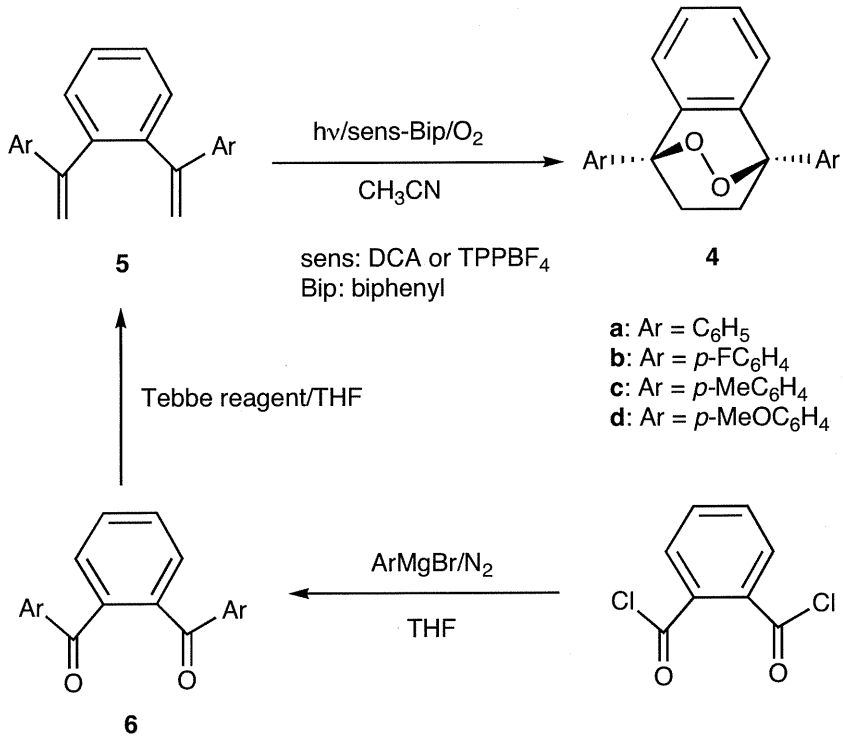
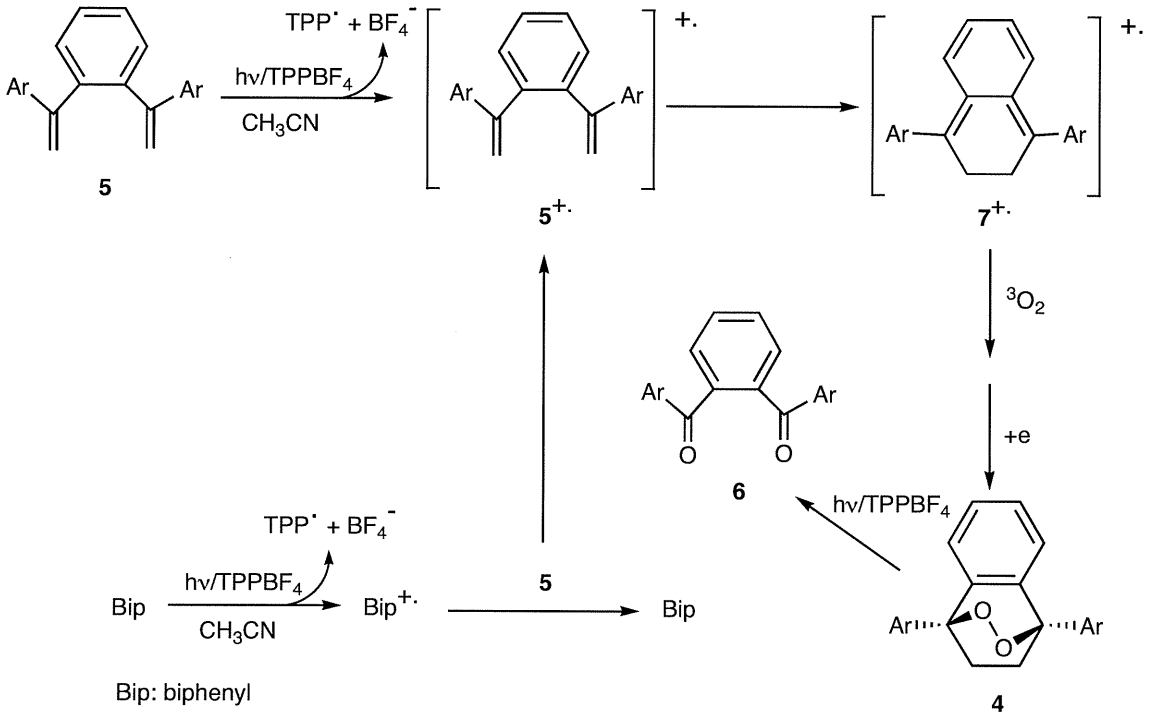

Ar
Ar
hν/sens-Bip/O2
CH3CN
Ar
O
O
Ar
5
sens: DCA or TPPBF4
Bip: biphenyl
4
a: Ar = C6H5
b: Ar = p-FC6H4
c: Ar = p-MeC6H4
d: Ar = p-MeOC6H4
Tebbe reagent/THF
ArMgBr/N2
THF
Cl
Cl
O
O
Ar
Ar
O
O
6

Scheme 2


Ar
Ar
5
TPP• + BF4-
hν/TPPBF4
CH3CN
5+·
7+·
3O2
+e
6
hν/TPPBF4
4
Bip
Bip+·
Bip
Bip: biphenyl

Scheme 3

Table 1. PET Oxygenation Reactions of 1,2-Bis(1-arylethenyl)benzenes **5**[a]

Run	Substrate[b]	Sensitizer[b]	Irradiation time (min)	Yield of products (%)[c]	
				4	**6**
1	**5a**	DCA	15	36	19
2	**5a**	DCA–Bip	10	49	12
3	**5a**	$TPPBF_4$	10	61	6
4	**5a**	$TPPBF_4$–Bip	5	83	3
5	**5b**	DCA	15	40	11
6	**5b**	DCA–Bip	10	44	11
7	**5b**	$TPPBF_4$	15	60	6
8	**5b**	$TPPBF_4$–Bip	5	66	2
9	**5c**	DCA	15	54	15
10	**5c**	DCA–Bip	10	68	11
11	**5c**	$TPPBF_4$	10	71	7
12	**5c**	$TPPBF_4$–Bip	5	88	4
13	**5d**	DCA	15	58	9
14	**5d**	DCA–Bip	10	58	9
15	**5d**	$TPPBF_4$	3	66	0
16	**5d**	$TPPBF_4$–Bip	3	67	0

[a] Oxygen-saturated acetonitrile solutions (10 ml) of **5** (0.20 mmol) containing the sensitizer or the sensitizer with Bip were irradiated by a 2 kW Xe lamp ($\lambda > 360$ nm).
[b] [**5**] = 20 mM; [$TPPBF_4$] = 2.0 mM; [DCA] = 0.2 mM; [Bip] = 60 mM.
[c] Isolated yield by silica gel TLC. **5a-d** were completely consumed.

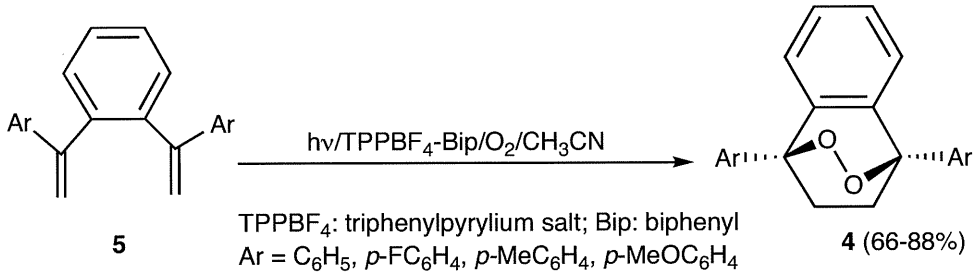
Table 2. *In Vitro* Antimalarial Activities of Cyclic Peroxides **1-4** against *P. falciparum* (FCR-3 strain) and Cytotoxicities against FM3A Cells[a]

Substrate	EC_{50} (M)		Selectivity[d]
	P. falciparum[b]	FM3A cell[c]	
4a	1.3×10^{-7}	1.0×10^{-6}	8
4b	1.7×10^{-7}	3.0×10^{-6}	18
4c	8.0×10^{-8}	1.9×10^{-5} (66%)[e]	>238
4d	1.1×10^{-7}	1.0×10^{-5}	91
1a[f]	1.0×10^{-6}	3.2×10^{-5}	>32
1b[f]	5.6×10^{-7}	1.8×10^{-5}	>32
1c[f]	5.0×10^{-7}	1.7×10^{-6}	3
1d[f]	1.2×10^{-6}	1.8×10^{-5}	>15
Artemisinin[g]	1.0×10^{-8}	1.0×10^{-5}	1000

[a] In vitro antimalarial activities and cytotoxicities are described in the Experimental Section.
[b] Chloroquine-sensitive (FCR-strain).
[c] Mouse mammary tumor FM3A cells in culture as a control for mammarian cell cytotoxicity.
[d] Selectivity = (mean of EC_{50} value for FM3A)/(mean of EC_{50} value for *P. falciparum*).
[e] Growth percent at the concentration indicated.
[f] Reference [13].
[g] Reference [7].

Applications of triphenylpyrylium salt-sensitized electron transfer photo-oxygenation reactions to the synthesis of benzo-fused 1,4-diaryl-2,3-dioxabicyclo[2.2.2]octanes as new antimalarial cyclic peroxides

Masaki Kamata, Jun-ich Hagiwara, Tomoko Hokari, Chiharu Suzuki, Ryohta Fujino, Sayaka Kobayashi, Hye-Sook Kim, Yusuke Wataya



5

$TPPBF_4$: triphenylpyrylium salt; Bip: biphenyl
Ar = C_6H_5, *p*-FC_6H_4, *p*-MeC_6H_4, *p*-$MeOC_6H_4$

4 (66-88%)

New antimalarial bicyclic peroxides **4** were synthesized by utilizing $TPPBF_4$-sensitized photoinduced electron transfer oxygenation reactions.

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Antimalarial activity of 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251) and its carboxylic acid derivatives

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Carbon radical
Plasmodium falciparum
Plasmodium berghei

ABSTRACT

Malaria is one of the world's deadliest diseases and is becoming an increasingly serious problem as malaria parasites develop resistance to most of the antimalarial drugs used today. We previously reported the *in vitro* and *in vivo* antimalarial potencies of 1,2,6,7-tetraoxaspiro[7.11]nonadecane (N-89) and 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251) against *Plasmodium falciparum* and *Plasmodium berghei* parasites. To improve water-solubility for synthetic peroxides, a variety of cyclic peroxides having carboxyl functionality was prepared based on the antimalarial candidate, N-251, and their antimalarial activities were determined. The reactions of N-89 and its derivatives with Fe(II) demonstrated a highly efficient formation of the corresponding carbon radical which may be suspected as a key for the antiparasitic activity.

