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Fluorinated Rhodacyanine (SJL-01) Possessing High Efficacy for Visceral Leishmaniasis (VL)

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Anti-Leishmania in vitro and in vivo activities of various rhodacyanine derivatives have been examined. Among them, the fluorinatied variant SJL-01 (**8**) showed IC_{50} of 0.011 μM against *Leishmania donovani* strain MHOM/ET/67/L82 (selective index of >15000) and 95−97% inhibition against *L. donovani* strain MHOM/ET/67/HU in female BALB/c mice by 1.3−12.5 mg/kg × 5 iv administrations. Negative results on chromosomal aberration test and in vitro micronucleus test suggest that compound **8** is a hopeful candidate for visceral leishmaniasis (VL).

Introduction

Leishmaniasis is one of the major target tropical diseases for the World Health Organization (WHO).[1] This serious tropical disease is caused by parasitic protozoa of the genus *Leishmania*.[2] Leishmania are obligate intracellular protozoan parasites of macrophages that are transmitted between hosts by the bite of female phlebotomine sand flies and are delivered to a host as mammal-infective metacyclic promastigotes along with the saliva of the sand fly and a mucin-rich gel produced by the parasites in the sand fly midgut.[3] It manifests mainly in three clinical forms: visceral leishmaniasis (VL[a]), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL), of which VL is the most severe form of the diseases. VL, also called Kala-azar or black fever, can have a fatality rate as high as 100% within two years if left untreated, and spontaneous cure is extremely rare.[4] VL is found throughout the intertropical and temperate regions and threatens ∼350 million people in 88 countries. Although few medicines are available for leishmaniasis, the most widely used drug is pentostam, containing the heavy metal antimony. Other medicines such as amphotericin B, liposomal amphotericin B, paromomycin, and miltefosine have individual problems such as toxicity, poor effect, or high cost. Development of other potential compounds including buparvaquone and aminoquinolines are underway. However, the emergence of drug resistant parasites has caused further serious problems for therapy and the invention of new types of medicines having novel chemical frameworks is highly desirable. On the basis of DLCs hypothesis targeting mitochondrial membrane,[5] we have searched hit compounds against leishmania parasites. In a previous paper, we reported that rhodacyanine dyes showed potent activity against *Leishmania major* causing CL.[6] Further studies of the activity of rhodacyanines against *L. donovani* that causes VL led us to the discovery of a promising candidate for VL. Here, we disclose our exciting findings.

Results and Discussion

Biological Results. In Vitro Anti-Leishmania Activity. Various rhodacyanine derivatives, including some that have been previously described[7−11] and also some newly synthesized candidates, were subjected to the screening test against *L. donovani*, which causes VL. Each candidate was evaluated for its in vitro activity against *L. donovani* strain MHOM/ET/67/L82 and for its inherent cytotoxicity against L-6 rat skeletal myoblasts (Table 1). The selectivity of a compound was defined as the ratio of the cytotoxicity/the inhibitory activity. The previously described **1** (MKT-077)[6,12] (Chart 1) exhibited significant activity with a selectivity factor of 450, compared with that of miltefosine, a drug that has been clinically applied to the treatment of leishmaniasis. Although **2** (SSJ-127)[11] could cure rodent malaria with sc administration, the in vitro activity was rather low against Leishmania parasites with low selectivity. Numerous derivatives of **2** having different alkyl substituents on three nitrogen atoms were prepared and their activities were tested. While compound **4** possessed relatively good inhibitory activity as indicated by its low IC_{50} value, its cytotoxicity was high. In compound **5**, replacement of the oxazolo[4,5-*b*]pyridine moiety with benzo[*d*]thiazole did not improve the activity. In compound **6**, substitution of an imine functionality provided better activity and selectivity in the in vitro test. Introduction of a chlorine atom on the right-hand ring

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[a] Abbreviations: VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; S9, a supplemented rat liver homogenate fraction; TMS, tetramethylsilane; L-6, a rat skeletal myoblast cell line; FBS, fetal bovine serum; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide.

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Table 1. Evaluation of Rhodacyanines: Axenic in Vitro Activity against *Leishmania donovani* and Cytotoxicity toward L-6 Myocytes

compd	*L. donvani* IC_{50} (μM)[a]	L-6 IC_{50} (μM)[b]	selectivity[c]
1	0.255	114.8	450
2	0.677	27.2	40
3	0.69	1.44	2.0
4	0.021	2.07	99
5	0.106	7.98	75
6	0.052	155.7	2987
7	0.028	21.2	757
8	0.011	>173.7	>15000
9	0.025	71.7	2870
10	0.02	84.5	4225
11	11.65	>169.1	>15
12	1.04	28.5	27
13	0.129	80.9	627
14	0.118	10.4	88
15	0.182	16.5	91
16	2.023	31.7	16
miltefosine	0.43	ND[d]	ND[d]

[a] Inhibitory activity of rhodacyanine drugs was assessed in an in vitro assay with *L. donvani* as described in the Experimental Section. IC_{50} values were determined as described, using calculations derived from *n* assays per drug concentration. [b] Cytotoxicity of each candidate was determined on the basis of IC_{50} values determined in an assay using L-6 myocytes, as described in the Experimental Section. [c] Selectivity was calculated as IC_{50} in the cytotoxicity assay/IC_{50} in the in vitro assay. [d] ND = not determined.

