

RESEARCH ARTICLE

# Effect of a bis-thiazolium compound on the biosynthesis of *Plasmodium falciparum* phospholipids

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## Abstract

In the eukaryotic cell, phospholipids can be biosynthesized by two pathways, one from choline and the other one from ethanolamine. The functional effectiveness of each pathway depends on the type of the cell. Thiazolium designed-drugs have shown, under *in vivo* conditions, antiplasmodial and antimalarial activities with inhibition of the phospholipids biosynthesis. This study aimed to discover the pathways involved in the biosynthesis of phospholipids in *Plasmodium* and deduce the biochemical steps inhibited by T4, a bis-thiazolium bromide drug. We compared the uptake of radiolabeled precursors and their selective incorporation in the phospholipids of cultured *Plasmodium*-infected and -uninfected erythrocytes which revealed that phosphatidylcholine of *Plasmodium* is synthesized both from choline and ethanolamine (4.7 vs 1.9 nmol/10<sup>10</sup> cells.h<sup>-1</sup>). T4 has no effect on the biosynthesis of phosphatidylethanolamine but T4 inhibited, in a selective way, the *in vitro* uptake of choline. However no enzymes in the biosynthesis of phospholipids seem to be inhibited by T4 but rather an inhibition of choline entry into the parasite.

**Keywords:** drug effect-metabolism- *Plasmodium falciparum*-phosphatidylcholine-phosphatidylethanolamine; T4; inhibition

**Abbreviations:** Cho, choline; CDP, cytidine diphosphate; CDP choline, cytidine diphosphate choline; CDP ethanolamine, cytidine diphosphate ethanolamine; CMP, cytidine 5-monophosphate; DAG, diacylglycerol; Etn, ethanolamine; PC, phosphocholine; PE, phosphoethanolamine; PtdCh, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; SEM, standard error of mean; NA, Nucleic Acid.

## Introduction

*Plasmodium* is the parasite responsible for malaria, the most wild spread infectious disease in the world. *P. falciparum* is the most deadly species among the four humans plasmodial infectious. Despite the huge efforts to control this disease, malaria still occurs as a public health problem in several countries. The different approaches to control this disease are vector control, vaccination, immunotherapy and chemotherapy. Chemotherapy approach progresses on different pathways and has indicated several potential biochemical targets [1-5]. However, many of these approaches need to be validated as effective and specific antiplasmodial biochemical targets.

Due to drug resistance of some strains, we constantly need to find out new more efficient drugs.

Several ammonium quaternary-derived drugs have shown an *in vivo* antimalarial activity in *Plasmodium*-infected rodents or monkeys [3, 6] or the mouse [3, 7]. Recent extended researches showed potent antimalarial activities with bis-thiazolium drugs [8]. Several bis-thiazolium derived-molecules such as T4, a methoxy-derived bromide bis-thiazolium, have shown an *in vivo* antimalarial activity. T4 exhibited, with *in vitro* test against *P. falciparum*, an IC<sub>50</sub> value of 0.65 nM and a good antimalarial property against *P. vinckei* (ED<sub>50</sub> = 0.14 mg/kg) [8]. T16, a structural analogue of T4, shows an inhibitory property on the choline uptake and an inhibition of phosphatidylcholine biosynthesis [9],

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indicating a potential mechanism of the antimalarial activity of the bis-thiazolium drug.

On the biological side, the erythrocyte is not expected to synthesize phospholipids, because of the lack of any machinery so that the growth of *Plasmodium* inside the erythrocyte requires an active production of phosphatidylcholine and phosphatidylethanolamine, the main

compounds for the cytoplasmic membrane of the parasite. Depending on the type of cell, several pathways, from choline or ethanolamine, have been shown to lead to these phospholipids. One of them, well known as the Kennedy pathway, consists in the phosphorylation of choline into phosphocholine (PC), then the transfer of cytidylmonophosphate onto the PC, leading to CDP-Choline. And finally,

