International Journal of Pharmaceutics 416 (2011) 402–409

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Cationic nanoemulsion as a delivery system for oligonucleotides targeting malarial topoisomerase II

F. Bruxel[a,b], S. Cojean[c], A. Bochot[a], H. Teixeira[b], C. Bories[c], P.-M. Loiseau[c], E. Fattal[a,*]

[a] Université Paris-Sud, CNRS UMR 8612, Physico-chimie - Pharmacotechnie - Biopharmacie, Faculté de Pharmacie, 5 Rue Jean-Baptiste Clément, Châtenay-Malabry F-92296, France
[b] Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Rio Grande do Sul (UFRGS), Av. Ipiranga 2752, 90610-000 Porto Alegre, Brazil
[c] Chimiothérapie Antiparasitaire, Univ Paris-Sud, CNRS UMR 8076, 5 Rue Jean-Baptiste Clément, Châtenay-Malabry F-92296, France

ARTICLE INFO

Article history:
Received 1 December 2010
Received in revised form 20 January 2011
Accepted 21 January 2011
Available online 1 February 2011

Keywords:
Oligonucleotides
Cationic nanoemulsion
DOTAP
Topoisomerase II
Plasmodium falciparum

ABSTRACT

A promising strategy based on the antisense oligonucleotides against the *Plasmodium falciparum* topoisomerase II has been considered using cationic nanoemulsion as oligonucleotide delivery system. Phosphodiester and chemically modified phosphorothioate oligonucleotides bearing negative charges were adsorbed on positively charged emulsion composed of medium chain triglycerides, egg lecithin, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and water, at different +/− charge ratios (positive charges from cationic lipid/negative charges from oligonucleotide): +0.5/−, +2/−, +4/− and +6/−. The physicochemical properties of the complexes were determined, as well as their stability in culture medium. Their interaction with erythrocytes through hemolysis, binding experiments and confocal microscopy were also evaluated. Finally, the *in vitro* evaluation of parasite growth and reinfection capacity was performed. The overall results showed that antisense oligonucleotides against *P. falciparum* topoisomerase II gene can be efficiently adsorbed onto a cationic nanoemulsion forming complexes. Whereas unloaded nanoemulsion displayed an hemolytic effect due to the presence of the cationic lipid, this was not the case of loaded nanoemulsion at low +/− ratios. Oligonucleotide-loaded nanoemulsions were found to be located inside the infected erythrocytes, inhibiting efficiently parasite growth (until 80%) and causing a delay in *P. falciparum* life cycle.

1. Introduction

Malaria is one of the most widespread parasitic diseases. It is currently endemic in more than 100 countries in tropical and subtropical areas. The mortality rate related to malaria is currently estimated at over one million people per year and has increased, most likely due to parasite drug resistance (WHO, 2008). Since the emergence of chloroquine resistance in the 1950s, antimalarial drug resistance for *Plasmodium falciparum*, is considered a major contributor to the global resurgence of malaria observed over the past 40 decades and one of the main hindrance for an effective control (Hastings et al., 2007; Na-Bangchang and Congpuong, 2007). The emergence of artemisinin resistance is one of the greatest threats to renewed efforts to eradicate malaria (Dondorp et al., 2009; Greenwood et al., 2008). There is therefore an urgent need for the development of new drugs for malaria treatment.

Antisense oligonucleotides (ON) have been used to selectively modulate the expression of genes as well as to inhibit protein synthesis. Over the past decade, they have been considered as a potential strategy for malaria, especially since several studies have shown that antisense ON targeting of different enzymes, antigens, and other targets can inhibit *in vitro P. falciparum* growth (Barker et al., 1996; Kanagaratnam et al., 1998; Noonpakdee et al., 2003; Wanidworanun et al., 1999). However, antisense ON-based therapy is limited by both rapid degradation of ON in biological fluids as well as their inability to efficiently cross cell membranes due to their hydrophilic character and high molecular structure (Opalinska and Gewirtz, 2002).

To circumvent these drawbacks, the association of ON with both polymer- or lipid-based colloidal carriers has been proposed. For instance, Föger et al. (2006) have recently described, for the first time, the incorporation of phosphorothioate antisense ON targeting malarial topoisomerase II into biocompatible chitosan nanoparticles. The complexes showed a more pronounced sequence specific inhibition of parasite growth compared to free antisense ON. Moreover, the association of ON with nanoparticles resulted in their protection against nuclease attack *in vitro*.

Lipid injectable emulsions have been used as sources of calories and essential fatty acids for patients for at least 40 years. Their low

* Corresponding author at: UMR 8612 CNRS, Faculté de Pharmacie - Université Paris-Sud 11, 5 Rue Jean-Baptiste Clément, Châtenay-Malabry F-92296, France. Tel.: +33 146835568; fax: +33 146619334.
E-mail addresses: elias.fattal@u-psud.fr, elias.fattal@cep.u-psud.fr (E. Fattal).

0378-5173/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2011.01.048

toxicity makes them a good alternative as an intravenous delivery system (Driscoll, 2006). Triglyceride-based emulsions stabilized by phospholipid and cationic lipid combinations have been studied as potential delivery systems for ON (Teixeira et al., 2003, 2001). ON molecules can be associated to oil droplets through ion-pair formation, due to the positively charged nanoemulsions and the negatively charged ON (Teixeira et al., 2001). More recently, the cationic lipid composition of an emulsion has been optimized to obtain the best conditions for the adsorption and release of a model ON (oligothymidilates) from nanoemulsions (Martini et al., 2007, 2008). Both electrostatic and hydrophobic interactions were found to play a role in the complexation process.