(compound **7**) enhanced the activity, while substitution of a fluorine atom at the same site resulted in excellent activity in the in vitro test, as well as a high selectivity factor for compound **8**, termed SJL-01. The corresponding maleate (**9**) and mesylate (**10**) derivatives showed good inhibitory activities. However, in **11**, the presence of an alcohol group at the right-hand ring reduced the activity dramatically. The replacement of the left-hand benzo[*d*]thiazole with oxazolo-[4,5-*b*]pyridine gave poor activity. Connecting the 1,3-dimethylbenzo[*d*]imidazole through a nitrogen (compound **13**) gave unsatisfactory results in the in vitro test. The same tendencies were observed on the tests of 3,5-dimethylthiazole **14**, 3,4-dimethylthiazole **15**, and the methylpyridine derivative **16**.

Macrophage in vitro screening, which is crucial to evaluate antileishmania activity, was then applied to several of the most promising rhodacyanines, but most compounds showed poor results as indicated by the IC_{50} values that exceeded the corresponding values in the axenic in vitro assay (e.g., compound **6**, entry 1 in Table 2). On the other hand, compounds **7** and **8** showed noteworthy activities (entries 2 and 3 in Table 2) compared with that of miltefosine.

In Vivo Anti-Leishmania Activity. On the basis of the results of the in vivo macrophage assays, compounds **7** and **8** were further evaluated by in vivo test using *L. donovani* strain MHOM/ET/67/HU in female BALB/c mice. As shown in Table 3, **8** showed better efficacy than **7** by ip administration (entries 1 and 2 in Table 3). Preliminary studies showed that no bioavailability was obtained by sc administrations of **8** and the corresponding mesylate **10**, and thus, the in vivo studies were carried out via iv administration. Excellent activities, ~95% inhibition, were observed by the 5 times treatments with compound **8** at dosages ranging from 1.3 to 12.5 mg/kg (entries 3−5). Even at a dose as low as 0.2 mg/kg × 5 of **8** gave 16.13% inhibition (entry 6), suggesting the existence of a dose dependent activity. The mesylate **10** given by iv administration (2 mg/kg × 5 iv treatment) was less active (entry 7) and a poor result when administered po (entry 8). Thus, the activity of compound **8** is much better than that of a conventional medicine, pentostam (entry 9), and comparable to that of amphotericin B (entry 10) and liposomal amphotericin B (entry 11).

Synthesis. All rhodacyanine derivatives were synthesized by an established method.[12,13] As an example, synthetic route of **8** is shown in Scheme 1. Thus, the reaction of rhodanine **17** with *N*,*N*-diphenylformamidine in DMF followed by treatment with acetic anhydride gave **18**, which was reacted with *N*-methyl-2-methylbenzothiazolium salt **19** to provide merocyanine **20**. After formation of **21**, obtained by the reaction of **20** with methyl *p*-toluenesulfonate, condensation of **21** with **22** in the presence of triethylamine followed by treatment with hydrochloric acid furnished **8**. The desired compound can be easily synthesized in excellent yield in six steps, affording crystalline compound. Highly pure product, mp 274.5−275.6 °C, is obtained by simple crystallization.

