Present Development Concerning Antimalarial Activity of Phospholipid Metabolism Inhibitors with Special Reference to *In Vivo* Activity

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The systematic screening of more than 250 molecules against Plasmodium falciparum in vitro *has previously shown that interfering with phospholipid metabolism is lethal to the malaria parasite . These compounds act by impairing choline transport in infected erythrocytes, resulting in phosphatidylcholine* de novo *biosynthesis inhibition.*

A thorough study was carried out with the leader compound G25, whose in vitro IC50 is 0.6 nM. It was very specific to mature parasites (trophozoïtes) as determined in vitro *with* P. falciparum *and* in vivo *with* P. chabaudi *-infected mice. This specificity corresponds to the most intense phase of phospholipid biosynthesis activity during the parasite cycle, thus corroborating the mechanism of action.*

The in vivo *antimalarial activity (ED50) against* P. chabaudi *was 0.03 mg/kg, and a similar sensitivity was obtained with* P. vinckei petteri, *when the drug was intraperitoneally administered in a 4 day suppressive test . In contrast,* P. berghei *was revealed as less sensitive (3- to 20-fold, depending on the* P. berghei *-strain). This difference in activity could result either from the degree of synchronism of every strain, their invasion preference for mature or immature red blood cells or from an intrinsically lower sensitivity of the* P. berghei *strain to G25. Irrespective of the mode of administration, G25 had the same therapeutic index (lethal dose 50 (LD50)/ED50) but the dose to obtain antimalarial activity after oral treatment was 100-fold higher than after intraperitoneal (or subcutaneous) administration. This must be related to the low intestinal absorption of these kind of compounds.*

G25 succeeded to completely inhibiting parasitemia as high as 11.2% without any decrease in its therapeutic index when administered subcutaneously twice a day for at least 8 consecutive days to P. chabaudi *-infected mice. Development of the pharmacological model has thus been fully validated with the malaria-infected-rodent model. Transition to human preclinical investigations now requires a synthesis of molecules which would permit oral absorption.*

Key words: malaria - *Plasmodium* - phospholipid metabolism - choline - pharmacology - chemotherapy

Membrane biogenesis accompanying malarial parasite growth requires adapted and intensive lipid synthesis due to the sole parasite machinery in a host cell devoid of any lipid synthesis pathway (Vial et al. 1990). A new therapeutic approach to this endemic disease, involving interference with parasite metabolic pathways, could provide a new range of antimalarial drugs with original structures and modes of action. We have developed a model to design antimalarial drugs based on interference with the essential phospholipid (PL) metabolism of *Plasmodium* during its intraerythrocytic cycle (Ancelin & Vial 1986, Ancelin et al. 1985, Vial et al. 1984). The most promising drug interference is choline transporter blockage, which provides *Plasmodium*

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with a supply of precursor for synthesis of phosphatidylcholine (PC), the major PL of infected erythrocytes. Choline entry is the rate limiting step of *de novo* biosynthesis, involving a carrier which is a very accessible target for blockage (Ancelin et al. 1991, Ancelin & Vial 1989). By a systematic screening of more than 250 molecules against *P. falciparum in vitro* we have previously shown that interfering with the transport of the polar head, choline, is lethal to the parasite (Vial et al. 1994). Twenty-four of these compounds possess antimalarial activity *in vitro* (expressed as IC50) lower than 80 nM. They are active against polypharmacoresistant strains and isolates in the same IC50 range. Ten compounds possess effective antimalarial activity *in vivo* against the *P. berghei* and *P. chabaudi* mouse system, with a therapeutic index (which is the ratio LD50 on ED50) of 8 to 40. Additionally, they also possess *in vitro* and *in vivo* antibabesia activity. The present paper will deal more particularly with one of the leader compounds G25, whose IC50 is 0.6 nM.

MATERIAL AND METHODS

Biological Materials - AB^+ human blood or AB^+ human serum came from the Blood Bank of Montpellier (France). Complete medium consisted of RPMI 1640 supplemented with 25 mM Hepes buffer, pH 7.4, and 10% AB^+ serum. The Nigerian strain of *P. falciparum* (Richards et al. 1977) was maintained by serial passages in AB^+ human erythrocytes suspended in complete medium at 37°C, using the petri dish candle-jar method (Jensen & Trager 1977). In some experiments, *P. falciparum* -infected erythrocytes were twice synchronized with 5% D-sorbitol as described previously (Lambros & Vanderberg 1979).

