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Anthracene-Polyamine Conjugates Inhibit *In Vitro* Proliferation of Intraerythrocytic *Plasmodium falciparum* Parasites

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Anthracene-polyamine conjugates inhibit the *in vitro* proliferation of the intraerythrocytic human malaria parasite *Plasmodium falciparum*, with 50% inhibitory concentrations (IC_{50}s) in the nM to μM range. The compounds are taken up into the intraerythrocytic parasite, where they arrest the parasite cell cycle. Both the anthracene and polyamine components of the conjugates play a role in their antiplasmodial effect.

Polyamines are present at high concentrations in rapidly proliferating cells, including cancer cells (1) and protozoan parasites (2), and are taken up from the external environment via specific transport mechanisms (3, 4). In cancer cells, the strategy of conjugating cytotoxic agents to polyamines with the aim of exploiting the polyamine uptake systems, as a means of facilitating their uptake, has proven effective (4). Here, a series of compounds containing cytotoxic anthracene (Ant) conjugated to a polyamine was investigated for their effect on the *in vitro* growth of the human malaria parasite *Plasmodium falciparum*.

P. falciparum (3D7) parasite cultures were maintained (5) and synchronized (6) as described previously. The anthracene-polyamine conjugates tested for their antiplasmodial effect were as follows: Ant-4 [Ant-CH_2-NH-$(CH_2)_4$-NH_2], a putrescine conjugate; Ant-44 [Ant-CH_2-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2], a homospermidine conjugate; Ant-444 [Ant-CH_2-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2], a homospermine conjugate; and 44-Ant-44 [NH_2-$(CH_2)_4$-NH-$(CH_2)_4$-NH-CH_2-Ant-CH_2-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2], a bis-homospermidine conjugate (7) (Table 1). A polyamine analog, N^1-methylHSpd [NH_2-$(CH_2)_4$-NH-$(CH_2)_4$-NH-CH_3], and an *N*-alkylated anthracene derivative, Ant-*N*-butyl [Ant-CH_2-NH-$(CH_2)_3$-CH_3] (8) served as controls (see structures in Table 3). The antiplasmodial activity of the compounds was determined after addition to "ring-stage" intraerythrocytic *P. falciparum* parasite cultures (200 μl at 1% hematocrit and 1% parasitemia in 96-well plates), which were then incubated for 96 h at 37°C. Parasite proliferation was assessed using the SYBR green I fluorescence assay as described elsewhere (9, 10). Statistical significance was assessed with a two-tailed *t* test (Graphpad Instat, version 3.06).

All four anthracene-polyamine conjugates tested inhibited the proliferation of *P. falciparum* parasites with IC_{50}s (i.e., the concentrations at which proliferation was inhibited by 50%) ranging from nM to low (<100) μM (Table 1). Ant-4 was the most potent of the compounds tested, with an IC_{50} of 0.64 ± 0.04 μM ($n = 9$; Table 1). The polyamine conjugates contain the same anthracene moiety, and the differential effects on parasite proliferation (Ant-4 > Ant-444 > Ant-44 > 44-Ant-44; Table 1) may therefore be attributed to the different conjugated polyamine moieties. The anthracene-putrescine conjugate Ant-4 showed significantly greater antiplasmodial potency than the *N*-alkylated anthracene derivative, Ant-*N*-butyl (IC_{50} = 1.54 ± 0.12 μM; $n = 4$; $P < 0.05$ [unpaired *t* test]), confirming a role for the polyamine moiety in the antiplasmodial effect of the anthracene-putrescine conjugate. Likewise, the finding that the antiplasmodial effect of N^1-methyl-HSpd (IC_{50} = 1.50 ± 0.12 mM; $n = 3$; $P < 0.05$ [unpaired *t* test]) was much less than that of Ant-4 indicates the importance of the anthracene moiety in the antiplasmodial action of the conjugate. Thus, both the anthracene and polyamine components play a role in the antiplasmodial effect of the conjugates.

