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BASIC BIOCHEMICAL INVESTIGATIONS AS RATIONALE FOR THE DESIGN OF ORIGINAL ANTIMALARIAL DRUGS. AN EXAMPLE OF PHOSPHOLIPID METABOLISM

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The future of antimalarial chemotherapy is particularly alarming in view of the spread of parasite cross-resistances to drugs that are not even structurally related. Only the availability of new pharmacological models will make it possible to select molecules with novel mechanisms of action, thus delaying resistance and allowing the development of new chemotherapeutic strategies. We reached this objective in mice. Our approach is hunged on fundamental and applied research begun in 1980 to investigate the phospholipid (PL) metabolism of intraerythrocytic Plasmodium. *This metabolism is abundant, specific and indispensable for the production of* Plasmodium *membranes. Any drug able to interfere with this metabolism blocks parasitic development. The most effective interference yet found involves blockage of the choline transporter, which supplies* Plasmodium *with choline for the synthesis of phosphatidylcholine, its major PL, this is a limiting step in the pathway. The drug sensitivity threshold is much lower for the parasite, which is more dependent on this metabolism than host cells. The compounds show* in vitro *activity against* P. falciparum *at 1 to 10 nM. They show a very low toxicity against a lymphoblastoid cell line, demonstrating a total absence of correlation between growth inhibition of parasites and lymphoblastoid cells. They show antimalarial activity* in vivo, *in the* P. berghei *or* P. chabaudi*/mouse system, at doses 20- to 100- fold lower than their acute toxicity limit. The bioavailability of a radiolabeled form of the product seemed to be advantageous (slow blood clearance and no significant concentration in tissues). Lastly, the compounds are inexpensive to produce. They are stable and water-soluble.*

Key words: malaria – *Plasmodium* – phospholipid – pharmacology – biosynthesis – lipids

The malaria story has been too macabre, and has been going on far too long now. The end of the story has been announced often, but each time it is put off. Today, no one will seriously risk announcing the disappearance of the killer *Plasmodium* in the near future.

As for malaria vaccine, "the main obstacle to a malaria chemotherapy is the malaria parasite, itself". Indeed, the parasite has managed to escape conventional weapons and even the newest ones, sometimes even before they come out.

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The world urgently needs to definitively rid itself of this deadly parasite and end the story. Since the present array of antimalarial drugs is extremely limited, with the parasite continuously developing new defense strategies, and even cross-resistance, there is no doubt that new strategies must be set forth. However, the parasite will be able to find unsuspected defenses at any moment. In this context, no one can reasonably consider reaching a drug-infallibly level. The best strategy would be to identify various Achilles' heels in *Plasmodium*, to be able to design subtle weapons against them. Increasing the pharmacological targets will consequently increase the weapon battery against this clever enemy.

Empirical screening of compounds is costly and not particularly effective, but may uncover some active compounds. Nevertheless, consid-

ering that resistance is rapidly developed for molecules whole mechanisms of action are related (Peters, 1990), new pharmacological models are absolutely essential for the selection of molecules with novel mechanisms of action to delay resistance. Investigations of parasite mebatolisms, to obtain a deeper understanding of the murderer's makeup and behavior, are the best ways of rationally developing new chemotherapeutic methods.

Our "Interactions Membranaires" laboratory is specifically devoted to the study of biological lipid functions and membrane functions. Our research on malaria was initiated in 1980 and aimed pinpointing everything that could be known about *Plasmodium* lipids with the declared goal of finding new pharmacological targets to destabilize the parasite. We first carried out fundamental research on *Plasmodium* lipids — what are they, where do they come from? Phospholipids (PLs) were soon found to be of major interest since the considerable amounts were biosynthesized by the parasite. In 1984, we effectively demonstrated with commercially available molecules that altering the PL biosynthetic pathway of the parasite caused an irremediable injury which was a life threat it could not recover from (Vial et al., 1984a). It was evidence that this metabolism was quite essential for the parasite and encouraged us to dig deeper through characterization of numerous enzymatic steps and limiting steps of some metabolic pathways. We were attempting to define what might be done to disorganize the metabolism. At this point, vital tools were unavailable, the pharmacology of the PLs did not exist. It was thus necessary to find scientists who could synthesize new molecules at a sustained rate, and hopefully become interested in our problem. This was achieved in 1986 with the Structural Organic Chemistry laboratory at our University. Unfortunately, despite the synthesis of about 100 new compounds, no appreciable improvement in the antimalarial activity of the compounds was reached until April, 1990.

Only at this time did we take a great leap forward with new molecules acting *in vitro* against *P. falciparum*, in the nanomolar range. We immediatly verified that they were able to cure mice from malarial infections of *P. berghei* or *P. chabaudi*. Thus, in addition to defining new pharmacological targets, perseverance was also required. Today, there are good chances that we will able to develop a drug (Vial et al., 1991a) but we are aware that this is only an option. It would be very exciting to succeed in our approach, though we know that the way to reach the final stage is long and intrinsically full of potential pitfalls.

AN OVERVIEW OF LIPIDS INVOLVED IN THE INTRAERYTHROCYTIC STAGE OF *PLASMODIUM*

Growth of the parasite within the host cell and the subsequent formation of up to 6-20 merozoites requires a great increase in total membranes. Four types of membranes are of particular interest in the infected erythrocyte: the erythrocyte plasma membrane, the parasitophorous vacuole membrane, the parasite plasma membrane (pellicle) and the internal parasite membranes (Aikawa, 1988).

