Parasitol Res
DOI 10.1007/s00436-007-0815-1

ORIGINAL PAPER

Trypanocidal activity of extracts and compounds from the stem bark of *Anogeissus leiocarpus* and *Terminalia avicennoides*

Mohammed N. Shuaibu · Ponchang T. A. Wuyep · Tetsuo Yanagi · Kenji Hirayama · Akitoyo Ichinose · Takashi Tanaka · Isao Kouno

Received: 8 November 2007 / Accepted: 15 November 2007

Abstract The antitrypanosomal activity of methanolic extracts of *Anogeissus leiocarpus* and *Terminalia avicennoides* were evaluated in vitro against four strains of *Trypanosoma* species with minimum inhibitory concentration (MIC) value range of 12.5–50 mg/ml. Successive fractionations of the two plant extracts in water, butanol and ethyl acetate gave a range of activity (MIC, 20 to ≥50 μg/ml). Activity-guided and chromatographic analysis of butanolic fractions on Sephadex LH-20 column followed by high-performance liquid chromatography, nuclear magnetic resonance analysis and both ultraviolet and thin layer chromatography revealed hydrolysable tannins with a range of activity (MIC, 7.5–27.5 μg/ml or 14–91 μM). Effect of the compounds on fibroblasts did not reveal serious toxicity at moderate concentration but is concentration dependent.

M. N. Shuaibu · T. Tanaka · I. Kouno
Natural Product Chemistry,
Graduate School of Biomedical Sciences,
Nagasaki University,
Nagasaki-shi, Japan

M. N. Shuaibu (✉) · K. Hirayama
Department of Immunogenetics, Institute of Tropical Medicine,
Nagasaki University,
1-12-4 Sakamoto,
Nagasaki-shi 852-8523, Japan
e-mail: nshuaibu@yahoo.com

T. Yanagi
Animal Research Centre for Tropical Infections,
Institute of Tropical Medicine, Nagasaki University,
1-12-4 Sakamoto,
Nagasaki-shi 852-8523, Japan

A. Ichinose
Electron Microscopy Central Laboratory,
Institute of Tropical Medicine, Nagasaki University,
1-12-4 Sakamoto,
Nagasaki-shi 852-8523, Japan

P. T. A. Wuyep
Department of Biological Sciences, Ahmadu Bello University,
Zaria, Nigeria

Introduction

African trypanosomiasis also known as sleeping sickness is caused by *Trypanosoma* transmitted by tsetse flies. It is a debilitating disease whose major burden is on the rural poor in the endemic sub-Saharan Africa (Welburn et al. 2006). In human, the disease takes two forms, chronic Gambian sleeping sickness caused by *Trypanosoma brucei gambiense* and acute Rhodesian sleeping sickness caused by *Trypanosoma brucei rhodesiense* with over 50 million people at risk of acquiring the infection living in and around the 200 tsetse flies foci in the 37 endemic African countries (WHO 1998).

The current available chemotherapies for these parasitic diseases are far from satisfactory. The available drugs against these diseases are limited and most have been in use for more than 50 years. Limited efficacy, drug resistance, cost and toxic side effects are the main drawbacks of most of the drugs. Moreover, no vaccine is available yet against any of these diseases, which makes chemotherapy the only available option to control the infections. The nature that frequently unleashes a barrage of new and frightening diseases against humans also provides the wherewithal to help conquer the diseases that it sets loose (Crump 2006). Traditional medicine has long focused on the power of natural products to treat and cure diseases. In Africa, just like other parts of the world, the extensive use of plants in folk medicine has been documented. In general, the influence of natural products upon drug discovery is

impressive and a number of clinically active drugs are either natural products or have a natural product pharmacophore (Koehn and Carter 2005). The need to study medicinal plants in detail from various points of view, to discover new therapeutically active compounds, therefore becomes imperative. The present study, therefore, discusses the in vitro anti-trypanosomal activity of methanolic extracts of *Anogeissus leiocarpus* and *Terminalia avicennoides* and investigates the activity of the successive solvent fractions and the identified compounds on four strains of *Trypanosoma* species.

Materials and methods

Plant extracts preparation, fractionation and isolation

Air-dried, powdered stem bark (1.2 kg each) of *T. avicennoides* and of *A. leiocarpus* were macerated in methanol at room temperature for 24–48 h. The methanolic crude extract yield (162.13 and 114.1 g) for *T. avicennoides* and *A. leiocarpus*, respectively, was suspended in water and successively partitioned with butanol and ethyl acetate. The insoluble precipitate formed during the solvent partitioning was dissolved in dimethyl sulfoxide (DMSO). Five grams of the butanolic fraction was chromatographed on the Sephadex LH-20 column with water containing increasing proportions of methanol (0–100%, 20% stepwise elution) to give six fractions. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis indicated that fraction (fr.) 1 (0.74 g, 15%) contained sugars and triterpene glycosides; fr. 2 (0.63 g, 13%) was further separated by silica gel column chromatography to give two compounds. Nuclear magnetic resonance (NMR) spectral analysis revealed that one was ellagic acid rhamnoside and the other was triterpene glycoside; however, further structural elucidations were not completed. Fr. 3 (0.31 g, 6%) was a mixture of tannins and phenol carboxylic acids. Fr. 4 (0.40 g, 8%) was a mixture of flavogallonic acid bislactone (co-TLC, HPLC) and punicalagin (HPLC) and fr. 5 (1.30 g, 26%) contained ellagic acid (TLC, HPLC) and tannins detected as a broad hump of the baseline on HPLC analysis. The major constituent of fr. 6 (0.69 g, 13.8%) was found to be terchebulin by NMR spectral comparison and unknown tannins and phenol carboxylic acid were also detected (Tanaka et al. 1986a, b; Lin et al. 1990), while the butanolic fraction of *A. leiocarpus* revealed castalagin as the major compound along with ellagic and flavogallonic acids (Tanaka et al. 1996).

Spectral analysis

^{1}H and ^{13}C NMR, ^{1}H–^{1}H correlated spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear single quantum coherence and heteronuclear multiple bond correlation spectra were recorded with a Unity *plus* 500 spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra were also measured using a JEOL JMN-AL400 (JEOL, Tokyo, Japan) operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Fast atom bombardment mass spectra were recorded on a JMS-700 N spectrometer (JEOL, Tokyo, Japan), and *m*-nitrobenzyl alcohol or glycerol was used as the matrix.

Chromatography and HPLC analysis

Column chromatography was conducted on Sephadex LH-20 (Pharmacia Fine Chemicals). TLC was performed on 0.2-mm-thick precoated Kieselgel 60 F_{254} plates (Merck) with benzene/ethyl formate/HCO_2H (1:7:1, *v/v*) with spots detected by ultraviolet (UV) illumination, sprayed with 2% ethanolic $FeCl_3$ or 10% H_2SO_4 reagent. Analytical HPLC was performed on a Cosmosil $5C_{18}$-AR II column (Nacalai Tesque; 250×4.6-mm inner diameter), with gradient elution at 10–30% (30 min) and 30–75% (15 min) of CH_3CN in 50 mM H_3PO_4 (flow rate, 0.8 ml/min; detection, JASCO photodiode array detector MD-910).

Assay for trypanocidal activities

The methanolic extracts and the isolated compounds were dissolved in DMSO at a stock solution of 5 or 10 mg/ml and diluted to concentrations ranging from 1.0 to 1,000 μg/ml. Different concentrations were assessed for trypanocidal activities in either 24- or 48-well plates in a 24–48-h time course. Minimum inhibitory concentrations (MICs) were each determined under inverted microscope for the different parasites. *Trypanosoma brucei brucei* GUTat3.1, *T. b. gambiense* Wellcome strain, *T. b. rhodesiense* IL2343 and *Trypanosoma evansi* bloodstream form trypomastigotes at a density of 7.5×10^{4} were maintained in HMI-9 medium (Hirumi and Hirumi 1994) supplemented with 10–15% heat-inactivated fetal calf serum (HyClone) at 37°C in a 5% CO_2–air mixture. HMI-9 is a bloodstream form of African trypanosome growth supporting medium which consists of Iscove's modified Dulbecco's medium supplemented with fetal bovine serum and all other growth supporting factors.

Different concentrations of the extracts were added and the growth and viability of the cells were monitored microscopically and by counting using haemocytometer. Drug sensitivity was expressed relative to the growth of control cells. The MICs were then determined. MIC is expressed as the lowest concentration of compound at which no trypanosome with normal morphology or motility can be found compared to control cultures after 24-h incubation.

Morphological studies

Light microscopy Giemsa staining and electron microscopy were used to study the morphological changes in the parasites provoked by the compounds. Parasites were cultured in 24- or 48-well culture plates in the presence and absence of different concentrations of the compounds for 24 h. Smears of the parasites were prepared 24 h after exposure to the compounds on glass slides, air-dried, fixed in methanol and finally stained with 5% Giemsa for 30 min. Slides were then washed with tap water, dried and observed under a microscope.

Scanning electron microscopy Parasites were incubated with different concentrations of extracts or compounds for 6–24 h. Aliquots of the parasite-containing medium were pipetted on to a 1-cm^2 glass slide coated with poly-*L*-lysine and fixed for 15 min. The slides were then transferred into a 0.2-M cacodylate buffer, pH 7.4. Thereafter, the samples were then dehydrated in a graded ethanol series (30% to absolute) and then isopentenyl acetate. The samples were then critically dried in liquid CO_2 and finally coated with Au–Pd in an ion sputter, kept overnight and viewed on JSM-840 AN scanning microscope.

In vitro toxicity

Toxicity toward newborn mouse brain-derived cells (NBMH) was assessed with cells plated in 48-well plates at 10^5 cells per well. Stock cell cultures were maintained in 25-cm^3 flasks and subcultured to the appropriate split ratio by mild trypsinisation once in 7–10 days. The cells were allowed to settle and start confluence formation for 24–48 h. Microscopically, the fibroblasts had a normal appearance and showed normal cell growth rates. The concentration of a compound which provoked a >75% reduction in cell viability compared to the control cells after 24-h incubation was considered as MIC for the cells. After adherence, the medium was removed and replaced by media containing the different concentrations of the extracts. The plates were incubated for 24–96 h at 37°C in a humidified 5% CO_2 incubator. Control cells were incubated with culture medium alone and with solvent, DMSO, at a final concentration of less than 4%. The wells were assessed microscopically for cell growth and MIC was determined. A week after incubation with the different extracts, the medium was replaced with a fresh one and cell growth was monitored. Selectivity indices were calculated by dividing the MIC values of the NBMH versus the MIC values for the parasites.

Results and discussion

Studies have shown that plants are used in traditional medicine in Africa to treat trypanosomiasis in humans and animals (Akah et al. 1998; Atawodi et al. 2002; Adamu et al. 2005). Some of these plants have been evaluated for trypanocidal activity but few were phytochemically validated vis-à-vis their active compounds. We evaluated the in vitro effect of methanolic extracts of *A. leiocarpus* and *T. avicennoides*, their active butanolic fractions and the isolated compounds with MIC values ranging from 25 to 50 μg/ml, ≤12.5 to 17.5 μg/m and 7.5 to 31.5 μg/ml (14 to 91 μM), respectively, against four strains African *Trypanosoma* (Tables 1 and 2).

The promising result displayed by the extracts of these plants and subsequent successive fractionations in butanol, ethyl acetate and water deserved further analysis. Phytochemical analysis of the extracts showed aqueous and butanolic fractions to exhibit better activity. Although substantial amount of phytochemical research has been carried out on these plants, there are little or no reports on the effect of their hydrolysable tannins on parasites in vitro. In some reports (Vonthron-Sénécheau et al. 2003) tannins are usually removed because of the general belief that they are nonselective inhibitors of enzymes. However, in this report and like that of Asres et al. (2001), we did not remove the tannins. This is because the traditional practitioner's prescription on local consumption of these extracts does not exclude the tannins. We followed the fractions with a better activity and identified hydrolysable tannins to display some trypanocidal effect. These fractions showed

Table 1 Trypanocidal activity of fractions from *A. leiocarpus* and *T. avicennoides* against four strains of *Trypanosoma*

Plant species	Minimum inhibitory concentrations (μg/ml)					
	Fraction	Tbb	Tbg	Tbr	Te	SI
A. leiocarpus	MeOH	37.5	50.0	37.5	25.0	30–60
	EtOAc	≤50	≥50.0	>25	37.5	30–60
	BuOH	<<25	<<25.0	20	<<25.0	60–75
	Water	≥25	≥25.0	25	≥25.0	60
	DMSO	>50	≤25.0	25	<25.0	30–60
T. avicennoides	MeOH	12.5	17.5	12.5	12.5	86–120
	EtOAc	37.5	25.0	25	≤50	30–60
	BuOH	≤50	≤50	25	≤50	30–60
	Water	<25	<<25.0	20	<<25.0	60–75
	DMSO	≤25	≥25	20	≤50	30–75

Table shows the MIC values of the different fractions of *A. leiocarpus and T. avicennoides* against blood stream forms of four different species of *Trypanosoma*. Each experiment was carried out in duplicate and repeated at least two or three times and averages were taken and reported. All MIC values for the fractions against NBMH were above ≥1,500 μg/ml.

