Selenium-induced apoptosis-like cell death in *Plasmodium falciparum*

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SUMMARY

Plasmodium falciparum has for some time been developing resistance against known anti-malarial drugs, and therefore a new drug is urgently needed. Selenium (Se), an essential trace element, in the form of inorganic Se, selenite (SeO_3^{2-}), has been reported to have an anti-plasmodial effect, but its mechanism is still unclear. In the present study, we evaluated the anti-plasmodial effect of several Se compounds against *P. falciparum in vitro*. The anti-plasmodial effect of several Se compounds was analysed and their apoptosis-inducing activity was evaluated by morphological observation, DNA fragmentation assay and mitochondrial function analysis. SeO_3^{2-}, methylseleninic acid, selenomethionine and selenocystine have anti-plasmodial effects with 50% inhibition concentration at 9, 10, 45, and 65 μM, respectively, while selenate and methyl-selenocysteine up to 100 μM have no effect on parasite growth. The effective Se compounds caused the parasites to become shrunken and pyknotic and significantly increased mitochondrial damage against *P. falciparum* compared to the untreated control. In conclusion, SeO_3^{2-}, methylseleninic acid, selenomethionine and selenocystine have anti-plasmodial activities that induce apoptosis-like cell death in *P. falciparum*, and the anti-plasmodial effects of Se seem to be based on its chemical forms. The apoptosis-like cell-death mechanism in *P. falciparum* can be beneficial to respond to the growing problem of drug resistance.

Key words: selenium, malaria, *Plasmodium falciparum*, apoptosis, drug, DNA fragmentation.

INTRODUCTION

Malaria, a parasitic disease caused by infection with *Plasmodium* sp., causes 2 million deaths worldwide annually (Breman, 2009). Many drugs have lost their usefulness against *Plasmodium falciparum* due to growing drug resistance (Epstein, 1999). Even with the current multidrug therapy programme, *P. falciparum* has developed drug resistance against the latest anti-plasmodial drug from the artemisinine family (Rogers *et al*. 2009). Therefore, a new anti-plasmodial agent is urgently needed for a global malaria control programme.

The biological effects of selenium (Se), an essential trace element in animals, are based on its wide variety of chemical forms (Alexander, 2007). Inorganic Se compounds, SeO_3^{2-} and selenate (SeO_4^{2-}), are endogenously reduced to selenide (H_2Se) (Suzuki and Ogra, 2002), and during the reduction of the compounds, Se produces oxygen radicals (Yan and Spallholz, 1993). Selenomethionine (SeMet), a selenoamino acid, is metabolized in mouse hepatic cells through an enzymatic process (Suzuki *et al*. 2007). Organic Se, methylseleninic acid (MSeA), and methylselenocysteine have been used to generate methylselenol (CH_3SeH) (Suzuki *et al*. 2008), the most biologically active chemical form of Se against human cancer cells (Wang *et al*. 2002; Zeng *et al*. 2006). However, Se metabolism in *P. falciparum* is still unknown.

A previous study by Taguchi *et al*. (2004) was originated with the notion that Se, in the form of sodium selenite (SeO_3^{2-}), had the ability to generate reactive oxygen species (ROS) (Davis and Spallholz, 1996), which would increase the oxidative stress level and allow SeO_3^{2-} to have anti-plasmodial effects. The study demonstrated that, indeed, SeO_3^{2-} exhibited an anti-plasmodial effect against drug-resistant *P. falciparum*. A decrease in a reduced type of glutathione (GSH) in parasitized red blood cells (pRBCs) was also observed, indicating oxidative stress involvement in the process. The cytoplasm of dead *P. falciparum* was shown to be shrunken after SeO_3^{2-} treatment. The characteristics of the morphology of these parasites are similar to those of human cancer cells undergoing apoptotic cell death (Vermeulen

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et al. 2005), which leads us to suspect that apoptosis is the anti-plasmodial mechanism of SeO_3^{2-}. In this study, we investigated the anti-plasmodial effects of several Se compounds, inorganic Se, SeO_3^{2-}, SeO_4^{2-}, Se amino acids, SeMet, selenocystine, as well as CH_3SeH precursors, MSeA and MSeCys. Furthermore, we conducted DNA fragmentation and mitochondria function analysis to investigate the involvement of apoptosis in Se anti-plasmodial mechanisms.

MATERIALS AND METHODS

Plasmodium falciparum *continuous culture*

Chloroquine-resistant *P. falciparum* strain K-1 was grown asynchronously following the modified method of Trager and Jensen (Taguchi *et al*. 2004) in disposable culture dishes (Greiner, Frickenhausen, Germany) under a controlled atmosphere of 5% CO_2 and 5% O_2 at 37 °C. The parasite was grown in RPMI-1640 medium (Sigma–Aldrich, St Louis, MO, USA) containing 10% type B or O human serum (serum type showed no significant difference to parasite growth), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Wako, Osaka, Japan), 25 μg/ml gentamycin (Sigma–Aldrich, St Louis, MO, USA), 25 mM sodium bicarbonate (Wako, Osaka, Japan), and human type O red blood cells (RBCs) to make a final 5% haematocrit mixture.

Selenium compounds

SeO_3^{2-} and MSeA were purchased from Sigma (St Louis, MO, USA). L-selenocystine (selenocystine) was purchased from Acros Organics (Gael, Belgium). SeO_4^{2-}, methylseleno-L-cysteine (MSeCys), and seleno-L methionine (SeMet) were purchased from Wako (Osaka, Japan). Selenocystine and SeMet were dissolved in 3% hydrochloric acid (HCl) and kept as a 1 M stock solution at −80 °C until use. SeO_3^{2-}, MSeA, SeO_3^{2-}, and MSeCys were dissolved in milli-Q (Millipore, Tokyo, Japan) and kept as a 1 M stock solution at −80 °C until use.