Male Swiss mice (from C.E.R. Janvier, France) weighing 30-40g were used. They were challenged either by intraperitoneal or intravenous administration of 10^7 parasitized red blood cells obtained from donor mice with parasitemias of 15-40% in the ascending phase. In order to obtain highly synchronized *P. vinckei petteri* infected cell suspensions, the mice were intraperitoneally challenged with thawed infected blood, as described previously (Montalvo-Alvarez et al. 1988).

P. chabaudi and *P. berghei* NC were provided by Prof Camus (Lille, France), *P. berghei* N1 (Keyberg 173) (Peters et al. 1975) was provided by Prof Peters (London, U.K.), and *P. vinckei petteri* (279 BY, RCA) (Carter & Walliker 1975) by Dr Irène Landau (Paris, France).

IN VITRO ANTIMALARIAL ACTIVITY

In vitro antimalarial activity was measured according to the method of Desjardins, in which the infected cell suspensions (final hematocrit 0.5-2%, parasitemia 0.3-0.6%) were preincubated for 48 h in the presence of the drug before the addition of the radioactive precursor [^{3}H] hypoxanthine at 1 µCi/well (Desjardins et al. 1979) . In some experiments *P. falciparum* -infected erythrocytes were synchronized twice or 3-times with 5% D-sorbitol (Lambros & Vanderberg 1979). At time 0, one ml of infected erythrocytes at 1-1.5% hematocrit and 0.3- 0.5% initial parasitemia (100% rings) was in each well of a 24-well plate; 500 µl of the drug (at 3-fold the final concentration) was added for the indicated time when the parasites were either in the ring stage (t0), trophozoite stage (t0+24h) or schizont stage (t0+ 36 h). Then, the drug was removed from each well by two changes of culture medium using fresh medium without the drug and infected cell suspensions were further incubated for 12 h. After that time, cell suspensions of every well were transferred in 96-well plates (200 µl/well) and 30 µl of [^{3}H]hypoxanthine (1.5 µCi/well) was then added to each well. Incubations were finally stopped by filtration at time 72 h. Results are expressed as percent of the hypoxanthine incorporation observed in control wells containing parasites cultures in the absence of the drug. Results are exprssed as the mean ± S.E.M. of 3 independent experiments carried out in triplicate.

IN VIVO ANTIMALARIAL ACTIVITY

Mice were inoculated intravenously with 10^7 infected erythrocytes at t0. Animals were then injected subcutaneously twice a day with increasing doses of G25 (5 doses from 0.01 to 0.9 mg/kg dissolved in 0.25 ml 0.9% NaCl). Doses were given for 4 or 8 consecutive days beginning either on the day of infection, at t0 + 2 h or at increasing periods, until t0 + 4 days. For each set of parasitemia, 3 animals were treated with a given dose of drug, whereas controls corresponded to the mean parasitemia of 6 mice. Parasitemia was determined daily between t0 and t0+12 days, and then twice a week for 60 days to assess recrudescence. Curative properties of the drug were evaluated by comparing the

survival time of infected and treated mice with that of untreated control mice. The drug was considered active when the survival time of a mouse treated with G25 was two times higher than that of an untreated, i.e. generally 14-16 days. Treatment was considered curative when no parasite could be detected after 60 days. Parasitemia appearence and deaths which occurred after 16 days were attributed to recrudescence. ED50, i.e. 50% suppression parasite was estimated from a plot of log dose against activity by comparison with control animals treated with 0.25 ml of 0.9% NaCl solution. Results are expressed as mean ± S.E.M.

RESULTS AND DISCUSSION

TIME-COURSE FOR G25 *IN VITRO* PARASITE GROWTH INHIBITION AND STAGE SPECIFICITY

Addition of a lethal dose of G25, at 20 nM, i.e. 20-fold the IC50 for pulse-inhibitory periods to synchronous cultures showed that inhibition by G25 occurred quickly (around 5 h) when applied to maturing parasites, trophozoite or schizont stages (data not shown). It was also effective on young ring forms but to lesser degree, requiring a longer incubation time to exert its effect (around 10 h). Indeed, after 3 h incubation in the presence of 20 nM G25, parasite growth was inhibited by 92 and 79 % for trophozoite and schizont stages, respectively, whereas ring stage viability was inhibited by 40%, and even after 20 h incubation, ring viability inhibition was never complete (-60%).

