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DISCOVERY OF NEW ANTI-PROTOZOAN AGENTS HAVING NOVEL MODE OF ACTION

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Abstract – Malaria, leishmaniasis, African sleeping sickness (African trypanosomiasis) and Chagas disease (American trypanosomiasis) are caused by different protozoan parasites. Although many people suffer from these diseases in tropical and subtropical areas, efficient medicines against these protozoan diseases are very few or absent. Efforts to develop new drugs against these neglected diseases led us to the discovery of SSJ-127 (**62**), which cured malaria and African trypanosomiasis mouse models by treatment with injection, SJL-01 (**74**) as a hit compound for leishmaniasis, and SSJ-183 (**109**) as a candidate against malaria, respectively. These compounds displayed novel modes of actions different from those of conventional medicines.

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1. INTRODUCTION

A large number of diseases caused by various parasites are known in tropical and subtropical regions. There are three main classes of parasites associated with diseases in humans: protozoa, helminths, and ectoparasites. Malaria, leishmaniasis, African sleeping sickness, and Chagas disease, caused by protozoan parasite infection, are target diseases for a special Program for Research and Training in Tropical Diseases (TDR) executed by World Health Organization (WHO).[1] Protozoa are microscopic, one-celled organisms that live in the blood or tissues of humans and are transmitted to other humans by an arthropod vector such as a mosquito or fly. Protozoan diseases are sometimes referred to as neglected diseases, since these are illnesses most often occurring in developing countries. Although many people suffer seriously from these sicknesses, these orphan diseases are frequently ignored by pharmaceutical industries in developed countries. Therefore, aid from not-for-profit public-private organizations such as Medicines for Malaria Venture (MMV), Drugs for Neglected Diseases initiative (DNDi), Institute for Oneworld Health, and so on, are critically needed for the development of new medicines.[2] We have worked toward the discovery of new drug candidates against protozoan diseases and would like to describe our ongoing findings. Since protozoan diseases are not common in developed countries, we present here a brief description of them.

1.1 Maralia

In tropical and subtropical regions, malaria is one of the most perilous infectious diseases. Each year, about 500 million cases of malaria occur, and nearly 1 million people die, the majority of them being young children and pregnant women.[3-7] Because of global warming, even inhabitants of temperate zones are in danger of exposure to malaria infection. Malaria is caused by protozoan parasites of the genus *Plasmodium*. Human malaria cases are generally brought about by four species, including *P. falciparum, P. vivax, P. ovale* and *P. malariae*.[4] Different animals are infected by different species of *Plasmodium*. For example, *P. berghei, P. yoelii* and *P. chabaudi* are known as rodent malaria, and do not infect humans. The most serious forms to humans are caused by *P. falciparum* and *P. vivax*. Malaria parasites are transmitted

by the bites of female *Anopheles* mosquitoes. The life cycle of malaria parasites is complex and they multiply within red blood cells of the host. Malaria transmission can be reduced with mosquito nets and insect repellents. No vaccine is currently available, and structures of typical medicines against malaria are shown in Figure 1.

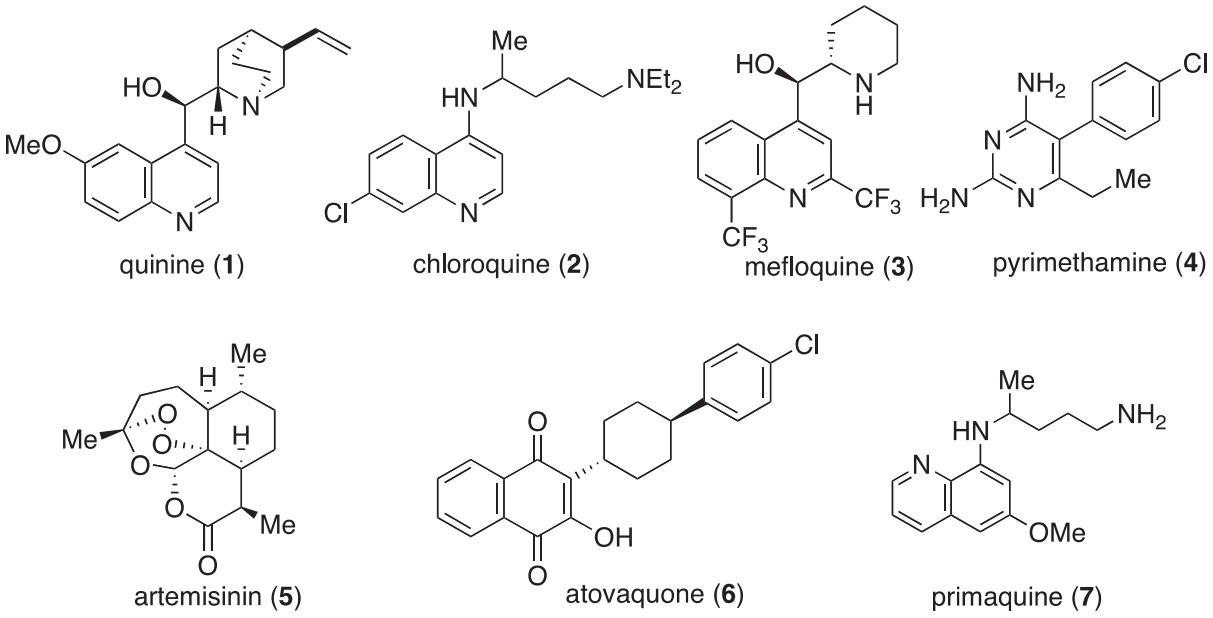


Figure 1. Typical medicines for malaria

Quinine (**1**), a cinchona alkaloid, is a well-known antimalarial agent, but its activity against the malaria parasite is not very high. The requirement that this drug be administered by injection is a serious drawback for its use as a tropical medicine. On the other hand, the synthetic compounds, chloroquine (**2**), mefloquine (**3**) and pyrimethamine (**4**) are orally active. Unfortunately, the appearance of parasite strains that are resistant to these drugs has introduced a serious problem in malaria treatment. Artemisinin (**5**), a sesquiterpene, also known as qinhaosu, has been isolated from *Artemisia annua* and is active against the drug resistant form of *P. falciparum*. Treatments containing an artemisinin derivative (artemisinin-combination therapy, ACTs) are standard treatment worldwide for the *P. falciparum* malaria. On the Thai-Cambodian border, artemisinin is losing its potency due to a drug-resistant form of the malaria parasite.[8] Atovaquone (**6**), an analog of ubiquinone acting on mitochondria, is available as a combination preparation with proguanil, but its cost as an anti-malarial agent is high. Primaquine (**7**) in combination with quinine or chloroquine is mainly used to treat malaria transmitted by *P. vivax*. Primaquine remains the only licensed drug that can provide a radical cure of the *P. vivax* malaria but **7** produces hemolytic anemia in individuals with glucose-6-phosphate deficiency.[5]

1.2. Leishmaniasis

Leishmaniasis is one of the major target protozoan diseases for WHO. This serious tropical disease is caused by parasitic protozoa of the genus *Leishmania*.[9] *Leishmania* are obligate intracellular protozoan parasites of macrophages that are transmitted between hosts by the bite of female phlebotomine sand flies and are delivered to a host as animal-infective metacyclic promastigotes along with the saliva of the sand fly and a mucin-rich gel produced by the parasites in the sand fly midgut.[10] Leishmaniasis threatens about 350 million people in 88 countries around the world. As many as 12 million patients are currently infected, with over 1 million estimated new cases occurring every year. It manifests mainly in three clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL), of which VL is the most severe form of the diseases. VL, also called Kala-azar or black fever, is a fatal disease with an estimated incidence of 500,000 cases per year. Over 90% of VL cases occur in India, Bangladesh, Nepal, Brazil, and Sudan. Diagnosis and treatment of VL have always been difficult, but recent development of new medicines has improved the situation, although the development of new, more effective drugs with less toxicity is urgently required. Examples of drugs for leishmaniasis are shown in Figure 2.

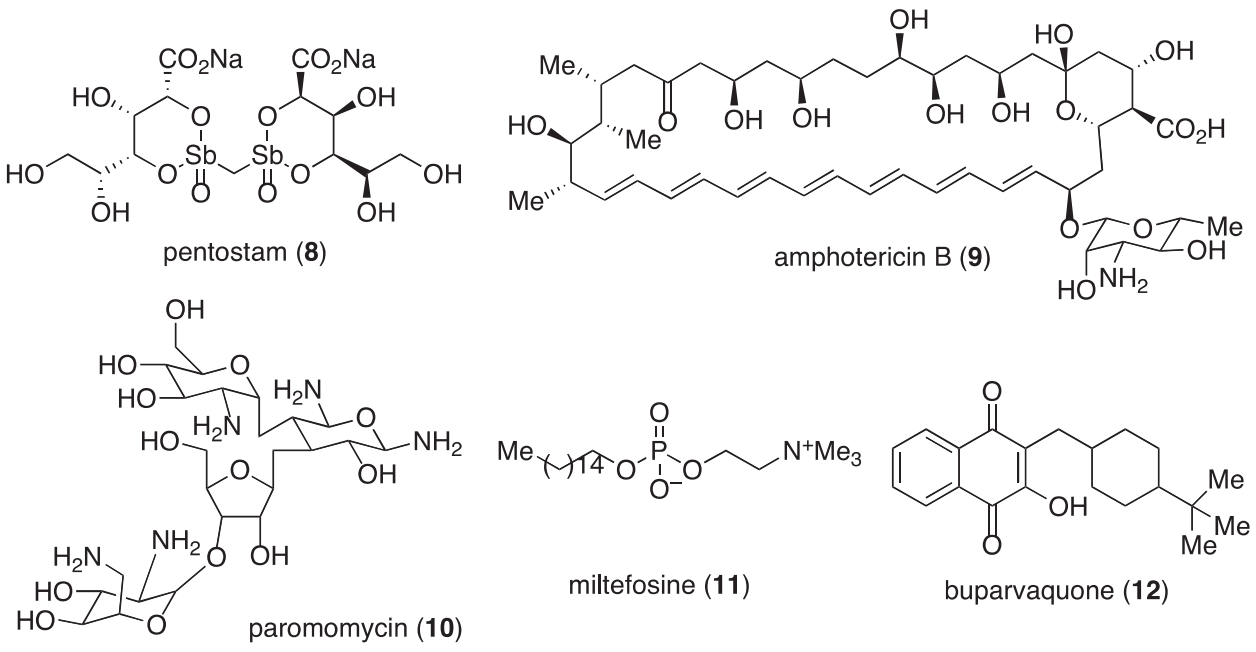


Figure 2. Medicines for leishmaniasis

Although several medicines are available for leishmaniasis, the most widely used drug is pentostam (**8**) containing pentavalent antimony. Other medicines such as amphotericin B (**9**), liposomal amphotericin B and paromomycin (**10**) also require injection administration. Only miltefosine (**11**) is orally active but the activity is not very high. All agents have individual problems such as toxicity, poor effects, or high cost.

