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Plasmodium berghei proteome changes in response to SSJ-183 treatment

Jun Lu *, Chika Arai, Abu Bakar Md, Masataka Ihara *

Drug Discovery Science Research Center, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan

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ABSTRACT

The benzo[*a*]phenoxazine derivative, SSJ-183 has shown excellent anti-malarial efficacy and safety. However, its mechanism of action is unclear. We investigated the effect of SSJ-183 on the rodent malarial parasite, *Plasmodium berghei*. We analyzed changes in protein expression in the erythrocytic cycle of *P. berghei* with or without 18 h of SSJ-183 treatment by two-dimensional gel electrophoresis. We confirmed results with matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight tandem mass spectrometry. After treatment, seven main proteins were significantly down-regulated, and two were up-regulated; results were reproduced in three independent tests. Some of these proteins were hypothetical parasite proteins or unnamed host products. However, three proteins were identified as a heat shock protein, a disulfide isomerase precursor, and berghepain-2 from *P. berghei*. All three showed reduced expression after SSJ-183 treatment. This suggested that SSJ-183 was a good anti-malarial drug candidate because it targeted parasite chaperone proteins.

1. Introduction

Malaria remains one of the world's most prevalent infectious diseases and leads to both mortality and morbidity.[1] Although malaria has been eradicated in some parts of the world, at present, 40% of the world's population lives in malarial regions. The estimated 515 million cases of human malaria reported each year are generally caused by four species, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. All are transmitted by the bites of female anopheline mosquitoes.[2] Until recently, chloroquine was the most widely used drug to treat and prevent malaria infections. However, resistance to chloroquine and sulfadoxine-pyrimethamine has perpetuated the on-going burden of malaria.[3] Novel, effective, safe, and inexpensive drugs are needed to control malaria. In a previous search for new anti-malarial agents, we synthesized benzo[*a*]phenoxazines. One derivative, SSJ-183, showed an IC_{50} value of 0.0076 μM against *P. falciparum* K1, and an IC_{50} value of 55.7 μM for toxicity in L-6 rat myoblasts, with a selectivity index of 7334. SSJ-183 achieved a cure after three oral doses of 100 mg/kg to mice infected with the ANKA strain of *Plasmodium berghei*. SSJ-183 was proven safe in a single dose toxicity test, a chromosome aberration test, in vitro and in vivo micronucleus tests, an hERG assay, and a photo-toxicity test. Based on these findings, SSJ-183 appears to be a promising candidate therapy for malaria.[4] However, the mechanism of action of SSJ-183 is unclear. In this report, we demonstrated that SSJ-183 targeted heat shock protein, disulfide isomerase precursor, and berghepain-2. The inhibition of these proteins may partly explain its anti-malarial effects.

2. Results and discussion

2.1. In vivo anti-malarial activity

In this study, we used a rodent malaria parasite species, *P. berghei*, as a model system to address issues relevant to the human-infectious species *P. falciparum* and *P. vivax*.[5] We infected mice with the NK-65 strains of *P. berghei* in order to measure the in vivo anti-malarial activities of candidate compounds. In a previous study, we found that a single 100 mg/kg dose of SSJ-183 administered after one day of infection was able to inhibit over 99.9% of *P. berghei* at day 4, and extended the mean survival of infected mice from approximately six days (untreated control) to 14.6 days. This suggested that SSJ-183 had a strong inhibition effect on the initial infection state. In the current study, we examined whether SSJ-183 could inhibit *P. berghei* in the moderate to severe infection states. We infected mice by inoculating intravenously with 1×10^6 parasitized erythrocytes. After four days, the mice exhibited 10–25% parasitemia (Fig. 1). Then, we administered a 1×50 mg/kg oral dose of SSJ-183 in a non-solubilizing, standard suspension that comprised 0.5% (w/v) hydroxypropyl methylcellulose, 0.5% (v/v) benzyl alcohol, and 0.4% (v/v) Tween 80 in water.[6] In preliminary test we found 100 mg/kg was too effective to collect parasite protein, so we use 50 mg/kg dose. Then, 18 h later, the infection ratios were measured. The control (untreated group) parasitemia had increased to 30–40%, but that

* Corresponding author. Tel./fax: +81 3 5498 6391.
E-mail address: m-ihara@hoshi.ac.jp (M. Ihara).

