

Antimalarial Activity of the Bisquinoline *trans*- N^1,N^2 -Bis(7-Chloroquinolin-4-yl)Cyclohexane-1,2-Diamine: Comparison of Two Stereoisomers and Detailed Evaluation of the *S,S* Enantiomer, Ro 47-7737

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The *S,S* enantiomer of the bisquinoline *trans*- N^1,N^2 -bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine, Ro 47-7737, is significantly more potent against chloroquine-resistant *Plasmodium falciparum* than the *R,R* enantiomer and the previously described racemate. Both the enantiomers and the racemate are more potent inhibitors of heme polymerization than chloroquine, and their activities are probably mediated by inhibition of this parasite-specific process. The *S,S* enantiomer, Ro 47-7737, was studied in more detail and proved to be a potent antimalarial in the treatment of *P. vivax* ex vivo and *P. berghei* in vivo. Its suppression of *P. berghei* growth in a mouse model (50% effective dose, 2.3 mg/kg of body weight) was equal to that of chloroquine and mefloquine, and Ro 47-7737 was found to be more potent than these two drugs in the Rane test, in which the curative effect of a single dose is monitored. The dose at which 50% of animals were permanently cured (34 mg/kg) was markedly superior to those of chloroquine (285 mg/kg) and mefloquine (>250 mg/kg). When administered orally at 50 mg/kg, Ro 47-7737 also showed a faster clearance of parasites than either chloroquine or mefloquine, and unlike the other two compounds, Ro 47-7737 showed no recrudescence. In a study to compare prophylactic efficacies of oral doses of 50 mg/kg, Ro 47-7737 provided protection for 14 days compared to 3 days for mefloquine and 1 day for chloroquine. The good curative and prophylactic properties of the compound can be explained in part by its long terminal half-life. The ability to generate parasite resistance to Ro 47-7737 was also assessed. With a rodent model, resistance could be generated over eight passages. This rate of resistance generation is comparable to that of mefloquine, which has proved to be an effective antimalarial for many years. Toxicity liabilities, however, ruled out this compound as a candidate for drug development.

The spread of chloroquine-resistant *Plasmodium falciparum* malaria is severely limiting our ability to treat malarial infection (27, 31). Mefloquine is rapidly becoming the first-choice drug for antimalarial prophylaxis (15), but resistance to this compound has been reported, especially in southeast Asia (5, 6, 13), and its cost prohibits its widespread use in many areas where malaria is endemic. Chloroquine is believed to exert its activity by inhibiting hemozoin formation in the digestive vacuole of the malaria parasite (4, 23), though this theory is opposed by some (1) and other possibilities have also been postulated (22). The ability of chloroquine to inhibit heme polymerization in the parasite is believed to be enhanced by the concentration of chloroquine in the parasite, and it is estimated that millimolar levels may be present in the digestive vacuole of the parasite at physiologically relevant concentrations in blood of around 30 nM (32). The cause of chloroquine resistance is unknown, but it is clearly associated with alterations in membrane-associated transport processes, resulting in a reduced uptake of the drug into the parasite and/or an increased efflux of the drug from the parasite (30).

Chloroquine analogs containing two quinoline rings linked by a basic substituent, termed bisquinolines, have long been known to possess antimalarial efficacy (25). At the time this efficacy was discovered, there was no justification to pursue these compounds as antimalarials because of the success being achieved with chloroquine. Interest in these compounds was regenerated when several bisquinolines that had good efficacies against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* malaria were described (28, 29). The best of these bisquinolines was *trans*- N^1,N^2 -bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine, also termed WR 268668, but it was synthesized and studied only in its racemic form. It was later discovered that the racemate exhibited a significant degree of cross-resistance with chloroquine when tested against a series of *P. falciparum* isolates (2), limiting its interest as a chemotherapeutic agent.

We have been interested in quinoline analogs which are effective against both chloroquine-resistant and chloroquine-sensitive *P. falciparum* malaria (20) and which probably mediate their actions by inhibition of heme polymerization (4). As an extension to this work we prepared the enantiomeric forms of the bisquinoline *trans*- N^1,N^2 -bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine and discovered that the *S,S* form overcame chloroquine resistance far better than the *R,R* form or

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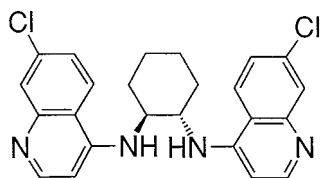


FIG. 1. Structure of Ro 47-7737, the *S,S* enantiomer of bisquinoline *trans*-*N*¹,*N*²-bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine.

the previously reported racemate (7). Here we more fully report this work and assess the antimalarial properties of the *S,S* enantiomer, Ro 47-7737 (Fig. 1), in more detail. We utilized in vitro studies of the most virulent human pathogen, *P. falciparum*, ex vivo studies of another important human pathogen, *Plasmodium vivax*, and in vivo studies of two rodent-specific species, *Plasmodium berghei* and *Plasmodium yoelii* subsp. NS. Particular attention was paid in the course of these studies to comparing the activity of Ro 47-7737 with those of chloroquine and mefloquine, the two major drugs currently available for malaria therapy and prophylaxis. Finally, pharmacokinetic and toxicology studies were performed to assess whether the compound can be considered a drug development candidate.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Fluka (Buchs, Switzerland) or Sigma (Buchs, Switzerland) and were at least of analytical grade. Cell culture reagents were purchased from Gibco BRL Ltd. (Paisley, Scotland). Tritiated hypoxanthine was purchased from Amersham, Little Chalfont, Buckinghamshire, United Kingdom.

Compounds. (1*S*,2*R*)-*N*¹,*N*²-Bis-(7-chloroquinolin-4-yl)-cyclohexane-1,2-diamine, Ro 48-6910; (1*R*,2*R*)-*N*¹,*N*²-bis-(7-chloroquinolin-4-yl)-cyclohexane-1,2-diamine, Ro 47-4577; and (1*S*,2*S*)-*N*¹,*N*²-bis-(7-chloroquinolin-4-yl)-cyclohexane-1,2-diamine, Ro 47-7737, were all prepared according to the reported procedure (29) and patents (7, 28), starting from the commercially available (1*S*,2*R*)-(+)-cyclohexane-1,2-diamine, (1*R*,2*R*)-(–)-cyclohexane-1,2-diamine, and (1*S*,2*S*)-(–)-cyclohexane-1,2-diamine, respectively. The corresponding dihydrochlorides were prepared by treatment of a hot 10% solution of the free bases in 2-propanol with 2.5 equivalents of gaseous HCl in 2-propanol. The specific rotation of the dihydrochloride salt of Ro47-7737 was $[\alpha]_D^{20} = +755.5^\circ$ (methanol concentration = 0.2 g/100 ml).

Heme polymerization assay. The heme polymerization assay was performed as a variation of the assay previously described (4, 24). In brief, an acetonitrile extract of trophozoite lysate was incubated overnight at 37°C (with or without inhibitors) on a GREINER microtiter plate in 500 mM sodium acetate, pH 4.8, with 140 μ M [¹⁴C]hemin (specific activity, 0.16 μ Ci/ μ mol) in a final volume of 100 μ l. Unincorporated heme was removed from the insoluble hemozoin by filtration through a Millipore Multiscreen microtiter plate (model MHVB N45) with the filtration system EVENT 4160 (Eppendorf). The microtiter plates were washed with 1 ml of 2% sodium dodecyl sulfate in 0.1 M sodium bicarbonate buffer, pH 9.1, and with 1 ml of 50 mM Tris-HCl, pH 7.5. The amount of membrane-bound insoluble hemozoin was determined by scintillation counting with a TopCount Microplate scintillation counter (Packard). The compounds were added to the reaction mixture as dimethyl sulfoxide (DMSO) solutions with up to a maximum concentration of DMSO of 25%.