The uptake of Ant-4 into parasitized erythrocytes was investigated by taking advantage of the inherent fluorescence of anthracene. Deconvolution fluorescence microscopy of parasitized erythrocytes preincubated with Ant-4 for 1 h at 37°C revealed intense fluorescent staining of the parasite cytosol and nucleus, with fluorescence observed neither in the erythrocyte compartment of infected erythrocytes nor in uninfected erythrocytes (see Fig. S1 in the supplemental material). This preferential accumulation of Ant-4 into parasitized erythrocytes, with little uptake into uninfected erythrocytes, was confirmed with flow cytometry (not shown).

Polyamines are taken up into the intraerythrocytic malaria parasite via a membrane-potential dependent mechanism and accumulate within the parasite (3). The interaction between the anthracene-polyamine conjugates of interest here and the mechanisms involved in polyamine uptake and accumulation in the parasite was investigated by testing the effect of anthracene-polyamine conjugates on the uptake of radiolabeled polyamines, measured as described elsewhere (3). The putrescine conjugate Ant-4, the homospermidine conjugates Ant-44 and 44-Ant-44, and the homospermine conjugate Ant-444 (each at a concentration of 500

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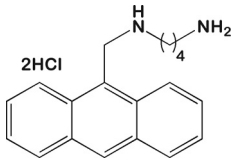
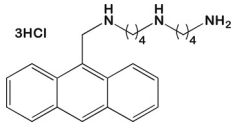
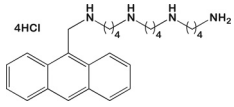
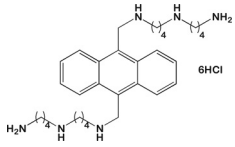
TABLE 1 IC_{50}s for the inhibition of growth of *P. falciparum* parasites, CHO cells, and various human cancer cell lines by anthracene-polyamine conjugates[a]

Characteristic	Value(s)			
	Ant-4 	Ant-44 	Ant-444 	44-Ant-44 
Polyamine-conjugated group[b]	R-putrescine	R-homospermidine	R-homospermine	bis-homospermine
IC_{50} (μM ± SE)				
P. falciparum	0.64 ± 0.04 (n = 9)	4.3 ± 0.3 (n = 5)	1.71 ± 0.24 (n = 6)	21 ± 5 (n = 2)
CHO[c]	7.7	0.45	10.6	1.1
HepG2	14.1 ± 0.5 (n = 3)	ND	ND	ND
HL-60[d]	20	ND	ND	ND
L1210[e]	6.3	0.3	7.5	1.5
SI				
CHO/Pf	12	0.1	6.23	0.05
HepG2/Pf	22	NA	NA	NA
HL-60/Pf	31	NA	NA	NA
L1210/Pf	10	0.07	4.4	0.075

[a] *P. falciparum*-infected erythrocytes (initially at the ring stage) were incubated with anthracene-polyamine conjugates over a range of concentrations at 37°C for 96 h, and the IC_{50}s (expressed here as μM values) were determined using the malaria SYBR green I fluorescence assay. Data are derived from n independent triplicate experiments (indicated in parentheses) ± standard errors (SE) or were taken from published work as indicated. L1210 cells, mouse leukemia cells; HL-60, human leukemia cells; HepG2, human hepatocellular liver carcinoma cells; Pf, *P. falciparum* parasites; SI (selectivity index) = (IC_{50} for Ant-4 against CHO, HL-60, L1210, or HepG2 cells)/(IC_{50} for Ant-4 against *P. falciparum* parasites). ND, not determined; NA, not applicable.

[b] "R" indicates the anthracene-conjugated moiety (7).

[c] Data are from reference 14.

[d] Data are from reference 13.

[e] Data are from references 15 and 23.

μM) all inhibited the uptake of both [^{3}H]putrescine and [^{3}H]spermidine into mature isolated trophozoite-stage *P. falciparum* parasites over 30 min. Putrescine uptake was reduced by 20% to 30% by the anthracene-polyamine conjugates, whereas spermidine uptake was reduced by 60% to 95% (see Fig. S2 in the supplemental material). The data are consistent with the polyamine-anthracene conjugates competing with the transport and/or intracellular accumulation of polyamines in the parasite.