The partial inhibition obtained with ring forms led us to investigate the sensitivity of the parasite at different stages. Increasing drug concentrations were applied to synchronized *P. falciparum*-infected erythrocyte suspensions for 3 h 30, allowing the determination of the IC50 for each stage (Fig. 1). Against schizonts, IC50 was 9 nM and against trophozoite stages, G25 was highly efficient with an IC50 of 0.7 nM. The ring stages were much less sensitive to the drug (IC50 of 160 nM) than the mature stages (trophozoites) by more than 2 orders of magnitude.

After 7 h incubation at 10 nM with the drug, ring stages (whose viability at this concentration was not significantly altered), (see Fig. 1), showed no significant ultrastructural alteration whereas trophozoites (whose viability was practically totally inhibited at this concentration) exhibited deep ultrastructural modifications. They consisted in unusual thickenings on the nuclear envelope and mainly on the endoplasmic reticulum which appeared deeply invaginated and dilated. The other membrane structures, such as mitochondria, parasitophorous and parasite membranes, digestive vacuole, did not show any morphological alterations.

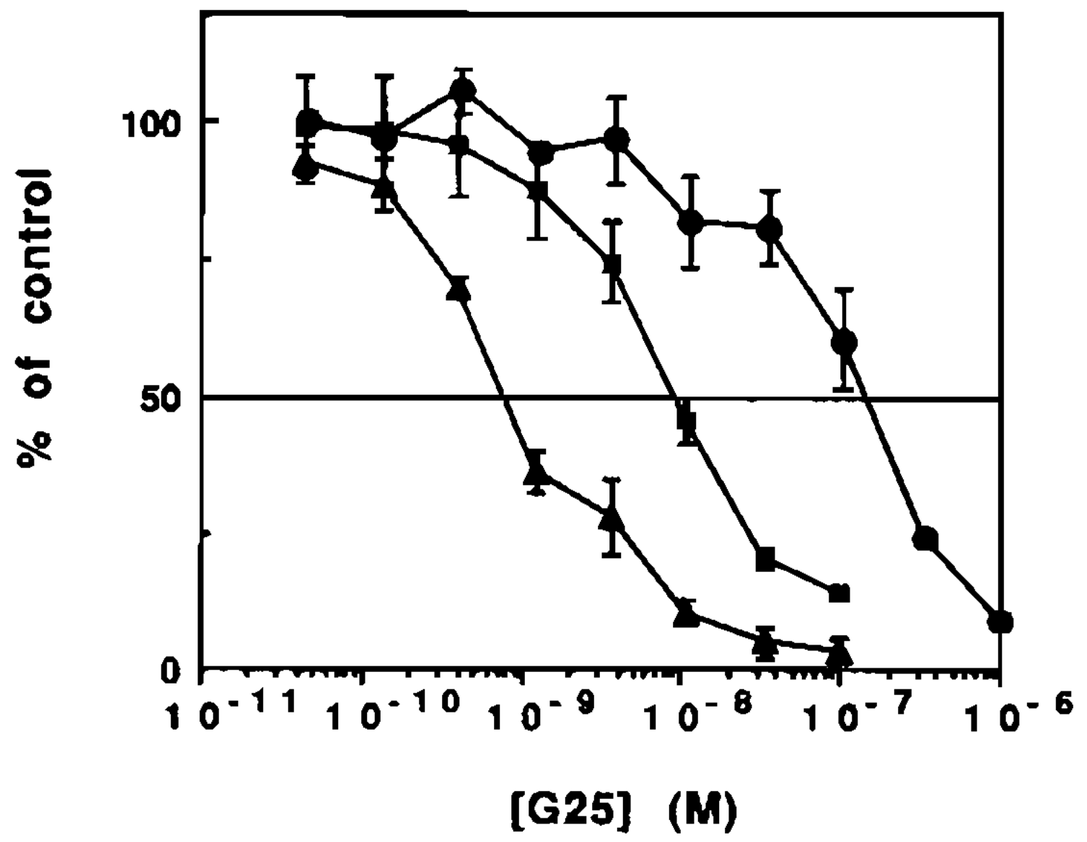

Fig. 1: G25 *in vitro* antimalarial activity as a function of parasite stage. *P. falciparum*-infected erythrocytes were twice synchronized with 5% D-sorbitol at time -43 h and -1h (Lambros & Vanderberg 1979). Drug was added for 3 h 30 when the parasites were either in the ring stages (•), trophozoite stage (▲) (+24 h) or schizont stages (■) (+36 h).

Blockage of choline transport (located in the plasmic membrane) leads to an impairment of choline supply to the parasite and consequently no more precursor is available for PC biosynthesis. In eukaryotes, endoplasmic reticulum is generally described as the organelle where *de novo* PL biosynthesis take place. For PC *de novo* biosynthesis, the 2 last enzymes of the pathway, choline phosphotransferase and the active form of choline phosphate cytidylyltransferase are present in endoplasmic reticulum (Kent et al. 1991).

In *Plasmodium*, we have previously checked that after 5 h incubation, G25 inhibited specifically PL biosynthesis compared to the other macromolecule biosynthesis such as nucleic acids or proteins. Inside PL metabolism, it has no effect on ethanolamine incorporation into PE up to 10^{-4} M, but was specific for *de novo* PC biosynthesis from choline.

The specificity for mature parasites corresponded to the more intense phase of phospholipid biosynthesis activity during the parasite cycle (Vial et al. 1982), and affects the endoplasmic reticulum thus corroborating the mechanism of action.

IN VIVO ANTIMALARIAL ACTIVITY AGAINST MURINE PARASITES

