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Inhibition of invasion and intraerythrocytic development of *Plasmodium falciparum* by kinase inhibitors

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Abstract. We have examined the effects of seven protein kinase inhibitors (staurosporine, genistein, methyl 2,5-dihydroxycinnamate, tyrphostins B44 and B46, lavendustin A and R03) on the erythrocytic cycle of the malaria parasite, *Plasmodium falciparum*. One (staurosporine) strongly inhibits serine/threonine kinases, but the remainder all exhibit a strong preference for tyrosine kinases. We have been able to discriminate between effects on invasion and on intraerythrocytic development. All reagents impeded development of intraerythrocytic parasites, though at widely differing concentrations, from the sub-micromolar to the millimolar. Several inhibitors, including staurosporine, also reduced invasion. The phosphatase inhibitor, okadaic acid, had a strong inhibitory effect both on invasion and development. The regulation of malaria development by phosphorylation or dephosphorylation reactions at several points in the blood-stage cycle is implied.
Key words. Malaria; *Plasmodium falciparum*; kinase inhibitors; okadaic acid.

Invasion of the red cell by the malaria parasite involves a sequence of steps, including secretion of rhoptry contents, hydrolysis of glycosyl phosphatidylinositol anchors, and proteolysis of unidentified substrates (see ref. 1 for review). Development of the internalised parasite follows a complex programme of events and is marked by massive membrane traffic between the parasite and the host cell membrane,[2,3] insertion of parasite-derived proteins in the latter[4] and extensive phosphorylation of both parasite[5] and host-cell[6] proteins. Several kinases have been identified in *Plasmodium falciparum*.[7–10] One might therefore expect that the blood-stage cycle of the parasite would be vulnerable to disruption by kinase inhibitors. A study by Ward et al.[11] has demonstrated that invasion of red cells by *P. knowlesi* is totally inhibited by micromolar concentrations of the kinase inhibitor, staurosporine, and that this acts at the stage of entry. Thus attachment and junction formation proceed normally, but the invasion sequence is arrested at this point. No other information on the influence of kinase inhibitors on the erythrocytic cycle appears as yet to be available. Here we examine how a series of kinase inhibitors affects invasion and development of *P. falciparum* in culture, and show differential effects of different inhibitors on both.

Materials and methods

Plasmodium falciparum parasites were grown in continuous culture[12] and synchronised by the sorbitol method.[13] For preparation of merozoites, mature schizonts were isolated from cultures by centrifugation on a Percoll cushion[14] and passed through a 23 G needle. Red cells were separated from the merozoites by low-speed centrifugation (1500 rpm) and the supernatant was used to inoculate target cell suspensions. Invasion was assessed by counting a minimum of 1000 cells on Giemsa-stained slides. Anomalies of development were observed on the same cell population, and in general, such parasites are retarded in development compared to the controls (i.e. 0 μM of inhibitor), their morphology is contracted and they stain much more intensely.

Okadaic acid was obtained from Sigma; the kinase inhibitors, staurosporine, genistein, methyl-2,5-dihydroxycinnamate (a stable analogue of erbstatin), tyrphostin B44, tyrphostin B46, and lavendustin A were from Calbiochem-Novabiochem Ltd. (UK), and R03 was a gift from Hoffman La Roche. Of these, staurosporine is predominantly a serine/threonine kinase inhibitor, whereas the remainder (some of which have additional metabolic actions) act primarily on tyrosine kinases. Stock solutions of inhibitors were prepared in dimethylsulphoxide and diluted into the culture medium. Solvent blanks (i.e. 0 μM of inhibitor) were performed as controls. In some experiments the kinase inhibitors were tested on parasites obtained directly from synchronised stock cultures, in others the late stage parasites were purified first (see results for details). The following inhibitors, genistein (250 μM), staurosporine (1 μM) and okadaic acid (1 μM) were incubated with red cells for 1 h at 37 °C before use in invasion assays.

