A new aminomethylphenol, JPC-2997, with high in vitro and in vivo antimalarial activity against blood stages of Plasmodium

Geoffrey W. Birrell¹#, Marina Chavchich¹#, Arba L. Ager³, Hong-Ming Shieh², Gavin D. Heffernan², Wenyi Zhao², Peter E. Krasucki², Kurt W. Saionz², Jacek Terpinski², Guy A. Schiehser², Laura R. Jacobus², G. Dennis Shanks¹, David P. Jacobus² and Michael D. Edstein¹

¹Department of Drug Evaluation, Australian Army Malaria Institute, Enoggera, Brisbane, Queensland 4051, Australia
²Jacobus Pharmaceutical Company, Princeton, New Jersey, USA
³University of Miami, Miami, Florida, USA

*Corresponding author:
Dr Mike Edstein; Telephone: +61-7-33324930; Fax: +61-7-33324800 and E-mail: Mike.Edstein@defence.gov.au

# These authors contributed equally to this study

Short Running Title: A new aminomethylphenol effective against Plasmodium

Keywords: Antimalarial drug discovery, Plasmodium, in vitro drug testing, pharmacokinetics, JPC-2997
4-((tert-Butyl)-2-((tert-butylamino)methyl)-6-(6-(trifluoromethyl)pyridin-3-yl)phenol (JPC-2997) is a new aminomethylphenol compound that is highly active in vitro against the chloroquine-sensitive D6, the chloroquine-resistant W2, and the multidrug-resistant TM90-C2B Plasmodium falciparum lines, with 50% inhibitory concentrations (IC\textsubscript{50}) ranging from 7 nM to 34 nM. JPC-2997 is >2,500 less cytotoxic (IC\textsubscript{50} values >35 µM) to human (HepG2 and HEK293) and rodent (BHK) cell lines compared with the D6 parasite line. In comparison to the chemically related WR-194,965, a drug that had advanced to clinical studies, JPC-2997 was 2-fold more active in vitro against P. falciparum lines and 3-fold less cytotoxic than WR-194,965. The compound possesses potent in vivo suppression activity against P. berghei with an ED\textsubscript{50} (50% effective dose) of 0.5 mg/kg/day following oral dosing in the Peters 4-day test. The radical curative dose of JPC-2997 was remarkably low at a total dose of 24 mg/kg using the modified Thompson test. JPC-2997 was effective in curing three Aotus monkeys infected with a chloroquine and pyrimethamine-resistant strain of P. vivax malaria at a dose of 20 mg/kg daily for 3 days. At the doses administered, JPC-2997 appeared to be well tolerated in mice and monkeys. Preliminary studies of JPC-2997 in mice show linear pharmacokinetics over the range 2.5 to 40 mg/kg, a clearance of 7.2 L/h/Kg, a high volume of distribution of 509 L/kg and an elimination half-life of 49.8 hours. The high in vivo potency data and lengthy elimination half life of JPC-2997 suggest that it is worthy of further preclinical assessment as a partner drug.
INTRODUCTION

Malaria remains one of the deadliest diseases in tropical countries with about 219 million cases in 2010 and an estimated 660,000 deaths, primarily in children from sub-Saharan Africa (1). Antimalarial drug resistance is a serious global health threat. To combat the development and spread of multiple drug resistance, artemisinin combination therapies (ACTs) such as artemether-lumefantrine, artesunate-amodiaquine and dihydroartemisinin-piperaquine are now recommended worldwide for first-line treatment of uncomplicated

*Plasmodium falciparum* malaria (2). Although ACTs remain highly effective, reports of *P. falciparum* resistance to artemisinins from four Southeast Asian countries (Cambodia, Myanmar, Thailand and Vietnam) are alarming (3-6). This has led to a concerted effort by WHO and national Ministries of Health to execute containment efforts in the affected countries. However, there is a genuine concern that artemisinin resistance will spread in a similar pattern to that of chloroquine and antifolate resistance from Southeast Asia to Africa in the 1970s and 1980s. In addition to the potential demise of the artemisinins, there is concern for the development and spread of resistance to the partner drugs. Thus, to avert a global health disaster there is an urgent need to develop new and effective antimalarial drugs as new partner drugs or to supplement existing ACTs.

In the 1960s and 1970s, there was renewed interest in antimalarial chemotherapy research due to the development and spread of chloroquine resistance. During this period, the US Army Research Program in Malaria tested over 250,000 compounds for antimalarial activity (7). By 1979, 43 different compounds from this program were selected for clinical trial evaluation. One of these, (4-(tert-butyl)-2-(tert-butylaminomethyl)-6-(4-chlorophenyl)phenol (WR-194,965), an aminomethylphenol, was highly potent
against chloroquine-sensitive and chloroquine-resistant *P. falciparum* lines, with IC$_{50}$ values in the low nanomolar range (8). In the rodent-*P. berghei* model, WR-194,965 had an ED$_{50}$ of 2.2 mg/kg/day using the Peters 4-day test (9). WR-194,965 at an oral dose of 27 mg/kg produced a 90% cure in Aotus monkeys infected with the multidrug-resistant *P. falciparum* Vietnam Smith strain (10).