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1. Introduction

Malaria is one of the world's deadliest diseases and is becoming an increasingly serious problem as malaria parasites develop resistance to drugs such as chloroquine and mefloquine. Hence, WHO recommends ACT (artemisinin-based combined therapy) since 2000 as a response to drug-resistant *Plasmodium falciparum* [1]. However, there are some reports of ACT-resistant *P. falciparum* [2–4], and parasite recrudescence is common following artemisinin monotherapy [5,6]. There is, therefore, considerable urgency to develop new classes of antimalarials [7]. Artemisinin (compound 1 in Fig. 1) is a 1,2,4-trioxane clinically used for the treatment of multidrug-resistant *P. falciparum* malaria. Artemisinin and its derivatives are known to show excellent antimalarial activities in infected mice [2]. We previously developed a convenient synthetic route to the spiro-peroxide, 1,2,6,7-tetraoxaspiro [7.11]nonadecane (N-89; compound 2 in Fig. 1), and found that this compound possesses antimalarial activities *in vitro* and *in vivo* comparable to those of artemisinin [8]. We recently reported the *in vitro* and *in vivo* antimalarial activities of 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251; compound 6 in Fig. 1) against *P. falciparum* and *P. berghei* parasites [9]. The N-251 showed high activities both in the *in vitro* and the *in vivo* tests (EC_{50} 0.023 μM; ED_{50} 15 mg/kg, per oral). The effects were similar to that of artemisinin *in vitro* and greater than artemisinin's activity *in vivo* (p.o.). In addition, administration of N-251 to mice bearing 1% of parasitemia (per oral 68 mg/kg, 3 times a day for 3 consecutive days) resulted in a dramatic decrease in the parasitemia: all the five mice given N-251 were cured without any recrudescence, with no toxicity in the 60 days of experiment.

We report herein the preparation of a variety of water-soluble carboxylic acid derivatives based on N-251 and their antimalarial activities. We also discuss the action mechanisms of these new agents.

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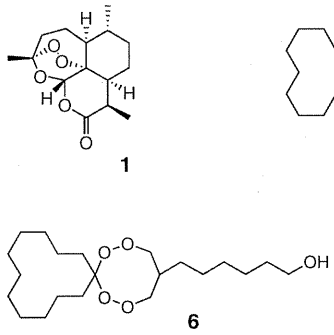

Fig. 1. Structures of the endoperoxide compounds artemisinin (compound 1), N-89 (compound 2) and N-251 (compound 6).

2. Materials and methods

2.1. Preparation of N-251 (compound 6) and its carboxylic acid derivatives

Synthesis and properties of N-251 (compound 6) and its carboxylic acid derivatives are described in the Supplementary material.

2.2. Reaction of spiro-peroxide (compound 15) with $FeSO_4/CuCl_2$

To a solution of $FeSO_4 \cdot 7\ H_2O$ (556 mg, 2 mmol.) and $CuCl_2$ (673 mg, 5 mmol) in H_2O (10 mL)–DMSO (20 mL) was added dropwise a solution of the spiro-peroxide (compound 15) (249 mg, 0.5 mmol) in CH_3CN (30 mL)–benzene (10 mL) during 1 h under an argon atmosphere and the mixture was stirred at room temperature for an additional 17 h. Then, the mixture was extracted with ether and the extract was dried over anhydrous $MgSO_4$ and evaporated. The residue was separated by column chromatography on silica gel. The first fraction (elution with ether–hexane, 5:95) gave ketone (compound 14) (24 mg, 26%). From the second fraction (elution with ether–hexane, 15:85) 12-chlorododecanoic acid (compound 13) (83 mg, 70%) was obtained. The third fraction (elution with ether–hexane, 80:20) was a complex mixture of unidentified products containing a keto-ol (compound 20) (60 mg). Elution with ethyl acetate gave a diol (compound 19) (83 mg, 55%).

The third fraction obtained above was dissolved in CH_2Cl_2 (5 mL) and to this solution a mixture of acetic anhydride (101 mg, 1 mmol) and triethylamine (204 mg, 2 mmol) was added in CH_2Cl_2 (5 mL) during 5 min and then the mixture was stirred at room temperature for 2 days. The mixture was poured into aqueous $NaHCO_3$ and the organic products were extracted with ether. By column chromatography of the residue on basic alumina (elution with ether–hexane, 30:70) an unsaturated aldehyde compound 21 (22 mg, 14% based on the used peroxide compound 15) was obtained. Subsequent elution with ether–hexane (30:70) resulted in the recovery of a mixture of unidentified products (40 mg). Spectroscopic data of compounds 13, 19, and 21 were indicated in the Supplementary material.

2.3. In vitro and in vivo antimalarial activities

The antimalarial activity of reagents against the *P. falciparum* FCR-3 strain *in vitro*, and the cytotoxicity to mouse mammary FM3A cells in culture were determined as described earlier [8,9]. The *in vivo* antimalarial activity was assessed using ICR mice infected with *P. berghei* (strain NK65). The animal studies were performed in accordance to the relevant laws and institutional guidelines. We used the protocols described previously for drug candidates [8,9]. Briefly, various concentrations of the test compounds, prepared in several solvents, were administered daily via indicated routes, to groups of five mice for four consecutive days beginning on the day of infection (to determine the ED value and survival time).

3. Results and discussion

3.1. Preparation of N-251 and its carboxylic acid derivatives

The strategy for the synthesis of the prodrug antimalarials based on N-89 (compound 2) was the introduction of a carboxyl group to the terminal carbon of the alkyl chain in N-89 at the 4-position. We hoped that this would not induce any significant decrease of the antimalarial activity and thus offer wider choice of suitable drugs. The key in the synthesis was the Ag_2O-promoted nucleophilic substitution of 1,3-diiodopropane derivative (compound 4) with bis-hydroperoxide (compound 3) to give the cyclic peroxide (compound 5) [8] (Fig. 2). Subsequent deprotection provided the 6-hydroxyhexyl-substituted spiro-peroxide (N-251; compound 6).

The reaction of N-251 (compound 6) with succinic anhydride gave the carboxylic acid compound 7, while oxidation of the alcohol (compound 6) with Jones reagent resulted in the formation of the alternative compound 8. Similarly, the carboxylic acids (compounds 9 and 10) were prepared from 4-allyl-1,2,6,7-tetraoxaspiro [7.11]nonadecane (Fig. 3 and Supplementary material).

Since the solubility of these carboxylic acids (compounds 7 to 10) in 5% aqueous $NaHCO_3$ was low (ca. 1 mg/1 mL), the dicarboxylic acid (compound 12) having a better solubility was prepared as illustrated in Fig. 4. Thus, oxidation of the alkene (compound 11) [10], followed by the reaction of the derived diol with 2 equiv. of succinic anhydride gave the desired dicarboxylic acid (compound 12), which showed an excellent solubility in 5% aqueous $NaHCO_3$ (>70 mg/1 mL).

3.2. Antimalarial activity of N-251 and its carboxylic acid derivatives

The antimalarial potencies of the alcohol (N-251; compound 6) and the carboxylic acids, compounds 7 to 10 and 12, *in vitro* against chloroquine-sensitive *P. falciparum* FCR-3 strain were determined, and the results are shown in Table 1. The activities of the carboxylic acid derivatives (compounds 7 to 10 and 12) were only moderate (EC_{50} 0.44 μM ~ 0.11 μM) and compound 11, precursor of compound

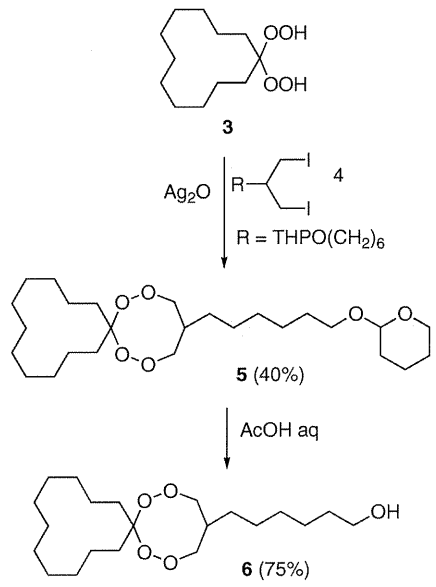

Fig. 2. Synthesis of the N-89 alcohol derivative 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-l4-yl)hexan-1-ol (N-251) (compound 6).

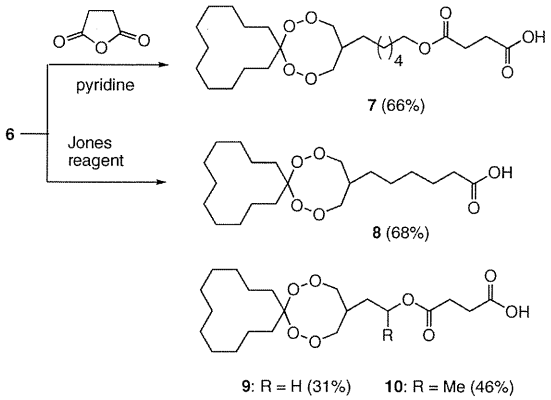

Fig. 3. Synthesis of the N-89 carboxylic acid derivatives (compounds 7 to 10).

12, has similar antimalarial activity compare to carboxylic acid derivatives.

The *in vivo* antimalarial activities of the parent spiro-peroxide N-89 and the alcohol derivative N-251, as measured in mice according to a published protocol [8,9], are shown in Table 2. With intraperitoneal (i.p.) and oral (p.o.) administrations, the N-251 showed strong activities similar to those of N-89 and artemisinin. Consistent with this, by the administration of N-251 (i.p.; 100 mg/kg/day; 4 days), malaria parasites disappeared from the blood. All 5 mice were confirmed to live over 60 days, indicating a complete cure. By intravenous administration (dissolved in a mixture of 10% Cremophor®, 10% ethanol and 80% saline), N-89 and N-251 showed similar antimalarial activities in infected mice, producing complete cure in four of five mice.

It is also interesting to note that the N-89 shows high antimalarial activity by intramuscular (i.m.) and subcutaneous (s.c.) injection. These data indicate that N-89, and probably N-251 as well, can be used in several ways of injection, a fact convenient for use in malaria-endemic areas.

In contrast, the p.o. and i.p. activities of the carboxylic acids, compounds 7, 10 and 12, dissolved in 5% aqueous $NaHCO_3$, were only moderate. In the case of the highly soluble dicarboxylic acid (compound 12), the intravenous (i.v.) administration was possible. However, the activity was moderate (ED_{50}: 50 mg/kg, ED_{90}: >100 mg/kg), with the mean survival time of the infected mice being around 200% compared to the parasite-infected mice without any administration. These results suggest that further modification of the structure may be required to develop a candidate of orally and/or intravenously active antimalarial drug having a reasonable solubility in aqueous solution.

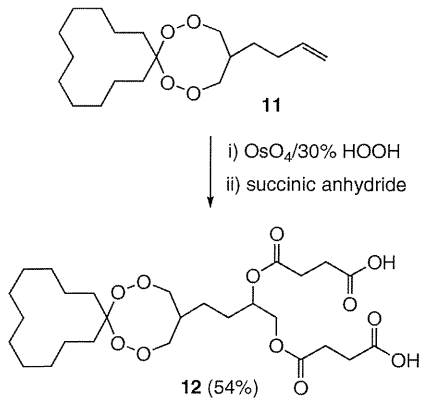

Fig. 4. Synthesis of the N-89 carboxylic acid derivative (compound 12).

Table 1
In vitro antimalarial activities of cyclic peroxides against *P. falciparum* and cytotoxicities against FM3A cells.