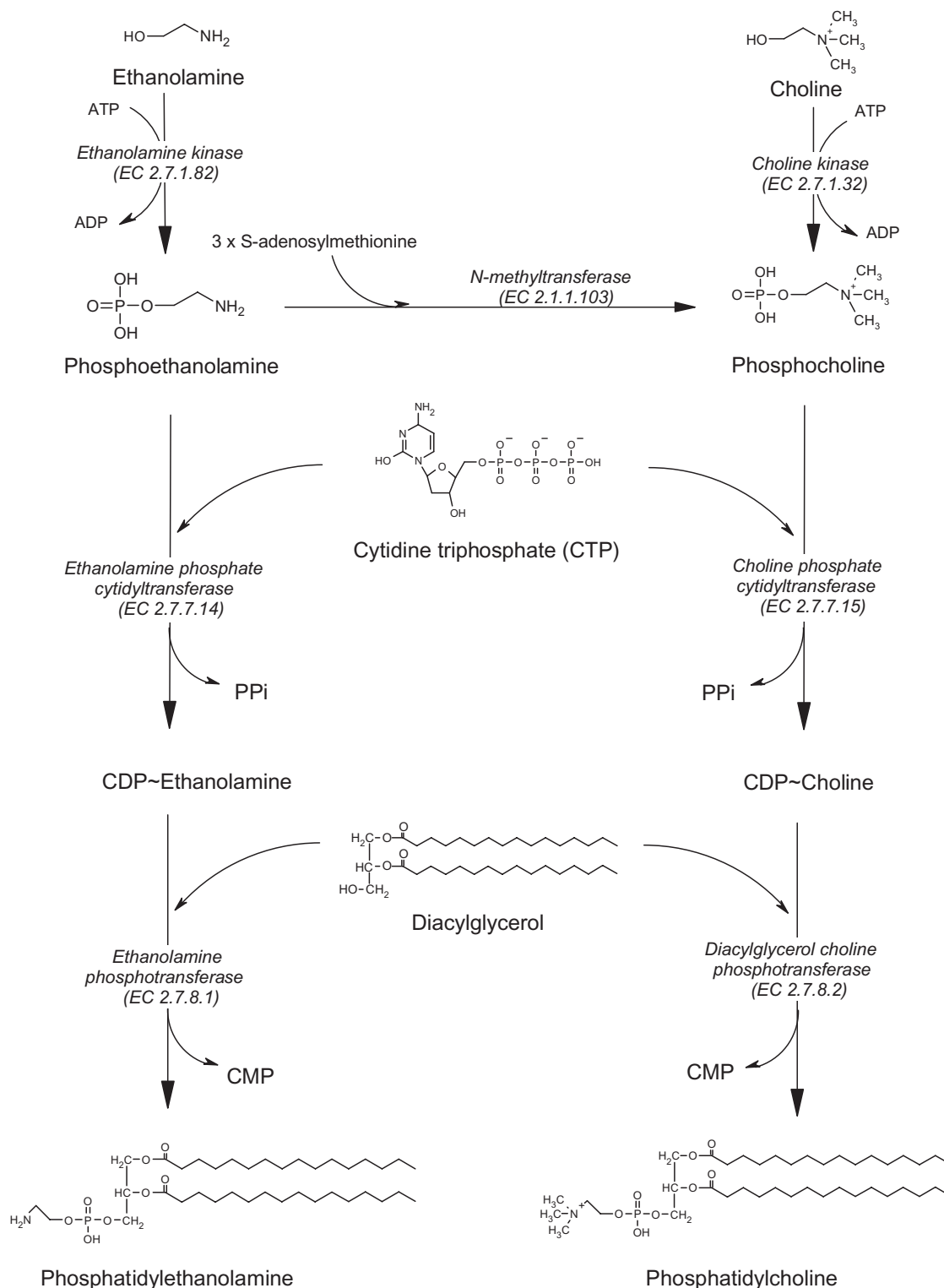


Figure 1. Biosynthesis of phosphatidylcholine and phosphatidylethanolamine.

the CDP-Choline is transferred onto a diacylglycerol (DAG) to get the PtdCho. Another pathway is the tri-methylation of the phosphoethanolamine (PE) into the PC and then conversion via the PtdCho via the CDP-Cho. The last one is the tri-methylation of phosphatidylethanolamine (PtdEtn) into PtdCho (Figure 1). The involvement of each pathway depends on the cell type. Little is known on the functional metabolic pathways used by *Plasmodium* to synthesize PtdCho and PtdEtn.

In this work we aimed to find out the biochemical mechanism and the interference of T4 on PtdCho and PtdEtn metabolisms in *Plasmodium*.