Summary

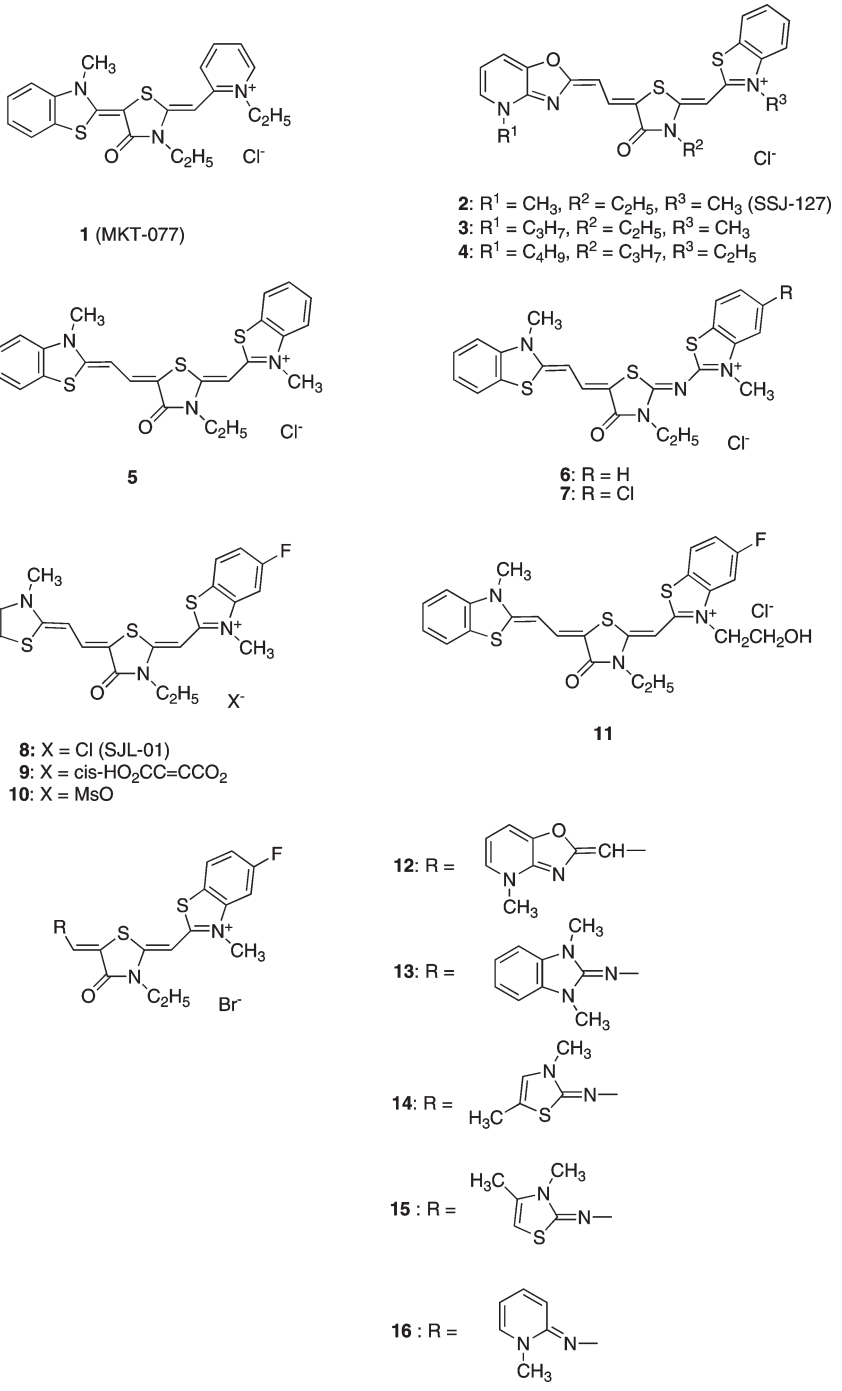
The present research led us to the discovery of compound **8**, which showed extraordinary efficacy in an in vivo test via iv administration. The activity was much better than pentostam and was comparable to that of amphotericin B, which is toxic and expensive. Therefore, the preclinical and clinical development of **8** aimed at leishmaniasis is important for the therapy of this neglected disease. Furthermore, in vitro micronucleus test of **8** showed a negative result at 2 μM in the absence of S9 (a supplemented rat liver homogenate fraction) and 31.3 μM in the presence of S9, while negative results were obtained at 5.19 μM (−S9) and at 3.97 μM (+S9) by the chromosomal aberration test; the results are consistent with a safe profile against genetic toxicity. Thus, compound **8** is very hopeful candidate for VL.

Experimental Section

Chemistry. General Details. Starting materials were obtained from Wako and Aldrich and used as received. Melting points were determined on a Yanaco apparatus and are uncorrected. NMR spectra were recorded on a Bruker 400 NMR spectrometer; TMS was used as an internal standard for ^{1}H NMR, and the solvent peak was used as an internal standard for ^{13}C NMR. Absorption spectra were scanned on JASCO V-550 UV−vis spectrophotometer. IR spectra were determined on a JASCO FT/IR-200 spectrophotometer. Mass spectra were recorded with JEOL JMS-600 and Shimadzu LCMS-2010 EV spectrometers. The purities (>98%) of products were determined by the LCMS spectrometer using Capcell pak C18 MGII 3 μm, 3.0 mm i.d. × 150 mm (Shiseido) eluting with 0.2 mL/min of 0.1% trifluoroacetic acid−acetonitrile (2:3 v/v) and monitoring with total ion current and UV at 254 nm.

2-((3-Ethyl-5-(2-(3-methylbenzo[*d*]thiazol-2(*3H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo-[*d*]thiazol-3-ium Chloride (8). Under an argon atmosphere, a mixture of **20** (0.5 g, 1.5 mmol), methyl *p*-toluenesulfonate (0.84 g, 4.5 mmol), and DMF (1.5 mL) in toluene (0.5 mL) was stirred at 115 °C for 6 h. After being cooled to ambient temperature, a mixture of **22** (0.51 g, 1.5 mmol) in acetonitrile (50 mL) was added to the resulting mixture. The mixture was stirred for 12 h at 75 °C. The precipitate formed was collected and washed with acetonitrile and ethyl acetate to give the tosylate as a dark-green solid (0.64 g, 65.8% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.20 (dd, J = 8.8 and 5.2 Hz, 1H), 7.82 (d, J = 7.7 Hz, 1H), 7.71 (dd, J = 9.9 and 2.1 Hz, 1H), 7.57 (d, J = 13.2 Hz, 1H), 7.49

Chart 1. Known and New Rhodacyanines



(d, J = 8.0 Hz, 2H), 7.42−7.18 (m, 4H), 7.11 (d, J = 8.0 Hz, 2H), 6.69 (s, 1H), 5.90 (d, J = 13.2 Hz, 1H), 4.15 (q, J = 7.1 Hz, 2H), 3.96 (s, 3H), 3.65 (s, 3H), 2.29 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H). MS (ESI^+) m/z: 482.1 [M^+].

