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Potent Antimalarial Activities of Polyether Antibiotic, X-206

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In the course of our screening program to discover antimalarial antibiotics, which are active against drug resistant *Plasmodium falciparum in vitro* and rodents infected with *P. berghei in vivo*, from the culture broth of microorganisms, we found a selective and potent active substance produced by an actinomycete strain K99-0413. It was identified as a known polyether antibiotic, X-206. We also compared the *in vitro* antimalarial activities and cytotoxicities of 12 known polyethers with X-206. Among them, X-206 showed the most selective and potent inhibitory effect against both drug resistant and sensitive strains of *P. falciparum*. Comparison of biological activities and ion-affinities of the above antibiotics suggests that monovalent cations play an important biological role for the intracellular growth of *P. falciparum* in parasitized erythrocytes. Moreover, X-206 showed potent *in vivo* antimalarial activity on the rodent model, though the therapeutic window was narrow compared with its selective toxicity *in vitro*. These observations are the first report of antimalarial activity of X-206.

Malaria has had a resurgence in many tropical areas. This disease now occurs in more than 90 countries worldwide, and it is estimated there are over 500 million clinical cases and 2.7 million malaria-caused deaths per year[1]. Furthermore, the spread of *Plasmodium falciparum* strains resistant to antimalarials such as chloroquine indicates that new antimalarials having novel structures and mechanisms of action are needed for chemotherapeutic control of this disease. In the course of our screening program to discover antimalarial antibiotics, which are active against drug resistant parasites *in vitro* and *in vivo* from soil microorganisms, an actinomycete strain K99-0413 was found to produce a known polyether antibiotic, X-206 (Fig. 1)[2]. 4~5 years ago, GUMILA *et al.* reported that 22 ionophore compounds including 17 polyether antibiotics showed *in vitro* antimalarial activities against drug sensitive strain of *P. falciparum* and 7 polyether antibiotics among them showed *in vivo* antimalarial activities[3,4].

Here, we describe the results of the sensitivity tests of the K1 and FCR3 strains against known antimalarial drugs under our *in vitro* assay condition, the antimalarial activities and cytotoxicities of 13 polyether antibiotics including X-206 *in vitro*, and the evaluation of X-206 on the rodent malaria model *in vivo*.

Material and Method

Chemicals

X-206 was purified in our Institute from the culture broth of an actinomycete strain K99-0413 as shown below. Culture mycelium of strain K99-0413 was extracted with acetone and concentrated *in vacuo* to remove acetone. The resulting aqueous solution was further extracted with *n*-hexane. The broth filtrate extracted with *n*-hexane was combined with the mycelial extract and concentrated. The combined extract was chromatographed on a silica gel column, and X-206 was eluted with $CHCl_3$-MeOH (100 : 1). Octacyclomycin was purified from the culture broth of a *Streptomyces* sp. No. 82-85[5]. Artemether and artesunate were generous gifts of WHO/TDR. Dianemycin and lysocellin were obtained from the antibiotic library of Research Center for Biological Function of our Institute. RPMI-1640 (with glutamine) and MEM media were