Peroxides	EC_{50} values (μM)		
	P. falciparum[a]	FM3A[b]	Selectivity[c]
6[d]	0.023	8.0	348
7	0.36	7.2	3
8	0.17	5.6	40
9	0.29	7.2	25
10	0.16	5.6	35
11	0.11	5.5	50
12	0.44	18.0	41
2[d]	0.025	8.2	328
Artemisinine	0.015	10.0	667
Artesunate	0.017	3.0	176

In vitro antimalarial activities and cytotoxicities were determined by the protocol previously reported [8,9].
[a] Chloroquine sensitive FCR-3 strain.
[b] Mouse mammary tumor FM3A cells in culture as a control for mammalian cell cytotoxicity.
[c] Selectivity = [mean of EC_{50} for FM3A cells]/[mean of EC_{50} for *P. falciparum*].
[d] The *in vitro* antimalarial activities and cytotoxicities were reported previously [8,9].

3.3. Preclinical safety properties of N-251

Nonclinical safety studies were performed for N-251 (compound 6) under Good Laboratory Practice (GLP) guidelines. The results are shown in Table 3. The lethal doses of N-251 for mouse, rat and dog are over 1000 mg/kg, indicating that N-251 is little toxic in mammals. Moreover, no mutagenicity or clastogenicity was detected for N-251. In addition, no toxicity to respiratory or central nervous system was observed. These results showed that N-251 would be as safe as artemisinin in its clinical application.

3.4. Reaction of spiro-peroxides with $FeSO_4/CuCl_2$

The mechanism of action of peroxide antimalarials involves interaction of the drugs with heme, that induces hemoglobin degradation

Table 2
In vivo antimalarial activities of cyclic peroxides against *P. berghei*-infected mice.

Peroxides	Route	ED_{50}, mg/kg	ED_{90}, mg/kg
2[a]	i.p.[b]	12	20
2[a]	p.o.[c]	20	40
2[a]	i.v.[d]	12	20
2	i.m.[e]	19	40
2	s.c.[f]	20	40
6[a]	*i.p.*[b]	15	26
6[a]	p.o.[c]	15	40
6[a]	i.v.[d]	22	45
7	p.o.[c,g]	37	78
10	p.o.[c,g]	41	>100
12	i.v.[d,g]	50	>100
12	i.p.[b,g]	62	>100
Artemisinin[a]	i.p.[b]	5	13
Artemisinin[a]	p.o.[c]	30	89

Various concentrations of the test compounds were prepared in olive oil (i.p. and p.o.), 25% polyethylene glycol (s.c.), or 10% cremophor–10% ethanol–80% saline (i.v.). The test compounds were administered to groups of five mice once a day starting on day 0 (mice were infected with *P. berghei* on day 0), and the administration was continued on day 1, day 2, and day 3. Parasitemia levels were determined on the day following the last treatment (on day 4). ED values of antimalarial activities indicated above were estimated by the previously reported protocol [8,9].
[a] The *in vivo* antimalarial activities were reported previously [8,9].
[b] Intraperitoneal (i.p.).
[c] Per oral (p.o.).
[d] Intraveneous (i.v.).
[e] Intramuscular (i.m.).
[f] Subcutaneous (s.c.).
[g] Solution in 5% $NaHCO_3$ solution.

Table 3
Preclinical safety tests of N-251 (compound 6) under Good Laboratory Practice (GLP).

Lethal dose (mouse; single application; p.o.)	>2000 mg/kg
Lethal dose (rat; single application; p.o.)	>2000 mg/kg
Lethal dose (dog; single application; p.o.)	>1000 mg/kg
Mutagenicity (*Salmonella typhimurium, Escherichia coli*)	Negative
Clastogenicity (CHL/U cells)	Negative
Toxicity to respiratory system (rat; 1000 mg/kg single application; p.o.)	Negative
Toxicity to central nervous system (rat; 1000 mg/kg single application; p.o.)	Negative

GLP guidelines are provided by the Ministry of Health, Labour and Welfare, Japan.

[11–15]. The interaction is believed to produce a range of potentially toxic oxygen- and carbon-centered-radicals. Several researchers have studied biomimetic Fe(II)-mediated decomposition to stimulate events in the ferrous-rich parasite food vacuole. To see the behavior of these new cyclic peroxides prepared in this study toward Fe(II), reactions with $FeSO_4$ were undertaken in the presence of $CuCl_2$, which is known to capture the resulting carbon radicals to produce corresponding alkyl chlorides [16]. The reactions of bis-hydroperoxide (compound 3) or sprio-peroxide (compound 2) gave in each case the corresponding chloride (compound 13) together with cyclododecanone (compound 14) (Fig. 5). It is interesting to note that the product compositions are very similar, suggesting that these two substrates, compounds 2 and 3, react in a similar fashion. To obtain information for the mode of decay of the tetroxocane moiety in spiro-peroxide, the spiro-peroxide (compound 15) having a higher molecular weight was prepared and the reduction was conducted. A mixture of compounds 13 (70%), 14 (24%), and the diol 19 (55%) was obtained, together with a complex mixture of products containing keto-ol (compound 20). Dehydration of compound 20 gave the unsaturated aldehyde (compound 21) in 14% yield based on compound 15 (Fig. 6).

Fe(II) is most likely to attack the peroxide bond of compound 15 from the less hindered direction [17] to give mainly the oxy radical (compound 16). Subsequent C–C bond fission produces the carbon radical (compound 17) (path a in Fig. 6), which is in turn captured by $CuCl_2$ to give finally 12-chlorododecanoic acid (compound 13), together with diol (compound 19) and/or keto-ol (compound 20). Alternatively, cleavage of the C–O bond in the oxy radical (compound 16) (path b) may occur to give cyclododecanone (compound 14) (path b). The high yield of compound 13 demonstrates that formation of the carbon radical (compound 17) from a spiro-peroxide (compound 15) is an efficient process, which would be a reason for the substantial antimalarial activity of this type of cyclic peroxides.

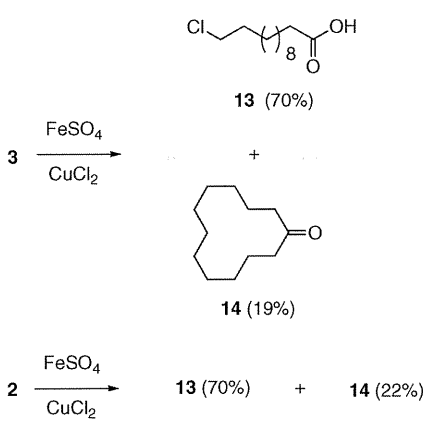

Fig. 5. Reaction of spiro-peroxides, N-89 (compound 2) and its derivative compound 3, with $FeSO_4/CuCl_2$.

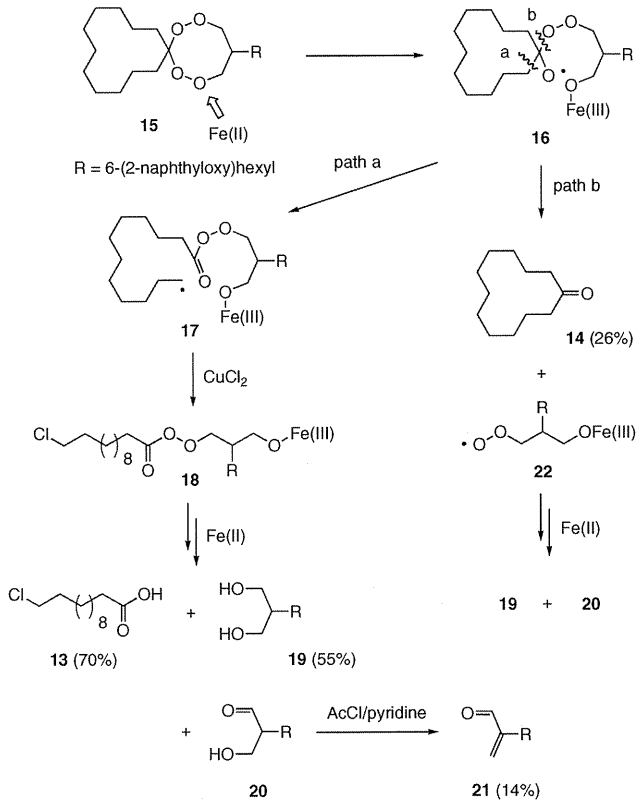

Fig. 6. Reaction of spiro-peroxide (compound 15) with $FeSO_4/CuCl_2$.

3.5. Conclusions

The N-251, preparable at a kilogram scale, can be used to synthesize the corresponding carboxylic acid derivatives without destroying the critical pharmacophore peroxide bond. These water-soluble spiro-peroxides were orally and intravenously effective against malaria, although they were so only moderately. Efficient generation of the corresponding carbon radicals from spiro-peroxides on reaction with Fe(II) would be the origin of antimalarial activity of N-89 and the related spiro-peroxides. We are going to attempt identification of N-251 molecular target(s) that would involve understanding of resistance mechanisms. We consider that N-251 deserves more extensive clinical evaluation, desirably including future trials in the human.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.parint.2011.08.017.

References

[1] Guidelines for the treatment of malaria. Second ed. Geneva: World Health Organization; 2010.
[2] White NJ. Qinghaosu (artemisinin): the price of success. Science 2008;320:330–4.
[3] Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. The New England Journal of Medicine 2008;359:2619–20.
[4] Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J. Artemisinin resistance in *Plasmodium falciparum* malaria. The New England Journal of Medicine 2009;361 455–46.
[5] McIntosh HM, Olliaro P. Artemisinin derivatives for treating uncomplicated malaria. Cochrane Database of Systematic Reviews 2000;2 CD000256.
[6] Codd A, Teuscher F, Kyle DE, Cheng Q, Gatton ML. Artemisinin-induced parasite dormancy: a plausible mechanism for treatment failure. Malaria Journal 2011;10:56.
[7] Sadanand S. Malaria: an evaluation of the current state of research on pathogenesis and antimalarial drugs. The Yale Journal of Biology and Medicine 2010;83:185–91.
[8] Kim HS, Nagai Y, Ono K, Begum K, Wataya Y, Hamada Y, et al. Synthesis and antimalarial activity of novel medium-sized 1,2,4,5-tetraoxacycloalkanes. Journal of Medicinal Chemistry 2001;44:2357–61.
[9] Sato A, Hiramoto A, Morita M, Matsumoto M, Komichi Y, Nakase Y, et al. Antimalarial activity of endoperoxide compound 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl) hexan-1-ol. Parasitology International 2011;60:270–3.
[10] Nagayama S, Endo M, Kobayashi S. Microencapsulated osmium tetroxide. A new recoverable and reusable polymer-supported osmium catalyst for dihydroxylation of olefins. Journal of Organic Chemistry 1998;63:6094–5.
[11] Posner GH, O'Neill PM. Knowledge of the proposed chemical mechanism of action and cytochrome p450 mechanism of antimalarial trioxanes like artemisinin allows rational design of new antimalarial peroxides. Accounts of Chemical Research 2004;37:397–404.
[12] Robert A, Dechy-Cabaret O, Cazelles J, Meunier B. From mechanistic studies on artemisinin derivatives to new modular antimalarial drugs. Accounts of Chemical Research 2002;35:167–74.
[13] Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, et al. Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 2003;424: 957–61.
[14] Wu Y. How might qinghaosu (artemisinin) and related compounds kill the intraerythrocytic malaria parasite? A chemist's view. Accounts of Chemical Research 2002;35:255–9.
[15] Haynes RK, Ho WY, Chan HW, Fugmann B, Stetter J, Croft SL, et al. Highly antimalaria-active artemisinin derivatives: biological activity does not correlate with chemical reactivity. Angewandte Chemie (International Ed in English) 2004;43:1381–5.
[16] Nonami Y, Baran J, Sosnicki J, Mayr H, Masuyama A, Nojima M. Reaction of highly methylated 2-methylenecycloalkyl hydroperoxides with $FeSO_4/CuCl_2$. Remarkably efficient 5-*endo-trig* or 6-*endo-trig* cyclization of the intermediate carbon radicals. Journal of Organic Chemistry 1999;64:4060–3.
[17] Abe M, Inakazu T, Munakata J, Nojima M, et al. ^{18}O-tracer studies of Fe(II)-induced decomposition of 1,2,4-trioxolanes (ozonides) derived from cyclopentenes and indenes. Inner-sphere electron transfer reaction of the peroxide linkage. Journal of the American Chemical Society 1999:6556–62.

