Supporting Information

Selective Accumulation of Rhodacyanines in the Plasmodial Mitochondria is Related to the Growth Inhibition of Malaria Parasites

Daiki Morisaki, Hye-Sook Kim, Hiroshi Inoue, Hiroki Terauchi, Shusuke Kuge, Akira Naganuma, Yusuke Wataya, Hidetoshi Tokuyama, Masataka Ihara, and Kiyosei Takasu

Contents:

Microscopic images of intracellular distribution of 1a in malaria-infected erythrocytes (*Plasmodium falciparum* FCR-3).

Microscopic images of triple stain experiment of *P. falciparum*-infected erythrocytes with rhodacyanine **1a**, DAPI (4',6-diamino-2-phenylindole) and Diff-Quik reagent is summarized in Figure S1.

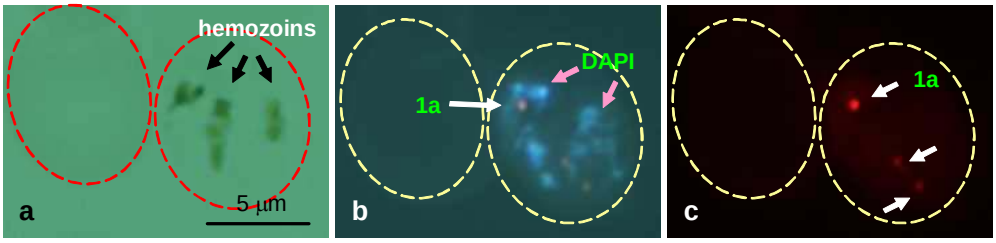


Figure S1. A solution of DMSO of **1a** (10 μM; final concentration is 2.0 x 10^{-6} M) and a solution of 4',6'-diamidino-2-phenylindole (DAPI; 10 μM) were incubated with *P. falciparum*-infected erythrocytes on the medium (RPMI 1640) at 37 °C for 1 h. Microscopic examination was carried out after Diff-Quik stained thin blood smears.

(a) Bright-field image; Red broken lines (left) corresponds to a normal red blood cell, and right one corresponds to malaria infected erythrocyte. Black stains (arrow) correspond to hemozoins in food vacuoles. (b) Fluorescent image through DAPI filter; Blue stains correspond to parasitic nuclei stained by DAPI (pink arrows), Pale orange spots correspond to fluorescence of **1a** (white arrow). (c) Fluorescent images through FITC filter; Red spots correspond to fluorescence of **1a** (white arrows).

Table S1. In vitro antimalarial activity and fluorescent property of tested compounds.

compound	EC_{50} (nM)[a]	λ_{em} (nm)[b]	Φ_F[c]
1a	21	516	2.1×10^{-4}
1b	22	NT[d]	NT[d]
1c	78	NT[d]	NT[d]
1d	680	560	7.9×10^{-4}
2	4,000	518	6.1×10^{-3}

[a] In vitro antiplasmodial activity against *P. falciparum* K1. [b] Maximum with highest wavelength of emission spectra (excitation: λ_{ex} = 495 nm) in MeOH (1.0×10^{-6} M) at 20 °C. [c] Determined relative to fluorescein. [d] Not tested.

Double Stain Experiments.

Microscopic images of double stain experiment of *P. berghei*-infected erythrocytes with fused-rhodacyanine **3** and DAPI (4',6-diamino-2-phenylindole) is summarized in Figure S2.

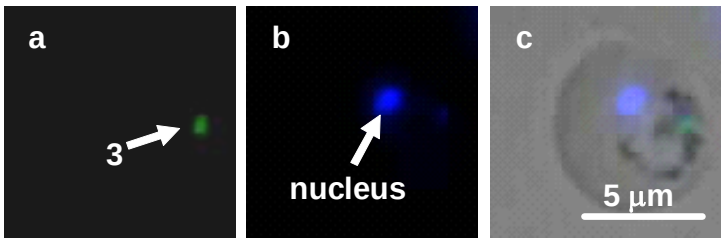


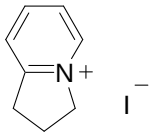
Figure S2. Fluorescent microscopic image of the intracellular distribution of **3** and DAPI in *P. berghei*- infected. (a) Through FITC filter; **3** (green spot). (b) Though DAPI filter, parasitic nuclei stained by DAPI. (c) Superimposed.