G25 *in vivo* antimalarial activity was determined with 3 different rodent strains according to a 4-day suppressive test in which parasites were inoculated intraperitoneally and drug was administered twice a day in the intraperitoneal mode. *In vivo* antimalarial activity (ED50) against *P. chabaudi* was 0.03 mg/kg, whereas *P. berghei* appeared to be 3- to 9-fold less sensitive depending on the *P. berghei*-strain, NC (Lille, INSERM U42, Prof Camus) and N1 (Keyberg 173) (Peters, et al., 1975) respectively (data not shown). Separated experiments carried out with *P. vinckei petteri* showed a sensitivity to G25 very similar to that of *P. chabaudi* (Landau et al. personal observation). LD50 was 1.2 mg/kg, which corresponds to a therapeutic index of 40.

It should be noted that intravenous parasite inoculation led to a 2-3-fold increase in the ED50 (not shown). Furthermore, the susceptibility of *P. chabaudi* to G25 progressively decreased as a function of time, when the strain was maintained by serial passages . As an example, ED50 was 10-fold increased (from 0.03 to 0.3 mg/kg) over a 2 year period of serial passages. After thawing of a stock strain frozen 2 years before, the initial susceptibility to the drug was partly restored. These variations are probably intrinsic to this *P. chabaudi* strain.

Results obtained *in vivo* with the various murine *Plasmodia* species also indicate that there are variations in the ED50 according to the *Plasmodium* species invading rodents. These rodent species differ in their ability to invade mature or immature erythrocytes and in their degree of synchronism which could probably, at least a part account for the observed differences. G25 appeared less efficient against *P. berghei* than against *P. chabaudi* or *P. vinckei petteri* (the most synchronized and showing a preference for mature erythrocytes). An intrinsically lower sensitivity of *P. berghei* to G25 could also be evoked.

ACTIVITY AS A FUNCTION OF DRUG ADMINISTRATION ROUTE, NUMBER OF DOSES AND PARASITE STAGES

G25 antimalarial activity was evaluated against *P. chabaudi* as a function of the drug administration mode, i.e. intraperitoneally, subcutaneously or orally. Irrespective of the route of administration, very steep curves were obtained with activity occurring over one order of magnitude (data not shown). The same activity was obtained whether the drug was administered intraperitoneally or subcutaneously (ED50 = 0.3 mg/kg). By contrast, the dose needed to obtain antimalarial activity after oral treatment was 100-fold higher than after subcutaneous administration. Irrespective of the mode of administration the same therapeutic index was obtained. Indeed, the difference obtained in ED50 relative to the administration mode, was also observed in the lethal doses (LD50). This must be related to the low intestinal absorption of this kind of chemical compound.