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Antimalarial activity of endoperoxide compound 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol

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ABSTRACT

Plasmodium falciparum, the major causative parasite for the disease, has acquired resistance to most of the antimalarial drugs used today, presenting an immediate need for new antimalarial drugs. Here, we report the *in vitro* and *in vivo* antimalarial activities of 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251) against *P. falciparum* and *Plasmodium berghei* parasites. The N-251 showed high antimalarial potencies both in the *in vitro* and the *in vivo* tests (EC_{50} 2.3×10^{-8} M; ED_{50} 15 mg/kg (per oral)). The potencies were similar to that of artemisinin *in vitro* and greater than artemisinin's activity *in vivo* (p.o.). In addition, N-251 has little toxicity: a single oral administration at 2000 mg/kg to a rat gave no health problems to it. Administration of N-251 to mice bearing 1% of parasitemia (per oral 68 mg/kg, 3 times a day for 3 consecutive days) resulted in a dramatic decrease in the parasitemia: all the 5 mice given N-251 were cured without any recurrence, with no diarrhea or weight loss occurring in the 60 days of experiment. N-251 deserves more extensive clinical evaluation, desirably including future trials in the human.

1. Introduction

Malaria, caused by infection with *Plasmodium* parasites, is a serious health threat to people worldwide. *Plasmodium falciparum*, the major causative parasite for the disease, has acquired resistance to most of the antimalarial drugs used today [1–3]. Therefore, there is an immediate need for new antimalarial drugs, hopefully accompanied by the understanding of their actions.

The Chinese herb, *Artemisia annua*, has been used as a folk remedy for malarial infection and its active component artemisinin was isolated and its structure was established in 1972 [4,5]. Its use as antimalarial drug began in 1979 and was proved to be very effective in killing *P. falciparum* in humans [4,5]. Its action is rapid and has been used widely for controlling malaria. However, *P. falciparum* resistant to artemisinin and artemisinin-based combination therapy has recently emerged around the border of Cambodia-Thailand [5–7]. In addition, a persistent disadvantage of artemisinin is that despite rapid action, it cannot prevent recurrence of the disease after the apparent eradication of the parasites in patient's blood.

Fifteen years ago, we began a search for new antimalarial medicines by utilizing artemisinin as a lead compound. We hypothesized that the intramolecular peroxide structure of artemisinin must be important for its biological actions, and performed synthesis of about 500 compounds many of them bearing such peroxide structures. Extensive selection by *in vitro* and *in vivo* antimalarial tests resulted in finding 1,2,6,7-tetraoxaspiro[7.11]nonadecane (N-89) (Fig. 1) as a highly promising antimalarial agent [8]. N-89 showed strong antimalarial activities both in the *in vitro* and the *in vivo* assays: EC_{50} 2.5×10^{-8}; ED_{50} 20 mg/kg per oral (*cf* Table 1). A remarkable feature of N-89 is its simple chemical structure with no asymmetric carbons. In extension of the discovery of N-89, we have prepared a large number of its derivatives and now wish to report that 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251), bearing a functional side chain hydroxyl group that allows derivatization, is as potent as N-89 against malaria parasites. This finding has opened up the way to explore mechanic aspects of this class of agents; i.e. the prodrug nature of N-89 is expected to be useful in such

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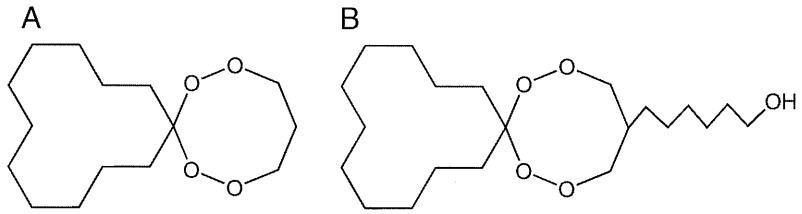

Fig. 1. Structures of the endoperoxide compounds N-89 and N-251. Chemical structures of N-89 (A) and N-251 (B).

studies. Indeed, by using the side chain '–OH' as a handle, we are now executing such investigations (work to be published).

2. Experimental procedures

2.1. General

Artesiminin and Chremophor® EL were obtained from Sigma-Aldrich, St. Louis, MO, USA. N-89 was synthesized as described previously [8]. N-251 was synthesized by replacing the hydrogen at C-4 of N-89 with hexanol [details to be published elsewhere]. Dimethylsulfoxide (DMSO) and olive oil were obtained from Wako, Osaka, Japan. Parasites have been maintained by weekly passage of 1×10^6 infected red blood cells into a healthy mouse by the intraperitoneal (i.p.) route.

2.2. Antimalarial activity and cytotoxicity in vitro

The antimalarial activity of reagents against the *P. falciparum* FCR-3 strain *in vitro*, and the cytotoxicity to mouse mammary FM3A cells in culture were determined as described earlier [9]. Stock solutions of the reagents in DMSO at various concentrations were used for these experiments.

Samples of parasitized human erythrocyte suspension (995 µl each) containing 3% hematocrit and 0.3% parasitemia were placed in wells of 24-well plates. To them were added 5 µl of drug-solutions of desired concentrations, and the plates were incubated for 72 h at 37 °C, during which no medium change was done. The numbers of parasitized erythrocytes were then recorded by microscopic observation of the smears of each well-sample, with the use of Diff-Quick Staining Kit (Sysmex International Reagents Co., Ltd., Kobe, Japan) to stain the parasite nuclei. The results are expressed as the effective concentration that produced 50% decrease in parasitemia relative to the control (with DMSO only). All of the test compounds were assayed in duplicate at each concentration. All data points represent the mean of three experiments and the results are represented by their averages.

For the measurement of cytotoxicity, mouse mammary cell FM3A was cultured as described before [8]: the cell density was adjusted to 5×10^4 cells/ml, and 995 µl samples of this suspension were placed in individual wells of 24-well plates. Five µl-samples of test compound in DMSO were added to these wells, and the plates were incubated at 37 °C in 5% CO_2 for 48 h. Duplicate wells were used for each drug concentration. Cell numbers were measured using a Particle Counter COULTER Z1 (Beckman Coulter, Miami, FL, USA). "Selective toxicity" means [EC_{50} to cell]/[EC_{50} against parasite].

2.3. In vivo antimalarial activity

The animal studies were performed in accordance to the relevant laws and institutional guidelines. The *in vivo* antimalarial activity was assessed using ICR mice infected with *Plasmodium berghei* (strain NK65). We used the protocols described previously for drug candidates [9]. Two tests were performed; one, administration of drugs at the time of the start of parasite infection, and another, a curative test, i.e., administration of drugs after the parasitemia has been established.

Beginning on the day of infection, various doses of drug as solutions were given to the groups of mice (5 in each group) either *i.p.* (as solution in DMSO), *p.o.* (as solution in olive oil) or *i.v.* (as solution in ethanol and Chremophore®, see footnote of Table 1) once a day for consecutive 4 days (i.e., until the third day after the infection). On the fourth day, blood samples from the animals were taken and the percentage of infected erythrocytes (the parasitemia level) was recorded. The parasitemia level in the control mice, for which olive oil only was administered, was 44.2%. The dose of the drug to reduce this level to its half was designated as ED_{50}. Parasitemia levels were monitored for 2 months. When no parasites were detected on the 60th day, we judged that parasite eradication was attained in these mice. The control mice lived 6.5 days on average after the infection.

In the curative test of the drug for *P. berghei* NK65 strain-infected mice, the mice were randomly sorted into groups of five, infected with the parasite, and kept until 1% of the red blood cells had been infected, and then the administration of N-251 dissolved in olive oil was started. The oral administration with 100 µl of the drug at a dose of

Table 1
Antimalarial activity of endoperoxide, N-251.

Compound	*P. falciparum* EC_{50} (M)	FM3A cell EC50 (M)	Selective toxicity [a]	*i.p.*[b]		*p.o.*[c]		*i.v.*[d]	
				ED_{50} (mg/kg)	ED_{90} (mg/kg)	ED_{50} (mg/kg)	ED_{90} (mg/kg)	ED_{50} (mg/kg)	ED_{90} (mg/kg)
N-89	2.5×10^{-8}	8.2×10^{-6}	328	12	20	20	40	12	20
N-251	2.3×10^{-8}	8.0×10^{-6}	348	15	26	15	40	22	45
Artemisinin	1.5×10^{-8}	1.0×10^{-5}	667	5	13	30	89		NT[e]

[a] EC_{50} of FM3A cell/EC_{50}, of *P. faiciparum*.
[b] Solvent: DMSO.
[c] Solvent: olive oil.
[d] Solvent: 10:1080(v/v/v)Cremophor®ELI polyoyethyleneglycerol 35 triricinoleate): ethanol: saline.
[e] NT: not tested.

68 mg/kg was performed three times a day, during three consecutive days. Experiments included a control group receiving the olive oil only. The antimalarial activity was evaluated by counting parasitemia in blood smears during 60 days after the parasite inoculation.

3. Results and discussion

3.1. In vitro antimalarial activity of N-251 against P. falciparum

A simple *in vitro* test for the drug, using *P. falciparum* in culture and using mouse mammary tumor FM3A cells as a model for the mammal host-cell gave promising results for N-251 as shown in Fig. 2. The EC_{50} against the parasite was 2.3×10^{-8} M, while the EC_{50} to FM3A was a higher concentration 8.0×10^{-6} M; the selective toxicity was thus 348 fold. Similar high selectivity was observed for N-251 against other strains of malaria parasites, the chloroquine-resistant K1 and the mefloquine-resistant strain that has been established in our laboratory (data not shown). These results suggested that N-251 is highly toxic to malaria parasites but only little toxic towards mammals; therefore, it is a good candidate as a safe antimalarial medicine. We also did a trial in which a single large dose of N-251 (2000 mg/kg) was given orally to a rat, and noted that it did not cause any untoward health harm as judged both by observation and by subsequent autopsy.

3.2. In vivo antimalarial activity of N-251 against P. berghei-infected mice

For the *in vivo* effect of N-251, the 4-day suppressive tests for mice were performed. In this protocol, infection and drug-administration were begun simultaneously (the antimalarial effect).