For *Plasmodium*, membranes are important in parasite maturation, in nutrients and catabolite exchanges with the surrounding medium, in interactions with the host immune system, but also in the access of antimalarial drugs to the intracellular parasite.

Consequently, in malaria-infected erythrocytes, the total lipid content (a major component of all biological membranes) is 3- to 7-fold greater than that of normal erythrocytes (Holz, 1977; Sherman, 1979, 1984; Vial et al., 1990a). For the present study, concerning *Plasmodium* lipids, we will generally deal with PLs, cholesterol and to a lesser extent with other neutral lipids.

Several studies measuring the cholesterol content of *Plasmodium*-infected erythrocytes have reported increased concentrations of their sterols, following parasitization. However, in rodents, results are complicated by the presence of reticulocytes, which appear at the same time as the parasite. Reticulocytes contain internal membranes that can account for cholesterol increase. In mature mammalian erythrocytes, this cholesterol increase only appears in some monkeys, with decreases sometimes being observed (Holz, 1977; Vial et al., 1990a).

Cellular cholesterol in the infected cells could be derived from two sources. However, published evidence now suggests that the malarial parasite is incapable of *de novo* synthesis of sterols at any parasite developmental stage (Trigg, 1968; Cenedella et al., 1969; Holz, 1977; Vial et al., 1984c), and that cholesterol is almost non-existent in fractions enriched with pure parasites (Rock et al, 1971; Wunderlich et

al., 1991). On the other hand, it is well known that erythrocyte cholesterol is in dynamic equilibrium with cholesterol in plasma lipoproteins, thus exchanges could occur between the parasite, the erythrocyte and plasma. Therefore, any factor that modifys cholesterol exchange between the erythrocyte and plasma, such as parasite invasion or maturation, could account for the reported increase.

Profound changes in plasmatic cholesterol have been reported, representing liver damage that occurs late in parasite infection (Eisenberg et al., 1975; Angus et al., 1971a, Maurois et al., 1981, Dei-Cas et al., 1986). Quantitative chances in the distribution of cholesterol-rich lipoproteins involving increased LDL and decreased HDL (Dei Cas et al., 1986; Sainte Marie & Vial, unpublished), associated with an inhibition of serum lecithin-cholesterol acyl transferase (Maurois et al., 1985), lead to an increase in the free cholesterol, at the expense of cholesterol ester (Angus et al., 1971b; McClean et al., 1976; Holz, 1977; Majumdar et al., 1981; Ononghbu & Onyeneke, 1983). The free cholesterol of plasma lipoprotein can then be readily exchanged with red blood cells, and this is probably the reason for the observed increase. However, we have shown that *P. knowlesi* and *P. falciparum* infected erythrocytes exchange cholesterol with plasma at the same rate and level as uninfected cells (Vial et al., 1984c).

To summarize, cholesterol increase of the erythrocyte *in vivo* is not specific to the infected erythrocyte, but also concerns the uninfected erythrocyte. This increase merely reflects an elevated plasma-free cholesterol, and is largely due to the severe dyslipoproteinemia which accompanies that malaria infection.

Only traces of other neutral lipids are found in normal erythrocytes and are probably parasite-associated, for example diacylglycerol, triacylglycerol and unesterified fatty acids (FAs). The increase, expressed in percentage, is extremely high, but the final amounts remain very low (Holz, 1977; Sherman, 1979).

Increased total lipid content of infected erythrocytes can be largely attributed to an increase in PL content. We would like to emphasize that this increase in the PL content of erythrocytes after infection is a general characteristic, for all *Plasmodium* species. This increase varies according to the parasitic stage, and in the last form, just before merozoite release, infected cells contain as much as 400 to 600% more PLs than normal erythrocytes (Holz, 1977; Vial et al., 1990a).

The plasma membrane of the human erythrocyte contains four major PLs, phosphatidylcholine (PC), phosphatylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM). During infection, PC and PE account for more than 80% of the PLs in the infected erythrocyte. This percentage is even higher in the parasite-enriched fraction. Phosphatidylinositol (PI) which is a minor PL in normal erythrocytes, shows the greatest increase when expressed as a percentage after infection. On the other hand, PS and SM, become virtually absent from plasmodial membranes.

Accompanying the qualitative and quantitative changes in PLs are changes in the amounts and proportions of fatty acids (FA). The most common FAs in normal erythrocytes are palmitic acid (16:0, i.e. 16 carbon atoms in the carbon chain, and no double bond), oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4). For most *Plasmodium* species, the most notable changes are a striking increase in octadecenoic acids, 18:1 and 18:2, and a decrease in arachidonic acid, a polyunsatured FA. We carried out a complete study in *P. knowlesi*-infected erythrocytes by determining the FA composition of individual PLs and neutral lipids. The linoleic acid content was higher in the PLs and neutral lipids (Beaumelle & Vial, 1986). We found that FA pattern were not as altered in choline-containing PLs (PC and SM) as in anionic PLs (PS and PI).