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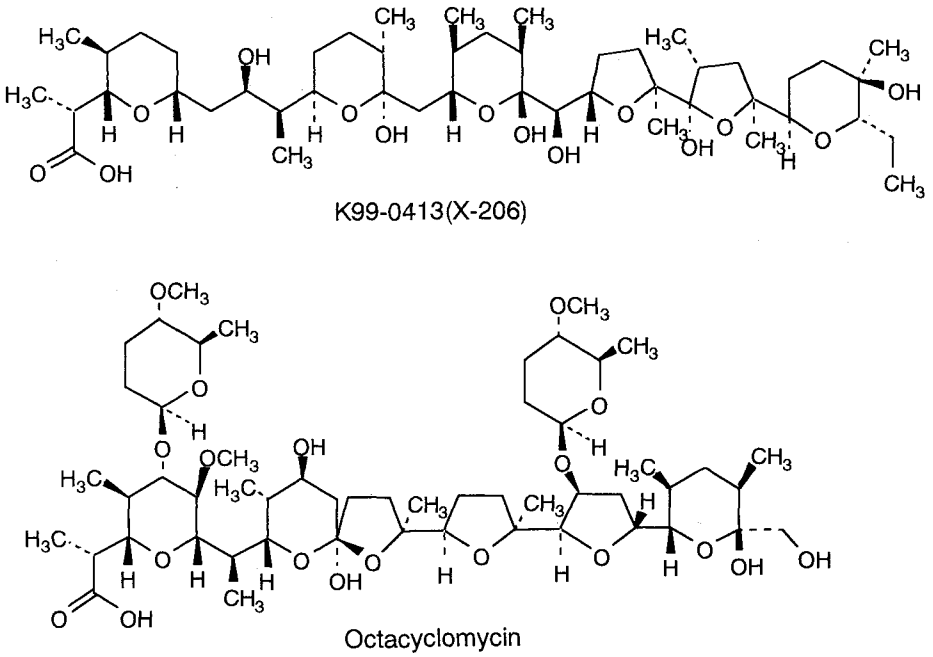
Fig. 1. Structures of X-206 and octacyclomycin.



obtained from Life Technologies (Gland Island, N. Y. USA). Fetal Calf Serum (FCS) was obtained from Hyclone Laboratories Inc. (Logan, Utah USA). Malstat reagent was obtained from Flow Inc. (Portland, OR. USA). MTT reagent was obtained from Research Organics Inc. (Cleveland, OH. USA). Other chemicals were commercially available and analytical grades.

In Vitro Anti-malarial Assay against *P. falciparum*

Type A^+ human plasma and erythrocytes were obtained from healthy volunteers in the Research Center for Clinical Pharmacology of our Institute. *P. falciparum* strains K1 (drug resistant) and FCR3 (drug sensitive) were generous gifts of Prof. K. KITA (The University of Tokyo). The parasites were grown based on a method described by TRAGER and JENSEN[6]. *P. falciparum* strains were cultured in the human erythrocytes in RPMI medium (RPMI-1640 with 25 mM HEPES buffer, 24 mM $NaHCO_3$, 0.2% glucose, 0.05% L-glutamine, 50 μg/ml hypoxanthine, and 25 μg/ml gentamicin) supplemented with 10% human plasma at 37°C, under 93% N_2, 4% CO_2, and 3% O_2. Anti-malarial activity of the test compound have been achieved by dose response curve using the parasite lactate dehydrogenase (pLDH) assay according to the method of MAKLER *et al.*[7]. One hundred ninety μl of asynchronous parasites (2.0% hematocrit and 0.5 or 1% parasitaemia) was seeded in a 96-well microplate, and 10 μl of a test compound solution (dissolved in 25% ethanol or 5% dimethylsulfoxide: DMSO) was added. After incubation at 37°C for 72 hours under 93% N_2, 4% CO_2, and 3% O_2, the plate was immediately frozen at −20°C for 18 hours. The plate was then thawed at 37°C, and 20 μl of the haemolyzed parasite suspension was transferred to another plate containing 100 μl of Malstat reagent. The plate was further incubated for 15 minutes at room temperature, and 20 μl of a 1:1 mixture of nitroblue tetrazolium and phenazine ethosulfate (2 mg and 0.1 mg/ml respectively) was added to each well. After incubation for 2 hours at room temperature in the dark condition, the blue formazan product was measured at 655 nm by an iEMS microplate reader MF (Labosystems, Helsinki, Finland). The 50% inhibitory concentration (IC_{50}) value was estimated from a dose response curve.