Experiment Section:

Chemistry

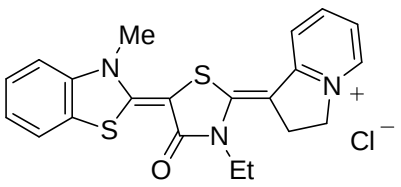
General Remarks: All reactions were carried out in oven-dried glassware under a positive pressure of argon unless otherwise noted. ^{1}H-NMR spectra were recorded using a JEOL JNM-AL-400 at 400 MHz. ^{13}C-NMR spectra were recorded using a Varian Gemini 2000 at 75 MHz or a JEOL JNM-AL-400 at 100 MHz. Chemical shifts for ^{1}H-NMR were reported in parts per million (ppm) downfield from tetramethylsilane as the internal standard and coupling constants are in Hertz (Hz). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet and m = multiplet. Chemical shifts for ^{13}C-NMR are reported in ppm, relative to the central line of a triplet at 77.0 ppm for deuteriochloroform. Infrared (IR) spectra were recorded on a SHIMADZU FTIR-8300 Spectrometer and are reported in wavenumbers (cm^{-1}). Analytical thin layer chromatography (TLC) separations were performed on Merck precoated analytical plates, silica gel 60 F_{254}. Fast atom bombardment (FAB) mass spectra were obtained by using JEOL JMA DX-303. UV/Vis spectroscopic studies were performed on a JASCO UV/Vis spectrophotometer. Compounds **1a**-**1d** and **2** were prepared according as the reported procedure.[1-4]

2,3-Dihydro-1*H*-indolizinylium Iodide (8).



A mixture of 2-(3-hydoroxypropyl)pyridine (**7**) (686 mg, 5.00 mmol), I_2 (2.56 g, 10.0 mmol), PPh_3 (2.64 g, 10.0 mmol) and imidazole (687 mg, 10.0 mmol) in toluene (20 mL) was stirred for 1 h at ambient temperature. To the reaction mixture was added Na_2SO_3 (3.0 g) in water（10mL）. The solution was extracted with AcOEt three times. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo to give **8** (990 mg, 80%) as dark brown oil. Its spectral data were identical with the reported ones.[5] The product was used without further purification in the next step.

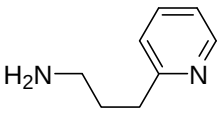
1-[3-Ethyl-5-(3-methyl-3*H*-benzothiazole-2-ylidene)-4-oxothiazolidin-2-ylidene]-2,3-dihydro-1*H*-indolizinylium Chloride (3).



To a suspension of 8 (0.766 g, 3.10 mmol) and 9 (1.53 g, 3.10 mmol) in MeCN (15 mL) was added NEt_3 (0.13 mL, 9.3 mmol) dropwise, and the

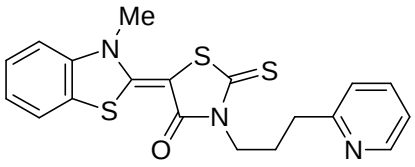
mixture was stirred for 3 h at 70 ^{o}C. After the mixture was cooled to ambient temperature, to the resulting mixture was added AcOEt giving red precipitates, which was collected and washed with AcOEt. The precipitates were dissolved in MeOH-$CHCl_3$ (1 : 1 v/v). The resulting solution was passed through the anion exchange resin Amberlite IRA-400(Cl^-), and the resin was washed with MeOH. After concentration of the elution in vacuo, the residue was crystallized from MeOH-AcOEt to give **3** (0.786 g, 59% in 2 steps) as red solids. Mp. 278 °C (decomp.); ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 8.70 (1H, d, *J* = 5.0 Hz), 8.28 (1H, t, *J* = 7.5 Hz), 8.45 (1H, d, *J* = 7.4 Hz), 7.64 (1H, d, *J* = 6.4 Hz), 7.57 (1H, d, *J* = 7.4 Hz), 7.48-7.40 (2H, m), 7.28 (1H, t, *J* = 7.4 Hz), 4.80 (2H, t, *J* = 8.2 Hz), 4.12 (2H, q, *J* = 7.4 Hz), 4.06 (3H, s), 3.52 (2H, t, *J* = 8.2 Hz), 1.12 (3H, t, *J* = 7.4 Hz); ^{13}C-NMR (100 MHz, DMSO-*d*6) δ 165.9, 164.7, 164.6, 152.4, 144.3, 143.5, 141.0, 140.3, 126.7, 125.4, 1232., 121.8, 120.4, 119.0, 106.6, 96.2, 80.8, 54.8, 34.3, 27.0, 14.5; IR (KBr) 3392, 1652, 1629, 1488, 1446, 1419, 1304 cm^{-1}; LRMS (*m*/*z*) 394 (M^+); Anal. Calcd for $C_{21}H_{20}ClN_3OS_2 \cdot H_2O$, C, 56.30; H, 4.95; N, 9.38. Found C, 56.36; H, 5.02; N, 9.29.

