Bulletin of the World Health Organization, 59 (3): 449-458 (1981)

Development of new derivatives of primaquine by association with lysosomotropic carriers

A. TROUET,[1] P. PIRSON,[2] R. STEIGER,[3] M. MASQUELIER,[3] R. BAURAIN,[3] & J. GILLET[4]

On the basis of the drug-carrier concept of chemotherapy, we entrapped primaquine in liposomes, and linked it to an amino acid (leucine), and to peptides (alanyl-leucine and alanyl-leucyl-alanyl-leucyl) as intermediate steps in the synthesis of covalent primaquine–glycoprotein conjugates that would be selectively recognized by hepatocytes.

The therapeutic activity of these compounds was tested in mice infected with sporozoites of Plasmodium berghei. *Causal prophylatic cures were obtained after a single intravenous injection of primaquine–liposomes (60–70 mg of primaquine/kg of bodyweight) and lower doses (35 mg of primaquine/kg of bodyweight) of ala-leu-primaquine and ala-leu-ala-leu-primaquine.*

The administration of such high doses was only possible as a result of the decreased toxicity of primaquine when entrapped in liposomes and confirms the validity of the drug-carrier concept for the treatment of malarial infections. The improved chemotherapeutic index of ala-leu-primaquine and ala-leu-ala-leu-primaquine resulted from their decreased toxicity and increased chemotherapeutic activity. These peptide derivatives are probably acting as pro-drugs of primaquine.

Primaquine is an important causal prophylactic agent used for the destruction of exoerythrocytic forms of *Plasmodium vivax* and *P. ovale*. However, the prophylactic and therapeutic applications are limited by its toxic side-effects (*1*).

We proposed the hypothesis that the selectivity and activity of antiprotozoal drugs, such as primaquine, could be increased significantly by linking them to lysosomotropic carriers (*2*). These carriers would promote the uptake of the drug by the infected host cells, e.g., the hepatocytes in the case of the exoerythrocytic stage of malarial infection, and reduce the exposure of sensitive uninfected cells.

Our objective was to prepare new carrier-linked derivatives of primaquine that would enable us to cure *P. berghei*-infected mice by the administration of a single dose. The validity of using mice infected with the sporozoite form of the malarial parasite for testing causal prophylactic antimalarial activity has been established previously (*3*, *4*).

The ideal primaquine–carrier complex should fulfil two important criteria:

(1) The carrier should be recognized and taken up selectively by the hepatocytes. Two types of carrier seem to comply with this condition—liposomes, which have been shown to be captured preferentially by liver and spleen (*5*, *6*), and glycoproteins, such as asialofetuin (*7*).

(2) The drug–carrier link should remain stable in the bloodstream and be cleaved inside the lysosomes of the target cells after endocytosis. This cleavage should release the drug in its active form.

The preparation of liposomes with entrapped primaquine presented no technical difficulties. Anti-leishmanial drugs have already been entrapped in liposomes and used with great success (*8–10*).

The synthesis of an adequate link between primaquine and glycoproteins was, however, more difficult to achieve. Primaquine can be linked via its free NH_2 group to a carboxylic side-chain (aspartic or glutamic acid) in the carrier protein by the formation of an amide bond (Fig. 1). However, this bond is not an ideal substrate for peptidases, and the adjacent carrier may cause steric hindrance. Thus, primaquine would probably not be released as such, but would retain a β-aspartyl or γ-glutamyl residue. This problem can be resolved by intercalating one amino acid or an oligopeptide spacer between the drug and the protein, and under these conditions, primaquine would be released

[1] Professor, Université catholique de Louvain, International Institute of Cellular and Molecular Pathology (ICP), 75, avenue Hippocrate, B-1200 Brussels, Belgium.

[2] Boursier IRSIA, ICP, Brussels, Belgium.

[3] Investigator, ICP, Brussels, Belgium.

[4] Professor, Ecole de Santé publique, Université catholique de Louvain, B-1200 Brussels, Belgium.

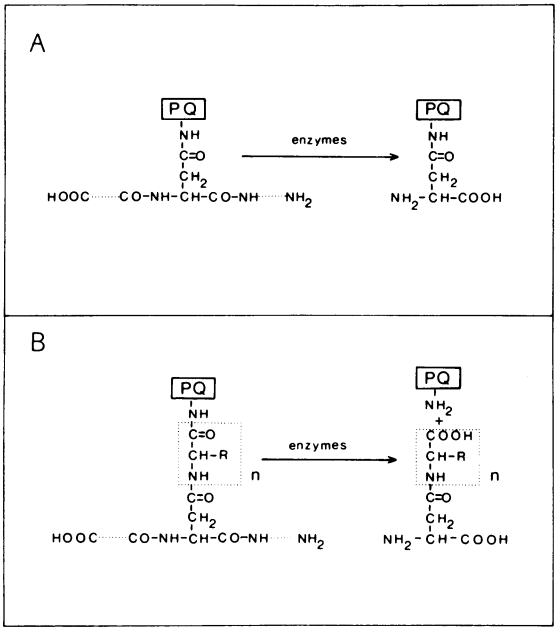


Fig. 1. Schematic illustration of the effect of an amino acid or peptide spacer on the enzymatic hydrolysis of primaquine from a PQ–protein conjugate. A: Direct linkage of primaquine to a protein; B: Primaquine linked to a protein via a spacer arm of *n* amino acids.