In a human-challenge study, 750 mg WR-194,965 was orally administered every 12 h for 36 h to six human volunteers infected with the Vietnam Smith strain. Four of the six patients were cured. The two subjects who recrudesced (at 29 and 40 days post-treatment) were successfully cured with mefloquine, and subsequent *in vitro* tests showed that the parasites from these patients were sensitive towards WR-194,965 (11). Attractive features of WR-194,965 are a non-quinoline structure and good tolerance, but a major disadvantage was limited potency which led to a cessation in its clinical development by the US Army. Recently, Powles et al. (12) from Merck Research Laboratories (Rahway, NJ) reported on the *in vitro* and *in vivo* efficacy of MK-4815 (2-aminomethyl-3,5-di-tert-butyl phenol) confirming the utility of the same basic aminoethyl phenol structure and demonstrating an improvement in potency over WR-194,965.

We have conducted a comprehensive structure-activity relationship (SAR) study of this chemical series. This program led to the identification of 4-(tert-butyl)-2-((tert-butylamino)methyl)-6-(6-(trifluoromethyl)pyridin-3-yl)phenol (JPC-2997), a new aminomethylphenol which addresses the potency problems of WR-194,965. The SAR study will form the basis for a future publication. The structures of WR-194,965 and JPC-2997 are shown as Fig. 1.
In this study, we describe the *in vitro* antimalarial and cytotoxicity of JPC-2997 and its *in vivo* antimalarial efficacy against *P. berghei* in mice and *P. vivax* in monkeys. Preliminary pharmacokinetic properties of JPC-2997 in mice and monkeys are also presented.

**MATERIAL AND METHODS**

**Drugs.** 4-(tert-Butyl)-2-((tert-butyramino)methyl)-6-(6-(trifluoromethyl)-pyridin-3-yl)phenol (JPC-2997 hydrochloride) and 4-(tert-butyl)-2-(tert-butylaminomethyl)-6-(4-chlorophenyl)phenol (WR-194,965 hydrochloride) were provided by The Jacobus Pharmaceutical Company and their synthesis will be described in detail elsewhere. Dihydroartemisinin was obtained from Vital Health Care (Mumbai, India) and Central Pharmaceutical Factory No. 1 (Hanoi, Vietnam), chloroquine disphosphate and mefloquine hydrochloride were obtained from Sigma (St. Louis, MO), atovaquone from GlaxoSmithKine (Middlesex, UK), pyronaridine tetraphosphate from (Shin Poong Pharm, Seoul, South Korea) and piperaquine tetraphosphate from Waterstone Technology (St.Carmel, IN) and Central Pharmaceutical Factory No. 1 (Hanoi, Vietnam).

**Parasites.** Three laboratory-adapted *P. falciparum* lines were used in this study: D6 from Sierra Leone is chloroquine and pyrimethamine sensitive but mefloquine resistant, W2 from Indochina is chloroquine and pyrimethamine resistant, and TM90-C2B from Thailand is atovaquone, chloroquine, and mefloquine resistant. Isolates were cultured in RPMI-1640-LPLF media,(Gibco, Invitrogen Corporation, CA) containing 5.97 g/L HEPES free acid (Sigma St. Louis, MO) , 2.0 g/L D-glucose, 0.05 g/L hypoxanthine, 40 mg/L gentamycin with pH adjusted to 6.9 and freshly supplemented with 0.21% sodium bicarbonate and 10% human plasma. Cultures were
maintained at 1-8% parasitemia and 4% hematocrit (O(+) red blood cells) in an atmosphere of 90% N₂, 5% O₂, 5% CO₂, at 37°C (13).

**In vitro antimalarial activity.** For in vitro antimalarial activity assessment, WR-194,965 and JPC-2997 compounds were dissolved in DMSO, dihydroartemisinin in 100% methanol, whereas piperaquine and chloroquine were dissolved in 50% methanol/50% water. All drugs were subsequently diluted in culture media (without hypoxanthine) containing 10% human plasma. Residual methanol and DMSO concentrations were found to be too low to inhibit parasite growth (data not shown). Parasite susceptibility testing to JPC-2997 and chloroquine was carried out by measuring the inhibition of the radioactive [³H]-hypoxanthine uptake (14). Briefly, highly synchronous ring stage parasite cultures produced by several rounds of D-sorbitol selection (15) were exposed to ten 2-fold dilutions of the compounds (in triplicate) in 96-well flat bottom microtiter plates at 37°C for a total of 48 h. Each well contained 100 µL of parasite culture at 2% hematocrit and 1% parasitemia in culture media prepared as described above but lacking hypoxanthine. A 20 µL (0.2 µCi) aliquot of [³H]-hypoxanthine was added to each well ~24 h after commencement of the assay. Parasite DNA was harvested onto glass fiber filters, and radioactivity was counted to generate [³H]-hypoxanthine uptake (counts per minute) versus drug concentration (log values) curves. IC₅₀ values were determined using a nonlinear regression analysis (GraphPad Prism V5.0, GraphPad Software, Inc., CA) and were defined as the concentrations producing 50% inhibition of uptake of [³H]-hypoxanthine by parasites when compared with compound-free samples (controls).

**In vitro cytotoxicity assay.** Compounds were tested for in vitro cytotoxicity against two human cell lines: HEK293 (human embryonic kidney), HepG2 (human hepatocellular carcinoma) and BHK (baby hamster kidney) by
the alamarBlue® (Invitrogen Corporation, CA) fluorescent cell viability assay (16). Cell cultures were maintained in RPMI-1640 media (Sigma, MO) containing 10% fetal bovine serum and 0.03% L-glutamine (termed complete media) in 75 cm² flasks at 37°C with media changed twice weekly. Cells from 60–80% confluent cultures were trypsinised, washed in complete media, and plated at 5 × 10³ cells per well in 135 μL of complete medium in 96-well flat-bottom plates for 24 h at 37°C prior to the addition of the compounds.

