Pharmacodynamics of Doxycycline in a Murine Malaria Model[▽]

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Doxycycline is reported to impair second-generation parasite schizogony. The effects of doxycycline alone and combined with dihydroartemisinin were investigated in a murine malaria model. Doxycycline lowered the rate of parasite growth within 2 days, with maximum effect in 6 days. Addition of dihydroartemisinin led to an additive antimalarial effect.

Tetracyclines are relatively potent antimalarial drugs with slow/delayed onsets of action (8, 10, 17, 19) and low parasite reduction ratios (20). Hence, it is recommended that tetracyclines be used in combination with fast-acting antimalarials, such as quinoline or artemisinin drugs, for extended durations of at least 7 days (14, 17, 20–22).

Doxycycline (DOX) is widely used in malaria prophylaxis and is acceptable for long-term therapy, except in children and pregnant women (22). Furthermore, DOX combined with quinine is indicated for standby emergency treatment of falciparum malaria (22), and DOX has demonstrated efficacy in combination with artesunate, albeit with a 20% recrudescence rate that was attributed to an inadequate dosage of one or both drugs (13).

Despite the limited clinical role, in vitro and animal investigations have shown that DOX and other tetracyclines are effective antimalarial drugs against drug-resistant parasite strains (4, 10, 12). However, combination studies of DOX and artemisinin drugs have produced conflicting reports of additive (9, 23) and synergistic (7, 19) effects, thus highlighting the difficulty in predicting clinical outcomes from in vitro experiments and animal studies.

While differentiation between synergistic and additive effects may be problematic, interpretation of pharmacodynamic data should be more feasible with the recent elucidation of the mechanism of action of tetracyclines (8). Dahl et al. (8) showed that DOX caused a parasite organelle, the apicoplast, to become dysfunctional in progeny parasites. Late-stage parasites were most susceptible to DOX, and the drug effect was evident in mature parasites from the second generation, which did not rupture to release viable merozoites.

With a background of conflicting in vitro data, clinical observations of delayed effect, and the recently reported mechanism of action, we investigated the effect of DOX alone and in combination with dihydroartemisinin (DHA) in a murine malaria model. The method used in our study was adapted from the Rane test (15) in order to evaluate the drug effect on parasite elimination in an established infection and to obtain pharmacodynamic data from a treatment model.

DOX hyclate (molecular weight = 512.9) was obtained from Sigma Aldrich, Castle Hill, NSW, Australia. DHA was obtained from Dafra Pharma, Turnhout, Belgium. All general laboratory chemicals were of analytical grade (Sigma-Aldrich Chemical Co., Milwaukee, WI; BDH Laboratory Supplies, Poole, England; Merck Pty Limited, Kilsyth, Victoria, Australia).

This study was approved by the Curtin University Animal Experimentation Ethics Committee. Male Swiss (5- to 6-week-old) and BALB/c (7- to 8-week-old) mice were obtained from the Animal Resource Centre (Murdoch, Western Australia) and housed at 22°C in a 12-h light/dark cycle, with free access to sterilized commercial food pellets (Glen Forrest Stockfeeders, Perth, Western Australia) and acidified water (pH 2.5).

Plasmodium berghei ANKA parasites were obtained from the Australian Army Malaria Research Institute (Enoggera, QLD, Australia) and maintained by weekly blood passage in BALB/c mice. An inoculum of 10^7 parasitized erythrocytes per 100 µl was prepared by dilution of blood harvested from BALB/c mice in citrate-phosphate-dextrose solution (sodium citrate [30 g/liter], sodium dihydrogen phosphate [0.15 g/liter], dextrose [2 g/liter]) and used to infect the experimental (Swiss) mice.

Peripheral blood smears were prepared two or three times per day from tail vein bleeds until the time of euthanasia (>40% parasitemia, >10% reduction in mouse body weight in less than 24 h, or termination of the experimental protocol). The thin films were fixed in methanol (3 min) and then stained with May Grunwald Giemsa, using a Hema-Tek staining machine (AMES, Elkhart, IL). Parasitemia was determined by counting 30 or 100 fields of view for >0.5% or <0.5% infected erythrocytes, respectively, under 100× oil immersion light microscopy (Leica DMLS microscope; Leica Microsystems, Gladsville, NSW, Australia). This procedure ensured a limit of detection of approximately 0.002% parasitemia.