Standard questions concerning the actual composition of the erythrocytic membrane after infection and of the parasite membranes (*i.e.* parasitophorous vacuolar membrane, parasite membrane and organelle membranes) have only been partially answered and with contradictory results. Concerning the lipid composition of the individual parasite membrane, the questions are generally still unresolved, due to the absence of techniques to isolate each of them. What we can state is that the global lipid composition is quite distinct from that of host erythrocytes (Rock et al., 1971; De Zeeuw et al., 1972; MacClean et al., 1976b; Holz, 1977: Van der Schaft et al., 1987, Wunderlich et al., 1991).

Reliable methods for isolating highly purified host cell membranes (Vidal et al., 1989a),

or parasites from infected blood (Wunderlich et al., 1987), have recently emerged and provide original information on possible alterations in host cell lipids. No differences have been found between normal and infected host erythrocyte membranes, concerning cholesterol content and lipid class composition (Gupta & Mihra, 1981; Joshi et al., 1986; Van der Schaft et al., 1987; Joshi & Gupta, 1988; Wunderlich et al., 1991). Nevertheless, thorough studies have revealed several changes in FA composition and particularly in PL molecular species in host cell membranes after infection and even differences between uninfected and normal erythrocytes (Simoes et al., 1990; Beaumelle & Vial, 1988c).

The capacity of parasites to induce subtle but marked changes in host cells membranes could modulate lipid bilayer properties (fluidity for instance), to possibly increase membrane permeability in order to gain better access to nutrients. It should be stressed that PLs are known not to exchange naturally between membranes. Then, lipids dynamics between the parasite and the erythrocyte membrane remain to be clarified.

Other studies reporting decreased SM content, a decline in the cholesterol/PL ratio and reduced exchange rate of cholesterol in the host membrane of *P. falciparum*-infected cells (Sherman & Maguire, 1990) conflict with those reported above, which showed unaltered cholesterol content and class distribution of PLs. Accusing dirty membranes of causing normal cholesterol levels does not stand (Sherman & Maguire, 1990) since contamination by the parasite would actually have lowered the cholesterol and SM content of this membrane due to the quasi-absence of both compounds in the parasite (see above). Although in our opinion this loss of cholesterol probably does not occur *in vivo* in the host membrane, it probably reveals modified properties of the infected erythrocytes (Vial et al., 1990b). This critical question will only be resolved by applying new original fractionation methods.

PLs are asymmetrically distributed over the two leaflets of the erythrocytic membrane. The external leaflet of the membrane primarily consists of the bulk of PC and SM, while the inner leaflet of the erythrocyte membrane is enriched in anionic PLs, with most of the PE and all of the PS (Op den Kamp, 1979). There are also contradictory results in this area concerning the eventual change in distribution after infection (see Vial et al. 1990a). The answer is crucial since the presence of PS in the external leaflet could play a role in the pathophysiology of malaria. Here again, only new and original methodology could offer alternative and definitive answers.

ORIGIN OF NEW PHOSPHOLIPID MOLECULES

The PL composition of the parasite is quite distinct from that of the host erythrocyte. The ways in which the intracellular parasite obtains the PLs required for biogenesis of its membranes is rather clear today.

The first possible source of PLs for the intracellular parasite, would involve the exchange of preformed PLs from the plasma or the host erythrocyte. Some studies have shown that the addition of radiolabelled natural PL molecules or fluorescent analogs (Mikkelsen et al., 1988; Haldar et al., 1989) either as vesicles (Van der Schaft et al., 1987; Moll et al., 1988), or radiolabelled HDL (Grellier et al., 1991) resulted in their appreciable uptake by the infected erythrocytes. The rate and amplitude of the exchange increase with maturation of the parasite (Van der Schaft et al., 1987; Moll et al., 1988). The internalized molecules go to the parasite, where they can be metabolized, in the case of PS and PE, by decarboxylation and methylation respectively. These phenomena can be enhanced by the presence of a PL exchange protein (Van der Schaft, 1987; Moll et al., 1988). On the other hand, the exchange of FA from one PL to another, which would involve phospholipase activity, has never been observed (Van de Schaft, 1987; Moll et al., 1988), except in one report (Grellier et al., 1991). However, in this latter study, the possibility that such a process took place outside of the infected cells cannot be ruled out.

There are rapid movements of PLs between the erythrocytic plasma membrane and the intracellular parasite, at least when additional PLs are introduced into the host membrane. In the absence of transfer proteins, PL vesicles could fuse with the external membrane or enter by endocytosis. A PC-specific transfer protein catalyzes a one-to-one exchange process whereas the nonspecific lipid transfer protein may mediate a net transfer of exogenous PLs. The rapid translocation of PL from the outer

monolayer of the plasma membrane to the inner monolayer (flip movement) (Beaumelle et al., 1988) does not necessarily indicate an enhanced transbilayer flip-flop of endogenous PL since disequilibrium may provide the driving force for an inward movement of the exogenous molecule. Generally, the exchange of preformed PLs cannot account for all PLs found in infected cells, since PE, PI and PS are almost completely absent in normal plasma, nor are they to be found in the plasma of infected animals (Angus et al., 1971b; Holz, 1977; Stocker et al., 1987, Vial et al., 1990a).

For its essential source of lipids, *Plasmodium* possesses all of the enzymatic machinery required for PL synthesis to ensure its development, whereas its host (the mature mammalian erythrocyte) is totally devoid of such metabolic capabilities (Van Deenen & De Gier, 1975). The parasite depends on the host for supplies of PL precursor molecules, polar head groups and FAs that it cannot synthesize.