## Materials and methods

### Chemical materials

The drug T4, in a bis-thiazolium bromide, was synthesized in-house and the stocks of samples were provided by the research unit UMR CNRS 5810 in France. Labeled precursors, [1-<sup>3</sup>H]ethan-1-ol-2-amine, [methyl-<sup>3</sup>H]choline and [<sup>3</sup>H]hypoxanthine were purchased from Amersham™ corp. The chemical materials were finally used diluted in a solution of RPMI 1640 obtained with Gibco™. When necessary, the organic solvent was previously dried in a nitrogen stream before dissolved in RPMI 1640.

### In vitro-cultured *P. falciparum*

The 3D7 strain of *P. falciparum* was provided by Dr B. Pradines (IMTSSA in Marseille France). The infected-erythrocytes were incubated in a modified RPMI 1640, freed of choline and ethanolamine, obtained from Gibco™ and supplemented with 25 mM HEPES (pH 7.6). The parasitemia was 12 % with a 2.5 % hematocrit in a total amount of 6.10<sup>6</sup> infected cells. A 100 μL volume suspension of culture were distributed in microwell plate and incubated at 37°C in a 5 % CO<sub>2</sub> atmosphere.

### Assessing T4 activity on infected-erythrocyte

We assessed the activity of T4 on the uptake of labeled-radioactive precursors by the infected-erythrocyte. At time 0 min, a ranging concentration of 50 μL of T4 [4.6 x10<sup>-9</sup> to 10<sup>-5</sup> molar] was added in the suspension of cultured *Plasmodium*. At time 60 min, were added 50 μL solutions of radioactive [<sup>3</sup>H]-choline and radioactive [<sup>3</sup>H]-ethanolamine (in a final concentration of 2.5 μM) each with a specific activity of 2 Ci/mmol. In control test was added [<sup>3</sup>H]-hypoxanthine (1μCi per well). The final volume of the suspension in each microwell was 200 μL. Metabolism reactions were stopped at time 240 min in a 4°C atmosphere. All the suspensions were passed through a cell harvester and macromolecules were transferred onto glass fiber filters. Radioactive precursors incorporated in macromolecule were measured with a beta scintigraph counter from Beckman™.

The uptake of choline, ethanolamine and hypoxanthine in macromolecule is expressed as percent of control (assay without T4). Each well of the assay was performed in triplicate.

### T4 effects on phospholipids biosynthesis

*P. falciparum*-infected erythrocytes in 1.25 x10<sup>6</sup> total infected cells were incubated in a final volume of 200 μL of modified RPMI 1640™ choline-free, supplemented with 25 mM HEPES (pH 7.6) and with choline (5 μM; 1.95 Ci/mmol), ethanolamine (2 μM; 2.29 Ci/mmol) and hypoxanthine (1 μCi/sample-test). Solution of T4 was added in ranging concentrations as described above. Suspension of cells was incubated for 4 h at 37 °C in a 5 % CO<sub>2</sub> atmosphere. Reactions were stopped at 4 °C and cultures were washed with NaCl (0.9%). Phospholipids were extracted according to the Folch protocol [10]. The organic phase and water phase were separately fractionated by thin-layer chromatography. Migrated spots were revealed with ninhydrine and iodine vapor. Fractionated spots were identified with known control samples and were scraped off. Then the radiolabeled silica were measured with a liquid scintillation counter from Beckman™. Results are expressed as percentage of control infected erythrocytes without T4. Results are means of duplicate tests.

## Results

Because of the lack of its own machinery, the erythrocyte is not expected to synthesize macromolecule like phospholipids or DNA. The *Plasmodium* which infects the erythrocyte has to synthesize macromolecules for its own growth. We assessed the activity of T4 on phospholipids metabolism by measuring the uptakes of choline and ethanolamine and their respective incorporation in the macromolecules of *Plasmodium*.

