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A new class of potent antimalarials and their specific accumulation in infected erythrocytes

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Abstract:

During asexual development within erythrocytes malaria parasites synthesize considerable amounts of membrane. This activity is an attractive target for chemotherapy as it is absent from mature erythrocytes. We found compounds inhibiting phosphatidylcholine biosynthesis *de novo* from choline were potent antimalarial drugs. The lead compound, G25, potently inhibited *in vitro* growth of the human malaria parasites *Plasmodium falciparum* and *P. vivax* and was 1000-fold less toxic to mammalian cell lines. A radioactive derivative specifically accumulated in infected erythrocytes to levels several hundredfold higher than in the surrounding medium, and very low dose G25 therapy completely cured monkeys infected with *P. falciparum* and *P. cynomolgi*.

The widespread resistance of malaria parasites to most common antimalarials, and cross-resistance to structurally unrelated drugs, emphasizes the need for new chemotherapeutic targets (1). While parasitizing red blood cells, the parasite invests heavily in membrane biogenesis, making phospholipid (PL) biosynthesis essential for intra-erythrocytic development (2), while these activities are absent from mature erythrocytes (3). Phosphatidylcholine (PC) is the major PL of infected erythrocytes. It is mainly synthesized from plasma-derived choline by the parasite enzymatic machinery and is an attractive target for antimalarial chemotherapy (2). In an attempt to inhibit PC synthesis in malaria parasites, we designed and synthesized a series of compounds based on their capacity to mimic choline structure (4). They were optimized for *in vitro* antimalarial activity and there was a very close correlation between the inhibition of parasite growth *in vitro* and specific inhibition of parasite PC biosynthesis *de novo* from choline (5). The compounds do not interfere with other types of PL synthesis, such as that of phosphatidylethanolamine or macromolecules like DNA (5). Toxic activity is highest against mature parasites, when PL synthesis is maximal (5). We hypothesize that the most likely drug target is the choline transporter, either on the surface of the infected erythrocyte or in the plasma membrane of the intracellular parasite (6). Structure-activity relationship (SAR) studies revealed the importance of the cationic group volume. Duplication of this group (bisquaternary ammonium salts) separated by a sufficiently long alkyl chain dramatically increases antimalarial activity to within the low nanomolar range (7). This defined the lead compound G25 (1,16-Hexadecamethylenebis(N-methylpyrrolidinium) dibromide) (7), which we analyze here.

G25 was assessed against *P. falciparum* strains with pre-existing resistance against chloroquine (CQ), quinine, (Q), mefloquine, and pyrimethamine (8, 9) and against four clinical isolates with varying resistance to CQ, Q and cycloguanil from patients in different African countries (10). G25 was effective against all strains/isolates tested, with 50% inhibitory concentrations (IC₅₀) ranging between 1 and 5.3 nM. Steep dose-response curves with IC₉₀/IC₅₀ ratios below three were consistently observed (9), indicating inhibition of a specific target and not a general cytotoxic effect. To assess the effect of G25 in the host, we measured IC₅₀ levels of G25 against Jurkat lymphoblast, U937 macrophage and MEG-01

megakaryoblast human cell lines (9). The IC₅₀ obtained was 3 to 4 orders of magnitude higher than the IC₅₀ against *P. falciparum*. No mutagenic activity (Ames test, (11)) with or without metabolic activation was evident at the highest nontoxic concentration (77 μM).

Antimalarial activity (4 days suppressive test of Peters (12)) and acute toxicity was first determined after intraperitoneal administration of G25 in *P. chabaudi* infected mice (13). Rapid and total clearance of parasites was observed with a 50% effective dose (ED₅₀) of 0.08 mg/kg and a 50% lethal dose (LD₅₀) of 1.4 mg/kg, indicating a therapeutic index (TI) of 17 in this animal model. These results prompted us to test G25 *in vivo* against human malaria parasites in *Aotus* monkeys (13). Animals infected with the *P. falciparum* FVO strain (14, 15) generally develop fulminating parasitemia (Fig. 1A, monkey M100) and if not treated die with parasitemia above 10-20%. Animals were initially treated when parasitemia was approximately 0.1%, around 4 days post-infection. G25 was administered intramuscularly (i.m.) twice daily for 8 days. Three monkeys treated with 0.2 mg G25 per kg body weight (9), were cured 4 days after treatment started and showed no recrudescence at 60 days follow-up (e.g. M151, Fig. 1A). Similar clearance was noted after treatment with quinine (M150) and Fansidar (9).

To determine the lowest effective G25 dose, three monkeys were treated with decreasing doses of G25 at 0.1% parasitemia. At G25 doses of 0.09 and 0.03 mg/kg, parasite clearance occurred at day 4 (Fig. 1B), without recrudescence over 60 days. At a dose of 0.01 mg/kg, parasitemia initially decreased but then rose again at day 2.5. At day 7 and beyond, parasitemia was only detectable in thick blood films, suggesting that 0.01 mg/kg G25 is close to the threshold of therapeutic activity. We determined the LD₅₀ in rats to be 1.4 mg/kg and the maximal tolerable G25 dose under subacute conditions in *Macaca fascicularis* monkeys and beagle dogs to be around 0.9 mg/kg (9). Since *Aotus* monkeys were cured with 0.03 mg/kg, the TI in *P. falciparum*-infected *Aotus* monkeys is at least 30, indicating high *in vivo* efficacy of G25 against *P. falciparum*. In further experiments, G25 efficacy was tested at about 100-fold higher initial parasitemia (5 to 14%). A total of nine *Aotus* monkeys were treated i.m. with 0.2 mg/kg G25 twice daily over 8 days. Eight monkeys cleared the parasites 4 to 5 days after the first dose (e.g., M169, Fig. 1C, (9)), similar to control animals treated with Fansidar (9). One monkey died after 2 days at 19%