The results in Fig. 3A show that orally administered N-251 possesses an antimalarial effect with an ED_{50} value of 15 mg/kg. It should be noted that artemisinin showed an ED_{50} value at 30 mg/kg in a comparable experiment we performed (Table 1). The results given in Table 1 also show the high antimalarial effects of N-251, N-89, and artemisinin when they were administered *i.v.* and *i.p.* These results indicate that the antimalarial effect of N-251 is equivalent or higher in comparison to artemisinin.

The survival of mice as a function of time after treatment is shown in Fig. 3B. The no-drug and 0.5 mg/kg administered mice all died on day 4.

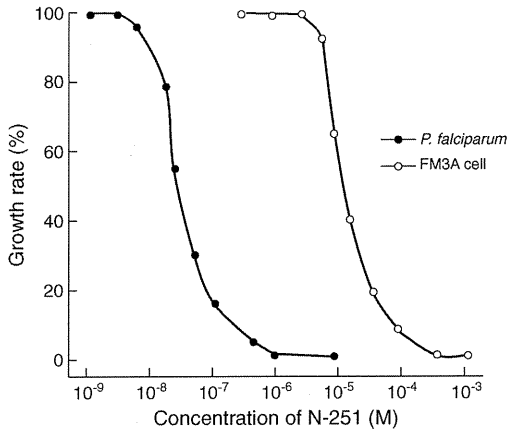

Fig. 2. In vitro antimalarial activity in *P. falciparum* and toxicity in mammalian cells. In vitro antimalarial activity and cytotoxicity of the N-251 compound on the *P. falciparum* FCR-3 strain (closed circles) and FM3A cells (open circles) are shown. The EC_{50} value was calculated from a semilog graph of the percentage of parasite growth against the N-251 concentration.

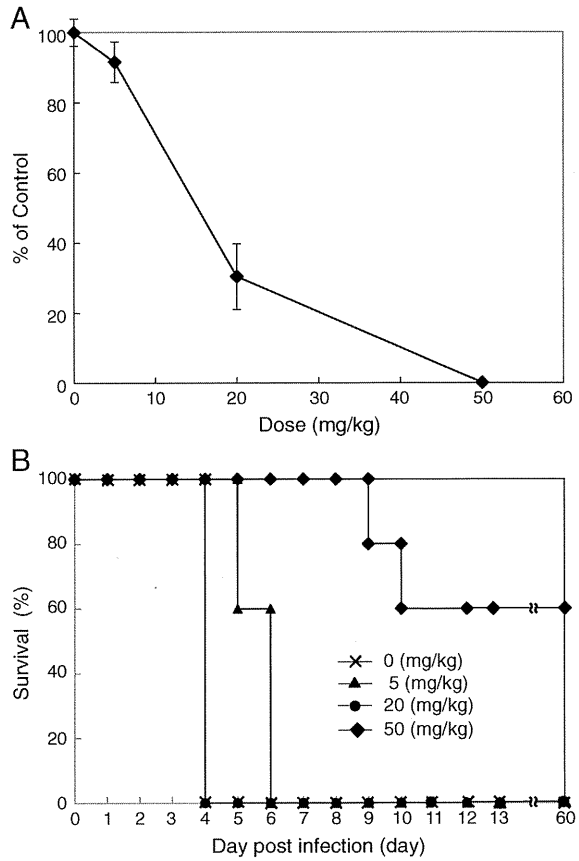

Fig. 3. In vivo antimalarial activity of N-251 against *P. berghei*-infected mice. *P. berghei*-infected mice were treated with N-251 at 0, 5, 20, 50 mg/kg, by oral route. (A) Curve for determination of ED_{50} value. (B) Survival rate. Results are for groups of 5 mice each. Two more repetitions of the experiments were performed, giving similar results.

In the 20 mg/kg group, however, two mice died on day 5, and the remaining three died on day 6, a result indicating life-prolongation by N-251. In contrast, the group receiving 50 mg/kg N-251 had one mouse that died on day 9 and another on day 10, and the remaining three mice showed complete disappearance of parasite in their blood and continued to live on day 60. It is remarkable that no recurrence of malaria had been observed in these surviving mice. These results indicate that N-251 possesses a strong antimalarial activity *in vivo*.

3.3. Cure for malaria in mice

As described above, the curing potency of N-251 against malaria was demonstrated by the 4-day *in vivo* tests. We then proceeded to explore curing mice of malaria by oral administration of N-251. The mice (5 in each group) were injected with *P. berghei*, diagnosed as malaria sickening after 70 h by confirmation of 1% of red blood cells as bearing the parasite(s), and then given N-251 orally at 68 mg/kg, three times a day for consecutive 3 days. Fig. 4 shows the time course of parasitemia level in the red blood cells (Fig. 4A), together with the surviving animal numbers (Fig. 4B). A small increase in the infected-red blood cell counts, which was noted at 8 h after N-251 administration, changed into a decrease at 16 h, and a complete disappearance resulted at 48 h in all treated mice. In the control mice, the parasites continuously increased to reach higher than 60% on the 6th day, and all the 5 mice died on that day. In sharp contrast, all the 5 mice given N-251 continued to live at the 60th day, and no parasites were detected in the blood of these mice. We

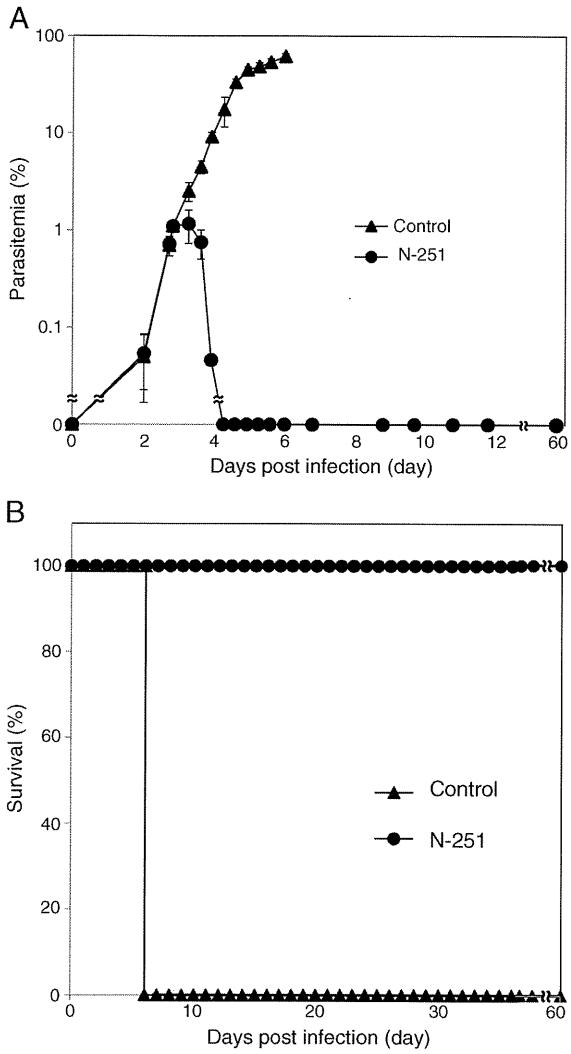

Fig. 4. Curative effect of N-251 in parasite-infected mice. Control (triangles); no-drug and/or N-251 (circles) at 68 mg/kg/3 times a day for 3 consecutive days, by oral route. (A) Parasitemia. Values represent the means ± standard error of parasitemia in five mice. (B) Survival rate. Results are for groups of 5 mice each. Two more repetitions of the experiments were performed, giving similar results.

concluded that a 100% cure for the 1% parasites-infected mice was achieved without any recurrence. It is remarkable that no side effects, such as diarrhea and weight loss, occurred. It should be noted that artemisinin is known to be unable to completely kill the parasite in the blood, and its effect has a short life [7]. In this regard, N-251 seems to be superior to artemisinin. It should also be remarked that a single-drug therapy was shown to be possible with N-251. It is hopeful that N-251 could be effective in curing malaria in the human. We are now going to attempt preclinical trials of N-251 that would involve pharmacokinetics studies and safety evaluations.

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References

[1] Ridley RG. Medical need, scientific opportunity and the drive for antimalarial drugs. Nature 2002;415:686–93.
[2] Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434:214–7.
[3] Mita T, Tanabe K, Kita K. Spread and evolution of *Plasmodium falciparum* drug resistance. Parasitol Int 2009;58:201–9.
[4] Klayman DL. Qinghaosu (artemisinin): an antimalarial drug from China. Science 1985;228:1049–55.
[5] White NJ. Qinghaosu (artemisinin): the price of success. Science 2008;320:330–4.
[6] Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 2008;359: 2619–20.
[7] Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 2009;361:455–67.
[8] Kim HS, Nagai Y, Ono K, Begum K, Wataya Y, Hamada Y, et al. Synthesis and antimalarial activity of novel medium-sized 1,2,4,5-tetraoxacycloalkanes. J Med Chem 2001;44:2357–61.
[9] Kim HS, Shibata Y, Wataya Y, Tsuchiya K, Masuyama A, Nojima M. Synthesis and antimalarial activity of cyclic peroxides, 1,2,4,5,7-pentoxocanes and 1,2,4,5-tetroxanes. J Med Chem 1999;42:2604–9.

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Schistosomicidal and antifecundity effects of oral treatment of synthetic endoperoxide N-89

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ABSTRACT

1,2,6,7-Tetraoxaspiro[7.11]nonadecane (N-89) is a chemically synthesized compound with good efficacy against malaria parasites. We observed strong anti-schistosomal activities of N-89 both *in vitro* and *in vivo*. In a murine model with experimental infection of *Schistosoma mansoni*, orally administered N-89 at the dose of 300 mg/kg resulted in a significant reduction in worm burden (63%) when mice were treated at 2-weeks postinfection. Strong larvicidal effects of N-89 were confirmed *in vitro*; schistosomula of *S. mansoni* were killed by N-89 at an EC50 of 16 nM. In contrast, no significant reduction in worm burden was observed when N-89 was administered at 5 weeks postinfection *in vivo*. However, egg production was markedly suppressed by N-89 treatment at that time point. On microscopic observation, the intestine of N-89-treated female worms seemed to be empty compared with the control group, and the mean body length was significantly shorter than that of controls. Nutritional impairment in the parasite due to N-89 treatment was possible, and therefore quantification of hemozoin was compared between parasites with or without N-89 treatment. We found that the hemozoin content was significantly reduced in N-89 treated parasites compared with controls ($P<0.001$). The surface of adult worms was observed by scanning and transmission electron microscopy, but there were no apparent changes. Taken together, these observations suggested that N-89 has strong antischistosomal effects, probably through a unique mode of drug efficacy. As N-89 is less toxic to mammalian host animals, it is a possible drug candidate against schistosomiasis.

1. Introduction

Schistosomiasis is a parasitic disease caused by trematode flatworms of the genus Schistosoma that is common in many tropical countries and affects more than 200 million people living in conditions of poor sanitation and/or with less developed social infrastructure [1–3]. The World Health Organization (WHO) is leading the global strategy of schistosomiasis control, with a focus on morbidity control through chemotherapy. Praziquantel (PZQ) is a safe and effective drug for schistosomiasis and has been the drug of choice since the late 1970s. This has raised concerns about the development of drug resistance, and suggestive cases of PZQ-resistant parasites have been reported in *Schistosoma mansoni* from African countries [4–6]. Therefore, the development of new antischistosomal drugs is a matter of priority, and new candidate compounds have been reported [7–9].