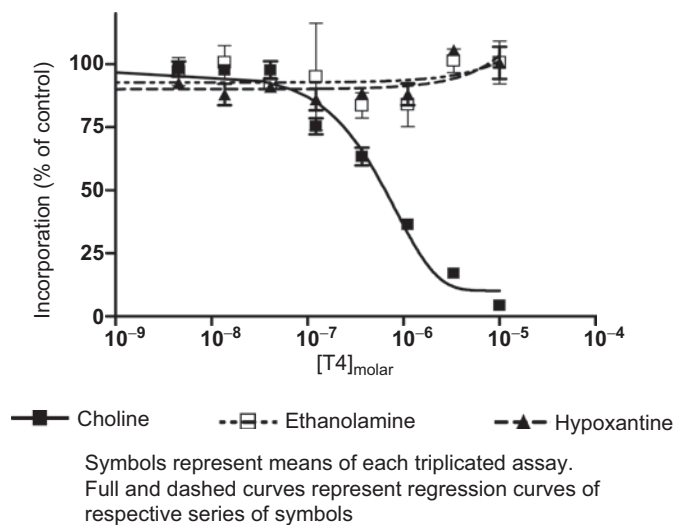
### T4 activity

We performed assays with asynchronous culture of *Plasmodium*, containing merozoites, trophozoites, young and later schizontes. The uptake of Etn in control test (15.9 nmol/10<sup>10</sup> cells) is 6 times superior to the uptake of Cho (2.6 nmol/10<sup>10</sup> cells).

Compared with control, T4 inhibits the uptake of choline in *Plasmodium* with a dose-response effect. The concentration of T4 which inhibits 50 % of choline uptake [IC<sub>50(Cho)</sub>] is about 5.7 x10<sup>-7</sup> M. Whereas, the uptake of ethanolamine is about 100 % of control test and evolves as a plateau whatever is the concentration of T4. This means that T4 has no inhibitory effect on the uptake of ethanolamine. In the same way, the uptake of hypoxanthine is constant and about 100 % of the control test uptake whatever is the T4 concentration. The shape of the curves from the T4 concentration 1.23 x10<sup>-7</sup> M, testifies to a selective action of the drug on the phospholipids metabolism (Figure 2).

### Incorporation of precursors in phospholipids

These assays were performed in order to emphasize the nature of the phospholipids in which precursors are incorporated and also to find out the biochemical pathway which is targeted by T4, in the metabolism of phospholipids. In this metabolism only the Kennedy pathway is known to lead choline to phosphatidylcholine. But



**Figure 2.** Effects of T4 on choline, ethanolamine and hypoxanthine uptakes in Plasmodium.

*P. falciparum*-infected erythrocytes, 3D7 strain, (12 % parasitemia, 2.5 % hematocrit in  $6 \times 10^6$  total infected cells) were incubated in a final volume of 200  $\mu$ L of modified RPMI 1640 choline-free supplemented with 25 mM Hepes (pH 7.6) and the indicated concentration of the compounds T4 (at time 0). Suspensions were incubated at 37 °C in a 5 % CO<sub>2</sub> incubator. Incorporation of choline, ethanolamine and hypoxanthine were assessed by adding [3H]-Choline (2.5  $\mu$ M, 2 Ci/mmol), [3H]-ethanolamine (2.5  $\mu$ M, 2 Ci/mmol) and [3H]-Hypoxanthine (1  $\mu$ Ci per sample test) at time 60 min for 3 h. Reactions were stopped at 4 °C and radioactive incorporation was measured after filtration using cell harvester. Incorporations of the labeled precursors are expressed as percent of control (without drugs) and are means of triplicate values  $\pm$  SEM. (Choline and ethanolamine incorporation in control-test were respectively: 2.6 and 15.9 nmol/ $10^{10}$  cells; The T4 inhibitory concentration of 50% choline uptake is  $5.7 \times 10^{-7}$  M).

**Table 1.** Precursors incorporation in phospholipids of infected-erythrocyte for control-test (without T4). (Experiment performed according the protocol reported in Figure 3 legend).

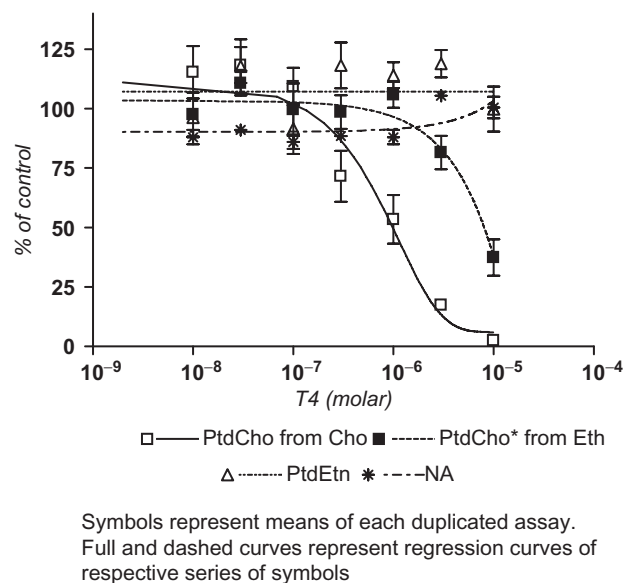
	Mean $\pm$ SEM (nmol/ $10^{10}$ cells/h)		
	Phospholipids	PtdEtn	PtdCho
Choline	20.0 $\pm$ 1.7	nd	20.0 $\pm$ 3.9
Ethanolamine	117 $\pm$ 10.5	30.6 $\pm$ 5.9	9.3 $\pm$ 2.2

nd : not detected

ethanolamine is expected to be synthesized into PtdEtn or PtdCho.