DOX hyclate was dissolved in water (11.25 mg/ml; injection volume, 200 µl) and administered by intraperitoneal injection at a dose of 90 mg/kg of body weight (equivalent to 78 mg/kg DOX; molecular weight = 444.4). DHA was dissolved in a 60:40 mixture of dimethyl sulfoxide-polysorbate 80 (7.5 mg/ml; injection volume, 100 µl) for a dose of 30 mg/kg. Drug administration commenced with DHA 64 h after inoculation (antic-

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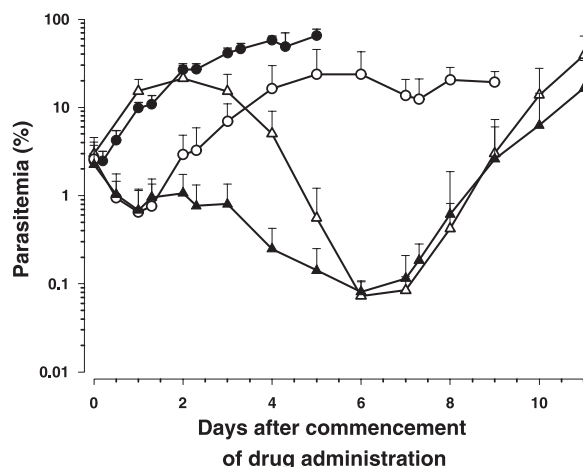
FIG. 1. Parasitemia-time profile for Swiss mice following doses of DOX hyclate (90 mg/kg at 1, 12, and 24 h; $n = 10$; △), DHA (30 mg/kg at 0 h; $n = 5$; ○), and a DOX-DHA combination (same doses; $n = 10$; ▲). Control mice ($n = 7$; ●) received the vehicle. All doses were given by intraperitoneal injection, commencing 64 h after inoculation with 10^7 $P.$ $berghei$-parasitized erythrocytes. Data are shown as percentages of infected erythrocytes (total parasitemia) plus standard deviations, commencing from the time of drug administration.

ipated parasitemia, 2 to 5%), and three doses of DOX were given at 1, 12, and 24 h to avoid adverse effects associated with concurrent drug administration. The control, DHA, DOX, and DOX-DHA groups comprised 7, 5, 10, and 10 mice, respectively.

Statistical analysis and representation was performed using SigmaStat 2004 and SigmaPlot 2004 (SPSS Inc., Chicago, IL). Data are given as means and standard deviations unless otherwise indicated.

Consistent with our previous work (11), DHA administration resulted in a prompt decline in parasitemia to a nadir approximately 24 h after dosing (Fig. 1). Preliminary single-dose DOX studies were based on previous reports of DOX in murine malaria (2, 3) but demonstrated weak effect up to 180 mg/kg of DOX (data not shown) and signs of discomfort in the mice at the highest dose. A three-dose regimen of DOX at 90 mg/kg per dose subsequently demonstrated a potent, delayed antimalarial effect with no observed adverse effects. A DOX-DHA combination was found to provide an apparent additive antimalarial effect (Fig. 1).

There was no significant difference between each group in the mean parasitemia levels at the time of initial dosing: control, 2.5% ± 0.5%; DOX, 3.0% ± 1.0%; DHA, 2.6% ± 2.0%; DOX-DHA, 2.2% ± 1.5% parasitized erythrocytes (Fig. 1). The nadir of parasitemia was significantly lower in the DOX (0.06% ± 0.02%) and DOX-DHA (0.06% ± 0.03%) groups than in the DHA (0.53% ± 0.45%; $P < 0.001$; one-way analysis of variance) group, but there was no significant difference between DOX and DOX-DHA. The median time to the nadir was significantly longer for DOX and DOX-DHA (6 days) than for DHA (1 day).