The above tosylate (0.5 g, 0.76 mmol) was stirred in methanol (50 mL) at 80 °C for 30 min, and then concentrated hydrochloric acid (about 2.3 mL) was slowly added to the mixture. After the mixture was stirred for 30 min, the precipitate was filtered and washed with methanol to give **8** as a dark-green solid (0.39 g, 99% yield). Mp 274.5−275.6 °C. UV−vis (H_2O): λ (nm) (log ε, L mol^{-1} cm^{-1}): 528 (4.54), 356 (4.23). IR ν (neat, cm^{-1}): 2972, 1685, 1525, 1471, 1376, 1362, 1314, 1276, 1198, 1055, 1033, 941, 891, 819, 747. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.27 (dd, J = 8.9 and 5.2 Hz, 1H), 7.83 (d, J = 7.7 Hz, 1H), 7.75 (dd, J = 9.9 and 2.3 Hz, 1H), 7.59 (d, J = 13.2 Hz, 1H), 7.43−7.31 (m, 3H), 7.28 (t, J = 6.0 Hz, 1H), 6.72 (s, 1H), 5.88 (d, J = 13.2 Hz, 1H), 4.17 (q, J = 7.1, Hz, 2H), 3.98 (s, 3H), 3.68 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 164.0, 163.4, 163.0, 161.5, 157.3, 141.6, 134.6, 127.5, 125.0, 124.2, 123.8, 122.6, 121.5, 113.6, 113.4, 112.4, 102.3, 102.1, 101.7, 90.9, 86.5, 34.8, 32.9, 12.4. MS (ESI^+) m/z: 482.1 [M^+]. Anal. Calcd for $C_{24}H_{21}ClFN_3OS_3 \cdot 2H_2O$: C, 51.97; H, 5.04; N, 7.55. Found: C, 52.02; H, 4.55; N, 7.58.

2-((3-Ethyl-4-oxo-5-(2-(4-propyloxazolo[4,5-*b*]pyridin-2(4*H*)-ylidene)ethylidene)thiazolidin-2-ylidene)methyl)-3-methylbenzo-[*d*]thiazol-3-ium Chloride (3). Dark-blue solid, mp 218−220 °C. IR ν (KBr cm^{-1}): 3392, 1663, 1524, 1466, 1130. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.20 (dd, J = 21.5 and 7.2 Hz, 2H), 7.95 (d, J = 8.3 Hz, 2H), 7.71 (m, 1H), 7.54 (t, J = 7.6 Hz, 1H), 7.27−7.17 (m, 1H), 6.73 (s, 1H), 5.55 (d, J = 13.7 Hz, 1H),

4.48−4.34 (m, 2H), 4.17 (dd, J = 14.0 and 7.0 Hz, 2H), 4.07 (s, 3H), 2.00−1.86 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H), 0.93 (t, J = 7.1 Hz, 3H). MS (ESI^+) m/z: 477.2 (M^+).

2-((5-(2-(4-Butyloxazolo[4,5-*b*]pyridin-2(4*H*)-ylidene)ethylidene)-4-oxo-3-propylthiazolidin-2-ylidene)methyl)-3-ethylbenzo[*d*]thiazol-3-ium Chloride (4). Black solid, mp 246−248 °C. IR ν (KBr cm^{-1}): 3417, 1667, 1515, 1464, 1130. ^1H NMR (400 MHz, DMSO-d_6) δ: 8.20 (dd, J = 17.4 and 6.8 Hz, 2H), 7.96 (d, J=8.4 Hz, 2H), 7.72−7.67 (m, 1H), 7.53 (t, J = 7.7 Hz, 1H), 7.29−7.16 (m, 1H), 6.70 (s, 1H), 5.55 (d, J = 13.7 Hz, 1H), 4.72 (q, J = 7.1 Hz, 2H), 4.51−4.34 (m, 2H), 4.14 (t, J = 7.1 Hz, 2H), 1.96−1.80 (m, 2H), 1.75−1.64 (m, 2H), 1.40−1.29 (m, 5H), 0.94 (m, 6H). MS (ESI^+) m/z: 519.3 (M^+).

Table 2. Inhibition by Rhodacyanines against *Leishmania donovani* in an in Vitro Activity Assay in Macrophages

entry	compd	*L. donvani* IC_{50} (μM)[a]
1	**6**	4.691
2	**7**	0.08
3	**8**	0.353
4	miltefosine	0.811

[a] IC_{50} values for each candidate were calculated from an in vitro assay conducted with macrophages, as described in the Experimental Section.