The characteristics of the antiplasmodial effect of the polyamine-anthracene conjugates were investigated in more detail using flow cytometry to monitor the progression of the parasite cell cycle in the presence and absence of the most potent of the conjugates, Ant-4. Experiments commenced with ring-stage parasites (1% hematocrit and 5% parasitemia), with cultures maintained at 37°C and monitored for 48 h. In untreated parasitized erythrocytes, nuclear division occurred as expected, with a reduction in the percentage of parasites with a single nucleus (ring stage and early trophozoite stage, corresponding to the G_1 phase of the cell cycle) and an increase in the percentage of parasites with multiple nuclei (schizonts, corresponding to the S phase of the cell cycle) after 24 h. After 48 h, the untreated parasites were again predominantly a single-nucleus ring-stage population, as the parasites reinvaded new erythrocytes and commenced the subsequent cycle (Table 2). In contrast, Ant-4-treated intraerythrocytic *P. falciparum* parasites remained at the initial single-nucleus stage throughout the 48 h of incubation period with no nuclear division, indicating disruption of the parasite's cell cycle within the first 24 h of exposure (Table 2). Binding and stabilization of DNA by polyamines play an important role in DNA replication and nuclear division during cell cycle progression (11). The inability of intraerythrocytic *P. falciparum* parasites to replicate their nuclei after Ant-4 treatment is consistent with Ant-4 acting at the level of the parasite's DNA. The putrescine moiety of Ant-4 may act by displacing the functional polyamines from DNA and delivering the anthracene moiety to the DNA, where the planar, polycyclic ring structure of anthracene intercalates tightly but reversibly between DNA base pairs (12), inhibiting DNA synthesis and inducing DNA damage, as has been described previously for HL60 cells (13).

Any effective antimalarial must exert selective toxicity for the parasite relative to the cells of the human host. The finding here that Ant-4 exerts a more potent antiplasmodial effect than Ant-44 contrasts with the previous finding that the proliferation of mammalian L1210 and CHO cells is inhibited more effectively by Ant-44 than by Ant-4 (14, 15). The relative potencies of the polyamine-anthracene conjugates for *P. falciparum* compared to mammalian cells are shown in Table 1. For the purpose of this study, the toxicity of Ant-4 to mammalian cells was investigated using human hepatocellular liver carcinoma cells (HepG2), with cell proliferation assessed using a lactate dehydrogenase assay (10). The IC_{50}s obtained here for HepG2 cells, together with those

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TABLE 2 Flow cytometric analysis of the effect of Ant-4 on nuclear division in intraerythrocytic *P. falciparum* parasites[a]

Treatment	HPT[b]	% cells/population ± SE				
		1N	2N	3N	4N	>4N
Control	4	88.4 ± 0.2	3.6 ± 0.04	0.21 ± 0.02	0.07 ± 0.01	0.075 ± 0.01
	24	28.5 ± 2.1	36.8 ± 1.8	20.4 ± 2.5	6.4 ± 1.0	0.43 ± 0.05
	48	60.5 ± 0.5	22.0 ± 0.6	9.9 ± 0.5	2.7 ± 0.1	2.6 ± 0.1
Ant-4 (0.64 μM, 1 × IC_{50})	4	88.7 ± 0.4	3.2 ± 0.1	0.12 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
	24	28.1 ± 0.8	39.8 ± 0.2	19.7 ± 0.5	3.6 ± 0.02	0.17 ± 0.05
	48	54.7 ± 0.8	18.4 ± 0.3	7.5 ± 0.2	3.9 ± 0.2	12.4 ± 1
Ant-4 (3.2 μM, 5 × IC_{50})	4	88.7 ± 0.1	3.5 ± 0.1	0.1	0.03 ± 0.03	0.03 ± 0.01
	24	87.3 ± 0.3	3.9 ± 0.03	0.17 ± 0.02	0.02 ± 0.02	0.03
	48	74.1 ± 0.7	8.5 ± 1.2	0.35 ± 0.10	0.05 ± 0.01	0.02 ± 0.01