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Results

The results of three experiments are summarised in table 1. For treatment with methyl 2,5-dihydroxycinnamate, tyrphostin B46, lavendustin A and R03, Percoll-purified late-stage parasites were diluted with normal red cells, while for genistein and staurosporine, rupturing late-stage parasite cultures were used. In all cases the starting parasitaemias (i.e. at 0 h) and those after a continuous 19 h exposure to inhibitor are shown. All seven kinase inhibitors assayed affected the erythrocytic cycle of *P. falciparum*, though at widely differing concentrations, and their effect on late-stage parasites was particularly marked in the higher concentration range (e.g. methyl-2,5-dihydroxycinnamate at 250 µM). Parasite death, sometimes accompanied by lysis of the host cell, ensued at concentrations above about 200 µM of genistein, 50 µM methyl 2,5-dihydroxycinnamate, 200 µM tyrphostin B46 and 10 µM R03. To test whether or not the sensitivity of *P. falciparum* to kinase inhibitors was stage-specific we exposed synchronised young ring-stage parasites to staurosporine and genistein (table 2). Both arrested intraerythrocytic development at the late ring or young trophozoite stage, though the required concentrations were respectively 1 and 200 µM. By contrast no effect on parasitaemia or development could be discerned after preincubating the target cells for 1 h at 37 °C with either inhibitor.

From the levels of parasitaemia, and making allowance for the number of mature parasites that had not ruptured, it was possible to judge whether the inhibitors also suppressed invasion. The counts suggested that lavendustin A did not affect invasion, whereas sufficient concentrations of methyl 2,5-dihydroxycinnamate and tyrphostin B46 (as well as its analogue, tyrphostin B44, data not shown) reduced it by 50% or more. To confirm this, several of the inhibitors were tested specifically for their effects on invasion by exposing only purified merozoites and target cells to the inhibitors. The results are shown in table 3, which confirms that the above two kinase inhibitors, as well as staurosporine, do indeed hinder invasion by directly acting on processes that occur within the merozoite after it has left the parent schizont. The viability of the merozoites is indicated by the control values for invasion in table 3.

We also examined the effect of the phosphatase inhibitor, okadaic acid, on the system. At a concentration of 10 nM in the medium this caused a 50% reduction in invasion (table 4). Preincubation of the target cells with 1 µM okadaic acid for 1 h at 37 °C led to a much smaller effect (ca. 20% reduction of invasion). When trophozoite-infected cells were similarly incubated, a

Table 1. Effect of kinase inhibitors on *P. falciparum* parasitaemias[a] after 19 h exposure.

Inhibitor	conc. µM	rings	troph	schiz
Methyl 2,5-dihydroxycinnamate	0	294	2	2
	25	232	2	4
(1r 169t 10s)[b]	50	202	18	16
	250	2	142	12
Tyrphostin B46	0	294	2	2
	100	90	50	22
(1r 169t 10s)[b]	200	22	70	10
	600	4	100	0
Lavendustin A	0	254	0	0
	50	254	4	4
(1r 169t 10s)[b]	100	222	22	14
	200	184	36	18
R03	0	294	2	2
	1	310	16	0
(1r 169t 10s)[b]	10[c]	2	76	22
Genistein	0	312	50	2
	20	332	44	2
(60r 50t 6s)[b]	100	236	48	4
	200	114	58	6
Staurosporine	0.0	146	30	0
	0.1	146	40	0
(52r 25t 8s)[b]	1.0	84	16	2

[a]Intraerythrocytic parasites per 1000 cells, differentially scored as rings, trophozoites (troph) and schizonts (schiz), after a 19 h incubation with kinase inhibitors or DMSO controls (i.e. 0 µM of kinase inhibitor).
[b]Starting parasitaemias at 0 h (rings (r), trophs (t), schiz (s)), i.e. when the inhibitors were first added.
[c]At an R03 concentration of 50 µM no invasion could be detected, and some 50% of the parasites were lysed.