In vivo activity was then investigated with *P. chabaudi* -infected mice as a function of parasite development and repeated drug administration. When administered once a day, the drug appeared slightly more active when applied to parasites at the trophozoite stage than at ring or schizont stages. When the drug was administered twice a day, and provided that the drug was administered when parasites were in the mature form, ED50 was 4-fold lower. No significant improvement was obtained when drug was given 3 times a day.

CURATIVE G25 ACTIVITY ON *P. CHABAUDI*- INFECTED MICE AND THERAPEUTIC INDEX AS A FUNCTION OF PARASITEMIA

The conditions of the modified 4 day suppressive Peters test we used are more representative of the prophylactic properties of the drug than of its curative properties. Indeed, the drug was administered only a few hours after infected cell inoculation. We thus investigated the curative effect of G25 when tested against mice with high parasitemia.

Preliminary experiments had shown that G25 when administered once a day for 8 days succeeded in a definitive cure of *P. chabaudi* - infected mice at 1% parasitemia, without recrudescence (not shown). Further experiments were then carried out to determine whether G25 could be curative at parasitemia higher than 10 % and to investigate which dose and duration of treatment were required for a definitive cure. Our purpose was also to determine whether at very high parasitemia, the therapeutic index was decreased, that is whether it was necessary to increase G25 doses to obtain the inhibition of parasitemia during severe infections.

For these experiments, increasing doses of G25 (from 0.01 to 0.9 mg/kg) were administered twice a day in the subcutaneous mode to *P. chabaudi* -in-

fected mice either on the day of infection at t0 + 2 h (P=0.05%), at t0+ 2 days (P=3.5%) or at t0+3 days (P=11.2%). After a 4 day treatment G25 exerted its antimalarial *in vivo* activity over a narrow range of concentrations between 0.1 and 1 mg/kg (one order of magnitude). Regardless of initial parasitemia (0.05, 3.5 or 11.2%), the ED50 were very close (between 0.3 and 0.5 mg/kg), and there was no significant decrease in the therapeutic index (Fig. 2). Antimalarial activity was also obtained after only 3 days of treatment and for the same range of G25 doses.

Parasitemia was followed as a function of time, for the highest tested G25 dose (Fig. 3). Untreated mice (control) generally died 6 to 7 days after the beginning of the experiment (between t0+ 7 and t0+ 8). At the lowest initial parasitemia (0.05%), after 4 day treatment the parasitemia was quite low (0.3 ± 0.06 compared to 31.7 ± 0.9 in the control) (Fig. 3A). At higher initial parasitemias, 3.5 or 11.2 %, at least 2 days were necessary to obtain a decrease in parasitemia compared to controls, but after 3 days of treatment, reduction in the parasitemia was observed (Fig. 3 B and C).

If the therapeutic window for treated mice, lasted for only 4 days (open symbols) a recrudescence was observed, even at low parasitemia (see the progressive rise in parasitemia Fig. 3 A and B). By contrast, irrespective of the initial parasitemia, a definite curative effect was obtained without recrudescence after 60 days, provided the treatment lasted for at least 8 consecutive days (closed symbols). Par-