as such. Experiments to link antitumoral anthracyclines, such as daunorubicin, to protein carriers have shown that a tetrapeptide spacer arm is required between the drug and the carrier in order to give a high yield of free anthracyclines in the presence of lysosomal hydrolases (*11*), and that the first amino acid linked to the drug should be leucine (*12*).

In this paper we report results obtained with primaquine entrapped in liposomes, which have been published partially elsewhere (*13*), and the interesting chemotherapeutic properties of the amino acid and peptide derivatives of primaquine, which were synthesized in order to link the drug to hepatotropic carriers, such as asialofetuin.

MATERIALS AND METHODS

Experimental malaria infection

Sporozoites of *P. berghei* were isolated, as described previously (*13*), from homogenates of *Anopheles stephensi* 18 days after a blood meal on parasitized mice. Male TB_{Esp} mice, weighing 18–22 g, were then infected intravenously with 10 000–30 000 of these sporozoites within 35 minutes of the dissection of the mosquitos.

Primaquine–liposome preparation

Small multilamellar liposomes containing primaquine disphosphate[a] in the aqueous phase (PQ-lip) were prepared according to the method of Bangham et al. (*14*). The liposomes consisted of chromatographically pure phosphatidyl choline, phosphatidyl serine, and cholesterol, in a molar ratio of 4:1:5, incorporating 97 ± 11 g of primaquine/mol of total lipid.

Amino acid and peptide derivatives of primaquine

N-L-leucyl primaquine (leu-PQ) was synthesized by the reaction of the *N*-carboxyanhydride derivative of L-leucine with primaquine, as described elsewhere for the synthesis of the amino acid derivatives of anthracyclines (*12*).

N-L-alanyl-L-leucyl primaquine (ala-leu-PQ) was prepared by the reaction of leu-PQ with the *N*-trityl alaninate of *N*-hydroxysuccinimide (*15*, *16*).

N-L-alanyl-L-leucyl-L-alanyl-L-leucyl primaquine (ala-leu-ala-leu-PQ) was synthesized as described for ala-leu-PQ by using ala-leu-PQ and leu-ala-leu-PQ successively as the starting material.

Labelled primaquine

^{3}H-labelled primaquine, [^{3}H]PQ, with a specific radioactivity of 148 MBq (4 mCi)/mmol was prepared by catalytic exchange in tritiated aqueous medium.

Enzymatic hydrolysis of amino acid and peptide derivatives of primaquine

Aliquots of 0.4 ml of the derivatives were incubated for various times at a concentration of 0.65 mmol/litre, in the presence of 0.3 ml of 25 mmol/litre cysteine, 0.3 ml of 0.5 mol/litre phosphate buffer (pH 6), 0.15 ml of a purified lysosomal fraction (*17*) at a protein concentration of 5.5 g/litre, and 0.35 ml of water.

Analogous incubations were performed in the presence of 100 ml of calf serum per litre of phosphate buffered saline (0.15 mol/litre), at pH 7.4.

The parent drug and digestion products were analysed by high-pressure liquid chromatography, after extraction of 0.1 ml of the sample with 0.1 ml of 0.1 mol/litre borate buffer at pH 10.2, and 1.8 ml of chloroform–methanol (volume ratio, 4:1).

Aliquots of 20 µl of the organic phase were then injected into a Hewlett Packard chromatograph (model 1080) fitted with a prepacked silica gel column (Lichrosorb Si 60-7 µ 250 x 4.5 mm)[b], and eluted with chloroform, methanol, glacial acetic acid, and 0.3 mmol/litre $MgCl_2$ (volume ratio, 720:210:40:30) at a flow-rate of 1 ml/min and a pressure of 6.9×10^3 kPa.

[a] From Aldrich Chemical Co., Milwaukee, WI, USA.

[b] From Merck, Darmstadt, Federal Republic of Germany.

The eluate was monitored for absorption at 254 nm using a spectrometer.[c]

Analysis of plasma samples

[^{3}H]PQ or [^{3}H]PQ-lip was injected intravenously into TB_{Esp} male mice at a dosage of 0.01 mg of primaquine and 0.097 μmol of lipid per g of body weight, in a total volume of 0.4 ml, to give a theoretical plasma concentration of 195 mg of primaquine per litre at time 0 (assuming a plasma volume of 0.0352 ml per g of bodyweight). Liposomes were labelled by the addition of [^{14}C]dipalmitoyl phosphatidyl choline (1850 MBq (50mCi)/mmol) to the lipid mixture described above.