NBMH Newborn mouse heart fibroblast, *SI* selectivity index—the ratio of MIC of NBMH versus MIC of the parasite (MIC of NBMH/MIC of parasite), *Tbb Trypanosoma brucei brucei*, *Tbg T.b. gambiense*, *Tbr T.b. rhodesiense*, *Te T. evansi*

Table 2 Trypanocidal activity of compounds from *A. Leiocarpus* and *T. avicennoides* against *Trypanosoma* strains

Isolated compound	Minimum inhibitory concentrations in micrograms per milliliter (μM)				
	Tbb	Tbg	Tbr	Te	SI
Castalagin	22.5 (24)	27.5 (29)	27.5 (29)	31.5 (34)	48–67
Ellagic acid	≥25 (83)	nd	≥37.5 (91)	nd	40–60
Flavogallonic acid	7.5 (16)	12.5 (27)	8.75 (19)	15 (32)	100–200
Punicalagin	≥15 (14)	18.5 (17)	22.5 (21)	27.5 (25)	55–100
Fraction 2	>50	>100	>160	>150	9.4–30
Terchebulin	27.5 (25)	31.5 (29)	27.5 (25)	≥25 (23)	48–60

Table shows the MIC values of the isolated compounds against blood stream forms of four different species of *Trypanosoma*. Each experiment was carried out in duplicate and repeated at least two or three times and averages were taken and reported. All MIC values for the compounds against NBMH were above 1,500 μg/ml. Values in bracket are concentrations in micromolar.
NBMH Newborn mouse heart fibroblast, *SI* selectivity index—ratio of MIC of NBMH (≥1,500 μg/ml) versus MIC of the parasite (MIC of NBMH/MIC of parasite), *Tbb Trypanosoma brucei brucei*, *Tbg T.b. gambiense*, *Tbr T.b. rhodesiense*, *Te T. evansi*, *nd* no data

activity of the order 20 to ≥50 μg/ml for both *A. leiocarpus* and *T. avicennoides* against both human and domestic animal pathogens (Table 2). HPLC analyses showed the aqueous and butanolic fractions of *A. leiocarpus* to contain mainly castalagin, flavogallonic acid and ellagic acid, with castalagin as the major compound. In addition, several unknown peaks arising from phenol carboxylic acids were also detected. The UV absorptions of the unknown peaks were closely related to that of ellagic acid, suggesting their structural similarities. On the other hand, *T. avicennoides* was observed to contain punicalagin, ellagic acid, flavogallonic acid and terchebulin. The purified and isolated hydrolysable tannins displayed trypanocidal activity as assayed in vitro with MIC values of 7.5–31.5 μg/ml (14–91 μM). The structures of the isolated compounds are shown in Fig. 1. Punicalagin, castalagin and the other related tannins have been reported to have numerous biological activities such as anticancer, antibacterial and antiparasitic (Nonaka et al. 1990; Yang et al. 2000; Asres et al. 2001; Kinjo et al. 2001; Taguri et al. 2004; Seeram et al. 2005). In other reports, Kubata et al. (2005) reported the trypanocidal effect of condensed tannins; however, this is the first report of the trypanocidal activity of punicalagin, flavogallonic acid and terchebulin from *T. avicennoides* and castalagin

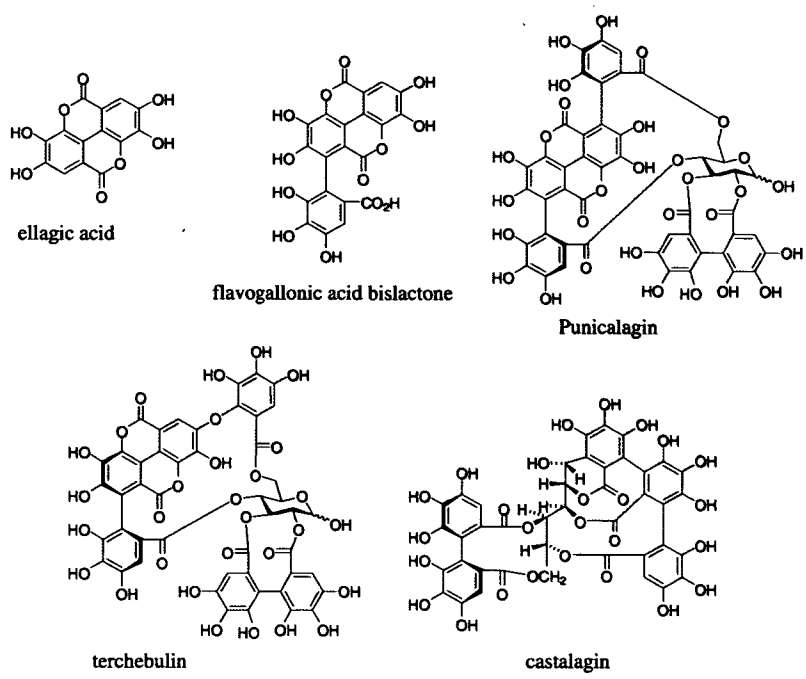


Fig. 1 Structures of the isolated compounds from *T. avicennoides* and *A. leiocarpus*

from *A. leiocarpus* on both the human and domestic animal pathogens causing trypanosomiasis, a result which is consistent with indigenous treatment of trypanosomiasis (Atawodi et al. 2002; Bizimana et al. 2006).

The effects of the extracts, fractions and those of the isolated compounds on the viability of the parasites and control mammalian cells were conducted by routine microscopic observation and parasite counting. It was observed to be concentration-dependent suppressive and the antiproliferative effect and the parasites appear to be immobilised and eventually died as depicted by the light and scanning electron micrographs in Fig. 2. Compared with the untreated cells, extract-exposed cells exhibited morphological changes similar to the butanolic fraction and isolated compounds by extensive swelling and eventual disruption of the surface membrane structures leading to loss of cytoplasmic contents. This pattern, of mode of parasite death, was consistently observed with the crude extract, the butanolic fraction and the isolated hydrolysable tannins, and this therefore suggests the involvement of these tannins in the killing of the parasites in vitro. These plant materials are generally taken orally as water extracts or as decoctions for treatment of various diseases and we investigated their toxicity on mammalian cells in vitro. Fibroblasts were used for the general toxicity and, like that on the parasites, is concentration dependent and of the order of ≥1,500 μg/ml as depicted in Fig. 3. We observed no serious toxic effect even at concentrations up to ≥1,500 μg/ml, a concentration which is about 200-fold higher than the MIC with lowest value ≤7.5 μg/ml. Previous reports have shown

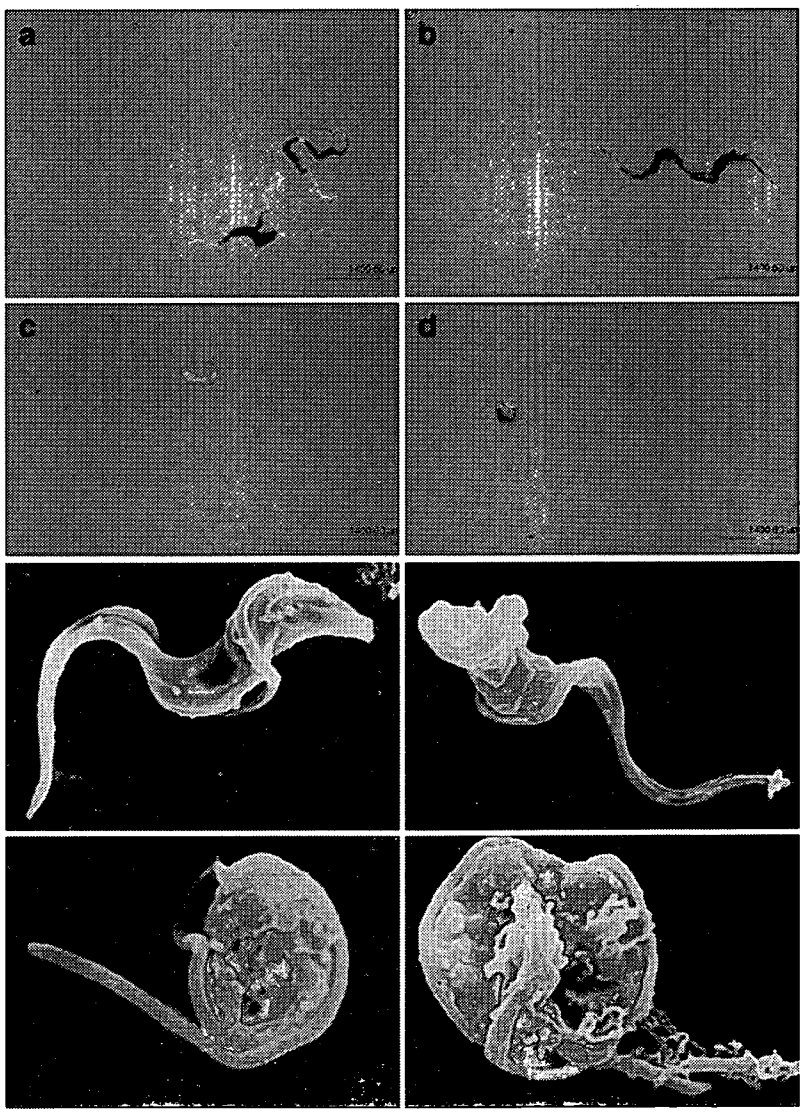


Fig. 2 Figure shows the effect of MIC of castalagin from *A. leiocarpus* extract on the morphology of *T.b. rhodesiense*. Parasites appear to be immobilised and eventually died as depicted by the light and scanning electron micrographs. **a–d** Light micrographs of *T.b. rhodesiense*. Parasites were cultured in 24-well culture plates in the presence and absence of different concentrations of the compounds. Smears of the parasites were prepared on glass slides, Giemsa stained and observed under a microscope; **e–h** scanning electron micrographs for *T.b. rhodesiense*. Parasites were aliquoted on polylysine-coated glass slide, fixed and dehydrated. The sample was critically dried, coated with Au–Pd and viewed on scanning microscope. **a, e** Control parasite without castalagin; **b, f** 30–60 min after exposure to castalagin; **c, g** 12 h after exposure to castalagin; **d, h** 24 h after exposure

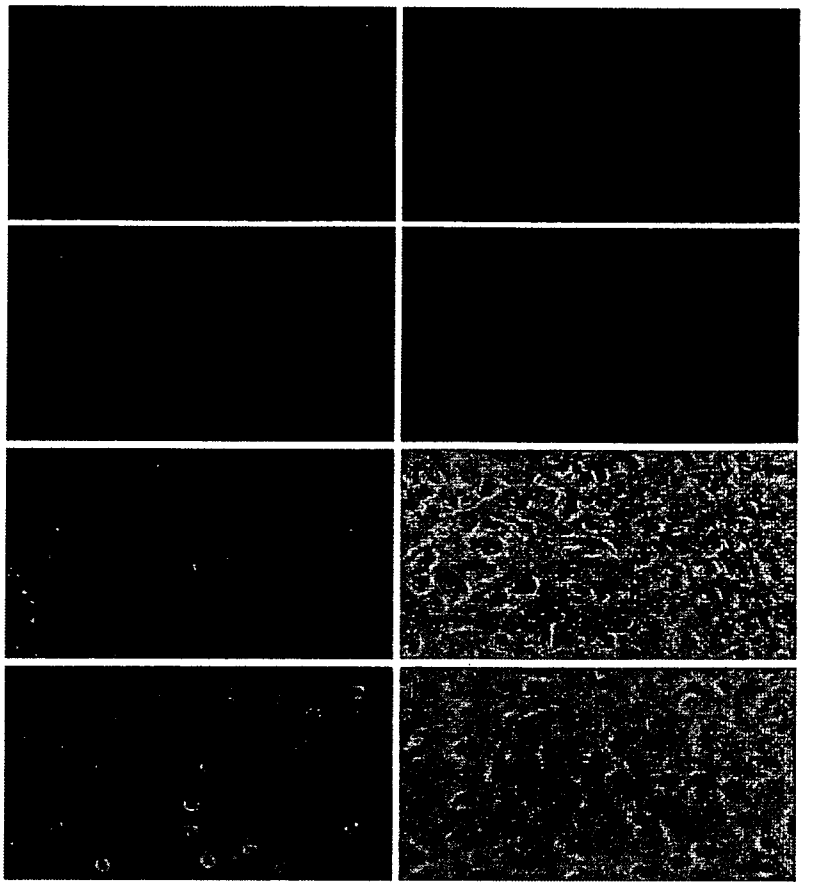


Fig. 3 Morphological appearance and viability of newborn mouse fibroblast following the in vitro incubation with the isolated compounds. **a** Normal control cells without compound; **b** +100 μg/ml castalagin; **c** +1,500 μg/ml castalagin; **d** +2,000 μg/ml *T. avicennoides* butanolic fraction; **e** +1,500 μg/ml punicalagin; **f** +2,000 μg/ml castalagin; **g** +2,000 μg/ml terchebulin; **h** +2,000 μg/ml flavogallonic acid

that higher concentrations of extracts of *Terminalia* species are tolerable in vivo, ≥100 mg/kg body weight (Abdullahi et al. 2001; Bizimana et al. 2006; Kamtchouing et al. 2006), which is consistent with the common practice of using these plants as chewing stick (Rotimi et al. 1988; Taiwo et al. 1999) and therefore corroborate with our in vitro toxicity study.

Bizimana et al. (2006) reported in vitro activities of *Afzelia africana*, *A. leiocarpus* and *T. avicennoides* against *T. b. brucei* (MIC, 1–10 μg/ml). This observation is in agreement with our result (≤12.5–17.5 μg/ml); however, assay system vis-à-vis incubation period and initial seeded parasite density per millilitre differed. Both aqueous and organic extracts from leaves, root and bark of *Terminalia* species were previously reported to have wide biological activities. In comparison with previously reported studies, *T. avicennoides* exhibited broader bactericidal, vibriocidal and antidiarrhoeal activities (Akinsinde and Olukoya 1995; Sanogo et al. 1998; Abdullahi et al. 2001; Iwalokun et al. 2001). Closely related species *Terminalia sericea* and *Terminalia superba* were reported to have antibacterial and anti-diabetic activities, respectively (Buwa and van Staden 2006; Kamtchouing et al. 2006). *A. leiocarpus* extract displayed significant in vitro activity against many clinically isolated bacterial strains and pathogenic fungi (Sanogo et al. 1998; Batawila et al. 2005). We also observed it to have promising trypanocidal activity with MIC values ranged from ≤25 to 50 μg/ml. Anti-parasitic activities of some members of Combretaceae were reported (Okpekon et al. 2004) and lower value of LC_{100} was observed for *T. brucei*.