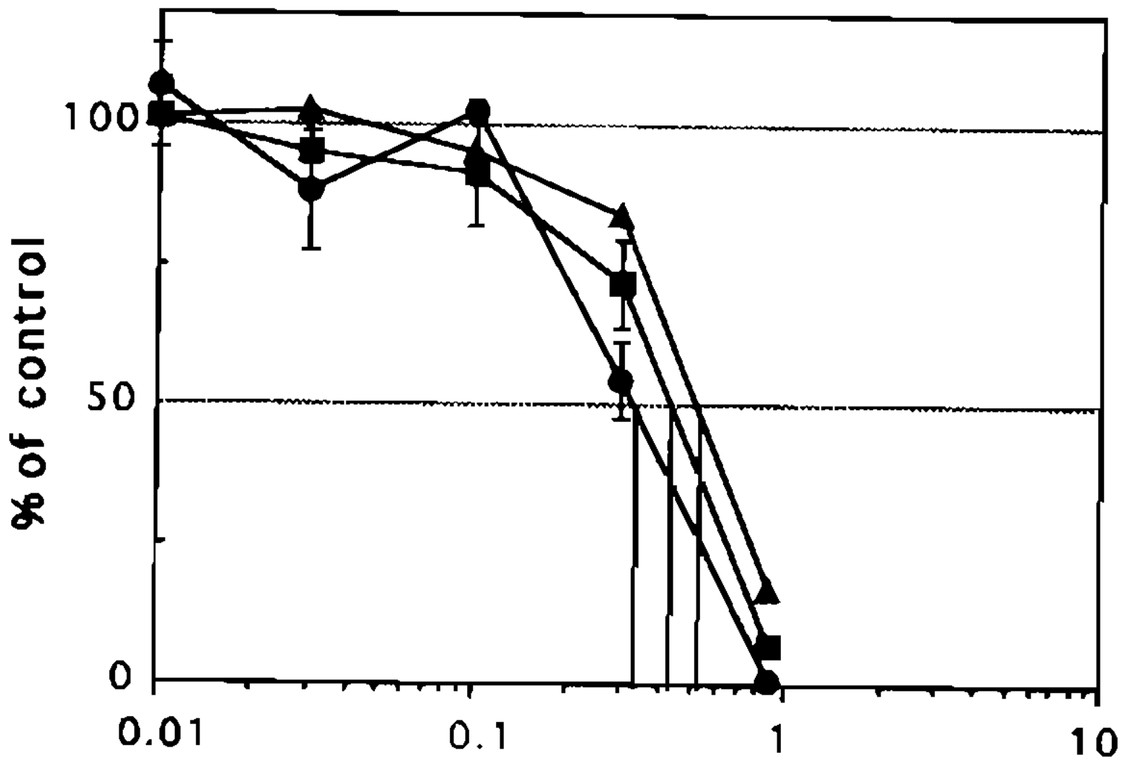

Fig. 2: *in vivo* G25 antimalarial activity against *P. chabaudi* -infected mice as a function of parasitemia. Mice were inoculated intravenously with 10^7 *P. chabaudi* -infected erythrocytes at t0. Animals were then subcutaneously dosed twice a day (at 8h30 and 17 h) with increasing doses (5 doses from 0.01 to 0.9 mg/kg) of G25 (dissolved in 0.25 ml 0.9% NaCl) for either 4 consecutive days beginning either on the day of infection, at t0 + 2 h (P=0.05%) (•), or at t0 + 2 days (P=3.5%) (■) or at t0 + 3 days (P=11.2%) (▲).

