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In Vitro and In Vivo Inhibition of Erythrocytic Development of Malarial Parasites by Docetaxel

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The stage-dependent susceptibility of *Plasmodium falciparum* to a short exposure to docetaxel (Taxotere) was evaluated by subjecting ring-infected, trophozoite-infected, and schizont-infected erythrocytes to a 5-h exposure to various concentrations of the drug. The schizont stage was shown to be the most sensitive stage; an inhibition of more than 60% of parasite development was observed at 10 nM. At this drug concentration, the development of the younger ring and trophozoite forms was unaffected. The in vivo antimalarial activity of docetaxel against the development in blood of old trophozoites of a species that causes malaria in rodents, *Plasmodium vinckei petteri*, was evaluated in IOPS-OF1 mice. Two tests were performed: the 4-day suppressive test, as described by Peters (W. Peters, p. 145–273, *in Chemotherapy, and Drug Resistance in Malaria*, vol. 1, 1987), and the effects of a single injection of docetaxel after inoculation of the parasites. A single injection of docetaxel at 40 mg/kg of body weight was sufficient to reduce drastically the level of parasitemia; 90% inhibition of the development of parasites in blood was observed 5 days after drug injection. This program avoided the toxicity observed when mice were treated with four injections of docetaxel. The possibility of using a single bolus of taxoids to treat malaria infections is discussed.

Malaria remains the major tropical disease, with more than 300 million people being infected and 3.5 million deaths annually (25). The widespread development of *Plasmodium falciparum* resistance to classical antimalarial agents such as chloroquine has strengthened the efforts to develop new chemotherapeutic strategies that are based on the selective inhibition of biological processes that are essential for parasite propagation but that do not have major effects on the host. Microtubules play a crucial role in the life cycle of malarial parasites. They are one of the major cytoskeletal components and are involved in key steps of the life cycle such as the formation of the mitotic spindle during parasite division (1, 12, 22), the exflagellation process during microgametogenesis (2, 24), and the differentiation of zygotes into ookinetes (7). During hepatic schizogony, more than 20,000 hepatic merozoites or exoerythrocytic merozoites are produced from one sporozoite, and during each erythrocytic schizogony, 20 to 24 new merozoites are produced in 48 h. Microtubules thus constitute targets of choice for reducing parasite propagation. Ultrastructural studies on mitotic spindle formation have only been conducted on sporogony (22) and hepatic schizogony (12). However, a recent immunofluorescence study has described the microtubular organization during the erythrocytic schizogony of *P. falciparum* (17), and studies with antimicrotubular drugs have pinpointed the essential role of microtubules during asexual schizogony (5, 27).

Among the numerous microtubular poisons, taxoids like paclitaxel (Taxol) (19, 28) and docetaxel (Taxotere) (4, 18) possess the unique property of stabilizing microtubules both in vivo (21) and in vitro (6, 20) by inhibiting their depolymerization to free tubulin. In vitro studies by us (23) and other investigators (16) have shown that taxoids are very effective agents against the development of chloroquine-susceptible and chloroquine-resistant strains of *P. falciparum*, with the dividing stages (schizonts) being the most sensitive stages of the erythrocytic cycle. Docetaxel was shown to be more active in vitro than paclitaxel against the same strain, i.e., against *P. falciparum* FcB_1/Colombia, the drug concentration inhibiting parasite development by 50% was 5 ± 1 nM for docetaxel, whereas it was 71 ± 8 nM for paclitaxel (23). Furthermore, short-term incubation of parasites with 30 μM docetaxel irreversibly blocked the in vitro development of *P. falciparum* schizonts (23). We hypothesized that a single dose of docetaxel applied at the time of parasite schizogony would drastically reduce the level of parasitemia observed in vivo with paclitaxel (16), but with a better effectiveness in view of the differences in the drug concentrations inhibiting parasite development by 50% observed in vitro. Such a chronotherapy might also reduce the toxicity that taxoids can produce. To assess the feasibility of such an approach, we first evaluated in vitro the effects of a short exposure to docetaxel on the erythrocytic development of the human malarial parasite *P. falciparum* and have shown that a 5-h exposure to docetaxel at a concentration as low as 10 nM was sufficient to irreversibly block the parasite schizogony. The in vivo antimalarial activity of docetaxel was then assessed on the blood stages of the rodent malarial parasite *Plasmodium vinckei petteri* (i) by the 4-day suppressive test, as described by Peters (15), and (ii) by determining the effects of a single injection of docetaxel on blood stages of *P. vinckei petteri* 2 or 26 h after parasite inoculation.