We will hereafter limit our statements to *Plasmodium* species that invade mature mammalian erythrocytes, such as *P. knowlesi* and *P. falciparum* (see Vial & Ancelin 1991b for an overview). The host cells are incapable of *de novo* synthesis of FA, cholesterol, or PLs. They slowly exchange lipids only to renew their own lipids which are exclusively located in the plasma membrane.

We separately examined the metabolism of various PL molecule constituents using the appropriate radioactive precursors, with two objectives: (i) to explore every possible biosynthetic pathaway; (ii) to quantify, as far as possible, each of the metabolic pathways to define their relative proportions, under physiological conditions. Moreover, to evaluate the significance of each metabolic pathway, we compared the overall PL increase during one *P. knowlesi* or *P. falciparum* cycle to the sum of the synthesis measured in each pathway. This showed that biosynthetic activities of *Plasmodium* can fulfill most of the theoretical PL parasite requirements.

This has led to a complete scheme of PL metabolism (Vial et al., 1990a; Vial & Ancelin, 1991b), a simplified version is shown in Fig. 1. Generally, *Plasmodium* shows a bewildering variety of PL biosyntheses. PC can be synthesized through a *de novo* pathway, but also by methylation of PE, which itself can originate from *de novo* biosynthesis or through the lipid metabolism of serine (see below). Important additional points can be summarized as follows:

– Plasma FAs and lysoPLs are both sources of FAs required by the parasite, and they act by a competitive mechanism (Vial et al., 1982a).

– The metabolic pathway for the *de novo* biosynthesis of PE is distinct from the metabolic pathway leading to *de novo* biosynthesis of PC. Indeed, we have shown that the phosphorylation of choline and ethanolamine are catalyzed by two different enzymes (Ancelin & Vial., 1986a; Ancelin et al., 1986).

– The enzymes catalyzing the final steps in the *de novo* biosynthesis of PC and PE are cholinephosphotransferase and ethanolaminetransferase, respectively. These enzymes are highly selective for individual molecular species of diacylglycerol. This suggests that substrate specificity is responsible for the various molecular species of parasite PLs (Vial et al., 1984b).

– The malarial parasite can biosynthesize PS. However, its level in the parasite is quite low because it is only an intermediate in PE and PC biosynthesis (Vial et al., 1982a). Moreover, the lipid metabolism of serine in PE also involves direct decarboxylation of serine, with subsequent utilization of the formed ethanolamine for the synthesis of an appreciable amount of PE (Elabbadi et al., unpublished results).

– The phosphoinositide cycle is present within *Plamodium*. This cycle might supply intracellular second messengers, which are known to affect intracellular calcium and protein kinase C activities. The function of this cycle in the biology of the parasite has yet to be clarified (Vial & Ancelin, 1991b).

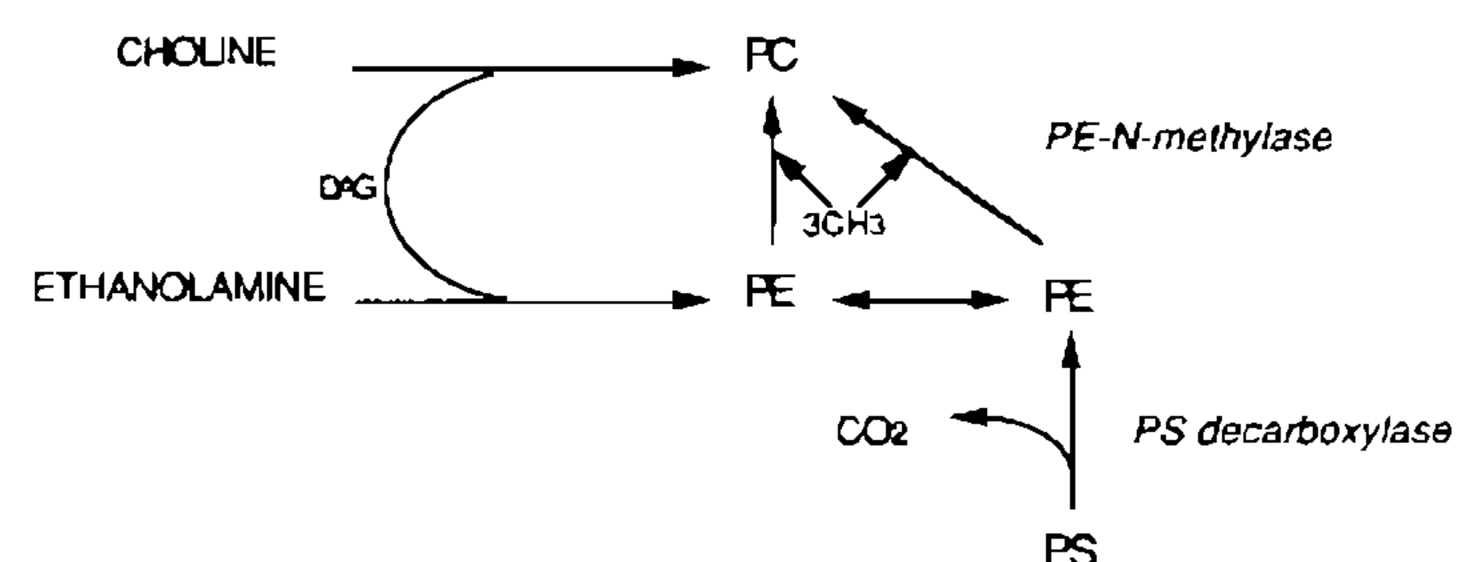

Fig. 1: simplified scheme of the pathways of phosphatidylcholine and phosphatidylethanolamine biosynthesis in *Plasmodium*.