The purpose of this study was to evaluate the potential of a cationic nanoemulsion (NE) as a delivery system for antisense ON targeting *P. falciparum* topoisomerase II. The ON sequences used here were previously described to be specific by Noonpakdee et al. (2003) and Föger et al. (2006). Phosphodiester antisense ON (PO) and chemically modified phosphorothioate antisense ON (PS) were complexed to the cationic emulsion. The physicochemical properties of the whole colloidal system as well as its interaction with erythrocytes though hemolysis, binding experiments and confocal microscopy were determined. Finally, we have evaluated the potential of such a system to inhibit *in vitro* parasite growth.

2. Materials and methods

2.1. Materials

Medium-chain triglycerides (MCT) (Lipoid AG, Germany), egg lecithin – Lipoid E-80 (Lipoid AG, Germany), dioleoyltrimethylammonium propane (DOTAP) (Sigma, USA), Nile red (Sigma, USA), and glycerol (Merck, Brazil) were used for the preparation of NE. Ultrapure water was obtained from a Milli-Q apparatus (Millipore, France). The ON sequences were obtained as antisense (5′-ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT-3′) or sense (5′-AGA ATC GTA TGG TTC AAA AGA ATA TTA CAT-3′) within the structural region of *P. falciparum* topoisomerase II. Phosphodiester antisense ON (PO, MW = 9146 g/mol), phosphodiester sense ON (sPO, MW = 9261 g/mol), full chemically modified phosphorothioate antisense ON (PS, MW = 9610 g/mol), full chemically modified phosphorothioate sense ON (sPS, MW = 9727 g/mol), 5′-end covalently conjugated with fluorescein isothiocyanate phosphodiester antisense ON (FITC-PO) and phosphorothioate antisense ON (FITC-PS) were purchased from Eurogentec (Angers, France). 5′-end labeled ^{33}P-phosphodiester antisense ON (^{33}P-PO) or ^{33}P-phosphorothioate antisense ON (^{33}P-PS) were synthesized using T4 polynucleotide kinase (Biolabs, U.K.) and ^{33}P ATP (Isotopchim, France).

2.2. Preparation and characterization of NE

NE composed of 8% (w/w) MCT, 2% (w/w) egg lecithin, 0.132% (w/w) DOTAP, 2.25% (w/w) glycerol and water to 100% (w/w) were prepared through spontaneous emulsification as previously described (Martini et al., 2008). Briefly, a limpid ethanolic solution containing the oily phase components was slowly added to a water phase containing glycerol, under moderate magnetic stirring. The excess of solvents mixture (ethanol/water) was then removed under reduced pressure at 50 °C until the desired final volume (5 ml). The final cationic lipid concentration was 2 mM, as previously optimized (Martini et al., 2008). The mean droplet size and zeta potential were determined through photon correlation spectroscopy and electrophoretic mobility (Zetasizer Nano ZS, Malvern Instrument, UK), respectively, at 20 °C. The NE were adequately diluted in water for size and polydispersity index (PDI) determinations or in 1 mM NaCl solution for zeta potential measurements.

2.3. Preparation and characterization of NE/ON complexes

Antisense ON (PO and PS) adsorption on cationic NE was performed at the end of the manufacturing process, resulting in NE/ON (NE/PO and NE/PS) complexes. Increasing concentrations of NE were added to water solutions of PO and PS at 10 μM (final concentration) and incubated during 15 min at room temperature, as described elsewhere (Teixeira et al., 1999). The physicochemical properties and morphology were evaluated (as described in Section 2.2) for NE/PO and NE/PS complexes. They were prepared at four different +/− charge ratios (ratios calculated between the number of positive charges from the cationic lipid present in NE, and the number of negative charges from the phosphate groups of ON) that are +0.5/−, +2/−, +4/− and +6/−.

2.4. Hemolysis experiments

Experiments were performed using both non-parasited and *P. falciparum*-infected erythrocytes. Blood was obtained from healthy O^+ human blood and erythrocytes were collected after centrifugation. A suspension of erythrocytes (5%) in RPMI medium (Invitrogen, France), supplemented with 25 mM HEPES (Sigma, France), 25 mM $NaHCO_3$ (Sigma, France), 0.5% Albumax (Invitrogen, France) and hypoxanthine 367 μM (Sigma, France) was prepared (same conditions as for the *in vitro* experiments) and carefully distributed in 24-well plates. Three sets of experiments were performed with NE/ON complexes: (a) NE/PO and NE/PS complexes obtained at different +/− charge ratios (+0.5/−, +2/−, +4/−, and +6/−), in a final PO and PS concentrations of 10 μM were incubated with non-infected erythrocytes for 15 min; (b) an hemolysis kinetics was performed on the complex at a charge ratio of +4/−, which was incubated with non-infected erythrocytes for 5, 15, 30, 60, and 180 min; (c) the complex at a charge ratio of +4/− was incubated with *P. falciparum*-infected erythrocytes for 15 min and 44 h. Besides, the hemolytic effect of unloaded NE was also evaluated over infected and non-infected erythrocytes for 44 h. The samples were then centrifugated and supernatants were analyzed with respect to hemoglobin release by measuring the absorbance at 570 nm. NaCl 0.9% (w/v) and Triton 100 (5%, w/v) were used as negative and positive controls, respectively. Results were expressed as the percentage of the amounts of hemoglobin release caused by complexes as percent of the total amount.