This study complements the recent elucidation of the mechanism of action of tetracyclines (8), showing that the response to DOX is delayed by at least 24 to 48 h (one to two erythrocytic cycles for $P.$ $berghei$), consistent with an

effect on progeny parasites. Furthermore, we have demonstrated a strategy for pharmacodynamic evaluation of antimalarial combinations and shown that the murine malaria treatment model could be valuable when the effect of individual components cannot be investigated in clinical trials or where a potent drug obscures the response of apparently weaker partner drugs (16).

The DOX doses used in our study were consistent with previous reports for a $P.$ $berghei$ model (2, 3). For example, six doses of 32 mg/kg DOX over 3 days were ineffective but six doses of 128 mg/kg (over 3 days) showed good antimalarial efficacy (2). By comparison, human doses of DOX for the treatment of falciparum malaria are in the order of 3 to 4 mg/kg/day for 7 days, in combination with other antimalarial drugs (14, 22). Pharmacokinetic and pharmacodynamic relationships are not well established for tetracyclines (1), and this is particularly evident in the case of DOX and malaria. In human studies, the mean half-life of DOX has been reported to range from 14 to 20 h (1). However, Newton et al. (14) reported that the half-life was 10.5 to 11.6 h in malaria infection, with doses given every 24 h (the erythrocytic cycle for $P.$ $falciparum$ is 48 h). In the present murine malaria study, we adopted the same strategy as Andersen et al. (2) and administered DOX every 12 h (the erythrocytic cycle for $P.$ $berghei$ is 24 h). Hence, 12-hourly dosing of DOX in the murine model is comparable to 24-hourly dosing in humans, in relation to the erythrocytic cycle of the parasite. By contrast, the pharmacokinetic relationship is less clear as pharmacokinetic properties for DOX in murine malaria have not been reported, although the half-life in healthy mice is approximately 2.8 h (5, 6) and evidence of allometry in DOX pharmacokinetics has been reported (18). Further studies of pharmacokinetic-pharmacodynamic relationships and interspecies scaling for DOX in malaria will be required to link murine data to human dosage regimens.

The murine malaria treatment model provides an understanding of drug efficacy in the context of an established infection and can be used for single (11) or multiple-dose investigations, as shown in the present study. By comparison, the Peters 4-day test evaluates drug efficacy according to suppression of malaria infection, with drug administration commencing at the time of parasite inoculation (15). Previously, Chawira et al. (7) used the Peters test and reported that a combination of artemisinin and tetracycline was synergistic in suppressing malaria infection with chloroquine-sensitive and chloroquine-resistant $P.$ $berghei$. However, in our model, DOX lowered the rate of parasite growth and the maximum observed effect was 6 to 7 days after commencing drug treatment. Addition of DHA as a potent, rapid-acting partner drug resulted in a prompt decline in parasite density that was sustained by the concurrent administration of DOX. These data indicate that in a murine malaria treatment model, DOX and DHA have an additive antimalarial effect.