Artemisinin-derivatives (ADs) are compounds extracted from the plant *Artemisia annua* used in traditional Chinese herbal medicine, which have strong malaricidal effects [10–13]. Recent studies clearly showed that these compounds also had strong effects against schistosome parasites [14,15]. The most notable difference between PZQ and ADs is the developmental stages of the parasite at which the drugs show efficacy [16,17]. Adult worms are highly sensitive to PZQ, while the larval stages are less sensitive to the drug [18,19]. On the other hand, ADs are effective mainly against the larval stage parasites, while adult worms are less sensitive to treatment with these drugs. In this sense, PZQ is a therapeutic drug, while ADs are drugs for prophylaxis [20]. Therefore, it is recommended to use a combination of the two drugs [21,22].

Although the mechanism of the efficacy has not yet fully been elucidated, peroxide bridge is necessary for antimalarial activities of ADs [10]. Previously, we reported that synthetic endoperoxide (1,2,6,7-tetraoxaspiro[7.11]nonadecane: N-89) [23] has high antimalarial activity against *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo*, and it shows low levels of cytotoxicity in mice and rats (LD50: >2000 mg/kg) [23–25]. ADs are structurally complicated and their chemical synthesis is

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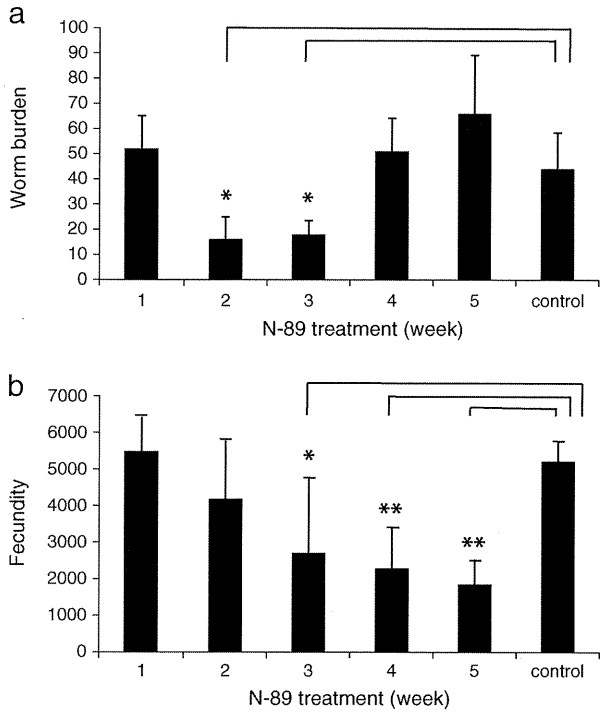

Fig. 1. *In vivo* effects of N-89 to *S.mansoni*. *S. mansoni*-infected mice were orally treated with N-89 from week 1 through week 5 postinfection. (a) *Y*-axis shows the number of worms that were collected by perfusion 9 weeks postinfection (**P*<0.001). (b) *Y*-axis shows the number of eggs produced per female worm. (**P*<0.05, ***P*<0.001).

not easy. On the other hand, N-89 is a compound with a relatively simple structure and is inexpensive to mass produce [23–25]. If N-89 also has strong effects against schistosome parasites, this will allow a new strategy of schistosomiasis control using a lower cost agent.

In this study, we found strong effects of N-89 against *S. mansoni* both *in vitro* and *in vivo*. The efficacies of N-89 were almost comparable to those of ADs. However, N-89 had additional effects that were not reported in the case of ADs, suggesting that N-89 may be a novel compound with unique antischistosomal activities.

2. Materials and methods

2.1. Parasites and animals

Puerto Rican strain *S. mansoni*, which was kept in our laboratory, was used for the present study. Female 5-week-old BALB/c mice were purchased from CLEA (Tokyo, Japan).

2.1.1. In vivo treatment of S. mansoni-infected mice with N-89

For *in vivo* study, mice were infected with 180 cercariae by the standard method in which mice were percutaneously exposed *via* the tail to cercariae for 1 h at room temperature [14]. BALB/c mice infected with *S. mansoni* were orally treated with N-89 suspended in olive oil at a dose of 300 mg/kg twice a day for two consecutive days. Mice were divided into 6 groups and treated with N-89 at various time points, *i.e.*, from week 1 through week 5 postinfection. To analyze parasite egg burden, eggs were recovered from the liver and intestine by the method reported previously [26]. Briefly, chopped liver and intestine were digested in 4% KOH at 37 °C for 1 h. After incubation, the digested samples were centrifuged at 1500 rpm for 5 min at room temperature, and pellets were resuspended in distilled water. Eggs were counted under a light microscope. Effects on pathological lesions after N-89 treatment were determined by observation of egg granulomas formed in the liver. Liver sections of Azan staining were prepared, and granuloma size was measured by using Image J image processing software (NIH). The mean size of 100 granulomas formed around a single egg in N-89 treated mice was compared to that in control (olive oil-treated mice). In addition, we calculated the body length of the worms using Image J. All *in vivo* experiments were approved by the Committee of Animal Rights and Ethics, Tokyo Medical and Dental University.

2.1.2. In vitro treatment of S. mansoni with N-89

As N-89 seemed to be effective against larval stage parasites, we prepared schistosomula from the lungs of mice and incubated them in

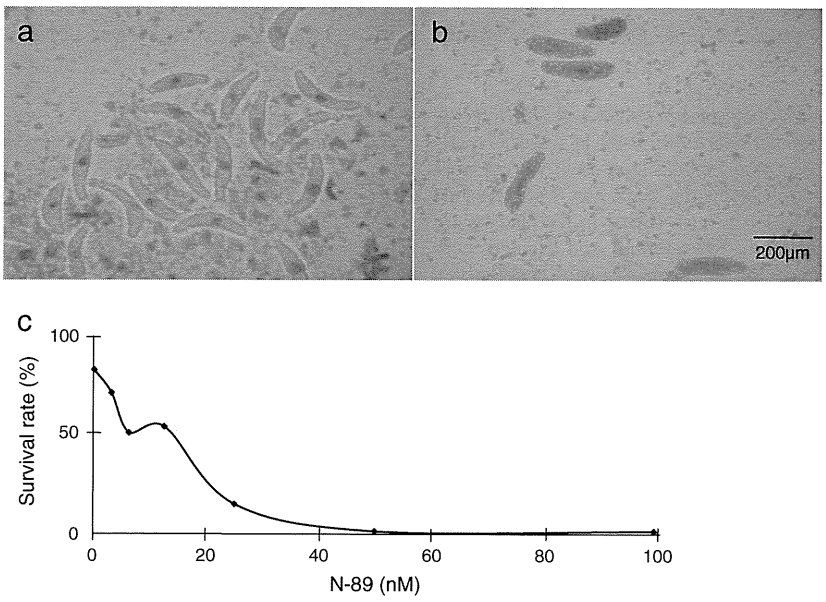

Fig. 2. Schistosomicidal effects of N-89 *in vitro*. (a) 14-day schistosomula were round-shaped and in a state of continuous contraction and extension when they are alive in the medium containing DMSO (2.5%) alone. (b) Schistosomula treated with 50 nM of N-89 were stiff and easily stained with trypan-blue. (c) Y-axis indicates the survival rate of 14-day schistosomula after treatment with serial dilutions of N-89.

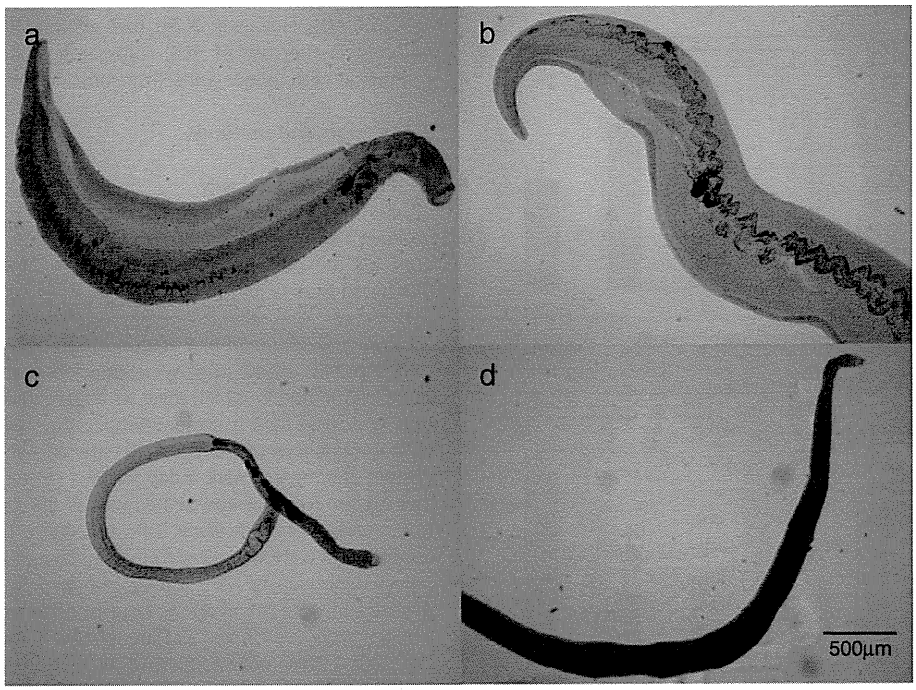

Fig. 3. Light microscopic observation of adult parasites after *in vivo* treatment with N-89. *S. mansoni*-infected mice were treated with or without N-89 5-weeks postinfection. Worms were collected 2 weeks after the treatment. 7-week *S. mansoni* worms were stained with hematoxylin–carmine solution. A male worm from mice treated with N-89 (a), a male worm from control mice (b), a female worm from mice treated with N-89 (c), a female worm from control mice (d).

RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% FBS (JRH Biosciences, Kansas, MO), 150 U/ml of penicillin, and 150 μg/ml of streptomycin (Gibco, Gaithersburg, MD) in 24-well plates (Greiner, Ulm, Germany). N-89 was dissolved in dimethylsulfoxide (DMSO) and added 25 μl to the plates which contains 1 ml of RPMI at various concentrations from 3.12 to 100 nM. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 7 days. Survival of the treated schistosomula was determined by trypan blue dye-exclusion test. Based on the observations, we calculated the EC_{50} of N-89 against schistosomula of *S. mansoni in vitro*.

2.2. Morphological observation of adult parasites after treatment with N-89 in vivo

To observe the morphological changes after N-89 treatment, infected BALB/c mice were administered orally with N-89 at 5 weeks post-infection at a dose of 300 mg/kg, and 2 weeks later adult worms were recovered by portal perfusion. Recovered parasites were washed thoroughly with 0.85% NaCl and 0.45% Na-citrate in distilled water, and paired worms were fixed in 70% ethanol and stained with hematoxylin–carmine solution for light microscopic observation. Parasites were observed by scanning electron microscopy and transmission electron microscopy (Hitachi, Tokyo, Japan) according to the method reported previously [27,28].