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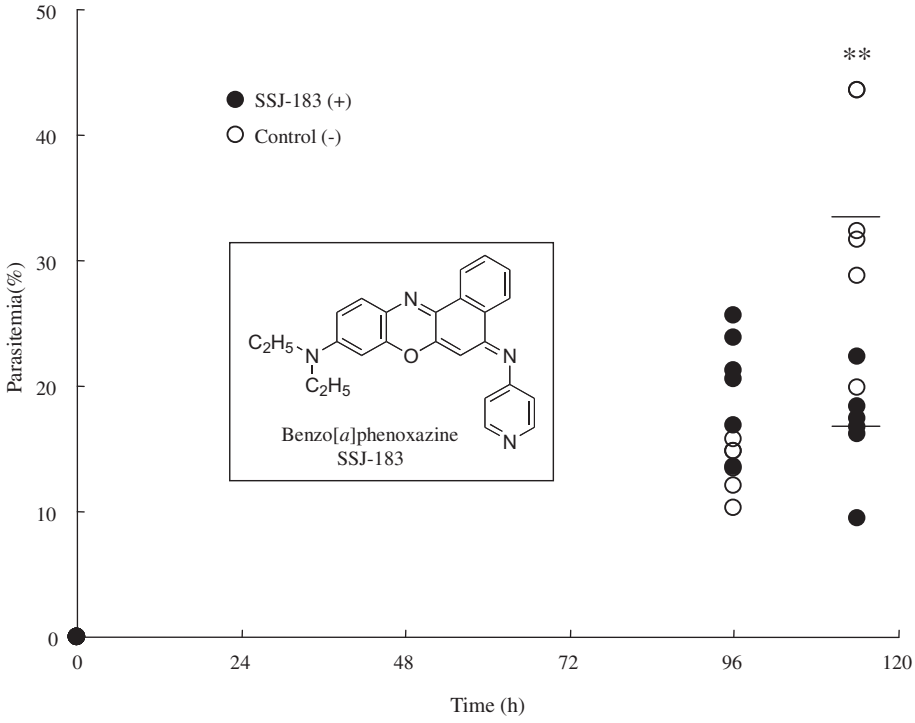

Figure 1. The comparison of parasitemia in ICR mice untreated (*open symbols*) or treated (*filled symbols*) with SSJ-183. All mice were infected with 1×10^6 *P. berghei* parasitized erythrocytes at day 0. After four days, six mice were orally administered 50 mg/kg SSJ-183, and six mice received no treatment (controls). After another 18 h, parasitemia was determined for all the mice. Significant different between means of controls and SSJ-183 treatment are indicated, $^{**}P$ <0.001. (Inset) Chemical structure of SSJ-183.

of the SSJ-183 treated group remained at the 20% level or decreased to 5–10% (Fig. 1). Thus, the parasite growth was controlled by SSJ-183, even under conditions of moderate to severe infection.

Next, we isolated blood proteins at this time point to investigate whether any changes occurred in protein expression in response to SSJ-183 treatment.

2.2. Proteome analysis

Proteins separated by two-dimensional electrophoresis (2-DE) were stained with SYPRO-Ruby and detected with an Anatech imaging system. The images were analyzed with Progenesis software. Comparisons between treated and untreated groups showed that seven protein spots were significantly down-regulated, and two protein spots were up-regulated with SSJ-183 treatment (Fig. 2). Almost these spots are reproducible at three different tests. In fact, there were more tiny protein spots whose expression levels changed, but we only selected nine reproducibly distinguishable spots for matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight tandem mass spectrometry (MALDI-TOF-MS). Some of these proteins were hypothetical parasite proteins, some were known or unnamed host products, and three proteins were identified as a heat shock protein (HSP), a disulfide isomerase precursor, and *P. berghei* berghepain-2. The latter three proteins had decreased expression levels in response to SSJ-183 treatment (Table 1). This suggested that SSJ-183 targeted these proteins in vivo to inhibit the growth of malarial parasites. It is also very interestingly to find *Mus musculus* beta-actin increase in parasite proteins, for many intracellular pathogens hijack host cell actin or its regulators for cell-to-cell spreading.[7] However, in this study we focus on the parasite proteins to discussion.