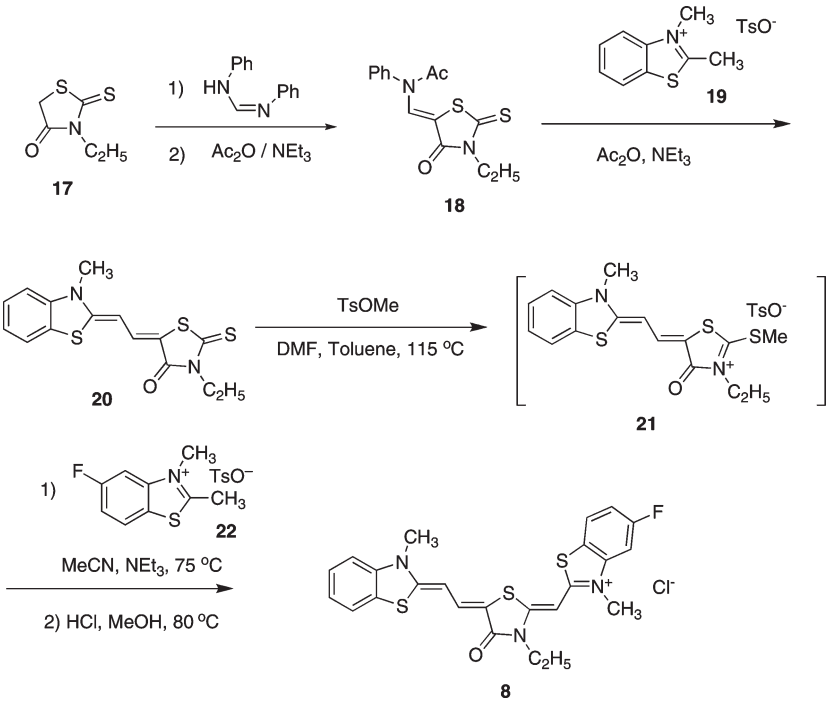
2-((3-Ethyl-5-(2-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)methyl)-3-methylbenzo[*d*]thiazol-3-ium Chloride (5). Dark-blue solid, mp 255−257 °C. UV−vis (MeOH): λ (nm) (log ε, L mol^{-1} cm^{-1}): 594 (5.05), 376 (4.57). IR ν (neat, cm^{-1}): 2968, 1689, 1531, 1470, 1376, 1349, 1317, 1277, 1226, 1193, 1148, 1089, 1059, 1033, 984, 909, 816, 743, 723. ^1H NMR (400 MHz, DMSO-d_6) δ: 8.24 (d, J = 7.7 Hz, 1H), 7.85 (dd, J = 15.3 and 8.2 Hz, 2H), 7.70−7.65 (m, 1H), 7.63 (d, J = 13.2 Hz, 1H), 7.54 (t, J = 7.7 Hz, 1H), 7.46−7.36 (m, 2H), 7.27 (ddd, J = 8.2, 6.5, and 2.0 Hz, 1H), 6.74 (s, 1H), 5.93 (d, J = 13.2 Hz, 1H), 4.16 (q, J = 7.0 Hz, 2H), 4.05 (s, 3H), 3.71 (s, 3H), 1.28 (t, J = 7.0 Hz, 3H). MS (ESI^+) m/z: 464.1 [M^+].

2-((3-Ethyl-5-(2-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)amino)-3-methylbenzo[*d*]thiazol-3-ium Chloride (6). Dark-blue solid, mp 242−244 °C. UV−vis (MeOH): λ (nm) (log ε, L mol^{-1} cm^{-1}): 566 (4.91), 388 (4.07). IR ν (neat, cm^{-1}): 1663, 1633, 1556, 1522, 1461, 1363, 1318, 1285, 1238, 1191, 1145, 1057, 1020, 992, 903, 817, 758, 733. ^1H NMR (400 MHz, DMSO-d_6) δ: 8.26 (d, J = 7.7 Hz, 1H), 7.93 (dd, J = 15.0 and 7.7 Hz, 2H), 7.82 (d, J = 13.4 Hz, 1H), 7.78−7.20 (m, 1H), 7.60 (dd, J = 15.0 and 7.7 Hz, 2H), 7.52−7.44 (m, 1H), 7.37−7.30 (m, 1H), 6.12 (d, J = 13.4 Hz, 1H), 4.08 (q, J = 7.1 Hz, 2H), 4.03 (s, 3H), 3.80 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H). MS (ESI^+) m/z: 465.1 [M^+].

Table 3. In Vivo Activity against *Leishmania donovani* HU3 in BALB/c Mice

entry	compd	dosing regimen ((mg/kg)/day)	inhibition (%)	95% confidence limit
1	**7**	50 (ip) × 5	18.2	19.2
2	**8**	50 (ip) × 5	31.43	12.2
3	**8**	12.5 (iv) × 5	95.40	5.4
4	**8**	4.1 (iv) × 5	97.15	3.51
5	**8**	1.3 (iv) × 5	94.87	6.3
6	**8**	0.2 (iv) × 5	16.13	5.1
7	**10**	2 (iv) × 5	46.36	7.0
8	**10**	50 (po) × 5	28.0	18.8
9	pentostam	15 (sc) × 5	62.04	14.7
10	amphotericin B	0.5 (iv) × 3	78.6	7.6
11	liposomal amphotericin B	1.5 (iv) × 3	95.53	3.2

Scheme 1. Synthesis of Compound **8**



5-Chloro-2-((3-ethyl-5-(2-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)amino)-3-methylbenzo[*d*]thiazol-3-ium Chloride (7). Dark-blue solid, mp 224−228 °C. IR ν (KBr, cm^{-1}): 1519, 1456. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.40 (br, 1H), 8.02−8.00 (m, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 13.2 Hz, 1H), 7.84−7.81 (m, 1H), 7.53 (t, J = 7.2 Hz, 1H), 7.37 (t, J = 8.0 Hz, 1H), 6.19 (d, J = 13.2 Hz, 1H), 4.11 (q, J = 7.2 Hz, 2H), 4.02 (s, 3H), 1.20 (t, J = 7.2 Hz, 3H). MS (ESI^+) m/z: 499 [M^+].

