N_4O_{15}$ $[M + H]^+$ 1047.6481; found 1047.6488.

Biology. *Compound Susceptibility Testing.* The susceptibility of different malaria strains to azithromycin analogues was determined using the tritiated hypoxanthine incorporation assay of Desjardins et al.,[21] as modified by Milhous et al.,[22] except that the drug exposure period was 48 h. The *P. falciparum* clones used were W2, D6, and TM91C235.[23] W2 from Vietnam is a mefloquine-sensitive strain resistant to chloroquine and pyrimethamine. D6 clone from West Africa is sensitive to all antimalarials, with a decreased in vitro sensitivity to mefloquine. TM91C235 is a strain from Thailand that is highly resistant to mefloquine and a number of other antimalarials. Compounds and control antimalarials (chloroquine, mefloquine, and azithromycin) were diluted 2-fold over 11 different concentration, and IC_{50} values were determined using a nonlinear logistic dose response program. The presented IC_{50} values represent averaged values in cases where multiple results were generated, and relative errors were usually <30% and did not exceed 50%, which is standard for this type of assay.

In Vivo Antimalarial Studies of P. berghei in Mice. The in vivo efficacies of the new compounds were determined by a modified Thompson test.[24] This test measures the survival of mice and parasite clearance following administration of the drug on days 3−5 postinfection. In brief, 5×10^6 *P. berghei* infected erythrocytes (KBG-173 strain) were inoculated into the intraperitoneal cavity of male mice that weighed 24−30 g. By day 3 postinfection, parasitemia ranged from 1.0% to 3.7%. Each drug suspended in peanut oil was administered subcutaneously (sc) twice daily from day 3 to day 5 postinfection. The volume of drug suspension given depends on the weight of the mouse and the drug concentration of the suspension. In general, the volume is given at 0.01 mL/g of body weight. Five mice were used in each dosage group. Blood films were taken on day 6 and biweekly for 31 days. Mice that were blood film negative on day 31 postinfection were considered cured. Compounds were considered active when the survival time of the treated mice was greater than twice that of the control mice (i.e., 12−14 days). Mice losing ≥20% of their body weights were sacrificed.

Cytotoxicity Assay. Two immortal cell lines, human monocytic leukemia (THP-1, monocyte) and human Caucasian hepatocyte carcinoma (Hep G2, epithelial), were obtained from the European Collection of Cell Cultures. Cells were maintained in completed RPMI 1640 (Cambrex) medium supplemented with 10% fetal bovine serum (Gibco) at 37 °C in 5% CO_2 atmosphere. Cells were passaged twice a week to keep them in rapid growth phase.

The compounds were examined for cytotoxic effects by continuous drug exposure by the CellTiter 96 Aqueous One solution cell proliferation assay (MTS Assay, Promega). A 15 μL amount of the MTS reagent was added directly to cell lines cultured in microtiter plates. After 1−4 h of incubation, the absorbance at 490 nm was recorded with a spectrophotometric plate reader (Ultra, TECAN). MTS assay measures cell viability through quantification of reducing equivalents such as NADH, using mitochondrial succinate dehydrogenase activity of living cells. The method was programmed for TECAN robotic system in GEMINI pipetting software.[25] The results were expressed as IC_{50} values.

Preliminary Pharmacokinetics. ***Metabolic Stability Assay.*** Selected compounds were evaluated in several secondary assays including metabolic stability assessments using in vitro liver microsomes. Sample stocks at 10 or 20 mM (depending on solubility) in DMSO were diluted to a final concentration of 1 μM into a mixture containing, 0.5 mg/mL of prewarmed pooled human or mouse liver microsomes (BD Gentest), 1.3 mM NADP (Sigma), 3.3 mM $MgCl_2$ (Sigma), and 0.1 M PBS, pH 7.4, using a TECAN Genesis robotic liquid handler. The reaction was started with the addition of 1 U/mL glucose 6-phosphate dehydrogenase G6PD. The mixture was incubated on a shaking platform at 37 °C, and aliquots were taken and quenched with the addition of an equal volume of cold acetonitrile at 0, 10, 20, 30, and 60 min. Samples were centrifuged at 3700 rpm for 10 min at 20 °C to remove debris. Sample quantification was carried out by LCMS, and metabolic half-life ($t_{1/2}$) was calculated by log plots of the total ion chromatograph area remaining.

Rat Pharmacokinetis. Male Sprague−Dawley rats weighing 250−300 g (IFFA CREADO, Lyon, France) were dosed in a crossover design. Each compound was administered intravenously at 10 mg/kg, followed by a 2 day washout period, and orally at 30 mg/kg free base equivalent.

Compounds were formulated in 1%/20% Encapsin (w/v) and saline. Blood samples were collected up to 24 h postdosing and analyzed by LCMS/MS. Pharmacokinetic parameters were calculated using WinNonlin (Pharsight, U.S.).

■ ASSOCIATED CONTENT

S Supporting Information. Additional spectroscopic information for selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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