HSPs are highly conserved, ubiquitous proteins that occur in most life forms. Their main role is to act as molecular chaperones by binding to nonnative proteins and facilitating refolding into the native state.[8] HSP70 proteins comprise a major heat shock protein family. Generally, HSP70 proteins are induced in response to stress; however, some HSP70 species are constitutively expressed in cells. It is becoming increasingly apparent that HSPs play an important role in the survival of *P. falciparum* against temperature changes associated with its passage from the cold-blooded mosquito vector into the warm-blooded human host.[9] There is growing evidence that HSPs from *P. falciparum* could also play a cytoprotective role in the life cycle of the parasite.[10] However, little information has been reported on HSPs from *P. berghei*. In this work, we identified an HSP from *P. berghei* that was down-regulated by SSJ-183 administration. Shonhai et al. (2007) proposed that 'perhaps most interesting is the possible development of drugs that target HSP70 function in *P. falciparum*. However, because of the high conservation between human HSP70s and *P. falciparum* HSP70s, the design of potential anti-malarials targeting *P. falciparum* Hsp70s is a real challenge'.[10] This work has taken the first step of that proposal with the demonstration that SSJ-183, a good drug candidate for malaria, targeted at least one HSP of *P. berghei*.

Another of the proteins identified was a disulfide isomerase precursor, which belongs to the protein disulfide isomease (PDI) family. There is little information on PDIs of malarial parasites. PDI is a chaperone protein, involved in protein quality control machinery.[11] It plays a major role in the re-arrangement of both intra-chain and inter-chain disulfide bonds, where it acts catalytically to initiate or reduce disulfide bonds. PDI is also expressed on the surfaces of cancer cells. In that context, inhibition of PDI led to reduced glioma cell migration and invasion.[12] Here, we postulate that PDI might be strongly expressed by invasive malarial parasites. Thus, inhibition of PDI by SSJ-183 could lead to reduced parasite migration and invasion. PDI may therefore represent a potential therapeutic target in malaria.

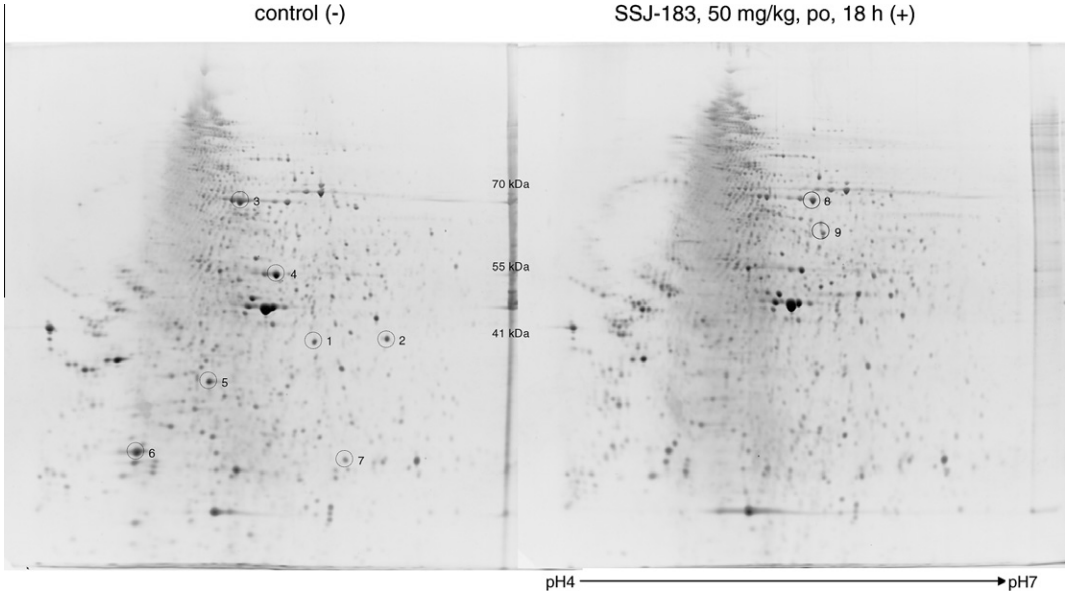

Figure 2. Two-dimensional electrophoresis pattern of *P. berghei* proteins from mice treated with or without SSJ-183. *P. berghei* proteins were separated by 2-D gel electrophoresis and stained with SYPRO-Ruby. (Left) *Dotted-line circles*: indicate spots down-regulated with SSJ-183 treatment. (Right) *Continuous line circles*: indicate spots up-regulated with SSJ-183 treatment. *Spot numbers*: refer to the identifications shown in Table 1.