2.5. Erythrocytes binding experiments

Prior to binding experiments, ^{33}P-PO and ^{33}P-PS were obtained as previously described (Aynie et al., 1996). Briefly, 5′-radiolabeling of ON (PO and PS) were carried out as follows: 5 μl of a solution of PO or PS (10 μM), 1 μl of T4 polynucleotide kinase, and 1 μl of β-33P-ATP (sp act 7.4 Bq/mmol) were incubated for 30 min at 37 °C. The reaction was stopped by heating the preparations at 80 °C for 15 min ^{33}P-PO and ^{33}P-PS was recovered after purification by exclusion chromatography and centrifugation at 2400 rpm for 1 min. The purity of ^{33}P-PO and ^{33}P-PS was evaluated using an automatic TLC-linear analyzer. The binding of complexes was assessed by three sets of experiments, as described in Section 2.4. After incubation, cells were separated by centrifugation and treated with Triton 100 (1%, v/v) at room temperature. The amount of radioactivity in the cell lysate was determined through liquid scintillation counting (LS6000 TA, Beckman-Coulter, USA), using Hionic-Fluor liquid scintillation cocktail (PerkinElmer, France). Results were expressed as the percentage of radioactivity found in cell pellets of the total amount of radioactivity with cells.

2.6. Stability of complexes in culture medium

Stability of complexes was carried out at 37 °C in supplemented RPMI culture medium (composition described in Section 2.4). The complexes tested where NE/PO and NE/PS at charge ratios of +0.5/−, +2/− and +4/−. The stability of unloaded NE (at the same proportion as present in the +0.5/−, +2/− and +4/− complexes) was also evaluated. Samples were diluted 10 times in the culture medium (as for the *in vitro* tests), whereas the mean droplet size and PDI were determined directly 15 min, 1, 8, 24 and 44 h after dilution.

2.7. Growth inhibition of P. falciparum in vitro

P. falciparum 3D7 strain was maintained in O^+ human erythrocytes in albumin RPMI supplemented medium (composition described in Section 2.4), under continuous culture using the candle-jar method (Trager and Jensen, 1976). The parasites were synchronized to the ring stage by repeated sorbitol treatment (Lambros and Vanderberg, 1979). A 5% (v/v) erythrocytes suspension with 0.5% parasitemia (number of parasites per 100 red blood cells) was incubated with the tested formulations: sense phosphodiester ON (sPO), sense phosphorothioate ON (sPS), antisense phosphodiester ON (PO) and antisense phosphorothioate ON (PS) solutions, NE/sPO, NE/sPS, NE/PO and NE/PS complexes in the charge ratio of +4/−. All of them were prepared at a concentration of ON of 10 μM (final concentration of 1 μM in the cell suspension). Parasites were also incubated with culture medium (negative control) or with 4 μM chloroquine (positive control) in 96-well culture plates. In addition, the unloaded NE was tested at the same concentration as the one present in the +4/− complexes (6.2 mg of inner phase/mL of culture medium). After 44 h incubation at 37 °C, the plates were subjected to 3 freeze–thaw cycles to achieve complete hemolysis. The parasite growth was determined by the ELISA-Malaria antigen test (DiaMed, France), for the detection of *P. falciparum* lactate dehydrogenase (pLDH), as well as by microscopic examination under oil immersion of Giemsa stained thin blood smears. Results were expressed as the percentage of reduction of parasite growth over the control receiving only the culture medium.

Parallel parasite cultures at 2% of parasitemia were used for the reinfection test. The NE/PO and NE/PS complexes were incubated with infected red blood cells containing parasites in early stage growth forms (ring) and/or mature growth forms (trophozoites) for 44 h. Blood smears were prepared after 24 and 44 h, by Giemsa staining. Parasitemia were determined by microscopic counting of at least 5000 erythrocytes under oil immersion.

2.8. Confocal fluorescence microscopy

Confocal laser scanning microscopy was used to determine the localization of FITC-PO and FITC-PS naked or complexed to NE (loaded with the lipophilic fluorescent Nile red) at +4/− charge ratio. Red blood cells infected or not with parasites at different stages of development were incubated 15 min with the tested formulations and then washed. The Glass slides were examined with a Zeiss LSM-510 confocal scanning laser microscope equipped with a 1 mW Helium Neon laser, using a Plan Apochromat 63× objective (NA 1.40, oil immersion). Fluorescence was observed with a long-pass 488 emission filter under 543 nm laser illuminations. The pinhole diameter was set at 71 μm. Stacks of images were collected every 0.42 μm along the *z*-axis.

Table 1
Size (nm) and zeta potential (mV) of NE and NE/ON complexes.

		Droplet size (nm)	PDI	Zeta potential (mV)
NE		142–200	<0.2	54 ± 4.3
NE/PO	+0.5/−	206 ± 23	<0.3	−20 ± 1
	+2/−	343 ± 239	<0.4	9 ± 18
	+4/−	216 ± 20	<0.3	37 ± 2
	+6/−	182 ± 33	<0.2	41 ± 1
NE/PS	+0.5/−	178 ± 6	<0.2	−23 ± 3
	+2/−	281 ± 172	<0.3	12 ± 19
	+4/−	204 ± 18	<0.3	38 ± 7
	+6/−	177 ± 7	<0.3	43 ± 6

2.9. Statistical analysis

Results were expressed as mean ± standard deviation of three independent experiments and were analyzed using the Student's *t*-test.

3. Results

3.1. Properties of NE and NE-ON complexes

Unloaded NE presented a droplet size of approximately 140–200 nm and a zeta potential positive value of about +55 mV (Table 1). The association of either PO or PS to NE has been performed prior to further utilization. The amount of PO or PS complexed to NE was close to 100% since no ^{33}P-PO or ^{33}P-PS radioactivity was found in the external aqueous phase after separation by ultrafiltration/centrifugation. Their size and zeta potential values are given in Table 1. The complexation of either PO or PS on NE at a +/− charge ratio higher than 4 led to positively charged complexes, while the complexes at +0.5/− charge ratio led to negatively charged ones. The zeta potential values of +2/− complexes were very close to zero, displaying important variations in their size between preparations. Indeed, these complexes exhibited the highest droplet size and PDI values, with the presence of some aggregates. In any case, differences between the physicochemical properties of NE/PO and NE/PS complexes were observed.