Human cell cultures

The non-cancerous cell line CHEK-1, an immortalized human oesophageal cell line established by the transduction of human papillomavirus type 16 E6/E7 into primary cultures of oesophageal keratinocytes (Sashiyama *et al*. 2002), and the HC human liver cell line were used in the experiment. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Tokyo, Japan) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Invitrogen, Tokyo, Japan).

Growth inhibitory effect of Se compounds against Plasmodium falciparum

Previous studies have reported that Se compounds at concentrations between 10 and 100 μM have a cytotoxic effect against *P. falciparum* (Taguchi *et al*. 2004) or against human cancer cell lines (Lee *et al*. 2005; Nian *et al*. 2009). Therefore, anti-plasmodial activities of SeO_3^{2-}, MSeA, SeMet, selenocystine, SeO_4^{2-}, and MSeCys were assessed by exposing *P. falciparum* to a medium containing either 10 or 100 μM of each Se compound. Unsynchronized parasite culture with initial parasitaemia 0·1% and 5% haematocrit was used. The culture medium containing the Se compound was changed, and the number of pRBCs was counted every 24 h. pRBCs were counted by making Giemsa-stained thin-smeared slides, and the number of pRBCs in 3000 RBCs was determined under a light microscope at 1000 times magnification. This experiment was terminated at 72 h. Each concentration was made in triplicate, and the experiment was performed 3 times. In the SeMet and selenocystine experiments, HCl was added to the culture medium to make a 0·0003% final concentration mixture as the untreated control.

Dose-dependent anti-plasmodial effects of Se compounds against Plasmodium falciparum

The Se compounds that showed anti-plasmodial activity (SeO_3^{2-}, MSeA, SeMet, and selenocystine) were evaluated for their dose dependency, and their 50% inhibition concentrations (IC50) were calculated by the probit method as described previously (Taguchi *et al*. 2004). Artemisinin (TCI, Tokyo, Japan) diluted in ethanol to make a final concentration of 0·002% in the culture medium and chloroquine diphosphate (Wako, Osaka, Japan) diluted in milli-Q were used for comparison. A parasite culture with 2% initial parasitaemia was prepared and incubated for 24 h with each of the effective Se compound concentrations (0, 10, 20, 40, 50, and 100 μM). Parasitaemia was evaluated by making Giemsa-stained thin-smeared slides and counting the number of pRBCs from 3000 RBCs at 24 h, as described above.

Morphology of Plasmodium falciparum *after Se compound treatment*

The morphology of parasite cells was observed after treatment with Se compounds using the calculated IC50 values for SeO_3^{2-}, MSeA, SeMet, and selenocystine or the highest concentration from this experiment (100 μM) for SeO_4^{2-} and MSeCys.

The morphology of the parasite cells was observed by making Giemsa-stained thin-smeared slides, as described above. The parasite cells were observed under a light microscope with a total magnification of

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1000 times. We also determined the proportion of shrunken parasites by counting the number of shrunken parasites in 200 parasites from 3 independent experiments.

Stage specificity of Se compounds

The stage specificity of each Se compound's IC50 was also evaluated, as described previously (Taguchi *et al*. 2004).

DNA fragmentation assay

Our current study showed the inhibition effect of selenium only at the 24 h time-point, therefore the DNA fragmentation and mitochondrial damage were performed at this same time point. When *P. falciparum* parasitaemia reached 8–9% in a 40 ml culture, the pRBCs were incubated with IC50-values of SeO_3^{2-}, MSeA, SeMet, or selenocystine for 24 h. The culture was centrifuged at 1500 ***g*** at 4 °C for 10 min, and the medium was discarded. Serum-free RPMI-1640 medium and 0·15% saponin solution were added to the cell pellet at a volume proportion of 3:4:1. This mixture was incubated for 15 min at 37 °C and washed 3 times with phosphate-buffered saline (PBS) at pH 7·2. After centrifugation at 1500 ***g*** at 4 °C for 10 min, the PBS was discarded, and the free parasite pellet was retained.

DNA was extracted from the free parasite cell by the phenol-choloroform extraction method (Dame *et al*. 1984). The free parasite cell pellet was incubated with 37·5 μl of lysis buffer (40 mM Tris-HCl, pH 8·0; 80 mM EDTA, pH 8·0; 2% sodium dodecyl sulfate; and 50 μM proteinase K (Takara, Shiga, Japan)) and 112·5 μl of milli-Q for 3 h at 37 °C in a water bath. Then 150 μl of milli-Q and 300 μl of buffer-saturated phenol (Invitrogen, Tokyo, Japan) were added, and the parasite suspension was centrifuged at 15 000 ***g*** at 4 °C for 10 min. The aqueous phase of the solution was carefully extracted into a new tube, and 300 μl of chloroform were added. After extraction of the aqueous phase, 3 μl of 20 mg/ml RNase A solution (Invitrogen, Tokyo, Japan) were added, and the solution was incubated for 30 min at 37 °C. The phenol and chloroform extraction process was performed once more. DNA was precipitated by adding 30 μl of 3 M sodium acetate and 750 μl of 99% ethanol and kept at −20 °C overnight. The solution was then centrifuged at 20 000 ***g*** at 4 °C for 15 min, and the supernatant was removed. The DNA precipitate was washed with 70% ethanol and dried at room temperature. The DNA precipitate was dissolved in milli-Q water.

Ten μg of parasite DNA from each Se compound-treated parasite sample were applied to a Multigel II Mini 10% polyacrylamide gel (Cosmo Bio Co., Ltd, Tokyo, Japan) and electrophoresed in a buffer solution containing 25 mM Tris and 192 mM glycine at 15 mA constant current. The DNA migration pattern on the gel was visualized using the 2D-Silver Stain Reagent Kit (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's manual.

