Infected Erythrocyte Choline Carrier Inhibitors: Exploring some Potentialities Inside *Plasmodium* Phospholipid Metabolism for Eventual Resistance Acquisition

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We have developed a model for designing antimalarial drugs based on interference with an essential metabolism developed by Plasmodium *during its intraerythrocytic cycle, phospholipid (PL) metabolism. The most promising drug interference is choline transporter blockage, which provides* Plasmodium *with a supply of precursor for synthesis of phosphatidylcholine (PC), the major PL of infected erythrocytes. Choline entry is a limiting step in this metabolic pathway and occurs by a facilitated-diffusion system involving an asymmetric carrier operating according to a cyclic model. Choline transport in the erythrocytes is not sodium dependent nor stereospecific as demonstrated using stereoisomers of α and β methylcholine. These last two characteristics along with distinct effects of nitrogen substitution on transport rate demonstrate that choline transport in the infected erythrocyte possesses characteristics quite distinct from that of the nervous system. This indicates a possible discrimination between the antimalarial activity (inhibition of choline transport in the infected erythrocyte) and a possible toxic effect through inhibition of choline entry in synaptosomes.*

Apart from the de novo *pathway of choline, PC can be synthesized by N-methylation from phosphatidylethanolamine (PE). There is a* de novo *pathway for PE biosynthesis from ethanolamine in infected cells but phosphatidylserine (PS) decarboxylation also occurs. In addition, PE can be directly and abundantly synthesized from serine decarboxylation into ethanolamine, a pathway which is absent from the host. The variety of the pathways that exist for the biosynthesis of one given PL led us to investigate whether an equilibrium can occur between all PL metabolic pathways. Indeed, if alternative (compensative) pathway(s) can operate after blockage of the* de novo *PC biosynthesis pathway this would indicate a potential mechanism for resistance acquisition. Up until now, there is no evidence of such a compensative process occurring in* Plasmodium*-infected erythrocytes under physiological conditions. Besides, the discovery of a highly parasite-specific pathway (serine decarboxylation and the presence of PS synthase) constitutes a very attractive and promising target, which could be attacked if resistances are built up against choline analogs. Indeed, potential inhibitors of the serine decarboxylase pathway could be very useful in acting instead of, or in synergy with, choline analogs.*

Key words: malaria - phospholipid metabolism - choline carrier - serine lipidic metabolism - resistance

The increasing polypharmacoresistance of malarial parasites to conventional drugs calls for new original molecules. From this perspective thorough biological and biochemical studies of the parasite would facilitate the discovery of specific metabolic pathways that might be exploited in the design of drugs capable of exterminating the parasite without injuring the host. This is the rationale of the approach we have developed, in choosing PL metabolism as a potential target. Indeed, this metabolism is very intense in the infected cell: after infection, there is an increase by as much as 6-fold in erythrocyte PL content. This large quantity of PL is needed by the parasite for the biogenesis of its membranes. To synthesize PL the parasite possesses a variety of complex metabolic pathways which are absent from the host, the mature mammalian erythrocyte (Vial & Ancelin 1992). But, the parasite depends on the host for the supplies of the polar head

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groups and fatty acid molecules that it cannot synthesize. Thus, many steps within the PL metabolism could be impaired and lead to parasite death. This includes access of the precursor molecules to the intracellular parasite as well as all the enzymatic steps involved in their metabolism (Vial et al. 1992).

PC is, along with PE, the major PL of the infected erythrocyte and we therefore chose PC de novo biosynthesis as a pharmacological target. Choline carrier is able to regulate and limit the supply of the extracellular nutrient choline to the parasite (Ancelin & Vial 1989). Besides, it is located in the erythrocyte plasmic membrane (extra parasitic) and thus constitutes a very accessible pharmacological target for blockage by inhibitors. The more specific the choline carrier characteristics of the infected erythrocyte are, the lower the toxic side-effects due to possible impairment by PL metabolism inhibitors of other enzymatic reactions involving choline as substrate will be.

Apart from by the *de novo* pathway, PC can be biosynthesized from PE, by N-methylation. The knowledge of the potential ways for resistance acquisition against PC metabolism inhibitors thus involves characterizing the various metabolic pathways involved in PL (and notably PC) metabolism and also investigating the possibility of an equibrilium between all of them.