3.2. Erythrocytes hemolysis

Interactions between NE/PO or NE/PS complexes and erythrocytes were first evaluated through hemolysis experiments (Fig. 1A and B). As it can be seen, between a NE/ON charge ratio of +0.5/− and +4/−, hemolysis was lower than 10% (Fig. 1A). However, increasing the NE/PO and NE/PS complexes charge ratio to +6/− induced a higher hemolytic effect that reached a value of 25% ($p < 0.05$). No differences were observed between NE/PO and NE/PS. Concerning the influence of time on hemolytic effect of complexes, as shown in Fig. 1B, for both NE/PO and NE/PS complexes at charge ratio +4/−, hemolysis reached quickly a maximum and did not change over time. Moreover, the hemolytic effect of complexes at a charge ratio of +4/− (9–12%) on *P. falciparum*-infected erythrocytes remained quite similar to the hemolytic effect on healthy erythrocytes (5–10%) even after an incubation time of 15 min or 44 h. Finally, the hemolytic effect of unloaded NE was significantly higher ($p < 0.05$) than those of the +4/− NE/PO and NE/PS complexes at the same concentration, achieving 30% and 42% hemolysis on non-infected and infected erythrocytes, respectively (data not shown).

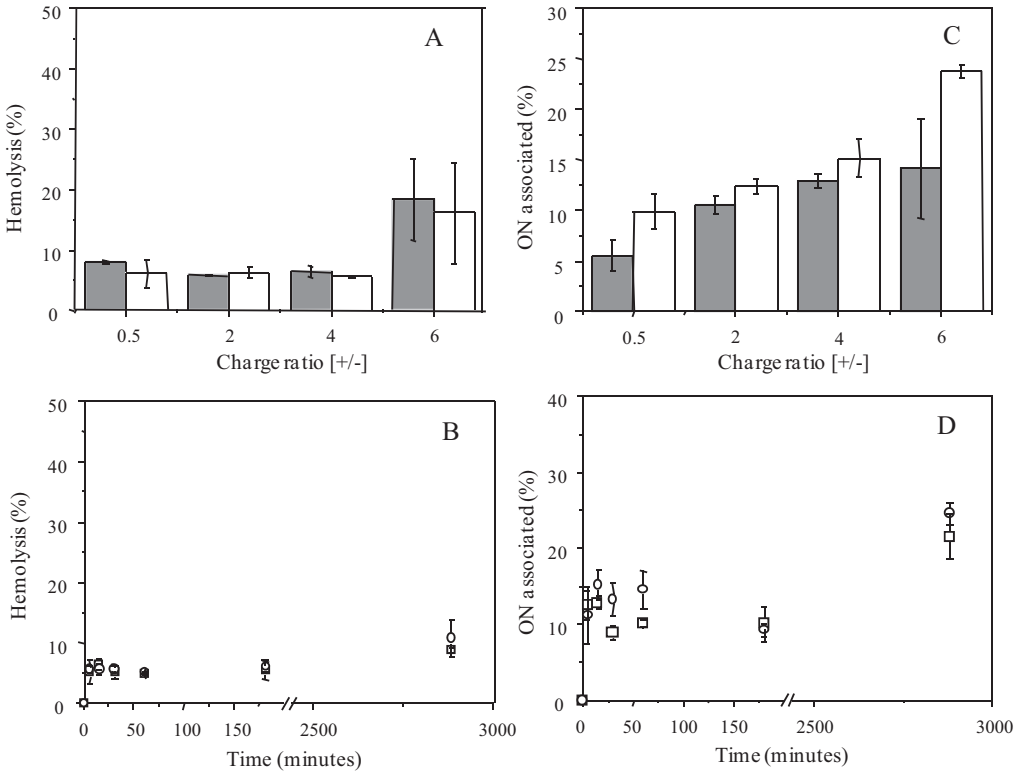

Fig. 1. Effect of the charge ratio of complexes and incubation time on hemolysis (A and B) and ON erythrocyte binding (C and D) with non-infected erythrocytes. NE/PO (gray bars) and NE/PS (white bars) complexes were obtained at different charge ratios (+0.5/−, +2/−, +4/− and +6/−) and incubated for 15 min with non-infected erythrocytes (A and C). NE/PO (squares) and NE/PS (circles) complexes were obtained at a charge ratio of +4/− and incubated for 2880 min with non-infected erythrocytes (B and D).

3.3. ON binding to erythrocytes

To evaluate cell binding of complexes, ^{33}P-labeled complexes were incubated with erythrocytes and the bound fraction was estimated after centrifugation. As the charge ratio increased, the radioactivity associated to erythrocytes increased, regardless the type of ON (Fig. 1C). Considering all charge ratios tested, the binding to erythrocytes was higher for NE/PS complexes ($p < 0.05$). Complexes obtained at a charge ratio of +4/− presented a binding over time comprised between approximately 10% and 15%. After 44 h of incubation, the values increased, regardless of the complex composition, by up to approximately 20% (Fig. 1D). Considering the binding of NE/PO and NE/PS complexes on *P. falciparum*-infected erythrocytes, the results were close to those observed with non-infected cells, with a binding remaining constant after 15 min or 44 h of incubation (approximately 14–17%) for both PO and PS.

