IN VIVO EVALUATION OF COMBINATION EFFECTS OF CHLOROQUINE WITH CEPHARANTHIN\textsuperscript{©} OR MINOCYCLINE HYDROCHLORIDE AGAINST BLOOD-INDUCED CHLOROQUINE-RESISTANT PLASMODIUM BERGHEI NK 65 INFECTIONS

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Abstract: The combination effects of chloroquine with Cepharanthin\textsuperscript{©} or minocycline hydrochloride were evaluated against a blood-induced infection with chloroquine-resistant \textit{P. berghei} NK 65 in ICR mice. The infected mice in an untreated control group showed a progressively increasing parasitemia leading to mouse death. A two-day dosage of 20 mg base/kg of chloroquine alone produced little effect against \textit{P. berghei} NK 65 infection, and all mice died from day 13 to 15 with an increasing parasitemia. A four-day dosage of 4 mg/kg of Cepharanthin\textsuperscript{©} alone produced no antimalarial activity, and all mice died by day 10. A four-day dosage of 50 mg/kg of minocycline hydrochloride alone produced a slight effect, but all mice died by day 18. Furthermore, mice given chloroquine in combination with Cepharanthin\textsuperscript{©} died from day 14 to 15. Mice given Cepharanthin\textsuperscript{©} plus minocycline hydrochloride also died from day 15 to 17. On the other hand, infected mice treated with chloroquine plus minocycline hydrochloride survived during the experiment. All mice treated with chloroquine alone, minocycline hydrochloride alone, chloroquine plus Cepharanthin\textsuperscript{©} or Cepharanthin\textsuperscript{©} plus minocycline hydrochloride showed low parasitemia levels during drug administration and a few subsequent days, but then malaria parasites re-increased in the bloodstream of the treated mice until death. On the other hand, malaria parasites in the mice given chloroquine plus minocycline hydrochloride decreased on day 6 and then could not be detected by microscopic examination during the observation period. This finding strongly suggests that the combination effects of chloroquine and minocycline hydrochloride are worthy of evaluation in human malaria. The results also clearly demonstrate the necessity and importance of in vivo experiments in estimating the activities of drugs.

Key words: \textit{Plasmodium berghei} NK 65, Cepharanthin\textsuperscript{©}, minocycline, antimalarial activity, chloroquine-resistance

INTRODUCTION

Malaria is one of the most important tropical diseases in the world. Chloroquine resistance in the human malaria parasite \textit{Plasmodium falciparum} arose first in South America and Southeast Asia (Harinasuta et al., 1962; Young and Moore, 1961). It has now spread to all parts of the world where malaria is endemic and poses a major threat to the elimination of the disease. Although there are several strategies for controlling the disease, chemotherapy is the primary defense against malaria, and thus the worldwide emergence of chloroquine-resistant variants of parasite has stimulated the development of new treatments for malaria (Payne, 1987; Winstanley, 2000). The development of a new drug, however, is an extremely expensive and time-consuming process, and hence there have been efforts to re-evaluate the antimalarial activities of certain drugs already accepted for clinical use in patients with various non-malarial infectious diseases. White and Olliaro (1996) recently advocated the utility of combination chemotherapy as a rational approach to the containment of drug-resistant malaria. These trials focused on the detection of compounds that display killing activity against multiple drug-resistant malaria parasites in vitro (Haruki et al., 2000; Lin et al., 2001). However, the question remains as to which in vitro antimalarial effects can be induced in vivo. Since the discovery of murine malaria parasite \textit{P. berghei} by Vincke and Lips (1948), the model of experimental murine malaria