SPECIFICITY OF CHOLINE CARRIER OF THE INFECTED ERYTHROCYTE VERSUS THAT OF THE NERVOUS SYSTEM: DISTINGUISHING BETWEEN ANTIMALARIAL ACTIVITY VERSUS POSSIBLE TOXIC EFFECT

Choline transport in infected erythrocytes was characterized in comparison with the choline transport of the nervous system. Newly synthesized compounds with potential antimalarial activity (Vial et al. 1994) are choline analogs and thus potentially capable of exerting a toxic effect in nervous system where choline and its metabolite, acetylcholine, are substrates for several reactions (High Affinity and Low Affinity Choline Transport in synaptosomes, HACT and LACT, respectively, choline acetyltransferase, acetylcholinesterase and cholinergic - muscarinic or nicotinic - receptors). We thus compared the characteristics of choline transport in the infected cell and that of the synaptosomes (HACT and LACT), the first step of choline metabolism in the nervous system, to distinguish the pharmacological effect from a possible toxic effect.

In the infected erythrocyte, choline entry occurs by a facilitated-diffusion system involving an asymmetric carrier which can be inhibited by N-ethylmaleimide when facing inwards (Ancelin et al. 1991). The carrier operates according to the cyclic model in which one substrate site is exposed on only one side at a time. Reorientation of the empty carrier is the rate limiting step of choline transport. Affinity for choline is the same as that of normal erythrocyte (Kt = 9 μM). After infection, the transport rate (Vm) is 10-fold higher than in normal erythrocytes (Ancelin et al. 1991).

In synaptosomes, choline can be transported by the HACT (Kt for choline = 1.6 μM), which is Na^+ dependent or by the LACT (Kt = 144 μM) which is Na^+ independent (Ferguson et al. 1991). Choline transport involves two steps, a recognition step of the carrier by the substrate, choline, and a translocation step of the substrate from outside to the interior of the cell. Choline stereoisomers at α or β position of the nitrogen (α or β -methylcholine), have distinct effects on these two steps and also depending on the HACT or LACT system. The HACT recognizes chiral compounds with a partial stereoselectivity, whereas it shows an absolute stereospecificity for the translocation step of both optically active substrates (α and β -analogues). Like HACT system, the enantiomers of α -methylcholine were distinguished by the Na^+ independent LACT (only (+) isomers being transported and not (-) isomers) but it exhibited no stereospecificity with respect to β-methylcholine, by contrast with HACT.

SODIUM DEPENDENCE OF CHOLINE TRANSPORT

The determination of the kinetic parameters of choline transport in erythrocytes was first performed by incubating the cells in buffers, differing by their ionic composition (see Table I). Considering that long term preincubation with extensive washing was required to completely deplete the cells of sodium and to supplement them in a buffer containing the desired cation (sodium, lithium, magnesium, or control), these experiments were only performed with fresh normal simian erythrocytes, since pure infected erythrocyte viability could be considerably altered under these drastic conditions.

Under our standard conditions in the physiological choline-free RPMI 1640 medium, the kinetic parameters of simian erythrocytes choline transport, Kt and Vm, were 7.2 ± 1.2 μM and 0.28 ± 0.05

TABLE I

Ionic dependence of kinetic parameters of choline transport in simian erythrocytes -Cells were preincubated for at least 5 h at 37°C with gentle stirring in the indicated choline-free medium consisting in RPMI 1640 or in a physiologic medium (M) containing 4 mM KCl, 1 mM CaCl2, 20 mM Hepes (pH 7.4) (A and B) and the indicated supplements consisting of 137 mM NaCl or LiCl, 70 mM Mg^{2+} or 250 mM sucrose. During this period, cells were pelleted and resuspended (8-fold) with fresh corresponding medium to allow complete ionic depletion. Then, cells were resuspended in the same medium and choline influx was measured by rapid mixing of 750 µl of suspension containing 1.8 10^8 infected erythrocytes with 50 µl of [^{3}H] choline (0.07-0.2 Ci/mmol) at a final hematocrit of 3.4 %. After 6 min at 37°C, the reaction was stopped by adding an excess of cold incubation buffer, and cell suspensions were aliquoted and overlayed on ice-cold n-dibutyl phtalate and centrifuged at 10000 g for 10 s at 4°C as described previously (Ancelin, et al., 1991). Specific choline uptake was determined by substracting values obtained with incubations carried out in the presence of 1.2 mM choline. Kinetic parameters were determined by Lineweaver and Burk analysis. A and B correspond to two independent experiments (2 different monkeys) carried out to detect Na^+ dependence.