parasitemia, most likely owing to an irreversible clinical condition. Two monkeys were challenged 60 days after G25 therapy and found to remain susceptible to parasite infection, indicating that parasite clearance and absence of recrudescence was unlikely to be related to antiparasite immunity. Decreasing the G25 dose to 0.09 mg/kg in one monkey (9) or reducing the therapeutic window to only four days at 0.2 mg/kg in two monkeys (e.g., V59, Fig. 2C) also successfully cured highly infected monkeys without recrudescence. The permanent cationic charge of G25 did not lead us to expect good oral absorption. Nevertheless, oral administration of G25 at 4 mg/kg over 8 days at 4% initial parasitemia appeared fully effective (V51, Fig. 2C). Recent experiments with analogues of G25 designed for good oral bioavailability have shown much higher oral bioavailability in preliminary experiments in non-human primates (16), which is of importance for future first line antimalarials that must generate oral activity. G25 thus fully succeeded in curing *P. falciparum* infected monkeys even at high parasitemia without recrudescence.

The other major human malaria parasite *P. vivax* favors invasion of immature erythrocytes (reticulocytes). Since reticulocytes retain some metabolic activities, these may provide the parasite with compensatory mechanisms under drug pressure. We therefore investigated the activity of G25 against *P. vivax* and against the phylogenetically closely related non-human primate parasite *P. cynomolgi* (17), that also favors reticulocytes. The *in vitro* IC₅₀ values of G25 against *P. vivax* and *P. cynomolgi* were in the same range as against *P. falciparum* (9), indicating high sensitivity of reticulocyte-restricted parasites to this class of drugs.

For *in vivo* studies, we used *P. cynomolgi* in rhesus monkeys. G25 treatment was initiated on day 7 post-infection when parasitemia ranged between 0.2 and 0.5%. Five monkeys were treated (0.15 mg/kg i.m., twice daily, 8 days) and five remained untreated (Fig 2). Treated animals had a peak parasitemia of 0.45 - 1% just prior to administration of the second dose (12 hours post-treatment). From day 4 onwards, parasites were not detectable by thick film analysis. The five untreated monkeys showed a typical blood stage parasitemia development for *P. cynomolgi* M strain in rhesus monkeys (18, 19). A peak parasitemia ranging from 5 to 10% at 3.5 days after the start of treatment was observed, and all control monkeys showed a typical recrudescence (0.2-2.5% parasitemia) at day 10/11. In contrast, no recrudescence was detected in treated monkeys until the end of the experiment

at day 36. At day 3, blood samples obtained from all treated monkeys and from one untreated monkey were washed extensively to remove circulating G25 and cultured overnight to determine whether parasites could recover and develop further in fresh culture medium. Parasites from treated monkeys did not recover, whereas untreated parasites grew well, indicating G25 toxic activity was irreversible.

G25 thus fulfills essential *in vitro* and *in vivo* parameters for the development of a new class of antimalarials. G25 cured infected monkeys at doses of 0.03 mg/kg, far below those used with current antimalarials (chloroquine, quinine and mefloquine). The satisfactory TI is lower *in vivo* in the monkey model (>30) than *in vitro* (~2000). This difference is due to a specific interaction of G25 with the cholinergic system. Experiments with rodents indicate that at very high doses G25 provokes a rapid and transient hypoxia due to interference with respiratory muscles, a decrease in the respiration rate and concomitant strong hemoglobin oxygen desaturation (9). Indeed, the LD₅₀ could be increased ninefold if G25 was given while providing respiration assistance for the rat (9).

To study the interaction of the effectors with infected erythrocytes, we synthesized the tritium-labeled bisquaternary ammonium salt VB5-T (N,N,N',N'-tetramethyl-N,N'-di[2-N(2-(p-³H-benzoyl)benzoyl)aminoethyl]-1,12-dodecanediaminium dibromide) (9). VB5-T is a G25 analogue, predicted by our SAR studies and confirmed experimentally to have potent *in vitro* antimalarial activity (IC₅₀ of 18.3 ± 3.3 nM (SEM, n = 4)). Using enriched *P. falciparum* trophozoite preparations (20), we observed that almost 180-fold more VB5-T was accumulated in infected than in normal RBC (1253 ± 108 pmol and 7 ± 1 pmol per 10¹⁰ cells, respectively). We measured drug accumulation (9) at VB5 concentrations ranging between 0.002 μM and 25 μM after 10 min incubations (Fig. 3A). Accumulation was linear with increasing VB5 concentrations. No saturation was observed around the IC₅₀ of VB5 (Fig. 3A insert) or at concentrations up to 1000-fold above the IC₅₀ of VB5. In a kinetic analysis with 50 nM and 1 μM VB5, accumulation increased linearly over the 3.5 hours time course of the experiment (10). At the same time, the cellular accumulation ratio (CAR), i.e. the concentration ratio of cell-associated VB5 to that in the same volume of culture supernatant, increased linearly to a value of around 300. A steady state was observed only at 25 μM VB5 concentration, (plateau 1600 nmol/10¹⁰ cells after 2 hours,