2-(3-aminopropyl)pyridine (10)



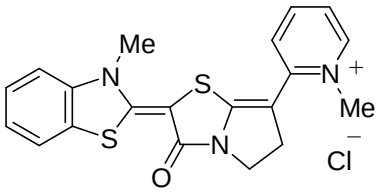
To a mixture of **7** (2.74 g, 20.0 mmol), PPh_3 (7.68 g, 30.0 mmol) and phthalimide (4.41 g, 30.0 mmol) in THF (50 mL) was added 40wt% diethyl azodicarboxylate in toluene (17.0 mL, 30.0 mmol) dropwise at 0 ^{o}C. The mixture was stirred for 5 h at the same temperature. The mixture was concentrated and the resulting residue was diluted with AcOEt. After the solution was extracted with 1 M HCl aq twice, the combined aqueous layers were neutralized with sat. $NaHCO_3$ and extracted with $CHCl_3$ three times. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo affording yellow oil. The crude residue was purified by column chromatography on silica gel (AcOEt) to give phthalimide (2.00 g, 7.15 mmol). Next, a mixture of the phthalimide and NH_2NH_2 monohydrate (0.50 mL, 10 mmol) in EtOH (50 mL) was refluxed for 1 h. After the reaction mixture was filtered, the mother liquid was concenctrated under reduced pressure to give **10** (0.929 g, 49% in 2 steps) as yellow oil, whose spectral dara were identical with the reported ones.[6]

5-(3-Methyl-3H-benzothiazol-2-ylidene)-3-(3-pyridin-2-ylpropyl)-2-thioxothiazolidin-4-one) (13).



To a mixture of **10** (602 mg, 4.87 mmol) and NEt_3 (1.8 mL, 13 mmol) in MeOH (15 mL) was added CS_2 (0.30 mL, 4.89 mmol) dropwise at ambient temperature, and the mixture was sturred for 1 h. To the mixture was added methyl bromoacetate (0.50 mL, 5.0 mmol), and the mixture was stirred for further 6 h at the same temperature. Concentration of the resulting mixture gave crude **11** as red solids, which was used in the next step without purification. To a mixture was the whole amount of crude **11** and **12** (1.79 g, 4.89 mmol) in MeCN (30 mL) was added NEt_3 (2.1 mL, 15 mmol) at ambient temperature, and the mixture was stirred for 4 h. The generated orange precipitates were collected by filtration. The residue was washed with MeCN to give **13** (0.361 g, 22% in 2 steps) as orange solids. Mp. 228-230 ^oC (decomp.); ^1H-NMR (400 MHz, DMSO-*d*6) δ 8.47 (1H, d, *J* = 5.0 Hz), 7.92 (1H, d, *J* = 7.4 Hz), 7.70-7.64 (2H, m), 7.51 (1H, t, *J* = 7.3 Hz), 7.35 (1H, t, *J* = 7.3 Hz), 7.29 (1H, d, *J* = 7.4 Hz), 7.19 (1H, dd, *J* = 7.3, 5.0 Hz), 4.12 (2H, q, *J* = 7.4 Hz), 3.96 (3H, s), 2.77 (2H, t, *J* = 7.6 Hz), 2.11 (2H, m); ^{13}C-NMR (100 MHz, DMSO-*d*6) δ 176.7, 160.3, 154.5, 148.9, 140.2, 136.3, 127.2, 125.4, 124.0, 122.5, 122.4, 121.2, 112.1, 97.3, 83.0, 43.2, 34.7, 32.5, 26.3; IR (KBr) 1645, 1514, 1469, 1417, 1340 cm^{-1}; LRMS (*m*/*z*) 400 (M^+).

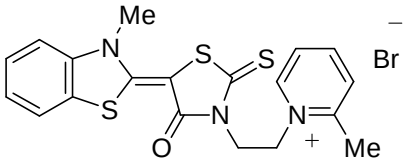
1-Methyl-2-[2-(3-methyl-3*H*-benzothiazol-2-ylidene)-3-oxo-2,3,5,6-tetrahydropyrrolo[2,1-b]thiazol-7-yl]pyridinium Chloride (4).