Table 1
Protein identification and expression compared between control and SSJ-183 treated groups

Spot no.	NCBI accession no.	Name	Expression levels	Ratios[a]	Species	pI[b]	Mr[c]	Matched peptide	Sequence coverage[d]
1	gi68071723	Heat shock protein	Down regulated	8.6	*P. berghei*	5.15	72,130	19	27%
2	gi49868	Put. beta-actin (aa 27–375)	Down regulated	7.2	*Mus musculus*	5.78	39,446	18	56%
3	gi68071723	Heat shock protein	Down regulated	3.5	*P. berghei*	5.15	72,130	29	45%
4	gi68070467	Disulfide isomerase precursor	Down regulated	2.2	*P. berghei*	5.44	55,718	31	68%
5	gi17978639	Berghepain-2	Down regulated	10.6	*P. berghei*	6.02	54,898	[e]	[e]
6	gi68067763	Hypothetical protein	Down regulated	3.1	*P. berghei*	4.85	21,586	11	43%
7	gi68064486	Hypothetical protein	Down regulated	2.5	*P. berghei*	5.61	27,689	9	28%
8	gi74220592	Unnamed protein product	Up regulated	1.3	*Mus musculus*	5.22	70,876	24	42%
9	gi74005074	Unnamed protein product	Up regulated	1.6	*Mus musculus*	5.67	61,089	15	35%

All of these differentially expressed proteins are presented in Figure 2.
[a] Protein expression ratios represent control:SSJ-183 treated groups. The averages of triplicate experiments were used.
[b] pI: the calculated isoelectric point, as recorded in the NCBI protein database.
[c] Mr: the nominal mass, as recorded in the NCBI protein database.
[d] The percentage of total protein sequences covered by the identified peptides.
[e] MS/MS fragmentation of EYYYIIR found in NCBI gi17978639, berghepain-2.

Another of the proteins identified was berghepain-2, a cysteine protease of *P. berghei*. Proteases play a key role in the life cycle of malaria parasites. In erythrocytic-stage parasites, aspartic-, cysteine-, and metallo-proteases appear to participate in the hydrolysis of hemoglobin. This is required to provide amino acids for parasite protein synthesis.[13,14] Cysteine proteases of *P. falciparum*, known as falcipains, have been identified as hemoglobinases and potential drug targets.[13,15] Our results have supported that hypothesis. Reduced berghepain-2 expression was associated with SSJ-183 treatment.

3. Conclusion

Previously, proteomic screening had only been performed in vitro with *P. falciparum* to determine the mode of action of antimalarial drugs. In this study, we used an in vivo rodent malaria model to investigate the mechanism of action of a novel anti- malarial drug candidate, SSJ-183. At 18 h after SSJ-183 administration, the infection rate of *P. berghei* was significantly inhibited. We compared parasite proteins expressed in treated and untreated states by 2-DE. We found that expression levels of nine proteins changed, and these were analyzed by MALDI-TOF-MS. Among the proteins identified, we found the stress-related proteins HSP and PDI precursor, and the metabolic protein, berghepain. These proteins were strongly expressed in active parasites. Inhibition of these proteins by SSJ-183 led to reduced parasite survival. Therefore, HSP, PDI, and berghepain-2 represent potential therapeutic targets in rodent malaria.

4. Materials and methods

4.1. Drugs

The benzo[*a*]phenoxazine derivatives were synthesized as previously described.[4] SSJ-183 hydrochloride (here, abbreviated to SSJ-183) was used for the in vivo assay.

4.2. Animals

The animal study was approved by the Hoshi University Animal Experimentation Ethics Committee. All the animals were obtained

from Japan SLC, Inc. (Hamamatsu, Japan). Female ICR mice (19–25 g) were maintained at a constant temperature (23 °C) and relative humidity of approximately 55% with a standard 12 h light–dark cycle. Animals were fed a standard rodent diet (Labo. MR Stock, Nosan Corp., Yokohama, Japan) and allowed free access to tap water.