MATERIALS AND METHODS

Drug. Docetaxel (RP 56976-FCH 160), a hemisynthetic compound related to paclitaxel (11), was a generous gift from Rhône-Poulenc Rorer (Vitry-sur-Seine, France).

For in vitro studies, docetaxel was prepared as a 10 mM stock solution in dimethyl sulfoxide and was stored at −20°C. Before use, docetaxel was diluted into culture medium.

For in vivo experiments, docetaxel was first dissolved in Tween 80 and was then adjusted to a concentration of 8 mg/ml with 5% (wt/vol) glucose in water. The suspension was sonicated, and drug dilutions were made in 5% (wt/vol) glucose solution.

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***P. falciparum* culture and synchronization.** The chloroquine-resistant strain FcB_1/Colombia (H. G. Heidrich, Max-Planck-Institut, Martinsried, Germany) was maintained in continuous culture as described by Trager and Jensen (26). The culture medium was RPMI 1640 (Gibco Laboratories, Eragny, France) supplemented with 5% human type O+ serum–11 mM glucose–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–44 mM sodium bicarbonate (pH 7.4)–60 μg gentamicin per ml. The culture was performed at 37°C erythrocytes (RBCs) with a 3 to 4% hematocrit and in an atmosphere of 3% CO_2–6% O_2–91% N_2. Under our culture conditions, the in vitro life cycle of this strain was 48 h.

Parasite synchronization was obtained by combining the gelatin flotation method (14) with sorbitol treatment (9). Mature schizont-infected RBCs were isolated and concentrated to 60 to 80% parasitemia with Plasmagel (Roger Bellon, Neuilly sur Seine, France). The schizonts were then put back in the culture and were diluted with fresh RBCs. After 5 h, reinvasion was stopped by lysing the remaining schizonts with 5% (wt/vol) sorbitol (in distilled water). Such a procedure allows one to obtain a homogeneous population of ring-infected RBCs in a 5-h window.

Docetaxel treatments of *P. falciparum* erythrocytic stages. Ring-infected RBCs (0 to 5 h old), trophozoite-infected RBCs (20 to 25 h old), and schizont-infected RBCs (37 to 42 h old) were incubated for 5 h at 37°C with docetaxel concentrations ranging from 30 to 0.01 μM. At the end of the incubation period, the cells were carefully washed with RPMI 1640 medium and were then maintained in culture medium without drug. Controls were performed with untreated synchronous cultures processed in the same way as the treated cultures. Giemsa-stained blood smears were prepared, and parasitemia was established on 2,000 cells by counting the number of new rings that formed during the second erythrocytic cycle. The inhibitory effect of docetaxel on parasite growth was determinated by comparing the level of parasitemia of the treated cultures with that of the control cultures. Each docetaxel concentration was tested in duplicate in two independent experiments.

Treatment of *P. vinckei petteri*-infected mice with docetaxel. (i) Inoculation of mice. Male IOPS-OF1 mice (Charles-River, Cleon, France) weighing approximately 20 g were inoculated with the strain 279 BY, which was isolated from the Central African Republic line of *P. vinckei petteri* (3). The blood of a donor mouse with a level of parasitemia of about 30% with parasites at the old trophozoite stage was collected in an heparinized syringe. The parasitized blood was diluted in physiological saline solution (0.9% NaCl) to a density of 5×10^7 RBCs/ml. At day 0, the mice were inoculated intraperitoneally with 0.2 ml of the parasite suspension (10^7 parasites).