A mixture of **13** (0.302 g, 0.750 mmol) and methyl *p*-toluenesulfonate (3.0 mL, 15 mmol) in DMF (8.0 mL) was stirred for 7 h at 80 ^oC. After the mixture was diluted with AcOEt, red solids were precipitated. The precipitates were collected by filtration and dried. To a suspension of the precipitates in MeCN (20 mL) was added NEt_3 (0.60 mL, 4.3 mmol) at ambient temperature, and the mixture was stirred for 3 h at 70 ^oC. Dilution of the mixture with AcOEt gave red precipitates, which was collected and washed with AcOEt. A solution of the precipitates in MeOH was passed through the anion exchange resin Amberlite IRA-400(Cl^-), and the resin was washed with MeOH. After concentration of the elution in vacuo, the residue was crystallized from AcOEt to give **4** (74 mg, 23% in 3 steps) as red solids. Mp 252-254 ^oC (decomp.); ^1H-NMR (300 MHz, DMSO-*d*6) δ 8.57 (1H, d, *J* = 6.3 Hz), 8.16 (1H, d, *J* = 7.6 Hz), 7.85-7.70 (2H, m), 7.55-7.40 (4H, m), 4.13 (3H, s), 3.96

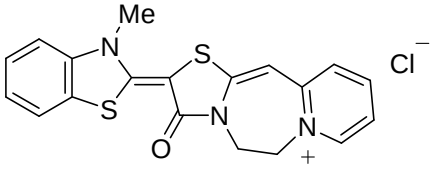
(3H, s), 4.04 (2H, t, J = 8.2 Hz), 3.93 (3H, s), 3.59 (2H, d, *J* = 8.2 Hz); IR (KBr) 1697, 1508, 1473, 1418 cm^{-1}; LRMS (*m*/*z*) 380 (M^+); Anal. Calcd for $C_{20}H_{18}ClN_3OS_2$, C, 57.75; H, 4.36; N, 10.10. Found C, 57.45; H, 4.70; N, 9.98.

2-Methyl-{2-[5-(3-methyl-3H-benzothiazol-2-ylidene)-4-oxo-2-thioxothiazolidin-3-yl]ethyl}pyridinium Bromide (17).



A mixture of 2-bromoamine hydrobromide (**14**) (0.791 g, 2.00 mmol) and 2-picoline (10 mL) was stirred for 5 h at 130 ^oC. After dilution with AcOEt giving precipitates, the resulting precipitates were collected by filtration and washed with AcOEt to afford **15** (0.92 g, 64%) as white solids, which was used in the next step without furthe purification. To a solution of 15 (0.791 g, 2.00 mmol) and NEt3 (1.8 mL) in MeOH (15 mL) was added CS_2 (0.13 mL, 2.00 mmol) dropwise at ambient temperature, and the mixture was sturred for 1 h. To the mixture was added methyl bromoacetate (0.20 mL, 2.0 mmol), and the mixture was stirred for further 6 h at the same temperature. Concentration of the resulting mixture gave crude **16** (0.73 g) as red solids, which was used in the next step without purification. To a mixture was the whole amount of crude **16** and **12** (0.73 g, 2.0 mmol) in MeCN (15 mL) was added NEt_3 (0.90 mL, 6.0 mmol) at ambient temperature, and the mixture was stirred for 3 h. The generated orange precipitates were collected by filtration. The residue was washed with MeCN to give **17** (0.221 g, 15% in 2 steps) as orange solids. Mp 263 ^oC (decomp.); ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 8.73 (1H, d, *J* = 5.5 Hz), 8.43 (1H, d, *J* = 8.5 Hz), 8.00 (1H, d, *J* = 8.0 Hz), 7.91-7.90 (2H, m), 7.70 (1H, d, *J* = 8.5 Hz), 7.54 (1H, t, *J* = 7.8 Hz), 7.37 (2H, t, *J* = 7.8 Hz), 4.96 (2H, t, *J* = 6.0 Hz), 4.61 (2H, t, *J* = 6.0 Hz), 3.94 (3H, s), 2.99 (3H, s); ^{13}C-NMR (100 MHz, DMSO-*d*6) δ 187.2, 164.2, 155.8, 155.2, 146.1, 145.8, 140.1, 129.9, 127.4, 125.6, 125.3, 124.3, 122.5, 112.4, 82.2, 54.7, 42.8, 34.6, 19.5; IR (KBr) 1635, 1519, 1483, 1421, 1346 cm^{-1}; LRMS (*m*/*z*) 400 (M^+).