Although this is not the first validation of the folkloric use of these plants and the observed activity of the isolated compounds may not be comparable to the existing drugs, this finding based on bioactivity-guided and phytochemical studies on different strains of African *Trypanosoma* undoubtedly confirms the involvement of these compounds (hydrolysable tannins) in the killing of the parasite.

Aknowledgement This work was supported by the Japanese Society for the Promotion of Science (JSPS). MNS is a JSPS fellow (ID #: 05166) and wishes to thank Pandey Kishor of the Department of Protozoology, Institute of Tropical Medicine, Nagasaki University and Dr. Haruna Danwanka, Mallam Farouq and Sabo Miri of Abubakar Tafawa Balewa University, Bauchi, Nigeria.

References

Abdullahi AL, Agho MO, Amos S, Gamaniel KS, Wambebe C (2001) Antidiarrhoeal activity of the aqueous extract of *Terminalia avicennoides* roots. Phytother Res 15:431–434

Adamu HM, Abayeh OJ, Agho MO, Abdullahi AL, Uba A, Dukku HU, Wufem BM (2005) An ethnobotanical survey of Bauchi State herbal plants and their antimicrobial activity. J Ethnopharmacol 99:1–4

Akah PA, Orisakwe OE, Gamaniel KS, Shittu A (1998) Evaluation of Nigerian traditional medicines: II. Effects of some Nigerian folk remedies on peptic ulcer. J Ethnopharmacol 62:123–127

Akinsinde KA, Olukoya DK (1995) Vibriocidal activities of some local herbs. J Diarrh Dis Res 13:127–129

Asres K, Bucar F, Edelsbrunner S, Kartnig T, Hoger G, Thiel W (2001) Investigations on antimycobacterial activity of some Ethiopian medicinal plants. Phytother Res 15:323–326

Atawodi SE, Ameh DA, Ibrahim S, Andrew JN, Nzelibe HC, Onyike EO, Anigo KM, Abu EA, James DB, Njoku GC, Sallau AB (2002) Indigenous knowledge system for treatment of trypanosomiasis in Kaduna state of Nigeria. J Ethnopharmacol 79:279–282

Batawila K, Kokou K, Koumaglo K, Gbeassor M, de Foucault B, Bouchet P, Akpagana K (2005) Antifungal activities of five Combretaceae used in Togolese traditional medicine. Fitoterapia 76:264–268

Bizimana N, Tietjen U, Zessin KH, Diallo D, Djibril C, Melzig MF, Clausen PH (2006) Evaluation of medicinal plants from Mali for their in vitro and in vivo trypanocidal activity. J Ethnopharmacol 103:350–356

Buwa LV, van Staden J (2006) Antibacterial and antifungal activity of traditional medicinal plants used against venereal diseases in South Africa. J Ethnopharmacol 103:139–142

Crump A (2006) New medicines from nature's armamentarium. Trends Parasitol 22:51–54

Hirumi H, Hirumi K (1994) Axenic culture of African trypanosome bloodstream forms. Parasitol Today 10:80–84

Iwalokun BA, Gbenle GO, Adewole TA, Akinsinde KA (2001) Shigellocidal properties of three Nigerian medicinal plants: *Ocimum gratissimum, Terminalia avicennoides,* and *Momordica balsamina*. J Health Popul Nutr 19:331–335

Kamtchouing P, Kahpui SM, Djomeni Dzeufiet PD, Tedong L, Asongalem EA, Dimo T (2006) Anti-diabetic activity of methanol/methylene chloride stem bark extracts of *Terminalia superba* and *Canarium schweinfurthii* on streptozotocin-induced diabetic rats. J Ethnopharmacol 104:306–309

Kinjo J, Nagao T, Tanaka T, Nonaka G, Okabe H (2001) Antiproliferative constituents in the plant 8. Seeds of *Rhynchosia volubilis*. Biol Pharm Bull 24:1443–1445

Koehn FE, Carter GT (2005) The evolving role of natural products in drug discovery. Nat Rev Drug Discov 4:206–220

Kubata BK, Nagamune K, Murakami N, Merkel P, Kabututu Z, Martin SK, Kalulu TM, Huq M, Yoshida M, Ohnishi-Kameyama M, Kinoshita T, Duszenko M, Urade Y (2005) *Kola acuminata* proanthocyanidins: a class of anti-trypanosomal compounds effective against *Trypanosoma brucei*. Int J Parasitol 35:91–103

Lin T, Nonaka G, Nishioka I, Ho F (1990) Tannins and related compounds. CII. Structures of terchebulin, an ellagitannin having a novel tetraphenylcarboxylic acid (terchebulic acid) moiety, and biogenetically related tannins from *Terminalia chebula* RETZ. Chem Pharm Bull 38:3004–3008

Nonaka G, Nishioka I, Nishizawa M, Yamagishi T, Kashiwada Y, Dutschman GE, Bodner AJ, Kilkuskie RE, Cheng YC, Lee KH (1990) Anti-AIDS agents, 2: inhibitory effects of tannins on HIV reverse transcriptase and HIV replication in H9 lymphocyte cells. J Nat Prod 53:587–595

Okpekon T, Yolou S, Gleye C, Roblot F, Loiseau P, Bories C, Grellier P, Frappier F, Laurens A, Hocquemiller R (2004) Antiparasitic activities of medicinal plants used in Ivory Coast. J Ethnopharmacol 90:91–97

Rotimi VO, Laughon BE, Bartlett JG, Mosadomi HA (1988) Activities of Nigerian chewing stick extracts against *Bacteroides gingivalis* and *Bacteroides melaninogenicus*. Antimicrob Agents Chemother 32:598–600

Sanogo R, Crisafi G, Germano MP, De Pasquale R, Bisignano G (1998) Evaluation of Malian traditional medicines: screening for antimicrobial activity. Phytother Res 12:S154–S156

Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, Heber D (2005) In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 16:360–367

Taguri T, Tanaka T, Kouno I (2004) Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. Biol Pharm Bull 27:1965–1969

Taiwo O, Xu H-X, Lee SF (1999) Antibacterial activities of extracts from Nigerian chewing sticks. Phytother Res 13:675–679

Tanaka T, Nonaka G, Nishioka I (1986a) Tannins and related compounds. XLI. Isolation and characterization of novel ellagitannins, punicacorteins A, B, C and D, and punigluconin from the bark of *Punica granatum* L. Chem Pharm Bull 34:656–663

Tanaka T, Nonaka G, Nishioka I (1986b) Tannins and related compounds. XLII. Isolation and characterization of four new hydrolyzable tannins, terflavins A and B, tergallagin and tercatain from the leaves of *Terminalia catappa* L. Chem Pharm Bull 34:1039–1049

Tanaka T, Ueda N, Shinohara H, Nonaka G, Fujioka T, Mihashi K, Kouno I (1996) C-glycosidic ellagitannin metabolites in the heartwood of Japanese chestnut tree (*Castanea crenata* Sieb. et Zucc.). Chem Pharm Bull 44:2236–2242

Vonthron-Sénécheau C, Weniger B, Ouattara M, Tra Bi F, Kamenan A, Lobstein A, Brun R, Anton R (2003) In vitro antiplasmodial activity and cytotoxicity of ethnobotanically selected Ivorian plants. J Ethnopharmacol 87:221–225

Welburn SC, Coleman PG, Maudlin I, Fevre EM, Odiit M, Eisler MC (2006) Crisis, what crisis? Control of Rhodesian sleeping sickness. Trends in Parasitol 22:123–128

World Health Organization (1998) Control and surveillance of African trypanosomiasis. WHO Technical Report Series 881. Geneva, Switzerland

Yang LL, Lee CY, Yen KY (2000) Induction of apoptosis by hydrolyzable tannins from *Eugenia jambos* L. on human leukemia cells. Cancer Lett 157:65–75

Available online at www.sciencedirect.com
ScienceDirect
Mitochondrion 7 (2007) 125–132
Mitochondrion
www.elsevier.com/locate/mito

Mitochondria and apicoplast of *Plasmodium falciparum*: Behaviour on subcellular fractionation and the implication

Tamaki Kobayashi [a], Shigeharu Sato [b], Shinzaburo Takamiya [c], Kanako Komaki-Yasuda [d], Kazuhiko Yano [d], Ayami Hirata [e], Izumi Onitsuka [e], Masayuki Hata [a], Fumika Mi-ichi [a], Takeshi Tanaka [a], Toshiharu Hase [f], Atsushi Miyajima [e], Shin-ichiro Kawazu [d], Yoh-ichi Watanabe [a], Kiyoshi Kita [a,*]

[a] *Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Buinkyo-ku, Tokyo 113-0033, Japan*
[b] *Division of Parasitology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK*
[c] *Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[d] *Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[e] *Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*
[f] *Division of Enzymology, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

Received 19 April 2006; accepted 21 September 2006
Available online 9 December 2006

Abstract

The mitochondrion and the apicoplast of the malaria parasite, *Plasmodium* spp. is microscopically observed in a close proximity to each other. In this study, we tested the suitability of two different separation techniques – Percoll density gradient centrifugation and fluorescence-activated organelle sorting – for improving the purity of mitochondria isolated from the crude organelle preparation of *Plasmodium falciparum*. To our surprise, the apicoplast was inseparable from the plasmodial mitochondrion by each method. This implies these two plasmodial organelles are bound each other. This is the first experimental evidence of a physical binding between the two organelles in *Plasmodium*.

Keywords: *Plasmodium falciparum*; Mitochondrion; Apicoplast; Fluorescence-activated organelle fractionation

1. Introduction

Malaria, by far the most important tropical parasitic disease, is caused by a group of parasites *Plasmodium* spp. belonging to the phylum Apicomplexa. Currently, various anti-malarial drug resistant parasite strains are reported and there is a long way for the development of vaccine. Emergence of insecticide resistant mosquito vector limits the current control schemes as well (Greenwood et al., 2005). In order to control this world problem, studies seeking for unique properties of the parasite are indispensable.

Previous study reported malaria parasites obtain most of their energy from glycolysis, if not all (Roth et al., 1988) and malaria parasite possesses one mitochondrion with various shapes at different stages of the intra-erythrocytic development and it is acristae (Slomianny and Prensier, 1986). Mitochondria of *Plasmodium* species carries 6-kb genome, which is the smallest mitochondrial genome ever been reported and encoding only 3 open reading frames with homology to classical mitochondrial protein, cytochrome *c* oxidase subunit I, cytochrome *c* oxidase subunit III and cytochrome *b*, as well as abbreviated rRNA genes (Vaidya et al., 1989; Feagin, 1992). Thus this

Abbreviations: DHOD, dihydroorotate dehydrogenase; FOS, fluorescence-activated organelle sorting; RBC, red blood cell.

* Corresponding author. Tel.: +81 3 5841 3526; fax: +81 3 5841 3444.
E-mail address: kitak@m.u-tokyo.ac.jp (K. Kita).

1567-7249/$ - see front matter © 2006 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2006.11.021

organelle heavily depends on most of the proteins and all tRNAs supplied from the outside.

Biochemical analysis suggested that *Plasmodium falciparum* might lack TCA cycle in the erythrocytic stage (see the review by Sherman, 1979). Recent completion of malaria genome project has revealed that the genes necessary for a complete TCA cycle were present in *P. falciparum* (Gardner et al., 2002). However, it still remains unclear whether the TCA cycle is responsible for the further oxidation of glycolysis product. Nevertheless, the activity of the electron transport chain and the membrane potential of this organelle are indispensable for the survival of the parasite. For example, dihydroorotate dehydrogenase (DHOD) involved in the parasite's *de novo* biosynthesis of pyrimidine requires the functional electron transport chain on the mitochondrial membrane as the electron disposal sink (Gutteridge and Trigg, 1970; Gero et al., 1984; Prapunwattana et al., 1988). More recent study showed that the membrane potential of mitochondria formed by respiration is essential for parasite growth (Srivastava et al., 1999) and complex III (ubiquinol-cytochrome *c* reductase) inhibitor, atovaqone, an anti-malarial that is currently in use is reported to disrupt mitochondrial membrane potential resulting in parasite growth reduction (Srivastava et al., 1997).

Aikawa (1966) carried out an extensive morphological study by electron microscope and found a distinctive organelle in the cell of the malaria parasite. This organelle is multi-membrane bound, always observed adjacent to the mitochondrion (see the review by Bannister et al., 2000). Later, a non-mitochondrial extra-chromosomal DNA encoding a set of genes characteristic of the plastid genome was found in apicomplexan parasites including *Plasmodium* spp. *Toxoplasma gondii* and *Theileria* spp. (Wilson et al., 1996; Kohler et al., 1997) localized the plastid genome-like DNA to the multi-membrane organelle in *T. gondii* by *in situ* hybridization, revealing that the distinctive multi-membrane organelle is the plastid of the apicomplexan parasite. The apicomplexan plastid, which is non-photosynthetic, is often called "the apicoplast" for abbreviation. The genome of the apicoplast is one of the smallest known plastid genomes (Wilson et al., 1996; Gardner et al., 2005). The apicoplast depends heavily on proteins imported post-translationally from the cytosol (see the review by Ralph et al., 2004), as does the mitochondrion.

