Synthesis and Antimalarial Efficacy of Aza-Fused Rhodacyanines in Vitro and in the *P. berghei* Mouse Model

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Received May 25, 2006

Several aza-fused rhodacyanines were synthesized and assessed for their in vitro and in vivo antimalarial activities against *Plasmodium falciparum* K1 and *P. berghei*. All synthetic compounds showed strong selective antimalarial in vitro activity. Class II azarhodacyanines, **3**, consisting of four heterocyclic units, were found to display good parasitemia suppression and low acute toxicity in vivo. Among them, **3c** appeared to be the most effective at a dose of 20−25 mg kg^{-1} day^{-1} (ip).

Malaria remains a major health problem in many developing countries. It affects mainly the population living in tropical and subtropical areas. Annually, it is estimated that there are 300−500 million cases of malaria leading to 1−2 million deaths, most of which are children under 5 years old.[1] One of the major reasons for the morbidity and mortality is the widespread emergence of drug-resistant strains of the parasite. Particularly, the efficacy of clinically available antimalarials, such as chloroquine (CQ[a]), primaquine, and pyrimethamine, is dramatically decreasing.[2,3] Therefore, new antimalarial drugs representing a new class of molecular framework and displaying novel mechanism of action compared to clinically used drugs are urgently needed.[4−6] We previously reported that rhodacyanine dyes (**1**),[7] having a π-delocalized lipophilic cationic (DLC) structure,[8,9] showed strong antimalarial activity in vitro against *Plasmodium falciparum* (drug-sensitive FCR-5 strain) with high selective activity (Figure 1).[10−12] However, further investigations by us revealed that rhodacyanines, such as MKT-077 (**1a**)[7,10] and MKH-57 (**1b**),[10] showed poor antimalarial activity in vivo using the rodent malaria parasite *P. berghei*. In our preliminary animal experiments it was observed that accumulation of **1** in tissue levels would prevent drug accumulation into malaria-affected erythrocytes. To improve the bioavailability by changing the rhodacyanine skeleton, we have designed aza-fused analogues **2** and **3** (Figure 2) as a second generation of antimalarial rhodacyanines. In this communication, we report the synthesis of two types of azarhodacyanines and the evaluation of their antimalarial activity in vitro and in vivo.

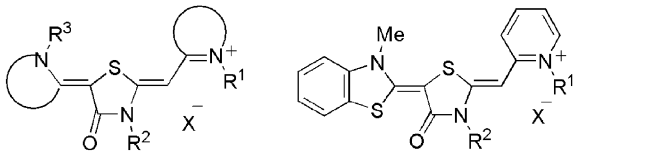

Figure 1. General and typical structures of rhodacyanine.

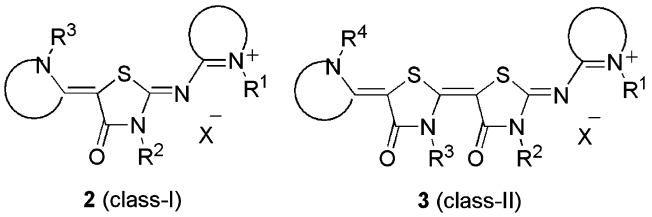

Figure 2. General structures of azarhodacyanines **2** (class I) and **3** (class II).

Chemistry

In general, rhodacyanines are composed of three linearly linked heterocycles involving rhodanine skeleton as a central core, and the rhodanine ring is tethered with the right cationic heterocycle by an sp^2 carbon atom, whereas the designed azarhodacyanines **2**[13] possess imine function instead of olefinic tether in the original rhodacyanines **1** because introduction of a heteroatom often leads to improve water solubility and/or hydrophilicity.[10] In this study, we also designed four-rings-linked analogues **3**, which have two rhodanine moieties flanked by two heterocyclic rings. Synthesis of **2** was accomplished according to the reported procedures with some modifications.[10,12] Scheme 1 illustrates a typical procedure for the synthesis of **2a**. Condensation of methylthionium salt **4** with 3-ethylrhodanine (**5**) in the presence of triethylamine afforded merocyanine **6**. *S*-Methylation of **6** using methyl *p*-toluenesulfonate was further carried out, and the resulting thioiminium salt **7** was condensed with 2-aminomethylpyridinium salt (**8**) in the presence of triethylamine, followed by the treatment with ion-exchange resin, to provide the desired azarhodacyanine **2a** (class I).