Fig. 3B). VB5-T accumulation was essentially irreversible; indeed, infected RBC that had been loaded with VB5, washed and re-incubated in fresh medium, retained 95% and 87% of VB5 after 3 hours at 37°C and 4°C, respectively (Fig. 3C). Accumulation was not due to a metabolization-dependent concentration gradient. In fact, HPLC analysis of infected RBC loaded for 1 hour at 37°C with 250 nM VB5-T revealed that more than 80% of the total radioactive material eluted at the same retention time as co-injected non-radioactive VB5-H preparation (9). Finally, VB5-T is not substantially accumulated in human CD4⁺ lymphocytes (CAR of 1.4 and 5.3 after 10 and 60 min, respectively (9)), corroborating the absence of toxicity of G25 in this cell line (9).

Initial fractionation studies using saponin lysis (21) indicated that more than 70% of accumulated VB5-T was recovered in the 100,000g pellets, while less than 20% was present in the erythrocyte cytosol fraction; the parasite cytosol appeared to be virtually VB5-T free (10). VB5-T did not co-purify with hemozoin crystals (22) and Ro40-4388, an inhibitor of hemoglobin proteolysis that interferes with the accumulation of the important antimalarial chloroquine (23), had no effect on VB5 accumulation or on G25 *in vitro* antimalarial activity (at 5 µM Ro40-4388, (10)), indicating that the mode of action of quaternary ammonium salts is likely to be independent of hemoglobin degradation. Furosemide (24), an inhibitor of the parasite-induced new permeation pathway (NPP) (25), had no effect on VB5 accumulation (at 100 µM furosemide, (10)) indicating that VB5 accumulation does not involve NPP. This is in contrast to pentamidine, an antiprotozoal drug of the diamidine compound class (26). Our current hypothesis is that VB5-T accumulates in association with the extensive membrane network surrounding the parasite (27), leading to concentration of the drug at the parasite surface, where it might interfere with choline uptake and consequent PC synthesis by the parasite.

The *in vivo* properties of anti-PL effectors against human malaria in non-human primates indicate that these compounds fulfill many criteria for potential clinical development. Concentration of bisquaternary ammonium salts through accumulation in infected erythrocytes could explain both their potent activity and their specificity for parasitized red blood cells.

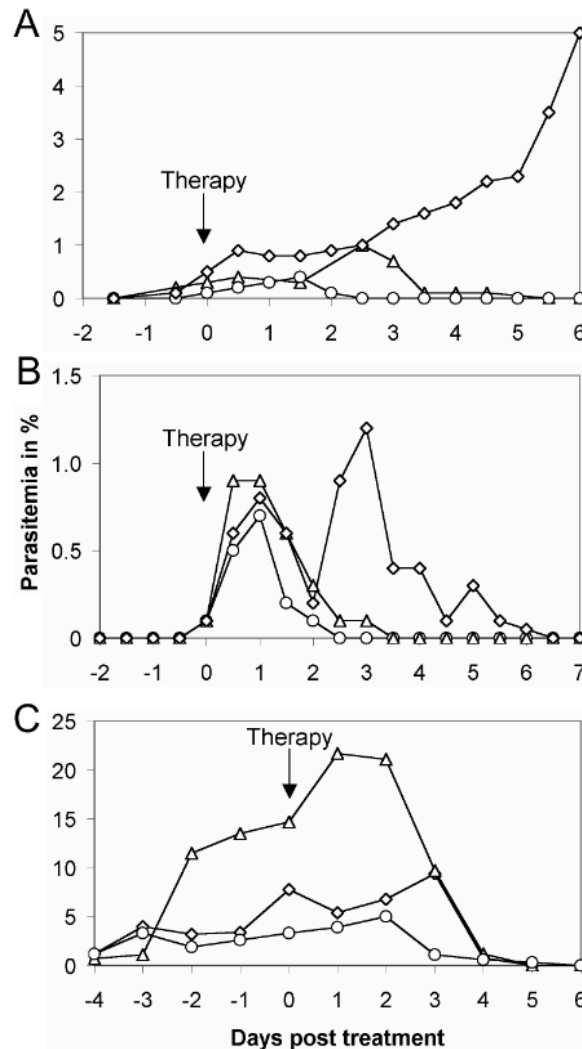


Fig. 1. *In vivo* efficacy of G25 against *P. falciparum* in *Aotus* monkeys.

Aotus lemurinus griseimembra monkeys were inoculated intravenously with 10^5 *P. falciparum* FVO-infected RBC isolated from a donor monkey. Parasitemia was determined by analysis of Giemsa-stained thin films. Antimalarial treatments were started either as soon as patent parasitemia became detectable (generally on day 4-6 post-infection, A and B) or later at higher initial parasitemia (C). The start of therapy was defined as day 0. G25 in saline was administered i.m. at 8 am and 8 pm for 8 days. A. Monkey M151 (circles) was treated with 0.2 mg/kg G25, and M150 (triangles) with a single dose of 30 mg quinine (i.v.). An infected untreated control monkey M100 (diamonds) showed typical development

with steadily rising parasitemia. This animal was cured on day 7 at 8% parasitemia with a single oral dose of 25 mg/kg Fansidar. B. The G25 doses were lowered to 0.09 mg/kg (M67, circles), 0.03 mg/kg (M74, triangles) and 0.01 mg/kg (M156, diamonds). C. Representative data from monkeys treated at initial parasitemia above 5%. G25 was given at doses of 0.2 mg/kg twice daily either for 8 days (M169, diamonds) or for only 4 days (V59, triangles); monkey V51 (circles) received oral G25 doses of 4 mg/kg twice daily for 8 days. All animals were tested for parasites up to 60 days post-treatment using both Giemsa stained thick and thin films and a highly sensitive PCR method based on amplification of species specific sequences from small ribosomal RNA genes, as described previously (30). All animals remained parasite free.