For biochemical studies of each organelle of *Plasmodium* spp., it is necessary to obtain the pure sample. Fry and Beesley (1991) reported a method to prepare the mitochondria from *Plasmodium* spp. by Percoll density gradient centrifugation. Takashima et al. (2001) reported another preparation method using nitrogen cavitation. The mitochondrial preparation by the latter method exhibited a significantly higher succinate dehydrogenase activity than that by the former method (Takashima et al., 2001). By contrast, no method for preparing the plasmodial apicoplast with a significant purity has been reported.

In this study, we combined nitrogen cavitation method with two different fractionation methods, Percoll density gradient centrifugation or fluorescence-activated organelle sorting (FOS), to prepare the mitochondrion of higher purity from *P. falciparum*. Surprisingly, we found that the mitochondrion and the apicoplast were recovered in the same fraction by each fractionation methods, most likely because the two organelles are bound each other. To our knowledge, this is the first report that suggests the presence of a physical connection between the mitochondrion and the apicoplast of *P. falciparum*.

2. Materials and methods

2.1. Parasite cultivation and handling

Plasmodium falciparum (Honduras-1 strain and 3D7 strain) was cultured following the method reported by Trager and Jensen (1976) with modifications. The culture was maintained with 3% hematocrit type A human red blood cell (RBC) in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) type A human serum. Prior to the preparation of crude mitochondrial fraction, parasites were synchronised by 5% (w/v) sorbitol as it was described previously (Lambros and Vanderberg, 1979).

2.2. Preparation of the crude P. falciparum mitochondria fraction

Plasmodium falciparum-infected RBC were collected by centrifugation at 800*g* for 10 min at 4 °C (LX-120, TOMY) when parasitemia is more than 5% but not exceeding 10%. Parasite was mainly trophozoite stage as it was confirmed by observing the Giemsa's stained smear. Trophozoite stage was considered because DHOD-specific activity is the highest (F. Mi-ichi, Personal communication). The crude *P. falciparum* were disrupted by nitrogen cavitation as described (Takashima et al., 2001). The pellet obtained after the centrifugation at 23,000*g* for 20 min at 4 °C (Himac CR22, HITACHI) was suspended in 200–400 μl MSE buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA (Dojin), 3 mM Tris–HCl; pH 7.4) and characterized as a crude mitochondrial fraction (Fig. 1).

2.3. Subcellular fractionation of P. falciparum mitochondria with the Percoll density gradient centrifugation

The crude mitochondrial fraction prepared from *P. falciparum* Honduras-1 strain (1.5 mg protein) was brought to a total volume of 8 ml in 23% (v/v) Percoll (GE Healthcare) in MSE. Percoll sample was centrifuged at 100,000*g* for 1 h at 4 °C (Himac SCP70H, HITACHI, rotor No. RP40). Together with the mitochondrial fraction, beads marker provided by the manufacture was centrifuged in parallel to confirm the formation of gradient and density of each fraction. The gradient was fractionated from top to bottom with glass Pasteur pipette (400 μl/ fraction). The formation

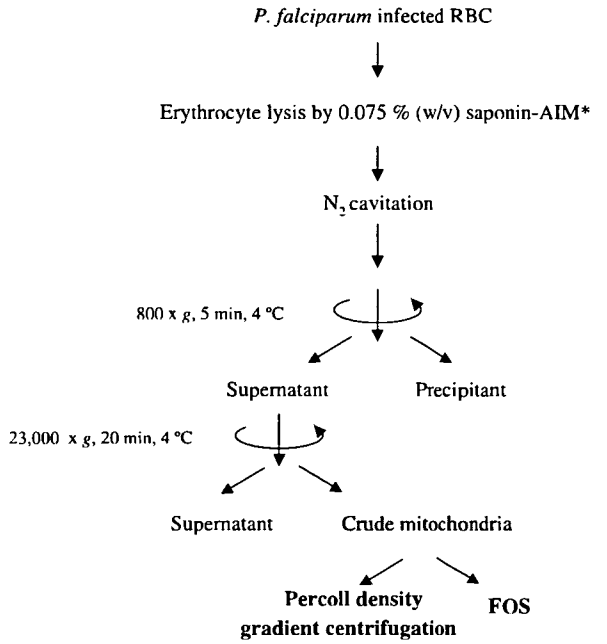


Fig. 1. Crude mitochondria preparation from *P. falciparum* *AIM (120 mM KCl, 20 mM NaCl, 10 mM Pipes, 1 mM $MgCl_2$, 5 mM glucose; pH 6.7).

of the gradient can be affected by factors such as the angle of the rotor. Therefore, it is critical to monitor the formation of the desired gradient by using the density marker beads all the time when one uses different centrifuge and rotor.

DHOD activity was measured to determine the fraction containing mitochondria (Mi-Ichi et al., 2005). DHOD assay was performed at 25 °C using UV3000 spectrophotometer (SHIMADZU) with 1 ml of the reaction mixture containing 45 μM 2,6-dichlorophenolindophenol (DCIP) (Sigma), 100 μM ubiquinone-2 (Sigma) and 2 mM KCN in 30 mM Tris–HCl (pH 8.0). The reaction was initiated by adding 500 μM dihydroorotate and the production of reduced DCIP was monitored at 600 nm (ε=21 mM^{-1} cm^{-1}).

2.4. Protein assay

Protein concentration of *P. falciparum* sample was determined by Bradford method (Bradford, 1976) using Bio-Rad protein assay reagent according to the manual provided by the manufacturer, with bovine serum albumin (PIERCE) as a standard. For the sample that contains Percoll, Percoll was precipitated under the presence of 250 mM NaOH and 0.025% (w/v) Triton X-100 as it was reported (Vincent and Nadeau, 1983) prior to the protein assay.

2.5. Western blot analysis

As Percoll interferes with SDS–PAGE, the fraction of interest was diluted with MSE buffer up to 8.5 ml and centrifuged at 220,000*g* for 1 h at 4 °C (CP10α, 70H, HITACHI) to remove Percoll. Samples were collected as floating pellet and suspended in MSE up to 1.5 ml. Suspended pellet was transferred to 1.5 ml tube and centrifuged at 20,000*g* for 10 minutes at 4 °C (TOMY MX-160). The pellet was re-suspended in MSE buffer. Prepared samples were then run on 12.5% polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked with 5% (w/v) non-fat skim milk powder in 0.5% (v/v) Tween–Tris-buffered saline and afterwards probed with polyclonal antibodies specific to mitochondrion or apicoplast. For the detection of mitochondrion and apicoplast, serum against recombinant *P. falciparum* iron–sulfur cluster subunit of complex II (rPfIp) and ferredoxin (rPfFd) were raised in mouse and rabbit, respectively. The dilution for the first antibody was 1:2000 for anti-rPfIp serum and 1:1000 for anti-rPfFd serum.

2.6. Electron microscopic observation of the subcellular-fractionated sample by the Percoll density gradient centrifugation

Percoll was removed from the sample as described above. Obtained pellet was suspended in fixative solution containing 2% (v/v) glutaraldehyde in 20 mM sodium phosphate buffer (pH 6.8): MSE buffer = 1:1. The sample was fixed overnight at 4 °C and subsequently washed with 20 mM Sodium phosphate buffer (pH 6.8): MSE buffer = 1:1 for three times. The fixed sample was then dehydrated and embedded in resin. Serial sections were cut and observed by transmission electron microscope (HITACHI H-7100).

2.7. GFP fusion constructs and P. falciparum transfection

The expression vector, pSSPF2/PfHSP60-GFP (Sato et al., 2003) was transfected to *Escherichia coli* DH5α and the transfected *E. coli* was grown in the terrific broth. Plasmids were collected by centrifugation and was purified using Plasmid Maxi Kit (Qiagen). The plasmid was verified with restriction digestion using *Bgl*II and *Xho*I, followed by agarose gel electrophoresis.

The transfection of *P. falciparum* 3D7 was done following the previous report (Sato et al., 2003) with slight modifications. 100 μl infected RBC at approximately 10% ring stage parasitemia was suspended in three volumes of cytomix (van den Hoff et al., 1992) containing 50 μg of plasmid DNA. Total 400 μl of the RBC suspension was electroporated in a 0.2 cm cuvette using Gene Pulser II (Bio-Rad) (0.31 kV, 975 μF). After the transfection, parasites were maintained in the medium supplemented with 5 nM WR99210 as it was described previously (Sato et al., 2003).

2.8. Immunofluorescent studies

Infected erythrocytes and mitochondrial fraction was observed using confocal microscope LSM510 (Zeiss).

Parasites expressing fluorescent protein were incubated for 30 min at 37 °C with MitoTracker Red CM-H_2XRos (Molecular Probes) diluted to 100 nM in culture medium. After the incubation, the culture was once washed with culture medium or AIM medium before microscopy observation.

2.9. Flow cytometry analysis and organelle sorting of transfected parasites

Plasmodium falciparum 3D7 strain expressing GFP was homogenized by nitrogen cavitation method and the crude mitochondrial fraction was prepared according to the procedure described in the previous section. The prepared mitochondrial fraction was analyzed and sorted by EPICS ALTRA (Beckman Coulter). The size of the sorted particles was determined by Flow Cytometry Size calibration Kit (Molecular Probes). Sorted sample were centrifuged and the precipitant was suspended with MSE.

2.10. Detection of organelle by PCR

The primer set to detect apicoplast genome was for the large subunit of rRNA gene, 5′-GAC CTG CAT GAA AGA TG-3′ and 5′-GTA TCG CTT TAA TAG GCG-3′ as it was described previously (Tan et al., 1997). The primer set to detect mitochondria genome was for the subunit I of Complex IV (cytochrome *c* oxidase), 5′-GAC CCA ACA TTT GCA GGA GAT C-3′ and 5′-CAT CAA TGG CAG CAT TAC CTA A-3′. The reaction mixture was prepared with 1 μl of the above mentioned sorted sample (out of final volume 100 μl) or diluted crude mitochondrial fraction, 1× PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 1.25 U *Taq* DNA polymerase (Invitrogen), 1.5 mM $MgCl_2$, 200 μM dNTP and 0.25 μM of each primer in a final volume of 50 μl. The volume of samples was determined not to reach the saturation after the PCR cycle.

The PCR was carried out using the following conditions: pre-heating at 95 °C, 3 min; denaturation at 95 °C, 30 s; annealing at 50 °C, 30 s; elongation at 72 °C, 1 min for 30 cycles followed by incubation at 72 °C for 10 min after the final cycle. For the amplification of mitochondrial genome and apicoplast genome, TEMP CONTROL PC-700 (ASTEC) and GeneAmp® PCR system 9700 (Perkin-Elmer) was used, respectively. Amplified products were then analyzed on a 1% (w/v) agarose gel.

In the experimental procedure, chemicals used were a special grade and were purchased from Wako unless otherwise stated.

3. Results

3.1. The mitochondria and apicoplast co-fractionated by the Percoll density gradient centrifugation

Previously, Fry and Beesley (1991) reported a method to prepare plasmodial mitochondria using a density gradient in 22% (v/v) Percoll formed by centrifugation at 10,000*g* for 5 min. As this method has been successfully used in other laboratories (Wilson et al., 1992; Krungkrai, 1995; Krungkrai et al., 1997), we preliminarily tested if this method is directly applicable to improve the purity of mitochondria prepared by nitrogen cavitation method, which showed higher enzyme activities of mitochondria than those of previous method (Takashima et al., 2001).

We found method reported by Fry and Beesley (1991) is not sufficient to separate mitochondria as g-force was too low and the time was too short (data not shown). Thus, we examined different conditions of Percoll density gradient centrifugation that might improve the current mitochondria sample. To estimate the purity of the obtained mitochondria, activity of mitochondria-specific enzyme, dihydroorotate dehydrogenase (DHOD), which is localized in the inner membrane of the mitochondria was measured. The increase in DHOD-specific activity indicates the enrichment of mitochondria.

We optimized the condition to be 23% (v/v) Percoll centrifuged at 100,000*g* for 1 h at 4 °C using Honduras-1 strain. The gradient was confirmed by the control marker beads in each experiment as it is shown in Fig. 2A. After the Percoll density gradient centrifugation of crude mitochondria sample, prominent brown float and dark brown precipitant were observed (Fig. 2A). The density gradient sample was fractionated and recovery profile of the total DHOD activity after the gradient centrifugation showed two peaks (Fig. 2B). The first peak (fractions 8–10) was sharp and more than twice as high total activity as the broad second peak (fractions 12–16). However, owing to the high amount of proteins recovered, the specific activity in the first peak was not improved. By contrast, the second peak of the total activity showed a significantly higher specific activity; the value of fraction 13 (66.2 ± 27.7 nmol/min/mg protein ($n = 3$)) was about 5 times higher than that of the initial sample (12.4 ± 3.01 nmol/min/mg protein ($n = 3$)). Same results were obtained when the fractionations were performed using 3D7 strain.

To assess the degree of contamination of other cell components, the fractions obtained by the Percoll density gradient centrifugation were analysed by electron microscopy. Fractions 13 and 14, which exhibited the highest DHOD-specific activity, contained a number of mitochondria with double membrane (Fig. 3C and D). Interestingly, another type of multi-membrane-bound organelle was also observed adjacent to the mitochondrion (Fig. 3D). A number of hemozoin particles were found in the crude mitochondria preparation before Percoll density gradient centrifugation (Fig. 3A). Fractions 13 and 14 was virtually free from hemozoin particle (Fig. 3B), although other fractions were prevailed by those bodies (Fig. 3E–G). This suggests that the food vacuole was successfully separated from the mitochondrion by the density gradient centrifugation in 23% (v/v) Percoll.