Assay medium	K_t (µM)	V_m (nmol/10^{10}cells/min)
RPMI[a]	7.2 ± 1.2	0.28 ± 0.05 (n=5)
Medium M supplemented with		
A.		
Sucrose	7.1	0.23
Na+	11.2	0.27
Li+	11.9	0.21
B.		
Na+	9.0	0.24
Mg2+/sucrose[b]	7.0	0.24

[a] standard assay conditions (Ancelin et al. 1991). [b]In these experiments, to avoid complications arising from a change in the chloride distribution ratio, isotonic MgCl2 was replaced by a buffer containing 70 mM MgCl2 and 70 mM sucrose, thus keeping tonicity and concentration of external chloride the same as in all the other buffers used.

nmol/10^{10} cells/min (n = 5), respectively (Ancelin, et al., 1991). Replacing RPMI medium by sucrose (in the absence of Na^+) did not result in any significant alteration either in the carrier's apparent affinity for choline or in the Vm.

Adding Na^+ to the incubation medium provoked an increase in the Kt value, by 25 to 50 %, depending on the experiments (B or A, respectively) without any significant modification in the Vm. When the external Na^+ is replaced by another monovalent cation, such as Li^+, the apparent affinity of the influx of choline is not significantly modified compared to the Kt in the presence of Na^+, but the unidirectional influx was slightly decreased (-24%). Replacing external Na^+ by divalent cation (Mg^{2+}) did not result in any significant alteration either in the carrier's apparent affinity for choline or in the Vm. Hence, choline transport rate in erythrocytes is not dependent on sodium by contrast with HACT (Wheeler 1979), but the carrier affinity for choline is dependent on monovalent cations. In fact, these results are consistent with the idea that monovalent cations can compete with choline for the transport site (with a Ki value in the molar range) (data not shown), leading to the conclusion that the choline carrier may be described as a cation carrier with a high affinity for choline (around 8 µM), very weak affinities for monovalent cations (Li^+>Na^+) and no affinity for divalent cations (Mg^{2+}) as already described for human normal erythrocytes (Martin 1972).

STEREOSPECIFICITY OF CHOLINE TRANSPORT IN ERYTHROCYTES AND EFFECTS OF CHOLINE SUBSTITUTION

The stereospecificity and effect of substitutions at position α or β of the nitrogen of choline were then studied on choline transport in normal and

P. knowlesi-infected simian erythrocytes. Results were compared with the data of the literature concerning choline entry in the synaptosomes (Ferguson et al. 1991) (Table II).

For infected erythrocytes, β-methylcholine was more active than the α-analogue for choline transport inhibition (IC50 differed by 3 to 13-fold). The same relative sensitivity was obtained with normal erythrocytes as compared with infected erythrocytes, i.e. β-methylcholine being a better inhibitor than α -methylcholine (by 9 to 38-fold). By contrast, for the HACT synaptosome system, α-methylcholine was a better inhibitor than the β-analogue as attested by a difference both in IC50 and also Ki (by 4 to 5-fold).

For infected and also normal erythrocytes, irrespective of the analogue, α or β, no significant difference was noted in the IC50 between the different stereoisomers (≤ 2.5x). By contrast, for HACT, a high stereospecificity was demonstrated either with α analog or β analog which showed the following order of activity: R(+)α > S(-)β (by 8-fold) and S(+)β>R(-)β (by 6-fold).

Thus, choline transport in infected erythrocytes is not stereospecific as it is in the synaptosomes of the nervous system. Furthermore, substitutions at the α or β position of the nitrogen (see Table II), or modifications in the steric hindrance of the nitrogen (data not shown) have quite distinct consequences on choline transport in infected erythrocytes or in the synaptosomes (HACT and LACT). Indeed, milder constraints are known to exist upon binding in system in which transport is coupled to the movement of sodium ions (e.g. HACT) than upon facilitated transport (erythrocytes) (Krupka 1990).