2-((3-Ethyl-5-(2-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo-[*d*]thiazol-3-ium Maleate (9). Dark-green solid, mp 260.1 −260.8 °C. UV−vis (H_2O): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 526 (4.68), 355 (4.37). IR ν (neat, cm^{-1}): 2972, 2937, 2866, 1684, 1526, 1472, 1376, 1360, 1346, 1314, 1275, 1198, 1055, 1032, 1013, 940, 891, 818, 747. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.28 (dd, J = 8.8 and 5.1 Hz, 1H), 7.99 (dd, J = 9.9 and 1.9 Hz, 1H), 7.89 (d, J = 7.8 Hz, 1H), 7.75 (d, J = 13.2 Hz, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.49 (dd, J = 17.0 and 8.3 Hz, 2H), 7.33 (t, J = 7.6 Hz, 1H), 6.78 (s, 1H), 6.03 (d, J = 13.2 Hz, 3H), 4.19 (q, J = 7.1 Hz, 2H), 4.06 (s, 3H), 3.78 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H). MS (ESI^+) m/z: 482.3 [M^+].

2-((3-Ethyl-5-(2-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo[*d*]thiazol-3-ium Methanesulfonate (10). Dark-green solid, mp > 300 °C. UV−vis (H_2O): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 524 (4.83), 355 (4.52). IR ν (neat, cm^{-1}): 2971, 2937, 1682, 1525, 1470, 1376, 1358, 1317, 1276, 1197, 1057, 1033, 939, 891, 819, 747. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.29 (dd, J = 8.8 and 5.2 Hz, 1H), 8.01 (dd, J = 9.9 and 2.0 Hz, 1H), 7.90 (d, J = 7.8 Hz, 1H), 7.76 (d, J = 13.2 Hz, 1H), 7.61 (d, J = 8.3 Hz, 1H), 7.49 (m, 2H), 7.34 (t, J = 7.8 Hz, 1H), 6.77 (s, 1H), 6.05 (d, J = 13.2 Hz, 1H), 4.19 (q, J = 7.1 Hz, 2H), 4.05 (s, 3H), 3.77 (s, 3H), 2.29 (s, 3H), 1.26 (t, J = 7.1 Hz, 3H). MS (ESI^+) m/z: 483.2 [M^+].

5-Fluoro-2-((3-(2-hydroxyethyl)-5-(2-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)ethylidene)-4-oxothiazolidin-2-ylidene)methyl)-3-methylbenzo[*d*]thiazol-3-ium Chloride (11). Dark-green solid, mp 277.0−277.7 °C. UV−vis (H_2O): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 600 (3.95), 379 (3.50). IR ν (neat, cm^{-1}): 3668, 2921, 2868, 1732, 1524, 1498, 1472, 1371, 1345, 1314, 1269, 1249, 1192, 1145, 1059, 1033, 1015, 819, 751. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.29 (dd, J = 8.9 and 5.2 Hz, 1H), 7.98 (dd, J = 10.0 and 2.2 Hz, 1H), 7.89 (d, J = 7.8 Hz, 1H), 7.74 (d, J = 13.2 Hz, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.54−7.43 (m, 2H), 7.32 (t, J = 7.7 Hz, 1H), 6.93 (s, 1H), 6.02 (d, J = 13.2 Hz, 1H), 5.19 (br, 1H), 4.78 (t, J = 4.4 Hz, 2H), 4.16 (q, J = 6.9, 6.9 Hz, 2H), 3.87 (t, J = 3.9 Hz, 2H), 3.78 (s, 3H), 1.24 (t, J = 7.1 Hz, 3H). MS (ESI^+) m/z: 512.1 [M^+].

2-((Ethyl-5-(2-(4-methyloxazolo[4,5-*b*]pyridin-2(4*H*)-ylidene)-ethylidene)-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo[*d*]thiazol-3-ium Bromide (12). Dark-green solid, mp 286−288 °C. UV−vis (MeOH): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 616 (5.00), 382 (4.33). IR ν (neat, cm^{-1}): 2982, 2819, 1771, 1747, 1715, 1697, 1682, 1648, 1542, 1524, 1489, 1338, 1227, 1033, 835. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.21 (dd, J = 8.5 and 5.2 Hz, 1H), 8.18−8.16 (m, 1H), 8.13 (d, J = 5.2 Hz, 1H), 8.05−7.79 (m, 2H), 7.37 (dt, J = 8.7, 8.5, and 1.9 Hz, 1H), 7.27−7.10 (m, 1H), 6.67 (s, 1H), 5.47 (d, J = 13.6 Hz, 1H), 4.15 (q, J = 7.2 Hz, 2H), 4.08 (s, 3H), 3.99 (s, 3H), 1.24 (t, J = 7.2, Hz, 3H). MS (ESI^+) m/z: 467.1 [M^+].