Development of other potential compounds including buparvaquone (**12**) and aminoquinolines are underway.

1.3. African Sleeping Sickness (African Trypanosomiasis)

Human African trypanosomiasis (HAT), also known as sleeping sickness, is an endemic disease of people and animals, caused by protozoa of the species *Trypanosoma brucei* and transmitted by the tsetse fly. HAT is a fatal disease if untreated. In 2008, this disease caused 48,000 deaths, but it is believed that many cases go unreported. African trypanosomiasis symptoms occur in two stages. The first stage is known as the hemolymphatic phase and if left untreated, the disease moves into the second stage, called the neurological phase. The arsenic compound melarsoprol (**13**) and eflornithine (**14**) are used for the second stage of HAT, but they are highly toxic. Pentamidine (**15**) is a treatment for the first stage, and other compounds including ascofuranone (**16**) are currently being examined in order to develop more effective medicines.

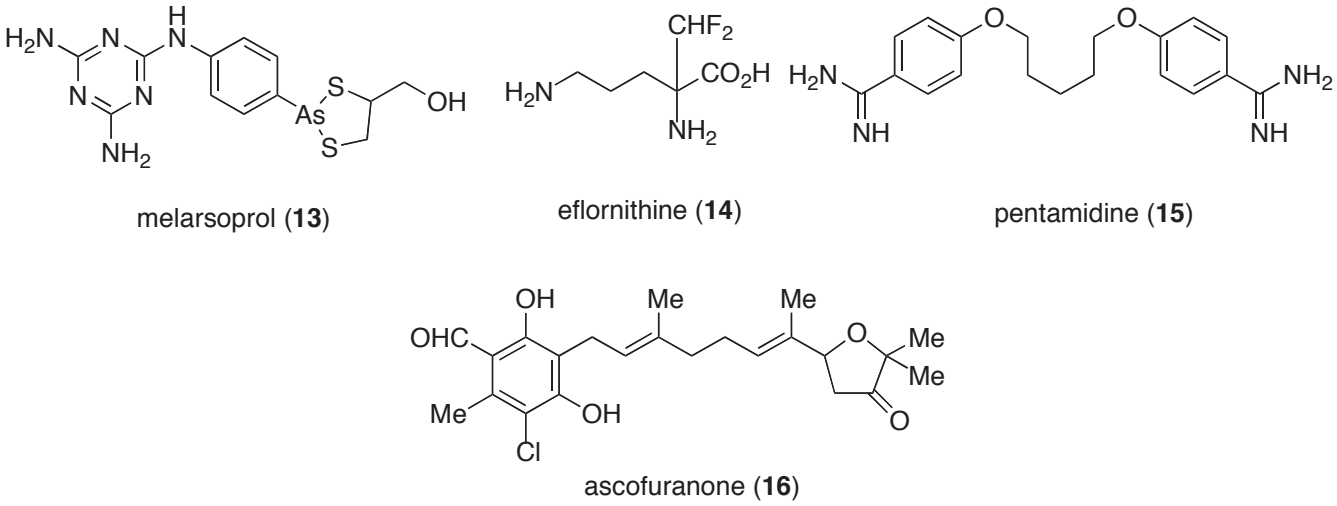
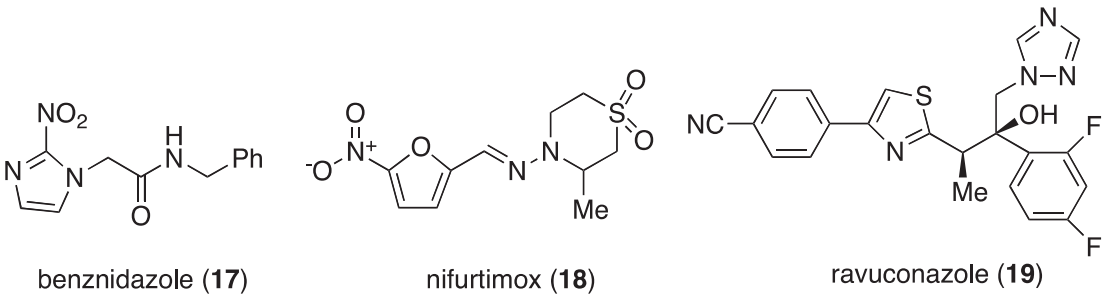


Figure 3. Medicines for African sleeping sickness

1.4. Chagas Disease (American Trypanosomiasis)

Chagas disease, also called as American trypanosomiasis, is a tropical disease caused by the flagellate protozoan *T. cruzi*, transmitted to humans and mammals by the triatomine vector. It is estimated that as many as 8 to 11 million people in Mexico, Central America, and South America are infected with this disease. Chagas disease is a chronic illness leading to death. Benznidazole (**17**) and nifurtimox (**18**) are used as treatments, but they have various side effects. Development of a prodrug of ravuconazole (**19**) is now in progress.



benznidazole (**17**) nifurtimox (**18**) ravuconazole (**19**)

Figure 4. Medicines for Chagas disease

1.5. Demands for New Medicines

There are too few medicines for treating protozoan diseases and the emergence of drug resistant parasites has caused further problems for therapy. The development of new types of medicines having novel chemical frameworks and new modes of action are extremely desired. Oral administration is preferred over injectable drugs due to the poor medical conditions in tropical and subtropical regions. Additionally, the new medicines should be available in large quantities at low cost.

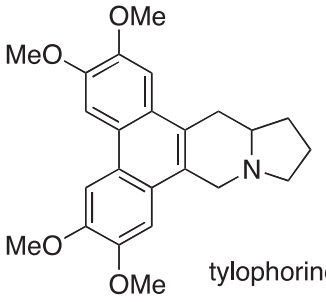
In this context, we began a research program in 1996 that focused on developing new medicines for protozoan diseases. Herein, we present the details of our progress.

2. RANDOM SCREENING

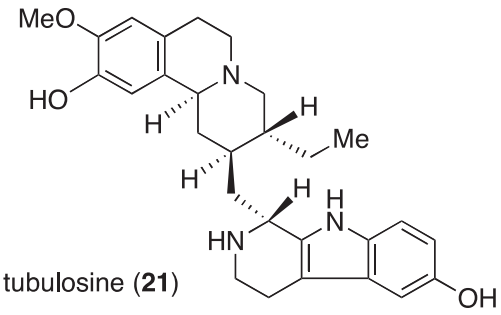
More than 150 compounds were selected from the compound library in our group at Tohoku University and Professor Wataya's group at Okayama University conducted the screening tests. Inhibitory activity (as IC_{50} values) against *P. falciparum* (chloroquine-sensitive FCR-3 strain) and evaluation of the cytotoxicity against FM3A were determined. Of these test compounds, the results of only four compounds (**20** – **23**) are shown together with the activities of quinine and chloroquine (Table 1). The selectivity of each compound was defined as the ratio of the cytotoxicity / the inhibitory activity.

Table 1. Evaluation of compounds (**20** – **23**): *in vitro* inhibitory activity against *P. falciparum* and cytotoxicity towards FM3A cells

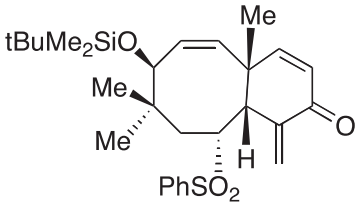
compounds	*P. falciparum* IC_{50} (μM)	FM3A IC_{50} (μM)	selectivity
tylophorine (**20**)	0.18	0.01	0.06
tubulosine (**21**)	0.025	0.38	15.2
22	0.019	0.05	2.63
homoprotoberberine (**23**)	0.40	37	92.5
quinine (**1**)	0.11	100	909
chloroquine (**2**)	0.018	32	1,778



tylophorine (**20**)



tubulosine (**21**)



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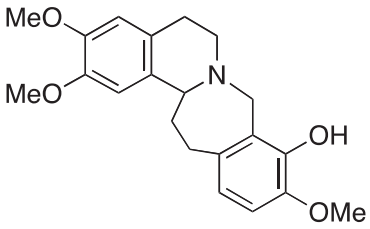
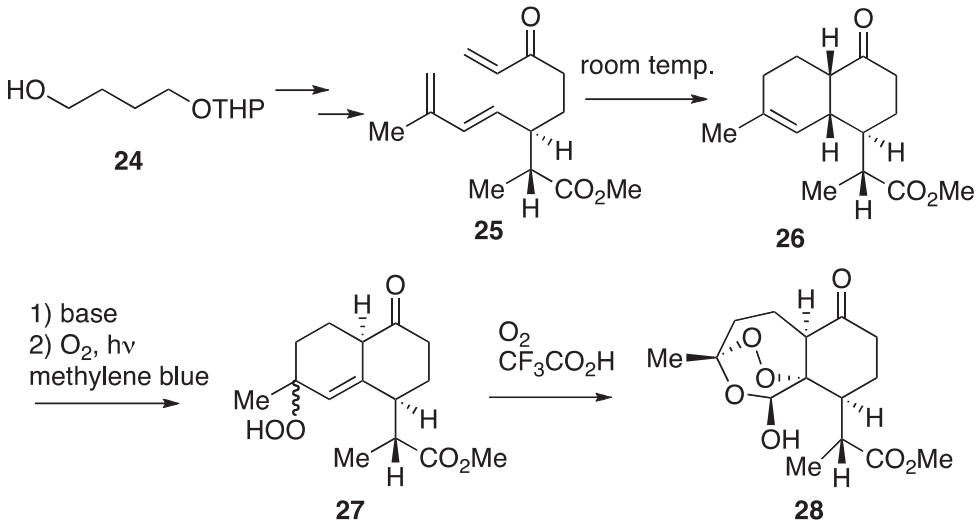


homoprotoberberine (**23**)

Racemates of the synthetic alkaloids tylophorine (**20**) and tubulosine (**21**) exhibited comparable inhibition activities against *P. falciparum* as those of quinine and chloroquine, but their cytotoxicities against the mouse FM3A cell were high. As a result, the corresponding selectivity was low. Although the inhibitory activity of **22** was high, the selectivity was poor.[11] Since homoprotoberberine derivatives showed reasonable activity with low cytotoxicity, many analogues were synthesized, but the best selectivity recorded by **23** was only 93.[12] Since selectivity values >100 were required for a promising hit compound in the screening assay, the random screening methodology was not effective.