Since the multi-membrane-bound organelle observed adjacent to the mitochondrion seemed to be the apicoplast,

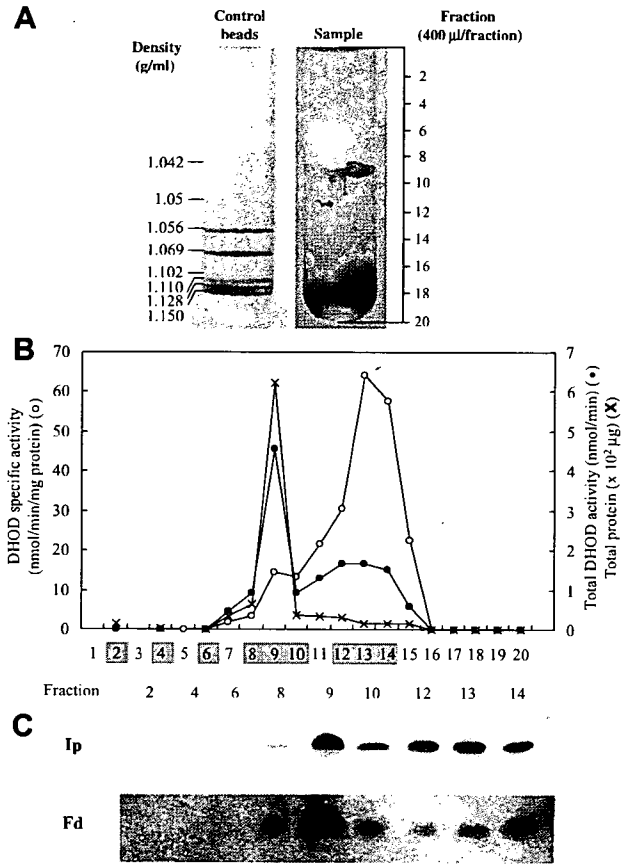


Fig. 2. Percoll density gradient centrifugation. (A) The formation of the gradient was confirmed by beads marker and the density of the control beads were indicated. *P. falciparum* crude mitochondrial fraction was applied to the Percoll density centrifugation forming the brown float and tight brown precipitant. The sample was fractionated from the top to the bottom as indicated. (B) The profile of total protein, DHOD-specific activity and total activity in each fraction of the 23% (v/v) Percoll density gradient centrifugation. The *x*-axis indicates the fraction number and those highlighted were analyzed by the Western blotting. The approximate location of each fraction is briefly indicated in (A). Total protein (X) DHOD total activity (●) and DHOD-specific activity (O). (C) The Western blot analysis of 23% (v/v) Percoll density centrifugation fractions. The localization of mitochondria and apicoplast were determined by using the specific antibodies. Antibody for mitochondria was for succinate dehydrogenase iron–sulfur cluster subunit (Ip) and antibody for apicoplast was for ferredoxin (Fd). eighty microliters of Percoll density fractionation sample was applied each lane.

we probed the fractions made after 23% (v/v) Percoll density gradient centrifugation with the antibody raised against a protein specifically localizing the mitochondrion or the apicoplast. As shown in Fig. 2C, the distribution profile of ferredoxin (Fd), an apicoplast-localizing protein (Vollmer et al., 2001), overlaps that of the iron-sulfer cluster subunit (Ip) of complex II (succinate-ubiquinone reductase), a mitochondrial integral membrane protein (Takeo et al., 2000). This implies even our improved Percoll density gradient centrifugation is not able to separate the mitochondrion from the apicoplast, although it is enough effective to remove the food vacuole or hemozoin particles.

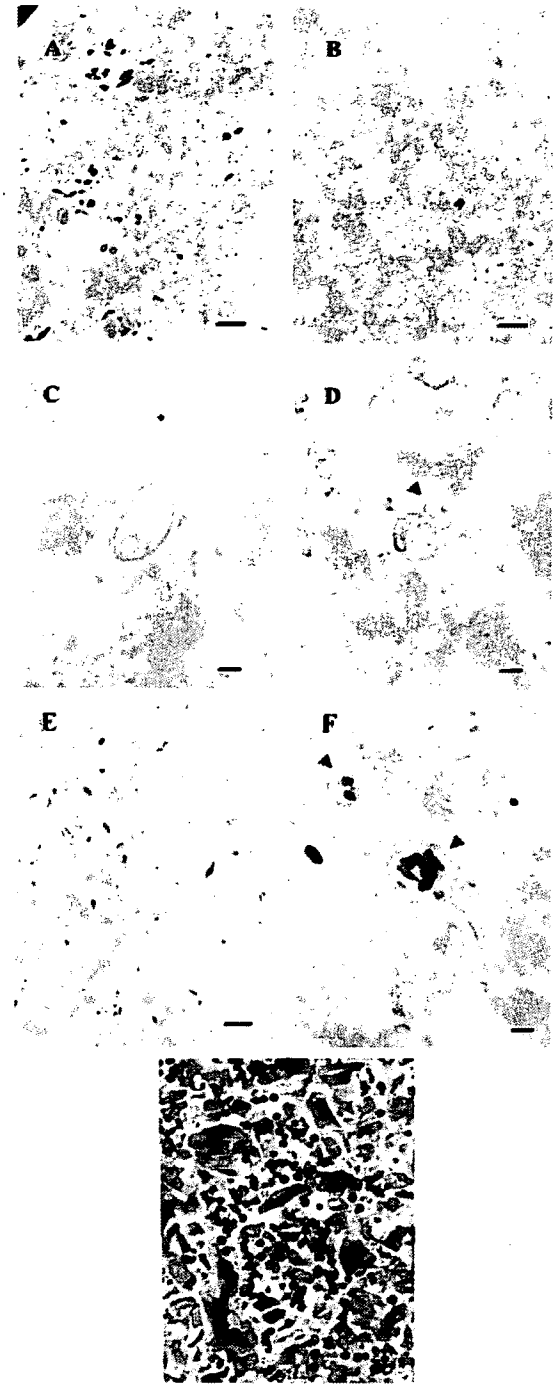


Fig. 3. Electron microscope observation (A) is the crude mitochondria fraction and (B) is the pooled peak fraction of DHOD-specific activity from fraction 13, and 14 after the Percoll density centrifugation (8000×). The dark particles in (A) are significantly reduced in (B), after the Percoll density gradient centrifugation. (C and D) Are observation of the peak DHOD-specific activity at the higher magnification (C; 30,000× and D; 15,000×). The double membrane-bound and multi-membrane-bound structures are indicated by arrows. (E and F) Are the observation of the brown float (fraction 9) at 8000× and 30,000×, respectively. Hemozoin contained in the membrane was observed and from its structure, it is concluded to be a food vacuole. (G) is the tight pellet and the characteristic structure shows it is hemozoin (30,000×). Scale bar; 1 µm for 8000×, 500 nm for 15,000× and 200 nm for 30,000× magnification.

3.2. The mitochondrion and apicoplast are physically bound to each other; co-fractionation of mitochondrion and apicoplast after the fluorescent-activated organelle sorting

As it was found that Percoll density gradient centrifugation is not suitable to remove the apicoplast from the mitochondrion, next we tested another separation technique based on a different physical property of the organelle to recover the apicoplast-free mitochondria from the crude mitochondrial sample.

Plasmodium falciparum 3D7 strain was transfected with a expression plasmid carrying a gene for a GFP derivative that localizes to the mitochondrion (Sato et al., 2003). Specific localization of GFP to the mitochondrion was confirmed by confocal microscope (data not shown) Then, the transfectant and non-transfectant parasites were disrupted by the nitrogen cavitation method and crude mitochondria sample was prepared as it is described in the material and method (Fig. 1). The expression of GFP in mitochondria did not affect the specific activity of mitochondria marker enzyme and therefore the property of mitochondria was not changed at least at the level of electron transfer. In addition, the particle size of the crude mitochondrial sample from GFP-expressing and non-GFP-expressing was not altered depending on the expression of GFP (Fig. 4, inserted figures).

The crude mitochondria fractions prepared from GFP expressing and non-GFP expressing *P. falciparum* was analyzed by flow cytometry. The crude mitochondria preparation from the GFP-expressing transfectant emitted significantly higher fluorescence compared to that of non-transfectant control (Fig. 4A and B). We recovered GFP-positive sample after the fluorescence-activated organelle sorting (FOS) and performed PCR to detect the presence of mitochondria using a specific primers for subunit I of cytochrome *c* oxidase, which is encoded on the mitochondrial DNA. Both the crude sample before FOS and the GFP positive fraction recovered after FOS gave a clear band, confirming the presence of the mitochondrial genomic DNA in these samples (Fig. 5, lanes 1 and 3). In addition, PCR product from apicoplast genomic DNA, large subunit of ribosomal RNA, were also detected (Fig. 5, lanes 2 and 4). This result implies that apicoplast were sorted together with mitochondria by FOS.

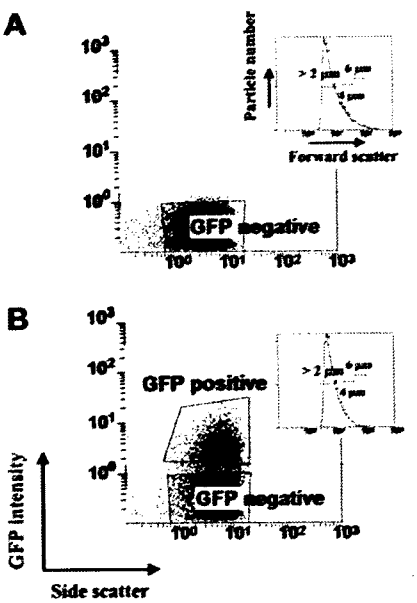


Fig. 4. FOS analysis of crude mitochondria fraction; control and GFP expressing sample. (A) The flow cytometry analysis of the crude mitochondrial fraction from control *P. falciparum* 3D7. The square "GFP negative" indicates the background. (B) The crude mitochondrial fraction prepared from GFP expressing transfectant. The size was determined by the calibration beads. The fraction of the sample expressing significantly high GFP signal were sorted as "GFP positive". The inserted figures show the particle size of the applied crude mitochondria sample.

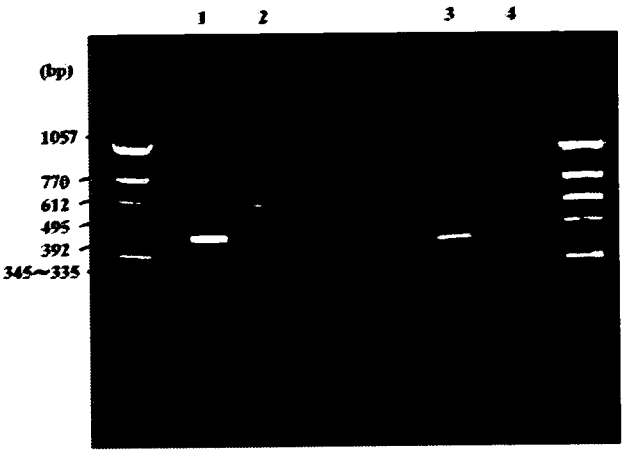


Fig. 5. PCR analysis of mitochondrial fraction and FACS sample. Lanes 1 and 2; crude mitochondria sample. Lane 1 is mitochondria genome-specific primer set and lane 2 is apicoplast genome-specific primer set. Lanes 3 and 4; after the organelle sorting. Lane 3 is mitochondria genome-specific primer set and lane 4 is apicoplast genome-specific primer set. For the detection of mitochondria, the primer set to amplify a part of gene encoding subunit I of cytochrome *c* oxidase was used. For the detection of apicoplast, a part of gene encoding large subunit of ribosomal RNA was used.

4. Discussion

The biochemical study of plasmodial mitochondria has been limited due to the difficulty to prepare sample with high quality and quantity. Previously we have reported the improved mitochondrial preparation from *P. falciparum* using nitrogen cavitation method (Takashima et al., 2001). In this study, we investigated the method to obtain a mitochondrial preparation of better quality by combining nitrogen cavitation with another fractionation method.

Percoll density gradient centrifugation is a well known method to prepare mitochondrial fraction from various organisms including *P. falciparum* and *Plasmodium yoelii* (Fry and Beesley, 1991). The method also has been used for the subcellular fractionation and purification of

mitochondrial enzymes from *Plasmodium* (Krungkrai, 1995, 1997). However, we have found that the purity of the mitochondrial sample prepared by the nitrogen cavitation method could not be improved by the method Fry and Beesley reported (1991). Instead, longer centrifugation with stronger force resulted in a better separation of the mitochondrion (23% (v/v) Percoll, 100,000*g* for 1 h). It is probably because each subcellular structure in the cell was less damaged by nitrogen cavitation in our method than the homogenization with glass-Teflon homogenizer by Fry and Beesley (1991). Indeed, the intact food vacuole containing haemozoin crystals was observed even after our fractionation method (Fig. 3F), and we previously noted that the mitochondrial activity represented by the succinate dehydrogenase was 2 to 3 times higher in of the crude mitochondrial preparation prepared by nitrogen cavitation (Takashima et al., 2001) than those prepared by other conventional methods (Suraveratum et al., 2000).

In this study, the DHOD-specific activity increased about 5 times after the Percoll density gradient centrifugation in the second peak (Fig. 2B), indicating that the contaminants were removed. Indeed, the observation by the electron microscope showed that hemozoin and food vacuole were significantly reduced after the Percoll density gradient centrifugation. Electron microscopic analysis confirmed that the mitochondrion with double membrane was enriched in the fractions forming the second peak (Fig. 3C and D).

