Mitotic inhibitors arrest the growth of *Plasmodium falciparum*

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We report that the mitotic inhibitor, vinblastin (VLB), is highly toxic to the malarial parasite, *Plasmodium falciparum*. In cultures in vitro growth is inhibited by 50% at a VLB level of about 28 nM, and totally abolished at a level of 100 nM. By tests on synchronized cultures we have found that the effect of VLB takes place at the trophozoite stage. Colcemid also inhibits schizogony with somewhat different kinetics. By mutagenesis with nitrosoguanidine followed by VLB selection we have isolated a VLB-resistant mutant which exhibits cross-resistance to vincristine. These data suggest a critical role of microtubules in the asexual schizogonic cycle of *P. falciparum*.

Malaria (P. falciparum) *Vinblastine* *Tubulin*

1. INTRODUCTION

The intraerythrocytic development of *Plasmodium falciparum* consists of an orderly sequence whereby the young ring form grows into a larger trophozoite with one or two nuclei, which within 24 h becomes a schizont with up to 24 nuclei, giving rise, upon bursting of the host red cell, to a similar number of merozoites ready to invade new red cells [1]. DNA synthesis is known to occur during the early trophozoite stage [2,3], but how nuclear division exactly takes place is unknown. Microtubular structures have been observed by electron microscopy within the nucleus of *P. gallinaceum* treated with pyrimethamine [4]. However, the role of these structures in schizogony is not yet clear. One possible approach to defining this role is the use of mitotic inhibitors. In cultures of *P. falciparum* we have found that vinblastine (VLB) totally and irreversibly interrupts the schizogonic cycle at the very low concentrations known to cause disruption of microtubules [5]. The action of the drug is entirely specific for the trophozoite stage of the parasite. Similar results are obtained with vincristine and with colcemid. We have also isolated a vinblastine-resistant mutant of *P. falciparum*.

2. MATERIALS AND METHODS

White cell-free cultures of *P. falciparum* [6] (the Wellcome-Liverpool strain was kindly supplied by Dr D.C. Warhurst of the Malaria Reference Laboratory of the London School of Hygiene and Tropical Medicine) were maintained in plastic petric dishes (Falcon Div., Becton Dickinson, USA) by the candle jar method [7]. Cultures were synchronized by the sorbitol method [7,8] and the resulting ring forms were sub-cultured in 30 × 10 mm petri dishes in complete medium (RPMI 1640 with 25 mM Hepes, 25 mM $NaHCO_3$, 50 μg/ml gentamycin sulphate and supplemented with 15% group AB human serum) or complete medium with the addition of the inhibitor to be

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tested. The initial parasitaemia in each case was about 1% and the haematocrit of the culture 5%. Parasite infection rates were determined by counting 5000 erythrocytes per slide on Romanowsky-stained smears prepared at appropriate intervals. All inhibition experiments were carried out at least three times, with duplicate dishes for each level of drug.

In order to test the effect of VLB on different stages of parasite development, synchronous cultures were subjected to 12 h pulses of 80 nM VLB in complete medium and levels of parasitaemia were determined at serial intervals.

In order to isolate vinblastine-resistant (VLB^R) mutants we have used the method described by Inselburg [9]. Ten 4 ml synchronized cultures in 60 mm petri dishes, with a parasitaemia of 8%, were treated for 24 h with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NG) (Sigma), at a final concentration of 9 μM, in complete medium. Exposure to the mutagen was started when parasites were between 10 and 25 hours old, so that NG was present during DNA synthesis. The cultures were then washed twice in incomplete medium and allowed to go through three cycles without selection. VLB at 160 nM was then added to the cultures when parasitaemia was 8%, and it was removed after 5 days, when parasitaemia was below 0.1%. Parasites started to reappear 7–14 days after the VLB was removed. From 14 days onwards cultures were maintained in 1.6 μM VLB.