3. SYNTHESIS OF ARTEMISININ DERIVATIVE

Since artemisinin (**5**) is easily available in large quantity from the plant *A. annua*,[13] synthesis of an analogue (**28**), which can not be readily prepared from the natural product, was investigated as shown in Scheme 1. The intramolecular Diels-Alder reaction of the triene **25**, which was stereoselectively synthesized from the alcohol **24**, occurred at room temperature to give the *cis*-octalone **26** in good yield. After conversion into the corresponding *trans* fused isomer, the irradiation of the product with a tungsten lamp in acetone containing a catalytic amount of methylene blue under atmospheric oxygen, followed by treatment of the formed hydroperoxide **27** with trifluoroacetic acid under atmospheric oxygen,[14] provided the desired peroxide **28** in a poor yield. The IC_{50} value of **28** against *P. falciparum* was 0.039 μM with a low cytotoxicity toward to FM3A cells, IC_{22}: 24 μM.[15] Although **28** gave a potent activity with high selectivity, the synthesis was too costly and would not provide the best method for the protozoan drug.



Scheme 1. Synthesis of a new artemisinin analogue

4. WORKING HYPOTHESIS FOR FURTHER RESEARCH

Malaria, leishmania and trypanosoma parasites are eukaryotes, which possess energy producing, and thus negatively charged organelles such as mitochondria and apicoplasts. Therefore, we focused on a concept proposed by Chen[16] in 1988; the accumulation of π-electron-delocalized lipophilic cations (DLCs) in the mitochondrion would lead to destruction of the organelle's function.

We observed that the IC_{50} value of the naturally occurring alkaloid MVC (**29**) against *P. falciparum* was enhanced from 5.0 μM to 0.13 μM by the transformation of the compound into its corresponding quaternary ammonium compound **30**.[17]

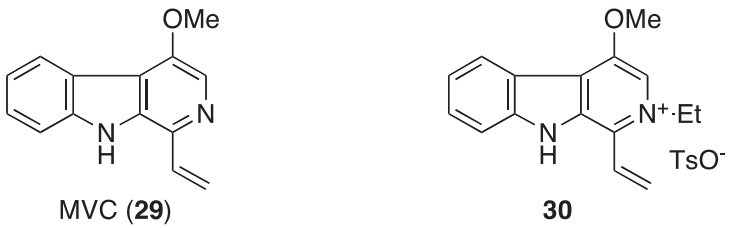


Figure 5. MVC (**29**) and the quaternary ammonium salt

5. RHODACYANINES

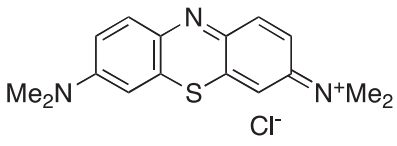
5.1. Antimalarial Activity

Based on the above working hypothesis, we searched hit compounds carrying quaternary ammonium cations as DLCs. Typical examples are shown in Table 2. Methylene blue (**31**) and rhodamine 123 (**32**) exhibited good activity against chloroquine sensitive *P. falciparum*, but the selectivities were not adequate.

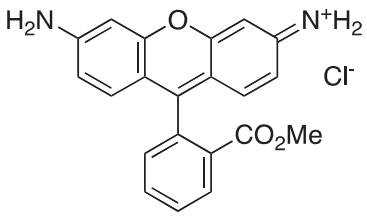
On the other hand, the activity of MKT-077 (**33**) was high, IC_{50} 0.07 μM, with low cytotoxicity, IC_{50} 15.0 μM, thus, the selectivity was 214.[18]

Table 2. Evaluation of DLCs **31** - **33**: *in vitro* activity against *P. falciparum* and cytotoxicity towards FM3A cells

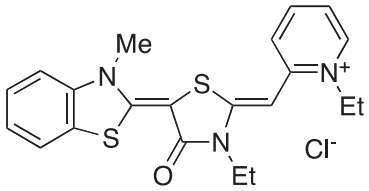
compounds	*P. falciparum* IC_{50} (μM)	FM3A IC_{50} (μM)	selectivity
methylene blue (**31**)	0.017	1.1	65
rhodamine 123 (**32**)	0.3	10	33
MKT-077 (**33**)	0.07	15	214



methylene blue (**31**)



rhodamine 123 (**32**)



MKT-077 (**33**)

Rhodacyanine derivative MKT-077 (**33**) had been developed as a novel anticancer agent, and it has been subjected to further clinical investigation for the treatment of solid tumors.[19,20] Rhodacyanines **34** consist of three linearly linked heterocyclic groups, in which the two terminal heteroaromatic rings (**A** and **C**) flank a rhodanine (4-oxothiazolidine) **B**-ring. The compounds are double conjugates of two different units, having parts comprised of neutral merocyanine (left, **A** ring) and cationic cyanine (right, **C** ring) structures.

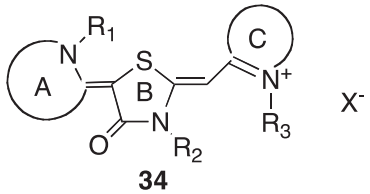


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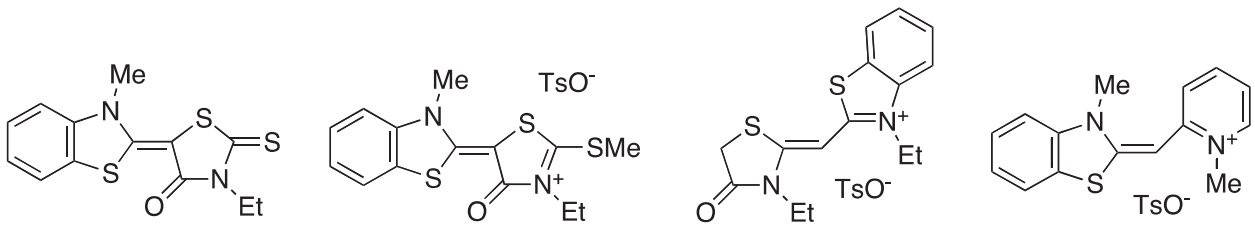
Figure 6. General formula of rhodacyanines

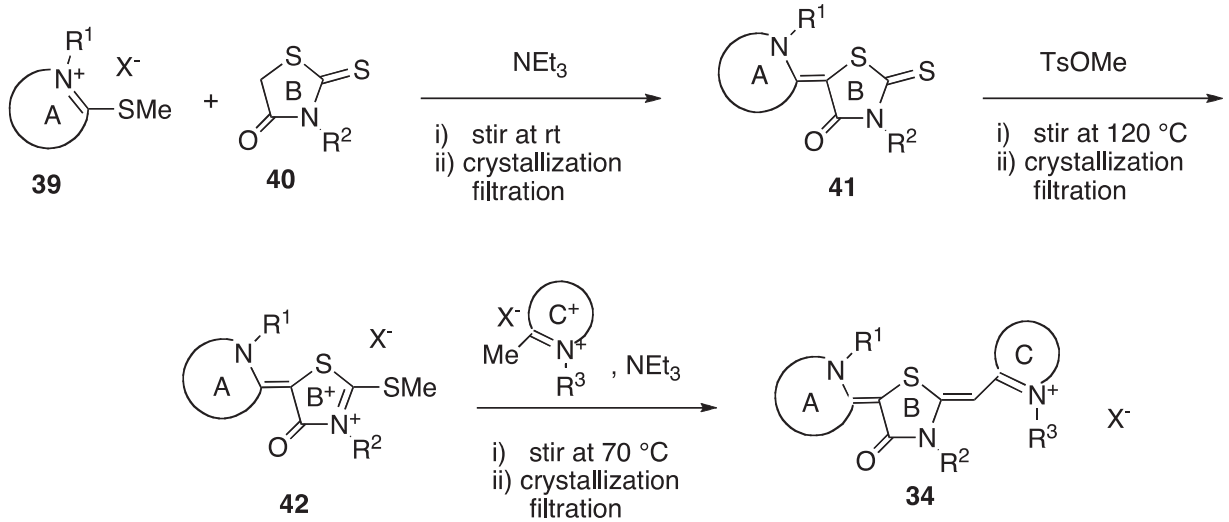
The requirement for each of the heterocyclic components of the rhodacyanines as shown in Table 3 was evidenced by the very low antimalarial activities of merocyanines **35** and **36**. Cyanines **37** and **38**, which have no merocyanine conjugation, display moderate activity against *P. falciparum*, but their potency is lower than that of **33**. Thus, tricyclic-rhodacyanine structures, containing both merocyanine and cyanine conjugation, are required for high antimalarial activity.