Unlike the food vacuole, the apicoplast was not separated from the mitochondrion after the Percoll density gradient centrifugation. It might be because the organelle, by chance, shares the similar density with the mitochondrion. We examined this possibility by a different fractionation method – fluorescence-activated organelle sorting (FOS). Single organelle sorting has been applied to various organelles to determine the characteristics of individual organelle (Böck et al., 1997). The use of fluorescent organelle cytometry attracted scientists' attention to study single mitochondria (Cavelier et al., 2000). In this study, we took the advantage of single organelle sorting to improve the purity of mitochondria sample. After the FOS, the presence of the mitochondrion and the apicoplast genomic DNAs in the GFP positive fraction recovered was confirmed by PCR with target specific primers. This result indicates that applying different mode of fractionation, mitochondria and apicoplast were, again, co-fractionated. Our results strongly suggest that the mitochondrion and the apicoplast of *P. falciparum* are bound to each other.

Up to date, study of mitochondrion and apicoplast of *Plasmodium* has been restricted to the whole cell, and physical interaction between two organelles had not been determined. The extensive genomic information from malaria genome project (Gardner et al., 2002) provided ideas about the putative metabolic pathway in mitochondria and apicoplast in *P. falciparum*. Among those pathways, heme biosynthesis is unique one as the pathway is predicted to begin in the mitochondrion but subsequent reactions are likely taken place in the apicoplast; 5-aminolevulinic acid (ALA), the first intermediate of the pathway, seemed to be synthesized in the mitochondrion via Shemin pathway but converted to porphobilinogen, the next intermediate, in the apicoplast. This peculiarity was experimentally confirmed by localizing ALA synthase and porphobilinogen synthase (ALA dehydratase), respectively (Sato et al., 2004). The parasite may have a requirement to locate these two organelles in a close proximity to facilitate transport of ALA from the mitochondrion to the apicoplast.

The mitochondrion and the apicoplast of *Plasmodium* spp. have been observed in a proximity to each other by electron microscopy and fluorescent microscopy (Aikawa, 1966; Hopkins et al., 1999; Sato et al., 2004; van Dooren et al., 2005), however the strength in the interaction might vary depending on the parasite stage. It is possible that interaction between apicoplast and mitochondrion to be disrupted in the process of mitochondria preparation.

Despite of extensive observations, it is still unknown if there is a significant structure directly connecting these organelles, like tight junction. These organelles can be connected indirectly, e.g., through the cytoskeleton. Both organelles were recovered not only in the fractions forming the second peak with the DHOD activity but also in those forming the first peak after our Percoll centrifugation (Fig. 2). This may suggest that the putative connection between the organelles has a complex structure involving plenty of auxiliary proteins in a physiological state, but can be reduced to a simple, basic structure that is still capable to hold both organelles together.

Acknowledgements

The authors thank late Dr. Masamichi Aikawa for comments on morphological observations by electron microscopy and wish to dedicate this paper to him. The blood and plasma used in this study is a kind donation from Tokyo Metropolitan Red Cross Blood Centre. WR99210, the drug used for the screening of transfected parasites was a kind gift from Jacobus Pharmaceutical Co., Inc. This study was supported by a Grant-in-Aid for scientific research on priority areas and for Creative Scientific Research from the Japanese Ministry of Education, Science, Culture, and Sports (13226015, 13854011, 17209013, 17590368, 18073004 and 18GS0314) and for research on emerging and re-emerging infectious diseases from the Japanese Ministry of Health and Welfare, and by British Medical Research Council.

References

Aikawa, M., 1966. The fine structure of the erythrocytic stages of three avian malarial parasites, *Plasmodium fallax*, *P. lophurae* and *P. cathemerium*. Am. J. Trop. Med. Hyg. 15, 449–471.

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., Mitchell, G.H., 2000. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. Parasitol. Today 16, 427–433.

Bock, G., Steinlein, P., Huber, L.A., 1997. Cell biologists sort things out: analysis and purification of intracellular organelles by flow cytometry. Trends Cell Biol. 7, 499–503.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cavelier, L., Johannisson, A., Gyllensten, U., 2000. Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. Exp. Cell Res. 259, 79–85.

Feagin, J.E., 1992. The 6-kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes. Mol. Biochem. Parasitol. 52, 145–148.

Fry, M., Beesley, J.E., 1991. Mitochondria of mammalian *Plasmodium* spp.. Parasitology 102, 17–26.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R.W., Fraser, C.M., Barrell, B., 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419, 498–511.

Gardner, M.J., Bishop, R., Shah, T., de Villiers, E.P., Carlton, J.M., Hall, N., Ren, Q., Paulsen, I.T., Pain, A., Berriman, M., Wilson, R.J.M., Sato, S., Ralph, S.A., Mann, D.J., Xiong, Z., Shallom, S.J., Weidman, J., Jiang, L., Lynn, J., Weaver, B., Shoaibi, A., Domingo, A.R., Wasawo, D., Crabtree, J., Wortman, J.R., Haas, B., Angiuoli, S.V., Creasy, T.H., Lu, C., Suh, B., Silva, J.C., Utterback, T.R., Feldblyum, T.V., Pertea, M., Allen, J., Nierman, W.C., Taracha, E.L., Salzberg, S.L., White, O.R., Fitzhugh, H.A., Morzaria, S., Venter, J.C., Fraser, C.M., Nene, V., 2005. Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. Science 309, 134–137.

Gero, A.M., Brown, G.V., O'Sullivan, W.J., 1984. Pyrimidine de novo synthesis during the life cycle of the intraerythrocytic stage of *Plasmodium falciparum*. J. Parasitol. 70, 536–541.

Greenwood, B.M., Bojang, K., Whitty, C.J., Targett, G.A., 2005. Malaria. Lancet 365, 487–498.

Gutteridge, W.E., Trigg, P.I., 1970. Incorporation of radioactive precursors into DNA and RNA of *Plasmodium knowlesi in vitro*. J. Protozool. 17, 89–96.

Hopkins, J., Fowler, R., Krishna, S., Wilson, I., Mitchell, G., Bannister, L., 1999. The plastid in *Plasmodium falciparum* asexual blood stages: a three-dimensional ultrastructural analysis. Protist 150, 283–295.

Kohler, S., Delwiche, C.F., Denny, P.W., Tilney, L.G., Webster, P., Wilson, R.J.M., Palmer, J.D., Roos, D.S., 1997. A plastid of probable green algal origin in Apicomplexan parasites. Science 275, 1485–1489.

Krungkrai, J., 1995. Purification, characterization and localization of mitochondrial dihydroorotate dehydrogenase in *Plasmodium falciparum*, human malaria parasite. Biochim. Biophys. Acta 1243, 351–360.

Krungkrai, J., Krungkrai, S.R., Suraveratum, N., Prapunwattana, P., 1997. Mitochondrial ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase: chemotherapeutic targets in malarial parasites. Biochem. Mol. Biol. Int. 42, 1007–1014.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. 65, 418–420.

Mi-Ichi, F., Miyadera, H., Kobayashi, T., Takamiya, S., Waki, S., Iwata, S., Shibata, S., Kita, K., 2005. Parasite mitochondria as a target of chemotherapy: inhibitory effect of licochalcone A on the *Plasmodium falciparum* respiratory chain. Ann. N. Y. Acad. Sci. 1056, 46–54.

Prapunwattana, P., O'sullivan, W.J., Yuthavong, Y., 1988. Depression of *Plasmodium falciparum* dihydroorotate dehydrogenase activity in *in vitro* culture by tetracycline. Mol. Biochem. Parasitol. 27, 119–124.

Ralph, S.A., van Dooren, G.G., Waller, R.F., Crawford, M.J., Fraunholz, M.J., Foth, B.J., Tonkin, C.J., Roos, D.S., McFadden, G.I., 2004. Metabolic maps and functions of the *Plasmodium falciparum* apicoplast. Nat. Rev. 2, 203–216.

Roth Jr., E.F., Calvin, M.C., Max-Audit, I., Rosa, J., Rosa, R., 1988. The enzymes of the glycolytic pathway in erythrocytes infected with *Plasmodium falciparum* malaria parasites. Blood 72, 1922–1925.

Sato, S., Rangachari, K., Wilson, R.J.M., 2003. Targeting GFP to the malarial mitochondrion. Mol. Biochem. Parasitol. 130, 155–158.

Sato, S., Clough, B., Coates, L., Wilson, R.J., 2004. Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite *Plasmodium falciparum*. Protist 155, 117–125.

Sherman, I.W., 1979. Biochemistry of Plasmodium (malaria parasite). Microbiol. Rev. 43, 453–495.

Srivastava, I.K., Rottenberg, H., Vaidya, A.B., 1997. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J. Biol. Chem. 272, 3961–3966.

Srivastava, I.K., Morrisey, J.M., Darrouzet, E., Daldal, F., Vaidya, A.B., 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome *b* in malaria parasites. Mol. Microbiol. 33, 704–711.

Slomianny, C., Prensier, G., 1986. Application of the serial sectioning and tridimensional reconstruction techniques to the morphological study of the *Plasmodium falciparum* mitochondrion. J. Parasitol. 72, 595–598.

Suraveratum, N., Krungkrai, S.R., Leangaramgul, P., Prapunwattana, P., Krungkrai, J., 2000. Purification and characterization of *Plasmodium falciparum* succinate dehydrogenase. Mol. Biochem. Parasitol. 105, 215–222.

Takashima, E., Takamiya, S., Takeo, S., Mi-ichi, F., Amino, H., Kita, K., 2001. Isolation of mitochondria from *Plasmodium falciparum* showing dihydroorotate dependent respiration. Parasitol. Int. 50, 273–278.

Takeo, S., Kokaze, A., Ng, C.S., Mizuchi, D., Watanabe, J.I., Tanabe, K., Kojima, S., Kita, K., 2000. Succinate dehydrogenase in *Plasmodium falciparum* mitochondria: molecular characterization of the SDHA and SDHB genes for the catalytic subunits, the flavoprotein (Fp) and iron–sulfur (Ip) subunits. Mol. Biochem. Parasitol. 107, 191–205.

Tan, T.M., Nelson, J.S., Ng, H.C., Ting, R.C., Kara, U.A., 1997. Direct PCR amplification and sequence analysis of extrachromosomal Plasmodium DNA from dried blood spots. Acta Trop. 68, 105–114.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Vaidya, A.B., Akella, R., Suplick, K., 1989. Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. Mol. Biochem. Parasitol. 35, 97–107.

van den Hoff, M.J., Moorman, A.F., Lamers, W.H., 1992. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res. 20, 2902.

van Dooren, G.G., Marti, M., Tonkin, C.J., Stimmler, L.M., Cowman, A.F., McFadden, G.I., 2005. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol. Microbiol. 57, 405–419.

Vincent, R., Nadeau, D., 1983. A micromethod for the quantitation of cellular proteins in Percoll with the Coomassie brilliant blue dye-binding assay. Anal. Biochem. 135, 355–362.

Vollmer, M., Thomsen, N., Wiek, S., Seeber, F., 2001. Apicomplexan parasites possess distinct nuclear-encoded, but apicoplast-localized, plant-type ferredoxin-$NADP^+$ reductase and ferredoxin. J. Biol. Chem. 276, 5483–5490.

Wilson, R.J., Fry, M., Gardner, M.J., Feagin, J.E., Williamson, D.H., 1992. Subcellular fractionation of the two organelle DNAs of malaria parasites. Curr. Genet. 21, 405–408.

Wilson, R.J.M., Denny, P.W., Preiser, P.R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D.J., Moore, P.W., Williamson, D.H., 1996. Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. 261, 155–172.

Review TRENDS in Parasitology Vol.23 No.5

Parasitology in Japan

Advances in drug discovery and biochemical studies

Kiyoshi Kita[1], Kazuro Shiomi[2] and Satoshi Ōmura[2]

[1] Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan
[2] Kitasato Institute for Life Sciences, Kitasato University and The Kitasato Institute, Minato-ku, Tokyo 108-8641, Japan

Japanese researchers continue to discover new means to combat parasites and make important contributions toward developing tools for global control of parasitic diseases. *Streptomyces avermectinius*, the source of ivermectin, was discovered in Japan in the early 1970s and renewed and vigorous screening of microbial metabolites in recent years has led to the discovery of new antiprotozoals and anthelminthics, including antimalarial drugs. Intensive studies of parasite energy metabolism, such as NADH–fumarate reductase systems and the synthetic pathways of nucleic acids and amino acids, also contribute to the identification of novel and unique drug targets.

Parasitology in Japan: pioneering aspects

Japanese researchers have had a long and successful history in the field of parasitology, perhaps best exemplified by the accomplishments of Kitasato. In establishing a personal and long-term collaboration with Koch and other European researchers and institutes, Kitasato paved the way for numerous compatriots to follow. He also recognized that the public and private sectors had differing but equally important contributions to make in the battle to understand and conquer parasitic diseases. The first man-made chemotherapeutic compound, trypan red, was discovered by Ehrlich and Shiga, who was sent to work alongside Ehrlich from Kitasato's research group in Japan [1]. Trypan red (Figure 1) was effective against trypanosomal infections of mice and its analog, suramin, is still an important drug in human African trypanosomiasis. In Japan, several key infectious diseases, such as malaria and schistosomiasis, have been eliminated and the first successful eradication of filariasis was accomplished in the 1970s by mass administration of the drug diethylcarbamazine [2,3]. Ivermectin, one of the most effective antiparasite drugs, also originated in Japan. This article focuses on recent progress in Japan on novel antiparasite-drug discovery and recent biochemical studies of parasite metabolism that indicate potential new drug targets (Table 1).