The choline carrier in the infected erythrocyte possesses characteristics quite distinct from that of the nervous system, concerning sodium dependence, stereospecificity and effect of nitrogen substitution. We have not studied the choline carrier characteristics of other eukaryotic cells. However, from a general point of view, the relatively high affinity for choline, energy independence (facilitated diffusion), constraints upon binding (as evidenced by the effects of various inhibitors, such as triethylcholine, 2 dimethylethanol or hemicholinium 3), makes choline transport in the erythrocyte quite distinct from that of others eukaryotic cell types (Krupka 1990, Lerner 1989, Wright et al. 1992). Besides, we have already described the total absence of correlation between concentrations of choline carrier inhibitors leading to inhibition of *in vitro* parasite growth and concentrations affecting viability of human lymphocytes (Vial et al. 1993). The sensitivity threshold to these pharmacological agents is much lower for the parasite, which is more dependent on PL metabolism than other host cells. This indicates a clear discrimination between the antimalarial activity

TABLE II

Stereospecificity of choline transport: effects of α- and β- methylcholine on the choline carrier of erythrocytes and rat cortical synaptosomes (HACT) -Normal simian erythrocytes (NE) or *Plasmodium knowlesi*-infected erythrocytes (1.8 10^8 cells) were first preincubated for 5 min at 37°C in choline free RPMI 1640 in the presence of increasing concentrations of the indicated methylcholine analogue. Choline influx was then initiated by the rapid addition of 8 μM [^{3}H]choline (5 Ci/mol). After 6 min at 37°C, the reaction was stopped at 4°C by adding an excess of cold special RPMI as described previously (Ancelin et al. 1991). Inhibition of choline influx was expressed as the concentration of the effector decreasing the entry of 8 μM-[^{3}H]choline entry by 50% (IC50). Results are mean of two independent experiments, carried out in triplicate

Methylcholine Analogues	IRBC	NE	HACT[a]	
	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	KI (μM)
R(+)-α[b]	320	420	6.4	7.5
S(-)-α	620	850	52	47.1
S(+)-β[c]	110	44	32	32.3
R(-)-β	50	22	190	190.3

[a:] results from (Ferguson, et al., 1991). [b,c:] corresponds to 1-hydroxy-2-N,N,N-trimethylpropylammonium and 2-hydroxy-1-N,N,N-trimethylpropylammonium iodide, respectively.

(choline transport in the erythrocyte) and the possible toxic effect (against others host cells).

PL METABOLISM: INTERREGULATIONS INSIDE INFECTED ERYTHROCYTE

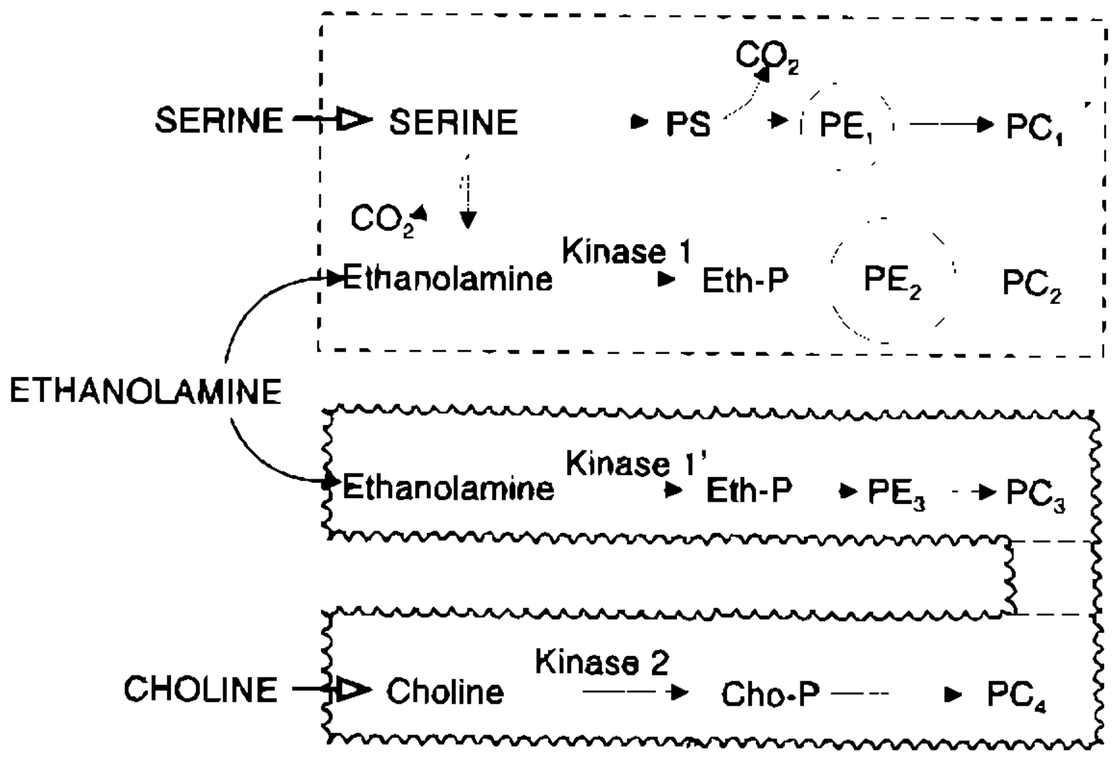
In *Plasmodium*-infected erythrocytes, various biosynthetic pathways coexist to synthesize one given PL, raising the question of whether an equilibrium might exist between all these metabolic pathways. Consequently, PL molecules might be furnished by any of these metabolic pathways. This evidences the necessity to determine whether or not the parasite would be able to compensate any blockage of one biosynthetic metabolic pathway (notably *de novo* PC biosynthesis by choline analogs) by favouring another alternate metabolic pathway. Such a compensation could lead to resistance induction against choline carrier inhibitors. An appropriate response would then be to block the other pathway involved in PC biosynthesis, thus totally locking the main pathways for PC biosynthesis. Of course this involves the identification and characterization of all the possible metabolic pathways for PL biosynthesis.