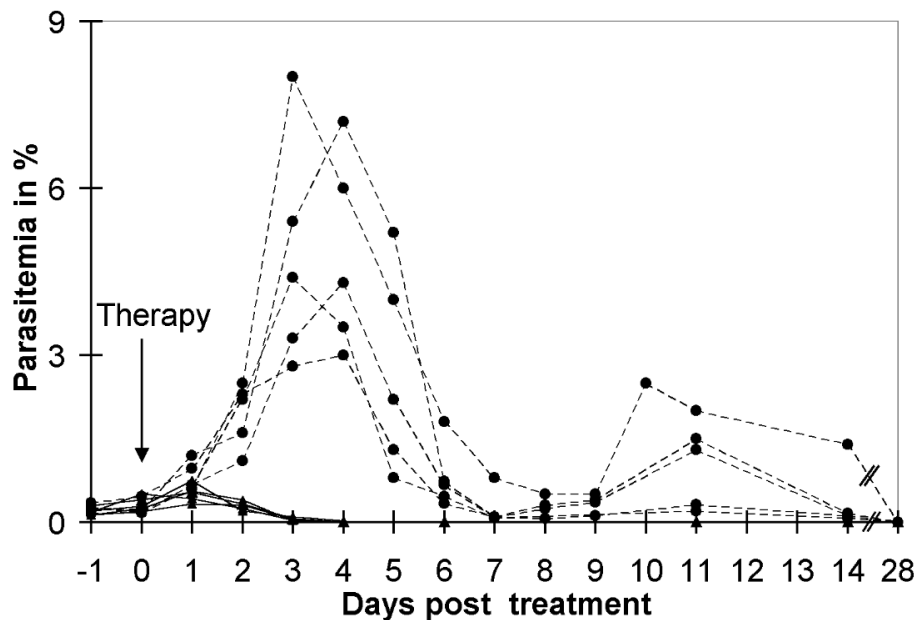


Fig. 2. Efficacy of G25 against *P. cynomolgi* infected rhesus monkeys.

An adult *Macaca mulatta* (rhesus) monkey was infected with *P. cynomolgi* M strain blood stage parasites. At 1% parasitemia, 10^6 parasites were used to infect 10 rhesus monkeys (3 - 6 years, sex matched into two groups of 5). At day 7 post-infection (parasitemia 0.2 - 0.5 %), treatments were started with G25 in saline (i.m., 0.15 mg/kg twice daily, 8 days)

(triangles). Control monkeys (circles) received saline injections. Parasitemia was determined on finger-prick blood using Giemsa-stained thin and thick blood films.

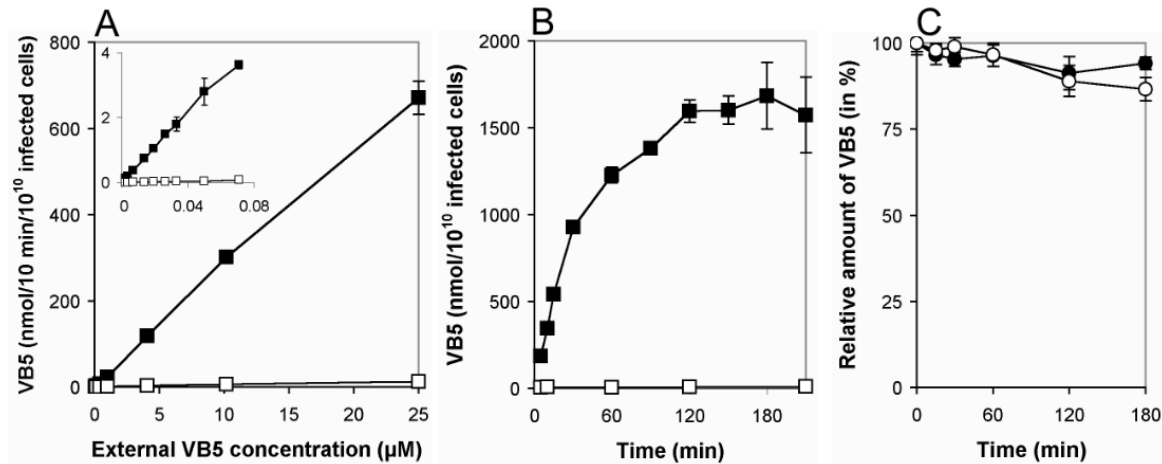


Fig. 3: Accumulation of VB5-T in infected erythrocytes.

A. Accumulation of VB5-T in infected (closed) and normal red blood cells (open) after 10 min at 37°C using a range of external concentrations. B. Kinetic analysis of VB5 accumulation at 25 μM in infected (closed) and normal (open) erythrocytes. C. Release of VB5-T from infected erythrocytes that had been preloaded for 10 min with 50 nM VB5-T. Cells were washed to remove excess VB5 and re-incubated in complete medium either at 37°C (closed) or 4°C (open). Quantities of VB5 remaining with the cells were expressed as a percentage of the amount after the wash (time = 0). In all graphs values are the mean ± SD of one representative experiment performed in triplicate. Experiments were repeated twice.