Cytotoxicity Tests on MRC-5 Cells

A human diploid embryonic cell line, MRC-5 was a generous gift of Dr. L. MAES (Tibotec NV, Mechelen, Belgium). The cytotoxicity of the test compound was measured by the colorimetric MTT assay[8,9] in 96-well microplates. In brief, 100 μl of MRC-5 cell suspension was added in 96-well microplates at 1×10^3 cells/well, and cultivated for 24 hours. Then 90 μl of standard culture medium (MEM+10% FCS) with or without 10 μl of test

compound solutions, which were dissolved in 25% ethanol or 5% DMSO were added to each well. The cultures were further incubated at 37°C under 5 % CO_2-95% air for 7 days, and 20 μl of MTT-PBS solution (5 mg/ml) was added to each well. The plate was then incubated at 37°C for 4 hours under 5% CO_2-95% air. Then the incubation medium was aspirated, and 100 μl of DMSO was added to solubilise the MTT formazan product. After mixing, absorbance at 540 nm was measured with an iEMS microplate reader MF. The 50% inhibitory concentration (IC_{50}) value was estimated from a dose response curve.

In Vivo Anti-malarial Assay with *P. berghei*

P. berghei strain N was a generous gift of Dr. W. PETERS (Northwick Park Institute for Medical Research, Middlesex, UK). *In vivo* anti-malarial activity was determined against rodent malaria-derived *P. berghei* strain N according to the 4-days suppressive test of PETERS *et al.*[10]. Male CD-1 (ICR) mice (Charles River Japan Inc., Japan) at weight of 18~20 g were inoculated with 10^6 parasitized red blood cells intravenously. Test compounds were dissolved in 10% DMSO solution and subcutaneously (s.c.) injected to the mice two hours after the infection (day 0). Test compounds were successively injected (s.c.) to the mice once a day for 3 consecutive days (Days 1~3). Five mice were tested at each dosage, and another 5 infected mice were injected with 10% DMSO-water as a control. The day after the last treatment (Day 4), thin blood films were made from the tail blood of the infected mice, and the parasitaemia was determined. The 50% effective dose (ED_{50}) was estimated from a dose response curve. The values were tested for statistical significance by Dunnett protocol.

Results and Discussion

Sensitivity of Plasmodial Strains against Antimalarial Drugs

The K1 strain which is known to be resistant to choloroquine, quinine and pyrimethamine. On the other hand, the FCR3 strain which is known to be susceptible to choloroquine, quinine and pyrimethamine. First, we studied the sensitivity of the K1 and FCR3 strains against known antimalarial drugs under our *in vitro* assay condition. The effects of 8 clinically used antimalarials on the drug resistant K1 strain and the drug sensitive FCR3 strain of *P. falciparum* were studied and their 50% inhibitory concentrations (IC_{50}s) were determined as shown in Table 1. Among the tested compounds, the derivatives from artemisinin, artemether and artesunate showed the most potent antimalarial activity against both strains. The drug effects of chloroquine, pyrimethamine, and trimethoprim against the K1 strain were lower about 12 fold, >12,820 fold and >714 fold, respectively, than those against the FCR3 strain. So the K1 strain exhibited resistance to chloroquine and also to pyrimethamine and trimethoprim, which are inhibitors of the parasite dihydrofolate reductase.

Table 1. Antimalarial activities of antimalarial drugs against FCR3 and K1 strains of *Plasmodium falciparum*.

	IC_{50} (nM)	
	FCR3 strain	K1 strain
Amodiaquine	14	41
Artemether	2.2	7.6
Artemisinin	18	24
Artesunate	2.7	11
Chloroquine	29	357
Primaquine	3,300	2,633
Pyrimethamine	7.8	>100,000
Quinine	600	780
Trimethoprim	140	>100,000

In Vitro Antimalarial Activities and Cytotoxicities of Polyether Antibiotics

The effects of 13 polyether antibiotics including K99-0413 (X-206) on *in vitro* antimalarial activity were evaluated by using the drug resistant K1 strain and the drug sensitive FCR3 strain of *P. falciparum*. Their IC_{50} values are listed in Table 2. All 13 compounds possessed antimalarial activities against both strains, with the IC_{50} values ranging between 0.15~587 nM and between 0.51~1,203 nM for the K1 strain and the FCR3 strain, respectively. Among the tested compounds, X-206 showed the highest antimalarial activities with the IC_{50} values of 0.15 nM and 0.51 nM for the K1 strain and the FCR3 strain, respectively. The antimalarial activity of X-206 on the K1 strain was 50 times more potent than that of artemether.