The incorporation of Cho into PtdCho is superior to the incorporation of Etn into PtdCho (4.7 vs 1.9 nmol/ $10^{10}$  cells.h<sup>-1</sup>). But the incorporation of Etn into PtdEtn is more superior (14.3 nmol/ $10^{10}$  cells.h<sup>-1</sup>) (Table 1).

As seen above, T4 inhibits the uptake of choline and its incorporation in the phospholipids PtdCho and PtdEtn. The activity of T4 mainly concerns inhibition of metabolized Cho into PtdCho and metabolized Etn into PtdCho. Compared with the pathway of PtdCho from Etn, a lower concentration of T4 can inhibit the pathway of PtdCho from Cho (Figure 3). The Kennedy pathway seems to be more sensitive to T4: the 50 % inhibitory concentration of PtdCho from Cho ( $IC_{50}$ ) is  $7.8 \times 10^{-7}$  M vs  $7.3 \times 10^{-5}$  M for PtdCho from Etn. T4 shows an inhibitory activity on the metabolism of



**Figure 3.** Effects of T4 on phospholipids biosynthesis and hypoxanthine incorporation by Plasmodium.

*P. falciparum*-infected erythrocytes (3D7 strain) (10 % parasitemia, 2.5 % hematocrit in  $5 \times 10^6$  total infected cells) were incubated in a final volume of 200  $\mu$ L of modified RPMI 1640 choline-free supplemented with 25 mM Hepes (pH 7.6) and [3H]-choline (20  $\mu$ M; 0.5 Ci/mmol) or [3H]-ethanolamine (2.2  $\mu$ M; 2.2 Ci/mmol). Suspension was incubated for 4 h at 37 °C in a 5 % CO<sub>2</sub> incubator. Reactions were stopped at 4 °C and wash with NaCl (0.9%). Phospholipids were extracted according Folch process (chloroform / methanol / water : 8/4/3) and fractionated by thin-layer chromatography. Identified fractionated spots were sp. and radiolabeled metabolites were measured by scintillation counting. Results are means of triplicate values  $\pm$  SEM.

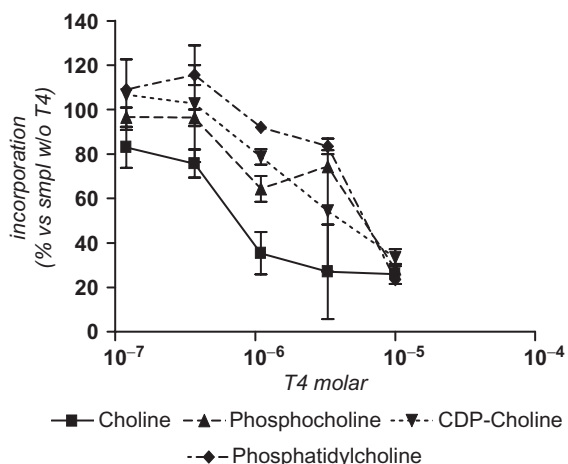
PtdCho with a selective activity on the PtdCho synthesized from Cho.

T4 has no inhibitory activity on the uptake of hypoxanthine and neither on its incorporation into nucleic acid. In the same way, the uptake of Etn is not influenced by T4. The amount of Etn incorporated in PtdEtn is significantly superior to 100 % of the control test without drug.