HOW PE AND PC ARE BIOSYNTHESIZED IN OTHER ORGANISMS?

In bacteria, PS formed via the *de novo* CDP-diacylglycerol dependent pathway is the exclusive source of PE and PC by successive decarboxylation and methylation respectively (Dowhan 1992, Vance & Vance 1991). In most mammalian cells, the CDP-polar head (ethanolamine or choline) pathways are regarded as the main routes for the biosynthesis of PE and PC, respectively (Vance 1991), except in some tissues (BHK-21, Chinese Hamster Ovary cells,...) where PS decarboxylation is the major pathway for PE biosynthesis (Miller & Kent 1986, Vance 1991, Voelker 1984). Let us also note that in the liver, PC synthesis by PE-N-methylation is substantial (Vance 1991). In the yeast, both pathways coexist and there is a control mechanism sensitive to the presence of soluble precursors of PL which contributes to the orientation of PL biosynthesis either towards PS de novo biosynthesis (as in bacteria), in the absence of polar heads such as inositol and ethanolamine (or choline), or towards CDP-polar head pathways, i.e. Kennedy pathways (as in higher eukaryotes) when these polar heads are present (Carman & Henry 1989).

THE VARIOUS PATHWAYS FOR PL METABOLISM IN *PLASMODIUM*

In *Plasmodium*, apart from the *de novo* pathway from choline, PC can also be synthesized by N-methylation from PE. For PE biosynthesis, several pathways also exist (Fig.). One pathway occurs via the de novo pathway (Kennedy pathway) from ethanolamine which enters the infected cell by passive diffusion in contrast with choline entry which involves a carrier (Ancelin et al. 1991). In this pathway, ethanolaminephosphate cytidylyltransferase is most probably the rate limiting step (unpublished observations). Two other pathways involve serine as precursor. The first one occurs via PS synthase and decarboxylase, leading to PS and PE, respectively, with the latter being then intensively methylated to PC. A second newly discovered pathway involves a direct decarboxylation of serine into ethanolamine, which is then phosphorylated into phosphoethanolamine and subsequently incorporated into PE. The serine decarboxylation step is absent from the host (Elabbadi et al. manuscript in preparation).



Biosynthetic routes for PL synthesis from serine and ethanolamine in Plasmodium-infected erythrocytes and possible intracellular compartmentation.

We have recently characterized PS synthesis in *Plasmodium*-infected erythrocyte (Elabbadi et al. manuscript in preparation). Rather than a base exchange mechanism, which is typical for PS biosynthesis in eukaryotic cells (Vance & Vance 1991), it involves a CDP-diacylglycerol dependent PS synthase activity, as it is the case in the prokaryotes (Dowhan 1992).

INTERREGULATIONS INSIDE PL METABOLISM

Faced with the bewildering variety of ways in which *Plasmodium* can synthesize a given PL, we have investigated whether an equilibrium could occur between all the various metabolic pathways leading to PE and PC. Indeed, it is particularly important for our pharmacological approach to determine whether there could be an alternative pathway able to compensate an inhibition of PC de novo biosynthesis by choline analogs. This could be especially important for potential resistances.