(ii) In vivo administration of docetaxel. The in vivo antimalarial activity of docetaxel was evaluated by two series of tests. In test 1, the 4-day suppressive test described by Peters (15), at 2 h after inoculation with *P. vinckei petteri*, mice were injected intraperitoneally with 0.1 ml of docetaxel solutions at different concentrations, giving doses ranging from 7.5 to 40 mg/kg of body weight. Eight animals were tested at each dose level. The injection of docetaxel was repeated daily for 3 consecutive days from day 0 of parasite injection. At the 5th day, thin blood smears were made from the tail blood of treated and untreated mice and the smears were stained with Giemsa. For the control group (six animals), docetaxel was omitted from the formulation and all the other steps were identical to those for the treated groups.

In test 2, single administration of the drug, mice (eight animals per group) received a single 0.1-ml intraperitoneal injection of docetaxel (40 mg/kg) at 2 or 26 h after the parasite injections. Five days later, blood smears were made from tail blood and the smears were stained with Giemsa.

For each experiment, the level of parasitemia was determined by counting 2,000 cells. Parasite growth inhibition by docetaxel was determined by comparison with the level of parasitemia in control untreated mice. All inhibition experiments were carried out in at least two independent experiments.

RESULTS AND DISCUSSION

Several investigations have shown that taxoids at nanomolar concentrations inhibit the in vitro development of *P. falciparum* when cultures were continuously exposed to the drug (16, 23). Schrével et al. (23) have also demonstrated that a 5-h exposure to 30 μM docetaxel is sufficient to block the development of *P. falciparum*, regardless of the period of the parasite's erythrocytic life cycle. Here, the efficacy of short exposures to docetaxel at lower concentrations was investigated on *P. falciparum* erythrocytic stages by a series of pulse experiments on 5-h synchronized cultures. The results, shown in Fig. 1, demonstrate that a short exposure of synchronous *P. falciparum* cultures to docetaxel inhibits parasite development in a concentration-dependent fashion. Furthermore, the susceptibility of *P. falciparum* toward docetaxel differed according to the erythrocytic stage. At 30 μM, the highest docetaxel concentration tested, 95% inhibition of ring development was observed. On the other hand, trophozoite development was inhibited at drug concentrations of 30 and 10 μM. Docetaxel was still more effective against the dividing forms (schizonts); at the lowest drug concentration tested, 0.01 μM, inhibition of schizont development of more than 60% was observed, whereas the development of the younger ring and trophozoite forms was unaffected at this concentration. Thus, a 5-h exposure to docetaxel at a concentration as low as 10 nM is sufficient to block the in vitro development of *P. falciparum* when the exposure is applied throughout the period of parasite division. To test whether the same may be true *in vivo*, we investigated the antimalarial activity of docetaxel on blood stages of a *Plasmodium* species that causes malaria in rodents, *P. vinckei petteri.*

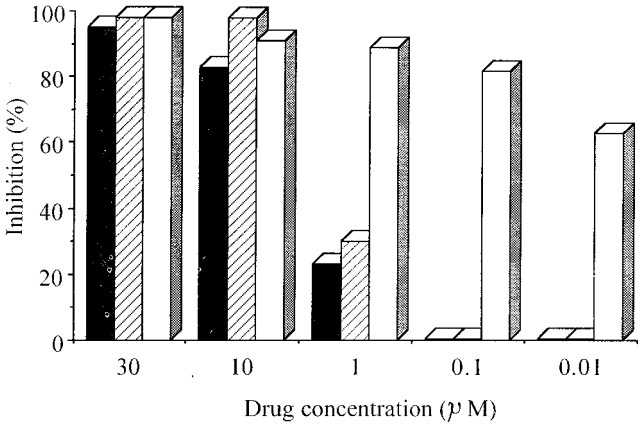

FIG. 1. Inhibitory effects of 5-h pulse exposure of *P. falciparum*-infected RBCs to docetaxel. The 5-h synchronized cultures at the ring stage (0 to 5 h; black bars), the trophozoite stage (20 to 25 h; open hatched bars), or the schizont stage (37 to 42 h; white bars) were incubated for 5 h at the indicated concentrations of docetaxel. After incubation, the cells were washed and resuspended in fresh culture medium. The level of parasitemia was determined on Giemsa-stained smears when parasites were at the ring stage of the second erythrocytic cycle in the control cultures. Parasite growth inhibition was determined by comparison of the level of parasitemia in treated cultures with that in untreated cultures processed in the same way as the treated cultures. Each bar corresponds to the mean of two experiments. For each experiment, the values are given in duplicate.