Table 3. Effects of skeletal composition on antimalarial activity

compound	framework	*P. falciparum* IC_{50} (μM)
33	**A-B-C**	0.07
35	**A-B**	>28
36	**A-B**	3.7
37	**B-C**	0.52
38	**A-C**	0.24



Although good activity and selectivity of **33** were observed, **33** showed a very poor efficacy upon *in vivo* testing using *P. berghei*. We next studied syntheses of various rhodacyanine derivatives, followed by biological evaluations. Most rhodacyanine derivatives can be prepared from commercially available starting materials within five steps. Generally, all synthetic intermediates as well as the rhodacyanines are easily crystallized compounds and the parallel combinatorial synthesis could be carried out by the combination of three components in one pot as shown in Scheme 2.[21]



Scheme 2. Parallel combinatorial synthesis of rhodacyanines

Further variations of rhodacyanines such as **43** and **44** (Figure 7) were prepared and their *in vitro* antimalarial activities against *P. falciparum* K1 (chloroquine-resistant strain) as well as their *in vivo*

activities against *P. berghei* (NK65 strain) in mice were determined.[22]

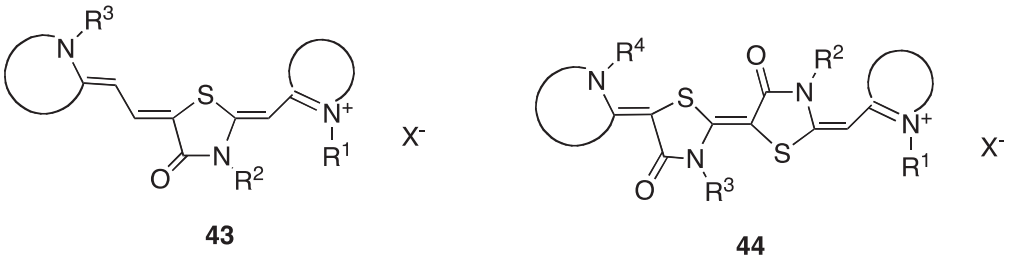


Figure 7. Other frameworks of rhodacyanines

Several aza-fused rhodacyanines including the two types of compounds **45** and **46** (Figure 8) were also synthesized and their antimalarial activities were evaluated.[23]

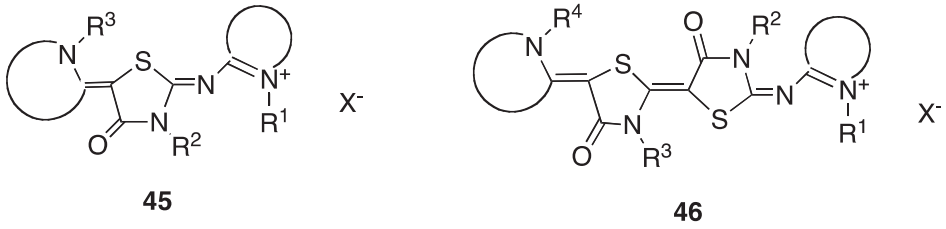


Figure 8. Two types of aza-fused rhodacyanines

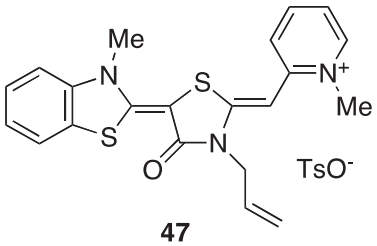
Synthesis of new types of rhodacyanines having rigid structures, as exemplified by **60** and **61**, and their biological evaluations have also been undertaken.[24]

In vitro activities of some typical examples against *P. falciparum* K1 (chloroquine-resistant strain) and cytotoxicity toward rat L-6 cells, carried out at the Swiss Tropical and Public Health Institute (Swiss TPH), are shown in Table 4.

Table 4. Evaluation of various rhodacyanine type compounds: *in vitro* inhibitory activity against *P. falciparum* K1 and cytotoxicity towards L-6 cells

compounds	*P. falciparum* K1 IC_{50} (μM)	L-6 IC_{50} (μM)	selectivity
47	0.019	110	5,789
48	0.04	100	2,500
49	0.015	>200	>1,333
50	0.13	76	585
51	0.35	42	120
52	0.012	30	2,500

53	0.012	20	1,700
54	0.71	78	110
55	0.0097	>200	20,619
56	0.0059	12	2,034
57	0.0042	11	2,619
58	0.01	46	4,600
59	0.023	12	522
60	0.062	160	2,581
61	0.046	150	3,261



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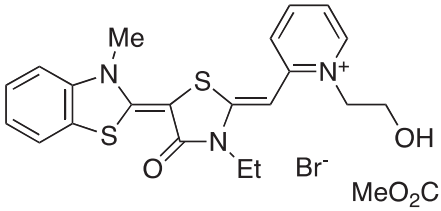

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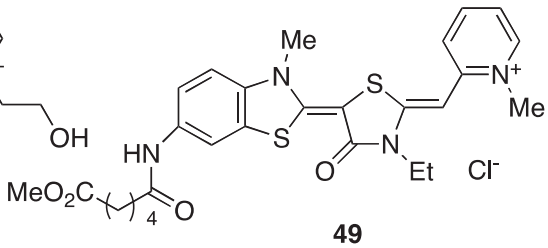

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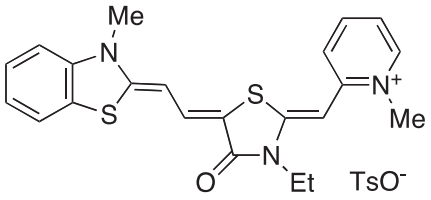

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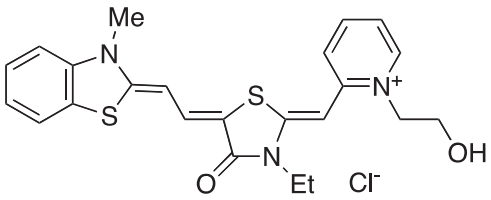

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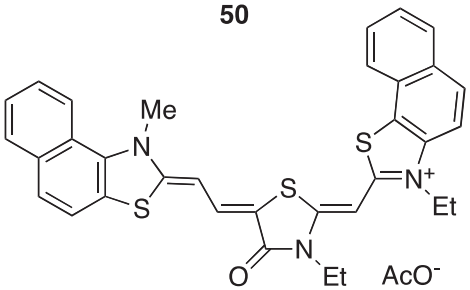

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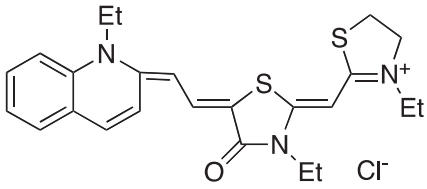

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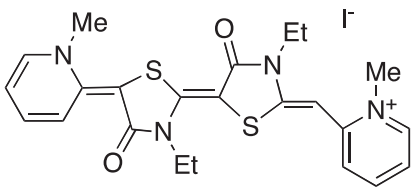

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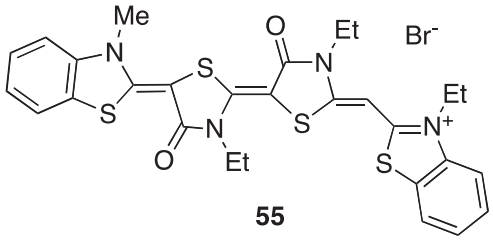

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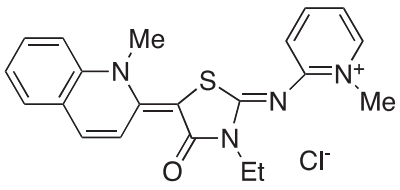

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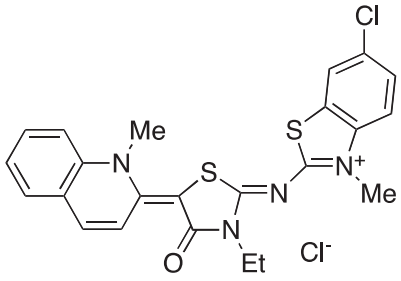

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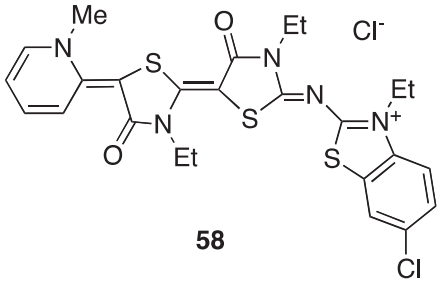

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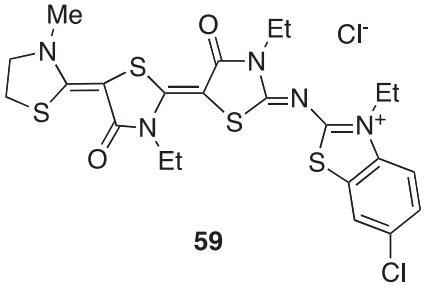

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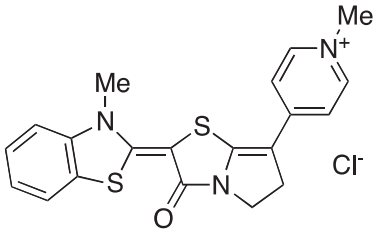

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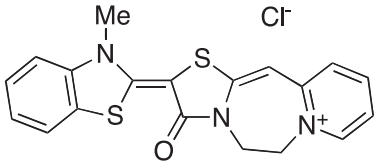

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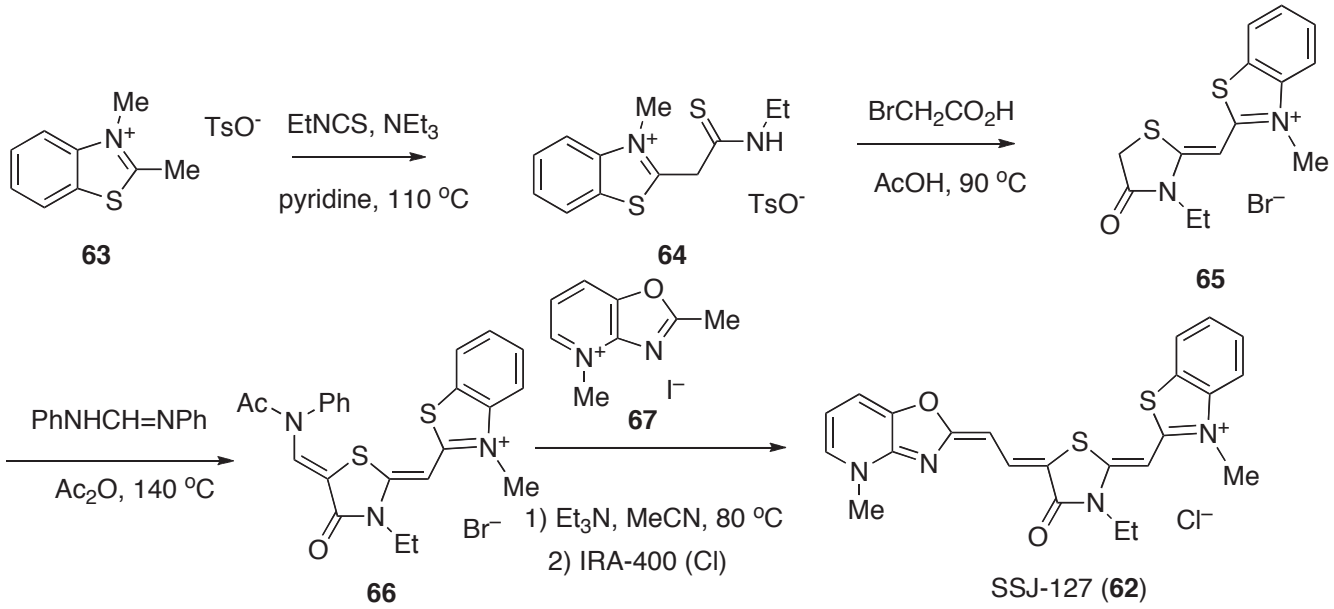
The allyl substituted rhodacyanine **47** showed potent inhibitory activity with a lower cytotoxicity; the selectivity was 5,789. The activity of **48** carrying a hydroxyl group was high enough and **49** possessing an amide functionality gave good *in vitro* activity. Although **50** and **51** having additional conjugation between the A and B rings exhibited somewhat lower activity, the analogues **52** and **53** showed higher activity. Activities of *bis*-rhodanine derivatives such as **54** and **55** were varied with structures of heterocyclic moieties at both edges. High *in vitro* activities were observed for aza-rhodacyanines **56** and **57**, although *bis*-rhodanine derivatives **58** and **59** showed lower activities. **60** and **61** having rigid structures also gave reasonable activities. Many rhodacyanines showed *in vivo* efficacy to some extent against *P. berghei via* injection treatment but cure was difficult. One rhodacyanine derivative, SSJ-127 (**62**), provided cure in an *in vivo* study by s.c. treatment as discussed below.