Triplicate 15 μL aliquots of JPC-2997 and WR-194,965 compounds and reference drugs chloroquine, dihydroartemisinin and piperaquine covering eleven 2-fold dilutions were added to the wells, mixed gently, and plates were incubated for further 72 h. Controls included compound-free wells with DMSO (vehicle) as positive controls and cell-free wells with media only, which were used for background subtraction.

Following incubation for 72 h, the culture medium was removed and 50 μL of 10% alamarBlue® in complete medium was added to each well for a further 2 h. The fluorescence from the wells was measured using a GENios Plus microplate reader and XFluor4 software, using a 530 nm excitation filter and a 595 nm emission filter. Data were obtained from at least two independent experiments for each cell line and analyzed using GraphPad Prism V5.0. The selectivity index (SI) was calculated using the IC₅₀ values derived from mammalian cells divided by the IC₅₀ values obtained against the *P. falciparum* D6 line.

**In vivo antimalarial studies of *P. berghei* in mice.** The *in vivo* efficacy of JPC-2997 and WR-194,965 was determined in three murine malaria models; the Peters 4-day test (17), the modified Thompson test (18) and the onset of action and time to recrudescence test (19, 20). The Peters 4-day test measures the suppressive activity of blood schizontocides over 4 days at a
tolerated dose that does not cause physical stress in healthy mice. Swiss
outbred ARC (Animal Resource Centre, Murdoch, Western Australia) female
mice 5-6 weeks of age (weight 24 to 32 g), in groups of six, were inoculated
intraperitoneally with $20 \times 10^6$ *P. berghei*-infected erythrocytes of the
chloroquine-sensitive ANKA strain. Untreated control mice typically die
between days 6 and 7 postinfection. Drugs were dissolved in Tween
80/ethanol (7:3, v/v), diluted 10-fold in distilled water, and administered
subcutaneously (s.c.) or orally (p.o.) at about 2 h after parasite inoculation
(D0) and daily for 3 consecutive days. Thin blood smears were made on D+4
and stained with Giemsa. The degree of infection (parasitemia expressed as
percentage of infected erythrocytes) was determined microscopically. The
ED$_{50}$ and ED$_{90}$ (50% and 90% effective doses) values were calculated by
nonlinear regression analysis using GraphPad Prism V5.0 and mean ED$_{50}$
and ED$_{90}$ values were based on at least two independent experiments.

The modified Thompson test measures the survivability of mice and
parasite clearance in an established infection following administration of the
drug at D+3 to D+5 postinfection. CD-1 male or female mice 5 weeks of age
(Charles River Laboratories, Wilmington, MA, weight 24 to 30 g), in groups of
seven, were infected with $5 \times 10^5$ parasitized erythrocytes of chloroquine–
sensitive *P. berghei* KBG-173 strain. By D+3 post-infection, parasitemia was
about 1%. This strain produces reliable parasitemia and death in CD-1 mice.
The strain is maintained *in vivo* in Swiss mice and has been used for more
than 20 years to test susceptibility to a wide range of antimalarial drugs.

Drugs were mixed in 0.5% hydroxyethylcellulose and 0.1% Tween 80 and
administered via oral gavage either once daily at 24 h intervals or twice daily
at 6 h apart on days 3, 4, and 5 postinfection. Blood smears were taken on
D+6 and twice a week thereafter, starting on day 10 until the end of the test
on day +31. Mortality data were tabulated for 31 days. Mice that survived for 31 days and were blood film negative were considered cured.

The onset of drug action and the time of recrudescence test measures the speed of parasite clearance by a candidate compound and the time that recrudescence occurs after a single dose. This was done by administering a single oral dose of 100 mg/kg at day +4 after infecting groups of ARC female mice (n=5) intraperitoneally with $10 \times 10^6$ *P. berghei*-infected erythrocytes (ANKA strain). Untreated control mice typically achieve a parasitemia of about 20% by day 4 postinfection. Drugs were mixed in ethanol/Tween 80/water (10:10:80, v/v/v). To monitor the reduction in parasitemia blood smears were collected at 12 h intervals for the first 96 h after dosing, and then daily from day 5 to 17, followed by collections every 2 to 3 days until day 30.

**Preliminary pharmacokinetics of JPC-2997 in mice.** Single dose escalating studies of JPC-2997 were carried out in healthy ARC female mice (aged 6 to 9 weeks, weighing between 23 and 40 g). Groups of five, were treated with 2.5, 5, 10, 20 and 40 mg/kg of JPC-2997. The mice, anesthetized with carbon dioxide, were sacrificed by cardiac puncture at 0 (before dosing) and at 1, 3, 6, 12, 24, 48, 72, 96, 120 h and then at days 7 and 14 following oral administration. Blood samples (~0.7 mL) were collected using lithium heparin as the anticoagulant. After separating 0.2 mL of blood, the remaining blood was centrifuged at 16,000 x $g$ at 4°C for 5 min and plasma separated. Blood and plasma samples were stored at -80°C until analysis and concentrations of JPC-2997 were measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS) (see supplemental material). The lower limit of quantification (LLOQ) of JPC-2997 in blood and plasma was 0.5 ng/mL, with an inaccuracy of <4.1%, using 50 µL of sample. The interassay precision of analysis (percent coefficient of variation [CV]) for JPC-2997 in
blood over the concentration range of 0.5 ng/mL to 2,000 ng/mL was <4.6% and in plasma over the concentration range of 0.5 ng/mL to 4,000 ng/mL was <9.4%.