P. falciparum synchronicity occurs normally in humans (8), and *P. vinckei petteri* was selected for study because of this similarity in synchronicity. Furthermore, *P. vinckei petteri* schizogony occurs 24 h after inoculation with frozen blood (13), and thus, the timing of the various parasitic stages can be set up precisely. Mice infected with 18-h-old trophozoites were treated with docetaxel by the 4-day suppressive test (15) with drug doses of 40, 30, 20, 10, and 7.5 mg/kg 2 h after parasite inoculation. The intraperitoneal injections of drug were repeated daily for 3 consecutive days, and blood smears were made on the 5th day. Results from a typical experiment are presented in Table 1. At the highest concentration tested, docetaxel had a high level of toxicity; two of the eight mice treated with 40 mg of the drug per kg were alive after the fourth injection. Total eradication of the parasite was observed in these mice, but they died 2 days later (data not shown). In the groups treated with docetaxel at 30 and 20 mg/kg, a drastic decrease in the level of parasitemia was observed, with inhibition of parasite development being more than 98 and 92%, respectively (Table 1). Under these test conditions, the docetaxel concentration causing 50% reduction of parasite development was 12 ± 1 mg/kg ($n = 3$). The strong inhibition of parasite development obtained at doses of 30 and 20 mg/kg led

TABLE 1. Inhibition of development of *P. vinckei petteri* blood stages by the 4-day suppressive test[a]

Drug concn (mg/kg) (days 0, 1, 2, 3)	Day 5		
	% Parasitemia	% Inhibition	Mortality (*n*/*N*)[b]
40	0	100	6/8
30	1.1 ± 0.7	98.0 ± 1.3	0/8
20	4.0 ± 2.6	92.7 ± 4.7	0/8
10	35.0 ± 8.3	42.9 ± 9.4	0/8
7.5	55.0 ± 3.4	2.4 ± 3.4	0/8
Control	55.7 ± 4.4		0/6

[a] The 4-day suppressive test was performed as described by Peters (15). Mice (eight animals per group) were inoculated with old trophozoites and were treated 2 h later with docetaxel at the indicated doses. The injections were repeated daily for 3 days. For the control group (six animals), docetaxel was omitted from the formulation and all the other steps were identical to those for the treated groups.

[b] Mortality is defined as *n*/*N*, where *n* is the number of dead mice and *N* is the number of mice in each group.

us to evaluate the effectiveness of a single drug administration. Docetaxel was administered either 2 or 26 h after parasite inoculation, when parasites began schizogony. In contrast to repetitive injections of docetaxel at doses of 40 mg/kg, no apparent toxicity was observed when a single dose of 40 mg/kg was injected. The inhibitory effects of a single injection of docetaxel are reproduced in Table 2. When docetaxel was injected 2 h after parasite inoculation, comparison of the level of parasitemia in treated and control mice 5 days later showed an inhibition of parasite development of about 90% (Table 2). This inhibition was similar to that observed when mice were treated with four injections of docetaxel at 20 mg/kg (Table 1). A single injection of docetaxel at 40 mg/kg 26 h after inoculation with old trophozoites reduced the level of parasitemia by 64% (Table 2). These findings are consistent with previous studies of Pouvelle et al. (16) on the in vivo effects of a single dose of paclitaxel (50 mg/kg) on the blood stages of *Plasmodium chabaudi adami*. Nevertheless, the two protocols used in the two studies are quite different and do not allow us to compare our results obtained with docetaxel with those obtained with paclitaxel. The higher degree of solubility of docetaxel in aqueous solutions compared with that of paclitaxel (10) may offer a better condition for the efficacy of docetaxel compared with that of paclitaxel. Furthermore, in the present series of in vivo experiments, injections of docetaxel were done intraperitoneally. So, it can be expected that intravenous inoculations, as applied in cancer therapy, might be more efficient against the development of malarial parasites in blood.

