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Discovery of Novel Benzo[*a*]phenoxazine SSJ-183 as a Drug Candidate for Malaria

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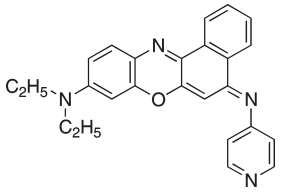
ABSTRACT Malaria is a serious infectious disease caused by protozoan parasites in tropical and subtropical regions. Even inhabitants of temperate zones are exposed to the danger of malaria infection because of travel and global warming. Novel, effective, safe, and inexpensive drugs are required to treat malaria and contribute to the global goal of eradication. A search for new antimalarial agents has been performed by the synthesis of new benzo[*a*]phenoxazines, followed by biological evaluations. The derivative SSJ-183 (**5**), having a 4-aminopyridine group, showed an IC_{50} value against *Plasmodium falciparum* of 7.6 nM and a selectivity index of > 7300. Cure was achieved by three oral doses of **5** at 100 mg/kg to mice infected with the *Plasmodium berghei* ANKA strain. The safety of **5** was supported by acute toxicity testing in mice with single doses up to 2000 mg/kg po, chromosome aberration test, in vitro as well as in vivo micronucleus tests, and phototoxicity studies in mice. Thus, **5** is a promising candidate as a new antimalarial agent.

KEYWORDS Antimalarial activity, benzo[*a*]phenoxazine, *Plasmodium falciparum*, *Plasmodium berghei*, oral administration



In tropical and subtropical regions, malaria is one of the most perilous infectious diseases caused by protozoan parasites. Each year, 500 million cases of malaria occur, and nearly 1 million people die, the majority of whom are young children and pregnant women.[1−4] Because of global warming, even inhabitants of temperate zones are in danger of exposure to malaria infection. No vaccine is currently available for malaria, and the resistance of the protozoa to clinically used chemotherapeutic agents is increasingly common. Thus, novel, effective, safe, and inexpensive drugs are highly desired to control malaria.

We have searched new hit compounds against malaria parasites from our compound library, taking into account the π-delocalized lipophilic cation (DLC) hypothesis[5] in which hydrophobic cations containing delocalized π-electrons are accumulated into mitochondria and inhibit metabolic activity. According to the working hypothesis, the rhodacyanine derivative MKT-077 (**1**)[6] (Figure 1) was first identified as a potential lead compound possessing potent in vitro activity against *Plasmodium falciparum*.[7] However, very poor efficacy was observed in vivo against *Plasmodium berghei*. Further searches based on the combinatorial synthetic approach[8] provided various in vivo active rhodacyanine derivatives.[9,10] Among them, SSJ-127 (**2**) showed cure of *P. berghei*-infected mice with subcutaneous administration,[11] but the rhodacyanine derivatives did not exhibit any oral activity. Phenoxazinium derivatives, a type of DLC exemplified by **3** and **4**, displayed potent in vitro activity and oral efficacy.[12−15] However, cytotoxicity was observed at low micromolar concentrations in several phenoxazinium derivatives.[12,14] Because the electrophilicity of the carbon atom at the 1 position in the phenoxazinium skeleton would be troublesome, we examined the addition of a benzene ring to the phenoxazinium framework. Thus, we have prepared and evaluated various benzo[*a*]phenoxazine derivatives. The corresponding salts of benzo[*a*]phenoxazines are a variant of DLC candidates. Herein, we disclose the details of the discovery and testing of SSJ-183 (**5**), having high in vitro