3. RESULTS

3.1. *Action of mitotic inhibitors*

When *P. falciparum* cultures are continuously exposed to VLB, a concentration-dependent inhibition of growth is observed (fig.1). Since the time course of the cycle is not affected, we are dealing with true inhibition, rather than with mere slowing down of the schizogonic process. In order to pinpoint the stage in the cycle at which VLB acts, we have also exposed cultures to pulses of the drug, rather than to its continuous presence (fig.2). It is seen clearly that VLB effectively abolished parasite growth only when added during the trophozoite stage. The fact that inhibition is not as complete as when VLB is present throughout the cycle can be entirely accounted for by the fact that synchrony is never quite perfect. Even when the

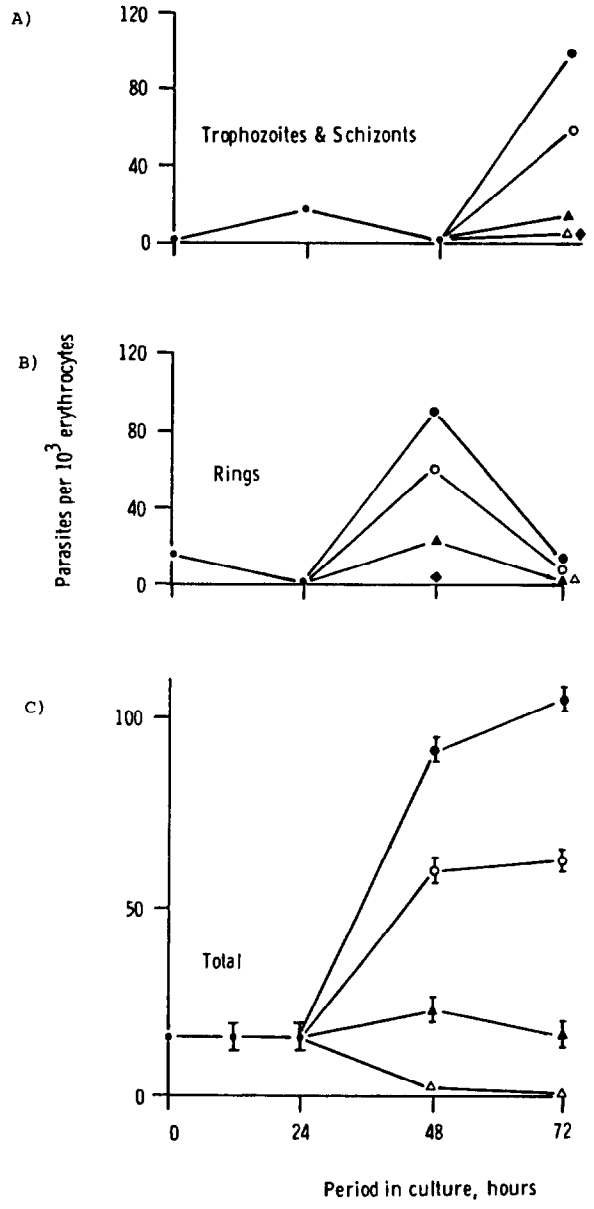

Fig.1. Inhibition of *P. falciparum* growth by vinblastine. VLB powder (Ely Lilly & Co.) was dissolved in RPMI 1640 at 1 mg/ml, and used within 24 h. The bottom panel (C) shows total parasites, while the upper panels (A and B) show individual stages as indicated. Points up to 24 h are averages for all parallel cultures. Vertical bars in the bottom panel indicate standard deviations for duplicate dishes. (●) Control and 10 nM VLB; (○) 20 nM VLB; (▲) 40 nM VLB; (△) 100 nM VLB.