– It has long been suspected that phospholipase A2 is present in *Plasmodium*. The function of the enzyme has even been suggested, i.e. the formation of lysoderivatives at the schizont stage, whose lytic properties could induce the release of the merozoite. However, we have never found such an enzyme in *Plasmodium* by using a number of different methods. Thus the presence of an active deacylation/reacylation cycle of PLs in *Plasmodium* is unlikely. On the other hand, we found very high lysophospholipase activity. This enzyme could allow the use of lysoPLs from the plasma, by releasing their FA part, or it could destroy any lysoPL whose presence might disrupt the infected erythrocyte (Vial et al., 1982a, 1989b, 1990a).

VALIDATION OF PL METABOLISM AS A PHARMACOLOGICAL POINT OF IMPACT

One of the fundamental goals in the biochemical study of *Plasmodium* is the discovery of metabolic pathway that might be considered in the design of drugs capable of exterminating the parasite without injuring the host.

The development of membranes is a crucial step in the generation of the earliest forms of life. Without it, cellular life is impossible. Membranes enclosing every cellular compartment define perimeters and maintain the essential differences between its contents and the environment. However, biological membranes are more than a passive barrier. They are also highly selective filters that maintain unequal concentrations of ions on either side, and allow nutrients to enter and waste products to leave the cells.

We have just seen that PLs are the main constituents of plasmodial membranes, as well as the extent and the specificity of PL biosynthetic activity of *Plasmodium*, carried out in host erythrocytes devoid of any PL metabolism. This killer parasite uses a variety of complex metabolic pathways but, to ensure its development, it depends on the host for supplies of the polar head groups and FA molecules that it cannot synthesize. From these results, an original approach to malarial chemotherapy should arise.

At that point, the only large problems was that the pharmacology of PL was almost nonexistent. Under these conditions, initial experiments were carried out with commercially available compounds, in two phases: (i) the antimalarial activity of some *commercial* analogs of polar heads of PLs or FAs were first evaluated and (ii) their antimalarial activity was related to interference with PL metabolism. It was indeed crucial to verify the mechanism of action of these compounds (disturbance of PL metabolism) and to determine their precise biochemical points of impact.

We first tested the effect of amino alcohols close to choline or ethanolamine, i.e. usually retaining the free hydroxyl. They stopped parasite growth and the EC_{50} of the most active compounds against *P. falciparum* was about 50 µM *in vitro*. A much more detailed study of the mechanism of growth inhibition of one of the compounds showed that a false PL (false metabolite) had been synthesized by parasite, with concomitant dramatic blockage of *de novo* PE synthesis without any simultaneous modification of protein or nucleic acid synthesis or glucose metabolism (Vial et al., 1984a).

We then studied the effects of larger molecules having 1 or 2 quaternary ammoniums, acting as potential choline analogs. These compounds were stronger inhibitors of parasite growth with EC_{50} of about 0.5 to µM. The antimalarial activity of the compounds could not have resulted from a hemolytic effect (doses causing 50% hemolysis were nearly 1000 times higher than the observed EC_{50}) nor from general disturbance of the membrane properties of the host erythrocyte (Ancelin et al., 1985). On the other hand, the drugs selectively inhibited *de novo* PC synthesis by blocking choline entry into infected erythrocytes. By contrast, they showed no effects on the synthesis of other PLs, proteins, or nucleic acids (Ancelin & Vial, 1986b) and did not cause the formation of false metabolites. These commercial compounds helped to validate our chemotherapeutic hypothesis, thus we were able to initiate a collaborative chemical synthesis program.

Finally, infected erythrocytes were known to have a large capacity for taking up FAs and incorporating them into PLs with high specificity (Beaumelle & Vial, 1986, 1988b). We demonstrated the effective inhibition of *Plasmodium* growth *in vitro* by FA analogs (EC_{50} of 7 to 90 µM), which was closely correlated with acyl-CoA synthetase inhibition (Beaumelle & Vial, 1988a).

We were thus able to clearly establish the validity of our hypothesis that PL biosynthesis

in malaria-infected cells is of such high intensity and specificity that the essential reactions involved are crucial for parasite development. PL metabolism of infected cells is a potential novel target for a new chemotherapy of malaria. We therefore assumed that any compound affecting PL biosynthesis was a potential inhibitor of parasite growth.

We also should stress that due to the possibility of affecting the incorporation of different precursors (FAs or the various polar heads) as well as their various metabolic pathways, there is a wide range of other potential means of altering metabolism. This notably includes the methylation pathways of PE into PC, and the lipid metabolism of serine. How vital these two pathways are for parasite survival remains to be demonstrated.

DEVELOPMENT OF THE PHARMACOLOGICAL MODEL; WHERE WERE ARE NOW

Hence, any compound that affects PL biosynthesis is a potential inhibitor of parasite growth, which also means that the PL metabolism of *Plasmodium* constitutes a realistic target for new malarial chemotherapy.