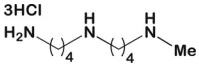
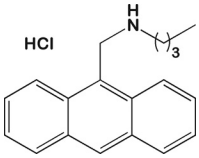
[a] *P. falciparum-infected* erythrocytes initially at the ring stage were incubated at 37°C either with or without Ant-4 for the times specified, and nuclear division was monitored using SYBR green I fluorescence. Ring- or early-trophozoite-stage intraerythrocytic parasites contain 1 one nucleus (1N), early schizonts contain 2 nuclei (2N), and later multinucleated schizonts contain at least 3 nuclei (3N, 4N, and >4N). The data represent averages of the results of three independent experiments and are shown ± SE.
[b] HPT, hours posttreatment.

obtained previously for cancerous (HL-60 and L1210) and noncancerous (CHO) mammalian cells (13, 15), were used to calculate a "selectivity index" (SI) = (IC_{50} against mammalian cell line)/(IC_{50} against *P. falciparum* parasites) for each of the different *P. falciparum*/mammalian cell combinations. The SI values for Ant-4 (>10) show selectivity toward *P. falciparum* parasites (16, 17) and are significantly lower than that for chloroquine against HepG2 cells relative to W2 (chloroquine-resistant) *P. falciparum* parasites (SI = 4,200) (18). The SI values for the other anthracene-polyamine conjugates were below 10 (Table 1).

The predicted physicochemical and absorption, distribution, metabolism, and excretion (ADME) properties of the anthracene-polyamine conjugates were calculated using the Discovery Studio Modeling Environment (Accelrys Software Inc., release 3.0) and indicated that all compounds, except 44-Ant-44 (molecular weight > 500), are Lipinski's "Rule-of-5" compliant (19) (Table 3). Ant-4 has good aqueous solubility (20) and oral bioavailability (human intestinal absorption) (21), is predicted to cross the blood-brain barrier, with no significant metabolism by cytochrome P450 (CYP2D6), and, finally, has no significant plasma protein binding (Table 3) (22).

In summary, the polyamine-anthracene conjugates represent novel chemical structures that show a potent antiplasmodial effect, with at least one such compound, Ant-4, showing the requisite selectivity for *P. falciparum* in preference to mammalian cells.

TABLE 3 Physicochemical and ADME properties of the anthracene-polyamine conjugates predicted by the Discovery Studio Modeling Environment (Accelrys Software Inc.)[a]

Conjugate	MW[b]	No. of H-bond donors	No. of H-bond acceptors	AlogP[c]	HIA[d]	Aqueous solubility level[e]	Blood brain barrier level[f]	CYP2D6 prediction[g]	Plasma protein binding prediction[h]
Ant-4	280	2	0	0.8	0	4	2	No	No
Ant-44	353	3	0	−0.2	1	5	3	Yes	No
Ant-444	424	4	0	−1.2	2	5	4	Yes	No
44-Ant-44	527	6	0	−4.1	3	5	4	Yes	No
N^1-methylHSpd	176	3	0	−3.6	3	5	4	No	No
									
Ant-*N*-butyl	264	1	0	3.2	0	2	0	Yes	Yes
									

[a] ADME, absorption, distribution, metabolism, and excretion.
[b] MW, molecular weight.
[c] Lipophilicity indicator (expected to be <5).
[d] Human intestinal absorption (HIA): 0 = good absorption, 1 = moderate absorption, 2 = low absorption, 3 = very low absorption.
[e] Drug likeness: 0 = extremely low, 1 = no (very low but possible), 2 = yes (low), 3 = yes (good), 4 = yes (optimal), 5 = no (too soluble).
[f] Penetration of blood-brain barrier: O = very high (brain-blood ratio greater than 5:1), 1 = high (brain-blood ratio between 1:1 and 5:1), 2 = medium (brain-blood ratio between 0.3:1 and 1:1), 3 = low (brain-blood ratio less than 0.3:1), 4 = undefined (outside 99% confidence ellipse).
[g] Inhibition of cytochrome P450 (CYP2D6): No = no inhibition, yes = inhibition.
[h] Plasma protein binding: No = no binding, Yes = binds to plasma proteins.

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Further exploration of members of this class of compound as antimalarials is under way.

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