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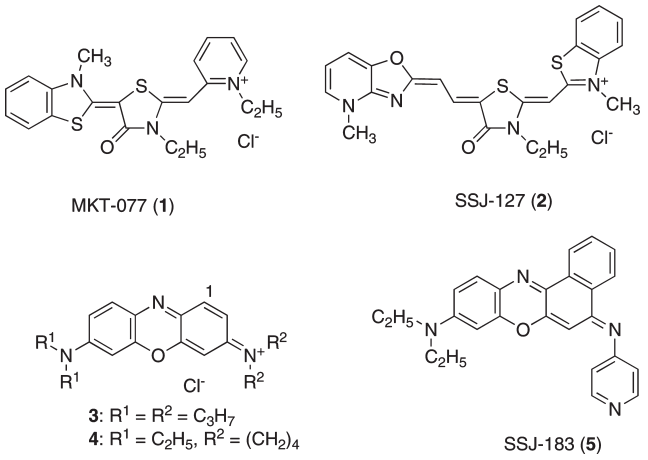
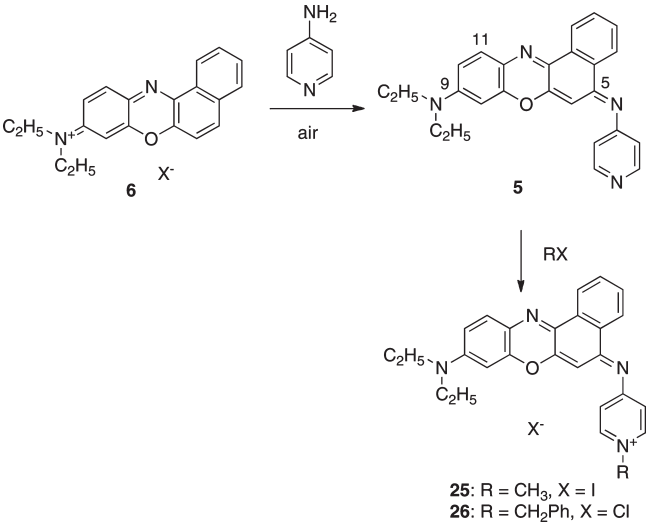


Figure 1. Structures of previous examples of antimalarial compounds and the candidate **5**.

Scheme 1. Synthesis of Benzo[*a*]phenoxazine **5** and Corresponding Quaternary Salts



and in vivo antimalarial activity when administered to *P. berghei*-infected mice via the oral route.

A number of benzo[*a*]phenoxazine derivatives have been prepared by known methods (Scheme 1).[16−20] As a typical example, the synthesis of **5** is shown in Scheme 1. Namely, the nitrate salt of **6** (X = NO_3)[16] was reacted with 4-aminopyridine for 24 h in refluxing ethanol. Chromatographic purification of the product on silica gel revealed **5**, mp 247−248 °C, in 38% yield. Notably, **5** is very stable as a free amine for a long period under ambient conditions. All tested compounds were prepared by the same method as described above. When **5** was converted into a salt by treatment with hydrochloric acid as usual, the elemental analysis of the salt, mp > 300 °C, indicated that the molecular ratio of **5** and hydrogen chloride must be 1:2.

Furthermore, the quaternary salts **25** and **26** were prepared by treatment of **5** with alkyl halides in acetonitrile under refluxing conditions. The physical and spectral data of new compounds **5** and **11**−**26**, prepared by the similar method, are recorded in the Supporting Information.

We evaluated compounds **5**−**26** (Figures 1 and 2) for their in vitro activity (IC_{50}) against *P. falciparum* K1, a strain resistant to chloroquine and pyrimethamine, and determined the cytotoxicity to mammalian cells. They were further screened by in vivo efficacy against *P. berghei* strain NK-65 via the oral route (po) according to established protocols (Table 1).[10] For the in vivo screening, test compounds were formulated in 5% glucose and 10% ethanol solution or in 7% Tween 80 and 3% ethanol solution to provide a dose of 100 mg/kg. The resulting suspensions were administered to female ICR mice as a single oral dose.