3.4. Complexes stability in culture medium

Considering the lowest percentage of hemolysis obtained for complexes at +0.5/−, +2/− and +4/− charge ratios, they were selected for a stability study, which consisted in measuring the droplet mean size and PDI changes over time. These experiments were carried out after sample dilution in albumin supplemented culture medium (in the same dilution factor as for the *in vitro* tests). Determination of the mean droplet size and PDI of +0.5/−, +2/− and +4/− NE/PO complexes showed that the mean droplet size of the positively charged complexes (+2/− and +4/− NE/PO) increased over the time (compared to time zero, $p < 0.05$), but remained between 150 and 280 nm (Fig. 2). At all charge ratios tested, the PDI was maintained below 0.2 until 8 h of incubation, increasing to over 0.3 only after 24 h. Unloaded NE, diluted in the same proportion, displayed the same stability profiles as the loaded ones.

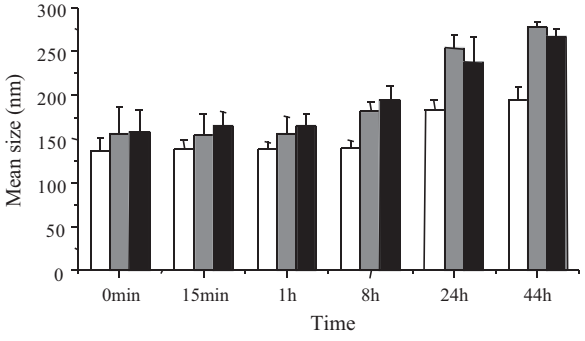

Fig. 2. Medium droplet size and polydispersity index (PDI) characterization of +0.5/− (white bars); +2/− (gray bars) and +4/− (black bars) charge ratio NE/PO complexes during time, after dilution in RPMI supplemented culture medium.

3.5. Growth inhibition of P. falciparum in vitro

Considered as the optimal formulations, +4/− NE/PO or PS complexes were chosen for the *in vitro* evaluation of *P. falciparum* growth, measured by the detection of the pLDH protein (Fig. 3A). Antimalarial effects of free 1 μM PO or PS (antisense) and sPO or sPS (sense control sequences), as well as +4/− NE/PO or NE/PS (antisense complexes) and NE/sPO or NE/sPS (sense control complexes), were evaluated in *P. falciparum* synchronized ring-stage parasites for 44 h. The final concentration in cell suspension was the same as for hemolysis, binding and stability experiments (1 μM). About 50% of inhibition of parasite growth was observed for the unloaded NE, used at the same amount as the one present in +4/− complex formation. Free PO, PS, sPO or sPS exhibited a low parasite growth inhibition (from 7 to 24%), regardless the type of ON tested. However, after they were adsorbed on the NE in a charge ratio of

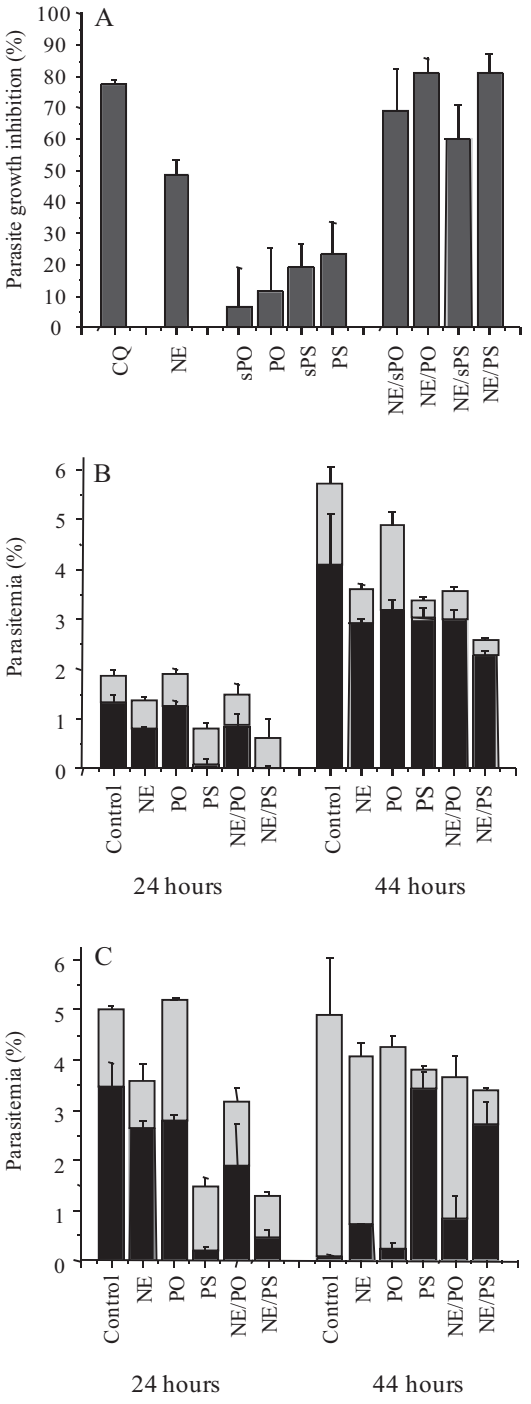

Fig. 3. (A) *P. falciparum* growth inhibition, quantified by the pLDH ELISA test, after 44 h of exposure to treatments (dark gray bars). (B) Reinfection test over ring stage form parasites, detected by microscopy counting (Giemsa staining) 24 and 44 h after treatment. (C) Reinfection test over mature (trophozoite and schizont forms) parasites, detected by microscopy counting (Giemsa staining) 24 and 44 h after treatment. All treatments were compared to a 100% of parasite growth control (Control). Chloroquine (CQ); nanoemulsion (NE); sense ON controls (sPO and sPS); antisense ON (PO and PS); sense NE/ON control complexes (NE/sPO and NE/sPS); antisense NE/ON complexes (NE/PO and NE/PS). Light gray bars: late stage parasites (schizonts and trophozoites); black bars: early stage parasites (ring).