2-((5-((1,3-Dimethyl-1*H*-benzo[*d*]imidazol-2(3*H*)-ylideneamino)-methylene)-3-ethyl-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo[*d*]thiazol-3-ium Bromide (13). Dark-blue solid, mp 274−276 °C. UV−vis (MeOH): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 512 (4.76), 350 (4.57). IR ν (neat, cm^{-1}): 1680, 1658, 1515, 1473, 1425, 1350, 1331, 1277, 1201, 1184, 1155, 1134, 1061, 997, 940, 884, 831, 756. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.75 (s, 1H), 8.30 (dd, J = 8.9 and 5.5 Hz, 1H), 7.97 (dd, J = 10.0 and 2.5 Hz, 1H), 7.63 (dd, J = 5.5 and 2.5 Hz, 2H), 7.46−7.39 (m, 3H), 6.71 (s, 1H), 4.18 (q, J = 7.0 Hz, 2H), 4.04 (s, 3H), 3.76 (s, 6H), 1.26 (t, J = 7.0, Hz, 3H). MS (ESI^+) m/z: 480.1 [M^+].

2-((5-((3,5-Dimethylthiazol-2(3*H*)-ylideneamino)methylene)-3-ethyl-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo[*d*]thiazol-3-ium Bromide (14). Dark-blue solid, mp 251−253 °C. UV−vis (MeOH): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 526 (4.86), 362 (4.56). IR ν (neat, cm^{-1}): 2975, 1659, 1584, 1528, 1489, 1417, 1389, 1333, 1279, 1204, 1148, 1061, 993, 979, 939, 893, 834, 755, 738. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.33 (dd, J = 8.9 and 5.2 Hz, 1H), 8.06 (s, 1H), 8.02 (dd, J = 9.9 and 2.4 Hz, 1H), 7.47 (dt, J = 8.9 and 2.4 Hz, 1H), 7.32 (d, J = 1.4 Hz, 1H), 6.74 (s, 1H), 4.15 (q, J = 7.0 Hz, 2H), 4.07 (s, 3H), 3.60 (s, 3H), 2.30 (s, 3H), 1.24 (t, J = 7.0, Hz, 3H). MS (ESI^+) m/z: 447.0 [M^+].

2-((5-((3,4-Dimethylthiazol-2(3*H*)-ylideneamino)methylene)-3-ethyl-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo[*d*]thiazol-3-ium Bromide (15). Dark-blue solid, mp 277−279 °C. UV−vis (MeOH): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 526 (4.92), 362 (4.64). IR ν (neat, cm^{-1}): 2916, 1681, 1584, 1525, 1474, 1416, 1375, 1328,1276, 1199, 1157, 1062, 1033, 869, 838, 749. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.34 (dd, J = 8.9 and 5.2 Hz, 1H), 8.18 (s, 1H), 8.03 (dd, J = 9.9 and 2.3 Hz, 1H), 7.48 (dt, J = 8.9 and 2.3 Hz, 1H), 6.83 (d, J = 1.1 Hz, 1H), 6.76 (s, 1H), 4.16 (q, J = 7.0 Hz, 2H), 4.08 (s, 3H), 3.62 (s, 3H), 2.30 (s, 3H), 1.25 (t, J = 7.0, Hz, 3H). MS (ESI^+) m/z: 447.0 [M^+].

2-((3-Ethyl-5-((1-methylpridin-2(1*H*)-ylideneamino)methylene)-4-oxothiazolidin-2-ylidene)methyl)-5-fluoro-3-methylbenzo-[*d*]thiazol-3-ium Bromide (16). Dark-blue solid, mp 256−260 °C. UV−vis (MeOH): λ (nm) ($\log \varepsilon$, L mol^{-1} cm^{-1}): 530 (4.74), 368 (4.41). IR ν (neat, cm^{-1}): 2975, 1649, 1519, 1448, 1333, 1274, 1203, 1163, 1061, 994, 938, 898, 835, 796, 763. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.7 (s, 1H), 8.29 (dd, J = 9.0 and 4.5 Hz, 2H), 7.99−7.88 (m, 2H), 7.65 (d, J = 9.0 Hz, 1H), 7.47−7.35 (m, 1H), 7.01 (t, J = 6.7, Hz, 1H), 6.68 (s, 1H), 4.17 (q, J = 7.0 Hz, 2H), 4.03 (s, 3H), 3.92 (s, 3H), 1.25 (t, J = 7.0, Hz, 3H). MS (ESI^+) m/z: 427.1 [M^+].

