Anthracene-polyamine conjugates inhibit *in vitro* proliferation of intraerythrocytic *Plasmodium falciparum* parasites

Jandeli Niemand[a], Pieter Burger[a], Bianca K Verlinden[a], Janette Reader[a], Annie M Joubert[b], Annette Kaiser[c], Abraham I Louw[a], Kiaran Kirk[d], Otto Phanstiel IV[e] and Lyn-Marie Birkholtz[a#]

[a] Departments of Biochemistry and [b] Physiology, University of Pretoria, Private Bag x20, Hatfield 0028, South Africa

[c] Institute for Pharmacogenetics, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany

[d] Research School of Biology, The Australian National University, Canberra, ACT, Australia 0200

[e] Department of Medical Education, University of Central Florida, 6850 Lake Nona Blvd, Orlando, FL 32827, USA

Abstract

Anthracene-polyamine conjugates inhibit the *in vitro* proliferation of the intraerythrocytic human malaria parasite, *Plasmodium falciparum*, with IC_{50} values in the nM-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.

Keywords: Malaria, *Plasmodium falciparum*, spermidine, putrescine, anthracene

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 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: 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 (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, 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, vs 3.06).

All four anthracene-polyamine conjugates tested inhibited the proliferation of *P. falciparum* parasites with IC_{50} values (i.e. the concentration 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} value 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-polyamine conjugate Ant-4 showed a 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. The finding that the antiplasmodial effect of N^1-methylHSpd (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 (Fig. S1). 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 radiolabelled 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 μM) all inhibited the uptake of both $[^3H]$putrescine and $[^3H]$spermidine over 30 min into mature, isolated trophozoite-stage *P. falciparum* parasites. Putrescine uptake was reduced by 20-30% by the anthracene-polyamine conjugates, whereas spermidine uptake was reduced by 60-95% (Fig. S2). 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, 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-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). By contrast, Ant-4 treated intraerythrocytic *P. falciparum* parasites remained at the initial single-nucleus stage throughout the 48 h 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 plays 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 a selective toxicity to the parasite, relative to the cells of the human host. The finding here that Ant-4 exerts a more potent antiplasmodium 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} value obtained here for HepG2 cells, together with those obtained previously for cancerous (HL-60 and L1210) and non-cancerous (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 show selectivity towards *P. falciparum* parasites within the accepted range (i.e. >10; (16, 17)) for antimalarial lead compounds (Table 1), while being significantly lower than that for chloroquine against HepG2 cells relative to W2 *P. falciparum* parasites (chloroquine-resistant; SI = 4200) (18). The SI values for the other anthracene-polyamine conjugates were below 10 (Table 1).

The predicted physicochemical and 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 Da), 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 2D6 and lastly 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* over mammalian cells. Further exploration of this class of compound as antimalarials is underway.

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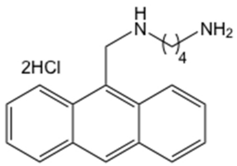
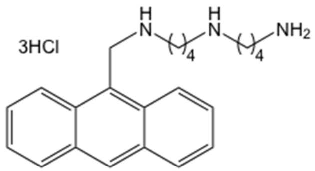
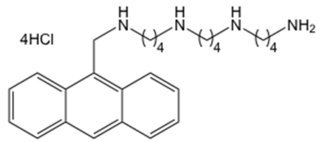
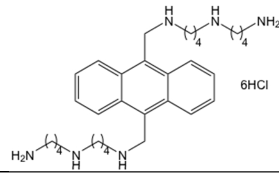
polyamine conjugates into cells containing active polyamine transporters. J. Med. Chem. 47:6055-6069.

Table 1: IC_{50} values for the inhibition of growth of *P. falciparum* parasites, CHO cells and various human cancer cell lines by anthracene-polyamine conjugates. *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} values (expressed here as μM) were determined using the malaria SYBR Green I fluorescence assay. Data are derived from n independent triplicate experiments (indicated in parentheses) ± S.E., or taken from published work as indicated.

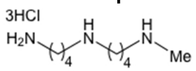
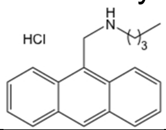
	Ant-4	Ant-44	Ant-444	44-Ant-44
				
Polyamine conjugated group[a]	R-putrescine	R-homospermidine	R-homospermine	R-bi-conjugated homospermine
IC_{50} values (μM)				
P. falciparum	0.64 ± 0.04 (n = 9)	4.3 ± 0.3 (n = 5)	1.71 ± 0.24 (n = 6)	21 ± 5 (n = 2)
CHO[b]	7.7	0.45	10.6	1.1
HepG2	14.1 ± 0.5 (n = 3)	ND	ND	ND
HL-60[c]	20	ND	ND	ND
L1210[d,e]	6.3	0.3	7.5	1.5
Selectivity indices				
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] R indicates the anthracene conjugated moiety (7); [b](14); [c] (13); [d](15); [e](23),

L1210 cells: mouse leukemia cells; HL-60: human leukemia cells; HepG2: human hepatocellular liver carcinoma cells; *P. falciparum* parasites: Pf; 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

Table 2: Physicochemical and ADMET properties of the anthracene-polyamine conjugates predicted by the Discovery Studio Modeling Environment (Accelrys Software Inc).

	MW	H-bond donor	H-bond acceptor	AlogP [a]	HIA [b]	Aqueous solubility level [c]	Blood brain barrier level [d]	CYP2D6 prediction [e]	Plasma Protein Binding Prediction [f]
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-mHSpd 	176	3	0	-3.6	3	5	4	No	No
Ant-*N*-butyl 	264	1	0	3.2	0	2	0	Yes	Yes

[a] Lipophilicity indicator, expected to be <5.

[b] Human intestinal absorption: 0 = Good absorption, 1 = Moderate absorption, 2 = Low absorption, 3 = Very low absorption.

[c] Drug-likeness: 0 = Extremely low, 1 = No, very low, but possible, 2 = Yes, low , 3 = Yes, good, 4 = Yes, optimal, 5 = No, too soluble.

[d] Blood brain penetration: O = very high penetrant (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 to 1:1), 3 = Low (Brain-Blood ratio less than 0.3:1), 4 = Undefined (Outside 99% confidence ellipse).

[e] Inhibition of Cytochrome P450 (2D6): No = no inhibition, Yes = inhibition

[f] Plasma protein binding: No = no binding, Yes = binds to plasma proteins

Table 3: Flow cytometric analysis of the effect of Ant-4 on nuclear division in intraerythrocytic *P. falciparum* parasites. *P. falciparum*-infected erythrocytes 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 multi-nucleated schizonts at least 3 (3N, 4N, >4N). The data are averaged from three independent experiments and are shown ± S.E.

		Percentage of cells/population				
Treatment	HPT[a]	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] Hours post-treatment