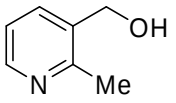
2-(3-Methyl-3*H*-benzothiazol-2-ylidene)-1-oxo-1,2,9,10-tetrahydro-3-thia-10a-aza-8-azoniabenzo[f]azulene (5).



To a solution of **17** (0.253 g, 0.511 mmol) in DMF (5 mL) was added methyl trifluoromethanesulfonate (0.60 mL, 3.0 mmol), and the solution was stirred for 2.5 h at 80 ^oC. After the mixture was diluted with AcOEt, red solids were

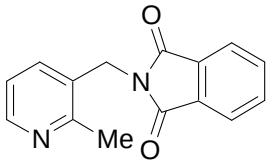
precipitated. The precipitates were collected by filtration and dried. To a suspension of the precipitates in MeCN (10 mL) was added NEt_3 (0.30 mL, 2.2 mmol) at ambient temperature, and the mixture was stirred for 4 h at 70 ^oC. Dilution of the mixture with AcOEt gave red precipitates, which was collected and washed with AcOEt. A solution of the precipitates in MeOH was passed through the anion exchange resin Amberlite IRA-400(Cl^-), and the resin was washed with MeOH. After concentration of the elution in vacuo, the residue was crystallized from AcOEt to give **5** (58 mg, 37% in 3 steps) as red solids. Mp 293 ^oC (decomp.); ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 8.38 (1H, d, *J* = 6.3 Hz), 8.01 (1H, d, *J* = 8.0 Hz), 7.88 (1H, d, *J* = 7.8 Hz), 7.57-7.55 (2H, m), 7.49 (1H, t, *J* = 7.8 Hz), 7.30 (2H, m), 6.23 (1H, s), 5.18-5.14 (1H, m), 5.08-5.03 (1H, m), 4.27-4.22 (1H, m), 4.00 (3H, s), 3.72-3.67 (1H, m); ^{13}C-NMR (100 MHz, DMSO-*d*6) δ 160.3, 154.5, 151.2, 150.1, 142.9, 140.9, 140.4, 127.1, 126.4, 125.5, 123.6, 122.1, 118.2, 111.7, 86.2, 79.8, 57.8, 45.0, 34.2; IR (KBr) 1651, 1624, 1581, 1489, 1419, 1357, 1344, 1302 cm^{-1}; LRMS (*m*/*z*) 382 (M^+); Anal. Calcd for $C_{19}H_{16}ClN_3OS_2 \cdot 2.5H_2O$, C, 51.04; H, 4.74; N, 9.15. Found C, 51.04; H, 4.76; N, 9.40.

3-Hydroxymethyl-2-methylpyridine (**S1**)



Ethyl nicotinate (**18**) (4.98 g, 30.1 mmol) was added to a suspension of $LiAlH_4$ (1.49 g, 39.2 mmol) in THF (50 mL) at 0 ˚C. The mixture was stirred for 1 h at the same temperature. The reaction was quenched by successive addition of water (1.2 mL), 10% NaOH (1.2 mL) and water (2.4 mL). After stirring for 5 min, reaction mixture was filtered through Celite. The filtrate was concentrated by evaporation under reduce pressure. The residue was purified by chromatography on silica gel (AcOEt : MeOH = 3 : 2) to afford **S1** (3.07 g, 83%) as a colorless oil. The spectral data were identical with reported ones.[7]

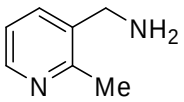
2-(2-Methylpyridin-3-ylmethyl)isoindole-1,3-dione (S2).



To a mixture of **S1** (2.45 g, 19.9 mmol), PPh_3 (7.68 g, 30.0 mmol) and phthalimide (4.41 g, 30.0 mmol) in THF (50 mL) was dropwise added 40% DEAD in toluene (17 mL, 30 mmol) at 0 °C, and the mixture was stirred for 5 h at 60 °C. The resulting mixture was concentrated under reduced pressure, and was dissolved with AcOEt (50 mL). The solution was extracted with 1 M HCl. After the aqueous layer was basicified with sat. $NaHCO_3$, the resulting mixture was extracted with $CHCl_3$. The combined organic layers were washed with brine, dried over Na_2SO_4, and