Antiprotozoan and metazoan compounds

In Japan, the search for antiparasitic drugs began in earnest in 1922 when Nishi *et al.* produced antimony sodium tartrate (Stibnal®) and showed its effect on *Schistosoma japonicum* [4]. The sodium salt proved less toxic than the previously reported potassium salt. Santonin was isolated from the flower of *Artemisia cina* and used for ascariasis control [5]. It was isolated by Nippon Shinyaku Company Ltd (http://www.nippon-shinyaku.co.jp/) from *Artemisia maritima* in the late 1920s and has been used commercially in Japan since 1940 [6].

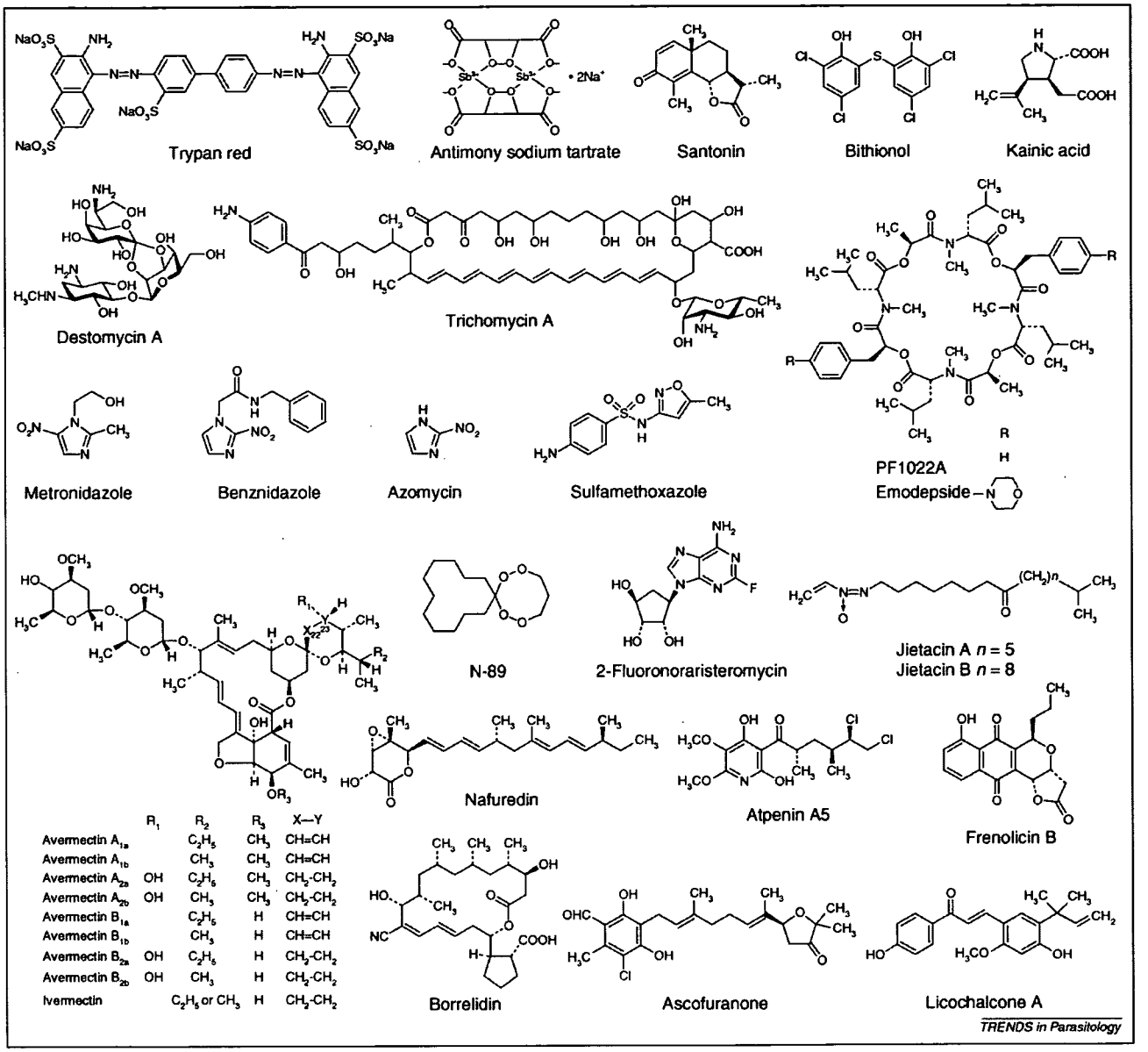
New antimetazoans

After World War II, Japan witnessed a flurry of intensive scientific activity and breakthroughs in the fight against parasitic diseases, which resulted in the development of several important antimetazoan-parasite drugs. For example, Yokogawa *et al.* discovered the effectiveness of bithionol in the chemotherapy of paragonimiasis [7] and it was widely used for trematode and cestode infections before praziquantel. Kainic acid, which was isolated from the red alga *Digenea simplex* by Murakami *et al.* [8], was used against some nematode infections as a mixture with santonin. Destomycin A, which is produced by the actinomycete *Streptomyces rimofaciens* and was isolated by Meiji Seika Kaisha Ltd (http://www.meiji.co.jp) [9], is an aminoglycoside that is used for the treatment of nematode infections in the veterinary field.

Isolated by Meiji Seika in the 1990s, PF1022A is a highly effective compound: a cyclic octadepsipeptide nematocide that is produced by the fungus *Rosellinia* spp. [10]. Emodepside, a semisynthetic derivative of PF1022A with a morpholine ring at each of the two D-phenyllactic acids in the para position [11], was later developed by Bayer HealthCare (http://www.bayerhealthcare.com), Meiji Seika and Astellas Pharma Inc. (http://www.astellas.com). Its target was identified as a novel 110 kDa heptahelical transmembrane receptor, named HC-110R, which is similar to mammalian latrophilins [12]. Latrophilins are latrotoxin (black widow spider venom) receptors and G-protein-coupled receptors that are implicated in the regulation of exocytosis. Latrotoxin also binds to HC110-R and causes influx of external Ca^{2+}. Emodepside works as an antagonist to latrotoxin signaling by impairing the influx of Ca^{2+}. It is highly effective against adult stages of the nematodes *Nippostrongylus brasiliensis* and *Strongyloides ratti* in rats and the nematode *Heligmosomoides polygyrus* in mice when used at an oral-dosage range of 1.0–10 mg/kg [11]. It can counteract resistance against the usual classes of anthelmintics and

Corresponding author: Ōmura, S. (omura-s@kitasato.or.jp).
Available online 23 March 2007.

www.sciencedirect.com 1471-4922/$ – see front matter doi:10.1016/j.pt.2007.03.005



	R_1	R_2	R_3	X—Y
Avermectin A_{1a}		C_2H_5	CH_3	CH=CH
Avermectin A_{1b}		CH_3	CH_3	CH=CH
Avermectin A_{2a}	OH	C_2H_5	CH_3	CH_2-CH_2
Avermectin A_{2b}	OH	CH_3	CH_3	CH_2-CH_2
Avermectin B_{1a}		C_2H_5	H	CH=CH
Avermectin B_{1b}		CH_3	H	CH=CH
Avermectin B_{2a}	OH	C_2H_5	H	CH_2-CH_2
Avermectin B_{2b}	OH	CH_3	H	CH_2-CH_2
Ivermectin		C_2H_5 or CH_3	H	CH_2-CH_2

Figure 1. Structures of antiparasitic compounds that were discovered by Japanese researchers. These compounds are either in current clinical use for parasite diseases or have antiparasitic activity *in vitro* and are good drug candidates. Refs: antimony sodium tartrate [4], ascofuranone [59–62], atpenin A5 [35], avermectins [19–23], azomycin [14,15], benznidazole [71], bithionol [7], borrelidin [38], destomycin A [9], emodepside [11,12], 2-fluoronoraristeromycin [18], frenolicin B [36], ivermectin [24–32], jietacins [33], kainic acid [8], licochalcone A [50], metronidazole [72], N-89 [17], nafuredin [34], PF1022A [10], santonin [5,6], sulfamethoxazole [16], trichomycin A [13], trypan red [1].

was launched in 2005 in Europe as a mixture with praziquantel to eliminate gastrointestinal helminths of cats.

New antiprotozoans

Japan has produced several important antiprotozoan drugs from natural sources. Trichomycin, isolated by Hosoya *et al.*, is used as an antitrichomonal and antifungal antibiotic [13]. It is a mixture of polyene macrolides that are produced by *Streptomyces hachijoensis*. The major component is a 38-membered ring heptaene macrolide, trichomycin A. Nitroimidazole group compounds, such as metronidazole and benznidazole, are derivatives of azomycin, which was isolated from the culture broth of *Streptomyces eurocidicus* by Umezawa *et al.* [14]. After the antitrichomonal activity of azomycin was reported, many such nitroimidazole compounds were then synthesized [15]. Sulfamethoxazole is a sulfa drug that was developed by Shionogi and Company (http://www.shionogi.co.jp) [16] and when mixed with trimethoprim is used as an antibacterial and used for the prevention and treatment of *Pneumocystis carinii*. Recently, several antimalarial candidates have been found by Japanese researchers. Cyclic peroxides with two peroxide groups in the same ring were synthesized by Wataya *et al.* and Nojima *et al.* to be analogous with artemisinin [17]. Among them, N-89 (1,2,6,7-tetraoxaspiro[7.11]nonadecane) showed antimalarial activity comparable to that of artemisinin both *in vitro* and *in vivo* and it cured infection with no parasite

Table 1. Japanese achievements in drug discovery and biochemical studies of parasites

	Year	Research group	Refs
Drug discovery			
Trypan red, the first man-made chemotherapeutic compound	1904	Ehrlich and Shiga	[1]
Antimony sodium tartrate for schistosomiasis	1922	Nishi and colleagues	[4]
Isolation of trichomycin from *Streptomyces hachijoensis*	1952	Hosoya and colleagues	[13]
Isolation of azomycin, the origin of nitroimidazoles	1953	Umezawa and colleagues	[14]
Isolation of kainic acid from *Digenea simplex*	1953	Murakami and colleagues	[8]
Synthesis of sulfamethoxazole	1957	Kano and Ogata	[16]
Bithionol for paragonimiasis	1962	Yokogawa and colleagues	[7]
Isolation of destomycin A from *Streptomyces rimofaciens*	1965	Kondo and colleagues	[9]
Isolation of avermectins from *Streptomyces avermectinius*	1979	Ōmura, Burg, Campbell and colleagues	[20,21]
Isolation of PF1022A, the origin of emodepside	1992	Sasaki and colleagues	[10]
Biochemical studies			
Anerobic respiratory chain of *Ascaris suum*	1984	Oya and colleagues	[39]
Trypanosome alternative oxidase and its specific inhibitor, ascofuranone	1996	Kita and colleagues	[59]
Pyrimidine biosynthetic gene cluster	1999	Aoki and colleagues	[51]
Unsaturated fatty acids essential for *Plasmodium* growth	2000	Mitamura and colleagues	[42]
Stage-specific isoforms of *A. suum* complex II	2003	Kita and colleagues	[41]
Peroxiredoxins in *Plasmodium*	2003	Kawazu and colleagues	[46]
Unique metabolism of sulfur-containing amino acids in *Entamoeba histolytica*	2003	Nozaki and colleagues	[63]

recrudescence or toxicity following oral administration in mice. Work has also indicated that *S*-adenosyl-L-homocysteine hydrolase might be a good target for antimalarial drugs. 2-fluoronoraristeromycin, designed by Kitade *et al.*, showed good selectivity for this enzyme in *Plasmodium falciparum* compared with that in humans [18].

Discovery of avermectins and other antiparasitics

Established by its namesake, The Kitasato Institute (KI: **http://www.kitasato.or.jp**) has a proud history of antibiotics discovery, including leucomycin (kitasamycin) and mitomycin C. Ivermectin is an endectocide that is active against a diverse range of nematodes, insects and arachnids and has its origins in the institute [19]. Ivermectin is now recognized by many as one of the most important drugs in human and animal health of the past century. In 1973, researchers at the KI started to screen nematocidal antibiotics in collaboration with Merck, Sharpe and Dohme Research Laboratories (MSD: **http://www.msd.com**). The Kitasato group isolated cultures from soil samples and undertook *in vitro* evaluation of the fermentation broths. In the MSD laboratories, promising cultures were screened using a novel model of helminth infection – mice infected with the nematode *Nematospiroides dubius*. The broth of strain MA-4680 exhibited potent anthelmintic activity [20]. This strain originated in a soil sample collected at Kawana, Shizuoka prefecture [21] and was classified as a new species of actinomycetes and named *Streptomyces avermectinius* (formerly *Streptomyces avermitilis*) [22]. Eight active components were isolated from the broth and named avermectins A_{1a}–B_{2b}. Compounds of the B series, which have a 5-hydroxy group, are markedly more active than those of the A series, which have a 5-methoxy group. Reduction of the 22,23-olefin of the most active avermectins B_{1a} and B_{1b} improved both the activity spectrum and the safety and the resulting 22,23-dihydro B_1 complex (as a mixture of 80% B_{1a} and 20% B_{1b}) was marketed as a veterinary drug under the generic and nonproprietary name ivermectin in 1981 [23].