We then investigated the cytotoxicities of the polyether antibiotics against a human diploid embryonic cell line MRC-5 to assess the cytotoxicity for host cells. Their IC_{50} values are also listed in Table 2. All the tested compounds possessed cytotoxicity against MRC-5 cells with the IC_{50} values ranging between 6.6~1,648 nM. Among them, monensin showed the highest cytotoxicity with the IC_{50} value of 6.6 nM. Whereas, lasalocid A, octacyclomycin, and

Table 2. Antimalarial activities and cytotoxicities of polyether antibiotics.

Compound	Antimalarial activity[A] IC_{50} (nM) for K1*	FCR3**	Cytotoxicity[B] IC_{50} (nM) for MRC-5 cells	Selectivity (B/A) K1	FCR3	Ion-affinity***
Class 1						
K99-0413(X-206)	0.15	0.51	551	3,673	1,080	$K^+ > Na^+$
Lonomycin A	2.2	13	94	43	7.2	$K^+ > Na^+$
Nigericin	2.7	19	100	37	5.3	$K^+ > Na^+$
Narasin	1.0	1.6	65	65	40	$K^+ > Na^+$
Salinomycin	1.4	1.4	104	74	74	$K^+ > Na^+$
Dianemycin	1.2	8.5	41	34	4.8	$Na^+ > K^+$
Monensin	0.9	0.9	6.6	7.3	7.3	$Na^+ > K^+$
Class 2						
Lysocellin	6.4	5.4	127	20	24	$K^+ > Na^+ > Mg^{2+}$ Ca^{2+}
Lasalocid A	65	29	1,648	25	57	$K^+ > Na^+$, $Ca^{2+} > Mg^{2+}$
Class 3						
A-23187	115	1,203	210	1.8	0.2	$Ca^{2+} > Mg^{2+}$
Indanomycin	587	567	506	0.9	0.9	$Ca^{2+} > Mg^{2+}$
Ionomycin	455	321	428	0.9	1.3	$Ca^{2+} > Mg^{2+}$
Unkown class						
Octacyclomycin	39	3.0	1,112	29	371	ND****

* drug resistance strain, ** drug sensitive strain, *** determined by PRESSMAN[12], WESTLEY[13] and BOLTE *et al.*[14], **** not determined.

X-206 showed lower cytotoxicities than the others with the IC_{50} values of 1,648 nM, 1,112 nM, and 551 nM, respectively. To compare the antimalarial activities and cytotoxicities, we introduced the selectivity indexes (cytotoxicity [IC_{50} for the MRC-5 cells]/antimalarial activity [IC_{50} for the K1 strain or the FCR3 strain]) as listed in Table 2. Among the tested compounds, X-206 showed the highest selectivity indexes with the ratios of 3,673 and 1,080 for the MCR-5 cells/K1 strain and the MCR-5 cells/FCR3 strain, respectively. Octacyclomycin also showed a rather high selectivity index with the ratio of 371 for the MCR-5 cells/FCR3 strain. The other compounds showed the indexes lower than 100.

Ionophore compounds including the polyether antibiotics have been reported to be potential antimalarial agents by GUMILA *et al.*[3,4] and by ADOVELANDE and SCHRÉVEL[11]. GUMILA *et al.* have reported *in vitro* antimalarial activities using drug sensitive strain of 22 ionophores including 17 polyether antibiotics, and they also evaluated the activity by the selectivity indexes[3]. Here, we studied antimalarial activities of 13 polyether antibiotics including 4 compounds (X-206, dianemycin, indanomycin, and octacyclomycin) that had not been tested by GUMILA *et al.* using both drug resistant and sensitive strains. Among them, X-206 showed the highest selectivity indexes for both strains, and octacyclomycin also showed good selectivity for the drug sensitive strain.