Mitochondria function analysis

Mitochondrial damage was evaluated by analysing the mitochondria transmembrane potential using Carbocyanine dye JC-1 of the mitochondria staining kit (Sigma–Aldrich, St Louis, MO, USA). Damaged mitochondria will lose their transmembrane potential and exhibit green monomers with an emission maximum of 527 nm; meanwhile, functioning mitochondria will retain their membrane polarization and exhibit red aggregates with an emission maximum of 590 nm. This knowledge permitted us to analyse the ratio of parasites with damaged and functioning mitochondria. The kit provided valinomycin, a mitochondrial dissipating agent to be used for comparison.

Mitochondria membrane staining was performed according to the manufacturer's instructions. Briefly, after treatment with IC50-values of SeO_3^{2-}, MSeA, SeMet, or selenocystine for 24 h, an unsynchronized parasite culture with 2% parasitaemia and 5% haematocrit, on a 96-well plate was centrifuged at 1500 ***g*** at 4 °C for 10 min, washed using RPMI solution, and incubated with 7·5 nM of JC-1 for 45 min at 37 °C. Quantitative analysis was performed using a multi-well plate reader (CytoFluor, Perspective Biosystems, Framingham, MA, USA) at a 490 nm excitation wavelength and a 530 nm emission wavelength for JC-1 monomers. JC-1 aggregates were measured at a 525 nm excitation wavelength and a 590 nm emission wavelength. The mitochondria function of parasites for each treatment group was then assessed by dividing the number of JC-1 aggregates by the value of the JC-1 monomer.

Haemolysis of human red blood cells by Se compound treatment

The haemolytic level was evaluated by measuring the haemoglobin (Hb) concentration released into the medium using a Haemoglobin B test kit (Wako, Osaka, Japan) according to the manufacturer's instructions. Briefly, after the incubation of RBCs with several concentrations of SeO_3^{2-} for 24 h, samples were centrifuged at 5000 ***g*** at 20 °C for 15 min. The supernatant was collected and analysed by measuring the absorbance at a wavelength of 550 nm with a reference wavelength of 600 nm. The absorbance was read using a microtitre plate reader (Becton-Dickinson, NJ, USA).

The same protocol was followed for experiments with MSeA, SeMet, selenocystine, SeO_4^{2-}, and MSeCys.

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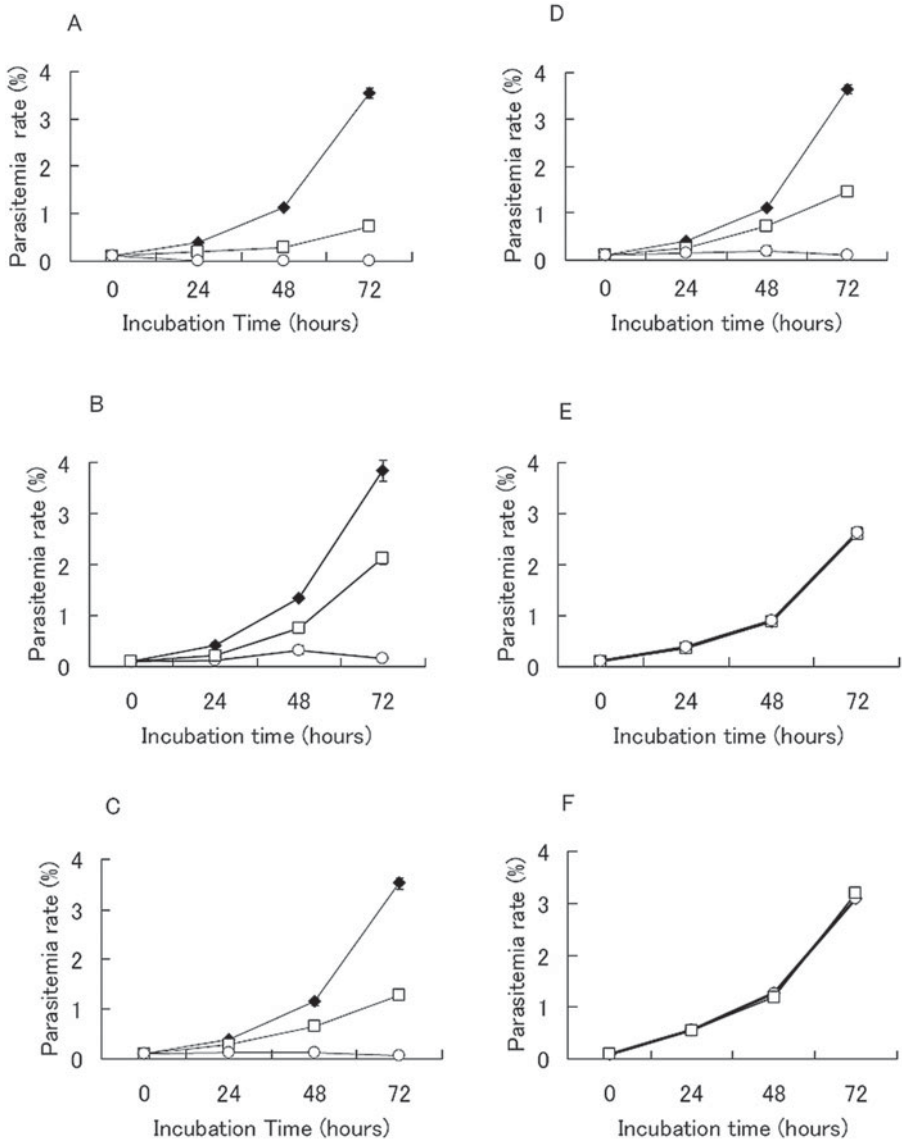

Fig. 1. Anti-plasmodial effect of selenium (Se) compounds, selenite (SeO_3^{2-}) (A), methylseleninic acid (MSeA) (B), selenomethionine (SeMet) (C), selenocystine (D), selenate (SeO_4^{2-}) (E), and methylselenocysteine (MSeCys) (F), on *Plasmodium falciparum*. Each Se compound was added to the culture medium to make a final concentration of 10 μM (white square) or 100 μM (white circle); meanwhile, the control group was treated only with the vehicle (black diamond). Parasitaemia (%) was determined every 24 h. Results are presented as means ± S.E.M. S.E. bars smaller than the symbols are not shown.