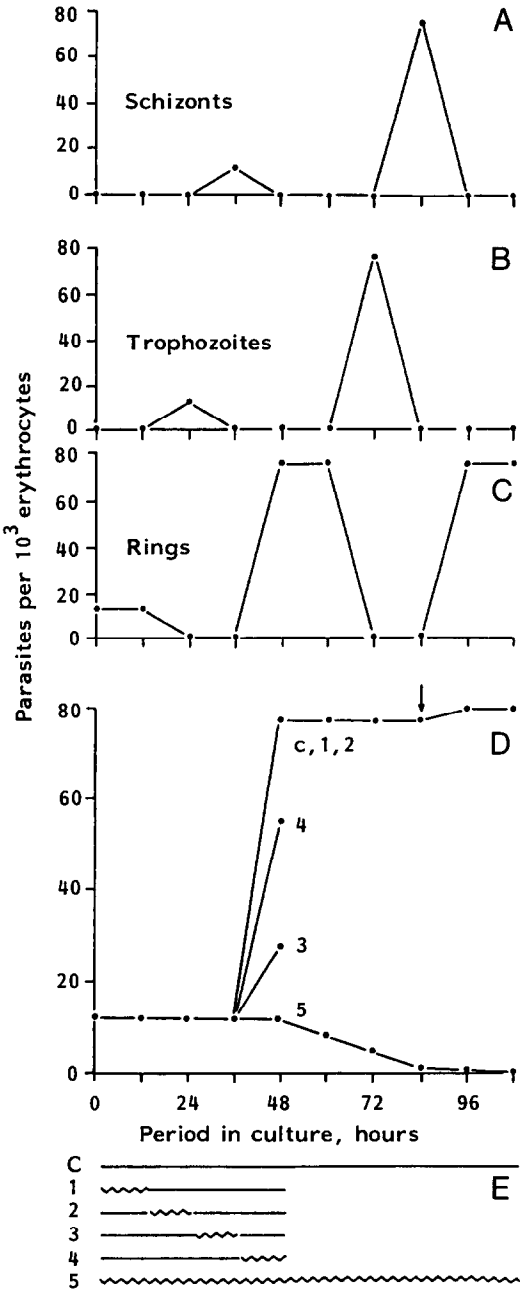

Fig.2. Stage-specificity of VLB action. Synchronized cultures (see fig.1) were subjected to 12 h pulses of 80 nM VLB. Top panels: (A–C) distribution of different forms of the parasite as a function of time in control cultures. Sharp successive peaks of trophozoites and schizonts indicate good synchrony of the cultures. (D) Marked differences in VLB effect depending on choice of timing of exposure to drug across the parasite cycle. The zig-zag lines at the bottom (E) indicate the period during which VLB was present in five otherwise identical dishes. These are identified by the numbers on the left, with which the growth curves are also labelled. Arrow at the top of panel D indicates dilution of the culture (1:6).

culture contained predominantly (87%) trophozoites, there were still about 13% ring forms present (see fig.2A), and these escaped the action of VLB.

Colcemid similarly inhibits growth of *P. falciparum*. However, the concentration required to achieve an effect comparable to that of VLB is about 1000-times higher (table 1). In addition, the nature of inhibition is different, because when the drug is removed after a 24-h exposure growth resumes with a 24-h delay (fig.3).

3.2. *Isolation of vinblastine-resistant mutants*

Mutagenesis with NG was carried out under conditions producing about 90% parasite mortality (see section 2). After NG treatment cultures were allowed to undergo three successive schizogonic cycles and they were then challenged with 1.6 μM VLB. Within three cycles parasites became undetectable by conventional microscopic examination, but they started to reappear after about 14 days, and continued to grow in VLB indefinitely (over 100 days). In order to make sure that VLB-resistance was due to a genetic and not to an adaptive change, the culture was split and one aliquot

Table 1

Drug sensitivity of *Plasmodium falciparum*

Drug	LD_{50}[a]	
	WL	VLB^R
Vinblastine	15–30 nM[b]	1.2–4.4 μM[b] (3.2 μM)[c]
Vincristine	7 nM	1 μM
Colcemid	50 μM	not done
Chloroquine	72 nM	45 nM

[a] Drug concentration causing 50% reduction in parasite level after 48 h exposure. Values are means of duplicate experiments unless stated otherwise

[b] Range of values in four sets of experiments

[c] After 30 days of growth in the absence of VLB

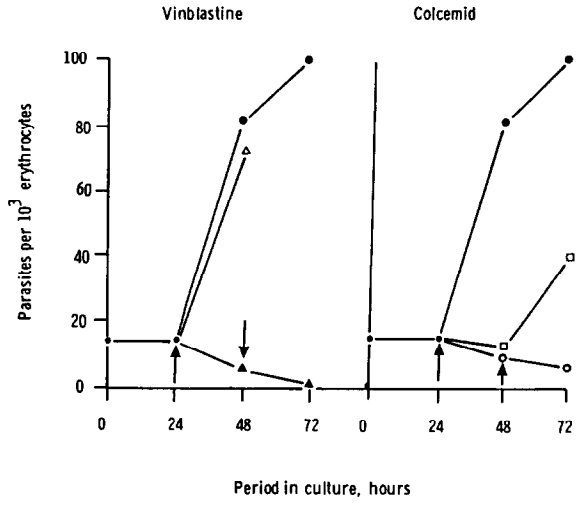

Fig.3. Plasmodiostatic effect of colcemid vs stage-specific killing by vinblastine. Left panel: (●) control culture; (△) VLB (80 nM) present only from 0 to 24 h; (▲) VLB (80 nM) removed at 48 h. Right panel: (●) control culture; (□) colcemid (500 μM) present only from 0 to 24 h; (○) colcemid (500 μM) removed at 48 h.