In this report we demonstrated that a short exposure of schizont-infected RBCs to a low concentration of docetaxel blocks the development of *P. falciparum* and that docetaxel administered as a single injection of 40 mg/kg is effective in vivo against the blood stages of *P. vinckei petteri*. These results indicate that a single injection of docetaxel is able to reduce significantly the level of parasitemia. Such a situation suggests that a clinical improvement in patients with severe cases of malaria can be expected by injection of docetaxel or other taxoids. Furthermore, the design of taxoids with lower toxicities could be useful for determining the feasibility and the efficiency of such a therapeutic approach since the mechanisms of these drugs, which stabilize the microtubules, is completely different from those of the usual antimalarial drugs.

TABLE 2. Inhibition of development of *P. vinckei petteri* blood stages by a single injection of docetaxel[a]

Time of one drug injection (day 0)	% Parasitemia	% Inhibition
A 2 h after parasite inoculation	5.1 ± 3.0	91.2 ± 5.6
B 26 h after parasite inoculation	21.1 ± 4.1	64.4 ± 8.9
C Control for series B	55.5 ± 4.8	

[a] Docetaxel was given at 40 mg/kg to different groups of mice (eight animals per group) 2 h (series A) or 26 h (series B) after inoculation with old trophozoites. For the control group (six animals), docetaxel was omitted from the formulation and all other steps were identical to those for the treated groups. The percentages of parasitemia and inhibition were determined on day 5.

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REFERENCES

1. **Aikawa, M., and R. Beaudoin.** 1968. Studies on nuclear division of a malarial parasite under pyrimethamine treatment. J. Cell Biol. **39:**749–754.
2. **Aikawa, M., C. Carter, Y. Ito, and M. M. Nijhout.** 1984. New observations on gametogenesis, fertilization, and zygote transformation in *Plasmodium gallinaceum*. J. Protozool. **31:**403–413.
3. **Caillard, V., A. Beauté-Lafitte, A. G. Chabaud, and I. Landau.** 1992. *Plasmodium vinckei petteri*: identification of the stages sensitive to Arteether. Exp. Parasitol. **75:**449–456.
4. **Denis, J. N., A. E. Greene, D. Guénard, F. Gueritte-Voegelein, L. Mangatal, and P. Potier.** 1988. A highly efficient, practical approach to natural taxol. J. Am. Chem. Soc. **110:**5917–5919.
5. **Dieckmann-Schuppert, A., and R. M. Franklin.** 1989. Compound binding to cytoskeletal proteins are active against *P. falciparum in vitro*. Cell Biol. Int. Rep. **13:**411–418.
6. **Gueritte-Voegelein, F., D. Guénard, F. Lavelle, M. T. Le Goff, L. Mangatal, and P. Potier.** 1991. Relationships between the structure of taxol analogues and their antimitotic activity. J. Med. Chem. **34:**992–998.
7. **Kumar, N., M. Aikawa, and C. Grotendorst.** 1985. *Plasmodium gallinaceum*: critical role for microtubules in the transformation of zygotes into ookinetes. Exp. Parasitol. **59:**239–247.
8. **Kwiatkowski, D., A. V. S. Hill, I. Sambou, P. Twumasi, J. Castracane, K. R. Manogue, A. Cerami, D. R. Brewster, and B. M. Greenwood.** 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet **336:**1201–1204.
9. **Lambros, C., and J. P. Vanderberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65:**418–420.
10. **Lavelle, F., C. Fizames, F. Gueritte-Voegelein, D. Guénard, and P. Potier.** 1989. Experimental properties of RP 56976, a taxol derivative. Proc. Am. Assoc. Cancer Res. **30:**2254.
11. **Mangatal, L., M. T. Adeline, D. Guénard, F. Gueritte-Voegelein, and P. Potier.** 1989. Application of the vicinal oxyamination reaction with asymmetric induction to the hemisynthesis of taxol and analogues. Tetrahedon **45:** 4177–4190.
12. **Meis, J. F. G. M., J. P. Verhave, P. H. U. Jap, and J. H. E. T. Meuwissen.** 1985. Transformation of sporozoites of *P. berghei* into exoerythrocytic forms in the liver of its mammalian host. Cell Tissue Res. **241:**353–360.
13. **Montalvo-Alvarez, A., I. Landau, D. Baccam, A. G. Chabaud, and H. Ginsburg.** 1988. Experimental modification of the circadian rhythm of *Plasmodium vinckei petteri* following cryopreservation: probable resistance of the merozoite to thawing. C. R. Acad. Sci. Paris III **307:**5–10.
14. **Pasvol, G., R. J. M. Wilson, M. E. Smalley, and J. Brown.** 1978. Separation of viable schizont-infected red cells of *Plasmodium falciparum* from human blood. Ann. Trop. Med. Parasitol. **72:**87–88.
15. **Peters, W.** 1987. Chemotherapy, and drug resistance in malaria, vol. 1, p. 145–273. Academic Press, Inc., New York.
16. **Pouvelle, B., P. J. Farley, C. A. Long, and T. Taraschi.** 1994. Taxol arrests the development of blood-stage *Plasmodium falciparum in vitro* and *Plasmodium chabaudi adami* in malaria-infected mice. J. Clin. Invest. **94:**413–417.
17. **Read, M., T. Sherwin, P. Holloway, K. Gull, and J. E. Hyde.** 1993. Microtubular organization visualized by immunofluorescence microscopy during erythrocytic schizogony in *Plasmodium falciparum* and investigation of post-translational modifications of parasite tubulin. Parasitology **106:**223–232.
18. **Ringel, I., and S. B. Horwitz.** 1991. Studies with RP 56976 (Taxotere): a semisynthetic analogue of taxol. J. Natl. Cancer Inst. **83:**288–291.
19. **Rowinsky, E. K., L. A. Cazenave, and R. C. Donehower.** 1990. Taxol: a novel investigational antimicrotubule agent. J. Natl. Cancer Inst. **82:**1247–1259.
20. **Schiff, P. B., J. Fant, and S. B. Horwitz.** 1979. Promotion of microtubule