At specific times, blood samples were taken from the femoral artery and transferred to tubes containing heparin. The plasma was separated by centrifugation. The radioactivity due to ^{3}H and ^{14}C was measured in a Packard LSC scintillation counter after adding 0.1 ml of the plasma to 5 ml of Aqualuma.[d] The results were calculated in disintegrations per minute (dpm)/ml, after correction for quenching, converted to μg of primaquine or μmol of lipid, and expressed as a percentage of the original dose injected.

Blood and tissue distribution

The mice were sacrificed after various time intervals and samples of blood, urine, and tissue (liver, spleen, kidney, heart, stomach, duodenum, and rectum) were taken and stored in 0.15 mol/litre NaCl at 0 °C. The tissues were homogenized using a Potter-Elvehjem homogenizer and sonicated for 30 sec at 50 W in a Branson B_{12} Sonifier. Blood samples stored in EDTA[e] were sonicated, as above. Then 100 μl of these tissue and blood samples were digested at 50 °C in 1 ml of a Lumasolve[d]–isopropanol mixture (volume ratio, 10:3), discoloured by incubating with 0.2 ml of H_2O_2 for 15 minutes, and finally added to 10 ml of a mixture, in a ratio of 9:1, of Lumagel[d] and 1 mol/litre HCl.

[^{3}H]PQ radioactivity was measured in a Packard scintillation counter (model LSC) and proteins were estimated by the fluorescamine method (*18*). The amount of blood contaminating the tissue samples was estimated by an immunological assay of serum albumin, using the Mancini technique (*19*). The amount of primaquine-associated radioactivity found in the tissues was expressed as nmol of primaquine/g of tissue protein, after correction for the radioactivity present in the blood that contaminated the tissue samples.

[c] Model LC-UV, Pye Unicam, Cambridge, England.
[d] From Lumac A.G., Basel, Switzerland.
[e] Ethylenediaminetetraacetic acid.

Chemotherapeutic parameters

After inoculation of the sporozoites, the parasitaemia was checked daily by making Giemsa-stained blood smears in order to estimate the infection rate (i.e., the proportion of mice that were infected).

The number of days that the mice survived was used to determine the median survival time (MST). The percentage increase in life span (ILS), as a result of treatment, was calculated as follows:

$$\left(\frac{\text{MST treated}}{\text{MST control}} - 1\right) \times 100.$$

At the end of the 50-day observation period, the percentage of long-term survivors (LTS) was established and blood from some of these survivors was inoculated into non-infected mice to confirm the absence of parasites.

Drug efficacy, expressed as the causal prophylactic activity (CPD_{50}), was calculated from the relationship between cure rate and administered dose.

The residual effect of PQ-lip on secondary blood parasites was examined by treating mice with PQ-lip 47 hours before an inoculation of mouse blood containing primary merozoites.

Toxicity parameters

The overall toxicity of primaquine and its derivatives was assessed by establishing the lethal dose in non-infected mice (LD_{50}). This is defined as the dose that kills 50% of the test animals.

The maximal tolerated dose (MTD) was defined as the highest dose that induced a weight loss of less than 5%, when administered to non-infected animals.

RESULTS

Toxicity of primaquine–liposomes

When primaquine incorporated in liposomes was administered intravenously, it was found to be about 3.5 times less toxic than free primaquine as expressed by its LD_{50}, and the ratio of the MTD for PQ-lip and free primaquine was 4.3:1 (Table 1).

Liposomes administered alone were not toxic, and the toxicity of primaquine given together with empty liposomes was unchanged.

Therapeutic efficacy of primaquine–liposomes

The best therapeutic effect was produced when mice were treated 3 hours after sporozoite inoculation (Table 2). At doses up to 25 mg/kg of body weight, primaquine and PQ-lip displayed similar therapeutic activities. At 30 mg/kg of body weight, primaquine

Table 1. Comparison of the toxicity and activity of primaquine and its derivatives[a]

Drug	MTD[b]	LD_{50}	CPD_{50}[c]	TI[d]
Primaquine	14	22	14	1.5
PQ-lip	60	79	15	5.1
Leu-PQ	22	27	16	1.7
Ala-leu-PQ	29	41	9	4.4
Ala-leu-ala-leu-PQ	$\geqslant 29$	$\geqslant 41$	7	$\geqslant 5.9$[e]

[a] All doses are expressed in mg of primaquine base equivalents/kg of body weight.
[b] Maximal tolerated dose.
[c] Causal prophylactic dose.
[d] Therapeutic index (LD_{50}/CPD_{50}).
[e] Preliminary results.

became toxic but the lower toxicity of PQ-lip allowed administration of higher doses, and all the animals given 60 or 70 mg/kg of body weight were cured. The CPD_{50} values of primaquine (14.2 mg of base/kg of body weight) and of PQ-lip (15.5 mg of base/kg of body weight) were not significantly different.