5.2. SSJ-127

SSJ-127 (**62**) was prepared starting with the benzo[*d*]thiazol-3-ium segment, as shown in Scheme 3.



Scheme 3. Synthesis of SSJ-127 (**62**)

Reaction of 2,3-dimethylbenzo[*d*]thiazol-3-ium tosylate (**63**) and ethyl isothiocyanate in the presence of triethylamine in pyridine gave the thioamide **64**. Without purification, **64** was treated with bromoacetic acid in acetic acid to afford the 4-oxothiazolidine **65**. Treatment of **65** with *N,N'*-diphenylformimidamide in acetic anhydride provided the phenylacetamidomethylenethiazolidine **66**. Reaction of **66** with the pyridinium iodide **67** in the presence of triethyamine in acetonitrile, followed by elution of the product through IRA-400 (Cl), gave SSJ-127 (**62**) in a reasonable yield. Thus, **62** was easily synthesized in five steps from known compounds using a standard synthetic procedure.[19]

IC_{50} values of SSJ-127 (**62**) against *P. falciparum* K1, *Trypanosoma cruzi, T. brucei rhodesiense*, *Leishmania donovani* and L-6 rat myocytes together with the selectivity index (SI) are shown in Table 5. SSJ-127 (**62**) showed activities at ten nM concentrations against *P. falciparum* K1 and *T. brucei rhodesiense* with good selectivity.[25]

Table 5. Antiprotozoal and Cytotoxic Activities (IC_{50} values μM) together with SI[a] of SSJ-127 (**62**)

P. falc. K1		*T. cruzi*		*T. b. rhod.*		*L. don.*, axenic		Cytotox. L-6
IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}
0.029	420	2.41	5.1	0.022	554	0.304	40.1	12.2

[a] Selectivity index (selectivity) = (IC_{50} value for L6)/(IC_{50} value for protozoan)

Next, *in vivo* experiments with SSJ-127 (**62**) were performed using *P. berghei* GFP ANKA strain at Swiss TPH and using *P. berghei* NK 65 strain at Hoshi University. Single subcutaneous (s.c.) administration of 100 mg/kg of **62** to NMRI mice (females) infected with *P. berghei* GFP ANAK exhibited 95% suppression after 4 days. A positive result, 40% suppression, was also obtained by a single p.o. administration of 100 mg/kg of **62** to the infected mice. A complete cure was observed by the *in vivo* test dosing **62** at Hoshi University. Namely, three times s.c. administrations of 40 mg/kg/d of **62** to ICR mice (males) infected with *P. berghe*i NK 65 strain provided 99.9% suppression and all treated mice survived until natural death.

A preliminary pharmacokinetic study using male rats, Crl:CD(SD), was carried out by intravenous (i.v.) and s.c. administrations. The results, analyzed by two-compartmental methods using the computer program 3P87, are summarized in Table 6. The s.c. bioavailability of **62** was determined to be excellent.

Table 6. Pharmacokinetic Parameters of SSJ-127 (**62**)

route	T1/2α (h)	T1/2β (h)	CL (L/h·kg)	V (L/kg)	T(peak) (h)	AUC0-24h (ng × h/mL)
i.v.[a]	0.055	4.17	11.35	4.45		88.08
s.c.[b]	0.79	5.22	4.34	66.09	5.36	4606.3

[a]dosage: 1 mg/kg
[b]dosage: 20 mg/kg

Furthermore, administration of SSJ-127 (**62**) resulted in a cure for the *in vivo* anti-trypanosomal tests against *T. brucei brucei* S427 in mice carried out at the Center for Basic Research, Kitasato University.[26] *T. brucei brucei* parasite causes animal African trypanosomiasis.

5.3. Anti-leishmanial Activity

Many rhodacyanines exhibited good activity against leishmania parasites. For example, the IC_{50} value of **68** against *L. major* causing cutaneous leishmaniasis (CL) was 0.012 μM,[27] a value that exceeds that of amphotericin B (**9**) whose IC_{50} was 0.14 μM.

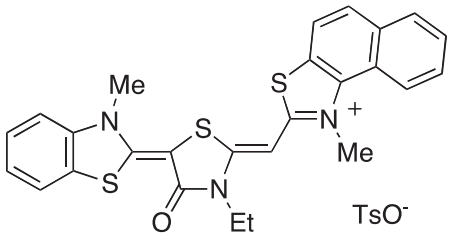


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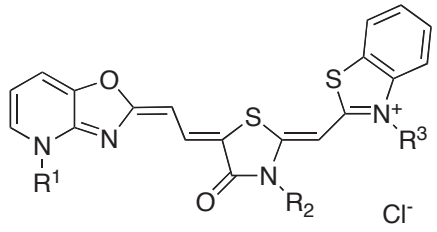
Figure 9. A rhodacyanine derivative

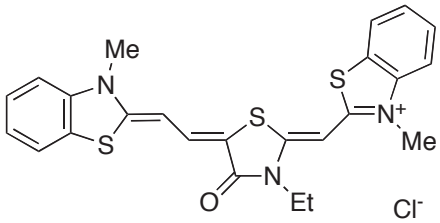
Various rhodacyanine derivatives were subjected to the screening test against *L. donovani*, which causes visceral leishmaniasis (VL). Each candidate was evaluated for its *in vitro* activity against *L. donovani* strain MHOM/ET/67/L82 and for its inherent cytotoxicity against L-6 rat skeletal myoblasts (Table 7). MKT-077 (**33**) exhibited ~ 2-fold greater inhibitory activity as compared with miltefosine (**11**) and with a selectivity factor of 450. Although SSJ-127 (**62**) could cure rodent malaria by s.c. administration, the *in vitro* activity was rather low against *Leishmania* parasites with low selectivity. Numerous derivatives of **62** having different alkyl substituents on three nitrogen atoms were prepared and their activities were tested. While compound **70** possessed relatively good inhibitory activity as indicated by its low IC_{50} value, its cytotoxicity was high. In compound **71**, replacing the oxazolo[4,5-*b*]pyridine moiety with benzo[*d*]thiazole did not improve the activity. In compound **72**, substitution of an imine functionality provided better activity and selectivity in the *in vitro* test. Introduction of a chlorine atom on the benzothiazole C ring (compound **73**) enhanced the activity, while substitution of a fluorine atom at the same site resulted in excellent activity in the *in vitro* test, as well as a high selectivity factor for compound **74**, termed SJL-01. The corresponding maleate (**75**) and mesylate (**76**) derivatives showed good inhibitory activities. However, in **77**, the presence of an alcohol group at the fluorobenzothiazole ring reduced the activity dramatically. The replacement of the left hand benzo[*d*]thiazole with oxazolo[4,5-*b*]pyridine gave poor activity (**78**). Connecting the 1,3-dimethylbenzo[*d*]imidazole through a nitrogen (compound **79**) gave unsatisfactory results in the *in vitro* test. The same tendencies were observed in the tests of 3,5-dimethylthiazole **80**, 3,4-dimethylthiazole **81**, and with the methylpyridine derivative **82**.[28]

Table 7. Evaluation of rhodacyanines: *in vitro* activity against *L. donovani* and cytotoxicity towards L-6 myocytes

compounds	*L. donovani* axenic IC_{50} (μM)	L-6 IC_{50} (μM)	selectivity
33	0.255	114.8	450
62	0.677	27.2	40
69	0.69	1.44	2.0
70	0.021	2.07	99
71	0.106	7.98	75
72	0.052	155.7	2,987
73	0.028	21.2	757
SJL-01 (**74**)	0.011	>173.7	>15,000
75	0.025	71.7	2,870
76	0.02	84.5	4,225
77	11.65	>169.1	>15
78	1.04	28.5	27
79	0.129	80.9	627
80	0.118	10.4	88
81	0.182	16.5	91
82	2.023	31.7	16
miltefosine (**11**)	0.43	-	-



69: $R^1 = C_3H_7$, R^2 = Et, R^3 = Me
70: $R^1 = C_4H_9$, $R^2 = C_3H_7$, R^3 = Et



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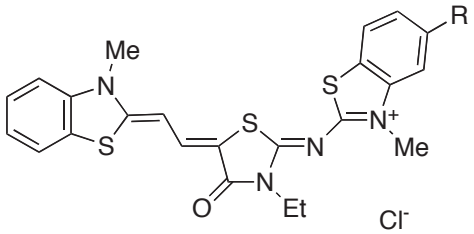
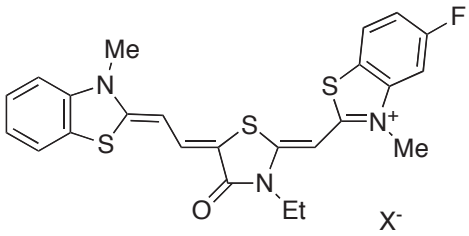
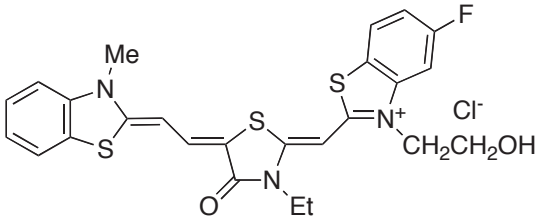