Pharmacokinetic parameters were maximum drug concentration \( [C_{\text{max}}] \), time to reach maximum drug concentration \( [T_{\text{max}}] \), area under the concentration-time curve from 0 h to the last data point \( [\text{AUC}_{0\to\text{last}}] \) and from the last data point to infinity \( [\text{AUC}_{\text{last}\to\infty}] \), terminal half-life \( [t_{1/2}] \), apparent oral clearance \( [CL/F] \), and apparent volume of distribution \( [V/F] \). These parameters were determined from the blood and plasma concentration-time data by noncompartmental methods (21). The blood to plasma concentration ratio was calculated using the ratio of \( [\text{AUC}_{\text{blood}}] \) to \( [\text{AUC}_{\text{plasma}}] \).

**In vivo efficacy of JPC-2997 in the Aotus monkey-Plasmodium vivax model.** Naive splenectomized Aotus monkeys (n=3), weighing between 960 and 1,126 g, were inoculated intravenously with \( 1 \times 10^6 \) parasites of the P. vivax AMRU1 strain, which is resistant to chloroquine (22). Three to four days after the onset of patency, monkeys were treated with 20 mg/kg JPC-2997 daily at 24 h intervals for 3 days by nasogastric tubulation. The drug was prepared and administered in 10% ethanol/1% Tween 80/89% distilled water. Heparinized blood samples (20 µL) were collected pre-dose on day 0 (i.e. immediately before JPC-2997 administration), on days 1 and 2 immediately before the next dose, and then on days, 3, 4, 7, 11, 14, 21, and 28 at the same time of day after starting treatment. JPC-2997 concentrations measured by LC/MS/MS. After treatment of monkeys, thick and thin blood smears were examined daily by counting parasitized erythrocytes against 500 leukocytes or 10,000 erythrocytes. When no parasites were detected, follow-up thick blood smears were examined twice a week for 62 days after starting treatment.
Animal Ethics. The animal studies for the Peters 4-day test, onset and duration assessment of antimalarial activity, monkey studies and pharmacokinetics of JPC-2997 were approved by the Army Malaria Institute Animal Ethics Committee in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The animal studies for the modified Thompson test were approved by the University of Miami Institutional Animal Care and Use Committee.

RESULTS

Aminomethylphenols are active against asexual blood stages of *P. falciparum* and have low cytotoxicity. The mean IC\(\text{50}\)s of JPC-2997 against the chloroquine-sensitive D6 and chloroquine-resistant W2 lines were 13 nM and 6.6 nM, respectively (Table 1). In comparison to WR-194,965, JPC-2997 was 2.3-fold and 4.2-fold more active against the D6 and W2 lines, respectively. JPC-2997 was less active against the multidrug-resistant TM90-C2B strain, with an IC\(\text{50}\) of 34 nM, but was 1.6-fold more active than WR-194,965. When compared to the standard antimalarial drugs, JPC-2997 was markedly more active than chloroquine but less active than dihydroartemisinin and piperaquine against the TM90-C2B *P. falciparum* line.

The mean IC\(\text{50}\) values for JPC-2997 determined in the cytotoxicity assay in three mammalian cell lines were relatively high (≥36 μM) resulting in SI values ≥2,769, which are similar to those of chloroquine. When compared with WR-194,965, the SI of JPC-2997 was about 3-fold higher. Overall, JPC-2997 has a high selectivity index when antimalarial activity is compared to mammalian cytotoxicity.

JPC-2997 is highly active *in vivo* against rodent malaria. In the Peters 4-day test, JPC-2997 suppressed the *P. berghei* ANKA strain in mice with an
ED$_{50}$ of 0.5 mg/kg/day following either subcutaneous or oral dosing (Table 2). JPC-2997 was 1.8-fold more active than either chloroquine (0.9 mg/kg/day) or piperaquine (0.9 mg/kg/day) but 2.5-fold less potent than dihydroartemisinin (0.2 mg/kg/day) after subcutaneous administration. When JPC-2997 was administered orally, it was more active than the reference standards; chloroquine (1.1 mg/kg/day) by 2.2-fold, dihydroartemisinin (1.3 mg/kg/day) by 2.6-fold and piperaquine (2.4 mg/kg/day) by 4.8-fold, based on their respective ED$_{50}$ values.

In the modified Thompson test, JPC-2997 produced 100% cure in mice infected with the $P$. berghei KBG-173 strain after single oral daily doses of 8 mg/kg for 3 days (total dose 24 mg/kg) (Table 3). In comparison, piperaquine was only 71% effective in curing mice when administered the same treatment regimen. Both JPC-2997 and piperaquine at a dose of 8 mg/kg given twice daily for 3 days were equally curative. WR-194,965 was 2-fold less active than JPC-2997 requiring a total dose of 96 mg/kg (i.e. 32 mg/kg twice daily x 3 days) to produce 100% cure (data not shown). In contrast to WR-194,965 and piperaquine, no mice were cured with chloroquine administered a total dose of 192 mg/kg and only 14% were cured with dihydroartemisinin at 192 mg/kg.