4.3. Parasite evaluations in infected mice

Peripheral blood smears were prepared with blood obtained from the tail veins of infected experimental mice.[16] The thin films were stained with Diff-Quick (Sysmex Corp., Kobe, Japan). Blood smears were examined under oil immersion at a magnification of ×100 with a Leica DM6000B microscope (Leica Microsystems K.K., Tokyo, Japan). Parasitemia was determined by counting over 10 fields of view.

4.4. In vivo anti-malarial activities

The anti-malarial efficacy of SSJ-183 was evaluated in vivo by infecting female ICR mice with rodent malaria *P. berghei* NK-65 ($n = 6$ in each group).[17] The mice were inoculated intravenously with 1×10^6 parasitized erythrocytes (resuspended in 200 μl of normal saline solution) on day 0. Drugs were administered daily by oral gavage (po) for each single dose. After 24 h (0–0.1% parasitemia) of infection, the dose was 100 mg/kg/day; after four days (10–25% parasitemia) of infection, the dose was reduced to 50 mg/kg/day. Control mice were treated with an equal volume of vehicle. The infection and suppression ratios were checked on day 4, 18 h after the po by comparing parasite growth between the control and treated groups. The suppression ratio = (parasitemia% control − parasitemia% sample)/parasitemia% control. All results are expressed as means ± standard deviation (SD). Significant different between sample means were determined using the Student *t* test.

4.5. Extraction of *P. berghei* protein

At 18 h after po, infected blood was collected from mice treated or untreated with SSJ-183, via cardiac puncture into a heparinized syringe. The blood samples were pooled among the SSJ-183 treated group ($n = 2$) and among the untreated group ($n = 2$). White blood cells were removed by centrifugation (700*g*, 5 min, 4 °C). Parasites were isolated and lysed by addition of lysis buffer [8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM dithiothreitol (DTT), 2% (v/v) pharmalyte 3–10 (GE Healthcare, Uppsala, Sweden)]; this was followed by three cycles of freeze–thawing (liquid nitrogen and 37 °C, respectively), and brief sonication before centrifugation at 16,000*g* at 4 °C for 20 min.[18] Aliquots containing 40 mg of parasites were combined with 100 μl of lysis buffer. Finally, 90 μl of supernatant was immediately applied to an isoelectric focusing gel for first dimensional electrophoresis.

4.6. Two-dimensional gel electrophoresis

P. berghei proteins were separated on 18-cm immobilized dry strips (GE healthcare, pH 4–7) gel with an isoelectric focusing system (Anatech Co., Ltd, Tokyo, Japan). The dry strip was soaked in rehydration buffer that contained 6 M urea, 2% (v/v) TritonX-100, 1% (v/v) pharmalyte 3–10, 13 mM DTT, and 2.5 mM acetic acid and incubated at 20 °C overnight. Isoelectric focusing was performed according to the manufacturer's instructions. After isoelectric focusing, proteins were reduced and alkylated by successive 15–30 min treatments with equilibration buffer that contained 0.5% (w/v) DTT, followed by 4.5% (w/v) iodoacetamide. Proteins were then resolved in the second dimension on an SDS–polyacrylamide gel (10% homogenous gel). Resolved proteins were stained with SYPRO-Ruby protein gel stain (PerkinElmer, Boston, US) for MALDI-TOF-MS analysis.[19]

4.7. In-gel digestion, peptide extraction, MALDI-TOF-MS analysis

Protein spots were excised from 2-DE gels, digested in-gel with trypsin (10 ng/μl in 50 mM NH_4HCO_3), and solvent-extracted, as described previously.[20] After extraction, peptide mixtures were dried and re-dissolved in 2 μl of 50% (v/v) acetonitrile, 5% (v/v) trifluoroacetic acid. Samples were then mixed with a saturated α-cyano-4-hydroxycinnamic acid (matrix) solution, and deposited on the MALDI-MS target plate according to a general two-layer method.[21] The MALDI-TOF-MS analysis of digested peptides was performed with an AXIMA Performance mass spectrometer (Shimadzu Crop., Kyoto, Japan).

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