In vitro measurement of *P. falciparum* parasite growth inhibition. Compounds were tested by the semiautomated microdilution assay against intraerythrocytic forms of *P. falciparum* derived from asynchronous stock cultures (3). The culture medium was a variation of that described by Trager and Jensen (26). It consisted of RPMI 1640 supplemented with 10% human type A⁺ serum, 25 mM HEPES, 25 mM NaHCO₃ (pH 7.3), and 100 μ g of neomycin/ml. Human type A⁺ erythrocytes served as host cells. The cultures were kept at 37°C in an atmosphere of 3% O₂, 4% CO₂, and 93% N₂ in humidified modular chambers.

Drug testing was carried out in 96-well microtiter plates. The compounds were dissolved in DMSO (5 mg/ml), prediluted in complete culture medium, and titrated in duplicate in serial twofold dilutions over a 64-fold range. After addition of the parasite cultures with an initial parasitemia (expressed as the percentage of erythrocytes infected) of 0.75% in a 2.5% erythrocyte suspension, the test plates were incubated under the conditions described above for 48 or 72 h. Growth of the parasites was measured by the incorporation of radiolabelled [³H]hypoxanthine added 16 h prior to termination of the test. Fifty percent inhibitory concentrations (IC₅₀) were estimated by Logit regression analysis.

Compounds were tested in vitro against various chloroquine-sensitive and drug-resistant strains of *P. falciparum*. The main reference strains used were K1

(Thailand; resistant to chloroquine) and NF54 (an airport strain of unknown origin that is sensitive to standard antimalarials). Other standard strains tested were (i) the chloroquine-sensitive strains FCH-5-C2 (clone of FCH-5; Tanzania), HB3 (Honduras; resistant to pyrimethamine), RF MEF3 (a laboratory strain made resistant to mefloquine), and Ro73 (Kenya) and (ii) the chloroquine-resistant strains RFCR-3 (The Gambia), ItG2F6 (Brazil), INDO (Indochina), W2 (Indochina), W2MEF (a mefloquine-resistant line derived from W2), 7G8 (Brazil), and T9/94 (Thailand). In addition, 32 isolates from Thailand (20) and 33 isolates from Tanzania (8, 20) were also tested. The Thai strains tested were T116, T120, TD378, TD379, TD380, TD381, TD385, TD386, TD388, TM01, TM02, TM03, TM04, TM05, TM06, TM08, TM19, TM20, TM25, TM28, TM41, TM43, TM52, TM53, TM56, TM57, TM58, TM62, TM67, TM69, and TM79. The Tanzanian strains tested were IFA002, IFA003, IFA004, IFA005, IFA006, IFA007, IFA009, IFA010, IFA011, IFA012, IFA013, IFA015, IFA016, IFA017, IFA018, IFA019, IFA020, IFA023, IFA051, IFA052, IFA059, IFA061, IFA070, IFA078, IFA092, IFA093, IFA111, IFA112, IFA120, IFA125, IFA127, IFA128, and IFA153.

In vitro measurement of parasite killing activity. To have a more precise picture of the in vitro activities of the compounds, we initiated experiments in which parasite survival (or killing) was monitored with Giemsa-stained blood smears at different time intervals rather than by measurement of growth inhibition (20). For these assays, the human serum supplement was replaced by 0.5% AlbuMax-I (Gibco BRL), a lipid-rich bovine serum albumin fraction (4).

Ex vivo activity against *P. vivax*. Due to the problems associated with long-term cultivation of *P. vivax*, caused by its requirement of reticulocytes, a short 11-h assay was employed. *P. vivax* (Ong) parasites were obtained by venipuncture from a synchronously developing asexual blood-stage infection in a splenectomized *Aotus boliviensis* monkey when the majority of the parasites had reached the mid-trophozoite stage of development. For comparison, *P. falciparum* 7G8 parasites from a synchronously developing culture were used to initiate an assay when the parasites had reached a similar stage of development. Leukocytes were removed by Plasmodipur treatment (9). Cultures were then started in triplicate in the presence of threefold drug dilutions of between 0.03 and 10 ng/ml and [³H]hypoxanthine at 20 μ Ci/ml, with a 0.3% parasitemia count and a 5% hematocrit count in 96-well plates. Culture conditions were similar to those described above for *P. falciparum*, except that the medium was supplemented with 20% human type A⁺B⁺ serum and 2 g of glucose/liter and 100 μ g of gentamicin/ml was incorporated as an antibiotic. After 11 h, the metabolic incorporation of radiolabel was measured and inhibition of parasite growth was determined.

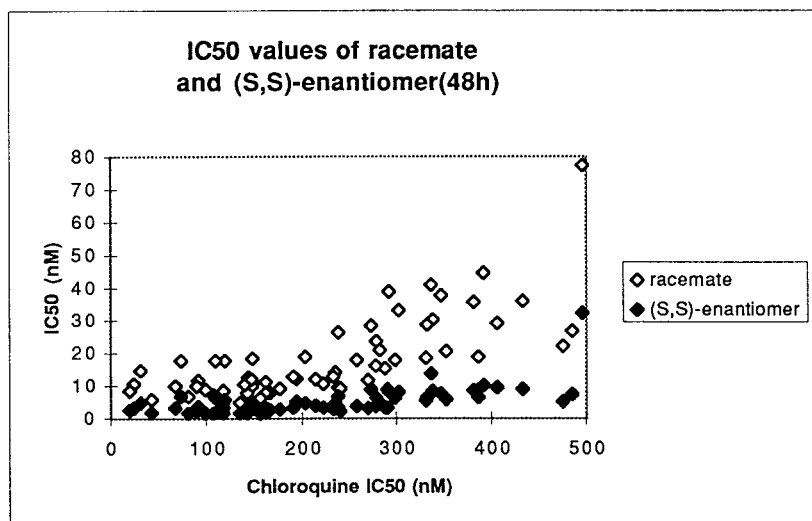
In vivo measurement of parasite growth and antimalarial activities of compounds. (i) Method of infection and treatment of animals. Male mice (Fü Albino, specific pathogen free) weighing 20 \pm 2 g were infected intravenously (i.v.) with 2×10^7 *P. berghei* ANKA strain-infected erythrocytes from donor mice on day 0 of the experiment. From donor mice with circa 30% parasitemia, heparinized blood was taken and diluted in physiological saline to 10⁸ parasitized erythrocytes/ml. An aliquot (0.2 ml) of this suspension was injected intravenously into experimental and control groups of mice. In untreated control mice, parasitemia rose regularly to 30 to 40% by day +3 after infection and 70 to 80% by day +4. The mice died between days +5 and +7 after infection. Throughout the experiments, mice were kept in groups of five animals in Makrolon type II cages in an air-conditioned animal room at 22 to 23°C. A diet with a *p*-aminobenzoic acid content of 45 mg/kg (Nafag; No. 9009 PAB-45) and tap water were available ad libitum.