The onset of action of JPC-2997 in the treatment of an established infection of $P$. berghei of about 20% parasitemia was compared with seven standard antimalarial drugs (Fig. 2). The order of parasitemia clearance and the time of first blood smear negative slides were: dihydroartemisinin (36 h), piperaquine (36 h), pyronaridine (36 h), amodiaquine (60 h), chloroquine (60 h), atovaquone (96 h), JPC-2997 (120 h) and mefloquine (120 h) (Fig. 2A). After clearance of parasitemia, recrudescences commenced at the following times: 2.5 days for dihydroartemisinin, 6 days for chloroquine, 8 days for both amodiaquine and atovaquone, 12 days for mefloquine and 20 days for JPC-
2997 (Fig. 2B). Of note only two of six mice treated with JPC-2997 recrudesced with the four mice remaining blood smear negative up to day 30 of follow-up. No mice treated with either piperaquine or pyronaridine recrudesced up to day 30 of observation.

**Preliminary pharmacokinetics of JPC-2997 in mice.** The mean blood and plasma concentration versus time profiles of JPC-2997 following single oral doses of 5 mg/kg and 20 mg/kg JPC-2997 are shown in Fig. 3. The pharmacokinetics of JPC-2997, following escalating single oral doses from 2.5 mg/kg to 40 mg/kg of JPC-2997 are summarized in Table 4, and plasma JPC-2997 concentration versus time profiles are shown at Fig. S1. Although the blood sampling collection times did not provide an opportunity to determine the maximum plasma concentration and the time it was reached, it appears that JPC-2997 is rapidly absorbed with the highest plasma concentrations measured at 1 h post dose. Plasma concentrations of JPC-2997 in mice declined biphasically with an elimination half-life of 49.8 h. The oral pharmacokinetics of JPC-2997 in mice over the dose range of 2.5 mg/kg to 40 mg/kg were in general linear (Table 4). JPC-2997 has a CL/F of 7.2 L/h/Kg with a V/F of 509 L/kg. In mice, blood concentrations of JPC-2997 paralleled those of plasma (Fig. 3) and the blood to plasma concentration ratio at various time points after the administration of 5 mg/kg or 20 mg/kg JPC-2997 was estimated at 0.7. The administered doses of JPC-2997 were well tolerated in the mice, with no observed adverse events such as vomiting, behavioral change or body weight change.

**JPC-2997 is highly potent in the treatment of malaria in the Aotus monkey-Plasmodium vivax model.** Monkeys were patent by day 5 after intravenous inoculation of the AMRU1 strain. When parasitemia reached between 31,691 and 51,620 parasites/µL of blood (geometric mean: 38,002
parasites/µL, n=3) the monkeys were treated with 20 mg/kg JPC-2997 per day for 3 days. The geometric mean parasitemia declined to 29,944 parasites/µL (range: 22,231 to 38,340) at 24 h and 11,371 parasites/µL (range: 3920 to 26,460) at 48 h after starting JPC-2997 treatment, a decrease in parasitemia of 21.2% and 70.1%, respectively, from the pre-dose parasitemia value. The majority of parasites showed an irregular microscopic appearance such as compact dark staining of cytoplasm and non-pigmented parasites consistent with being drug affected by day 2 posttreatment. By day 5 parasitemia had been cleared in all monkeys. Over a 62 day follow-up period there was no recurrence of parasites. The maximum mean (±SD) blood concentration of JPC-2997 was 744 ± 82 ng/mL at day 4 after starting treatment and by day 28 the concentration had declined to 171 ± 82 ng/mL, with an estimated elimination half-life of about 10.8 days (Fig. S2). The monkeys appeared to tolerate the treatment regimen well with no observed adverse events such as vomiting, behavioral changes or body weight change.

**DISCUSSION**

*In vitro* antimalarial and cytotoxicity analyses. In this study, we showed JPC-2997 to be about 2- to 4-fold more active *in vitro* than WR-194,965 against both drug-sensitive and multidrug-resistant *P. falciparum* lines. There was a lack of cross-resistance between JPC-2997 and chloroquine with increased activity of JPC-2997 against the chloroquine-resistant W2 line but similar activity in inhibiting the chloroquine-sensitive D6 line. In addition to its high antimalarial activity, JPC-2997 was several thousand-fold less cytotoxic in murine and human cell lines. The SI of JPC-2997 was about 3-fold less toxic than WR-194,965. Based on JPC-2997’s superior *in vitro* antimalarial activity and favourable cytotoxicity profile compared to WR-194,965, further
studies were performed to assess the *in vivo* antimalarial efficacy of JPC-2997 with comparisons to standard antimalarials.

**In vivo efficacy in murine *Plasmodium berghei* models.** The efficacy of JPC-2997 was demonstrated against patent infection in mice using two murine models. Studies against the chloroquine-sensitive *P. berghei* ANKA strain showed JPC-2997 to be highly active in suppressing infection in the Peters 4-day test, with mean ED<sub>50</sub> values of 0.5 mg/kg/day following either s.c. or p.o. dosing. When compared with chloroquine and piperaquine, the efficacy of JPC-2997 was about 2-fold higher. For comparison, Peters et al. observed similar activity between WR-194,965 (ED<sub>50</sub> of 2.2 mg/kg/day) (9) and chloroquine (ED<sub>50</sub> of 1.9 mg/kg/day) (17) following s.c. administration in mice infected with the chloroquine-sensitive *P. berghei* N strain.

In the modified Thompson test, JPC-2997 showed remarkably potent activity against the *P. berghei* KBG-173 strain, with a total dose of 24 mg/kg curing all infected mice and was better than piperaquine when given once daily for 3 days. JPC-2997 was more effective than WR-194,965 and considerably superior to either chloroquine or dihydroartemisinin when given twice daily for 3 days. In addition to JPC-2997’s impressive suppression and radical cure of blood stage murine malaria, the drug was effective in preventing recrudescence in 66% (four of six) of infected mice treated with a single oral dose of 100 mg/kg, whereas recrudescence occurred in all mice treated with amodiaquine, atovaquone, chloroquine, dihydroartemisinin and mefloquine. Overall, the oral potency of JPC-2997 is a major improvement over WR-194,965.