2.3. Quantification of hemozoin contents of S. mansoni

Hemozoin was extracted from *S. mansoni* and quantified by the method reported previously [29–31]. Protein contents of worm homogenates were measured using a protein assay kit (Bio-Rad, Hercules, CA). Infected mice were administered orally with N-89 (300 mg/kg) at 5 weeks postinfection, and 2 weeks later adult parasites were tested for hemozoin contents. The worms used for the tests were paired to compare worms in the same/similar developmental stages. For each experiment, 15 to 30 worms were used from each mouse. Worms were homogenized in 1 ml of PBS (pH 7.2), and centrifuged for 10 min at 10,000×*g*. Insoluble pellets were washed with 0.1 M sodium hydrogen carbonate, and then dissolved in 0.1 N NaOH. Hemozoin was converted to heme in this treatment, and we then measured the converted heme as hemozoin in accordance with the reagent manufacturer's protocol (Hemin, Sigma-Aldrich, St. Louis, MO). Heme was quantified spectrophotometrically by measuring absorbance at 405 nm. Hemozoin content in the parasite was expressed as ng heme/mg protein.

2.4. Statistical analysis

Statistical analyses were performed by Student's *t* test. In all analyses, $P<0.05$ was taken to indicate statistical significance.

3. Results

3.1. Schistosomicidal effects of N-89 in vivo

Reduction of worm burden was observed when mice were treated 2 or 3 weeks postinfection, and the maximum effect of N-89 driven reduction in worm burden was observed at 2 weeks postinfection compared with the olive oil control group (Fig. 1a). Schistosomicidal effects became less apparent at 3 weeks postinfection, and there was no detectable reduction in worm burden when mice were treated at 5 weeks postinfection. However, egg production per paired female worm was significantly reduced when mice were treated with N-89 at 5 weeks postinfection. Reduction in egg production per female worm in the N-89-treated group was statistically significant in comparison to the olive oil control group (Fig. 1b). These observations indicated that the larval stage is the target for the killing effect of N-89, while this agent showed inhibitory effects on fecundity of adult worms without killing the parasite.

3.2. In vitro effects of N-89 for schistosomula of S. mansoni

To confirm the direct effects of N-89 against the larval stage of *S. mansoni*, schistosomula were treated with serial dilutions of N-89 and

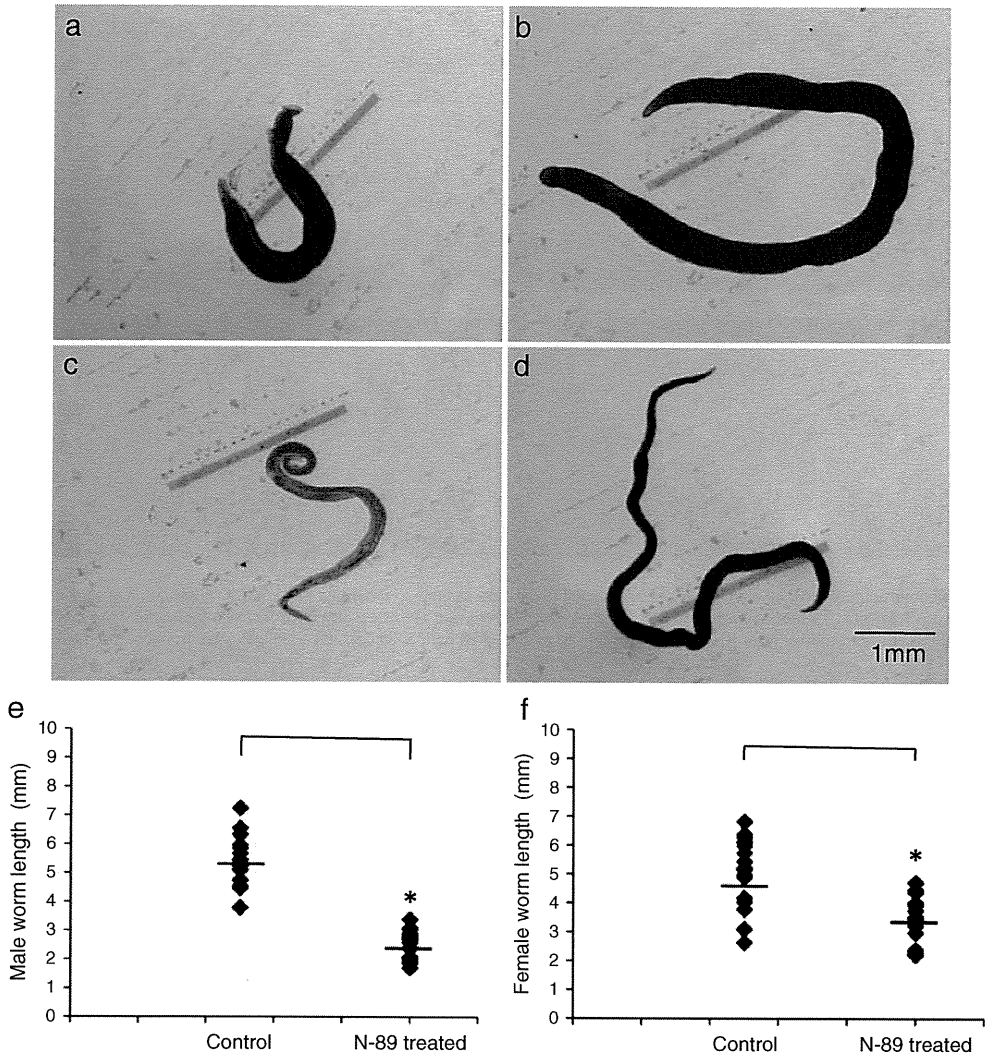

Fig. 4. The mean body length of the worms. All worms used were obtained in the same manner as described in Fig. 3. *Y*-axis indicates the length of male worms (a) (*$P<0.01$) and female worms (b) (*$P<0.01$).

cultured for 7 days *in vitro*. The schistosomicidal effects of N-89 were dose-dependent, and the EC_{50} against *S. mansoni* larvae was calculated as 16 nM (Fig. 2a–c). During the observation period, all schistosomula were alive and active under culture conditions containing DMSO alone (data not shown).

3.3. Pathological changes in the liver in infected mice treated with N-89

The sizes of granulomas formed around single schistosome eggs in N-89 treated mice was compared to that in control animals. The liver pathology of the mice treated at 5 weeks postinfection showed significantly smaller granulomas compared with controls ($P<0.001$) (data not shown).

3.4. Morphological changes of N-89 treated adult worms

To observe morphological changes of the parasite after N-89 treatment *in vivo*, we compared morphological profiles of the adult worms with or without N-89 treatment. The most obvious difference was noted in the intestine of female worms on light microscopic observation. Briefly, the dense substances, probably hemozoin, disappeared in N-89-treated worms (Fig. 3a–d). Furthermore, the mean body length of the treated worms was smaller than that of untreated controls (Fig. 4a–f). On TEM observation, the tegument morphology was compared between parasites with and without N-89 treatment. In both males and females, there were no marked differences between N-89-treated worms and control worms (Fig. 5a–d). In the SEM profiles, we found small surface changes, such as the disappearance of tubercles on the surfaces of males and shortened spines on females, but these changes were not as severe as the findings of previous studies for PZQ and ADs [28,32] (Fig. 5e–h).

3.5. Heme contents of adult parasites with and without N-89 treatment

As hemoglobin is the main source of nutrition for adult female worms, we measured hemozoin contents of parasites with and without N-89 treatment to examine whether nutritional impairment occurred in N-89-treated parasites. In the N-89-treated group, the mean heme content was 15 nmol heme/mg protein, while it was 89 nmol heme/mg protein in the untreated controls; this difference in heme content was statistically significant ($P<0.001$) (Fig. 6).

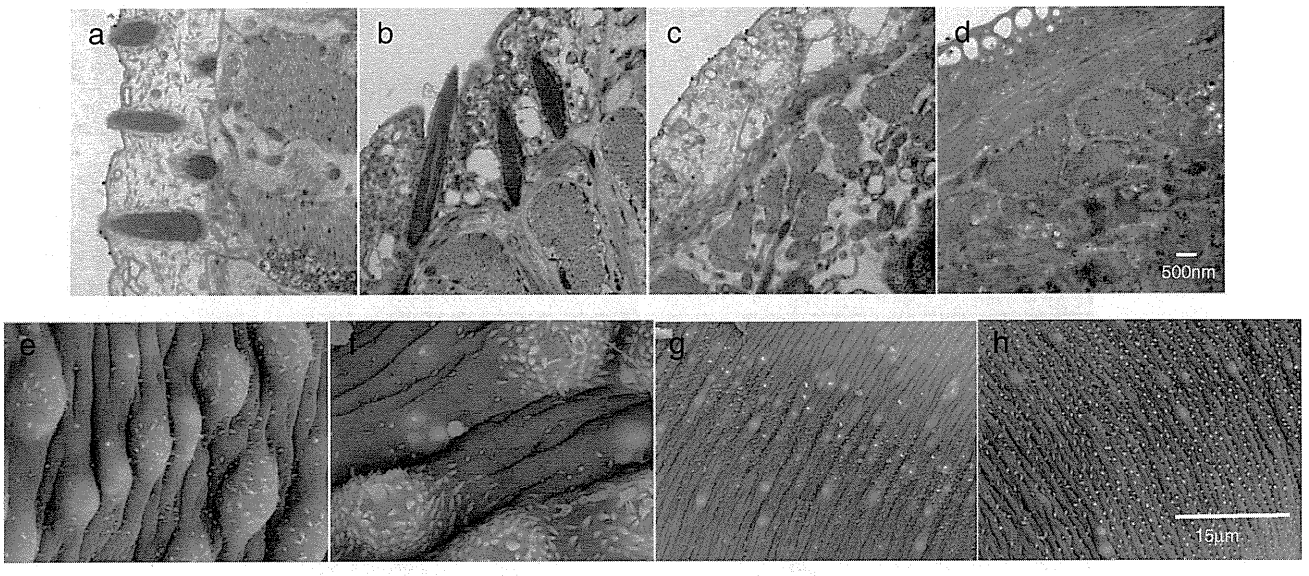

Fig. 5. EM observation of *S. mansoni* adult worms. All worms used were obtained in the same manner as described in Fig. 3. TEM observation of a male worm from mice treated with N-89 (a), a male worm from control mice (b), a female worm from mice treated with N-89 (c), and a female worm from control mice (d). SEM observation of a male worm from mice treated with N-89 (e), a male worm from control mice (f), a female worm from mice treated with N-89 (g), and a female worm from control mice (h).

4. Discussion

Rational drug design should be applied to develop new agents for use against schistosomiasis. As PZQ is the only drug available for controlling disease activity, the appearance of drug-resistant strains is a non-negligible concern. New drug candidates must be developed to address this concern, and ADs are promising candidates for this purpose. However, it should be noted that ADs are used for malaria therapy because of the recent WHO recommendation for use of artemisinin-based combination therapy (ACT). ADs are drugs prepared from plant materials. Due to their structural complexity, these compounds are not easy to chemically synthesize, and the distribution of the product depends on the supply of herbal plant materials. On the other hand, mass production of N-89 is not difficult, and it can be prepared at a much lower cost than ADs. No serious toxicity has been noted for N-89 in animal [23–25]. As N-89 is effective for reducing egg fecundity but not worm burden when it is administered 5 weeks post infection, it can supplement the effect of praziquantel that is effective for reducing worm burden.