(ii) Administration of compounds. Compounds were prepared at an appropriate concentration, either as a solution or as a suspension containing 3% ethanol and 7% Tween 80. They were administered either subcutaneously (s.c.) or per os (p.o.) in a total volume of 0.01 ml per gram of mouse. The activity of

TABLE 1. Comparative growth inhibitory activities at 48 and 72 h of the bisquinoline *S,S* enantiomer and the racemate against the chloroquine-sensitive parasite strain NF54 and the chloroquine-resistant strain K1

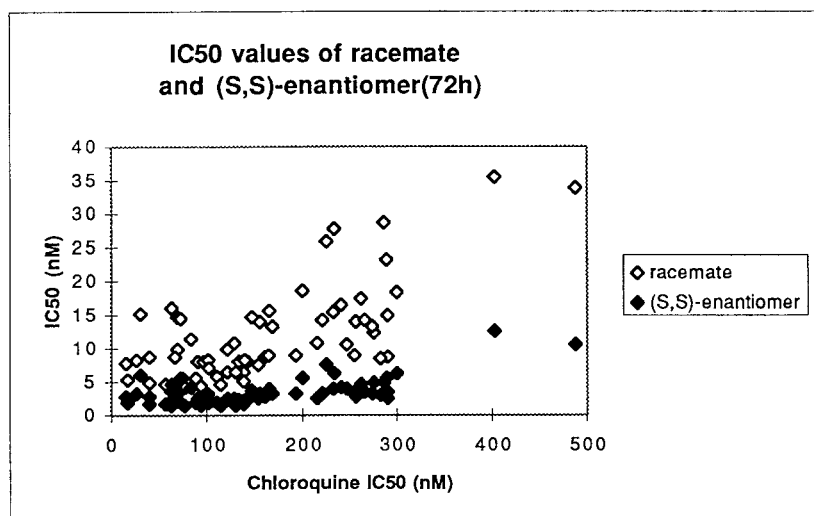
Compound	Mean IC ₅₀ (nM) \pm SD ^a for indicated strain at:			
	48 h		72 h	
	NF54	K1	NF54	K1
Ro 47-7737 (<i>S,S</i> enantiomer)	6 \pm 2	13 \pm 5	6 \pm 2	10 \pm 4
Ro 48-6910 (racemate)	12 \pm 6	30 \pm 17	13 \pm 5	22 \pm 10
Chloroquine	16 \pm 4	316 \pm 83	14 \pm 4	279 \pm 86
Mefloquine	56 \pm 5	18 \pm 7	56 \pm 20	14 \pm 5

^a Values were determined by measurement of [³H]hypoxanthine incorporation into growing cultures. The bisquinoline enantiomer and racemate were added as free bases. Chloroquine was added as the diphosphate salt. Mefloquine was added as the hydrochloride salt. All values are calculated from the data of at least 20 independent experiments.



Pearson correlation coefficient racemate = 0.73 ($p < 0.05$)

Pearson correlation coefficient enantiomer = 0.61 ($p < 0.05$)



Pearson correlation coefficient racemate = 0.71 ($p < 0.05$)

Pearson correlation coefficient enantiomer = 0.63 ($p < 0.05$)

FIG. 2. Comparative IC_{50} for the *S,S* enantiomer, Ro 47-7737, and the racemate, Ro 48-6910, against a range of *P. falciparum* strains of various chloroquine sensitivities.

the compound was determined by a variety of methods outlined in subsequent sections.

(iii) **ED_{50} and ED_{90} determinations.** Determinations of 50 and 90% effective doses (ED_{50} and ED_{90} , respectively) were made by a variation of the Peters et al. 4-day test (17) in which animals were treated with a single dose only, rather than with the four consecutive daily doses of the original method. Groups of five mice were treated once on day +1 (24 h after infection). On day +3 (48 h after treatment) blood smears of all animals were prepared and stained with Giemsa. Parasitemia was determined microscopically, and the difference between the mean value of the control group (taken as 100%) and those of the experimental groups was calculated and expressed as percent reduction. The ED_{50} and ED_{90} values were calculated by nonlinear fitting with the JMP statistical program (Statistical Analysis Institute, Cary, N.C.).

(iv) **Rane test.** The Rane test was carried out by a variation of the method described by Osdene et al. (14). Groups of five mice were given various single doses, either s.c. or p.o., at day +3 after infection. Antimalarial activity in this assay was expressed in terms of survival time and was measured in two ways. The

first measure used was the minimum effective dose (MED); this is defined as the dose at which the survival time of the animals is doubled compared to the survival time of an untreated control group. The second measure used was the 50% curative dose (CD_{50}); this is defined as the dose at which 50% of the mice survive for >60 days. Further information was obtained in this test by monitoring of parasitemia by microscopic examination of Giemsa-stained blood smears on the day at which survival time was doubled, usually day +12.

(v) **Onset of drug action and recrudescence.** The onset of drug action was determined after a single fixed p.o. dose of 50 mg/kg at day +3 after infection. The reduction in parasitemia was monitored 6 and 12 h after treatment, and the time of recrudescence was assessed by daily blood smears for 11 days, followed by intermittent assessment for up to 30 days (10).

(vi) **Prophylactic activity.** Prophylactic activities of the three compounds were compared after administering single doses of 50 mg/kg either s.c. or p.o. to different groups of five animals at various times before infection. All groups, including an untreated control group, were then infected at the same time. Parasitemia was determined for each animal on day +3 after infection, and

TABLE 2. Comparative growth inhibition at 48 h by the bisquinoline enantiomers and the racemate against 12 laboratory strains

Strain	IC ₅₀ (nM) at 48 h of:		
	Ro 47-7737 (<i>S,S</i> enantiomer)	Ro 47-4577 (<i>R,R</i> enantiomer)	Ro 48-6910 (racemate)
CQ sensitive strains			
NF54	5	7	7
FCH-5-C2	6	8	8
HB3	4	9	8
RF MEF3	6	7	8
Ro73	5	9	9
CQ resistant strains			
RFCR-3	13	43	25
ItG2F6	10	33	20
INDO	6	19	12
W2	10	48	22
7G8	13	42	26
W2MEF	4	10	7
T9/94	9	24	19

percent reduction of the level of parasitemia compared to levels for animals given no drug was determined (21).

Gametocytocidal action. Activity against the gametocytes of *P. berghei* clone I (obtained from D. Walliker, Edinburgh, Scotland) was determined according to the method of Peters and Robinson (18). Mice infected with *P. berghei* clone I and known to be carrying gametocytes were treated with a single dose of compound. One hour after drug administration, *Anopheles gambiae* mosquitoes were fed on the mice. Control mosquitoes were fed on infected mice which had not been treated. The gametocytocidal activity of the compounds were measured in two ways, namely, (i) by counting the number of developing oocysts on the midguts of the mosquitoes and (ii) by counting the number of infected mosquitoes.

Sporontocidal action. To determine sporontocidal action, the same procedure as described for the gametocytocidal assay was followed, except that *Anopheles gambiae* mosquitoes were fed on untreated gametocyte-carrying mice (19). The mosquitoes were then supplied with 0.05% solutions of test drug in 10% sucrose, which was renewed each day. Mosquitoes were dissected on the seventh day after the infection, and the oocysts were counted.

Determination of plasma compound levels required to confer prophylactic protection on *P. berghei*-infected mice. Groups of eight mice were treated orally with various doses of Ro 47-7737, mefloquine, or chloroquine. Because of their relative activities and different pharmacokinetic properties, Ro 47-7737 and mefloquine were given 48 h prior to infection while chloroquine was given 24 h prior to infection. Five animals per group were infected with *P. berghei* at time zero, and parasitemia was assessed microscopically on day +3 following infection. Results were expressed as percentage inhibition of parasitemia compared to the parasitemia of animals receiving no drug. The three remaining animals per group were not infected but were instead sacrificed at time zero for determination of plasma compound concentrations. Concentrations in plasma were determined by high-performance liquid chromatography (HPLC) (Ro 47-7737 and chloroquine) or high-performance thin-layer chromatography (mefloquine).