Empty liposomes had no therapeutic effect and did not change the activity of primaquine when administered as a mixture.

No significant residual therapeutic activity could be shown on the erythrocytic stage of the infection, and thus the curative effect of PQ-lip may be considered as true prophylatic activity against the pre-erythrocytic stage of *P. berghei.*

Table 2. Antimalarial effects of primaquine and primaquine-liposomes

Drug	Dose (mg of primaquine diphosphate/kg of body weight)	Time of administration[a]	No. of mice	Long-term survivors[b] (%)	Median survival time (days)[c]	Increase in life span (%)
Controls			67	0	11	0
Primaquine	20	+3	18	0	21	91
	25	+3	17	53	> 50	> 355
	30	+3	9	22	0	- 91[d]
	25	-1	8	0	25	126
	25	-3	7	14	20	81
PQ-lip	20	+3	14	29	23	109
	25	+3	18	50	39	255
	30	+3	20	50	41	273
	40	+3	24	88	> 50	> 355
	60	+3	8	100	> 50	> 355
	70	+3	12	100	> 50	> 355
	40	-3	8	38	32	113

[a] The number of hours after (+) or preceding (-) the sporozoite inoculation.
[b] Percentage of mice who survived to day 50.
[c] All infected, non-cured animals died before day 30.
[d] Caused by drug toxicity.

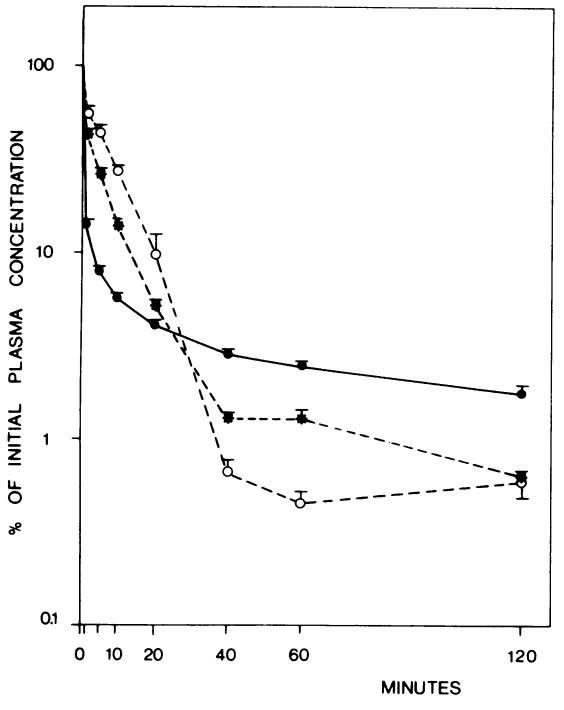


Fig. 2. Plasma concentration of primaquine and liposomal lipids after intravenous administration of free primaquine and primaquine entrapped in liposomes (mean ± SE, 9 experiments), expressed as a percentage of the initial plasma concentration.

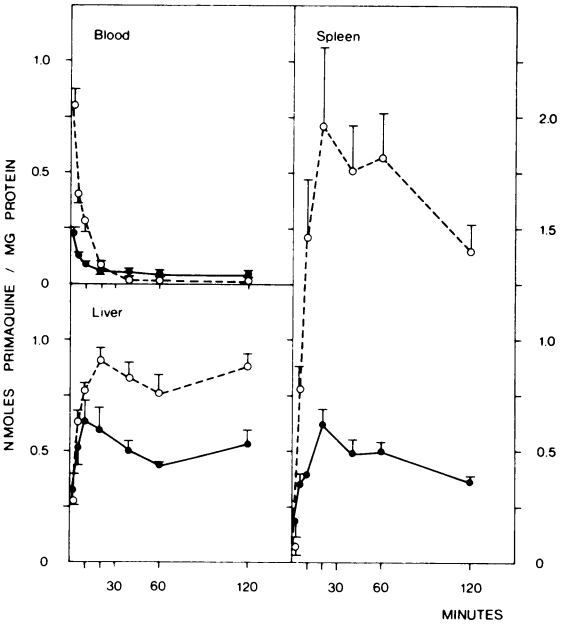


Fig. 3. Concentration of [3H]PQ-associated radioactivity in blood, liver, and spleen after intravenous administration of [3H]PQ, free (●——●) and entrapped in liposomes (o- - -o) (mean ± SE, 6 experiments).