Ivermectin is used for the control of economically important nematodes that infect the gastrointestinal tract, lungs and kidneys, in addition to other nematodes of livestock such as *Thelazia* and *Parafilaria*. It is also used against several arthropod parasites, including grubs, lice, mites and screw worms [24]. In companion animals, it provides good control of *Dirofilaria immitis*. Ivermectin was later found to be safe and highly effective in controlling some nematode infections in humans. Of greatest importance is that it proved highly efficacious against microfilariae of *Onchocerca volvulus*, which causes river blindness. Unique, pioneering and multifaceted public–private partnerships (PPPs) were established and eventually incorporated several United Nations agencies (**http://www.un.org**) and nongovernmental organizations. These PPPs enabled the donation of ivermectin (under the brand name Mectizan®) and led to a model for a highly successful mass drug administration (MDA) program that should culminate in the elimination of onchocerciasis in the endemic areas of Africa and the Americas [25]. Ivermectin is a remarkably safe and effective broad-spectrum drug. Side effects, when they do occur, are minimal and tolerable, usually resulting from inflammatory and immune defenses that are triggered by immobilized or dead microfilariae [26]. Therefore, ivermectin can be administered by nonmedical individuals after a modicum of training and can be used in MDA programs that are run by affected communities themselves. Although ivermectin is also effective against *Loa loa* [26], a few severe adverse events have occurred in individuals with high levels of *L. loa* microfilariae that have been treated with ivermectin [27]. Recently, ivermectin has also become an invaluable tool for a global elimination initiative against lymphatic filariasis, a disease that is caused by infection with *Wuchereria bancrofti* and *Brugia malayi* [26]. This means that the drug will be available free of charge in order to rid the world of two devastating diseases and to improve the lives of hundreds of millions of people. Ivermectin has now been found to be the most useful agent for strongyloidiasis [28]. In addition to the control of nematode infections, ivermectin also shows efficacy against human scabies, which is caused by infection with the ectoparasitic mite *Sarcoptes scabiei* [29].

Avermectin targets a parasite-specific glutamate-gated chloride channel [30], which it activates to cause neurological disruption of the parasite. Although avermectins also bind to γ-aminobutyric acid-gated and glycine-gated chloride channels in mammals, their affinity for invertebrate receptors is ~100-times higher. Mice that have the P-glycoprotein gene knocked out showed increased sensitivity to ivermectin [31]. This result established an important role for P-glycoprotein in the maintenance of the blood–brain barrier and indicated that the safety of ivermectin is because of the blood–brain barrier, in addition to the low affinity of the receptors.

In 2003, the Kitasato group elucidated the complete genetic sequence of *S. avermectinius* [32]. The genome is the largest bacterial genome yet sequenced (9026 kb) and encodes ≥7574 potential open reading frames. Analysis identified the gene cluster that is involved in secondary-metabolite production and sheds light on the biology of microbial secondary-metabolite synthesis at the genetic level, thus offering the prospect of creating some new metabolites.

After the discovery of avermectin, the Kitasato group isolated jietacins A and B from the culture broth of *Streptomyces* spp. using the pine wood nematode, *Bursaphelenchus lignicolus* [33]. Jietacins show a ~10-times higher nematocidal activity than avermectin B_{1a} against *B. lignicolus in vitro*. Nafuredin, produced by the fungus *Aspergillus niger*, was found during screening for an NADH–fumarate reductase inhibitor [34]. It inhibits helminth electron transport enzyme complex I and shows anthelmintic activity against *Haemonchus contortus* in sheep (see later). Atpenin A5 was discovered during the same screening and is the most potent complex II inhibitor. It might, therefore, be a useful tool for clarifying the biochemical and structural properties of complex II [35]. In the case of antiprotozoan compounds, frenolicin B, which is produced by *Streptomyces roseofulvus*, has potent anticoccidial activity in *Eimeria tenella* infection in chicks [36].

Building on the increased global awareness that the public and private sectors need to work together if the major communicable diseases are to be overcome, a PPP that includes leading Japanese pharmaceutical companies, the Japanese Ministry of Health, Labour and Welfare, and the World Health Organization was established in 1999 to discover new antimalarials [37] and is collectively known as the JPMW project. A screening center was established at the KI in which evaluation of antimalarial activity of compounds is carried out both *in vitro* and using the rodent malaria model. These compounds come from two sources: those donated from the chemical libraries of 14 Japanese companies and natural products from the KI. More than 30 000 compounds have been screened since 2000 and one compound, borrelidin, shows excellent antimalarial activity in both *in vitro* chloroquine (CQ)-susceptible and CQ-resistant *P. falciparum* models [38]. It also has antimalarial activity against rodent malaria. The effective dose is lower than that required for artemether, artesunate and CQ itself. The 90% effective dose (ED_{90}) values of orally-administered borrelidin, artemether, artesunate and CQ against CQ-resistant *P. yoelii*-infected mice were 1.1, 40, >50 and >100 mg/kg, respectively [38].

Elucidation of biochemical targets in protozoan and metazoan parasites

Parasites have developed a range of physiological functions that are necessary for their survival within the specialized environment of the host and studies of these parasitic adaptations have provided interesting biological discoveries [39]. Recent advances in biochemical and molecular biological approaches have provided new knowledge and led to many breakthroughs in the field of biological evolution and parasite diversity, with the novel understanding of unique aspects of parasite metabolism heralding promising targets for chemotherapy.

The anerobic respiratory chain of mitochondria in helminths

Recent research by Kita *et al.* on the respiratory chain of the parasitic helminth *A. suum* has shown that the mitochondrial NADH–fumarate reductase system (**Figure 2**) has an important role in the anerobic energy metabolism of adult parasites, in addition to unique features of the developmental changes that occur during their life cycle [39]. In this system, the reducing equivalent of NADH is transferred to the low-potential rhodoquinone (RQ) by the NADH–RQ reductase complex (complex I). This pathway ends with the production of succinate by the rhodoquinol–fumarate reductase activity of complex II [succinate–ubiquinone (UQ) reductase in aerobic respiration]. Electron transfer from NADH to fumarate is coupled to site I phosphorylation of complex I by generation of a proton-motive force. The difference in redox potential between the NAD^+–NADH couple ($Em' = -320$ mV) and the fumarate–succinate couple ($Em' = +30$ mV) is sufficiently high to drive ATP synthesis. The anerobic NADH–fumarate reductase system is found not only in nematodes but also in bacteria and many other parasites.

An anthelmintic compound, nafuredin, selectively inhibits helminth complex I at nanomolar concentrations [34]. Kinetic analysis showed that the inhibition by nafuredin is competitive against an exogenous RQ. These findings, coupled with the fact that helminth complex I uses both RQ and UQ as an electron acceptor, indicate that the structural features of the quinone reduction site of helminth complex I might differ from that of mammalian complex I. In fact, the inhibitory mechanism of quinazolines was competitive against RQ and partially competitive against UQ [40].

Amino *et al.* have demonstrated that *A. suum* mitochondria express stage-specific isoforms of complex II: the flavoprotein subunit and the small subunit of cytochrome *b* of the larval complex II differ from those of the adult enzyme, although two complex IIs share a common iron–sulfur cluster subunit [41]. Enzymatic assays indicated that *A. suum* complex IIs have different properties compared with complex IIs of mammalian hosts and that the larval complex II is able to function as a rhodoquinol–fumarate reductase. As mentioned earlier, the most potent inhibitor of complex II, atpenin A5, was found during screening of inhibitors for the *A. suum* complex II [35]. Osanai *et al.* obtained a crystal of adult complex II and an analysis of parasite-specific factors in the enzyme complex is now in progress.

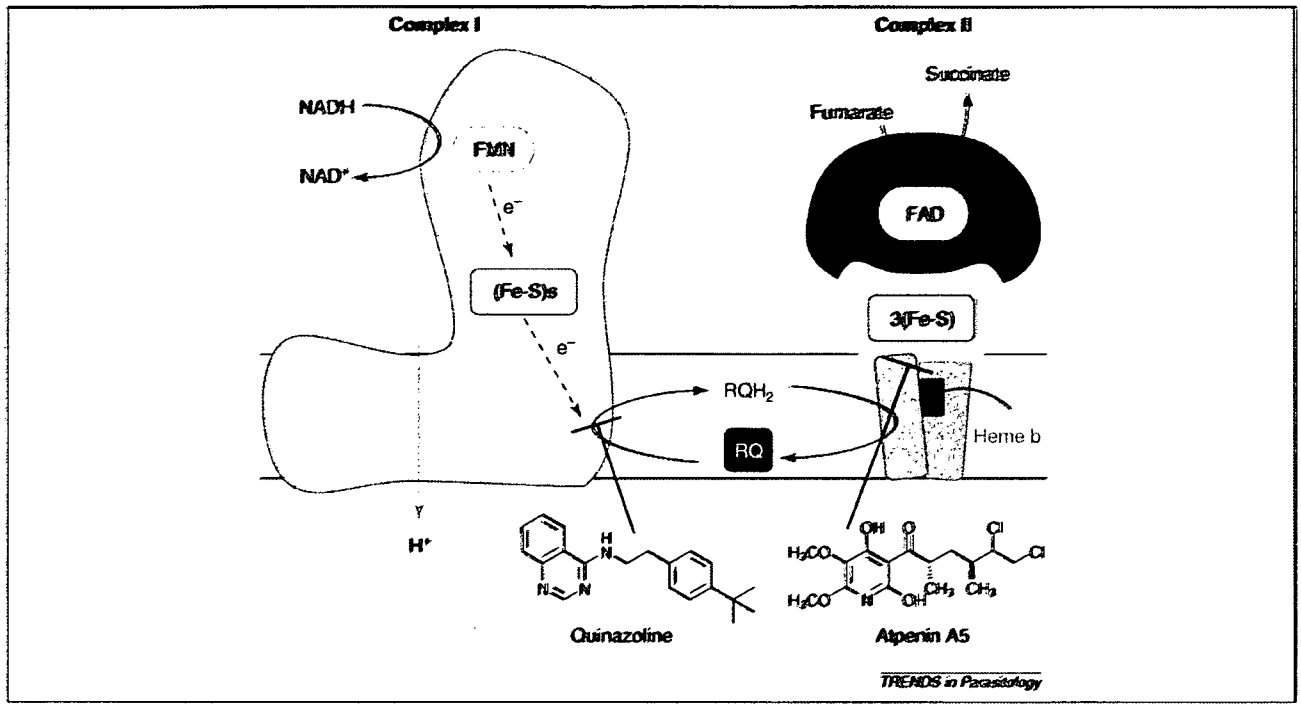


Figure 2. NADH–fumarate reductase system in *Ascaris suum* mitochondria. The respiratory inhibitors quinazoline [40] and atpenin A5 [35] inhibit *A. suum* complex I and complex II, respectively. Nafuredin (see Figure 1) also inhibits *A. suum* complex I [34]. Figure by K. Sakamoto, University of Tokyo.

Unique *Plasmodium* enzymes

P. falciparum is the causative agent for the most severe form of malaria in humans. Because clinical symptoms and complex pathogenesis are exclusively associated with the asexual multiplication of this parasite in erythrocytes, Mitamura and colleagues have focused on intra-erythrocytic proliferation of *P. falciparum* and lipid metabolism and trafficking in parasitized erythrocytes [42]. They found that limited combinations of saturated and unsaturated free fatty acids (the best combination of which is palmitic and oleic acids) are essential serum factors that are required for parasite growth to ensure complete intra-erythrocytic development and cell cycle progression [42]. This finding showed that scavenging free fatty acids from host serum is crucial for the survival of intra-erythrocytic *Plasmodium* parasites. Furthermore, Mitamura and colleagues have shown genetic evidence that *P. falciparum* diacylglycerol acyltransferase 1, a principal enzyme for triacylglycerol biosynthesis, seems to have a crucial role in the intra-erythrocytic proliferation of the parasite [43]. Their work on lipid metabolism and trafficking in *P. falciparum* has attracted attention with respect to both basic biology and its potential application for malaria chemotherapy [44].

Because the parasites do not possess catalase or glutathione peroxidase, the bulk of peroxide-reducing capacity in the cell seems to be provided by peroxiredoxins (Prx) [45]. Kawazu and his group reported that disruption of the gene that encodes the cytosolic 2-Cys Prx (PfTPx-1) in *P. falciparum* renders parasites hypersensitive to reactive oxygen and nitrogen species [46]. They also showed that the other cytosolic Prx (Pf1-Cys-Prx) might help to protect the parasite against oxidative stress that results from hemoglobin metabolism [47]. These findings indicate that parasites use the Prx proteins on different occasions for management of intracellular oxidative stresses during their intra-erythrocytic development [48]. How the parasite regulates proper expression of the antioxidant proteins under different physiological situations is a matter of great interest.

Differences between the mitochondria of malaria parasites and those of the host are also expected to indicate potential targets for chemotherapy. Using active mitochondria that have been isolated from *P. falciparum* [49], Miichi *et al.* investigated the licochalcone A-inhibited bc_1 complex (ubiquinol–cytochrome *c* reductase), in addition to complex II (succinate–ubiquinone reductase) of *P. falciparum* mitochondria [50]. Because the properties of the *P. falciparum* bc_1 complex are different from those of the mammalian host and are a target of atovaquone, chalcones are promising candidates for new antimalarial drugs.

Targets in trypanosomes and ameba

Nara and coworkers reported a novel, polycistronic pyrimidine-biosynthetic (*pyr*) cluster that contains five genes (*pyr1*, *pyr3*, *pyr6–5*, *pyr2* and *pyr4*) and encode all six enzymes that are involved in pyrimidine biosynthesis in *Trypanosoma cruzi* and *Leishmania* spp. [51]. Although most eukaryotes carry *pyr5–6*, kinetoplastids possess the conversely fused *pyr6–5*. This biosynthesis pathway is essential for protozoan survival [52,53]. In particular, the *pyr4*- and *pyr6*-encoded enzymes are promising drug targets because they differ biochemically from those of their mammalian-host counterparts, probably because these genes have been horizontally acquired [53–55]. Phylogenetic studies of the kinetoplastid *pyr6–5* indicated that