References and Notes

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8. *P. falciparum* was maintained under standard conditions (28) with human O⁺ erythrocytes in complete medium (RPMI 1640 medium with 10% human AB⁺ serum and 40 µg/ml gentamicin). Human cell lines were cultured as described (29).
9. Supplementary material showing *in vitro* G25 activities, all data on G25 treated monkeys, detailed G25 clinical effects, animal study protocols, VB5 accumulation assays and the chemical syntheses of VB5-T and VB5-H are available on Science Online.
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31. We are grateful to J. C. Doury, P. Ringwald, L. Basco, J. Le Bras, D. Warhurst, WHO, and the Walter Reed Army Institute of Research for testing the compounds in their laboratories and they are gratefully acknowledged for their much appreciated expertise and skillful work. We thank A. van der Wel, M. Dubbeld and S. Rivière for technical assistance and C. Ben Mamoun and R. G. Ridley for critical reading of the manuscript. These studies were supported by grants from the European Union (INCO-DEV, IC18-CT960056, QLK2-CT-2000-01166), CNRS/DGA (GDR 1077), MENRT (PRFMMIP and VIHPAL), and the UNDP/World Bank/WHO special program for Research and Training in Tropical Diseases. K. W. is supported by a Marie Curie fellowship of the European Union.

Supplementary Material

Supplemental Table 1.

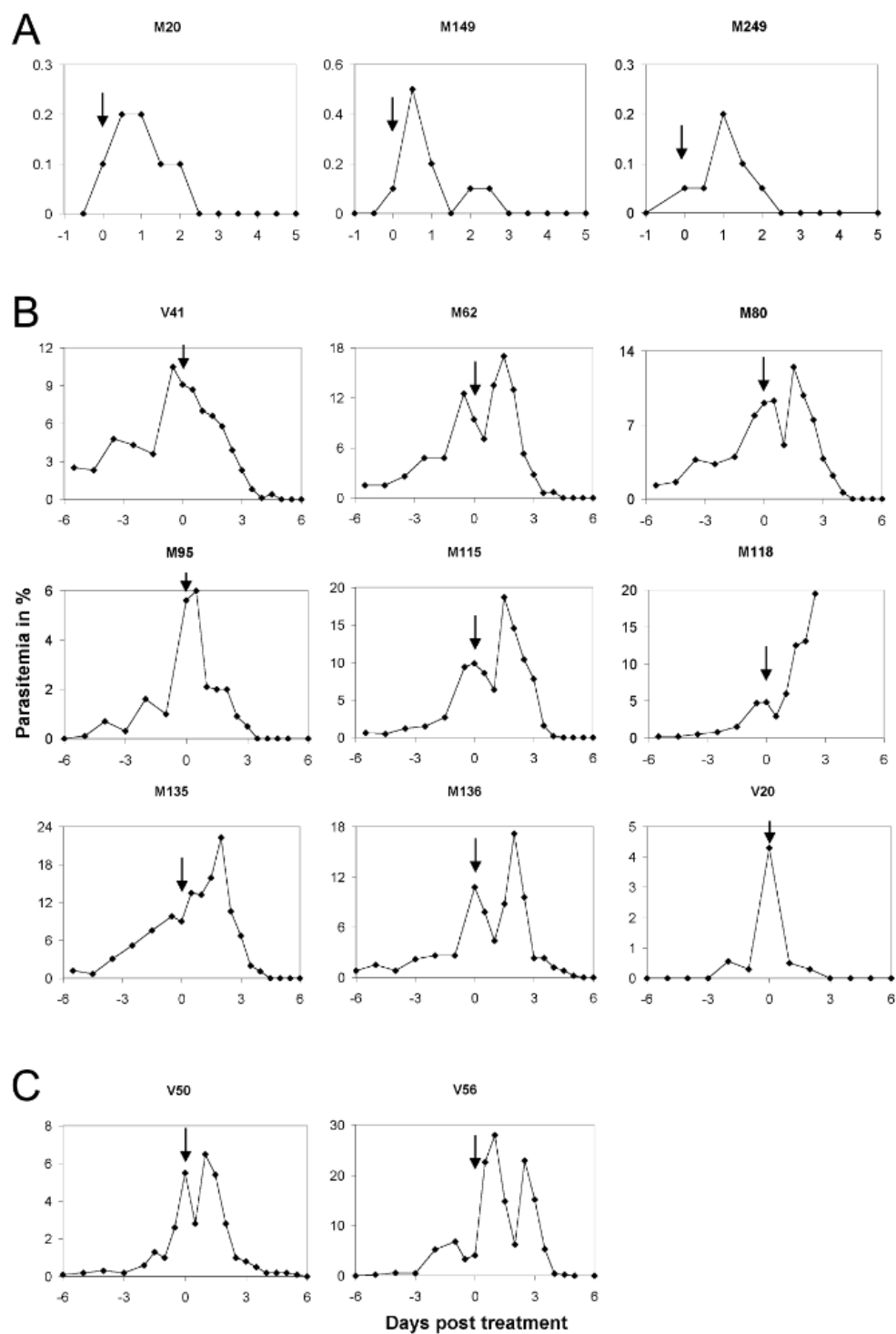
Toxic effect of G25 against malaria parasites and human cell lines *in vitro*