Pharmacokinetics of primaquine and PQ-lip

Fig. 2 illustrates the plasma half-life of primaquine when administered free and entrapped in liposomes. One minute after the injection of primaquine, the plasma concentration had fallen to 15% of its initial value, to give an estimated half-life ($T_{\frac{1}{2}}$) of 19 s. When the drug was administered as PQ-lip, the first phase of rapid elimination accounted for 45% of the injected dose and had a $T_{\frac{1}{2}}$ of 46 s. The remaining primaquine was eliminated more slowly, with a half-life of 5.3 min.

The lipids of PQ-lip were cleared from the plasma in two phases: the first phase was very rapid with a $T_{\frac{1}{2}}$ of 70 s and accounted for 45% of the lipids, while the second was slower, with a $T_{\frac{1}{2}}$ of 4.2 min. During the first 20 min there was a discrepancy between primaquine levels and the associated lipid constituents of the liposomes, suggesting that 10% of the primaquine initially contained in the liposomes dissociated rapidly upon intravenous injection.

Distribution of [3H]PQ and [3H]PQ-lip in the tissues

Of the total dose of labelled primaquine, 30% was found in the liver, 20 min after injection; less than 1% was found in the spleen, heart, stomach, duodenum, and rectum, while 4–5% was found in the kidneys and lungs, 20–120 min after administration. When given as PQ-lip, the amounts of ^{3}H in the liver and spleen increased to 50% and 4%, respectively, after 20 min, whereas the amounts found in the kidneys and lungs decreased. Fig. 3 illustrates the concentrations of [3H]PQ in blood, liver, and spleen, 1–120 min after intravenous injection of primaquine and PQ-lip. Table 3 gives the drug concentrations after 20 and 120 min in all tissues tested.

Knowledge of the distribution of primaquine in the tissues is essential for the evaluation of therapeutic activity and toxicity in each tissue. The amount of ^{3}H recovered was assumed to be proportional to the level of primaquine or its active metabolites. With free primaquine, the highest concentrations were recorded in the lungs (3700 nmol/g of tissue protein), and the liver and spleen (600 nmol/g of tissue protein). When incorporated into liposomes, the distribution of

Table 3. Distribution of [^{3}H]PQ in various tissues after intravenous administration [^{3}H]PQ and [^{3}H]PQ-lip

Tissue	Time after drug administration (min)	Primaquine[a]		PQ-lip[a]	
		Mean	S.D.	Mean	S.D.
Liver	20	589	113	907	53
	120	530	62	882	56
Spleen	20	616	65	1960	430
	120	364	26	1400	120
Heart	20	257	64	52	29
	120	286	62	42	19
Kidney	20	382	58	103	21
	120	523	57	114	9
Lungs	20	3706	1250	267	36
	120	3538	1564	118	24
Stomach	20	63	16	62	16
	120	37	5	84	31
Duodenum	20	38	9	38	10
	120	15	3	54	30
Rectum	20	131	79	22	8
	120	35	24	45	21

[a] Results are expressed in pmoles of primaquine diphosphate/mg of tissue protein (6 experiments).

primaquine was dramatically changed, giving concentrations as follows: spleen, 1900 nmol/g of protein; liver, 900 nmol/g of tissue protein; other tissues, less than 300 nmol/g of tissue protein. Thus, trapping primaquine inside liposomes leads to concentration of the drug in the spleen and, to a lesser extent, in the liver, while decreasing its uptake by the lungs, kidneys, and heart.

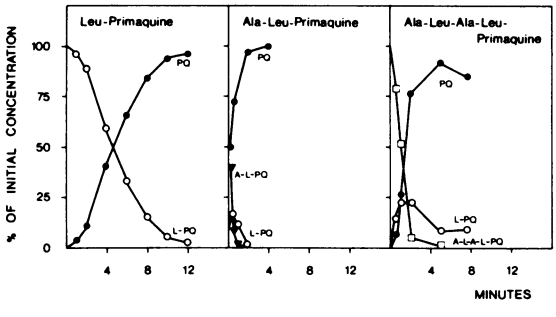


Fig. 4. *In vitro* hydrolysis of amino acid and peptide derivatives of primaquine by lysosomal enzymes at pH 6. The results are expressed as a percentage of the initial concentration of the derivative.

L-PQ = leu-PQ; A-L-PQ = ala-leu-PQ; A-L-A-L-PQ = ala-leu-ala-leu-PQ.