For the synthesis of **3** (class II), the promising method was the condensation of thioiminium salt **7** and cyanine **9**, which correspond to each double-heterocyclic unit on left and right sides, respectively. A typical procedure for the synthesis of **3a** is summarized in Scheme 2. Synthesis of **9** was accomplished by a three-step sequence from 2-aminopyridine (**10**), namely, the reaction of **10** with isothiocyanate to give thiourea **11**, which was transformed into rhodanine **12** by treatment with chloroacetic acid in refluxing EtOH.[14] Quaternarization of **12** by

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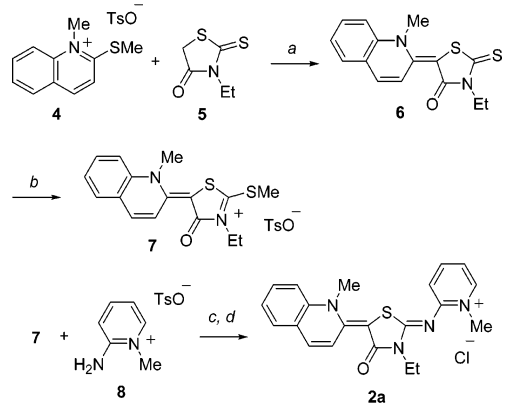
[a] Abbreviations: CQ, chloroquine; DLC, π-delocalized lipophilic cation; MSD, mean survival days.

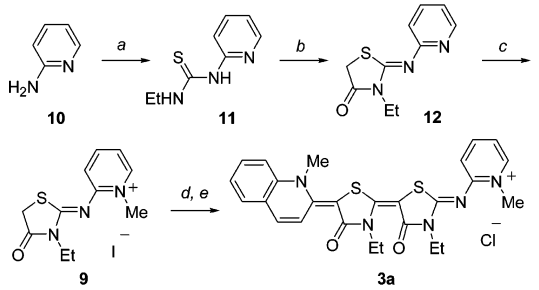
10.1021/jm0606241 CCC: $33.50
Published on Web 07/04/2006

Scheme 1. Synthesis of Class I Azarhodacyanine **2a**[a]



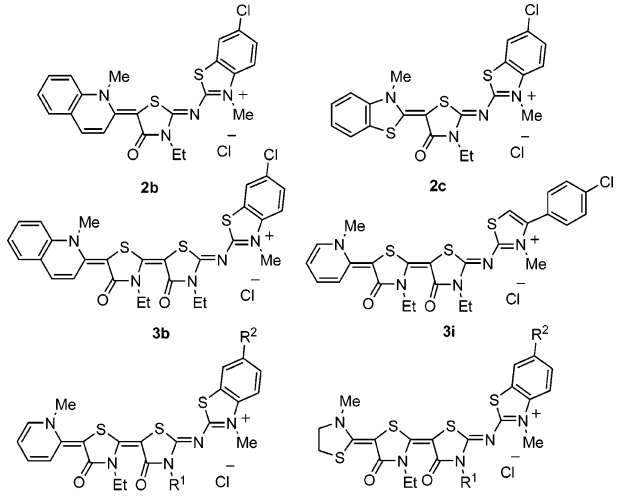
[a] Reagents and conditions: (a) NEt_3, MeCN, 10 °C; (b) *p*-TsOMe, DMF, 130 °C; (c) NEt_3, MeCN, 70 °C; (d) Amberlyte IRA-400(Cl), MeOH.

Scheme 2. Synthesis of Class II Azarhodacyanine **3a**[a]



[a] Reagents and conditions: (a) EtNCS, benzene, 80 °C; (b) $ClCH_2CO_2H$, NaOAc, EtOH, 100 °C; (c) MeI, MeCN, 70 °C; (d) **7**, NEt_3, MeCN, 70 °C; (e) Amberlyte IRA-400(Cl), MeOH.

Chart 1. Prepared Azarhodacyanines



3c: R^1 = Et, R^2 = Cl **3f**: R^1 = Et, R^2 = OMe **3j**: R^1 = Et, R^2 = Cl
3d: R^1 = Et, R^2 = H **3g**: R^1 = ^{i}Pr, R^2 = Cl **3k**: R^1 = allyl, R^2 = Cl
3e: R^1 = Et, R^2 = F **3h**: R^1 = allyl, R^2 = Cl **3l**: R^1 = Et, R^2 = OMe

methyl iodide furnished cyanine **9**. The condensation of **7** and **9** in the presence of triethylamine, followed by an ion exchange process, gave the desired **3a**. By use of the above two procedures, several azarhodacyanines **2a**−**c** and **3a**−**l** were synthesized (Chart 1).[15]