72: R = H
73: R = Cl



74: X = Cl (SJL-01)
75: X = *cis*-$HO_2CC{=}CCO_2$
76: X = MsO



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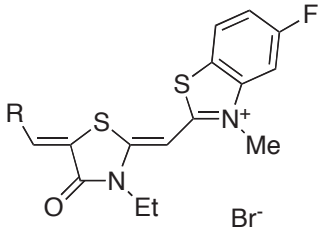
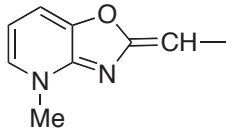


78: R =



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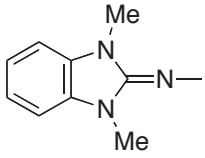
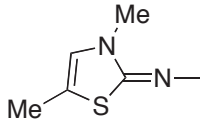
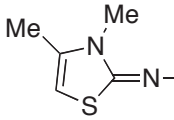


80: R =



81: R =



82 : R =

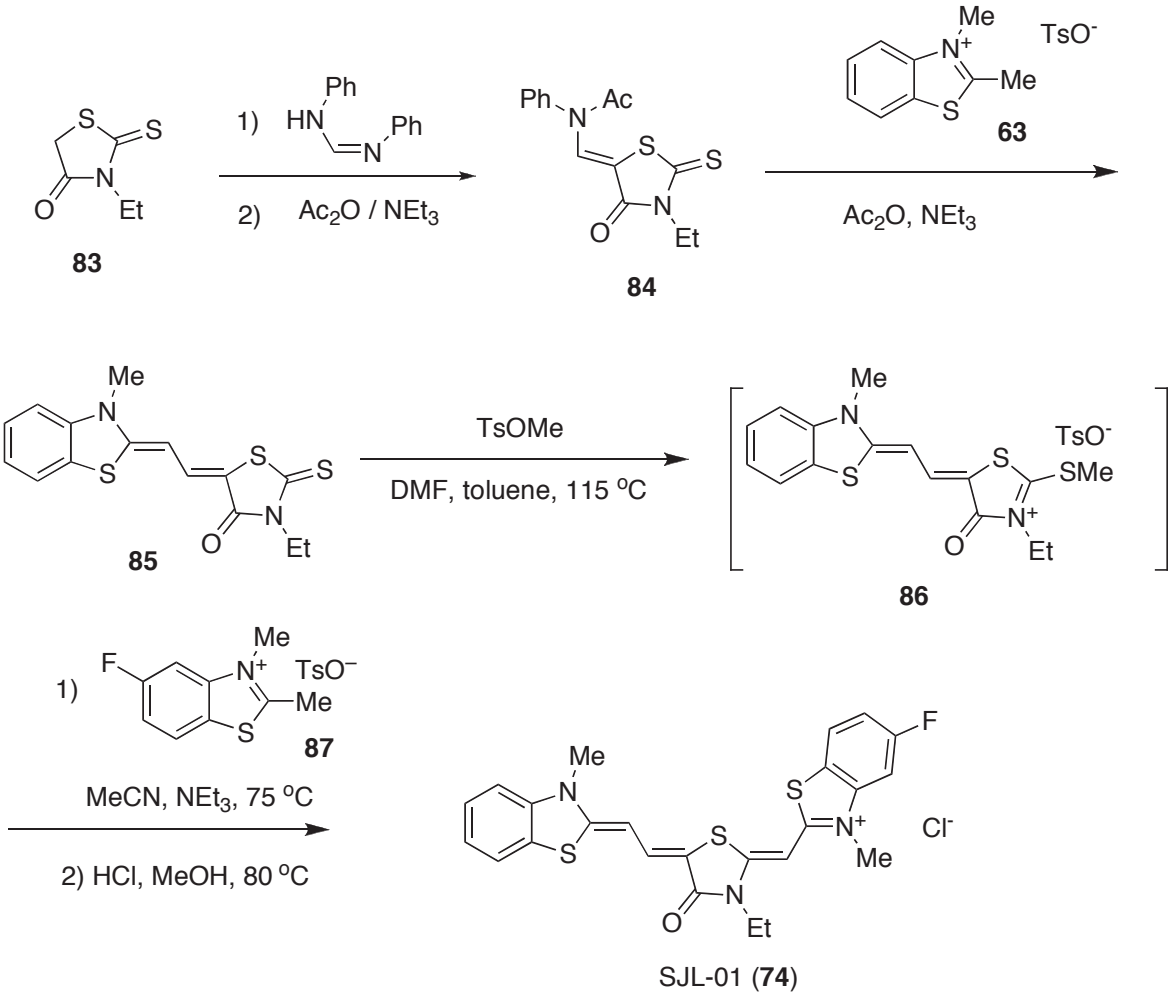
Macrophage *in vitro* screening, which is crucial to evaluate anti-leishmania activity, was then applied to several of the most promising rhodacyanines, but most of the compounds showed poor results as indicated by the IC_{50} value of compound **72** in Table 8. On the other hand, compound **74** showed noteworthy activities, compared with that of miltefosine. Thus, SJL-01 (**74**) was selected for *in vivo* evaluation.

Table 8. Inhibition by rhodacyanines against *Leishmania donovani* in an *in vitro* activity in macrophages

compound	IC_{50} *L. donovani* (μM)
72	4.691
74	0.353
Miltefosine (**11**)	0.811

5.4. SJL-01 (74) as an Anti-visceral Leishmaniasis (VL) Agent

Synthesis of **74** was carried out as shown in Scheme 4. Reaction of rhodanine **83** with *N,N'*-diphenyl formamidine in DMF, followed by treatment with acetic anhydride gave **84**, which was reacted with



Scheme 4. Synthesis of SJL-01 (**74**)

N-methyl-2-methylbenzothiazolium salt **63** to provide merocyanine **85**. After formation of **86**, obtained by the reaction of **85** with methyl *p*-toluenesulfonate, condensation of **86** with **87** in the presence of triethylamine, followed by treatment with hydrochloric acid, furnished **74**. The desired compound can be easily synthesized in excellent yield in six steps, affording a crystalline compound. The highly pure product, mp 274.5-275.6 ºC, was obtained by simple crystallization.[28]

On the basis of the results of the above *in vitro* macrophage assays, compound **74** was further evaluated by *in vivo* testing using *L. donovani* strain HU3 in female BALB/c mice, performed at the London School of Hygiene & Tropical Medicine. Since preliminary studies showed that no bioavailability was obtained by s.c. administrations of **74**, the *in vivo* studies were carried out *via* i.v. administration. Excellent activities, ~ 95% inhibition, were observed by the 5-times treatments with compound **74** at dosages ranging from 1.3 - 12.5 mg/kg (Table 9). Even at a dose as low as 0.2 mg/kg x 5 of **74** gave 16.13% inhibition, suggesting the existence of a dose dependent activity. The activity of compound **74** is much better than that of the conventional medicines, pentostam (**8**) and amphotericin B (**9**), and comparable to that of liposomal amphotericin B.[28]

Table 9. *In vivo* activity against *Leishmania donovani* HU3 in BALB/c mice

compound	dosing regimen (mg/kg/day)	inhibition (%)
74	12.5 (i.v.) x 5	95.40
74	4.1 (i.v.) x 5	97.15
74	1.3 (i.v.) x 5	94.87
74	0.2 (i.v.) x 5	16.13
pentostam (**8**)	15 (s.c.) x 5	62.04
amphotericin B (**9**)	0.5 (i.v.) x 3	78.6
liposomal amphotericin B	1.5 (i.v.) x 3	95.53

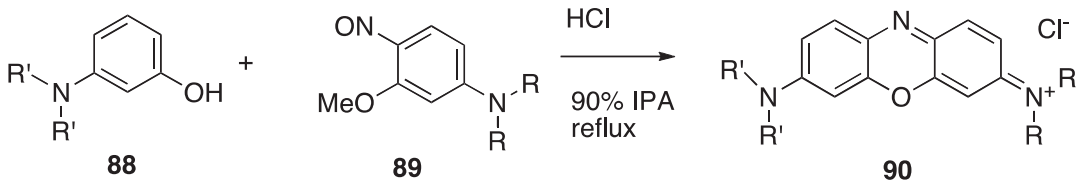
Preclinical and clinical development of **74** aimed at leishmaniasis is important for the therapy of this neglected disease. *In vitro* receptor binding assays were carried out against 80 receptors and very small inhibitions were observed at 1.0 μM except in the cases of four receptors, BZD (rat heart) (72%), M_1 (human recombinant) (60%), M_2 (human recombinant) (80%) and M_4 (human recombinant) (73%). Furthermore, the *in vitro* micronucleus test of **74** showed a negative result at 2 μM in the absence of S9 (a supplemented rat liver homogenate fraction) and 31.3 μM in the presence of S9, while negative results were obtained at 5.19 μM (-S9) and at 3.97 μM (+S9) by the chromosomal aberration test. It was concluded that **74** did not demonstrate mutagenic potential in the *in vitro* cell mutation assay. Furthermore, it was concluded that **74** did not show any evidence of causing an increase in the induction of micronucleated polychromatic erythrocytes or bone marrow cell toxicity. The *in vitro* acute effects of **74** on the hERG K^+ channel current, recorded from stably transfected HEK-293 cells, were evaluated at

nominal concentrations ranging from 1 μM up to 10 μM. Thus, compound **74** is very hopeful candidate for VL.