+4/−, there was a higher parasite growth inhibition (65-81%) for all complexes, compared to the free ON ($p < 0.05$). In addition, NE/PS complex significantly reduced parasite growth compared to the NE/sPS complex ($p < 0.05$), while NE/PO complexes did not (compared to NE/sPO complexes). All formulations tested presented antimalarial activity similar to that of chloroquine, used as positive control.

Besides the detection of parasite growth *in vitro* through the evaluation of pLDH protein, microscopic counts of all forms of parasites were performed in parallel cultures, after treatment with the tested formulations, over different stages of development: early stage (ring) forms (Fig. 3B) and mature or late stage (trophozoites and schizonts) forms (Fig. 3C). After 24 h of treatment, more pronounced inhibition effects on parasite growth were observed, while after 44 h these effects decreased. Even after treatment, the parasites persist in infecting new red blood cells, but a delay in the development cycle was observed after treatment with free PS or NE/PS complex. As shown in Fig. 3C, after 24 h of treatment, there was a reduction of ring forms levels and consequently, after 44 h, a reduction of also late stage forms of parasites, compared to control, receiving only culture medium.

3.6. Interaction with *P. falciparum*-infected erythrocytes by confocal microscopy

Cell and parasite uptake of NE/PO and NE/PS complexes was investigated by confocal microscopy studies. Non-infected red blood cells and erythrocytes infected with different stages parasites were incubated with the tested formulations for 15 min (Fig. 4). Fluorescence was only observed into the infected erythrocytes, mainly into those containing parasites at the late stages of development. Red and green fluorescence, from the labeled NE and ON, respectively, were co-located within the parasite (Fig. 4D). Moreover, the free ON was not observed within the erythrocytes, infected or not (data not shown).

4. Discussion

We have recently reported the adsorption of a linear homopolythymidilate ON (16-mer) on the o/w interface of NE (Martini et al., 2008). The main physicochemical characteristics of the NE obtained in this study are in accordance with that reported in previous studies using NE obtained through the spontaneous emulsification procedure under similar conditions (Fraga et al., 2008; Martini et al., 2008; Trimaille et al., 2001, 2003). As studied by Martini et al. (2008), the highest possible value of zeta potential obtained for these systems could be reached with 2 mM of the cationic lipid covering the surface and was, therefore, attributed to the presence of the lipid at the oil-in-water (o/w) interface of NE (Martini et al., 2008).

The first finding of the present study was to demonstrate the ability of such a system to bind a high length 30-mer ON bearing either PO or PS moieties since only small ON were previously complexed by NE. The results obtained suggest that complexation occurs, regardless the length of the ON, since similarly electrostatic interactions are involved in this process. For complexes with positive zeta potential values, no differences between the physicochemical properties of NE/PO or NE/PS complexes were observed ($p > 0.05$). Considering that PS differs from PO only by the modification of the phosphodiester linkage, where non-bonding phosphate oxygen is replaced by sulphur, the ability for ion pairing remained unchanged when high amounts of positive charges are available.

The +/− charge ratio indicates the amount of positive charges from the cationic lipid over the amount of negative charges provided by ON molecules. We studied NE/PO and NE/PS complexes at four different charge ratios (from +0.5/− to +6/−), with growing NE concentrations added to equal concentrations of ON. The +2/− NE/PO and NE/PS complexes presented evidences of instability after 30 min of complexation (data not shown), what could be

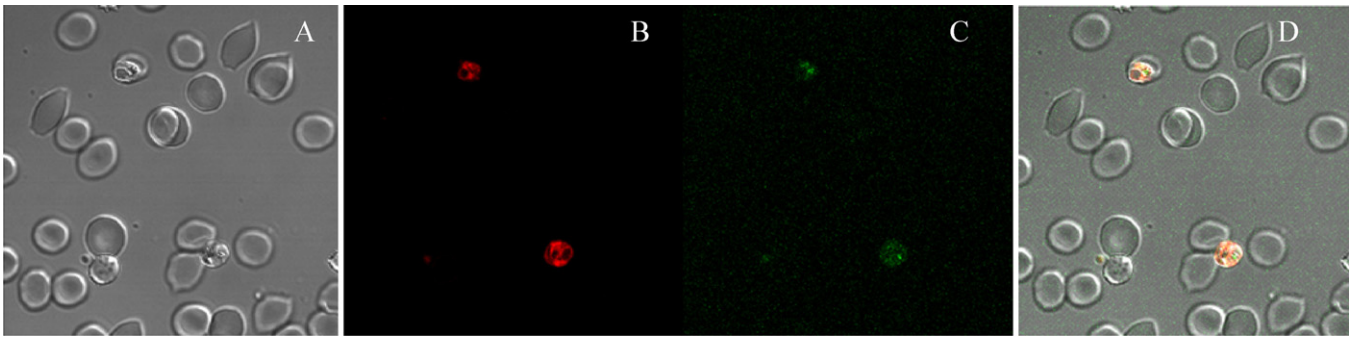

Fig. 4. Confocal microscopy images of *P. falciparum* infected-erythrocytes after incubation with dual labeled +4/− NE/PS complexes (fluorescein and Nile red) during 15 min. Bright field (A and D) and fluorescence images (B and C) recorded with 488 nm excitation and 543 nm emission wavelengths.

related to their low zeta potential values and high PDI as previously described (Martini et al., 2008; Roland et al., 2003; Teixeira et al., 1999).

The interactions of NE/PO and NE/PS complexes (obtained at different charge ratios) with erythrocytes were assessed through hemolysis, binding and confocal microscopy experiments. The binding experiments were performed so as to collect data on total cell binding (internalized and/or adsorbed at the outer cell membranes). As the charge ratio increased, binding to erythrocytes increased. Such a result could be attributed to the electrostatic interactions of the positive moieties of NE/PO and NE/PS complexes with negatively charged membranes of erythrocytes containing sialic acid residues (Van Damme et al., 1994). Similar features were previously reported for DNA-cationic liposomes complexes with however a higher percentage of binding (Sakurai et al., 2001a, 2001b). A plausible explanation for these differences could be the physicochemical properties of the carriers. In fact, nanoemulsions exhibit a lower density than liposomes, thus oil droplets can be efficiently separated from cells during binding experiments by centrifugation.