Our program has involved characterizing pharmacological targets (mainly the choline transporter) and the regular chemical synthesis of compounds acting as effectors of this metabolism. Pharmacological interference has been tested with 150 molecules (distributed in seven families), most of them synthesized by the Structural Organic Chemistry Laboratory. Several parameters have been examined with reference to the basic molecular skeleton $-N-CH_2-CH_2-OH$, such as the importance of the hydroxyl group and nitrogen, the length of the chain separating these two units and steric hindrance of the nitrogen atom. These results are described in a report that will be submitted for publication about one year after patent registration.

The pharmacological target – The most effective interference now appears to be blockage of *de novo* PC biosynthesis, more particularly the choline transporter, which supplies the choline precursor for the synthesis of this major *Plasmodium* PL. In fact, four steps take place between the uptake of extracellular choline and its incorporation into PC by the Kennedy pathway of *Plasmodium:* one transport step and three enzymatic steps. We observed that there are two limiting steps, carrier-mediated entry of choline into the cell, and cytidylyltransferase activity. However, cytidyltransferase is not saturated *in situ* by phosphorylcholine since the external choline concentration can regulate PC biosynthesis via the level of phosphorylcholine (Ancelin & Vial, 1989). For this reason, and considering the extracellular location of the transporter, which makes it more accessible than intracellular cytydyltransferase, we chose choline transport as a pharmacological target. Moreover, the compounds inhibiting this step proved to be the most active with respect to their EC_{50} and did not lead to potentially false metabolites.

We have shown that choline entry into infected erythrocytes involves an asymmetric carrier-mediated process which remains rigorously controlled during parasite development, allowing no free diffusion of the precursor. Our results are compatible with a cyclic carrier model in which one substrate site is exposed on one side at a time only (in either an outward-facing or an inward-facing conformation). After infection, the affinity of the transporter for choline does not change, but the entry rate (V_m) is 10-fold higher than that observed in the normal erythrocyte (Ancelin et al., 1991).

General properties of the molecules – The new molecules are inexpensive to produce. They are water-soluble, highly stable, and can be analyzed and assayed using conventional methods.

In vitro efficacy – The new molecules, whose structures have been optimized through structure/activity relationship studies (QSAR), show powerful antimalarial activity with 50% effective concentrations (EC_{50}) in the nanomolar range (Table).

These molecules top the list of three different families of compounds upon which chemical synthesis has been focused. The availability of different families of highly active compounds should increase the chances of finding a molecule that is both effective and physiologically acceptable.

Mechanisms of action – The compounds show specificity of action in two respects, i.e. specificity for the biosynthesis of PC from choline (among other PL metabolisms) and

TABLE

Antimalarial activity of our compounds *in vitro* against *Plasmodium falciparum* or *in vivo* against *P. berghei* or *P. chabaudi* the mouse and acute toxicity intraperitoneally

			Plasmodium	*berghei*	*Plasmodium*	*chabaudi*
Compounds	EC_{50}(nM)[1]	LD_{50}	ED_{50}[2]	TI[3]	ED_{50}	TI
V_2	43	13.4	0.8	17		
BC_{25}	0.27	10	0.5	20	0.22	45
BB_3	1.2	2.4	0.1	24	0.11	22
V_6	2.7	1.24	0.04	31	0.013	98
AQ3B	500	45	2.7	16		
BN_1	2600	28	3.4	8		
V_3	33	3.40-6	0.42	11		
AQ03	800	7.2	1	7		
BC_{26}	17	4.5	0.6	8		
V_{10}	1.1	0.2	0.05-0.1	≤ 4	0.023	10
DB_2	4.9	0.34	0.25	≤ 2		
BB4	29	0.6	> 0.3	≤ 2		
Chloroquine	15	69	2.05	34		

[1]EC_{50}: Efficient concentration$_{50}$ against *P. falciparum in vitro*
[2]Doses are expressed in mg/kg.
[3]TI = therapeutic index = LD_{50}/ED_{50}.

specificity relative to the synthesis of other macromolecules (nucleic acids and proteins), as evidenced by the fact that the PC_{50}* is well below the PE_{50} and NA_{50} in each case.

There is a very close correlation between the antimalarial activity of the compounds and the inhibition of PL metabolism (correlation factor of 0.88, for n = 47) (Fig. 2). Thus, PL metabolism is a very realistic target for new malaria chemotherapy.

The PC_{50} are in the same order of magnitude as the Ki obtained for the choline transporter. An excess of choline causes a significant shift in the curves of inhibition of PC metabolism.

Lastly, it is striking that the compounds show the same antimalarial activity on chloroquine-sensitive and polypharmacoresistant strains.

Specificity of action relative to other cell systems – There is a total absence of correlation between concentrations producing 50% inhibition of parasite growth *in vitro* (EC_{50}) and concentrations affecting the viability of a lymphoblastoid cell line (another cell system showing rapid division) (LV_{50}). Furthermore, comparison of the EC_{50} inhibiting *Plasmodium* growth and that inhibiting choline entry into synaptosomes of the nervous system reveals a different (or opposite) effect on the choline transporter in each of the cell systems.

These results provide clear evidence that the structural prerequisites for inhibition of PL metabolism are highly specific to infected cells and certain compounds could now be devoid of toxic effects.

Acute toxicity and efficacy in vivo – Acute toxicity (over four consecutive days, expressed as LD_{50}) and antimalarial activity (4-day test of Peters, 1970) were determined after intraperitoneal administration of the drug in mice.