6. PHENOXAZINIUM SALTS

Oral administration is important for antimalarial agents. We found that phenoxazinium salts exhibited high efficacy *via* oral administration (p.o.),[29] and excellent bioavailabilities of phenoxazinium salts were gained by p.o.[30] However, the development of 3,7-*bis*(dialkylamino)phenoxazinium derivatives as medicines was difficult because these compounds typically were purified with the aid of zinc chloride.[31] We found that symmetric and asymmetric 3,7-*bis*(dialkylamino)phenoxazinium derivatives with high purity could be obtained chromatography followed by large-scale crystallization.[32]

Some synthetic intermediates can be prepared easily by the palladium-catalyzed reaction.[33] 3,7-*bis*(Dialkylamino)phenoxazinium salts **90** were obtained by the reaction of 3-*N*,*N*-dialkylaminophenols **88** and *N*,*N*-dialkyl-3-methoxy-4-nitrosoanilines **89** in acidic solutions, and then purified by chromatography with a short silica gel column.



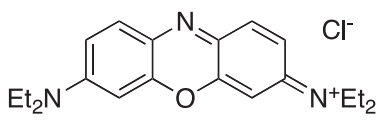
Scheme 5. Synthesis of phenoxazinium salts

Antiprotozoal and cytotoxic activities of the 3,7-*bis*(dialkylamino)phenoxazinium salts are shown in Table 10. Comparison of the IC_{50} value of **95** with the IC_{50} values of the other compounds suggests that the presence of a morpholino group might increase the polarity and hydrophilicity of those compounds, thereby decreasing their activities. However, the introduction of a morpholino group substantially reduced the toxicity, which suggests that increased polarity and hydrophilicity result in decreased toxicity. This phenomenon was evidenced by the high toxicity and low selectivity of **97**, in which long alkyl chain led to increased lipophilicity of the compound. Taking into consideration activity and toxicity, the compounds with a short alkyl chain (methyl and ethyl) are good antiprotozoal candidates, whereas the introduction of long alkyl chains or morpholino groups is not recommended. Symmetrical and asymmetrical structures did not have different activities, but a bulky wing structure affected both toxicity and efficacy. We hypothesize that the active center of these drugs might be the central tricyclic moiety and that a planar conformation of this structure is essential. Thus, the introduction of bulky groups might increase toxicity or decrease activity. Most phenoxaziniums showed good activities against *P. falciparum*. It was noteworthy that phenoxazinium derivatives had potent activity against *T. cruzi*.[34]

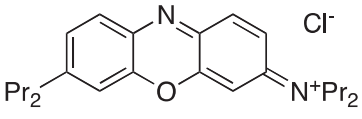
Table 10. Antiprotozoal activities and cytotoxicity (IC_{50} values μM) of phenoxazinium salts

compound	*P. falc.* K1		*T. cruzi*		*T. b. rhod.*		*L. don.*, axenic		Cytotox. L-6
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}
standard[a]	0.148	-	0.866	-	0.006	-	0.280	-	0.02
91	0.003	2410.0	0.036	185.4	0.228	29.4	0.214	31.3	6.7
92	0.002	165.0	0.017	23.6	0.023	17.4	0.002	165.0	0.4
93	0.003	1400.0	0.121	32.6	0.160	24.6	0.037	107.7	3.93
94	0.005	1050.0	0.081	67.7	0.154	35.6	1.136	4.8	5.47
95	0.431	66.5	7.090	4.0	8.735	3.3	5.182	5.5	28.62
96	0.006	4022.5	0.608	36.6	5.451	4.1	14.369	1.5	22.23
97	0.002	63.0	0.015	9.0	0.022	6.3	0.002	63.0	0.14

[a]Standards: chloroquine (*P. falciparum*), benznidazole (*T. cruzi*), melarsoprol (*T. b. rhodesiense*), miltefosine (*L. donovani*), and podophyllotoxin (L-6 cells, cytotoxicity)



basic blue 3 (**91**)



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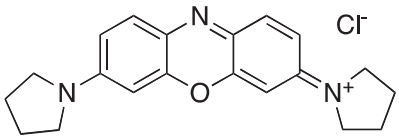

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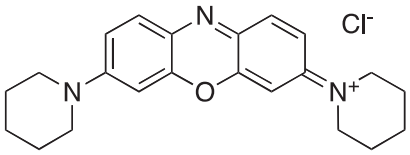

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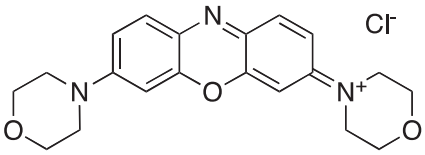

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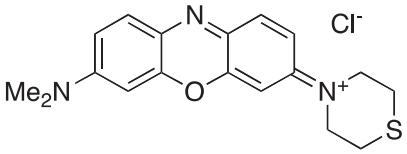

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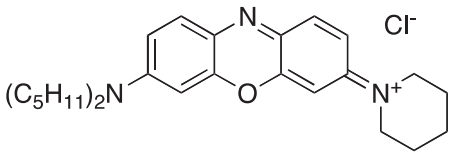


97

As compared to the phenoxazinium ions, the phenothiazinium ions such as methylene blue (**31**) displayed potent i*n vitro* activity against *P. falciparum* K1 but their cytotoxicities were generally high.[35]

7. BENZO[*a*]PHENOXAZINES

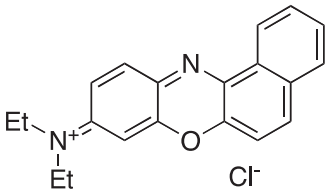
Although phenoxazinium salts showed good efficacy in *in vivo* tests by oral administration, the toxicity study gave unsatisfactory results. Since the electrophilicity of the carbon atom at the 1 position in the

phenoxazinium skeleton would be troublesome, we considered the effect of the addition of a benzene ring to the phenoxazinium framework. We therefore prepared and evaluated various benzo[*a*]phenoxazine derivatives. The corresponding salts of benzo[*a*]phenoxazines are a variant of DLC candidates but they would exist as a free base, non-DLC form, in the mammalian body. A number of benzo[*a*]phenoxazine derivatives were prepared by known methods[36-40] and then subjected to the screening test. The results of some benzo[*a*]phenoxazines against *P. falciparum* K1, cytotoxicity toward L-6 myoblasts, and *in vivo* activity against *P. berghei* NK-65 are summarized in Table 11.

Table 11. 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

Compound	*P. falc*. K1 IC_{50} (μM)	Cytotox. L-6 IC_{50} (μM)	Selectivity	Inhibition (%)	MSD*
98	0.606	3.82	6.3	-	-
99	0.868	65.9	76.0	15	6.3
100	0.0156	43.14	2,765	15	6.6
101	0.191	164.9	863	0	6.3
102	0.232	123.2	531	0	6.3
103	0.063	179.8	2,855	14.7	6.3
104	0.027	67.9	2,515	81	10.3
105	0.1125	167.9	1,493	11.4	6.3
106	0.005	13.6	2,734	3.3	6.3
107	0.015	15.8	1,052	>99.9	8.3
108	0.023	117.0	5,088	62.7	10.0
SSJ-183 (**109**)	0.0076	55.7	7,334	>99.9	14.6
110	0.0081	165.1	20,390	>99.9	10.0
111	0.05	67.1	1,342	15.3	6.3
112	0.195	63.8	327	5.6	6.3
113	0.029	96.9	3,342	97.9	7.3
114	0.18	86.6	481	99.7	13.0
115	0.017	14.8	887	>99.9	15.7
116	0.038	>190.2	>5,007	12.7	6.3
117	0.011	>190.2	>17,290	14.2	6.6
118	0.009	1.56	174	22.0	6.6
119	0.024	0.75	31	39	7.3

*MSD: mean survival days



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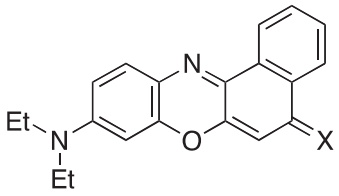
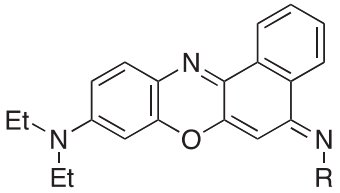
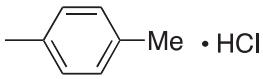
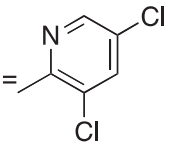


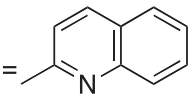
99: X = O
100: X = N •HCl

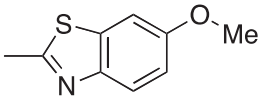


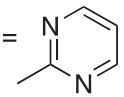
101: R = Ph• HCl

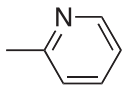
102: R =  • HCl

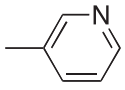
103: R = 

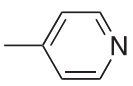
104: R = 

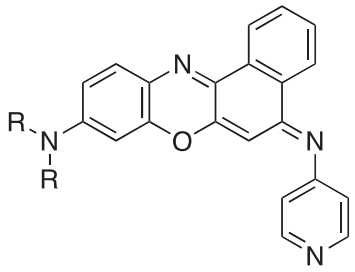
105: R = 

106: R = 

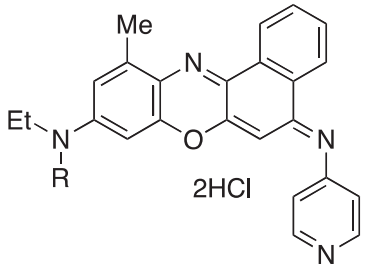
107: R = 

108: R = 

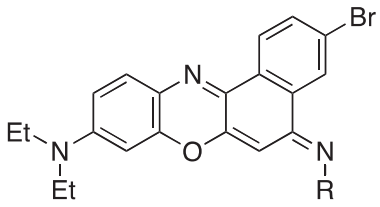
109: R = 

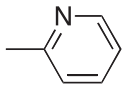


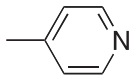
110: R = Me
111: R = Pr • 2HCl
112: R = Bu • 2HCl
113: R = $(CH_2)_2O(CH_2)_2$



114: R = Et
115: R = $CH_2CH_2NHSO_2Me$



116: R = 

117: R = 

Benzo[*a*]phenoxazinium **98**, having no substituent at the 6 position, showed weak activity against *P. falciparum* K1. The activity of Nile red (**99**) 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 (**100**) to 0.0156 μM. Although a low cytotoxicity was observed for **100**, the *in vivo* activity was poor.