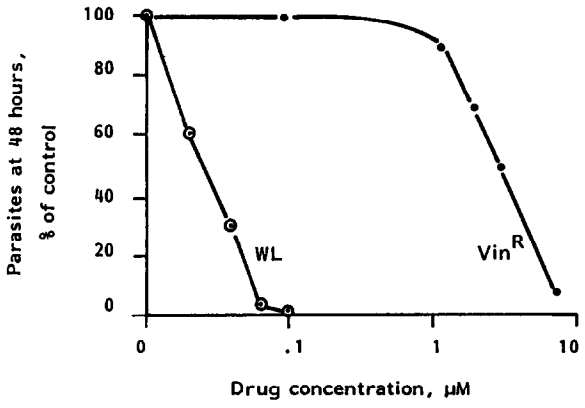

Fig.4. Dose-response curve of effect of anti-mitotic drugs on parasite growth. In the control culture parasites at 48 h were 92 per 1000 erythrocytes (= 100%).

was grown for 30 days in the absence of the drug. After repeated challenge, vinblastine-resistance was confirmed (see table 1). Our VLB^R mutant tolerates a drug concentration about 100-times higher than the strain from which it derives (fig.4). The VLB^R mutant exhibits cross-resistance also to vincristine. On the other hand, both strains have the same level of sensitivity to the well known antimalarial agent, chloroquine (table 1).

4. DISCUSSION

The best evidence thus far for the role of microtubular formations in nuclear division in *Plasmodia* has come from ultrastructural work carried out on *P. berghei*. Spindle structures have been observed both in ookinetes in the mosquito [10] and in exoerythrocytic stages in mouse liver [11]. By contrast, no information is available on the blood forms of *P. falciparum*. Our data indicate that VLB interferes with an event in the schizogonic cycle which takes place specifically at the trophozoite stage. In animal cells VLB is known to prevent the formation of mitotic spindle microtubules [12,13]. In order to corroborate the idea that the same may be true in *Plasmodium*, we have carried out similar experiments with other mitotic inhibitors such as vincristine and colcemid (which have been used in the treatment of human malaria [14]). We have found that parasite development is again blocked at the trophozoite stage. However, with colcemid higher concentrations are needed (50 μM for 50% inhibition), and the block is to some extent reversible upon removal of the drug.

Although it is possible that these agents affect *Plasmodium* by a different mechanism, the irreversibility of VLB action versus the reversibility of colcemid inhibition is consistent with the known effects of these agents on animal cells, whereby the former produces disruption of microtubules while the latter merely prevents polymerization of tubulins [12]. In addition, the observation that VLB blocks the cycle at very low concentrations suggests that its highly specific interaction with tubulin is involved. The most likely conclusion is that the spindle apparatus is, indeed, essential for nuclear division in the schizogonic cycle of *P. falciparum*. Previously, Sinden and Smalley [15] had reported a 'lethal effect' of colchicine in vitro on asexual forms from patients with clinical malaria, and the same agent has been used to synchronize cultures [16]. Taxol, an agent that inhibits dissociation of tubulin subunits [17] blocks replication of *Trypanosoma cruzi* [18]. No information was previously available on VLB in protozoa.

If susceptibility to microtubule reagents is taken

to indicate that these structures have a role in nuclear division, the timing of the latter would be very close to that of DNA synthesis [2]. This finding is consistent with previous evidence by Gutteridge and Trigg [3] that there is little or no G_2 phase in *P. knowlesi*, and it points to a significant difference in the division cycle between this parasitic protozoon and most animal cells. Since VLB is effective only against trophozoites it appears that tubulins are either present or accessible to the drug only at this stage.

The specificity of VLB action on *P. falciparum* is supported by our ability to isolate a drug-resistant derivative strain, under conditions which presumably selected for a single-step mutant. The mutant exhibits cross-resistance to the related drug, vincristine. We do not yet know whether VLB-resistance is due to decreased drug uptake by the parasite or to an intracellular effect. However, the mutational change is not unspecific, since sensitivity of the parasite to chloroquine is not affected. If the mutation involves microtubules, the VLB^R mutant might help to understand the function of these structures in the parasite cycle. Similar mutants have been described in a variety of organisms [19]. At a time when the progressive spread of drug-resistant *P. falciparum* is causing grave concern [20] it may be important to increase the range of agents that can block the parasite cycle in a characteristic way.

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