Table shows the LD_{50} (drug dose in mg/kg capable of inhibiting parasite growth *in vivo* by 50%) of some of our compounds, along with their therapeutic index (TI) (ratio of LD_{50}/ED_{50}).

*PC_{50}, PE_{50} and NA_{50} correspond to the drug concentrations capable of inhibiting 50% of PC, PE and nucleic acid synthesis from choline, ethanolamine and hypoxanthine, respectively.

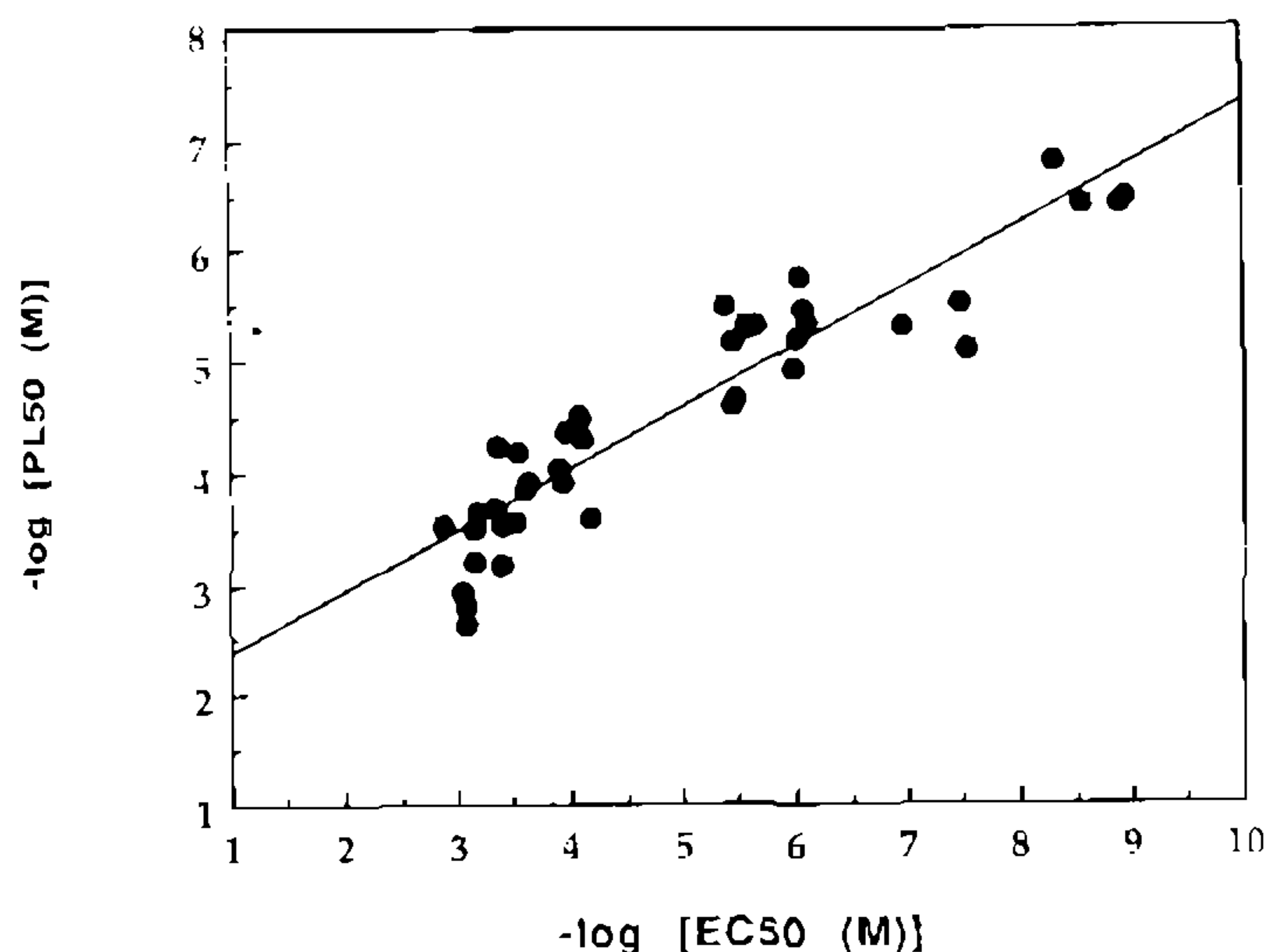

Fig. 2: correlation between phospholipid metabolism inhibition (expressed as PL_{50}) and parasite growth inhibition (expressed as EC_{50}). PL_{50} and EC_{50} correspond to drug concentrations resulting in 50% inhibition of PL metabolism and parasite growth, respectively.