Benzo[*a*]phenoxazinium **6**,[16] having no substituent at the 6-position, showed weak activity against *P. falciparum* K1. The activity of Nile red (**7**) possessing an oxygen substituent was also weak, while the introduction of a nitrogen functionality increased the potency and reduced the IC_{50} value of Nile blue A (**8**) to 0.0156 μM. Although a low cytotoxicity was observed for **8**, the in vivo activity was poor. The phenyl and tolyl derivatives **9**[16] and **10**[16] showed low activities in both in vitro (IC_{50}: 0.191 and 0.232 μM) and in vivo tests. Higher activities were achieved in the in vitro test of compounds possessing hetero aromatic rings on the nitrogen at the 6-position. Therefore, several derivatives **11**−**14** were prepared and evaluated, but their in vivo efficacies were unsatisfactory. Improved in vivo efficacies were observed for the compounds **15**, **16**, and **5** carrying a pyridine ring. Among them, **5** exhibited the best activity: an IC_{50} value of 0.0076 μM against *P. falcipaurm* K1, an IC_{50} value of 55.7 μM in the cytotoxicity test, a selectivity of 7334, and > 99.9% inhibition of *P. berghei* NK-65. In addition to the inhibition of parasitemia, the mean survival days (MSD) after a single dose were extended to 14.6 days as compared to approximately 6 days for an untreated control. Very similar results were obtained, when single oral doses of 100 mg/kg were administered to NMRI female mice infected with *P. berghei* ANKA strain[21] in three independent experiments (n = 3 mice per experiment, average inhibition = 99%, and average MSD = 16; data not shown).

Although numerous analogues were prepared and assessed, only the 4-aminopyridine derivatives are presented here. Thus, the dimethyl derivative **17** showed potent activity but a shorter survival as compared to **5**. With longer substituents at the 9-position, **18** and **19** were less active. Morpholine compound **20** displayed good activity. Furthermore, substitution of a methyl group at the 11-position improved the safety, and two derivatives **21** and **22** provided good in vivo efficacy. Because the hydrochlorides **21** and **22** gave similar in vivo activities as that of **5**, benzo[*a*]phenoxazine is apparently absorbed through the gut as the hydrochloride when administered po. The absence of cytotoxicity of compounds **23** and **24**, having a bromine atom on the A ring, was encouraging; however, both showed low in vivo activity, possibly due to their poor solubility and poor oral bioavailability. On the other hand,

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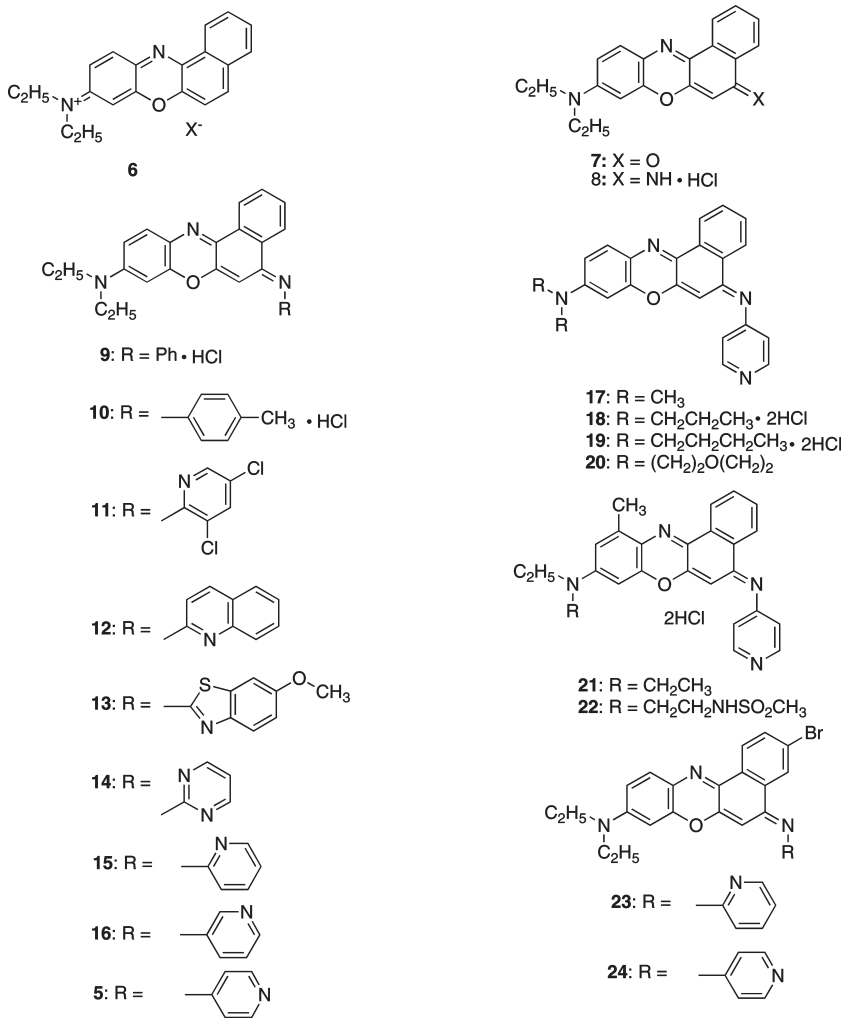