Biological Results and Discussion

The antimalarial potency of several synthetic azarhodacyanines was evaluated in vitro against *P. falciparum* K1 (CQ-resistant strain) according to the procedures described by Desjardins and co-workers.[16] Their cytotoxicities were determined using a rat skeletal myoblast cell line, L-6. Selectivity indices, defined as the ratio EC_{50}(L-6)/EC_{50}(*P. falciparum*), were determined. The biological results are summarized in Table 1. All compounds displayed strong antimalarial activity within the range 4−23 nM and excellent selectivity indices larger than 500. Among the tested compounds, class I compound **2b** showed excellent inhibitory effects against CQ-resistant *P. falciparum* with an IC_{50} of 4.4 nM (entry 2). Its antimalarial activity was estimated to be 4- and 30-fold stronger than those of rhodacyanine **1b** having methyne tether and CQ, respectively (entries 6 and 7). Class II compounds **3** displayed slightly lower antimalarial activity (EC_{50} = 10−23 nM) than **2**, but their cytotoxicity levels were low (entries 3−5). The in vitro results indicate that azarhodacyanines **2** and **3** would be new lead structures for antimalarial agents.

Table 1. In Vitro Antimalarial Activity and Cytotoxicity of Azarhodacyanines

		EC_{50} (nM)		
entry	compd	*P. falciparum*[a]	L-6	selectivity
1	**2a**	5.9	1.2×10^5	2.0×10^4
2	**2b**	4.4	1.1×10^4	2.5×10^3
3	**3c**	10	4.6×10^4	4.6×10^3
4	**3d**	22	1.4×10^4	6.4×10^2
5	**3j**	23	1.2×10^4	5.2×10^2
6	**1b**	19	1.0×10^5	5.0×10^3
7	CQ[b]	150		

[a] K1 (CQ-resistant) strain. [b] CQ = chloroquine.

Table 2. In Vivo Antimalarial Potency of Azarhodacyanines[a]

entry	compd	dose (mg kg^{-1} day^{-1})[b]	suppression (%)	MSD[c]
1	**2a**	10 (ip)	64.5	ND[d]
2	**2b**	10 (ip)	54.6	ND[d]
3	**2c**	10 (ip)	54.1	ND[d]
4	**2c**	25 (ip)	62.7	5.5
5	**3a**	25 (ip)	78.4	5.6
6	**3b**	25 (ip)	49.7	7.0
7	**3c**	25 (ip)	97.1	25.3
8	**3c**	100 (po)	41.7	6.5
9	**3d**	25 (ip)	96.4	22.0
10	**3e**	25 (ip)	79.5	10.8
11	**3f**	25 (ip)	98.3	6.3
12	**3g**	25 (ip)	95.5	7.7
13	**3h**	25 (ip)	95.7	18.0
14	**3i**	25 (ip)	95.6	5.3
15	**3j**	25 (ip)	88.4	16.0
16	**3k**	25 (ip)	73.1	12.8
17	**3l**	25 (ip)	95.0	5.0
18	**1b**	10 (ip)	27.0	ND[e]
19	CQ[e]	10 (ip)	90.6	22.7

[a] In vivo evaluation was carried out according as Peters' 4-day suppressive protocol using five ICR-mice. [b] ip = intraperitoneal administration; po = per os administration. [c] MSD = mean survival days. MSD for untreated mice (control) is ∼6 days. [d] ND = not determined. [e] CQ = chloroquine.

Next, in vivo evaluation of antimalarial efficacy of **2** and **3** was carried out using the rhodent malaria model *P. berghei* NK-65 (drug-sensitive strain) in mice. The experiments were performed according to Peters' 4-day suppressive test protocol.[17] The reduction of parasitaemia in % was determined by comparing the parasitemia of infected mice that were injected with the compounds for 4 days with the parasitemia of untreated control mice. Observation of malaria-affected mice after the end of treatment was continued in order to record their mean survival days (MSD). The in vivo results are summarized in Table 2. Carborhodacyanine **1b** showed only weak antimalarial activity at a dose of 10 mg kg^{-1} day^{-1} by intraperitoneal (ip) injection (entry 18), whereas the newly designed azarhodacyanines **2**

exhibited good parasitemia suppression (55−65%) at the same dose (entries 1−3). The results clearly indicate that the introduction of a nitrogen atom in the cyanine conjugation would increase antimalarial efficacy in vivo. However, several signs of acute toxicity, such as diarrhea and body weight loss, were observed with **2a**, **2b**, and **1b**. Moreover, doses higher than 25 mg kg^{-1} day^{-1} (ip) resulted in 100% fatality in mice within 24 h. In contrast, **2c** exhibited no apparent acute toxicity at a dose of 10 mg kg^{-1} day^{-1}. However, disappointedly, an increase of the dose (25 mg kg^{-1} day^{-1}) afforded only slightly improved activity of 63% suppression and obvious weight loss was observed (entry 4). Moreover, no survival effect of the treated mice was observed compared with the control (malaria-affected mice without drug treatment).