We also examined the hemolytic effect of NE/PO and NE/PS complexes. Irrespective of composition, complexes obtained at charge ratios up to +4/− shows similar hemolysis, below 10%. For the highest charge ratio tested (+6/−), as well as for unloaded NE, the hemolytic effect was significantly increased ($p < 0.05$), what could be related to the presence of more positive available charges for the interaction with red blood cells. Besides, it is also probably related to the nature of cationic lipid present in the NE. Previous results showed a higher hemolytic effect for oleylamine containing NE (data not shown). It has been well-documented that single-tailed cationic lipids were more toxic than their double-tailed counterparts since they can interact strongly with cell membranes and may interfere with membrane function through various mechanisms, consequently leading to cell toxicity (Lasch, 1995; Senior et al., 1991; Tang and Hughes, 1999).

For this reason, the NE/PO and NE/PS complexes obtained at the highest charge ratio, without a marked hemolytic effect were chosen for further studies. Thus, the hemolysis and binding of +4/− NE/PO and NE/PS complexes were evaluated over time in healthy and *P. falciparum*-infected erythrocytes. The results showed similar hemolytic effect and binding to infected or not erythrocytes, after both 15 min and 44 h of incubation, suggesting no marked effect of 2% of parasites on these parameters. Contrary, accentuated hemolysis was observed for unloaded NE in the presence of parasites. The designed incubation time of 44 h is related to *P. falciparum* life cycle, as for the following tests of *in vitro* evaluation of parasite growth.

In this context, it was necessary to verify the maintenance of physicochemical properties of NE/PO and NE/PS complexes in the presence of the culture medium, since it was supplemented with albumin, a serum protein. It is well known that adsorption of components on the surface of cationic systems can occur, depending on their charge density (Gessner et al., 2002; Luck et al., 1998). For comparison of the results, the same NE concentration was also diluted in culture medium, since different properties are observed with salinity and dilution variations (Rabinovich-Guilatt et al., 2004). Even though there was a small increase in the mean droplet size of positively charged +2/− and +4/− NE/PO complexes (zeta potential of about +10 to +40 mV), it was maintained under 300 nm. Thus, considering that the interaction with the red blood cells occurs from 15 min, the complexes in the three different charge ratios were considered stable.

The antisense ON sequence against topoisomerase II has been studied against *P. falciparum* K1 strain (Föger et al., 2006; Noonpakdee et al., 2003). Specific inhibition of parasite growth of 47% by free ON (Noonpakdee et al., 2003) and 87% by chitosan antisense nanoparticles (Föger et al., 2006) were obtained for this strain at an ON final concentration of 0.5 μM. In our study, at 1 μM final concentration, about 20–25% of inhibition was observed for free PO and PS, against about 80% for their complexes at the charge ratio of +4/− (Fig. 3A). Similar parasite growth inhibition was obtained for NE/PO and NE/PS complexes, corroborating the red blood cell binding results, since no difference in binding was obtained at this charge ratio (+4/−).

The antimalarial activity of +0.5/− and +2/− NE/PO and NE/PS complexes were also tested (preliminary results not shown), but unlike Föger et al. (2006), which had greater effect with negatively charged nanoparticles-ON, we found a better parasite growth inhibition by positively charged +4/− NE/ON complexes, which had a final positive zeta potential value (+30 mV). Unloaded NE led to a parasite growth of 50%. Considering that they have higher positive zeta potential (about +50 mV) compared to NE/PO and NE/PS complexes, their interaction with negatively charged cell membranes is enhanced. This interaction leads to a higher haemolytic effect. Therefore, unloaded NE may not be the best control for comparing the results.

Non-specific effects were observed for the free PO and PS, as already reported by other authors at high concentrations (Kanagaratnam et al., 1998; Ramasamy et al., 1996; Rapaport et al., 1992; Stein, 2001; Wanidworanun et al., 1999). Contrary to them, no inhibition of 3D7 *P. falciparum* strain was observed at concentrations lower than 1 μM of PO or PS, even for NE/PO or NE/PS complexes (preliminary results not shown). The non-specific effects have been characterized as similar to polyanions effects, such as dextran sulfate, by blocking the recognition of sialic acids of erythrocytes membrane by merozoites, during the reinfection pro-

cesses (Barker et al., 1996; Noonpakdee et al., 2003; Rapaport et al., 1992). However, in our studies these effects disappeared for NE/PS complexes, which were more effective that the NE/sPS complexes ($p < 0.05$) but not with the NE/PO complexes for which non-specific effects were still observed. This might be due to another mechanism such as a sequence non-specificity in the 3D7 strain, due to a different mRNA conformation, or a different protein expression profiling, for exemple (Koncarevic et al., 2007).

Finally, during the reinfection test, there was a higher reduction in parasitemia after treatment of erythrocytes infected by mature parasites compared to those infected by ring stage forms. Late stage forms seem to be more permeable than ring forms (Elford et al., 1985; Kirk, 2001), as observed by confocal microscopy, by both green (FITC-PO and FITC-PS) and red (loaded NE) fluorescence inside trophozoites parasitized erythrocytes.