Strain or clone / origin	Laboratory *	CQ IC ₅₀ (nM) †	additional resistance ‡	G25 IC ₅₀ (nM)	G25 IC ₉₀ (nM)
<i>P. falciparum</i>					
D6 / Sierra Leone	WRAIR	9 ^S	MFQ	5.3	10.5
FcM29 / Cameroon	CNRS 5539	260 ^R	nd	1.0	2.9
TM90C2B / Thailand	WRAIR	114 ^R	MFQ	1.9	5.5
W2 / Indochina	WRAIR	191 ^R	Q, Pyr	4.2	9.1
<i>P. vivax</i> (Chesson)	BPRC	12 ^S	nd	6.1	nd
<i>P. cynomolgi</i> (M-strain)	BPRC	4.5 ^S	nd	1.0	nd
Human cell lines					
Jurkat Lymphoblast	CNRS 5539	13000	-	23000	nd
U937 Macrophage	CNRS 5539	27000	-	7000	nd
MEG-01 Megakaryoblast	CNRS 5539	10000	-	6000	nd

* BPRC, Biomedical Primate Research Center, Rijswijk, NL; CNRS 5539, Montpellier, France; WRAIR, Walter Reed Army Institute of Research, Washington DC, on behalf of the World Health Organization.

† 50% and 90% inhibitory concentrations were determined for *Plasmodium* by [³H]hypoxanthine incorporation after contact of the cultures with the drug for 48 hours for *P. falciparum* (1, 2), and 30 hours for *P. vivax* and *P. cynomolgi* (3), and for human cell lines by [³H]thymidine incorporation after 24 hours of contact (4). Values are the mean of two to four independent experiments. ^S and ^R indicate sensitivity and resistance to the drug.

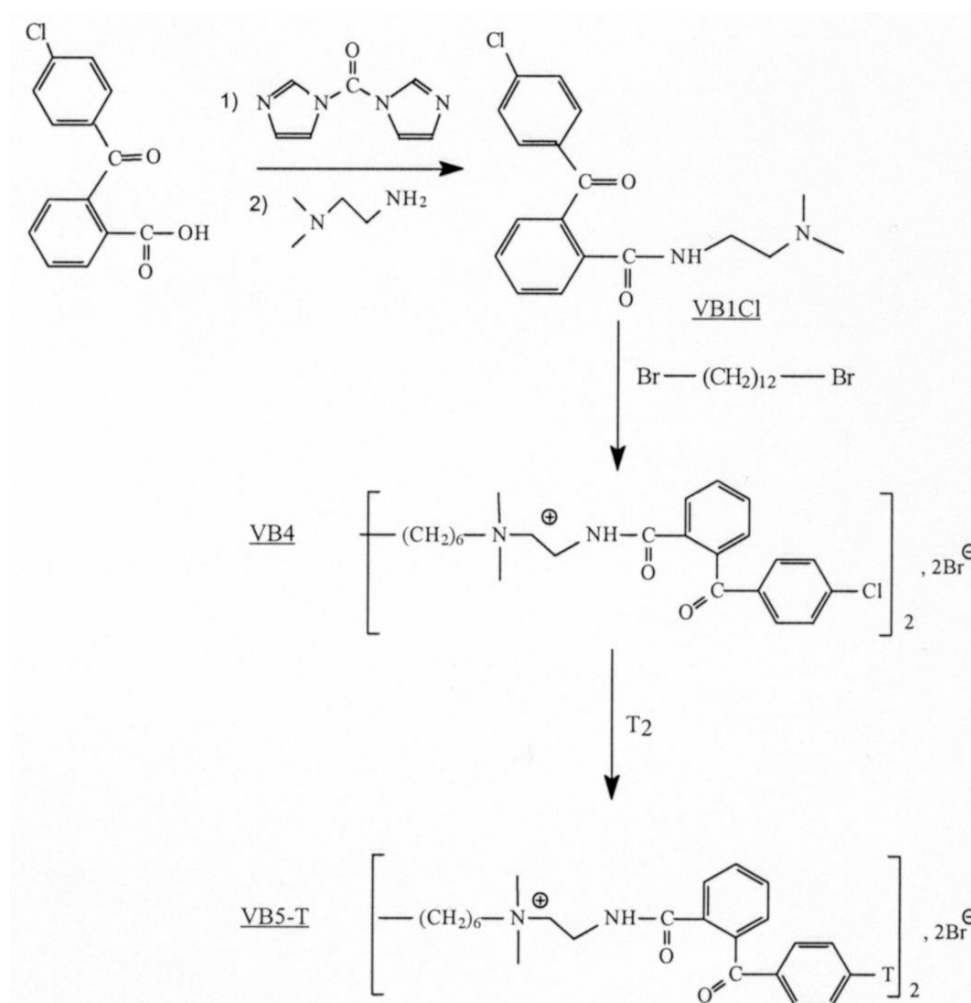
‡ CQ, chloroquine; Q, quinine; MFQ, mefloquine; Pyr, pyrimethamine; nd, not determined



Supplemental Figure 1:

In vivo efficacy of G25 against *P. falciparum* in *Aotus* monkeys.