Figure 2. Structures of tested benzo[*a*]phenoxazine derivatives.

high cytotoxicity was observed for the quaternary salts **25** and **26**.

To gain additional information on the in vivo efficacy of **5**, we carried out a dose−response experiment in NMRI female mice infected with *P. berghei* GFP ANKA strain[21] (Table 2). High efficacy was observed by the po route with cures achieved by oral administration of three daily doses of 100 mg/kg.

In other evaluations with compound **5**, we detected no lethality at doses up to 2000 mg/kg po using 20 mice. Furthermore, no effects were found at 1000 μM (− and + S9) in a chromosome aberration test, at 2.0 μM (− and + S9) in an in vitro micronucleus test and at 1000 mg/kg × 2 in an in vivo rat micronucleus test. No phototoxicity was detected in mice dosed at 300 mg/kg po. In binding assays against 80 receptors, only two human recombinant receptors, A_3 and D_3, were inhibited ~80% with 1 μM, and no inhibitions were noted with other receptors. The selectivity was further supported by ≥1000-fold higher IC_{50} values of **5** against three other protozoal parasites (36000 nM for *Trypanosoma brucei rhodesiense*, 11300 nM for *Trypanosoma cruzi*, and 6500 nM for *Leishmania donovani*) as compared to *P. falciparum* strain K1. Interestingly, the deep purple/blue color of the compound formulation was not detected in the urine, eyes, and organs of mice treated with a single oral dose of 100 mg/kg, although the prototype molecule, methylene blue, in this class stains tissues and urine. In vitro and in vivo activities of **5** are much better than those of methylene blue. Furthermore, no hemolysis was proved in blood taken from G6PD-deficient patient at Jichi Medical University, and the details will be discussed in the future.

In vivo pharmacokinetic studies in rats were carried out according to the reported procedure (Figure 3).[21] After oral dosing, **5** had a bioavailability of approximately 30%. After iv administration, the terminal half-life was approximately 5.5 h, and **5** demonstrated a high volume of distribution and high clearance.

In summary, in a search for novel antimalarial agents, new benzo[*a*]phenoxazines were synthesized and subjected to biological evaluation. The derivative **5** possessing the 4-aminopyridine moiety showed an IC_{50} value against

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Table 1. Evaluation of Benzo[*a*]phenoxazines: In Vitro Activity against *P. falciparum* K1, Cytotoxicity toward L-6 Myoblasts, and in Vivo Activity against *P. berghei* NK-65 (n = 3 Mice)