In vitro *hydrolysis of amino acid and peptide derivatives of primaquine*

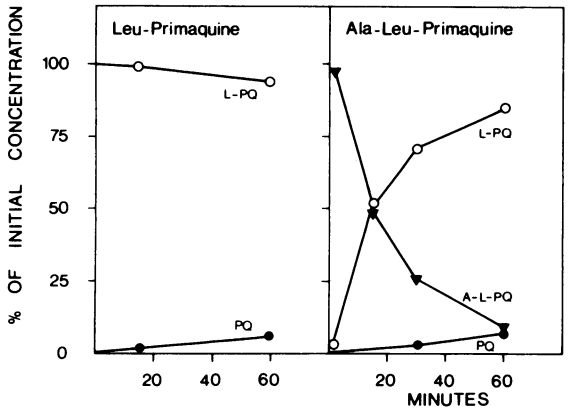
Leu-PQ, ala-leu-PQ, and ala-leu-ala-leu-PQ were rapidly hydrolysed by lysosomal enzymes to release 85–100% of the primaquine as free drug at pH 6. Similar results were obtained at pH 4.5 (Fig. 4).

In the presence of serum, leu-PQ was very stable, but ala-leu-PQ was slowly converted to leu-PQ—50% after 15 min and 80% after one hour (Fig. 5).

Toxicity and therapeutic efficacy of the amino acid and peptide derivatives of primaquine

Leu-PQ was as toxic and as active as primaquine, but ala-leu-PQ was significantly less toxic, both in terms of LD_{50} and MTD, thus enabling the administration of higher doses (Table 4).

The toxicity and activity studies of ala-leu-ala-leu-PQ are incomplete. Preliminary results indicated that its toxicity was equal to or lower than that of ala-leu-PQ, and that its activity seemed to be higher than that of ala-leu-PQ, leu-PQ, and free primaquine, even at non-toxic doses. The activity of these derivatives



L-PQ = leu-PQ; A-L-PQ = ala-leu-PQ.

Fig. 5. *In vitro* digestion of amino acid and peptide derivatives of primaquine in calf serum. The results are expressed as a percentage of the initial concentration of the derivative.

seemed to be minimal when administered before the sporozoite inoculation.

DISCUSSION

Primaquine

Except for the route of drug administration, the therapeutic model used in these studies was similar to that of Fink (*4*), and the CPD_{50} value of 14.2 mg of primaquine base/kg of body weight was higher than the 6.6 mg/kg of body weight reported by Fink, after intraperitoneal administration.

After intravenous injection of [^{3}H]PQ, 30% of the label was found in the liver while the concentration of label per g of tissue protein was greatest in the lungs, liver, spleen, and kidneys. Further studies should aim at identifying the parental compounds and

Table 4. Antimalarial effects of the amino acid and peptide derivatives of primaquine

Drug	Dose (mg of primaquine diphosphate/kg of body weight)	No. of mice	Long-term survivors[a] (%)	Median survival time (days)[b]	Increase in life span (%)
Controls		43	0	17	0
Primaquine	12.5	9	0	17	0
	25	9	67	> 50	> 194
Leu-PQ	20	15	13	25	39
	30	20	35	25	39
	35	20	75	> 50	> 194
	40	14	50	37	106[c]
Controls		101	0	18	0
Ala-leu-PQ	10.9	27	11	24	33
	12.5	9	56	> 50	> 178
	17.5	18	56	> 50	> 178
	25	9	100	> 50	> 178
	32.8	27	81	> 50	> 178
	35	18	100	> 50	> 178
Controls		10	0	20	0
Ala-leu-ala-leu-PQ	12.5	7	57	> 50	> 150
	25	7	86	> 50	> 150
	35	6	100	> 50	> 150

[a] Percentage of mice who survived to day 50.

[b] All infected non-cured animals died before day 30.

[c] Caused by drug toxicity.

metabolites of ^{3}H-labelled primaquine to enable this data to be related to the toxic and therapeutic effects of the drug. The high level of accumulation of [^{3}H]PQ in the liver probably explains the efficacy of primaquine in the exoerythrocytic stages of malaria. It is not yet known to what extent the accumulation involves the hepatocytes or the Kupffer cells, or whether the drug is localized preferentially in a particular cell compartment.

Primaquine–liposomes

Primaquine entrapped in liposomes was found to dissociate partially (10%) on intravenous injection. The plasma primaquine levels after such an injection initially fell more slowly than those after administration of free primaquine. The distribution of primaquine in tissue was also changed: the spleen and liver accumulated, respectively, 3 times and 2 times more drug from PQ-lip than from free primaquine. This is in agreement with data from other authors (*5*, *6*) showing a selective uptake of liposomes by liver and spleen. Other tissues such as lung, kidney, and heart accumulated between 30 and 5 times less drug from PQ-lip than from free primaquine. The distribution data indicate a possible explanation for the reduced toxicity of PQ-lip, related to the decreased uptake of the drug by non-target tissues which are known to be involved in the subacute toxicity of primaquine in other animal species (*20*).

Although the hepatic concentration of primaquine was almost doubled by the administration of PQ-lip rather than free primaquine, the CPD_{50} values and the chemotherapeutic activity of PQ-lip and free primaquine were very similar. This could be explained if the increased uptake of PQ-lip concerned mainly the Kupffer cells and not the hepatocytes which seem to be involved in the exoerythrocytic malaria infection.