Further investigation made clear that class II compounds **3** afforded much better results in antimalarial efficacy and toxicity, although biological results in vitro were inferior to class I. All compounds could be injected at a dose of 25 mg kg^{-1} day^{-1} (ip) and displayed moderate to excellent suppression levels at the dose. Compounds **3a** and **3b**, bearing a quinoline moiety at the left edge, showed 78% and 50% suppression levels, respectively, but considerable weight loss during the period of treatment was observed (entries 5 and 6, Table 2). Displacement of the pyridine ring for quinoline resulted in significant enhancement of antimalarial potency and survival effect. Compound **3c** resulted in 97% suppression at a dosage of 25 mg kg^{-1} day^{-1} by ip administration (entry 7). Although day-to-day weight loss caused by its acute toxicity was observed during the medication period, the mice still looked fine and their body weight was recovered after the treatment. Finally, the mice treated by **3c** survived for 25 days on average. It is noteworthy that the in vivo antimalarial potency of **3c** (25 mg kg^{-1} day^{-1}) is comparable to that of CQ at a dose of 10 mg kg^{-1} day^{-1} (entry 19). Replacement of chlorine atom at the benzothiazole ring (a heteroaromatic moiety at the right edge) with a hydrogen atom retains antimalarial efficacy and survival effect (entry 9), whereas the fluorinated analogue resulted in lower potency (entry 10). Interestingly and surprisingly, **3f** and **3i**, whose structures are analogous to that of **3c**, resulted in excellent inhibition of parasitic growth comparable to **3c**, but its acute toxicity was significantly high to result in a much earlier death of the animals (entries 11 and 14). A partial structure−activity relationship study has revealed that the substituent on the right rhodanine ring (see Figure 2) also influences the survival, although the antimalarial activity is retained (entries 7, 12, and 13). Displacement of the pyridine ring of **3c** with thiazolidine (**3j**) slightly decreased parasites in suppression and survival (entry 7 vs entry 15). Similar tendencies among structure, activity, and acute toxicity for thiazolidine analogues **3j**−**l** were observed (entries 15−17). Thus, the toxicity was considerably affected by R^1 and R^2 substituents (see Chart 1). We are now investigating structure−toxicity relationships uncovered thus far as well as conducting a pharmacokinetic study. It is noteworthy that **3c** has a 42% suppression level at a dosage of 100 mg kg^{-1} day^{-1} (po) although mice survival was not extended (entry 8). We believe the results indicate that azarhodacyanines have potential for oral bioavailability.

Finally, **3c** and **3d**, showing high antimalarial activity with low acute toxicity in vivo, were selected to investigate their dose response by ip administration. As shown in Table 3, significant improvements on suppression of parasitemia were observed for **3c** and **3d** with increased doses. Nevertheless, these compounds showed remarkable toxicity at a dose of more than 40 mg kg^{-1} day^{-1}, although growth of malaria parasites was almost completely suppressed. The results clearly demonstrate that ip administration of **3c** and **3d** at a dose of about 20 mg kg^{-1} day^{-1} resulted in the best suppression of parasitemia and the best mean survival of the mice.

Table 3. In Vivo Antimalarial Potency of **3c** and **3d** at Various Doses (ip)[a]

compd	dose (mg kg^{-1} day^{-1})	suppression (%)	MSD[b]
3c	10	79.8	11.8
	20	97.4	20.3
	40	99.1	7.0
3d	10	70.8	9.8
	20	92.4	19.0
	40	97.8	7.8
control		0	5.8

[a] In vivo evaluation was carried out according as Peters' 4-day suppressive protocol using five ICR-mice. [b] MSD = mean survival days.

In summary, we synthesized two classes of antimalarial azarhodacyanines **2** and **3** and evaluated the in vitro and in vivo antimalarial potency against *P. falciparum* (CQ-resistant K1 strain) and *P. berghei* (NK-65 strain). Both of them were found to possess promising in vitro potency with IC_{50} values ranging from 4 to 23 nM and good selectivity indices of more than 500. In vivo results indicated that class I azarhodacyanines **2** showed higher acute toxicity than **3** (class II). Partial SAR study for **3** suggested that substituents on the heterocyclic moieties remarkably influence acute toxicity in vivo. **3c** having pyridine and 6-chlorobenzothiazole moieties provided 97% suppression of parasites at a dose of 20−25 mg/kg without signs of high toxicity and significantly prolonged the survival of malaria infected mice. Currently, pharmacokinetic and pharmacodynamic studies of **3** are under consideration as well as the synthesis of improved azarhodacyanines with a reduced toxicity profile.