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has proved to be very convenient both for detection of anti-
malarial activity (Peters et al., 1975) and investigations into
the mode of action with P. falciparum in culture. Further-
more, the mouse infected with P. berghei is generally con-
sidered to be a valid model for the primary screening of
drugs for eventual use against human malaria (WHO,
1973). In 1971, attempts to confirm the antimalarial activity
of Artemisia annua extracts in mice infected with P. ber-
ghei led to the isolation of a plant constituent, artemisinin
(Klayman, 1985). The use of drug-resistant strains of P.
bergher can yield additional information concerning both
the mode of action of a compound, and its potential value
against drug-resistant strains of human malaria. Thus, we
utilized the murine model to investigate the effects of chlo-
roquine - in combination with drugs known to reverse chlo-
roquine resistance in vitro (Haruki et al., 2000; Lin et al.,
2001) - against blood-induced chloroquine-resistant P. ber-
ghei NK 65 infections in ICR mice.

MATERIALS AND METHODS

Animals and parasites

All animal experiments were performed according to the
Guidelines for Animal Experimentation, Hamamatsu
University School of Medicine. Outbred male ICR mice, 7
weeks old, purchased from SLC Inc. (Hamamatsu, Japan),
were used. Murine malaria parasites, chloroquine-resistant
Plasmodium berghei strian NK 65), were a gift from Pro-
fessor Y. Wataya (Okayama University, Japan). The blood
stage parasites were stored in a - 80°C deep freezer. For
experiments, the parasites from frozen stock were injected
into two mice. The mouse showing 10-15% of parasitemia
was bled under ether anaesthesia to collect the parasitized
blood. Experimental mice were given an intraperitoneal in-
jection of the 10^6 parasitized blood. To determine the effect
of the drug, treated mice were monitored for % parasitemia
and days of survival relative to control mice up to day 30
post infection. Thin blood smears from the tail vein were
prepared and Giemsa-stained throughout the observation
period.

In vivo antimalarial activity of chloroquine against
chloroquine-resistant P. berghei NK 65 infections

Twenty mice were infected intraperitoneally with 10^6
parasitized erythrocytes and divided into four per group, as
well as an untreated control group, for activity assay of
chloroquine diphosphate (Sigma Chemical Co., MO, USA).
From day 4 after injection, the mice were given chloroquine
orally at 20 mg base/kg body weight (kg) for 2 or 3 days, or
40 mg base/kg for 1 or 2 days, respectively, in the treated
groups. An equivalent volume of distilled water was given
orally to mice in the untreated, infected group for 3 days.

In vivo antimalarial activity of Cepharanthin® and minocy-
cline hydrochloride against chloroquine-resistant P. berghei
NK 65 infections

Twenty-eight mice were infected intraperitoneally with
10^6 parasitized erythrocytes and divided into four per group,
including an untreated control group, for activity assay of
the compounds in combination with or without chloroquine.
From day 4 after injection, the mice in three groups were
given chloroquine orally at 20 mg base/kg once a day for 2
days, and furthermore, to detect the activity of Cepharan-
thin® and minocycline hydrochloride in combination with
chloroquine, the mice in two out of three of the groups
treated with chloroquine received Cepharanthin® at 4 mg/kg
intrapertioneally once a day for 4 consecutive days and mi-
ocycline hydrochloride at 50 mg/kg intraperitoneally once
a day for 4 consecutive days. Furthermore, the mice in the
other three groups were given Cepharanthin® at 4 mg/kg,
minocycline hydrochloride at 50 mg/kg, or both drugs si-
multaneously, intraperitoneally once a day for 4 consecu-
tive days. An equivalent volume of water was given orally to
mice in the untreated, infected group for 4 consecutive days.

RESULTS

In vivo antimalarial activity of chloroquine against
chloroquine-resistant P. berghei NK 65 infections

As shown in Fig. 1A, mice in the untreated control
group died from day 9 to day 11 post infection after a grad-
ual body weight loss. Chloroquine treatment was somewhat
effective against the parasites but it did not entirely eradi-
cate them. All mice in the groups given the drug died after
a gradual body weight loss as follows: mice given a two-
day dosage of 20 mg base/kg died from day 12 to day 14,
those given a three-day dosage of 20 mg base/kg died from
In vivo antimalarial activity of Cepharanthin

Bloodstream of all treated mice and all eventually died. Two days. Malaria parasites, however, rebounded in the bloodstream of the control and treated group gradually increased and all of the mice died by day 11. All of the mice treated with chloroquine showed low parasitemia levels during drug administration and the following few days. Especially, malaria parasites in the mice given 50 mg/kg of minocycline hydrochloride decreased on day 6 but rebounded from day 10 in the bloodstream of all treated mice until death.