The therapeutic index of PQ-lip (Table 1) was, however, about 3 times higher than that of primaquine since its lower toxicity permitted the injection of a higher single dose which was capable of completely curing all infected mice.

Peptide derivatives of primaquine

The amino acid and peptide derivatives of primaquine were shown to have very interesting chemotherapeutic properties.

Leu-PQ, ala-leu-PQ, and ala-leu-ala-leu-PQ are suitable intermediate products for linking primaquine to proteins since they are hydrolysed rapidly by lysosomal enzymes to release free primaquine. However, leu-PQ was not hydrolysed in the presence of serum, while ala-leu-PQ and probably ala-leu-ala-leu-PQ were slowly hydrolysed to release leu-PQ (Fig. 2).

Since the toxic and chemotherapeutic activities of leu-PQ were very similar to those of primaquine (Table 1), it is likely that leu-PQ is either active by itself or is hydrolysed into primaquine within the cell (probably inside the lysosomes).

Ala-leu-PQ and ala-leu-ala-leu-PQ were less toxic and more active than primaquine and leu-PQ according to their MTD, LD_{50}, and CPD_{50} values (Table 2). Both derivatives were fully curative of infected mice after a single administration of 35 mg/kg of body weight.

Elucidation of the reasons why these derivatives are less toxic and more active than primaquine requires detailed *in vivo* pharmacokinetic and tissue distribution studies. However, we suggest that ala-leu-PQ and ala-leu-ala-leu-PQ are pro-drugs, which have to be hydrolysed to primaquine or leu-PQ in order to be active. This activation could occur either in the serum or inside the cells. The uptake and distribution of the pro-drugs in the tissue are different from those of primaquine, which may explain their different chemotherapeutic properties.

These results will promote the synthesis of other amino acid and peptide derivatives in a effort to modulate the rate and extent of hydrolysis of the derivatives in the serum, and in the cells.

Primaquine-protein conjugates

The synthesis of ala-leu-ala-leu-PQ will enable us to prepare conjugates of primaquine and proteins, such as asialofetuin which is selectively recognized by hepatocytes. This will allow us to check whether linking to selective carriers increases the chemotherapeutic activity of primaquine as well as decreasing its toxicity. We hope to prepare conjugates with a variety of action times by modifying the peptide arm as a function of its sensitivity to lysosomal hydrolases. This could lead to primaquine derivatives that would be active when administered several hours before the exoerythrocytic infection cycle.

ACKNOWLEDGEMENTS

We wish to thank Mr F. Herman, Mrs S. Cornelis-Marcelis, Miss Ch. de Ville de Goyet, and Mrs M. Debroux-Dechambre for their excellent technical assistance.

RÉSUMÉ

MISE AU POINT DE NOUVEAUX DÉRIVÉS DE LA PRIMAQUINE PAR ASSOCIATION AVEC DES SUPPORTS LYSOSOMOTROPES

Afin de diminuer la toxicité de la primaquine et d'en accroître l'activité, nous en avons préparé de nouveaux dérivés par incorporation dans des liposomes et par liaison à des acides aminés et à des peptides.

Lorsqu'elle est emprisonnée dans des liposomes multilamellaires, la primaquine devient nettement moins toxique, ce qui permet d'administrer des doses curatives à 100% de diphosphate de primaquine (60 et 70 mg/kg de poids corporel) en une seule injection intraveineuse pour traiter, chez des souris, des infections à *Plasmodium berghei* provoquées par des sporozoïtes.

Comme on peut l'évaluer par sa radioactivité, la ^{3}H-primaquine emprisonnée dans des liposomes a une hémikrèse (demi-vie) plasmatique plus longue que celle de la primaquine libre et s'accumule deux fois plus dans le foie, qui capte presque 50% de la dose injectée. Son absorption par la rate est augmentée de trois fois, tandis que les poumons, les reins et le cœur accumulent nettement moins de primaquine lorsqu'elle est emprisonnée dans des liposomes. Le degré maximal de réduction de l'absorption, à savoir environ 30 fois, est observé pour les poumons.

Ces résultats pharmacocinétiques peuvent expliquer la toxicité moindre de la primaquine emprisonnée dans des liposomes, pour autant que les mesures de radioactivité faites après administration de ^{3}H-primaquine concernent le médicament original ou ses métabolites actifs.