TABLE 3. Activities of Ro 47-7737, Ro 47-4577, and Ro 48-6910 against five sensitive strains and seven resistant strains

Compound	IC ₅₀ (nM) at 48 h against:				<i>r/s</i> ^a
	CQ sensitive strains		CQ resistant strains		
	Mean ± SD	Range	Mean ± SD	Range	
Ro 47-7737 (<i>S,S</i> enantiomer)	5 ± 1	4–6	9 ± 3	4–13	1.8
Ro 47-4577 (<i>R,R</i> enantiomer)	8 ± 1	7–9	33 ± 14	10–48	3.9
Ro 48-6910 (racemate)	8 ± 1	7–9	19 ± 7	7–26	2.4

^a The resistance index (*r/s*) represents the mean IC₅₀ of the resistant strains divided by the mean IC₅₀ of the sensitive strains.

TABLE 4. Parasite killing effects of Ro 47-7737, chloroquine, and mefloquine

Compound	Effect of compound ^a against strain:			
	NF54 (sensitive)		K1 (resistant)	
	Killing concn (ng/ml)	Time (days)	Killing concn (ng/ml)	Time (days)
Ro 47-7737	3	2	10	4
Chloroquine	30	5	300	5
Mefloquine	10	4	30	5

^a The minimum concentrations required to cause disappearance of parasites from culture and the times taken to achieve this are given.

In vivo generation of resistance to Ro 47-7737. In vivo studies of the generation of resistance to Ro 47-7737 were carried out with mice infected with chloroquine-sensitive *P. berghei* N and chloroquine-resistant *P. yoelii* subsp. NS (16, 17). The objective was to assess possible cross-resistance between the test compounds and chloroquine and to determine the potential for the parasites to develop resistance to the new compounds. As a preliminary step, a range of doses of a test compound is given once, s.c., to batches of mice on the day that the animals receive a standard infective inoculum. The delay in the time required for each batch of mice to develop a parasitemia of 2% compared with that in control animals (2% delay time) is assessed, and the dose that yields a delay of at least 4 days is selected for the next step. This consists of subinoculating blood from animals that have received the selected dose once the infections recrudescence and attain the 2% level. The recipients again receive the same, single dose on the day of infection, and the time to attain the 2% level compared with that of untreated controls in the same passage is recorded. Further passages are made from the mice with the recrudescence infections. The acquisition of resistance is indicated by a fall in the 2% delay time. Complete resistance is indicated by a 2% delay time that approaches or reaches zero.

Pharmacokinetics. (i) Animal experiments. All animals were obtained from Biological Research Laboratories (Füllinsdorf, Switzerland). During the experiments, the animals were housed in cages or in restraining jackets (dogs) under standard conditions (22 ± 2°C, 55% ± 10% relative humidity, 12-h light-dark cycle). Most of the time the animals had free access to food (standard diet) and tap water. For pharmacokinetic analysis, blood was collected in tubes containing EDTA as an anticoagulant and NaF for stabilization. Plasma was obtained by immediate centrifugation at 1,000 × g for 10 min at 5°C. Plasma samples were frozen at –20°C prior to HPLC analysis. All formulations were prepared freshly on the day of the experiment and were administered within 30 min after preparation.

Mouse. Male albino mice (specific-pathogen-free MoRo; weight, 30 to 40 g) were treated with Ro 47-7737 dissolved in a suitable volume of 0.9% saline solution (i.v.) or distilled water (p.o.). Volumes of 6.5 ml/kg or 0.2 ml/mouse were administered either i.v. by bolus injection into the tail vein or p.o. by gavage (nominal dose, 10 mg/kg). Blood samples (about 1 ml) were collected by heart puncture under CO₂ anaesthesia.

Rat. Male rats (RoRo; body weight, 250 to 300 g) were treated with Ro 47-7737 dissolved in a suitable volume of distilled water (11.7 mg/ml). At least 2 days before the administration of the compound, an indwelling catheter was implanted into the jugular vein for i.v. dosing and blood sampling. Compound was administered either i.v. via the jugular vein catheter or p.o. by gavage at a dose level of 10 mg/kg. Blood samples (approximately 0.8 ml) were collected from the jugular vein catheter at preselected time points.

Dog. Swiss male beagle dogs (body weight, 10 to 18 kg, 2 to 7 years) were treated with 1% Ro 47-7737 dissolved in 1 N lactic acid in 5% mannitol. The compound was given by short-term infusion (1 h) at 10 mg/kg into the cephalic vein. Blood samples (2 ml) were collected from the cephalic vein opposite to the cephalic vein used for infusion.

(ii) Analytics. Levels of Ro 47-7737 in plasma were determined by HPLC. Following addition of an internal standard and NaOH, plasma was extracted with a mixture of *n*-butyl chloride–methyl tertbutyl ether (9:1, vol/vol). The extract was evaporated, dissolved in 200 μl of a mixture of the mobile phase and the buffer used in the mobile phase (3:1, vol/vol), and then chromatographed on a C₁₈ reverse-phase column (5 μm of Inertsil ODS-2 [G. L. Science, Tokyo, Japan] or 5 μm of Nucleosil 100 [Macherey-Nagel, Düren, Germany]) protected by a C₁₈ reverse-phase guard column (5 μm of Lichrospher 100 RP-18) (E. Merck, Darmstadt, Germany) with, as the mobile phase, a mixture of acetonitrile and 100 mM NaH₂PO₄–75 mM NaClO₄ (pH 3; brought to this pH with 1 M H₂PO₄) at a ratio of 38:62, vol/vol. The injection volume was 150 μl. UV detection was performed at 335 nm. Under these conditions, Ro 47-7737 eluted after approximately 5.3 min. Dog plasma was used for the preparation of both calibration and quality control (QC) samples, while rat and mouse plasma samples were used for the preparation of QC samples only. The limit of quantification of the assay was 2.5 to 5 ng/ml with 250-μl plasma specimens.

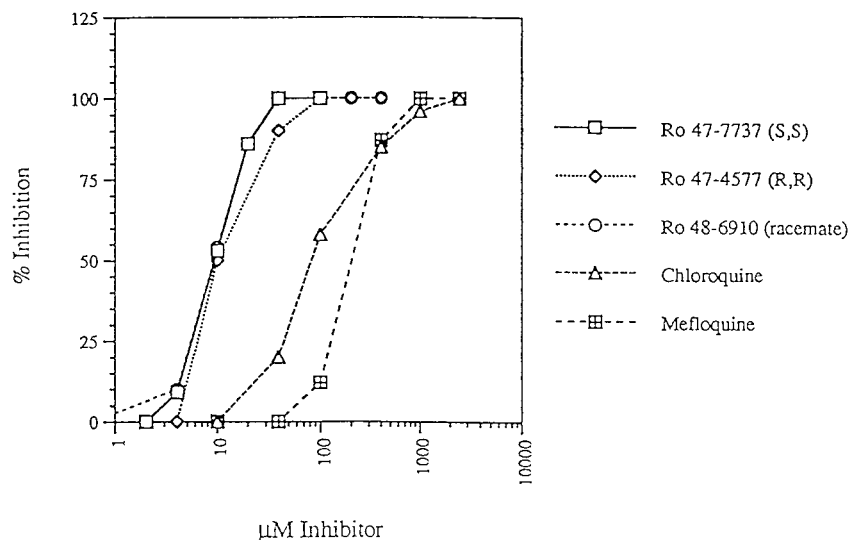


FIG. 3. Comparison of levels of inhibition of heme polymerization by the *S,S* enantiomer, Ro 47-7737, the *R,R* enantiomer, Ro 47-4577, and the racemate, Ro 48-6910, with those of chloroquine and mefloquine.