Combination effects of the compounds against chloroquine-resistant P. berghei NK 65 infections

Mice in the untreated control group died from day 9 to 11 post infection (Fig. 3A). A two-day dosage of 20 mg base/kg of chloroquine alone produced little effect against P. berghei NK 65 infection, and all of the mice died from day 13 to 15. Four daily doses of 4 mg/kg of Cepharanthin® alone produced no antimalarial activity, and all of the mice died by day 10. A four-day dosage of 50 mg/kg of minocycline hydrochloride alone produced a slight effect, but all of the mice died by day 18. Mice given Cepharanthin® plus minocycline hydrochloride died from day 14 to 17. Mice given chloroquine plus Cepharanthin® died from day 14 to 15. On the other hand, mice given chloroquine in combination with minocycline hydrochloride survived during the experiment. Malaria parasites appeared from day 4 in the bloodstream of the control and treated groups (Fig. 3B). Parasitemia levels of mice in the control and Cepharanthin®-treated groups gradually increased and all of the mice died. All mice treated with chloroquine alone, minocycline hydrochloride alone, Cepharanthin® plus minocycline hydrochloride and chloroquine plus Cepharanthin® showed low parasitemia levels during drug administration and the following few days. However, malaria parasites rebounded in the bloodstream of the treated mice until death. On the other hand, parasites in the mice treated with a combination of chloroquine and minocycline hydrochloride suppressed the multiplication of parasites during drug administration and the following few days. Especially, malaria parasites in the mice given 50 mg/kg of minocycline hydrochloride decreased on day 6 but rebounded from day 10 in the bloodstream of all treated mice until death.

In vivo antimalarial activity of Cepharanthin® and minocycline hydrochloride against chloroquine-resistant P. berghei NK 65 infections

Mice in the untreated control group died from day 8 to 10 post infection, showing a progressively increasing parasitemia (Figs. 2A and 2B). Four daily doses of 0.2, 2 or 4 mg/kg of Cepharanthin® from day 4 after parasite injection produced no antimalarial activity and all of the mice died by day 10, showing a pattern of parasitemia similar to that in the untreated control group (data not shown). A four-day dosage of 2.5 mg/kg of minocycline hydrochloride produced no effect, and all of the mice died by day 10. On the other hand, four daily doses of 25 or 50 mg/kg of minocycline hydrochloride produced a slight effect, but all of the mice died by day 17. Malaria parasites in the mice given a four-day dosage of 2.5 mg/kg of minocycline hydrochloride gradually increased until the mice died. A four-day dosage of 25 or 50 mg/kg of minocycline hydrochloride suppressed the multiplication of parasites during drug administration and the following few days. Especially, malaria parasites in the mice given 50 mg/kg of minocycline hydrochloride decreased on day 6 but rebounded from day 10 in the bloodstream of all treated mice until death.

Combination effects of the compounds against chloroquine-resistant P. berghei NK 65 infections