Experimental Section

Chemistry. Synthesis of 2a. To a mixture of **7** (244 mg, 0.50 mmol) and 2-amino-1-methylpyridinium *p*-toluenesulfonate (140 mg, 0.50 mmol) in acetonitrile (2.5 mL) was dropwise added triethylamine (0.21 mL, 1.5 mmol), and the mixture was stirred at 70 °C for 12 h. To the mixture was added ethyl acetate (2.5 mL), and the mixture was cooled to room temperature. After the mixture was stirred for 30 min at room temperature, the precipitate formed was collected and washed with CH_3CN/EtOAc (1:1, v/v) to give the crude product *p*-toluenesulfonate salt **8**. The crude residue was dissolved in $CHCl_3$/MeOH (1:1, v/v), and the solution was then passed through an anion-exchange resin (IRA-400(Cl)) by eluting with $CHCl_3$/MeOH (1:1, v/v). After concentration, recrystallization from MeOH/EtOAc yielded **2a** (147 mg, 71% yield) as orange solids: mp 239−240 °C; IR (KBr) 1612, 1508, 1481, 1394, 1165 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.74 (1H, d, J = 6.3 Hz), 8.33 (1H, dd, J = 8.1, 8.1 Hz), 7.98 (1H, d, J = 9.3 Hz), 7.90 (1H, d, J = 8.5 Hz), 7.86−7.72 (4H, m), 7.47 (2H, dd, J = 6.3, 8.1 Hz), 4.04 (3H, s), 4.01 (2H, q, J = 7.1 Hz), 3.94 (3H, s), 1.29 (3H, t, J = 7.1 Hz); MS (FAB^+) m/z 377 (M^+). Anal. ($C_{21}H_{21}ClN_4OS$) C, H, N.

Synthesis of 3a. To a mixture of **7** (122 mg, 0.25 mmol) in CH_3CN (2.5 mL) was dropwise added triethylamine (0.11 mL, 0.75 mmol). The mixture was stirred at 70 °C for 15 h and then cooled to room temperature. To this mixture was added EtOAc (3.0 mL), and the mixture was stirred for 1 h. The precipitate was collected and washed with CH_3CN to give the crude residue (mixed salt of iodide and *p*-toluenesulfonate). The crude was dissolved in $CHCl_3$/MeOH (1:1, v/v). The solution was passed through an anion-exchange resin (IRA-400(Cl)) and eluted with the same mixed solvent. After concentration, recrystallization from MeOH/EtOAc yielded **3a** (117 mg, 86%) as deep-purple solids: mp >300 °C; IR (KBr) 1653, 1616, 1483, 1435, 1396, 173 cm^{-1}; ^1H NMR (400

MHz, DMSO-d_6) δ 8.81 (1H, d, J = 6.3 Hz), 8.39 (1H, dd, J = 7.2, 8.7 Hz), 7.98−7.85 (2H, m), 7.84−7.70 (4H, m), 7.54 (1H, dd, J = 6.3, 7.2 Hz), 7.44 (1H, dd, J = 7.2, 7.5 Hz), 4.06 (3H, s), 4.04−3.97 (4H, m), 3.95 (3H, s), 1.28 (3H, t, J = 7.0 Hz), 1.24 (3H, t, J = 7.0 Hz); MS (FAB) m/z 504 (M^+). Anal. ($C_{26}H_{26}ClN_5O_2S_2 \cdot 0.5H_2O$) C, H, N.

Biological Assays. In vitro antimalarial assay, in vitro cytotoxic assay, and in vivo antimalarial assay were performed as reported previously.[9]

Acknowledgment. We thank Dr. Kozo Sato, Dr. Hiroshi Kitaguchi, and Dr. Masayuki Kawakami (Fuji Photo Film Co., Ltd.) for helpful discussions and for providing several compounds. This work was financially supported by a Grant-in-Aid from Japan Science and Technology Agency (JST) and a Grant-in-Aid from the Takeda Science Foundation.

Supporting Information Available: Synthetic procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) We do not assign all the geometries of synthetic azarhodacyanines in this study. Although there are several possible geometrical isomers of azarhodacyanines, the structures shown in the schemes and charts are depicted as a single geometrical isomer. It is well-known that the conjugated double bonds, such as the merocyanine and cyanine moieties, can be easily isomerized in the solution and that the trans geometrical isomers would be thermodynamic products.
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JM0606241