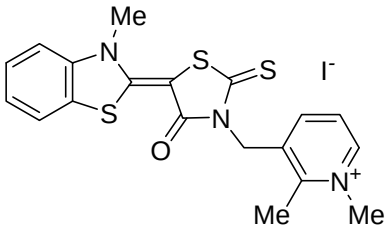
concentrated. The residue was purified by chromatography on silica gel (AcOEt) to afford **S2** (2.06 g, 62%) as white solids. The spectral data were identical with reported ones.[8]

3-Aminomethyl-2-methylpyridine (19)



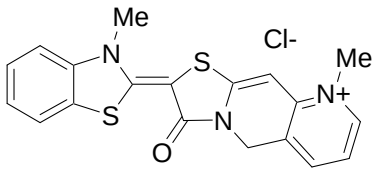
A mixture of **S2** (0.501 g, 3.76 mmol) and $NH_2NH_2 \cdot H_2O$ (1.0 g, 8.0 mmol) in EtOH (30 mL) was refluxed for 1 h. After the reaction mixture was cooled to 4 °C, and the precipitates were filtered off. The filtrate was concentrated under reduced pressure to give **19** (0.456 g; crude product) as a yellow oil, which was used in the next reaction without further purification. The spectral data were identical with reported ones.[9]

2-Methyl-3-{2-[5-(3-methyl-3*H*-benzothiazol-2-ylidene)-4-oxo-2-thioxothiazolidin-3-yl]methyl}pyridinium Iodide (S3)



To a mixture of the above crude product (**19**; 0.399 g) and NEt_3 (1.0 mL, 6.8 mmol) in MeOH (15 mL) was dropwised added CS_2 (0.20 mL, 3.2 mmol) at room temperature, and the mixture was stirred for 1 h. To the reaction mixture was added methyl bromoacetate (0.31 mL, 3.3 mmol) dropwise and stirred for 2 h at room temperature. The reaction mixture was concentrated under reduced pressure to give **20**. To the mixture of **20** and **12** (1.20 g, 3.27 mmol) in MeCN (30 mL) was added NEt_3 (1.0 mL, 6.8 mmol) dropwise at room temperature and stirred for 4 h. The orange precipitates were collected by filtration and washed with MeCN to give **21.** A mixture of the crude product **21** and MeI (0.42 mL, 3.5 mmol) in DMSO (5 mL) was sttired at 60 °C for 12 h. After cooling to room temperature, AcOEt (50 mL) was added to the reaction mixture. The red precipitates were separated from the solution by filtration. The red precipitates were washed with AcOEt to give **S3** as red solids. (0.534 g, 28% from **19**). Mp. 260 °C (decomp.), ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 8.24 (1H, d, *J* = 6.3 Hz), 7.96 (1H, d, *J* = 8.0 Hz), 7.89 (1H, d, *J* = 7.7 Hz), 7.67 (1H, d, *J* = 7.7 Hz), 7.57 (1H, m), 7.38 (1H, t, *J* = 7.7 Hz), 7.21 (1H, t, *J* = 7.7 Hz), 5.34 (2H, s), 4.06 (3H, s), 3.96 (3H, s), 3.52 (3H, s); ^{13}C-NMR (100 MHz, DMSO-*d*6) δ 187.1, 164.2, 155.4, 154.2, 145.2, 140.1, 134.6, 127.4, 125.3, 125.1, 124.3, 122.5, 119.2, 112.4, 82.8, 46.5, 44.3, 34.6, 16.6; IR (KBr) 1638, 1506, 1458, 1420, 1344, 1204 cm^{-1}; LRMS (*m*/*z*) 400 (M^+).

8-Methyl-2-(3-methyl-3*H*-benzothiazol-2-ylidene)-3-oxo-2, 3-dihydro-4*H*-1-thia-3a-aza-8-azoniacyclopenta[b]naphtha lene Chloride (6)