Toxicology. Two-week exploratory oral toxicity studies of male Wistar albino rats (six per group) and male and female beagle dogs (one per group) were performed. Ro 47-7737 was administered by gavage at equal volumes of 10 ml/kg as a suspension in a standardized vehicle containing sodium carboxymethyl cellulose. Daily doses of 0, 20, or 100 mg/kg (week 1) or 0, 30, or 150 mg/kg (week 2) were administered to rats, and doses of 0, 5, 20, or 100 mg/kg were administered to dogs. Basic mutagenicity testing included the Ames assay (Organisation for Economic Cooperation and Development guideline no. 471/472) and an in vitro micronucleus test (12) with Chinese hamster ovary cells. In addition, phototoxic potential was determined in vitro in a 3T3 mouse fibroblast Neutral Red uptake (NRU) assay by the methodology outlined in the ERGATT/FAME data bank of in vitro techniques in toxicology (INVTTOX protocol no. 78, 3T3 NRU phototoxicity assay, March 1994) and in vivo after single- and multiple-dose administration to hairless rats (11).

RESULTS

Stereochemistry affects growth inhibition of chloroquine-resistant strains. A comparison of the respective inhibitory properties of the *S,S* enantiomer, Ro 47-7737, and the racemate, Ro 48-6910, with chloroquine and mefloquine against the chloroquine-sensitive laboratory strain NF54 and the chloroquine-resistant laboratory strain K1 is shown in Table 1. The data confirm that the racemate is a potent inhibitor of both chloroquine-sensitive and chloroquine-resistant parasites (29) but demonstrate a superior potency for the *S,S* enantiomer.

In order to assess whether the potency of the *S,S* enantiomer, Ro 47-7737, was superior against a larger number of isolates, we determined the IC_{50} for the *S,S* enantiomer and the racemate against 13 laboratory strains, 32 Tanzanian iso-

lates, and 33 Thai isolates of various sensitivities to chloroquine. Among these isolates were highly chloroquine-resistant parasites displaying IC_{50} for chloroquine at 48 h of up to 500 nM (a parasite is deemed to be chloroquine resistant if it has an IC_{50} greater than 100 nM [2]). The 48- and 72-h IC_{50} of both compounds are compared to those of chloroquine in Fig. 2. The range of values obtained for the racemate against a range of chloroquine-resistant strains were similar to those previously reported by others (2). However, the *S,S* enantiomer, Ro 47-7737, consistently showed superior activity to the racemate for all chloroquine-resistant strains tested by a factor of 2 to 3, suggesting that it could prove to be a very potent antimalarial agent. The correlation coefficient of the IC_{50} with chloroquine was lower for the enantiomer than for the racemate, but both were statistically significant, suggesting a degree of cross-resistance with chloroquine even for the enantiomer.

A direct comparison of both the *S,S* and *R,R* enantiomers with the racemate was made in an experiment utilizing five chloroquine-sensitive strains and seven chloroquine-resistant strains. The results are shown in Tables 2 and 3. Once again, the *S,S* enantiomer gave the best results for both chloroquine-

TABLE 5. ED_{50} and ED_{90} for compounds administered s.c. and p.o. against *P. berghei*

Compound	Mean value (mg/kg) \pm SD for compound administered:			
	s.c.		p.o.	
	ED_{50}	ED_{90}	ED_{50}	ED_{90}
Ro 47-7737 ^a	0.7 \pm 0.0	1.0 \pm 0.2	2.3 \pm 2.2	4.6 \pm 3.7
Chloroquine ^a	1.5 \pm 0.3	3.3 \pm 0.5	2.4 \pm 0.2	5.0 \pm 1.0
Mefloquine ^b	3.0	3.9	3.3	6.4

^a Average values of at least three experiments.

^b Single experiment.

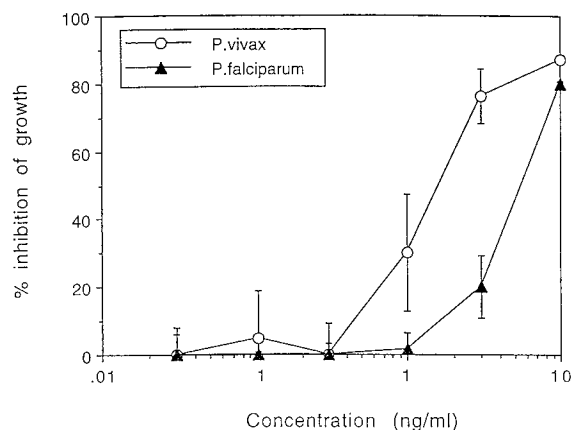


FIG. 4. Activity of the *S,S* enantiomer, Ro 47-7737, against *P. vivax* ex vivo.

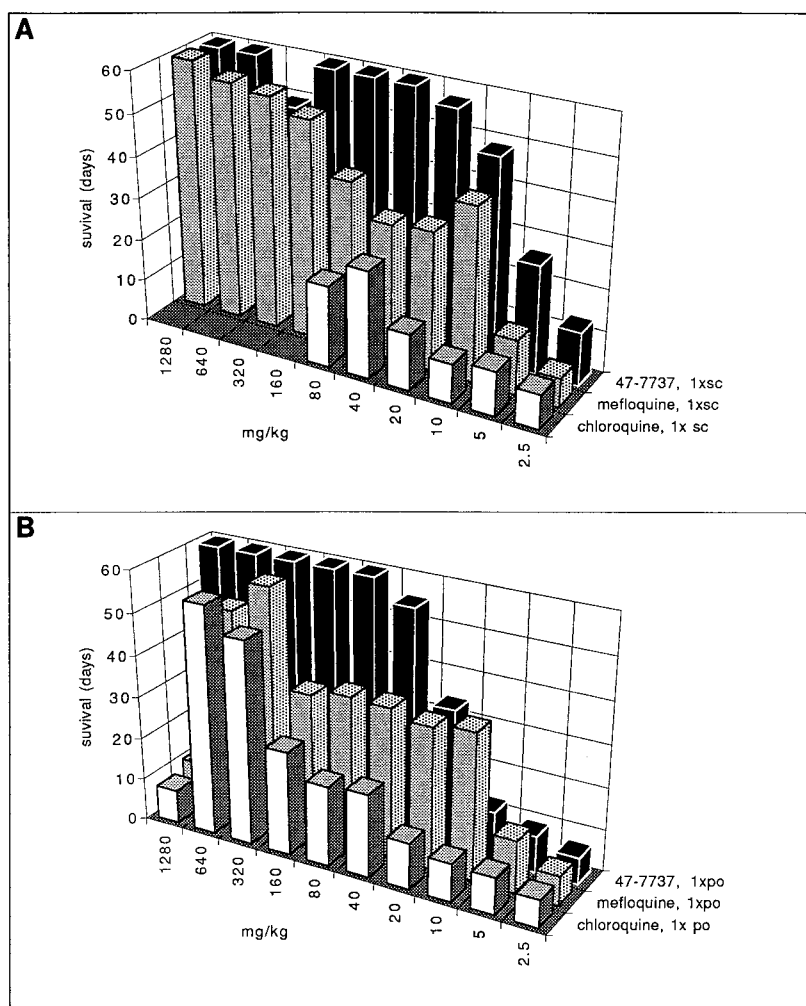


FIG. 5. Rane test. The survival of mice infected with *P. berghei* following treatment with single s.c. (A) and p.o. (B) doses on day 3 after infection is shown.

sensitive and, especially, chloroquine-resistant strains. For the chloroquine-resistant strains the IC_{50} of the racemate were intermediate between those of the *S,S* enantiomer and the *R,R* enantiomer, suggesting that the activity of the racemate is an additive effect of both the component enantiomers. A resistance index (29) was calculated for each compound from the combined data. The value obtained for the racemate, 2.4, is similar to a value of 2.1 calculated previously (29). The values for the *S,S* and *R,R* enantiomers were 1.8 and 3.4, respectively, suggesting that in addition to its superior absolute potency, the *S,S* enantiomer also showed a lower degree of cross-resistance to chloroquine.