Mice in the untreated control group died from day 9 to 11 post infection (Fig. 3A). A two-day dosage of 20 mg base/kg of chloroquine alone produced little effect against P. berghei NK 65 infection, and all of the mice died from day 13 to 15. Four daily doses of 4 mg/kg of Cepharanthin® alone produced no antimalarial activity, and all of the mice died by day 10. A four-day dosage of 50 mg/kg of minocycline hydrochloride alone produced a slight effect, but all of the mice died by day 18. Mice given Cepharanthin® plus minocycline hydrochloride died from day 14 to 17. Mice given chloroquine plus Cepharanthin® died from day 14 to 15. On the other hand, mice given chloroquine in combination with minocycline hydrochloride survived during the experiment. Malaria parasites appeared from day 4 in the bloodstream of the control and treated groups (Fig. 3B). Parasitemia levels of mice in the control and Cepharanthin®-treated groups gradually increased and all of the mice died. All mice treated with chloroquine alone, minocycline hydrochloride alone, Cepharanthin® plus minocycline hydrochloride and chloroquine plus Cepharanthin® showed low parasitemia levels during drug administration and the following few days. However, malaria parasites rebounded in the bloodstream of the treated mice until death. On the other hand, parasites in the mice treated with a combination...
of minocycline hydrochloride and chloroquine decreased on day 6 and then could not be detected by microscopic examination during the observation period.

**DISCUSSION**

Recently, drugs enhancing the sensitivity of parasites to chloroquine were studied in resistant strains of rodent and human *Plasmodium* (Martin et al., 1987; Bitonti and McCann, 1989; Peters et al., 1990; Kyle et al., 1993). In the present experiment, *P. berghei* NK 65 showed a chloroquine-resistant property, and hence this variant can be a useful model for research on drugs that enhance the sensitivity of parasites to chloroquine (Ishih et al., 2003).

Lin et al. (2001) re-evaluated the antimalarial effects of three tetracyclines accepted for clinical use in patients with various non-malarial infectious diseases, and they reported that minocycline was effective against chloroquine-resistant *P. falciparum* in vitro. Their electron microscopic examination revealed a number of electron dense vesicles with a single membrane bound in the cytoplasm of minocycline-treated parasites (Lin et al., 2001). The anti-parasite action of minocycline in vitro studies may be attributable to the high lipophilicity of this drug, but it is difficult to explain whether the structural changes resulted from the specific action mechanism of tetracyclines. In our *P. berghei* NK 65 infected ICR model, all mice given minocycline hydrochloride alone showed low parasitemia levels during drug administration and the following few days, indicating the inhibitory effects of minocycline on parasite growth, but they died after re-increase of malaria parasites. In contrast, the combination of chloroquine with minocycline hydrochloride eventually produced a killing activity against *P. berghei* NK 65 parasites, and consequently, all mice survived during the experiment. Kim et al. (1998) evaluated a combination effect of 5-fluoroorotate and sulfamonomethoxine using this malaria isolate and reported a potent synergistic activity. By definition, chloroquine resistance reversal agents specifically target the chloroquine resistance mechanism and do not enhance the baseline activity of chloroquine against parasites (Martin et al., 1987; Bitonti and McCann, 1989). In the present study, both chloroquine and minocycline hydrochloride showed moderate antimalarial activity against the present parasite isolate when given alone to the infected mice, and thus it remains unclear which drug enhanced the antimalarial activity of the other. This finding, however, strongly suggests that the combination of chloroquine and minocycline hydrochloride extends the clinical utility of chloroquine if the higher dose of minocycline hydrochloride is tolerated. Further studies are necessary to clarify the mechanisms of combination effects of these drugs.

Meanwhile, Haruki et al. (2000) reported that Cepharanthin\(^{\circ}\), widely used clinically in Japan, enhanced the activity of chloroquine against the resistant strain of *P. falciparum* in vitro. The mechanism by which Cepharanthin\(^{\circ}\) increases the potency of chloroquine is unclear, but it may be related to enhanced accumulation of chloroquine. In our model, however, Cepharanthin\(^{\circ}\) given approximately 20 times in clinical use (Harada et al., 2001) produced neither combination effects with chloroquine nor its intrinsic antimalarial activity, suggesting the difference between in vitro and in vivo assay. The present results indicate that differences in assay systems, such as in vitro and in vivo methods, might lead to different and conflicting results for the antimalarial activities of drugs, and they indicate the necessity for and importance of in vivo experiments in the estimation of the activities of drugs.

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REFERENCES