Aotus monkeys were infected, treated and analyzed as described in figure 1. The start of therapy was defined as day 0 (marked by an arrow). G25 in saline was administered i.m. at 8 am and 8 pm. A. Monkeys M149, M249 and M20 were treated at low initial parasitemia with 0.2 mg/kg G25 (M149 and M249 for 8 days, M20 for 4 days). B. All Monkeys were treated at high initial parasitemia with 0.2 mg/kg G25 for 8 days, except V20 that received a single oral dose of 25 mg/kg pyrimethamine/sulfadoxine (Fansidar). M118 died at day 2 at 19% parasitemia. C. Monkey V50 received 0.09 mg/kg G25 for 8 days and V56 0.2 mg/kg G25 for 4 days. All animals remained parasite free during a follow up period of 60 days.



Supplemental Figure 2:

Chemical synthesis of VB5-T and VB5-H

VB5-T (N,N,N',N'-tetramethyl-N,N'-di[2-N(2-(p-³H-benzoyl)benzoyl)aminoethyl]-1,12-dodecanediaminium dibromide) was prepared by multistep synthesis. Twenty-six mmol of 1,1'-carbonyldiimidazole was added to a solution of 25 mmol of 2-(4-chlorobenzoyl)benzoic acid in 30 ml of dioxane. The mixture was heated under reflux for 30 min, and left for 1 hour at room temperature. Then 25 mmol of N,N-dimethylethylenediamine was added and the mixture was heated under reflux for 10 min, and left at room temperature for 12 hours. The solvent was removed and the crude product was extracted with three portions of dichloromethane. The organic phase was dried over MgSO₄ and evaporated under reduced pressure. N-(2-dimethylaminoethyl)-2-(p-chlorobenzoyl) benzamide (VB1Cl) was obtained with 99% yield. 10 mmol of VB1Cl and 4 mmol of 1,12-dibromododecane were dissolved in 30 ml of ethanol and heated under reflux for 12 hours. The solvent was removed and the pasty residue was stirred with a portion of diethyl ether, filtered, and dried under reduced pressure. N,N,N',N'-tetramethyl-N,N'-di[2-N(2-(p-chlorobenzoyl)benzoyl)aminoethyl]-1,12-dodecanediaminium dibromide (VB4) was recrystallized from methanol/diethyl ether and the product was obtained with 10% yield (melting point 176-180°C). Product purity was assessed by HPLC analysis. A mixture of VB4 (4 μmol in 76 μl of methanol), Pd/Al₂O₃ (22 mg) and triethylamine (20 μl, 144 μmol) in 0.1 ml of methanol was placed in a tritiation reactor, frozen and connected to the automatic tritium gas transfer unit. After reaching a vacuum of 10⁻³ Torr, pure tritium gas was introduced and compressed to 800 mbar. The reaction mixture was brought to 50°C and stirred for 5 hours. The catalyst was filtered off, washed with a small amount of methanol and the solvent and triethylamine were evaporated. VB5-T was dissolved in 5 ml of methanol and the solvent evaporated to eliminate labile tritium atoms (cryodistillation). The total radioactivity recovered was 153.6 mCi. Quantitative and comparative determination (HPLC, UV) revealed 41.7 Ci/mmol (1543 GBq/mmol) specific activity. The radiochemical purity was greater than 95%. Aliquots of VB5-T in methanol were stored at -80°C. The non-radioactive analogue VB5-H was synthesized in the same way, except that in the initial step 2-benzoylbenzoic acid was used instead of 2-(4-chlorobenzoyl)benzoic acid, and that the last tritiation step was omitted.

Clinical effects of G25

For monkeys, the maximum tolerable dose was determined according to the Organization for Economic Co-operation and Development (OECD) Test Guidelines 420 and 425 (5) with slight modifications to maximally reduce the number of animals used. Animals were dosed in a stepwise procedure, using single animals, with the initial dose being chosen as the dose expected to produce some signs of toxicity. Careful clinical observation was made until the animal ceased to display any sign of toxicity and at least once daily thereafter for 2 weeks. Monkeys were tested with one single i.m. dose of 0.2, 0.55, 0.9 and 1.5 mg/kg G25. It is noteworthy that due to the absence of G25 cumulative toxic effects and short elimination half life of G25 (6 h, unpublished data), the values for toxicity are very close under acute (one single dose) and sub-acute (twice a day for 4 or 8 days) conditions.

The dose of 0.2 mg/kg was tested in three monkeys and no clinical sign of toxicity was observed. At 0.5 mg/kg, the symptoms consisted in weakness with a rapid (after 5-10 min) and transient (35-45 min) period of prostration during which the animal was weakly sensible to external stimuli. At acute doses above 0.9 mg/kg weakness can be concomitant with mydriasis, a transient muscular fasciculation and prolonged apnea (with pauses in breathing). Small rapidly contracting muscles (extraocular muscles, head and face) appeared to be impaired before slower contracting muscles (arms and legs) and intercostals muscles (respiratory muscles, diaphragm). In rats similar effects were observed after intravenous administration of 0.6 mg/kg G25, but not at 0.4 mg/kg, while at and above 0.8 mg/kg (i.v.) some animals died. G25 led to a rapid and very transient major hypoxia due to alveolar hypoventilation. Respiratory frequency decreased, respiratory rhythm became irregular with pauses in breathing. The respiratory troubles were concomitant with muscular spasms. Return to baseline respiration was concomitant with, or slightly consecutive to a period of prostration during which the animal was weakly sensible to external stimuli. A hypotensive effect occurred with an absence of adaptation of the cardiac frequency to arterial tension, but baseline tension was recovered after 5 min. No digestive phenomenon and no lachrymal secretion occurred. Micturition was not systematic. The same apparent symptoms were observed in mice. The effects of respiratory paralysis in rodents at 0.6 mg /kg (acute i.v.) were also observed in beagle dogs at 0.5 mg/kg (i.m.).