Comparative killing activity against chloroquine-resistant and -sensitive strains. The best of the bisquinoline enantiomers, Ro 47-7737, was also compared with chloroquine and mefloquine for its ability to kill *P. falciparum* parasites in culture, rather than just inhibit growth. Compound was added at concentrations of 1, 3, 10, 30, 100, and 300 ng/ml to both chloroquine-sensitive and chloroquine-resistant parasite cultures, and parasite levels were monitored microscopically over 7 days. The results of this study are summarized in Table 4, which shows the minimum concentration required to completely kill the parasites and the length of time required. The results were qualitatively similar to those obtained by measur-

ing IC_{50} . Ro 47-7737 was equal or superior to chloroquine and mefloquine against both strains of parasite. The minimum concentration of Ro 47-7737 required to completely kill the chloroquine-resistant parasite K1 was higher by a factor of 3 than that for the sensitive strain NF54 and also required 4 days instead of 2. A similar difference in potency against the two strains was observed for mefloquine.

Comparative inhibition of heme polymerization. It is believed that chloroquine, and possibly mefloquine, may exert its antimalarial activity through inhibition of heme polymerization. This process allows the detoxification of heme, released as a result of hemoglobin degradation, and leads to the formation of hemozoin, a crystalline pigment, in the lysosomal food vacuole of the parasite (4, 23). A comparison of the inhibitory activities of the bisquinoline enantiomers, chloroquine, and mefloquine is shown in Fig. 3. The bisquinoline compounds are most active, with IC_{50} equal to 10 μ M, compared with values for chloroquine of 80 μ M and for mefloquine of 200 μ M. No difference was observed between the two bisquinoline enantiomers.

Activity against *P. vivax*. Ro 47-7737, the most active bisquinoline enantiomer, was tested against synchronous, mid-trophozoite-stage cultures of both *P. vivax* and *P. falciparum*. This test was carried out over 11 h, during which maturation to

TABLE 6. Comparative results of the Rane test for compounds administered p.o. and s.c.

Compound	Route	MED (mg/kg)	CD ₅₀ (mg/kg)	Comment
Ro 47-7737	p.o.	10	34	Lethal at 1,280 mg/kg
	s.c.	2	12	
Chloroquine	p.o.	21	285	Lethal above 640 mg/kg
	s.c.	15	>80	Lethal above 80 mg/kg
Mefloquine	p.o.	5	>250	Lethal above 640 mg/kg
	s.c.	5	143	

the schizont stage occurred. The data for the compound are given in Fig. 4 and show that the compound was highly active against *P. vivax*, more so than against the *P. falciparum* strain 7G8. The activity of Ro 47-7737 against *P. vivax* was equivalent to that of chloroquine (not shown).

In vivo activity of Ro 47-7737 in comparison to those of chloroquine and mefloquine. The in vivo activity of the best bisquinoline enantiomer, Ro 47-7737, was compared to those of chloroquine and mefloquine in a series of assays with the murine *P. berghei* model.

Comparative ED₅₀ and ED₉₀. Ro 47-7737 exhibits a therapeutic in vivo activity similar to those of chloroquine and mefloquine against blood stages of *P. berghei*. Comparative ED₅₀ and ED₉₀ are shown in Table 5. The compound was slightly less potent when applied p.o. rather than s.c. This may indicate a reduced oral bioavailability of the compound compared to chloroquine and mefloquine.

Rane test. Compounds were administered both s.c. and p.o. at doses ranging from 2.5 to 1,280 mg/kg. The results are summarized in Fig. 5 and Table 6. Figure 5 demonstrates graphically that full protection is reached at lower doses of Ro 47-7737 (40 mg/kg s.c. and 80 mg/kg p.o.) than with either chloroquine or mefloquine, which barely achieve full survival even at the highest doses. This implies that Ro 47-7737 is not only more potent but also more efficacious. In addition, efficacy is maintained with Ro 47-7737 up to 1,280 mg/kg p.o. This efficacy is not observed for chloroquine or mefloquine, as animals start to suffer from acute toxicity from these compounds before such high doses are reached. Table 6 gives quantitative values by which these effects can be assessed. Of clinical relevance is a comparison of equieffective oral doses. The MED of Ro 47-7737 (10 mg/kg p.o.) was between the corresponding doses for chloroquine (21 mg/kg p.o.) and mefloquine (5 mg/kg p.o.). The CD₅₀ for Ro 47-7737 (34 mg/kg p.o.) was much lower than those for both chloroquine (285 mg/kg p.o.) and mefloquine (>250 mg/kg p.o.). This result indicates that the initial effect of the compound in terms of survival of the animal is similar to those of chloroquine and mefloquine but that the effect against the parasite is much more long-lasting. Moreover, the CD₅₀ for Ro 47-7737 is much lower than the toxic dose, which is not the case for chloroquine and mefloquine.

Onset of drug action and recrudescence. The comparative effects of single oral doses of 50 mg of Ro 47-7737, chloroquine, and mefloquine/kg on the parasitemia of mice suffering from a *P. berghei* infection are illustrated in Fig. 6. Two important aspects of Ro 47-7737 in comparison to chloroquine and mefloquine emerge from this experiment. First, the compound rapidly reduces parasitemia to zero, closely resembling chloroquine in its speed of action rather than the slower-acting mefloquine. Indeed, a close examination of the early time points shows that zero parasitemia is achieved even more

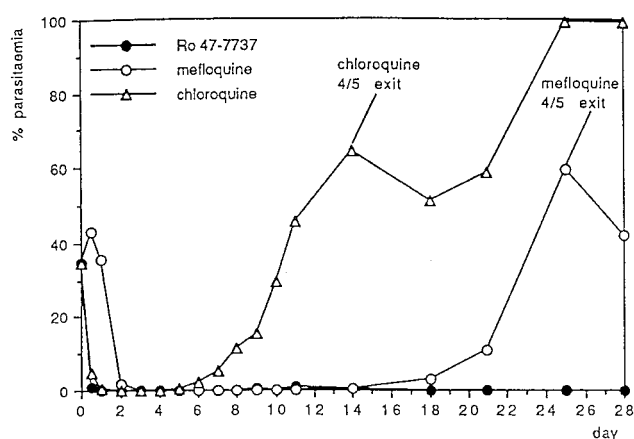


FIG. 6. Onset of drug action and suppressive activities of Ro 47-7737 and reference compounds against *P. berghei* in vivo. Parasitemia was monitored at various times after a single p.o. dose of 50 mg/kg at day +3 of infection. The term exit refers to the deaths of animals due to recrudescence parasitemia.

quickly for Ro 47-7737 than for chloroquine. Secondly, as already suspected from the Rane test, the parasitemia remains suppressed over a long period of time. For Ro 47-7737 there is no evidence of recrudescence after 28 days, compared to chloroquine, for which recrudescence occurs at day 6, or mefloquine, for which recrudescence occurs at day 18. There appears to be less danger of recrudescence with Ro 47-7737 than with either chloroquine or mefloquine after a single dose of 50 mg/kg.