The results of the present study suggest that N-89 is a novel antischistosomal compound with a unique mechanism of action compared to other drugs used to combat schistosomiasis, such as PZQ and ADs. Due to the structural similarity, we postulated that N-89 would have both antimalarial and antischistosomal effects in the same manner as observed for ADs. However, reference to previous publications regarding ADs indicated that there were marked differences in its anti-schistosomal effects. That is, N-89 showed two modes of antischisto-somal effect — larvicidal effects and antifecundity effects. Previous reports have indicated no such dual modes of drug efficacy for ADs [17]. Thus, it is possible that N-89 has functions distinct from those of ADs.

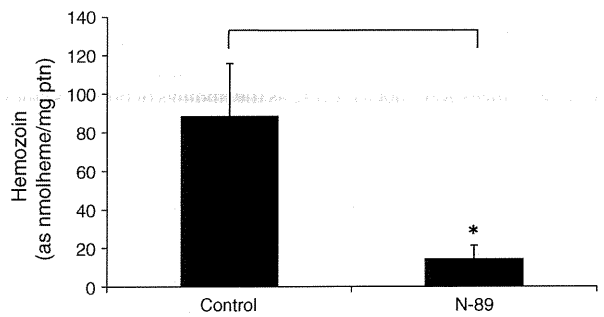

Fig. 6. Heme contents of adult parasites obtained after *in vivo* treatment with N-89.7-week worms collected in the same manner described in Fig. 3 were examined for quantification of hemozoin contents. Y-axis indicates the hemozoin contents (as nmol heme/mg protein) (*$P<0.001$).

It is still necessary to elucidate the detailed mechanisms of action for the two different effects of N-89. Considering the presence of endoperoxide structures in N-89, it is possible that oxygen stress generated by N-89 may be a factor involved in the schistosomicidal effects. Recent studies demonstrated the importance of the redox system for parasite survival [33,34]. However, no direct evidence in support of this possibility is available, nor killing effect of the worms was observed when *Sm*-infected mice were treated with N-89 at 5 week postinfection. In spite of this situation, we observed the reduction of egg fecundity. Morphological observations in the present study suggested that N-89 treatment induce nutritional deficits in the worms, as heme contents in N-89-treated female worms were significantly reduced compared to controls. This may be related to the antifecundity effect of the drug against female worms. It is well discussed that host hemoglobin derived from the host blood is essential for growth, development and reproduction of schistosomes [35,36]. It is possible that N-89 inhibits a process for hemoglobin usage in female worms, and more direct evidence may be obtained by testing the effects of N-89 on the biological pathways involved in hemoglobin uptake. It has been suggested that proteolysis of hemoglobin was important for worm development in male and female, and production of yolk protein in developing egg was also important for female worm [37]. The two modes of drug efficacy in N-89 raise questions regarding why the larval stages were destroyed, while the adult stage was resistant to this drug. In other cases, such as vaccine efficacy, lung stage parasites are the targets for the killing effects [7], although these are immune-mediated mechanisms. Analysis of the direct target molecules for N-89 could provide valuable information for the development of therapeutic strategies. Studies to elucidate these points using other approaches, such as proteomic analysis, are currently underway in our laboratory.

In conclusion, N-89 is a promising compound for use as an antischistosomal drug, which may supplement the effects of PZQ

through mutually different modes of efficacy. Strategies using N-89 as supplemental effect for praziquantel or ADs would be helpful to avoid the development of drug-resistance. Therefore, N-89 is a good candidate partner for its efficacy, safety, and its low cost of mass production.

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References

[1] Chitsulo L, Engels D, Montresor A, Savioli L. The global status of schistosomiasis and its control. Acta Trop 2000;77:41–51.
[2] Esrey SA, Potash JB, Roberts L, Shiff C. Effects of improved water supply and sanitation on ascariasis, diarrhoea, dracunculiasis, hookworm infection, schistosomiasis, and trachoma. Bull World Health Organ 1991;69:609–21.
[3] McCullough FS, Gayral P, Duncan J, Christie JD. Molluscicides in schistosomiasis control. Bull World Health Organ 1980;58:681–9.
[4] Fallon PG, Tao LF, Ismail MM, Bennett JL. Schistosome resistance to praziquantel: fact or artifact? Parasitol Today 1996;12:316–20.
[5] Liang YS, Coles GC, Doenhoff MJ, Southgate VR. In vitro responses of praziquantel-resistant and -susceptible *Schistosoma mansoni* to praziquantel. Int J Parasitol 2001;31:1227–35.
[6] Ismail M, Botros S, Metwally A, William S, Farghally A, Tao LF, et al. Resistance to praziquantel: direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers. Am J Trop Med Hyg 1999;60:932–5.
[7] Xiao S, Tanner M, N'Goran EK, Utzinger J, Chollet J, Bergquist R, et al. Recent investigations of artemether, a novel agent for the prevention of schistosomiasis japonica, mansoni and haematobia. Acta Trop 2002;82:175–81.
[8] Hamed MA, Hetta MH. Efficacy of citrus reticulata and mirazid in treatment of *Schistosoma mansoni*. Mem Inst Oswaldo Cruz 2005;100:771–8.
[9] Angelucci F, Sayed AA, Williams DL, Boumis G, Brunori M, Dimastrogiovanni D, et al. Inhibition of *Schistosoma mansoni* thioredoxin-glutathione reductase by auranofin: structural and kinetic aspects. J Biochem 2009;284:28977–85.
[10] Dhingra V, Rao KV, Narasu ML. Current status of artemisinin and its derivatives as antimalarial drugs. Life Sci 2000;66:279–300.
[11] Ridder S, Kooy F, Verpoorte R. *Artemisia annua* as a self-reliant treatment for malaria in developing counties. J Ethnopharmacol 2008;120:302–14.
[12] Krishna S, Bustamante L, Haynes RK, Staines HM. Artemisinins: their growing importance in medicine. Trends Pharmacol Sci 2008;29:520–7.
[13] Jonathan LV, Sarah AB, Reto B, Susan AC, Francis CKC, Jacques C, et al. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 2004;430:900–4.
[14] SH L, Kumagai T, Ai QH, Yan XL, Ohmae H, Yabu Y, et al. Evaluation of the anthelmintic effects of artesunate against experimental *Schistosoma mansoni* infection in mice using different treatment protocols. Parasitol Int 2006;55:63–8.
[15] Utzinger J, Xiao S, Keiser J, Chen M, Zheng J, Tanner M. Current progress in the development and use of artemether for chemoprophylaxis of major human schistosome parasites. Curr Med Chem 2001;8:1841–60.
[16] Xiao SH, Mei JY, Jiao PY. *Schistosoma japonicum*-infected hamsters (*Mesocricetus auratus*) used as a model in experimental chemotherapy with praziquantel, artemether, and OZ compounds. Parasitol Res 2011;108:431–7.
[17] Xiao SH. Development of antischistosomal drugs in China, with particular consideration to praziquantel and the artemisinins. Acta Trop 2005;96:153–67.
[18] Stelma FF, Talla I, Sow S, Kongs A, Niang M, Polman K, et al. Efficacy and side effects of praziquantel in an endemic focus of *Schistosoma mansoni*. Am J Trop Med Hyg 1995;53:167–70.
[19] Goennert R, Andrews P. Praziquantel, a new broad-spectrum antischistosomal agent. Z Parasitenk 1977;52:129–50.
[20] Xiao SH, Jennifer K, Jacques C, Juorg U, Yuxiang D, Yvette E, et al. *In vitro* and *in vivo* activities of synthetic trioxolanes against major human schistosome species. Antimicrob Agents Chemother 2007;51:1440–5.
[21] Xiao SH, Utzinger J, Chollet J, Tanner M. Effect of artemether administered alone or in combination with praziquantel to mice infected with *Plasmodium berghei* or *Schistosoma mansoni* or both. Int J Parasitol 2006;36:957–64.
[22] Inyang-Etoh PC, Ejezie GC, Useh MF, Inyang-Etoh EC. Efficacy of a combination of praziquantel and artesunate in the treatment of urinary schistosomiasis in Nigeria. Trans R Soc Trop Med Hyg 2009;103:38–44.
[23] Aly NS, Hiramoto A, Sanai H, Hiraoka O, Hiramoto K, Kataoka H, et al. Proteome analysis of new antimalarial endoperoxide against *Plasmodium falciparum*. Parasitol Res 2007;100:1119–24.
[24] Kim HS, Nagai Y, Ono K, Begum K, Wataya Y, Hamada Y, et al. Synthesis and antimalarial activity of novel medium-sized 1,2,4,5-tetraoxacycloalkanes. J Med Chem 2001;44:2357–61.
[25] Kim HS, Begum K, Ogura N, Wataya Y, Nonami Y, Ito T, et al. Antimalarial activity of novel 1,2,5,6-tetraoxacycloalkanes and 1,2,5-trioxacycloalkanes. J Med Chem 2003;46:1957–61.
[26] Sabah AA, Fletcher C, Webbe G, Doenhoff MJ. *Schistosoma mansoni*: chemotherapy of infections of different ages. Exp Parasitol 1986;61:294–303.
[27] Wang W, Itoh S, Matsuda A, Ichinose S, Shinomiya K, Hata Y, et al. Influences of mechanical properties and permeability on chitosan nano/microfiber mesh tubes as a scaffold for nerve regeneration. J Biomed Mater Res A 2008;84:557–66.
[28] Shaw MK, Erasmus DA. *Schistosoma mansoni*: structural damage and tegumental repair after in vivo treatment with praziquantel. Parasitology 1987;94:243–54.
[29] Oliveir MF, d' Avila JCP, Torres CR, Oliveira PL, Tempone AJ, Rumjanek FD, et al. Haemozoin in *Schistosoma mansoni*. Mol Biochem Parasitol 2000;111:217–21.
[30] Oliveira MF, d' Avila JC, Tempone AJ, Soares JB, Rumjanek FD, Fereira-P A, et al. Inhibition of heme aggregation by chloroquine reduces *Schistosoma mansoni* infection. J Infect Dis 2004;190:843–52.
[31] Correa Soares JB, Menezes D, Vannier-Santos MA, Ferreira-Perreira A, Almeida GT, Venancio TM, et al. Interference with hemozoin formation represents an important mechanism of schistosomicidal action of antimalarial quinoline methanols. PLoS Negl Trop Dis 2009;3:e477.
[32] Xiao S, Shen B, Chollet J, Utzinger J, Tanner M. Tegumental changes in adult *Schistosoma mansoni* harbored in mice treated with artemether. J Parasitol 2000;86:1125–32.
[33] Kumagai T, Osada Y, Kanazawa T. 2-Cys peroxiredoxins from *Schistosoma japonicum*: the expression profile and localization in the life cycle. Mol Biochem Parasitol 2006;149:135–43.
[34] Kumagai T, Osada Y, Ohta N, Kanazawa T. Peroxiredoxin-1 from *Schistosoma japonicum* functions as a scavenger against hydrogen peroxide but not nitric oxide. Mol Biochem Parasitol 2009;164:26–31.
[35] Zussman RA, Bauman PM, Petruska JC. The role of ingested hemoglobin in the nutrition of *Schistosoma mansoni*. J Parasitol 1970;56:75–9.
[36] Lawrence JD. The ingestion of red blood cells by *Schistosoma mansoni*. J Parasitol 1973;59:60–3.
[37] Sauer MCV, Senft AW. Properties of a proteolytic enzyme from *Schistosoma mansoni*. Comp Biochem Physiol 1972;42B:205–20.