Cytotoxicity of Se compounds against human cell lines

Cell proliferation inhibition analysis was performed with HC cell lines and CHEK cell lines in the presence of various concentrations of SeO_3^{2-} by a colourimetric methyl thiazolyl tetrazolium (MTT) assay, as described previously (Faried *et al*. 2006). Briefly, cells (2×10^4 cells in 50 μl/well) were plated in 96-well plates. After the initial cell seeding, different concentrations of SeO_3^{2-} were added and incubated for 24 h. Ten μl of WST-8 assay cell-counting solution (Dojindo Lab., Tokyo, Japan) were added to each well and incubated at 37 °C for 3 h. After the addition of 100 μl of 1 N HCl/well, the cell proliferation inhibition rate was determined by measuring the absorbance at a wavelength of 450 nm with a reference wavelength of 650 nm. The absorbance was read using a microtitre plate reader (Becton-Dickinson, NJ, USA).

The same protocol was followed for MSeA, SeMet, selenocystine, SeO_4^{2-}, and MSeCys experiments.

Statistical analyses

All data are presented as means ± S.E.M. from at least 3 sets of independent experiments. A one-way ANOVA test followed by the Dunnett Test was used for mitochondria function analysis. A P value <0·05 was considered statistically significant. IC50 and LD50 values were calculated using probit

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method. All statistical analyses were performed on R free software.

RESULTS

Inhibitory effect of Se compounds against Plasmodium falciparum *growth*

Figure 1 shows the growth inhibition effects of SeO_3^{2-}, MSeA, SeMet, selenocystine, SeO_4^{2-}, and MSeCys against *P. falciparum*. The parasitaemia rate of untreated control *P. falciparum* was increased from 0·1% at 0 h to 0·33–0·6% at 24 h, to 0·83–1·27% at 48 h, and to 2·63–3·97% at 72 h.

The parasitaemia rate of 10 μM SeO_3^{2-}-treated *P. falciparum* increased to 0·18% at 24 h, to 0·28% at 48 h, and to 0·72% at 72 h (Fig. 1A). The parasitaemia rate of 100 μM SeO_3^{2-}-treated parasites decreased to 0% at 24 h until 72 h (Fig. 1A). This result confirms that SeO_3^{2-} has an inhibitory effect against *P. falciparum* growth (Taguchi *et al*. 2004).

MSeA (Fig. 1B), SeMet (Fig. 1C), and selenocystine (Fig. 1D) at concentrations of 10 μM and 100 μM in 24-, 48-, and 72-h periods also demonstrated decreasing parasitaemia rates compared to their untreated controls. Unlike SeO_3^{2-}, they did not decrease the parasitaemia rate to 0%.

On the other hand, SeO_4^{2-} (Fig. 1E) and MSeCys (Fig. 1F) at 10 μM and 100 μM showed parasitaemia rates similar to those of the untreated control up to 72 h. Therefore, we conclude that SeO_4^{2-} and MSeCys have no inhibitory effects against *P. falciparum* growth at these concentrations.

Dose-dependent anti-plasmodial effects of Se compounds against Plasmodium falciparum

The growth-inhibition rates of SeO_3^{2-}, MSeA, SeMet, and selenocystine at several concentrations against *P. falciparum* growth after 24 h of incubation are shown in Fig. 2.

The inhibition rate of 10 μM SeO_3^{2-} against *P. falciparum* growth was 52·95%, while that of 20 μM SeO_3^{2-} was 80·72%. The inhibition rate of 40 μM SeO_3^{2-} reached 100%. SeO_3^{2-} concentrations of 50 μM and 100 μM showed results similar to that of 40 μM. These results show that the inhibition rate of SeO_3^{2-} reaches a plateau at 40 μM and the anti-plasmodial effects of SeO_3^{2-} are dose dependent. The 50% inhibition concentration (IC50) value of SeO_3^{2-} was calculated to be 9 μM.

MSeA, SeMet, and selenocystine showed increasing inhibition against *P. falciparum* growth with increasing concentration, although not one of them at any concentration reached the 100% inhibition level. The IC50-values of MSeA, SeMet, and selenocystine were calculated to be 10, 45, and 65 μM, respectively. As a comparison, IC50 values of artemisinin and chloroquine diphosphate were calculated to be 43 nM and 660 nM respectively (chloroquine diphosphate results were not fitted into Fig. 2 due to the concentration range difference with selenium compounds). The IC50-value of artemisinin was consistent with the previous report (Thanh *et al*. 2010).

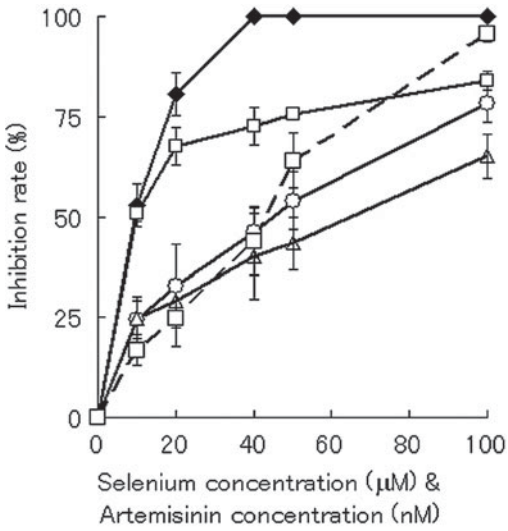

Fig. 2. Dose-dependent effect of various selenium (Se) compound treatments on *Plasmodium falciparum* in μM, selenite (SeO_3^{2-}) (black diamond), methylseleninic acid (MSeA) (white square), selenomethionine (SeMet) (white circle), selenocystine (white triangle) and artemisinin as comparison in nM (dashed line with white square). All parasitaemia results are divided by the control values to show the inhibition rate. Inhibition rates are presented as means ± S.E.M.