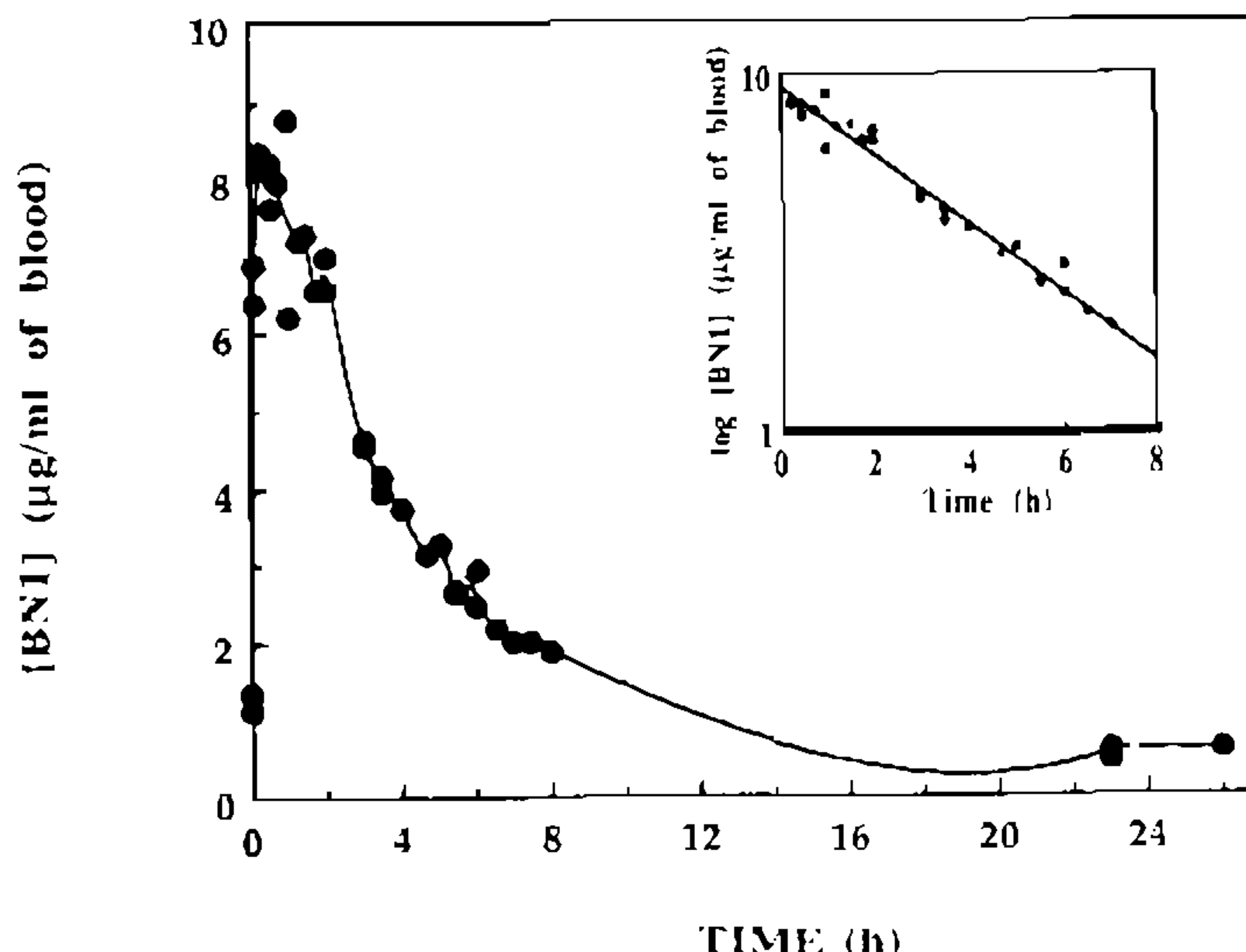

Fig. 3: plasma level or radiolabeled BN1 after subcutaneous administration in mice.

A rapid total disappearance of parasitemia was observed and the antimalarial efficacy *in vivo* can be considered to be satisfactory considering the therapeutic index of the first molecules tested (about 20 for 5 of the 12 molecules tested to date). When these active molecules were tested on *P. chabaudi*, another murine parasite, they showed the same activity, or even much higher, with a therapeutic index that reached 100.

In any case, it now appears possible to administer therapeutic doses of our molecules and our pharmacological approach has thereby become plausible and realistic.

Bioavailability – The fate of a home-made radiolabeled forms of a compound that is moderately active (BN1) (synthesized before discovery of the very active series), but very structurally similar, was determined after subcutaneous administration in the mouse. The compound rapidly appeared in the blood (10 to 15 min). In contrast, blood clearance was relatively slow, i.e. $t_{1/2}$ of the order of 3.5 h (Fig. 3), indicating that it takes 14 h for 94% of the drug to be eliminated.

When these results are extended to humans, taking into account differences in biological rhythms, we obtain a high value for the half-life, i.e. $t_{1/2}$ of the order of 24 hours, or 4 days for almost complete elimination of the drug. This could obviate the need for repeated administration of the drug.

Evaluation of the apparent volume of distribution in the mouse (Vd = 2.2 l/kg) indicated that there was no significant concentration of the compound in tissues.

CONCLUSION AND PROSPECTS

Our work involves a new approach to malarial chemotherapy. This approach has required extensive fundamental research (metabolic pathways and their limiting steps, characteristics of enzymes and transporter, pharmacological studies, mechanisms of action of the compounds, acute toxicity, screening of *in vitro* and *in vivo* activities), as well as the active synthesis of new molecules accompanied by structure/activity relationships. With the elements now at our disposal, we are optimistic about the possibility of creating a new drug. In addition to the curative or preventive treatment of malaria, the compounds will be tested for other properties and for their efficacy against other microorganisms. It goes without saying that the continuation of this program now requires industrial know-how to carry out certain studies (toxicity in different species, drug metabolism, design of a finished drug product, phase-I trials, etc.).

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