Relationship among Antimalarial Activities, Cytotoxicities and Ion-affinities of Polyether Antibiotics

GUMILA *et al.* suggested that the different ion-affinities of ionophores with cations reveal the different antimalarial activities of ionophores, and indicated that the ionophores specific to monovalent cations showed the most or more potent activity against *Plasmodium*[3]. We classified 12 compounds except octacyclomycin by their ion-affinities according to the results of PRESSMAN[12], WESTLEY[13], and BOLTE *et al.*[14] as shown in Table 2. Class 1 polyether antibiotics, which are specific to monovalent cations, had the highest antimalarial activities ($IC_{50} \leq 2.7$ nM for the K1 strain). Class 2 polyether antibiotics, which are able to complex with both mono and divalent cations, had moderate antimalarial activities ($IC_{50} \leq 65$ nM for the K1 strain). Class 3 polyether antibiotics, which are specific to divalent cations, had weak antimalarial activities ($IC_{50} \leq$ 587 nM for the K1 strain).

As for the selectivity indexes of the MRC-5 cells/K1 strain, class 1 polyether antibiotics except monensin showed good values ($\geq$34). Class 2 polyether antibiotics showed moderate indexes (20~25). Class 3 polyether

Table 3. *In vivo* antimalarial activities of X-206, artemether and artesunate against *P. berghei* strain N.

Compound	Route	ED_{50}(mg/kg)
X-206*	s.c.	0.53
Artemether	s.c.	1.5
Artesunate	s.c.	1.7

*toxic dose=3mg/kg

antibiotics showed the lowest selectivity indexes (0.9~1.8). The present results including the selectivity indexes of the MCR-5 cells/FCR3 strain were similar to the results of GUMIIA *et al.*[3]. Therefore, the monovalent cations may also play the important biological role for the intracellular growth of drug resistant *P. falciparum* in parasitized erythrocytes.

The complexation affinity for various cations of octacyclomycin had not been measured and therefore classified to the unknown class. Octacyclomycin is a diglycoside polyether antibiotic, and structurally similar to the monoglycoside polyether antibiotics, etheromycin and antibiotic-6016. Because etheromycin is specific for monovalent cations[15], and the antibiotic-6016 is able to complex with both mono and divalent cations[16], octacyclomycin may be classified to class 1 or 2 from the results of its antimalarial activity and cytotoxicity.

In Vivo Antimalarial Activities of X-206, Artemether and Artesunate in Mice

The subcutaneously administration of X-206 and the clinically used compounds, artemether and artesunate, were evaluated by using the *P. berghei* strain N (drug sensitive strain) infected mice. Their ED_{50} values are listed in Table 3. X-206 showed the highest *in vivo* antimalarial activities among the tested compounds, but X-206 was toxic beyond 3 mg/kg (data not shown). The acute toxicity (LD_{50}, s.c.) of X-206 for mice was 11 mg/kg, which has been reported by BERGER, *et al.*[2]. Therefore X-206 had a narrow therapeutic window in the rodent malaria model though it showed selective and potent antimalarial effect *in vitro*.

In vivo antimalarial activities of polyether antibiotics have been reported by RAETHER *et al.*[17], SCHILDKNECHT *et al.*[18], GUMILA *et al.*[4], and ADOVELANDE and SCHRÉVEL[11] using different test conditions. Salinomycin and lasalocid A were shown to be inactive or weakly active against the drug resistant strain of *P. berghei* in rats after oral or subcutaneous administration[17]. Three synthetic monensin urethane derivatives were shown to be moderately active against *P. berghei* in mice after oral administration[18]. Lonomycin, nigericin, narasin, monensin A, monensin methyl ether, lasalocid A, and 5-bromo lasalocid A were shown to be very active against *P. vinckei petteri* or *P. chabaudi* in mice after oral or intraperitoneal administration[4]. Recently, ADOVELANDE and SCHRÉVEL reported that the combination of nigericin and monensin using the lower doses showed a synergistic effect on the rodent malaria model *in vivo*[11]. These observations suggest the possibility of using X-206 in combination therapy for malaria chemotherapy.

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