Un dérivé comprenant un acide aminé (leu-primaquine) et deux dérivés peptidiques (ala-leu-primaquine et ala-leu-ala-leu-primaquine) ont été synthétisés comme produits intermédiaires pour lier la primaquine à des protéines supports. Ces dérivés présentent, par eux-mêmes, des propriétés chimiothérapiques très intéressantes et sont très probablement des médicaments précurseurs de la primaquine, qui doivent être hydrolysés et activés pour donner de la primaquine dans le sérum et/ou à l'intérieur des cellules. L'ala-leu-primaquine et l'ala-leu-ala-leu-primaquine sont au minimum deux fois moins toxiques que la primaquine et elles sont caractérisées par de faibles valeurs de la dose prophylactique causale (DPC_{50}), à savoir 11 et 7,5 mg base/kg, respectivement, contre 14 à 16 pour la primaquine, les primaquine–liposomes et la leu-primaquine. Lorsqu'elles sont administrées par voie intraveineuse, en une injection, à des doses équivalentes à 35 mg/kg de diphosphate de primaquine, l'ala-leu-primaquine et l'ala-leu-ala-leu-primaquine entraînent 100% de guérison chez des souris infectées par des sporozoïtes de *P. berghei*.

Nous procédons actuellement à la liaison de la primaquine, par l'intermédiaire d'une «entretoise» tétrapeptidique, à l'asialofétuine, qui est une glycoprotéine faisant l'objet d'une endocytose sélective par les cellules hépatiques; les propriétés chimiothérapiques et toxiques de ces conjugués seront déterminées chez des souris infectées par *P. berghei*.

REFERENCES

1. THOMPSON, P. E. & WERBEL, L. M. *Antimalarial agents*. New York, Academic Press, 1972.
2. TROUET, A. Increased selectivity of drugs by linking to carriers. *European journal of cancer*, **14**: 105-111 (1978).
3. GREGORY, K. G. & PETERS, W. The chemotherapy of rodent malaria. IX. Causal prophylaxis. Part 1: A method for demonstrating drug action on exoerythrocytic stages. *Annals of tropical medicine and parasitology*, **64**: 15-24 (1970).
4. FINK, E. Assessment of causal prophylactic activity in *Plasmodium berghei yoelii* and its value for the development of new antimalarial drugs. *Bulletin of the World Health Organization*, **50**: 213-222 (1974).
5. KIMELBERG, H. K. Differential distribution of liposome-entrapped [^{3}H] methotrexate and labelled lipids after intravenous injection in a primate. *Biochimica et biophysica acta*, **448**: 531-550 (1976).
6. JULIANO, R. L. & STAMP, D. The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs. *Biochemical and biophysical research communications*, **63**: 651-658 (1975).
7. ASHWELL, G. & MORELL, A. G. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Advances in enzymology*, **41**: 99-128 (1974).
8. BLACK, C. D. V. ET AL. The use of Pentostam liposomes in the chemotherapy of experimental leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **71**: 550-552 (1977).
9. ALVING, C. R. ET AL. Therapy of leishmaniasis: superior efficacies of liposome encapsulated drugs. *Proceedings of the National Academy of Sciences of the United States of America*, **75**: 2959-2963 (1978).
10. NEW, R. R. C. ET AL. Antileishmanial activity of antimonial entrapped in liposomes. *Nature (London)*, **272**: 55-56 (1978).
11. TROUET, A. ET AL. DNA, liposomes and proteins as carriers for antitumoral drugs. *Recent results in cancer research*, **75**: 229-235 (1980).
12. MASQUELIER, M. ET AL. Aminoacid and dipeptide derivatives of daunorubicin. I. Synthesis, physicochemical properties and lysosomal digestion. *Journal of medicinal chemistry*, **23**: 1166-1170 (1980).

13. PIRSON, P. ET AL. Liposomes in the chemotherapy of experimental murine malaria. *Annals of tropical medicine and parasitology*, **74**: 383-391 (1980).
14. BANGHAM, A. D. ET AL. Preparation and use of liposomes as models of biological membranes. In: Korn, S. ed., *Methods in membrane biology*. New York and London, Plenum Press, 1974, vol. 1, pp. 1-68.
15. STELAKATOS, G. C. ET AL. On the trityl method for peptide synthesis. *Journal of the American Chemical Society*, **81**: 2884-2887 (1959).
16. ANDERSON, G. W. ET AL. The use of esters of *N*-hydroxysuccinimide in peptide synthesis. *Journal of the American Chemical Society*, **86**: 1839-1842 (1964).
17. TROUET, A. Isolation of modified liver lysosomes. *Methods in enzymology*, **31**: 323-324 (1974).
18. STEIN, S. ET AL. Amino acid analysis with fluorescamine at the picomole level. *Archives of biochemistry and biophysics*, **155**: 203-212 (1973).
19. MANCINI, G. ET AL. Immunochemical quantitation of antigens by single radial immunodiffusion. In: *Immunochemistry*. Oxford, Pergamon Press, 1965, vol. 2, pp. 235-254.
20. LEE, C. C. ET AL. *Bulletin of the World Health Organization*, **59**: 439-448 (1981).