G25 probably acts as a neuromuscular blocker. An interaction with the cholinergic system (nicotinic receptors of neuromuscular junctions) is also suggested by the fact that intraperitoneal choline administration (150 mg/kg) to mice increased the G25 LD₅₀ 3 to 4-fold. Respiratory troubles were the first cause of mortality since in rats the LD₅₀ could be increased ninefold under assisted ventilation. Hence, in rodents as in monkeys, the effects are comparable regarding timing and development of symptoms with rapid and transitory effects (starting 10 min after injection and disappearing in less than one hour). No apparent cumulative toxic effect occurred after repeated administration (for 5 days), as also attested by the very close values obtained for LD₅₀ under acute and sub-acute conditions.

Animal study protocols

All primate experiments at the BPRC were conducted following Dutch and European laws on animal experimentation. These laws guarantee the qualification of researchers, veterinarian staff and animal caretakers involved in experimental studies and breeding of non-human primates. Primate study protocols were evaluated and approved by an independent animal care and use committee prior to start of the studies. Studies were monitored by a qualified independent veterinarian.

All primate experiments conducted at the Fundacion Centro de Primates were approved by the Animal Ethical Committee of Universidad del Valle, that is constituted according to the Colombian law. The Center uses the European Union "Primate Vaccine Evaluation Network" (PVEN) guidelines for the use of primates in biomedical research (6) and functions in accordance with the NIH guidelines for animal use.

All experiments at the UMR 5539 have been performed in the animal facilities of the CECEMA (Centre d'Élevage et de Conditionnement Experimental des Modèles Animaux) of the University of Montpellier II and in accordance with the current legislation (permission N° A34370).

VB5 accumulation assays in *Plasmodium* infected erythrocytes and human Jurkat cells

A fresh aliquot of VB5-T in methanol (specific activity 41.7 Ci/mmol) was diluted in complete medium and protected from light. Final methanol concentrations in the assay never exceeded 0.1%. For VB5 concentrations above 250 nM, VB5-T was mixed with VB5-H. Experiments were done with trophozoites of the Nigerian strain (7) at 8-13% parasitemia 24 h after the second sorbitol synchronization (8) and with normal erythrocytes. Assays were performed in triplicate in a final volume of 300 μ l and at 1.7% haematocrit in complete medium in siliconized 5 ml glass tubes. The exact number of cells per assay was determined by counting an appropriate dilution in a haemocytometer. Fifty μ l of VB5-T was added to 250 μ l of prewarmed cell suspension and incubated at 37°C. The reactions were stopped by adding 4 ml of ice cold RPMI and immediate centrifugation (200 g, 4°C). The cells were resuspended in 600 μ l of ice cold RPMI 1640 medium and transferred onto a cold oil cushion of 400 μ l dibutyl phtalate (Sigma) in a 1.5 ml microtube. Rapid centrifugation (13000 g, 15 s) collected all cells at the bottom of the tube, separated from the supernatant by the oil. The tubes were frozen in liquid nitrogen, the tube tip cut off and the oil carefully removed after thawing. The cells were dissolved in 75 μ l of "Solvable" tissue solubilizer (Packard), and bleached with 8 μ l 0.1 M EDTA (pH 8) and 30 μ l hydrogen peroxide as indicated by the manufacturer. Radioactivity was quantified using 3 ml UltraGold scintillation solution (Packard) in a scintillation counter (Beckman). Accumulated VB5-T was expressed as nmol/time/ 10^{10} cells. Accumulation of 100% parasitized erythrocytes was calculated as follows: [(P-N)/parasitemia]+N, where P and N are the quantities of VB5-T accumulated for equal numbers of cells in non-purified parasite cultures and normal erythrocytes, respectively.

VB5 accumulation assays with Jurkat cells were performed essentially as described for RBC, except that about 3×10^6 cells (corresponding to the same packed cell volume) were used per assay and that cells were separated from culture supernatants by centrifugation through a 80% dibutyl phtalate/20% dioctyl phtalate cushion. When incubated with 50 nM VB5-T at 37°C, 299 ± 17 and 897 ± 48 pmol VB5-T were accumulated per 10^{10} cells after 10 and 60 min, respectively (mean of three experiments, each performed in triplicate \pm SEM).

Recovery of accumulated VB5 from *Plasmodium* infected erythrocytes

To extract VB5-T that had accumulated in parasite cultures for 1 hour at 37°C, 10^8 cells were washed three times in excess phosphate buffered saline, suspended in 50 μ l H₂O and VB5-T extracted with 3 ml ethanol. After centrifugation, the supernatant was collected, the solvent evaporated and the residue solubilized in 100 μ l ethanol. More than 80% radioactivity accumulated by the cells could be recovered by this procedure. Aliquots of 2×10^6 disintegrations per minute together with 20 nmol of nonradioactive VB5-H were analyzed by HPLC using a 0.39/30 cm C18 column (Waters) over an acetonitrile/water gradient (30% to 70%) for 50 min at a flow rate of 1 ml/min. VB5-H was detected using a photodiode array detector; the tritium content was determined from 0.5 ml aliquots.

Supplementary References:

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