	IC_{50} (μM)[a]				
compound	*P. falciparum* K1	cytotoxicity L-6	selectivity[b]	inhibition (%)[c]	MSD
6; X = Cl	0.606	3.82	6.3	ND	ND
7	0.868	65.9	76.0	15	6.3
8	0.0156	43.14	2765	15	6.6
9	0.191	164.9	863	0	6.3
10	0.232	123.2	531	0	6.3
11	0.063	179.8	2855	14.7	6.3
12	0.027	67.9	2515	81	10.3
13	0.1125	167.9	1493	11.4	6.3
14	0.005	13.6	2734	3.3	6.3
15	0.015	15.8	1052	> 99.9	8.3
16	0.023	117.0	5088	62.7	10.0
5	0.0076	55.7	7334	> 99.9	14.6
17	0.0081	165.1	20390	> 99.9	10.0
18	0.05	67.1	1342	15.3	6.3
19	0.195	63.8	327	5.6	6.3
20	0.029	96.9	3342	97.9	7.3
21	0.18	86.6	481	99.7	13.0
22	0.017	14.8	887	> 99.9	15.7
23	0.038	> 190.2	> 5007	12.7	6.3
24	0.011	> 190.2	> 17290	14.2	6.6
25	0.009	1.56	174	22.0	6.6
26	0.024	0.75	31	39	7.3
chloroquine	0.019−0.066	ND[d]	ND	> 99.9	12.6

[a] Mean of two independent assays. [b] Calculated as IC_{50} for L-6 cells/IC_{50} for *P. falciparum*. [c] Parasitemia was determined on day 4 after infection. The difference between the mean value of the control group and those of treated groups is calculated and expressed as a percent relative to the control group. Single oral administration (100 mg/kg) was given on day 1. [d] ND, not determined.

Table 2. In Vivo Results for **5** Orally Administrated to n = 3 Mice/Dose Once Daily for Three Consecutive Days to *P. berghei* GFP ANKA Strain (Data from 2 to 3 Independent Experiments)

mg/kg	inhibition (%)[a]	MSD (% of cured animals)
3 × 100	> 99.9	> 30.0 (100%)
3 × 30	99	27.2 (78%)
3 × 10	26	4.0[b]

[a] Determined on day 4 postinfection. [b] Mice were euthanized on day 4 based on a lack of sufficient efficacy.

P. falciparum of 7.6 nM and a selectivity index of > 7300. Cure was achieved with three daily oral doses to mice infected with *P. berghei* ANKA strain at a dosage of 100 mg/kg. The safety of **5** was demonstrated by a single dose toxicity test, the highest dose being 2000 mg/kg, a chromosome aberration test, in vitro and in vivo micronucleus tests, and a phototoxicity test. Binding assays against 80 receptors supported a high selectivity. Urine and eyes were not colored during treatment, and no color was detected in organs after 7 days of administration. On the basis of the above findings, compound **5** appears to be a promising candidate for further testing. We have also developed a more efficient synthesis of **5**, and a report of this new synthesis as well as the investigation of the mechanism of action of **5** will be published in due course.

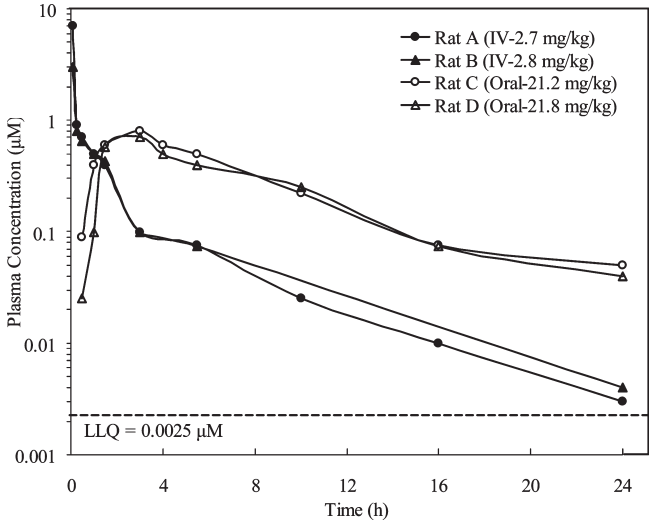

Figure 3. Plasma concentration vs time profile of **5** in male Sprague−Dawley rats following intravenous (2.8 mg/kg, filled symbols) and oral (21.5 mg/kg, open symbols) administration.

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SUPPORTING INFORMATION AVAILABLE General synthetic procedure, melting point, and spectral data for all new tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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