Biology. In Vitro Anti-Leishmanial Assay. The *Leishmania donovani* strain MHOM/ET/67/L82 was used. The strain was maintained in the Syrian Golden hamster. Amastigotes were collected from the spleen of an infected hamster and then grown in axenic culture at 37 °C in Cunningham SM medium, pH 5.4, supplemented with 10% heat-inactivated fetal bovine serum (FBS) under an atmosphere of 5% CO_2 in air. Stock drug solutions are prepared in 100% dimethyl sulfoxide (DMSO) at 10 mg/mL and heated or sonicated if necessary to dissolve the sample. Assays were performed in 96-well flat-bottom microtiter plates, with each well containing 100 μL of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution. After 72 h of incubation, the plates were inspected under an inverted microscope to ensure growth of the controls and sterile conditions. An amount of 10 μL of Alamar blue (12.5 mg of resazurin dissolved in 100 mL of distilled water) was then added to each well, and the plates were incubated for another 2 h. The plates were then read with a microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The decrease of fluorescence (i.e., inhibition) was expressed as a percentage of the fluorescence of control cultures and plotted against the drug concentrations. The IC_{50} value was calculated from the sigmoidal inhibition curve by the software program.

Cytotoxicity Assay. The L-6 rat skeletal myoblast cell line was grown in RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% FBS in T-25 tissue culture flasks at 37 °C in 5% CO_2 in air. The cultures were subpassaged three times a week, using trypsin to detach the cells, and split in a 1:2 or 1:3 ratio, depending on the density of the parent culture. Samples were cryopreserved at a low passage number. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL and heated or sonicated if necessary to dissolve the sample. Assays were performed in 96-well microtiter plates, each well receiving

100 μL of culture medium with 4×10^4 cells. After 24 h, the medium was removed from all wells and replaced by 100 μL of fresh medium in all wells except for those in row H of the plate. Fresh medium (150 μL) containing the highest drug concentration was added to wells of row H. Serial drug dilutions were prepared by transferring 50 μL from wells of row H to wells of row G. After gentle mixing, 50 μL from row G was transferred to row F, and so on. After 72 h of incubation the plates were inspected under an inverted microscope to ensure growth of the controls and sterile conditions. Then 10 μL of Alamar blue (12.5 mg of resazurin dissolved in 100 mL of distilled water) was added to each well and the plates were incubated for another 2 h. The plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Device Cooperation, Sunnyvale, CA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA).

Macrophage in Vitro Anti-Leishmania Assay. Primary peritoneal macrophages from NMRI mice are collected 1 day after stimulation of macrophage production with an intraperitoneal injection of 2 mL of a 2% potato starch suspension. All cultures and assays were performed at 37 °C under an atmosphere of 5% CO_2 in air. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL and heated or sonicated as necessary to dissolve the sample. Assays are performed in sterile 16-well chamber slides. To each well are added 100 μL of a murine macrophage suspension (4×10^5/mL) in RPMI 1640 medium containing bicarbonate and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) supplemented with 10% heat inactivated FBS (RPMI/FBS). After 24 h, 100 μL of a suspension containing amastigotes (1.2×10^6/mL) was added to each well, giving a 3:1 ratio of amastigotes/macrophages. The amastigotes were harvested from an axenic amastigote culture and suspended in RPMI/FBS. After 24 h, the medium containing free amastigotes was removed, the cells were washed once with medium, and fresh medium containing drug dilutions (four 3-fold dilutions for each compound) was added. Parasite growth in the presence of the drug was compared to control wells. After 4 days of incubation, the culture medium was removed and the slides were prepared by first fixing with methanol for 10 min and then staining with a 10% Giemsa solution. Infected and noninfected macrophages were counted in the control cultures and in those exposed to the serial drug dilutions. The results were expressed as percent reduction in parasite burden compared to control wells, and the IC_{50} was calculated by linear regression analysis (Microsoft Excel).

In Vivo Leishmaniasis Assay. The pentostam sensitive *L. donovani* (strain MHOM/ET/67/HU3) was used. Female specific pathogen-free BALB/c mice (6−8 weeks, about 20 g) and Syrian hamsters *Mesocricetus auratus* were employed. *L. donovani* amastigotes were isolated from the spleen of a heavily infected donor hamster, and an inoculum containing 7.5×10^7 amastigotes/mL in RPMI 1640 was prepared. Animals are infected intravenously (tail vein) with a 0.2 mL bolus (equivalent to 1.5×10^7 amastigotes) on day 0. Infected mice were randomly assorted into groups of five. On day 7 after infection one mouse was sacrificed, liver smears were taken, slides were methanol fixed and Giemsa stained, and infection was assessed. Experimental compounds are dissolved in 10% ethanol and 5% glucose solution. Groups of mice were weighed before and after treatment, and the % weight change was noted. Mice are treated intraperitonealy or intravenously once a day for 5 days on days 7−11 of infection (5 animals/group) using 50 mg of drug per kg per day (unless previous toxicity data indicate that a lower dose was preferable). On day 14 after infection, 3 days after the completion of treatment, livers were removed and weighed, and smears were methanol fixed and Giemsa stained. Parasite burdens were determined microscopically (×100, oil immersion) by counting the number of amastigotes per 500 liver cells. The results were expressed as the mean number of amastigotes per liver cell × mg of liver. The parasite burden of drug treated groups was compared with that of the untreated group, and the % inhibition was calculated.

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