For this purpose, we modulated the metabolic flux through one pathway (the de novo pathway) by the supply of increasing polar head concentrations, looking at the consequence on the possible alternative pathway. The only interregulations noted in *Plasmodium* concerned inhibition of the PE-N-methylase pathway by choline concentration higher than 100 μM choline, inhibition of PS synthesis by ethanolamine concentrations higher than 160 μM and an inhibition of *de novo* PC biosynthesis around 0.5 mM ethanolamine (Ancelin et al. manuscript in preparation). Considering the high (unphysiological) polar head concentrations required to produce these inhibitory effects, one can conclude that no effective coordinated regulation probably occurs in Plasmodium under physiological conditions. This would only occur if polar head concentrations are greatly increased in malaria infected plasma (which remains speculative).

Thus, the complex regulatory cascade responding to the presence of soluble PL precursors and capable of exerting control over the transcription of structural yeast genes (Carman & Henry 1989, Nikoloff & Henry 1989) does not seem to function in *Plasmodium*-infected erythrocytes.The absence of effective coordinated regulation in *Plasmodium* between PL metabolic pathways under physiological conditions was also confirmed in cases of specific blockage of *de novo* PC biosynthesis by choline analogs (absence of activation in PE-N-methylation) (Ancelin et al. 1985) or *de novo* PE biosynthesis interference with ethanolamine analogs (absence of activation of serine decarboxylation into PE) (Vial et al. 1984) at least after short-term incubations.

PL METABOLISM COMPARTMENTATION IN THE INFECTED ERYTHROCYTE

The above study shows an absence of interference between choline and serine metabolism, in contrast with the partial reciprocal mixing of PE biosynthesized from serine or ethanolamine in Plasmodium-infected erythrocytes. The possibility or not of an equilibrium existing between certain PL metabolic pathways supports the hypothesis that newly biosynthesized PL could consist partly of mixable or non-mixable metabolic pools leading to intracellular compartmentation according to their synthesis pathways (Elabbadi et al. manuscript in preparation). The pathway which occurs via PS synthase and decarboxylase, leads to a small pool of PE (PE1) which is highly methylated into PC (PC1) (see Fig.). The second pathway which involves direct decarboxylation of serine into ethanolamine, leads to a large pool of PE (PE2) whose methylation into PC is very low. The PE synthesized from serine supply a pool which is different from that resulting from the exogenous *de novo* biosynthesis (PE3). Altogether, this makes *Plasmodium*-infected erythrocyte a highly compartmented organism regarding PL metabolism.

CONCLUSION

Choline entry in *Plasmodium*-infected erythrocyte involves a specific carrier whose blockage by choline analogues is effectively lethal to the malarial parasite. Some of these compounds are capable of curing highly infected mice (with a parasitemia higher than 10%) (see Ancelin et al. in the same issue). Choline carrier in the erythrocyte was thus further characterized to identify some specific features, notably in comparison with the nervous system where choline is also substrate for various enzymatic reactions. The present study provides evidence that the choline carrier in the erythrocyte possesses specific characteristics regarding ionic dependence, stereospecificty, substitutions at the α or β position of the nitrogen which makes possible the tolerance of the host for these choline analogues.

At the present time, we are also currently evaluating the possible mechanisms that the parasite could develop to induce resistance against choline analogues. Predicting these mechanisms saves time in shortcutting resistances by developing other ways to attack this metabolism. The present study which concerns the various metabolic pathways leading to PC (from choline, ethanolamine and serine) is thus particularly relevant in the case of resistance induced against choline analogs that are inhibitors of de novo PC biosynthesis. At the present time, our

data provide no indication of the occurrence of compensative process in plasmodial PL metabolism.

Nevertheless, in the long term or after high drug pressure, the possibility that such a process could occur cannot be excluded. In this case, the addition of other inhibitors (for instance, serine lipidic metabolism inhibitors) could prevent the expression of the alternative pathways thus totally locking the main pathways for PC biosynthesis. Indeed, the highly parasite-specific pathway (serine decarboxylation) could constitute a very attractive and promising pharmacological target. If PS biosynthesis effectively proceeds in *Plasmodium* via the CDP-diacylglycerol *de novo* pathway as it is the case in procaryotes, this will also provide an additional potential target, absent from the eukaryotic host. Hence, if resistances are built up against choline analogs, analogs of serine that could be either potential inhibitors of the serine decarboxylase pathway or of the PS *de novo* biosynthesis could be very useful in acting instead of, or in synergy with, choline analogs.

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