**In vivo efficacy in the Aotus monkey-*Plasmodium vivax* model.** JPC-2997 at a dose of 20 mg/kg daily for 3 days was effective in curing Aotus monkeys infected with the *P. vivax* AMRU1 strain. The same regimen of
chloroquine does not cure Aotus monkeys infected with AMRU1 (22).

Clearance of *P. vivax* malaria by JPC-2997 was comparable to that observed against *P. berghei* taking about 4 to 5 days to clear infection. Although parasitemia clearance was not rapid in the splenectomized monkeys most parasites appeared drug affected 24 h to 48 h after starting treatment and in non-splenectomized monkeys parasite clearance might have been faster. We are planning to assess the *in vivo* efficacy of JPC-2997 against the chloroquine-resistant *P. falciparum* FVO strain in Aotus monkeys in the near future.

**Pharmacokinetic analysis.** Based on these favorable *in vivo* efficacy data, the pharmacokinetics of JPC-2997 in healthy mice showed linear kinetics between the oral dose range of 2.5 to 40 mg/kg. It appears to be well absorbed and exhibits biphasic decline in both blood and plasma concentrations with a relatively long elimination half-life of 49.8 h. When compared with other antimalarial drugs JPC-2997’s elimination half-life is longer than dihydroartemisinin (25 min) (23), amodiaquine (~7 h) (24), atovaquone (12 h) (25), and mefloquine (17 h) (26), comparable to chloroquine (47 h) (27) but shorter than piperaquine (15 days) (28). Unlike the quinoline antimalarials amodiaquine (29), chloroquine (30) and pyronaridine (31) that concentrate in erythrocytes, JPC-2997 does not appear to accumulate in erythrocytes, with a blood to plasma concentration ratio of 0.7. However, to accurately measure JPC-2997’s uptake into erythrocytes requires not only measurement of the blood to plasma concentration ratio but also knowledge of the unbound fraction of JPC-2997 in plasma, which is presently unknown and the hematocrit of the blood sample. JPC-2997 has a CL/F of 7.2 L/h/kg and its large V/F of 509 L/kg indicates extensive tissue distribution. From a pharmacokinetic perspective, the relatively long elimination half-life
and large volume of distribution justifies JPC-2997 being a potential partner
drug for antimalarial treatment or to be used alone for prophylaxis.

**Comparison with MK-4815.** JPC-2997 and MK-4815 have comparable *in*
vitro antimalarial activity in the low nanomolar range against a broad-spectrum
of *P. falciparum* lines, with varying levels of susceptibility to standard
antimalarials. However, JPC-2997 is at least 2-fold more potent than MK-4815
against *P. berghei* KBG-173 strain with no mice cured after a total dose of 48
mg/kg MK-4815 (16 mg x 3 days) using the modified Thompson test (data not
shown). In contrast to JPC-2997, MK-4815 (12) has a lower clearance and a
much shorter elimination half-life in mice (4.5 h versus 49.8 h) and monkeys
(14 h versus 259 h) suggesting that MK-4815 would have less posttreatment
prophylaxis activity than JPC-2997.

**Future application.** The quinoline derived Mannich bases amodiaquine
and pyronaridine are in current use for the treatment of uncomplicated *P.
falciparum* malaria as partner drugs with artesunate (2). Artesunate-
amodiaquine (ASAQ Winthrop®, Sanofi-Aventis) is a widely available ACT,
with high efficacy in Africa (32-34) but variable potency in Southeast Asian
countries because of tolerant amodiaquine strains (35). In the past,
amodiaquine administered alone for treatment or long-term prophylaxis of
malaria has been associated with severe adverse events of agranulocytosis,
hepatotoxicity and neurotoxicities (involuntary movements/dystonia) (36, 37).
These adverse events are believed to be associated with the oxidative
metabolism of amodiaquine forming toxic reactive iminoquinone metabolites
(38 39). Since, JPC-2997 is not a quinoline derivative, it does not form toxic
iminoquinone metabolites.

When compared with JPC-2997, amodiaquine is not as potent in
suppressing murine malaria in the Peters 4-day test with an *ED*$_{50}$ of 2.1
mg/kg/day (40) and in the present study it is less effective in preventing recrudescence of the *P. berghei* ANKA strain. These favorable *in vivo* efficacy findings for JPC-2997 suggest that it may be a suitable partner drug with artesunate or as an addition to an existing ACT such as dihydroartemisinin-piperaquine to inhibit potential development of drug resistance. The latter concept is worthy of further consideration as the elimination half-life of JPC-2997 lies between those of dihydroartemisinin and piperaquine.

In designing the next generation of drugs for malaria control and eradication the Medicines for Malaria Venture (MMV) has developed target candidate profiles (TCP) that provide a job description for a new molecule to enter clinical development (41). JPC-2997 fulfills TCP-2 definition of a long duration partner drug, capable of killing residual parasites that are not eliminated by a rapid asexual clearance TCP-1 compound. Furthermore, because of JPC-2997’s long half-life it may be a suitable TCP-4 candidate compound for chemoprotection.