To a DMF solution of **S3** (0.800 g, 1.52 mmol) was added TfOMe (0.6 mL, 4.9 mmol) and stirred for 2 h at 70 °C. After the mixture was cooled to room temperature, AcOEt (30 mL) was added to the reaction mixture. The red precipitates were separated from the solution by filtration. The red solid was dissolved in CH_3CN (10 mL). To the solution was added NEt_3 (1.0 mL, 6.8 mmol) dropwise and stirred at room temperature for 3 h. AcOEt was added to the reaction mixture. The red precipitates were separated by filtration. The resulted red precipitates were dissolved in MeOH. After the solution was mixed with Amberlite IRA-400(Cl) resin, the mixture was filtered. The resulted solution was concentrated under reduced pressure, and the residue was recrystallized from MeOH and AcOEt to give **6** (0.433 g, 71%) as red solids. Mp. 275 °C (decomp.); ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 8.17 (1H, d, *J* = 6.0 Hz), 7.89 (1H, d, *J* = 7.7 Hz), 7.82 (1H, d, *J* = 7.4 Hz), 7.59 (1H, d, *J* = 8.3 Hz), 7.49 (1H, t, *J* = 7.4 Hz), 7.30 (1H, t, *J* = 7.4 Hz), 7.14 (1H, t, *J* = 6.6 Hz), 6.06 (1H, s), 5.26 (2H, s), 3.98 (3H, s), 3.84 (3H, s). ^{13}C-NMR (100 MHz, DMSO) δ 163.4, 154.8, 154.5, 148.1, 141.5, 140.2, 137.2, 127.1, 125.4, 124.4, 123.6, 122.2, 117.8, 111.7, 82.4, 79.6, 44.7, 43.4, 34.2; LRMS (*m*/*z*) 366 (M^+). IR (KBr) 3422, 1663, 1521, 1495,1232, 1193, 756 cm^{-1}, Anal. Calcd for $C_{19}H_{16}ClN_3OS_2 \cdot 2.25H_2O$, C, 51.58; H, 4.67; N, 9.50. Found C, 51.30; H, 4.29; N, 9.15.

Measurement of Fluorescence

Fluorescent spectroscopic studies were performed on a SHIMADZU FR-5300PC. The slit width was 10 nm for both excitation and emission. The photonmultiplier voltage was 950 V. A solution of samples was prepared to be 1.0×10^{-6} M in MeOH containing 0.5% DMSO as a co-solvent. Relative quantum efficiencies of fluorescence of fluorescein derivatives were obtained by comparing the area under the corrected spectrum of the test sample excited at 495 nm in 0.1 M NaOH with that of a solution of fluorescein, which has a quantum efficiency of 0.90. The quantum efficiencies (Φ) of fluorescence were obtained with the following equation (F denotes fluorescence intensity at each wavelength and $\Sigma[F]$ was calculated by summation of fluorescence intensity. I was refractive index of solvent).

$$\Phi^{sample} = \Phi^{standard} ABS^{standard} \Sigma[F^{sample}] I^{standard} / ABS^{sample} \Sigma[F^{standarsd}] I^{sample}$$

Biological Experiment

General. The care and treatment of mice were in accordance with the guidelines (No. 141, 1987) issued by the Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Science and Technology.

Materials

Stock solution: All tested compounds were dissolved in DMSO to make a 5.0 x 10^{-3} M stock solution, which was diluted to the required concentration with MeOH for measurement.

Human malaria parasites: Chloroquine-sensitive *Plasmodium falciparum* (ATCC 30932, FCR–3 strain) was used in our study. *P. falciparum* was cultivated by a modification of the method of Trager and Jensen[1] using a 5% hematocrit of type A^+ human red blood cells suspended in RPMI 1640 medium (Gibco, NY) supplemented with heat-inactivated 10% type A^+ human serum. The plates were placed in a CO_2–O_2–N_2 incubator (5% CO_2, 5% O_2, and 90% N_2 atmosphere) at 37 °C, and the medium was changed daily until 5% parasitemia (which means that 5 parasite-infected erythrocytes in every 100 erythrocytes were existing).

Rodent malaria parasites: *P. berghei* (NK 65 strain) was used in our study. Five-week-old ICR male mice obtained in sterile containers from Japan SLC, Inc. (Hamamatsu, Japan) weighing 22—25 g were used. They were housed under a natural day-night cycle at 25 °C. The mice were randomly assigned to treated groups and housed in cages each containing five individuals. Parasites were collected by cardiac puncture in a heparinized syringe from a donor mouse harboring about 15% parasitemia. The blood was diluted with 0.9% NaCl solution to final concentrations of 1 x 10^6 infected erythrocytes / 0.2 mL of infecting suspension.

Observation of accumulation of fluorescent dyes in *P. berghei*

Plasmodium berghei (NK 65 strain) was used. Five-weeks-old ICR male mice obtained in sterile containers from Japan SLC, Inc. (Hamamatsu, Japan) were used. Fluorescent microscope (Laica FW4000) was used.