REFERENCES

1. **Agwuh, K. N., and A. MacGowan.** 2006. Pharmacokinetics and pharmaco-dynamics of the tetracyclines including glycylcyclines. J. Antimicrob. Chemother. **58:**256–265.
2. **Andersen, S. L., A. Ager, P. McGreevy, B. G. Schuster, D. Wesche, R. Kuschner, C. Ohrt, W. Ellis, R. Rossan, and J. Berman.** 1995. Activity of azithromycin as a blood schizonticide against rodent and human plasmodia in vivo. Am. J. Trop. Med. Hyg. **52:**159–161.
3. **Andersen, S. L., A. L. Ager, P. McGreevy, B. G. Schuster, W. Ellis, and J. Berman.** 1994. Efficacy of azithromycin as a causal prophylactic agent against murine malaria. Antimicrob. Agents Chemother. **38:**1862–1863.
4. **Basco, L. K., and J. Le Bras.** 1993. Activity in vitro of doxycycline against multidrug-resistant *Plasmodium falciparum.* Trans. R. Soc. Trop. Med. Hyg. **87:**469–470.
5. **Bocker, R., and C. J. Estler.** 1981. Comparison of distribution of doxycycline in mice after oral and intravenous application measured by a high-performance liquid chromatographic method. Arzneimittelforschung **31:**2116–2117.
6. **Bocker, R., L. Warnke, and C. J. Estler.** 1984. Blood and organ concentrations of tetracycline and doxycycline in female mice. Arzneimittelforschung **34:**446–448.
7. **Chawira, A. N., D. C. Warhurst, B. L. Robinson, and W. Peters.** 1987. The effect of combinations of qinghaosu (artemisinin) with standard antimalarial drugs in the suppressive treatment of malaria in mice. Trans. R. Soc. Trop. Med. Hyg. **81:**554–558.
8. **Dahl, E. L., J. L. Shock, B. R. Shenai, J. Gut, J. L. DeRisi, and P. J. Rosenthal.** 2006. Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum.* Antimicrob. Agents Chemother. **50:**3124–3131.
9. **Fivelman, Q. L., J. C. Walden, P. J. Smith, P. I. Folb, and K. I. Barnes.** 1999. The effect of artesunate combined with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* in vitro. Trans. R. Soc. Trop. Med. Hyg. **93:**429–432.
10. **Geary, T. G., and J. B. Jensen.** 1983. Effects of antibiotics on *Plasmodium falciparum* in vitro. Am. J. Trop. Med. Hyg. **32:**221–225.
11. **Gibbons, P. L., K. T. Batty, P. H. R. Barrett, T. M. E. Davis, and K. F. Ilett.** 2007. Development of a pharmacodynamic model of murine malaria and antimalarial treatment with dihydroartemisinin. Int. J. Parasitol **37:**1569–1576.
12. **Kaddu, J. B., D. C. Warhurst, and W. Peters.** 1974. The chemotherapy of rodent malaria, XIX. The action of a tetracycline derivative, minocycline, on drug-resistant *Plasmodium berghei.* Ann. Trop. Med. Parasitol. **68:**41–46.
13. **Looareesuwan, S., C. Viravan, S. Vanijanonta, P. Wilairatana, P. Charoenlarp, C. J. Canfield, and D. E. Kyle.** 1994. Randomized trial of mefloquine-doxycycline, and artesunate-doxycycline for treatment of acute uncomplicated falciparum malaria. Am. J. Trop. Med. Hyg. **50:**784–789.
14. **Newton, P. N., J. F. Chaulet, A. Brockman, W. Chierakul, A. Dondorp, R. Ruangveerayuth, S. Looareesuwan, C. Mounier, and N. J. White.** 2005. Pharmacokinetics of oral doxycycline during combination treatment of severe falciparum malaria. Antimicrob. Agents Chemother. **49:**1622–1625.
15. **Peters, W., and B. L. Robinson.** 1999. Malaria, p. 757–773. *In* O. Zak and M. A. Sande (ed.), Handbook of animal models of infection. Academic Press, San Diego, CA.
16. **Pukrittayakamee, S., R. Clemens, A. Chantra, T. Luknam, S. Looareesuwan, and N. J. White.** 2001. Therapeutic responses to antibacterial drugs in vivax malaria. Trans. R. Soc. Trop. Med. Hyg. **95:**524–528.
17. **Rieckmann, K. H.** 1983. Falciparum malaria: the urgent need for safe and effective drugs. Annu. Rev. Med. **34:**321–335.
18. **Riond, J. L., and J. E. Riviere.** 1990. Allometric analysis of doxycycline pharmacokinetic parameters. J. Vet. Pharmacol. Ther. **13:**404–407.
19. **Sponer, U., S. Prajakwong, G. Wiedermann, H. Kollaritsch, G. Wernsdorfer, and W. H. Wernsdorfer.** 2002. Pharmacodynamic interaction of doxycycline and artemisinin in *Plasmodium falciparum.* Antimicrob. Agents Chemother. **46:**262–264.
20. **White, N. J.** 1997. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. Antimicrob. Agents Chemother. **41:**1413–1422.
21. **White, N. J.** 2002. The assessment of antimalarial drug efficacy. Trends Parasitol. **18:**458–464.
22. **World Health Organization.** 2005. International travel and health, p. 132–151. World Health Organization, Geneva, Switzerland.
23. **Ye, Z., and K. Van Dyke.** 1994. Interaction of artemisinin and tetracycline or erythromycin against *Plasmodium falciparum* in vitro. Parasite **1:**211–218.