In summary, JPC-2997 is a highly potent compound against chloroquine-resistant *P. falciparum* lines with low cytotoxicity in mammalian cell lines. It possesses potent *in vivo* efficacy in the murine *P. berghei* and Aotus monkey-*P. vivax* models, with long-lasting action in preventing recrudescences. After oral dosing, JPC 2997 is rapidly absorbed with a relatively long elimination half-life in mice and monkeys. Overall, the *in vitro* and *in vivo* antimalarial potency of JPC-2997 and its favorable pharmacokinetic properties suggest that the compound is worthy of further studies including toxicological and metabolic evaluation and preclinical assessment.
ACKNOWLEDGMENTS

We wish to thank the technical excellence of Kerryn Rowcliffe for in vitro drug testing and Donna MacKenzie, Stephen McLeod-Robertson and Thomas Travers for rodent studies using the *P. berghei* ANKA strain and efficacy studies in Aotus monkeys. We thank Dr Ivor Harris and Dr John Hunter for veterinarian support towards the Aotus monkey study. We are grateful to the Australian Red Cross Blood Service for the provision of human blood and sera for in vitro cultivation of *P. falciparum* lines. The opinions expressed are those of the authors and do not necessarily reflect those of the Defence Health Service or any extant Australian Defence Force policy.

FUNDING

This research was funded by the Australian Defence Organization and The Jacobus Pharmaceutical Company Inc.

TRANSPARENCY DECLARATIONS


Note: Part of this work was presented previously by Dr David Jacobus at the annual meeting of the American Society of Tropical Medicine and Hygiene, November 11-17, 2013, Washington DC, USA.
REFERENCES


of uncomplicated *Plasmodium falciparum* among children under five in

33. Faye B, Offianan AT, Ndiaye JL, Tine RC, Toure W, Djoman K,
Sylla K, Ndiaye PS, Penali L, Gaye O. 2010. Efficacy and tolerability
of artesunate-amodiaquine (Camoquin plus) versus artemether-
lumefantrine (Coartem) against uncomplicated *Plasmodium falciparum*
Health 15:608-613.

34. Ndounga M, Mayengue PI, Casimiro PN, Loumouamou D, Basco
LK, Ntoumi F, Brasseur P. 2013. Artesunate-amodiaquine efficacy in
Congolese children with acute uncomplicated falciparum malaria in

35. Ratcliff A, Siswantoro H, Kenangalem E, Maristela R, Wuwung RM,
fixed-dose artemisinin combinations for drug-resistant falciparum and
vivax malaria in Papua, Indonesia: an open-label randomised

36. Hatton CS, Peto TE, Bunch C, Pasvol G, Russell SJ, Singer CR,
associated with amodiaquine prophylaxis against malaria. Lancet
1:411-414.

Amodiaquine induced agranulocytosis and liver damage. Br. Med. J.

38. Naisbitt DJ, Williams DP, O'Neill PM, Maggs JL, Willock DJ,
Pirmohamed M, Park BK. 1998. Metabolism-dependent neutrophil


**TABLE 1** In vitro antimalarial activities and cytotoxicity (IC₅₀) of JPC-2997 against *Plasmodium falciparum* and mammalian cell lines and selectivity index (SI).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>D6 (nM)</th>
<th>W2 (nM)</th>
<th>TM90-C2B (nM)</th>
<th>HEK293 (μM)</th>
<th>SI</th>
<th>HepG2 (μM)</th>
<th>SI</th>
<th>BHK (μM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPC-2997</td>
<td>13 ± 2</td>
<td>6.0 ± 1.7</td>
<td>34 ± 1</td>
<td>40 ± 5</td>
<td>3077</td>
<td>42 ± 10</td>
<td>3230</td>
<td>36 ± 9</td>
<td>2769</td>
</tr>
<tr>
<td>WR-194,965</td>
<td>30 ± 5</td>
<td>28 ± 4.0</td>
<td>53 ± 5</td>
<td>31 ± 3</td>
<td>933</td>
<td>28 ± 1</td>
<td>35 ± 1</td>
<td>1167</td>
<td></td>
</tr>
<tr>
<td>Piperaquine</td>
<td>23 ± 2</td>
<td>36 ± 5</td>
<td>22 ± 2</td>
<td>87 ± 27</td>
<td>3783</td>
<td>&gt;120</td>
<td>&gt;5217</td>
<td>&gt;120</td>
<td>&gt;5217</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>13 ± 1</td>
<td>143 ± 28</td>
<td>144 ± 28</td>
<td>51 ± 27</td>
<td>3923</td>
<td>27 ± 13</td>
<td>2077</td>
<td>33 ± 16</td>
<td>2538</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>1.9 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.7</td>
<td>4 ± 1</td>
<td>2105</td>
<td>30 ± 9</td>
<td>15789</td>
<td>107 ± 13</td>
<td>5632</td>
</tr>
</tbody>
</table>

Values are means ± SD, with n ≥ 2 observations; SI = IC₅₀ values for mammalian cell line against the *Plasmodium falciparum* D6 line.
### TABLE 2 In vivo antimalarial activity of JPC-2997 in mice infected with the *Plasmodium berghei* ANKA strain in the Peters 4-day test.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Compound</th>
<th>ED$_{50}$ (mg/kg/day)</th>
<th>ED$_{90}$ (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>JPC-2997</td>
<td>0.5 ± 0.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Chloroquine</td>
<td>0.9 ± 0.3</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Dihydroartemisin</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Piperaquine</td>
<td>0.9 ± 0.4</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>Oral</td>
<td>JPC-2997</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Chloroquine</td>
<td>1.1 ± 0.6</td>
<td>4.7 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Dihydroartemisin</td>
<td>1.3 ± 0.2</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Piperaquine</td>
<td>2.4 ± 0.9</td>
<td>6.7 ± 3.2</td>
</tr>
</tbody>
</table>

Values are means ± SD, with $n \geq 2$ observations.
### TABLE 3

*In vivo* efficacy of JPC-2997 and reference antimalarials administered orally either daily or twice daily over 3 days in mice infected with the *Plasmodium berghei* KBG-173 strain in the modified Thompson test.