When the parasitemia reached about 10%, the infected mice were anesthetized with Et_2O, and blood was collected in heparinized syringes by cardiac puncture. The blood was diluted 50

times with HBS (145 mM HCl, 10 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes-NaOH). Stock solutions of rhodacyanines (5.0 x 10^{-4} M) were made in DMSO. 0.99 mL of red blood cell suspension mixed with 0.01 mL stock solutions of rhdacyanines, to give a final rhodacyanines concentration of 5.0 x 10^{-6} M, was incubated at 38 °C. After 20 min incubation, the cells were chilled to 4 °C and washed two times with cold PBS (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$ and 10 mM NaH_2PO_4-Na_2HPO_4, pH 7.4). Cells stained with rhodacyanines were examined on a fluorescence microscope. Shutter speeds were 5 ms on bright field, 1000 ms on FITC filter, 100 ms on DAPI and Y7 filter.

In vitro* antimalarial assay. *P. falciparum

P. falciparum K1 strain (a clone originating from Thailand) was used in this study. The strain was maintained in RPMI 1640 medium with 0.36 mM hypoxanthine, supplemented with 25 mM HEPES, 25 mM $NaHCO_3$, neomycin (100 U/mL), and 5 g/L of Albumax II (lipid-rich BSA, GIBCO, Grand Island, NY, USA), together with 5% washed human A^+ erythrocytes. All cultures and assays were conducted at 37 ˚C under an atmosphere of 4% CO_2, 3% O_2, and 93% N_2. Cultures were kept in incubation chambers filled with the gas mixture. Subcultures were diluted to a parasitemia of between 0.1% and 0.5% and the medium was changed daily. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL and heated or sonicated if necessary to dissolve the sample. For the assay, the compound was further diluted in serum-free culture medium and finally to the appropriate concentration in complete medium without hypoxanthine. The DMSO concentration in the wells with the highest drug concentration did not exceed 1%.

Assays were performed in sterile 96-well microtiter plates, each well containing 0.2 mL of parasite culture (0.15% parasitemia, 2.5% hematocrit) with or without serial drug solutions. Seven 2-fold dilutions were used, covering a range from 5 to 0.078 μg/mL. Each drug was tested in duplicate and the assay was repeated for active compounds showing an EC_{50} below 1.0 μg/mL. After 48 h of incubation at 37 ˚C, 0.5 mCi 3H-hypoxanthine was added to each well.

Cultures were incubated for a further 24 h before being harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results are recorded as counts per minute per well at each drug concentration and expressed as percentage of the untreated controls. EC_{50} values are calculated from the sigmoidal inhibition curves.

Reference

1. Kawakami, M.; Koya, K.; Ukai, T.; Tatsuta, N.; Ikegawa, A.; Ogawa, K.; Shishido, T.; Chen. L. B. *J. Med. Chem.* **1998**, *41*, 130.
2. Takasu, K.; Inoue, H.; Kim, H.-S.; Suzuki, M.; Shishido, T.; Wataya, Y.; Ihara, M. *J. Med. Chem.* **2002**, *45*, 995.
3. Takasu, K.; Terauchi, H.; Inoue, H.; Kim. H.-S.; Wataya, Y.; Ihara, M. *J. Comb. Chem.* **2003**, *5*, 211.
4. Takasu, K.; Pudhom, K.; Kaiser, M.; Brun, R.; Ihara, M. *J. Med. Chem.* **2006**, *49*, 4795.
5. Nomura, T.; Iwaki, T.; Yasukata, T.; Nishi, K.; Narukawa, Y.; Uotani, K.; Hori, T.; Miwa, H. *Bioorg. Med. Chem.* **2005**, *13*, 6615.
6. Murphy, J. A.; Sherburn, M. S. *Tetrahedron* **1991**, *47*, 4077.
7. Ueyama, N.; Yanagisawa, T.; Kawai, T.; Somegawa, M.; Baba, H.; Mochizuki, S.; Kosakai, K.; Tomiyama, T. *Chem. Pharm. Bull*. **1994**, *49*, 1841.
8. Suh, Y. G.; Oh, U. T.; Kim, H. D.; Lee, W.; Park, H. G.; Park, O. H.; Lee, Y. S.; Park, Y. H.; Joo, Y. H.; Choi, J. K.; Lim, K. M. Kim, S. Y.; Kim, J. K.; Koh, H. J.; Moh, J. H.; Jeong, Y. S.; Yi, J. B.; Oh, Y. I. PCT Int. Appl. (2002), WO 2002016318: CAN 136:216541.
9. Schmitz, H.; Sattler, H. J.; Schunack, W. *Arch. Pharm*. **1975**, *308*, 969.