Morphology of Plasmodium falciparum *after Se compound treatment*

As shown in Fig. 3, we observed the morphology of *P. falciparum* after Se compound treatment. Ring-form, late trophozoite and schizont stages of *P. falciparum* were seen in the untreated control culture after 24 h of incubation (Fig. 3A). Shrunken and pyknotic parasite cells of either ring-form or late trophozoite/schizont stage were observed after incubation with IC50-values of SeO_3^{2-} (Fig. 3B), MSeA (Fig. 3C), SeMet (Fig. 3D), or selenocystine (Fig. 3E) for 24 h. The proportions of shrunken parasites after treatment with effective Se compounds are as follows: SeO_3^{2-}: 50·33%, MSeA: 23%, SeMet: 48·33%, selenocystine: 21·67%. On the other hand, parasite cells that were incubated with this experiment's highest concentration (100 μM) of SeO_4^{2-} (Fig. 3F) or MSeCys (Fig. 3G) for 24 h showed parasites similar to those in the control.

Stage specificity of Se compounds

The stage specificity experiments showed that SeO_3^{2-}, MSeA, SeMet, or selenocystine at their

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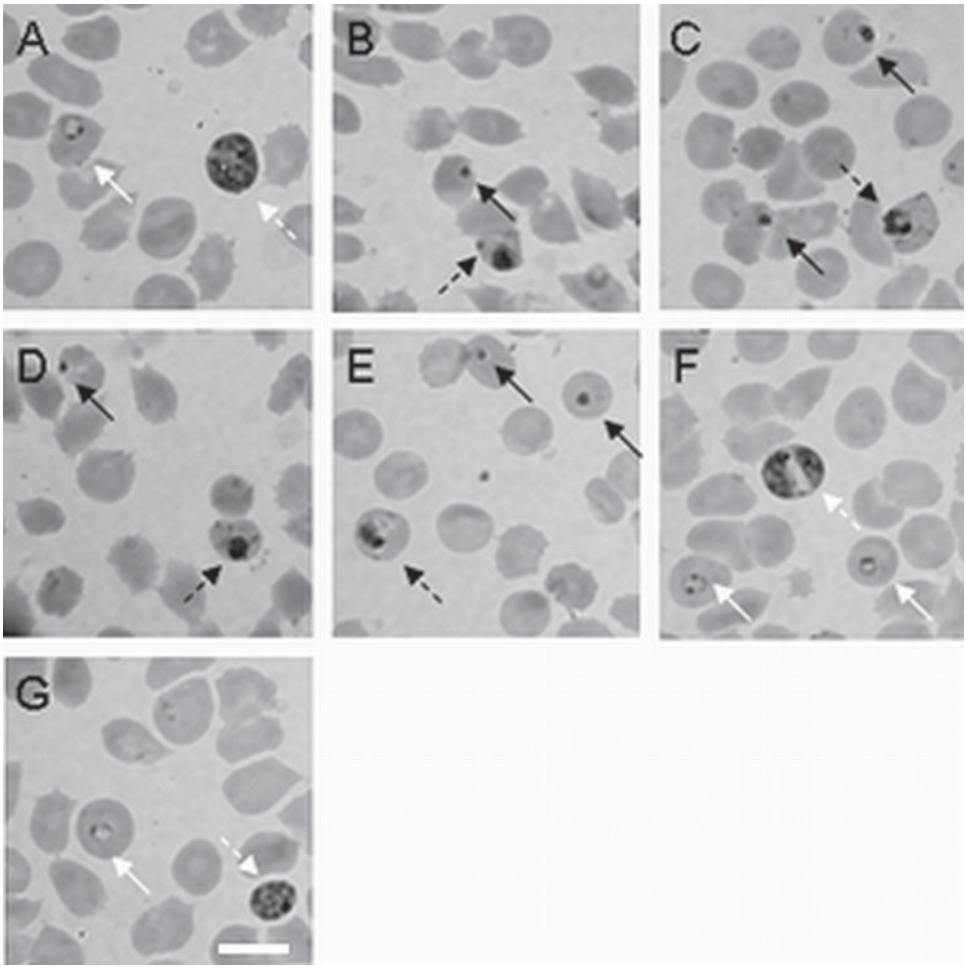

Fig. 3. Morphology of *Plasmodium falciparum* seen under light microscopy, after 24 h of incubation. Ring-form (solid white arrow) or late trophozoite/schizont (dashed white arrow) parasites from untreated parasite (A), or treated with 100 μM of either selenate (SeO_4^{2-}) (F), or methylselenocysteine (MSeCys) (G). Shrunken and pyknotic ring-form (solid black arrow) or late trophozoite/schizont (dashed black arrow) parasites after treatment with inhibition concentration 50% values of selenite (SeO_3^{2-}) (B), methylseleninic acid (MSeA) (C), selenomethionine (SeMet) (D), or selenocystine (E). Scale bar = 10 μm.

IC50 values have similar inhibiting effects on parasite growth at any developmental stage.

DNA fragmentation of Plasmodium falciparum *after Se compound treatment*

The results of the DNA fragmentation assay of *P. falciparum* after Se compound treatment are shown in Fig. 4. *Plasmodium falciparum* treated with SeO_3^{2-} showed a laddering pattern (Fig. 4B) at a region under 300 base pairs. Meanwhile, untreated parasites showed no ladder (Fig. 4A). MSeA (Fig. 4C), SeMet (Fig. 4D), and selenocystine (Fig. 4E) showed results similar to those for SeO_3^{2-}.