Prophylaxis. The relative abilities of Ro 47-7737, chloroquine, and mefloquine to protect mice prophylactically from *P. berghei* infection were tested with single doses of 50 mg/kg p.o. The results are shown in Fig. 7. With this dosing regimen, Ro 47-7737 protected mice from subsequent infection for up to 14 days. Mefloquine protected for up to 3 days, and chloroquine protected for only 1 day.

Plasma drug concentrations required for prophylactic effects. We determined the levels of Ro 47-7737, chloroquine, and mefloquine in the plasma of mice at the time of infection which suppressed the development of parasitemia (Fig. 8). The plasma Ro 47-7737 level required to provide prophylactic pro-

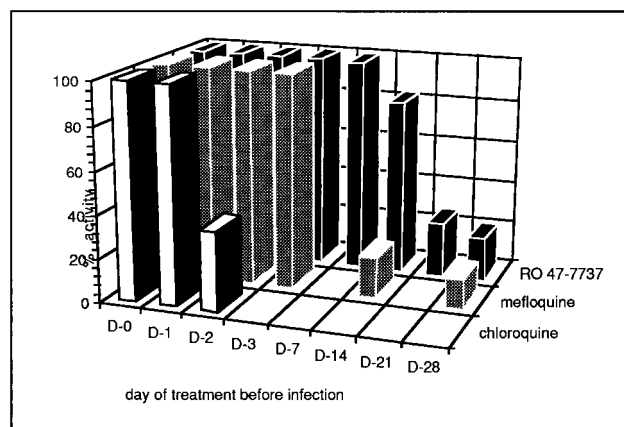


FIG. 7. Prophylactic assay comparing 50-mg/kg p.o. doses of Ro 47-7737, chloroquine, and mefloquine. The graph shows the prophylactic activities of the drugs in inhibiting parasite growth when given on the day of infection (day 0) and at different times before infection.

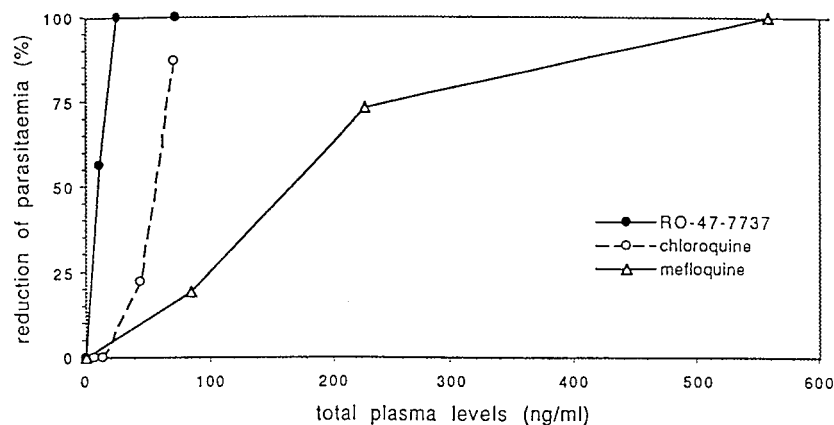


FIG. 8. Correlation of levels of compound in plasma at the time of infection and the degree of protection achieved for Ro 47-7737, chloroquine, and mefloquine.

tection is significantly less than those for both chloroquine and mefloquine. This probably reflects its superior antiparasitic activity (Table 1).

In vivo resistance generation. In *in vitro* studies no strains resistant to Ro 47-7737 could be generated (not shown). To assess the likelihood of resistance generation *in vivo*, we treated infected mice with subcurative doses of Ro 47-7737. Recrudescence parasites were then passaged to naive animals for further treatment and passage. The data obtained in this study are outlined in Fig. 9 and compared to results with mefloquine. Resistance to Ro 47-7737 developed slowly, requiring several passages. Chloroquine-resistant *P. yoelii* subsp. NS was totally resistant after eight passages in 63 days. Chloroquine-sensitive *P. berghei* N behaved similarly to *P. yoelii* subsp. NS, with a gradual reduction in response occurring over six passages and 45 days. These results are comparable to those obtained for mefloquine against chloroquine-resistant *P. yoelii* subsp. NS, to which resistance could be generated over five passages within 30 days.

Gametocytocidal and sporontocidal activities. Ro 47-7737, like chloroquine and mefloquine, demonstrated no gametocytocidal or sporontocidal activity.

Pharmacokinetic evaluation of Ro 47-7737. The pharmacokinetics of Ro 47-7737 have been assessed for mice, rats, and dogs following single *i.v.* (Table 7) and *p.o.* (Table 8) administration. Ro 47-7737 showed a long terminal half-life (>100 h) in all species tested, which is most likely caused by slow redistribution from a deep peripheral compartment(s). This hypothesis is supported by a large volume of distribution value for the compound against all species tested (ranging from 40 to 230 liters/kg). Ro 47-7737 showed good oral bioavailability in all species, despite some variability in the dog. Values obtained were as follows: mouse, 37%; rat, 54%; dog (data not shown), 11 to 90%. In general, underestimations of half-life and volume of distribution are possible for all animal species tested, since the characterization of the time course of the terminal concentration of the drug in plasma might be limited by the analytical assay used (limit of quantification, 2.5 to 5 ng/ml).

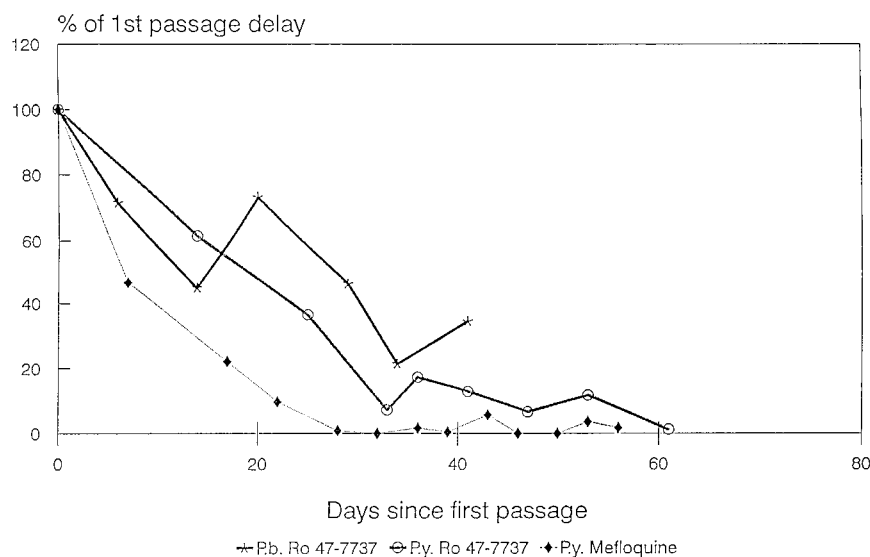


FIG. 9. Comparison of resistance development to Ro 47-7737 and mefloquine in mouse *P. berghei* N (P.b.) and *P. yoelii* subsp. NS (P.y.) models. Dosing of Ro 47-7737 for *P. berghei* N was 10 mg/kg, and that for *P. yoelii* subsp. NS was 100 mg/kg. Dosing of mefloquine for *P. yoelii* subsp. NS was 100 mg/kg.