#### Single Daily Dose

<table>
<thead>
<tr>
<th>Compound</th>
<th>Daily dose (mg/kg)</th>
<th>Total Dose Administered (mg/kg)</th>
<th>Median survival time (days)</th>
<th>Number of mice alive at day 31</th>
<th>(%) cured on day 31*</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPC-2997</td>
<td>8</td>
<td>24</td>
<td>&gt;31</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>JPC-2997</td>
<td>4</td>
<td>12</td>
<td>19</td>
<td>6/7</td>
<td>86</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>8</td>
<td>24</td>
<td>24</td>
<td>5/7</td>
<td>71</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>4</td>
<td>12</td>
<td>21</td>
<td>4/7</td>
<td>57</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>64</td>
<td>192</td>
<td>18</td>
<td>0/7</td>
<td>0</td>
</tr>
</tbody>
</table>

#### Twice Daily Dose

<table>
<thead>
<tr>
<th>Compound</th>
<th>Twice daily dose (mg/kg)</th>
<th>Total Dose Administered (mg/kg)</th>
<th>Median survival time (days)</th>
<th>Number of mice alive at day 31</th>
<th>(%) cured on day 31*</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPC-2997</td>
<td>8</td>
<td>48</td>
<td>&gt;31</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>JPC-2997</td>
<td>2</td>
<td>12</td>
<td>25</td>
<td>6/7</td>
<td>71</td>
</tr>
<tr>
<td>WR-194,965</td>
<td>8</td>
<td>48</td>
<td>23</td>
<td>4/7</td>
<td>71</td>
</tr>
<tr>
<td>WR-194,965</td>
<td>2</td>
<td>12</td>
<td>17</td>
<td>0/7</td>
<td>0</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>8</td>
<td>48</td>
<td>&gt;31</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>2</td>
<td>12</td>
<td>22.5</td>
<td>5/7</td>
<td>71</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>32</td>
<td>192</td>
<td>16</td>
<td>0/7</td>
<td>0</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>32</td>
<td>192</td>
<td>18</td>
<td>1/7</td>
<td>14</td>
</tr>
</tbody>
</table>

Single daily dose of WR-194,965 and chloroquine were not determined.

* Cured on day 31 indicates the % of mice that were blood film negative on day 31 after starting drug treatment.
TABLE 4 Pharmacokinetics of JPC-2997 in healthy mice administered single oral doses of JPC-2997 between 2.5 to 40 mg/kg.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>2.5 (mg/kg)</th>
<th>5 (mg/kg)</th>
<th>10 (mg/kg)</th>
<th>20 (mg/kg)</th>
<th>40 (mg/kg)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. at 1 h* (µg/L)</td>
<td>288</td>
<td>753</td>
<td>1,013</td>
<td>2,067</td>
<td>3,235</td>
<td>0.37 ± 0.08</td>
</tr>
<tr>
<td>Conc. / Dose (%)</td>
<td>0.38</td>
<td>0.50</td>
<td>0.34</td>
<td>0.34</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>AUC/Dose (%)</td>
<td>18.0</td>
<td>17.5</td>
<td>14.0</td>
<td>15.1</td>
<td>13.9</td>
<td>15.7 ± 1.9</td>
</tr>
<tr>
<td>AUC (µg.h/L)</td>
<td>13,490</td>
<td>26,213</td>
<td>41,847</td>
<td>90,370</td>
<td>166,370</td>
<td></td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>60.1</td>
<td>48.0</td>
<td>53.5</td>
<td>40.1</td>
<td>47.4</td>
<td>49.8 ± 7.5</td>
</tr>
<tr>
<td>V (L/kg)</td>
<td>533</td>
<td>441</td>
<td>615</td>
<td>427</td>
<td>531</td>
<td>509 ± 76.9</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>6.2</td>
<td>6.4</td>
<td>8.0</td>
<td>7.4</td>
<td>8.0</td>
<td>7.2 ± 0.9</td>
</tr>
</tbody>
</table>

* Highest plasma JPC-2997 concentration measured with the bleed schedule used.
FIG. 1 Chemical structure of (A) WR-194,965 and (B) JPC-2997
FIG. 2 (A) Onset of action and (B) recrudescence after a single oral dose of 100 mg/kg of AQ, amodiaquine; ATQ, atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; JPC-2997; MQ, mefloquine; PPQ, piperaquine and PRN, pyronaridine to groups of six mice on day 4 postinfection with *Plasmodium berghei* ANKA strain. The control (CTL) group of mice were administered the vehicle.
FIG. 3 Mean (SD) plasma and blood concentrations versus time of JPC-2997 in healthy mice administered a single oral dose of 5 mg/kg (plasma - closed triangle; blood - open triangle) and 20 mg/kg (plasma - closed circle; blood - open circle) JPC-2997. Inset are mean (SD) plasma and blood concentrations-time profiles of JPC-2997 over the first 48 h after dosing with 5 mg/kg and 20 mg/kg of JPC-2997.