Mitochondria function analysis of Plasmodium falciparum *after Se compound treatment*

There are several known apoptosis pathways, one of which involves permeabilization of mitochondria, which results in the loss of mitochondria transmembrane potential. As shown in Fig. 5, we explored the effects of SeO_3^{2-}, MSeA, SeMet, and selenocystine on *P. falciparum* mitochondria.

Untreated *P. falciparum* showed a JC-1 red aggregate/ JC-1 green monomer (R/G) ratio of 1·72. *Plasmodium falciparum* treated with IC50-values of SeO_3^{2-} showed a R/G ratio of 0·77 ($P < 0{\cdot}001$), which is a significantly lower ratio of parasites with functioning mitochondria to parasites with damaged mitochondria compared to the control. *Plasmodium falciparum* treated with IC50-values of MSeA, SeMet, or selenocystine showed R/G ratios of 1·39 ($P < 0{\cdot}05$), 0·87 ($P < 0{\cdot}001$), and 1·36 ($P < 0{\cdot}05$), respectively, and all results were significantly lower than that of the control. However, SeO_3^{2-} and SeMet showed visibly lower R/G ratios than MSeA and selenocystine. For comparison valinomycin, a mitochondria-damaging agent, at 1 nM showed R/G ratios of 1·38 ($P < 0{\cdot}05$) that is also significantly lower than the control. We have also evaluated RBCs only (5% haematocrit), without *P. falciparum* with the R/G ratio result of 0·53.

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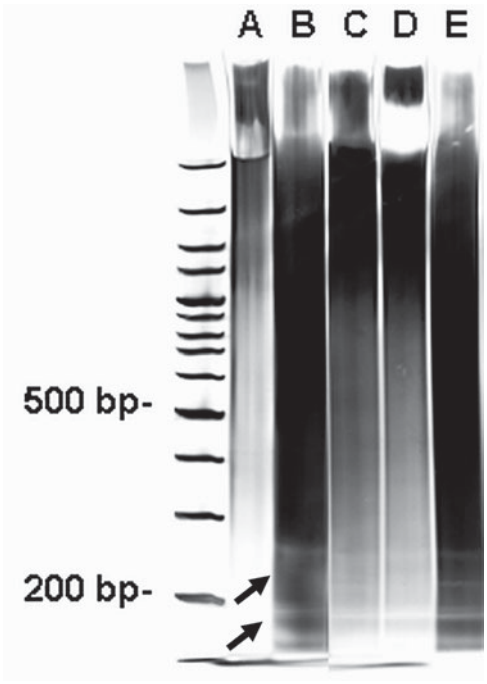

Fig. 4. DNA fragmentation assay of *Plasmodium falciparum* treated with various selenium compounds: Untreated parasites (A) or parasites incubated for 24 h with IC50-value of selenite (SeO_3^{2-}) (B), IC50-value of methylseleninic acid (MSeA) (C), IC50-value of selenomethionine (SeMet) (D), and IC50-value of selenocystine (E). Black arrows indicate the observed DNA ladder.

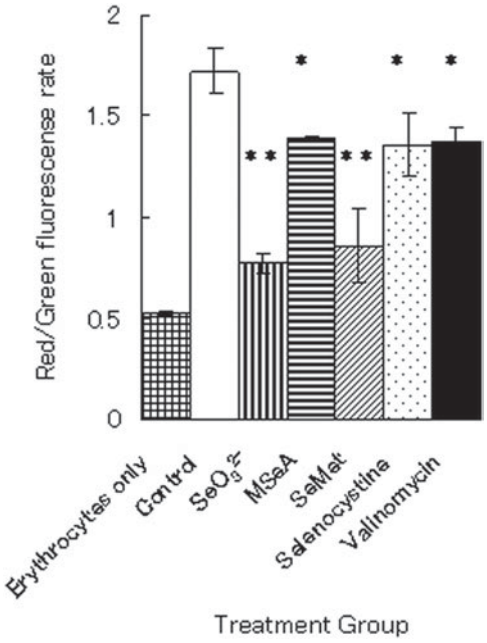

Fig. 5. Function analysis of *Plasmodium falciparum* mitochondria. Damaged mitochondria will lose their transmembrane potential and exhibit green monomers, while functioning mitochondria will retain their membrane polarization and exhibit red aggregates. Red/Green fluorescence rates show the number of parasites with functioning mitochondria compared to parasites with damaged mitochondria. Results are presented as means ± S.E.M. * $P<0{\cdot}05$, ** $P<0{\cdot}001$.

Effects of Se compounds on haemolysis of human red blood cells

The concentration of Hb released into the culture medium was determined in order to evaluate the toxicity of Se compounds against RBCs (Fig. 6). A significantly higher release of Hb into the medium compared to the control was considered to be a sign of toxicity against RBCs. RBCs treated with either milli-Q or 0·03% HCl solution (0 μM) were used as the control, and the Hb concentrations were 0·077 g/dl and 0·076 g/dl, respectively. RBCs treated with 400 and 200 μM SeO_3^{2-} caused release of 0·153 g/dl and 0·106 g/dl of Hb, respectively, into the medium, which was statistically significant compared to the control. One hundred to 10 μM SeO_3^{2-} resulted in a concentration no higher than 0·089 g/dl in the medium, which was not statistically significant.

MSeA at 400 μM caused 0·107 g/dl of Hb release, significantly higher than that of the control. Meanwhile, the MSeA at other concentrations did not show significantly higher Hb release compared to the control.

SeMet, selenocystine, SeO_4^{2-}, and MSeCys at all concentrations did not show a significantly higher Hb release compared to the control.