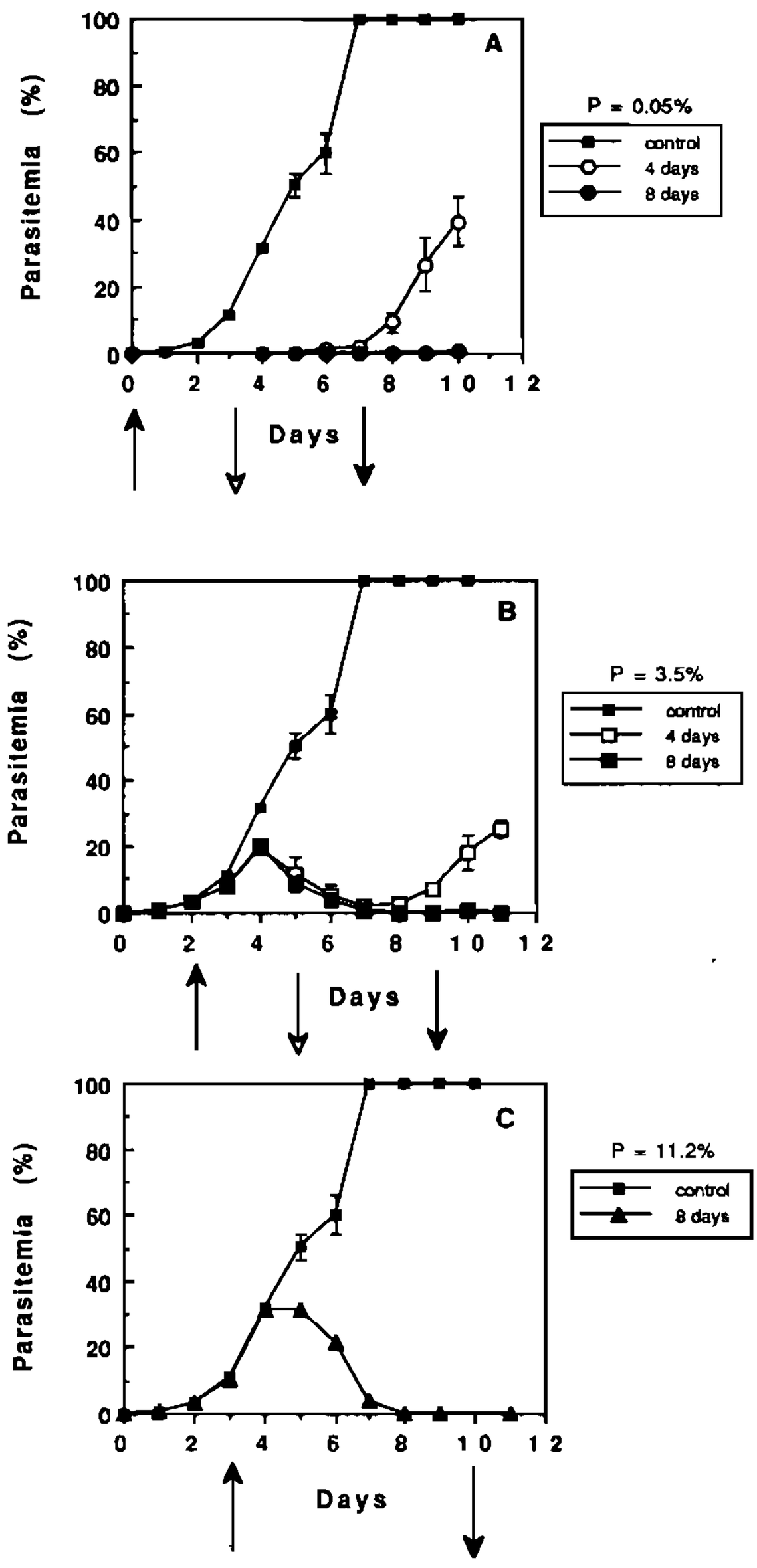

Fig. 3: curative G25 antimalarial activity against *P. chabaudi*-infected mice as a function of parasitemia and treatment window. Experiments were carried out as in Fig. 2 except that mice were dosed with 0.9 mg/kg G25 for either 4 or 8 consecutive days beginning either on the day of infection, (A) at t0 + 2 h (P=0.05%), or (B) at t0 + 2 days (P=3.5%) or (C) t0 + 3 days (P=11.2%). Black arrows correspond to the beginning (up direction) and the end (down) of the 8 day treatment, white arrows correspond to the end of the 4 day treatment.

ticularly spectacular is the decrease in parasitemia observed at the very high parasitemia of 11.2 % which was also without recrudescence after 60 days. (Fig. 3 C)

G25 succeeded fully in inhibiting parasitemia as high as 11.2% without any decrease in its therapeutic index (LD50/ED50). It is thus not necessary to increase doses to reduce parasitemia even for severe infections.

CONCLUSIONS AND PROSPECTS

Altogether, these results show that impairing PL metabolism by choline analogs is quite an original strategy considering notably the polypharmacoresistance of *Plasmodium*, as already demonstrated by the high efficiency *in vitro* against resistant isolates. Particularly interesting is the possibility of curing highly infected mice (11.2% parasitemia) without any modification in the therapeutic index. Development of our pharmacological model has now been fully validated with the malaria-infected mouse model, notably in curing highly infected mice without recrudescence. The next experiments will consist in testing curative G25 activity with *P.falciparum* -infected monkeys.

The transition to human preclinical investigations now requires synthesis of molecules with better tolerance and oral absorption. Indeed, the chemical structure of the molecules makes them poorly permeable to the intestinal barrier and hinders a further development of these compounds since oral administration appears a prerequisite for widespread use in uncomplicated malaria.

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