### Incorporation of precursors in the metabolites of phospholipids synthesis

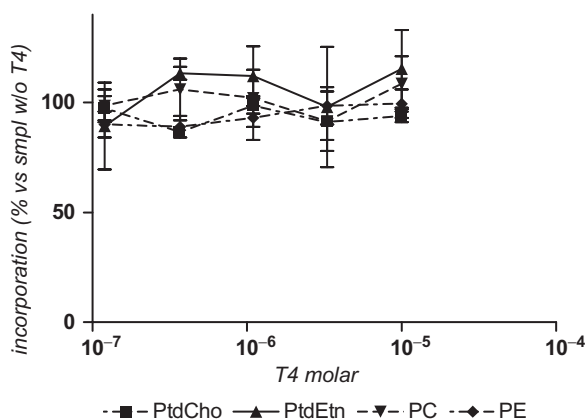
In order to find out the biochemical target of T4 in PtdCho and PtdEtn metabolism, we performed the following assays. We assessed the synthesis of all intermediates and final molecules in the metabolism of phospholipids i.e. the water-soluble molecules and organic-soluble ones from the *Kennedy pathway* (intracellular Cho, PC, CDP-Cho, and PtdCho) and those from the pathways starting with Etn and leading to PtdEtn or PtdCho (intracellular Etn, PE, PC, CDP-Etn, CDP-Cho). We expected to observe an increased synthesis of molecules on the upstream side of the eventual blocked pathway. On the contrary, we could expect a decrease on the backing side of the same blocked pathway. The amount of each synthesized molecules species was assessed after fractionation and quantification of the radioactivity.

T4 inhibited all the intermediate molecules of the metabolism of PtdCho from Cho. Intracellular Cho, PC, CDP-Cho



**Figure 4.** Effects of T4 on choline metabolism pathway.

*P. falciparum*-infected erythrocytes, 3D7 strain,  $2.7 \times 10^7$  total infected cells were incubated in a final volume of 200  $\mu$ L of modified RPMI 1640 choline-free supplemented with 25 mM Hepes (pH 7.6) and  $[^3\text{H}]$ -choline (32  $\mu\text{M}$ ; 2.95 Ci/mmol). T4 was added at the indicated concentrations. Suspension was incubated for 2 h at 37  $^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. Reactions were stopped at 4  $^{\circ}\text{C}$ . Phospholipids and water-soluble metabolites were separated according to the Folch extracting protocol. A thin-layer-chromatography was performed to fractionate the phospholipids and the aqueous metabolites, respectively in the organic phase and the supernatant aqueous phase. Revealed spots with iodine vapour and ninhydrine were identified with standards. Each spot was sp. off and measured by scintillation counting. Results are means of 4 values  $\pm$  SEM.



**Figure 5.** Effect of T4 on the ethanolamine metabolism pathway.

*P. falciparum*-infected erythrocytes (3D7 strain), hematocrit 1.5% and  $2.7 \times 10^7$  total infected cells obtained with the Vario Macs™ concentration protocol were incubated in a final volume of 200  $\mu$ L RPMI 1640 supplemented with 25 mM Hepes (pH 7.6) and  $[^3\text{H}]$ -ethanolamine (3.2  $\mu\text{M}$ ; 1.30  $\mu\text{Ci}$ ). T4 was added at the indicated concentrations. Suspension was incubated for 2 h at 37  $^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. Reactions were stopped at 4  $^{\circ}\text{C}$  and water-soluble metabolites were obtained from the aqueous supernatant according to the Folch protocol. A thin-layer-chromatography was performed to fractionate the aqueous metabolites. Spots revealed with iodine vapour and ninhydrine were identified by compared standards. They were sp. off and measured by scintillation counting. Results are means of duplicate values  $\pm$  SEM.

and PtdCho decreased according to the increase of T4 concentration (Figure 4). On the contrary, T4 has no effect on the incorporation of Etn in phospholipids intermediate

molecules. PE, PC, PtdEtn and PtdCho are not influenced by T4 whatever its concentration (Figure 5).

## Discussion

The modified medium, RPMI 1640 choline and ethanolamine-free, was labeled by adding a known amount of radioactive  $[^3\text{H}]$ -choline or  $[^3\text{H}]$ -ethanolamine so as to assess the accurate amount of choline and ethanolamine uptake as suggested by Elabbadi [11]. Incubation time, concentrations of choline and ethanolamine were chosen as to enable the biosynthesis of phospholipids. Therefore all the experimental conditions allowed an optimal growth of the parasites in a blank test [12]. The uptake of parasite is the uptake of the infected-erythrocyte complex deduced from the one of an uninfected-erythrocyte.

T4 is a bis-thiazolium derived molecule. T16 and T3 have shown antiplasmodial and potent antimalarial activities [9, 13] so we expected T4, a structural analogue of T16, to show a similar activity.