Toxicity of Se compounds against non-malignant human cell lines

As shown in Fig. 7, the toxicity of Se compounds against human cell lines was evaluated using 2 human non-malignant cell lines. Se compound treatment of HC cell lines (Fig. 7A) showed the following 50% lethal dose (LD50) values: SeO_3^{2-} 270 μM and selenocystine 190 μM. SeO_4^{2-}, MSeA, and SeMet showed no toxicity at any concentration. MSeCys did not reach an LD50 value up to 800 μM although it showed increased toxicity compared to the control.

The mortality of CHEK cell lines after Se compound treatment was investigated (Fig. 7B). SeO_3^{2-} and MSeA showed their LD50-values to be 100 μM and 800 μM, respectively. SeMet, selenocystine, SeO_4^{2-}, and MSeCys did not reach their LD50-values up to 800 μM although they showed some toxicity compared to the control.

DISCUSSION

In this study we demonstrated that 4 Se compounds were effective against *P. falciparum* growth. Based on their IC50-values, SeO_3^{2-} had the strongest antiplasmodial effect, followed by MSeA, SeMet, and selenocystine. Our study also showed that MSeA, SeMet and selenocystine inhibits all developmental stages of *P. falciparum* similar to SeO_3^{2-} (Taguchi *et al.* 2004), which is different from chloroquine which is known to affect mainly ring-form stages of *P. falciparum* (Orjih, 1997).

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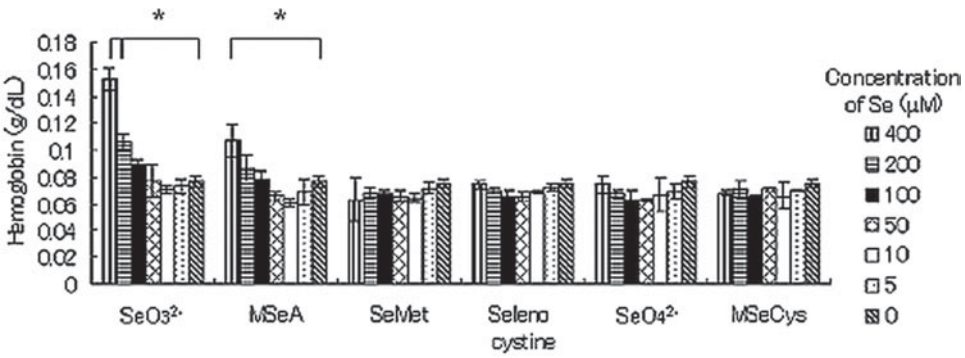

Fig. 6. Haemoglobin (Hb) concentration in spent medium after red blood cells (RBC) were treated with various selenium (Se) compounds: selenite (SeO_3^{2-}), methylseleninic acid (MSeA), selenomethionine (SeMet), selenocystine, selenate (SeO_4^{2-}), and methylselenocysteine (MSeCys). RBC treated only with a vehicle (0 μM group) is used as the control. All Se-treated groups are compared with the control. The higher Hb concentration compared to the control after Se treatment suggests higher haemolysis. Hb concentrations are presented as means ± S.E.M. * $P < 0{\cdot}05$.

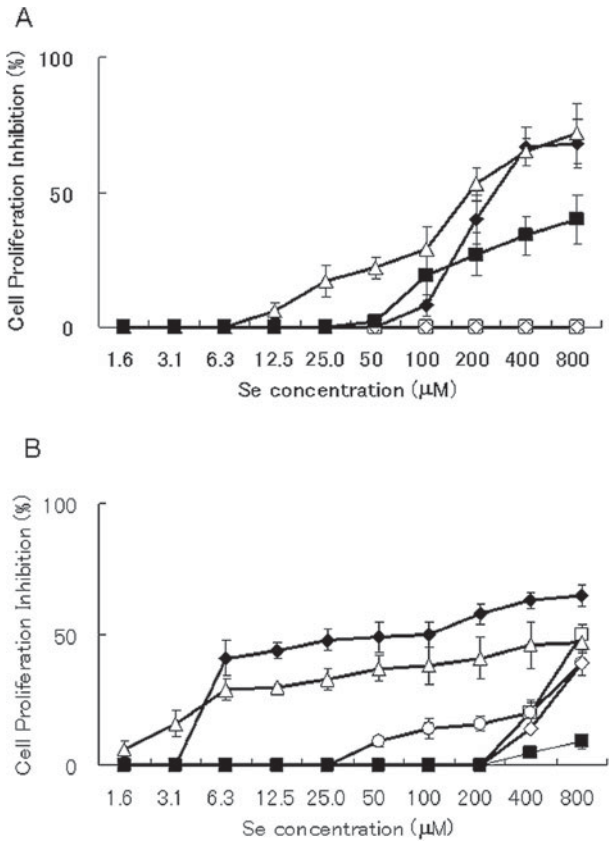

Fig. 7. Effect of 24-h treatment of various concentrations of various selenium (Se) compounds, selenite (SeO_3^{2-}) (black diamond), methylseleninic acid (MSeA) (white square), selenomethionine (SeMet) (white circle), and selenocystine (white triangle), selenate (SeO_4^{2-}) (white diamond), and methylselenocysteine (MSeCys) (black square), on human hepatic cell line (HC) (A) and human oesophagus cell line (CHEK) (B).

DNA fragmentation assay results showed a laddering pattern, but only at the region under 300 base pairs, thus we cannot clearly discern whether the ladder observed here is due to apoptosis-related DNA fragmentation or degrading DNA. However, shrunken and pyknotic parasites and increased mitochondrial damage were clearly shown after treatment with effective Se compounds. There are few conflicting reports on what are the definite signs of apoptosis (Baritaud *et al*. 2010; Marinho-Filho *et al*. 2010), our results include generally accepted signs of apoptosis (Faried *et al*. 2006; Menna-Barreto *et al*. 2007; Meslin *et al*. 2007), therefore they suggest that apoptosis-like cell death may be the anti-plasmodial mechanism of Se against *P. falciparum*.