assembly *in vitro* by taxol. Nature (London) **277:**665–667.
21. **Schiff, P. B., and S. B. Horwitz.** 1980. Taxol stabilizes microtubules in mouse fibroblast cells. Proc. Natl. Acad. Sci. USA **77:**1561–1565.
22. **Schrével, J., G. Asfaux-Foucher, and J. M. Bafort.** 1977. Etude ultrastructurale des mitoses multiples au cours de la sporogonie du *Plasmodium b. berghei*. J. Ultrastruct. Res. **59:**332–350.
23. **Schrével, J., V. Sinou, P. Grellier, F. Frappier, D. Guénard, and P. Potier.** 1994. Interactions between docetaxel (Taxotere) and *Plasmodium falciparum*-infected erythrocytes. Proc. Natl. Acad. Sci. USA **91:**8472–8476.
24. **Sinden, R. E., E. U. Canning, and B. Spain.** 1976. Gametogenesis and fertilization in *Plasmodium yoelii nigeriensis*: a transmission electron microscope study. Proc. R. Soc. London Ser. B **193:**55–76.
25. **Struchler, D.** 1989. Malaria: how much is there world wide? Parasitol. Today **5:**39–40.
26. **Trager, W., and J. B. Jensen.** 1976. Human malarial parasites in continuous culture. Science (Washington, D.C.) **193:**673–677.
27. **Usanga, E. A., E. O'Brien, and L. Luzzato.** 1986. Mitotic inhibitors arrest the growth of *Plasmodium falciparum*. FEBS Lett. **209:**23–27.
28. **Wani, M. C., H. L. Taylor, M. E. Wall, P. Coggon, and A. T. McPhail.** 1971. Plant antitumor agents. VI. The isolation and structure of a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. Am. Chem. Soc. **93:** 2325–2327.