The other known metabolites of choline are acetylcholine and betaine. They are known to be synthesized by superior mammalian cells, but *Plasmodium* is not expected to synthesize these two small molecules [14]. Whatever acetylcholine, betaine or other small derived molecules would have been synthesized, only the macromolecules like nucleic acid or phospholipids are expected to be retained on the fiber glass and therefore capable of measurement.

The known phospholipids in the cell structure of *Plasmodium* are mainly found in the cytoplasmic and the vacuole membranes. Therefore, the extracted lipid in the organic phase is expected to represent all the phospholipids from the cytoplasmic membrane of the parasite and the parasitophorous vacuole membrane. The water-soluble phase contains the non-lipid metabolites like intracellular Cho, PE, PC, CDP-Cho and CDP-Etn.

Since the experiments were performed on an asynchronous culture of *Plasmodium*, it means different levels of metabolism. Therefore, our results can be seen as the mean intensity of the metabolism in one parasite life cycle.

The metabolism of phospholipids is complex. It involves Cho, Etn and serine. Several pathways had been identified and their enzymes characterized [15-18]. These pathways are different according to the cell species. The tri-methylation of PtdEtn into PtdCho is known to occur in mammalian cells and yeast [19-21]. This pathway had been evoked in *Plasmodium* but had not been conclusively shown [14, 22]. Recently, Witola localized the phosphoethanolamine N-methyl transferase in the *Plasmodium* cell and therefore showed the coupling of Etn metabolism with the Kennedy pathway [23].

The  $IC_{50(\text{Cho})}$  of T4 is within the range of chloroquine or quinine  $IC_{50}$  [24]. This is in favor of the potential biochemical target of T4 on the choline metabolism.

Hypoxanthine was used to assess the metabolism of nucleic acid. The biosynthesis of nucleic acid is assumed

to be molecular evidence of the parasite livelihood. But nothing is known about the delay between the stop of nucleic acid biosynthesis and its outcome on cellular growth. When T4 inhibits the uptake of Cho with a dose-response effect, the parasites still incorporate hypoxanthine for the synthesis of DNA even though this DNA is not proved to be functional. At this average concentration of T4, Etn is still incorporated for the biosynthesis of phospholipids with a mean rate over 100 % (Figure 2). This means a selective action of the drug. This selective inhibition on Cho metabolism does not exclude any other activity on another vital metabolism of the parasite, (*ie*: heme detoxication). Therefore, a potential specific action of T4 needs to be clarified.

At the trophozoite and schizonte stage of *Plasmodium*, the phospholipids of the cytoplasmic membrane are predominantly PtdCho. But the incorporation of precursors into PtdEtn appears to be superior to their incorporation into PtdCho. This could mean a more active turnover of PtdEtn or a possible tri-methylation of PtdEtn into PtdCho as suggested by Vial [22].

T4 has a dose-response effect on the PtdCho coming from the Cho suggesting that the Kennedy pathway could be inhibited by the drug.

A significant amount of PC coming from labeled Etn means that the Etn pathway is coupled with the Kennedy pathway probably by the methylation of PE into PC as suggested by recent molecular biology studies [25, 26]. This coupling pathway, starting with Etn and leading to PtdCho, was not inhibited by T4 when the experiment was performed with a normal culture, medium containing Cho (Figure 4). On the contrary, T4 inhibited the biosynthesis of PtdCho coming from Etn when the experiment was performed in a modified medium freed from Cho (Figure 2). It means different effects of T4 on PtdEtn biosynthesis. Choline seems to play a role in the regulation of the PtdCho biosynthesis coming from Etn as suggested by Witola et al [27]. So the decrease of synthesized PtdCho from Etn observed in Figure 3 could be a combined effect of T4 and Cho deficiency. Besides this observation, the non inhibition of PtdCho coming from Etn means that the inhibitory activity of T4 does not come from a hypothetical blocking of CCT (*EC 2.7.7.15*) or CPT (*EC 2.7.8.2*). These two enzymes catalyze respectively the transformation of PC into CDP-Cho and the CDP-Cho into PtdCho.

To conclude, T4 shows a selective inhibition on the biosynthesis of PtdCho. There's a selective action on the Cho and the Etn pathways. Nevertheless, none of the enzymes catalyzing the biosynthesis of the phospholipids inside the parasite seem to be inhibited by the drug. But our results are in favor of a coupled pathways of Cho and Etn with a mutual interference on the biosynthesis of phospholipids.

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**Declaration of interest:** The authors report no conflicts of interest.

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