Although all 4 effective Se compounds caused mitochondrial damage, we observed that SeO_3^{2-} and SeMet induced visibly higher mitochondrial damage compared to MSeA and selenocystine, which suggests that the pathway induced by SeO_3^{2-} and SeMet may be more dependent on a mitochondrial loss of function than are MSeA and selenocystine. These findings suggest that the effects of Se against *P. falciparum* depend on their chemical forms.

Out of the 4 effective Se compounds, SeO_3^{2-}, MSeA, and selenocystine are also known to generate ROS *in vitro* in the presence of GSH (Kitahara *et al*. 1993; Spallholz *et al*. 2001; Taguchi *et al*. 2004), which is abundantly available in both RBCs and *P. falciparum* (Roth, 1987; Becker *et al*. 2003). Blood-stage *P. falciparum* lives in a pro-oxidant environment; therefore, it is vulnerable to an increase in oxidative stress (Muller, 2004). In human cancer cell lines, oxidative stress may initiate apoptosis by causing DNA damage (Ozben, 2006). These lead us to suspect that in the cases of SeO_3^{2-}, MSeA, and selenocystine, ROS production and increased oxidative stress are pathways of Se-induced apoptosis-like cell death in *P. falciparum*.

However, SeMet, which was found to be effective against *P. falciparum*, is not known to produce ROS in the presence of GSH (Spallholz *et al*. 2004), which suggests that another Se-induced apoptosis-like cell-death pathway is available independent of ROS production. It is worth noting that SeMet, as well as SeO_3^{2-}, MSeA, and selenocystine,

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has been reported to induce apoptosis in human cancer cells by generating an intermediate Se metabolite, H_2Se (Jackson and Combs, 2008) or CH_3SeH (Jiang *et al*. 2002; Hu *et al*. 2005), which activates the apoptosis cascade. This finding highlights the possibility of another apoptosis-like cell-death pathway in *P. falciparum* that depends on the generation of apoptosis-inducing Se metabolites.

The above discussion indicates that at least 3 patterns may be available for Se compounds to induce apoptosis-like cell death against *P. falciparum*. The SeO_3^{2-} anti-plasmodial pathway involves ROS production (Spallholz *et al*. 2001) and mitochondrial damage. The MSeA and selenocystine antiplasmodial pathways also involve ROS production (Kitahara *et al*. 1993; Spallholz *et al*. 2001) but cause less mitochondrial damage. On the other hand, SeMet induces apoptosis-like cell death without ROS production (Spallholz *et al*. 2004) but causes more mitochondrial damage.

Although the reasons for the inactivity of SeO_4^{2-} and MSeCys against *P. falciparum* are still unclear, a lack of enzymes that can metabolize SeO_4^{2-} and MSeCys to their active forms may be the explanation. SeO_4^{2-} (Bebien *et al*. 2002) and MSeCys (Abdulah *et al*. 2009) both show cytotoxic effects against human cancer cell lines, but enzymes such as selenate reductase and methionase are needed. Furthermore, the presence of these enzymes in either *P. falciparum* or RBCs has not been reported.

The toxicity experiment with SeO_3^{2-}, MSeA, SeMet, and selenocystine showed that their IC50-values against *P. falciparum* were lower than the LD50-values against 2 human cell lines, and that they caused no significant increase of haemolysis in RBCs. However, we also observed that SeO_3^{2-} and selenocystine caused a marked increased of toxicity against CHEK cell lines at concentrations as low as 6·3 μM. These findings suggest that while MSeA and SeMet at concentrations capable of inducing apoptosis in *P. falciparum* are unlikely to cause cytotoxic effects in the human host, SeO_3^{2-} and selenocystine may have some toxic effect on the host. In addition, we acknowledge that recent anti-malarial drugs are usually in the nanomolar range but, as stated in the Introduction section, selenium is an essential trace element, and in human selenium supplementation studies such as the SELECT study, selenium was used the micromolar range (Lippman *et al*. 2009).

There are some controversies regarding the occurrence of apoptosis in *P. falciparum*. Picot *et al*. (1997) and Meslin *et al*. (2007) reported that blood-stage *P. falciparum* treated with chloroquine or etoposide showed signs of apoptosis, while Nyakeriga *et al*. (2006), using the same treatment, did not find any signs of apoptosis, and Totino *et al*. (2008), using chloroquine or staurosporine, found autophagosomes, a sign of autophagic cell death. The current study cannot settle the controversies over the mechanism of cell death that occurs in *P. falciparum*, but the results support the occurrence of apoptotic cell death in blood-stage *P. falciparum*. In addition, our results indicate that apoptosis-like cell death can take place in chloroquine-resistant *P. falciparum*.

The property of the caspase family proteins to induce apoptosis in mammals has been well studied (Budihardjo *et al*. 1999; Wells and Mallucci, 2009). Several genes of metacaspase, a group of caspase-like proteins, have been reported in *P. falciparum* (Meslin *et al*. 2007) and *P. berghei* (Le Chat *et al*. 2007), but the proteins themselves have not been found, and the functions of these metacaspases are still unclear. Further studies that clarify the proteins involved in the apoptosis-like cell death of *P. falciparum* are needed.

In conclusion, the results of the present study show that SeO_3^{2-}, MSeA, SeMet, and selenocystine have anti-plasmodial effects by inducing apoptosis-like cell death in *P. falciparum*. In addition, Se anti-plasmodial properties and apoptosis-like cell-death pathways are dependent on their chemical forms. Next, we will confirm the involvement of ROS and the proteins involved in the Se-induced apoptosis-like cell death of *P. falciparum*. Further study on the Se-induced apoptosis-like cell-death mechanism in *P. falciparum* can be beneficial to tackle the growing problem of drug resistance.

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