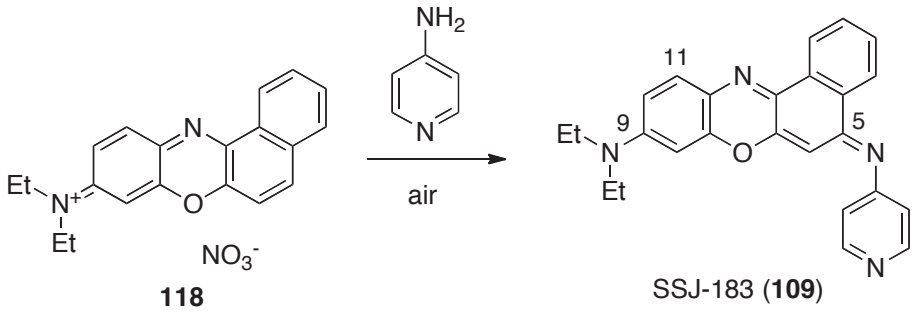
The phenyl and tolyl derivatives **101** and **102** showed low activities in both *in vitro* (IC_{50}: 0.191 and 0.232 μM) and *in vivo* tests. Higher activities were achieved in the *in vitro* test of compounds possessing hetero aromatic rings on the nitrogen at the 6 position. Therefore, several derivatives **103** – **106** were prepared and evaluated but their *in vivo* efficacies were unsatisfactory. Improved *in vivo* efficacies were observed for the compounds **107**, **108** and **109** carrying a pyridine ring, with **109** exhibiting the best activity among these three compounds. In addition to the inhibition of parasitemia, the mean survival days (MSD) after a single dose of **109** were extended to 14.6 days compared to approximately 6 days for an infected 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 in three independent experiments.

Although numerous analogues were prepared and assessed, only the 4-aminopyridine derivatives are presented here. Although the dimethyl derivative **110** showed potent activity, it produced shorter survival compared to **109**. With longer substituents at the 9 position, **111** and **112** were less active. Morpholine compound **113** displayed good activity. Furthermore, substitution of a methyl group at the 11 position improved the safety and two derivatives **114** and **115** provided good *in vivo* efficacy. The hydrochlorides **114** and **115** gave similar *in vivo* activities as that of **109**, suggesting that benzo[*a*]phenoxazine is apparently absorbed through the gut as the hydrochloride when administered p.o. Absence of cytotoxicity of compounds **116** and **117**, 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.[41]

On the basis of the above findings including other factors such as ease of preparation and toxicity, SJL-183 (**109**) was selected for the further study.

7.1. SSJ-183 (109) as an Anti-malarial Agent

SSJ-183 (**109**) can be prepared from the commercially available **118** in one step (Scheme 6). Notably, the compound **109** is very stable as the free amine for a long period under ambient conditions. An alternative and more effective synthetic method of **109** has been elaborated.[42]



Scheme 6. Synthesis of SSJ-183 (**109**)

SSJ-183 (**109**) exhibited potent activity: an IC_{50} value of 0.0076 μM against *P. falciparum* K1, IC_{50} of 55.7 μM in the cytotoxicity test, selectivity of 7334, and >99.9% inhibition of *P. berghei* NK-65. To gain additional information on the *in vivo* efficacy of **109**, we carried out a dose response experiment in NMRI female mice infected with *P. berghei* GFP ANKA strain (Table 12). High efficacy was observed by the p.o. route with cures achieved by oral administration of three daily doses of 100 mg/kg.

Table 12. *In vivo* results for **109** orally administrated to n=3 mice/dose once daily for three consecutive days to *P. berghei* GFP ANKA strain

mg/kg	Inhibition (%)	MSD (% of cured animals)
3 x 100	>99.9	>30.0 (100%)
3 x 30	99	27.2 (78%)
3 x 10	26	4.0

In other evaluations with compound **109**, we detected no lethality at doses up to 2,000 mg/kg p.o. using 20 mice. Furthermore, no effects were found at 1,000 μM (- and + S9) in a chromosome aberration test, at 2.0 μM (- and + S9) in an *in vitro* micronucleus test and at 1,000 mg/kg x 2 in an *in vivo* rat micronucleus test. No phototoxicity was detected in mice dosed at 300 mg/kg p.o. In binding assays against 80 receptors, only two human recombinant receptors, A_3 and D_3, were inhibited ~80% at 1 μM and no inhibitions were noted with other receptors. The selectivity was further supported by ≥1000-fold higher IC_{50} values of **109** against three other protozoal parasites (36 μM for *T. brucei rhodesiense*, 11.3 μM for *T. cruzi* and 6.5 μM for *L. donovani)* 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 **109** are much better than those of methylene blue. Furthermore, no hemolysis was observed in blood taken from G6PD deficient patient at Jichi Medical University.

In vivo pharmacokinetic studies in rats were carried out according to the reported procedure (Figure 10).[43] After oral dosing, **109** had a bioavailability of approximately 30%. After i.v. administration, the terminal half life was approximately 5.5 h and **109** demonstrated a high volume of distribution and high clearance.

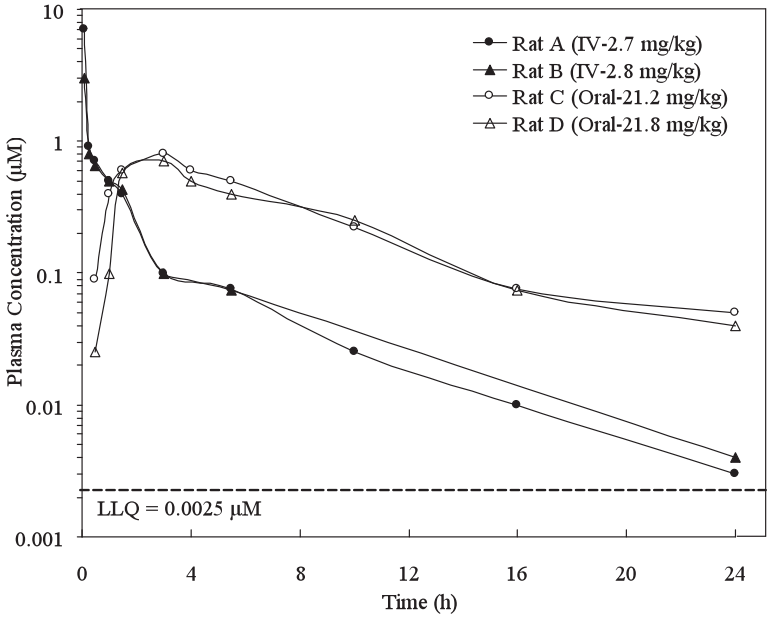


Figure 10. Plasma concentration versus time profile of **109** in male Sprague Dawley rats following intravenous (2.8 mg/kg, filled symbols) and oral (21.5 mg/kg, open symbols) administration

8. STUDY OF THE MODE OF ACTION

Rhodacyanines were accumulated into a special organelle, which was stained with fluorescence. Visualization of the fluorescent organelle showed it to be in close proximity to mitochondria.[44] SSJ-127 (**62**) was detected in mouse malaria parasites using fluorescence imaging *in vitro* and in the experimentally administered model. Selective accumulation of **62** in an organelle was observed in all blood stages of live malaria parasites. The organelle was clearly different from the mitochondrion and the nucleus in terms of morphology. The shape of the organelle changed during the asexual blood stages of the parasite. There was always a close association between the organelle and the mitochondrion. These results raised the possibility that SSJ-127 (**62**) accumulates in an apicoplast of the malaria parasite and affects protozoan parasite-specific pathways.[45]

Further studies for the determination of the 3-D structure of receptors against our compounds are now underway.

9. CONSIDERATION OF A MOLECULAR MODEL

CPS models for two compounds, SJL-01 (**74**) and SSJ-183 (**109**), are illustrated in Figure 11. The two molecules are similar in having two planes composed of heteroaromatic rings that are intersected by some degree of an angle, resulting in their asymmetric shapes. These phenomena may be important in creating the specific biological properties of the molecule.

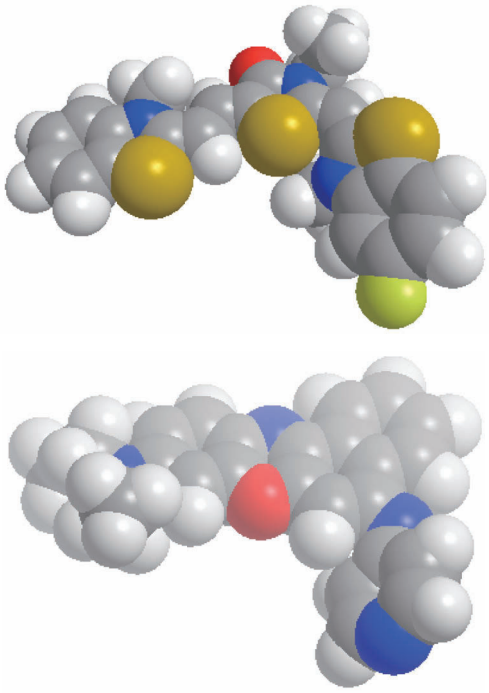


Figure 11. CPK models of SJL-01 (**74**) and SSJ-183 (**109**)

10. CONCLUSION

According to the working hypothesis based on DLC, several hopeful candidates have been discovered. Thus, rhodacyanine derivative SSJ-127 (**62**) cured rodent malaria (*P. berghei*) and *T. bucei brucei* infected mouse models. Fluorinated rhodacyanine SJL-01 (**74**) showed extraordinary efficacy against *L. donovani* in the *in vivo* test *via* i.v. administration. The activity of **74** was much better than or comparable to those of conventional medicines, which are toxic and expensive. Furthermore, benzo[*a*]phenoxazine derivative SSJ-183 (**109**) possessing the 4-aminopyridine moiety showed an IC_{50} value against *P. falciparum* of 7.6 nM and a selectivity index of >7,300. Cure was achieved with three daily oral doses to mice infected with *P. berghei* ANKA strain. Benzo[*a*]phenoxazines are not DLCs. Preliminary biological experiments clearly demonstrated that the target organelle for our compounds is not the mitochondrion but an organelle specific to protozoan parasites. Thus, high safety could be expected for these compounds. Further extensions of these studies are now in progress.

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