Table 2. Effect of kinase inhibitors on the development of *P. falciparum* ring-stage parasites[a].

Inhibitor	conc. µM	rings	troph	schiz
Control	0	9	224	16
Genistein	25	62	204	14
	250	8	196	0
Staurosporine	0.1	62	188	38
	1.0	0	250[b]	0

[a]Differential parasitaemia (see table 1 for definition) per 1000 cells after a 20 h incubation with kinase inhibitors. The starting parasitaemia was 26% ring-stage parasites.
[b]All parasites appear abnormal/dead.

Table 3. Effect of kinase inhibitors on invasion of red cells by merozoites.

Inhibitor	conc. µM	rings 20 h	rings 24 h
Staurosporine	0.0	16	11
	0.8	4	0
Methyl 2,5-dihydroxy-cinnamate	0	14	11
	10	8	8
	50	1	0
Tyrphostin B46	0	14	11
	10	8	7
	100	2	2

The ring counts are per 1000 cells and were taken after 20 h so that successful invasions could be unambiguously assayed as mature rings. Repeat smears were taken 4 h later. Controls correspond to 0 µM of kinase inhibitor.

Table 4. Effect of a 19 h exposure of okadaic acid on *P. falciparum* development/invasion

Inhibitor	conc. μM	rings	troph	schiz
Okadaic acid	0.00	196	0	0
	0.01	98	0	0
(0r 18t 5s)[a]	0.1	70	0	0
	1.0	20	0	4[b]
	5.0	4	2[b]	12[b]

[a]Starting parasitaemia (0 h), generated by Percoll-purified late-stage parasites diluted with normal red cells (see table 1 for definition).
[b]Abnormal/dead parasites.

40% reduction in the level of parasitaemia was observed after culturing for a further 19 h in inhibitor-free medium. Abnormal late forms were also apparent. A 1 h incubation of ring-infected cells had no discernible effect on parasite morphology over the same period.

Discussion

Staurosporine, alone among the inhibitors that we have tested, has a high degree of specificity for serine/threonine kinases, though in no case can it be assumed that at high inhibitor concentrations there are no secondary effects on other types of kinase. Like Ward et al.[11], working with *P. knowlesi*, we find that staurosporine inhibits invasion of *P. falciparum* and this is essentially complete at a concentration of 1 μM. Ward et al.[11] showed in addition that the inhibition occurred after attachment and before entry of the parasite.

This effect of staurosporine probably occurs, as Ward et al.[11] concluded, through a serine/threonine kinase, since it is antagonised by the phosphatase inhibitor, okadaic acid, which is essentially inactive towards phosphotyrosine phosphatases[15]. The effects of all the other inhibitors, which act specifically, or at least highly preferentially on tyrosine kinases, are manifested only at much higher concentrations. The concentrations required for inhibition do not necessarily reflect the affinity of the inhibitor for its target enzyme and could equally be limited by poor membrane penetration or, more probably, sequestration in the cytosol or membrane of the host cell or parasite through adventitious interactions with proteins. We cannot exclude that the inhibitors have poor affinity for the particular kinase(s) on which they act in the parasite. However, it is clear that phosphorylations are required for normal development. These may regulate enzymic activities, as the effect of staurosporine suggests, and more particularly initiation of signalling pathways, as indicated by the efficacy of the tyrosine kinase inhibitors.

It is established that numerous proteins are phosphorylated in the intraerythrocytic parasite[5], as well as some host-cell membrane proteins[10], notably protein 4.1[6]. Malaria parasites have been found to contain active cAMP-dependent and -independent kinases[7], a type I casein kinase[8], a serine/threonine kinase, PfPK2[9], and a unique calcium-dependent kinase, PfCPK, containing EF-hand motifs[16]. It has been suggested in the above studies that these enzymes may present a new target for chemotherapy.

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