TABLE 7. Animal pharmacokinetics of Ro 47-7737 after a single i.v. dose

Animal	Dose (mg/kg)	i.v. route	No. of animals	$t_{1/2}$ (h) ^a	CL (ml/min/kg) ^c	V_{ss} (liters/kg) ^d
Mouse	10	Bolus	39	(104)	15	106
Rat	8–10	Bolus	4	(50–70) ^b	50–70	200–250
Dog	10	1-h infusion	2	(135–135)	13–18	85–123

^a $t_{1/2}$, half-life. The reported values are probably underestimated due to inappropriate assay sensitivity.

^b After multiple-dose administration, a half-life of 350 to 400 h was found in rats.

^c CL, total clearance from plasma.

^d V_{ss} , volume of distribution at steady state.

The large volume of distribution, long terminal half-life, and reasonable bioavailability of Ro 47-7737 justified it as being a potential antimalarial prophylactic from a pharmacokinetic viewpoint.

Toxicity evaluation of Ro 47-7737. For rats, no clinical findings were noted during week 1, but a slight decrease in body weight gain was seen in the increased high-dose group during week 2. A slight reduction of sperm motility was noted at the end of the 2-week treatment period but was found to be fully reversible at the end of a 4-week treatment-free period. The main histopathological finding was granuloma formation in mesenteric lymph nodes in the high-dose group; no granulomas were seen in other lymph nodes. No relevant clinical findings were recorded for dogs receiving 5 or 20 mg/kg. The highest dose of 100 mg/kg was poorly tolerated, particularly by the male, and both animals in this group were sacrificed prematurely. Findings included salivation, vomiting, diarrhea, signs of abdominal pain, weight loss, and altered laboratory parameters. Post mortem examinations revealed multifocal hepatocellular necrosis, particularly in the high-dose male, and signs of disturbed spermatogenesis and atrophic changes of the prostate in males and follicular activation of the ovaries and endometrial activation of the uterus in females. No indication of mutagenic or clastogenic activity was seen in the mutagenicity tests. Ro 47-7737 was found to be phototoxic in vitro and also in vivo after multiple-dose administration.

DISCUSSION

Antiparasitic activity. This study demonstrates that the *S,S* enantiomer of the bisquinoline *trans-N¹,N²-bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine* is significantly more potent than the previously reported racemate (2, 29) and the *R,R* enantiomer. In particular, the *S,S* enantiomer, Ro 47-7737, is more active against chloroquine-resistant strains and shows a lower degree of cross-resistance to chloroquine. For these reasons we undertook an extensive parasitological evaluation of the Ro 47-7737's potential as an antimalarial drug.

TABLE 8. Animal pharmacokinetics of Ro 47-7737 after a single p.o. dose

Animal	Dose (mg/kg) ^a	No. of animals	C_{max} (ng/ml) ^b	T_{max} (h) ^c	$t_{1/2}$ (h) ^d	Bioavailability (%)
Mouse	10	26	123	5	(76)	37
Rat	8–10	4	30	2	(50)	54

^a The doses (in an aqueous solution) were calculated for the free base.

^b C_{max} , maximum concentration of Ro 47-7737 in plasma.

^c T_{max} , time to maximum concentration of Ro 47-7737 in plasma.

^d $t_{1/2}$, half-life. The values are probably underestimated.

The compound is inherently more potent than either chloroquine or mefloquine against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* erythrocytic stages grown in culture, and it is potent against the trophozoite stage of *P. vivax* in an ex vivo model. In a *P. berghei* murine model it shows suppression of growth at levels in plasma significantly lower than those for either chloroquine or mefloquine. The greater potency of Ro 47-7737, compared to chloroquine and mefloquine, at inhibiting heme polymerization may account for this superior antiparasitic activity. However, as the *S,S* and *R,R* enantiomers inhibit heme polymerization with equal potencies but differ in their abilities to inhibit chloroquine-resistant parasite growth, it is possible that transport factors affecting the compound's accumulation in the parasite may also play a role. The strong activity of Ro 47-7737 against trophozoite stages and its inactivity against gametocytes and oocyst formation are also consistent with heme polymerization as the target of the compound, as is its inactivity against liver stages (not shown).

Activity in clinically relevant in vivo models. The potent growth-inhibitory properties of Ro 47-7737 against erythrocytic stages probably account for the rapid clearance of parasitemia in vivo. In addition, several *P. berghei* models showed that the compound has good curative properties and good prophylactic potential. The CD_{50} in the Rane test was much lower than that for either chloroquine or mefloquine, and a dose of 50 mg/kg p.o. produced prophylactic protection for 14 days as opposed to 3 days for mefloquine and 1 day for chloroquine. We have confirmed that these curative and prophylactic properties are due to an exceptionally long half-life for this compound, estimated at 104 h in the mouse.

Issue of cross-resistance with chloroquine. There was a correlation between Ro 47-7737 and chloroquine IC_{50} , indicating a cross-resistance. Similarly, there was a reduced ability of the compound to kill the chloroquine-resistant K1 strain compared to its ability to kill the chloroquine-sensitive NF54 strain. We have also found that the activity of the *S,S* enantiomer, Ro 47-7737, against chloroquine-resistant K1 is enhanced by the addition of desipramine, an agent capable of reversing chloroquine resistance (not shown). These data suggest that a mechanism associated with chloroquine resistance also limits the activity of Ro 47-7737. However, the low absolute IC_{50} against a range of chloroquine-resistant *P. falciparum* strains suggested that Ro 47-7737 might overcome chloroquine resistance in the clinic if appropriate levels in plasma could be obtained at reasonable, nontoxic dosing levels and if resistance to Ro 47-7737 would not develop independently. An in vivo model of development of resistance to a chloroquine-resistant rodent malaria, *P. yoelii* subsp. NS, suggested that although resistance could be generated, this occurred at a rate similar to that of mefloquine, which has been a successful antimalarial drug for many years (15).

Pharmacokinetic and toxicological analyses. The pharmacokinetic evaluation of Ro 47-7737 suggested that the long-lasting activity of Ro 47-7737 results from its long terminal half-life. In addition, the compound exhibited sufficient oral bioavailability. However, toxicity liabilities were evident. The effect of Ro 47-7737 on the sex organs of juvenile dogs caused concern, though preliminary studies of other species suggested that this may be a species-specific effect. In addition, significant differences in the metabolic profile of Ro 47-7737 between dog liver microsomes and rat and human liver microsomes were observed, calling into question the dog as a valid species for toxicological evaluation (data not shown). On top of this concern, however, Ro 47-7737 proved highly phototoxic in both a cell-based assay and an in vivo assay with hairless rats. Because of the long half-life of this compound, its proposed use in

tropical climates, and the strong danger of related photocarcinogenicity side effects, the compound could not be considered for development.

Conclusion. Ro 47-7737 is a compound highly active against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* and the other major human malaria parasite, *P. vivax*. It is an orally active, fast-acting compound with a long-lasting effect and has powerful prophylactic properties. Toxicity liabilities, however, particularly phototoxicity and the danger of attendant photocarcinogenicity, rule it out as a drug development candidate.

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