5. Conclusions

NE provide a positively charged interface which allows the adsorption of ON, at different charge ratios. The adsorption of PO or PS remained quite similar, suggesting that it was governed by electrostatic interactions between positively and negatively charged moieties from cationic lipids and ON, respectively. The binding with erythrocytes increased proportionally to the +/− charge ratio of the complexes, with no marked hemolysis at low charge ratio (under +4/−). In such conditions, PO and PS can bind with *P. falciparum*-infected erythrocytes in a similar extent. ON most probably take advantage from the destabilization of the cell membrane due to the positive charges of NE, which could increase membrane permeability and allowed ON molecules to associate with cells. Our results showed that the association of ON to NE increases their activity against *P. falciparum* chloroquine-sensitive strain *in vitro*, validating the tested approach. Those systems are interesting for further studies in chloroquine-resistant strain, with the improvement of the ON sequence and backbone chemistry modification. Besides, studies including the determination of the mRNA and protein levels are in progress to better understand the results obtained. The optimized system will then be transposed to ON targeting *Plasmodium berghei* topoisomerase II for a further *in vivo* evaluation on the *P. berghei* mouse model.

Acknowledgements

The authors wish to thank CAPES/COFECUB (540/06) for their financial support, Valérie Nicolas from Plate-Forme d'Imagerie Cellulaire (IPSIT) of Université Paris-Sud 11, for the confocal microscopy analysis and the Centre National de Référence du Paludisme, AP-HP Hôpital Bichat-Claude Bernard Paris, France.

References

Aynie, I., Vauthier, C., Foulquier, M., Malvy, C., Fattal, E., Couvreur, P., 1996. Development of a quantitative polyacrylamide gel electrophoresis analysis using a multichannel radioactivity counter for the evaluation of oligonucleotide-bound drug carrier. Anal. Biochem. 240, 202–209.

Barker Jr., R.H., Metelev, V., Rapaport, E., Zamecnik, P., 1996. Inhibition of *Plasmodium falciparum* malaria using antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. U.S.A. 93, 514–518.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P., Lindegardh, N., Socheat, D., White, N.J., 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N. Engl. J. Med. 361, 455–467.

Driscoll, D.F., 2006. Lipid injectable emulsions: pharmacopeial and safety issues. Pharm. Res. 23, 1959–1969.

Elford, B.C., Haynes, J.D., Chulay, J.D., Wilson, R.J., 1985. Selective stage-specific changes in the permeability to small hydrophilic solutes of human erythrocytes infected with *Plasmodium falciparum*. Mol. Biochem. Parasitol. 16, 43–60.

Föger, F., Noonpakdee, W., Loretz, B., Joojuntr, S., Salvenmoser, W., Thaler, M., Bernkop-Schnürch, A., 2006. Inhibition of malarial topoisomerase II in *Plasmodium falciparum* by antisense nanoparticles. Int. J. Pharm. 319, 139–146.

Fraga, M., Laux, M., Dos Santos, G.R., Zandona, B., Dos Santos Giuberti, C., de Oliveira, M.C., da Silveira, M.U., Teixeira, H.F., 2008. Evaluation of the toxicity of oligonucleotide/cationic nanoemulsion complexes on Hep G2 cells through MTT assay. Pharmazie 63, 667–670.

Gessner, A., Lieske, A., Paulke, B., Muller, R., 2002. Influence of surface charge density on protein adsorption on polymeric nanoparticles: analysis by two-dimensional electrophoresis. Eur. J. Pharm. Biopharm. 54, 165–170.

Greenwood, B.M., Fidock, D.A., Kyle, D.E., Kappe, S.H., Alonso, P.L., Collins, F.H., Duffy, P.E., 2008. Malaria: progress, perils, and prospects for eradication. J. Clin. Invest. 118, 1266–1276.

Hastings, I.M., Korenromp, E.L., Bloland, P.B., 2007. The anatomy of a malaria disaster: drug policy choice and mortality in African children. Lancet Infect. Dis. 7, 739–748.

Kanagaratnam, R., Misiura, K., Rebowski, G., Ramasamy, R., 1998. Malaria merozoite surface protein antisense oligodeoxynucleotides lack antisense activity but function as polyanions to inhibit red cell invasion. Int. J. Biochem. Cell Biol. 30, 979–985.

Kirk, K., 2001. Membrane transport in the malaria-infected erythrocyte. Physiol. Rev. 81, 495–537.

Koncarevic, S., Bogumil, R., Becker, K., 2007. SELDI-TOF-MS analysis of chloroquine resistant and sensitive *Plasmodium falciparum* strains. Proteomics 7, 711–721.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. 65, 418–420.

Lasch, J., 1995. Interaction of detergents with lipid vesicles. Biochim. Biophys. Acta 1241, 269–292.

Luck, M., Paulke, B.R., Schroder, W., Blunk, T., Muller, R.H., 1998. Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics. J. Biomed. Mater. Res. 39, 478–485.

Martini, E., Carvalho, E., Teixeira, H., de Oliveira, M.C., 2007. Oligonucleotide adsorption on nanoemulsions obtained by spontaneous emulsification. Quim. Nova 30, 930–934.

Martini, E., Fattal, E., de Oliveira, M.C., Teixeira, H., 2008. Effect of cationic lipid composition on properties of oligonucleotide/emulsion complexes: physicochemical and release studies. Int. J. Pharm. 352, 280–286.

Na-Bangchang, K., Congpuong, K., 2007. Current malaria status and distribution of drug resistance in East and Southeast Asia with special focus to Thailand. Tohoku J. Exp. Med. 211, 99–113.

Noonpakdee, W., Pothikasikorn, J., Nimitsantiwong, W., Wilairat, P., 2003. Inhibition of *Plasmodium falciparum* proliferation in vitro by antisense oligodeoxynucleotides against malarial topoisomerase II. Biochem. Biophys. Res. Commun. 302, 659–664.

Opalinska, J.B., Gewirtz, A.M., 2002. Nucleic-acid therapeutics: basic principles and recent applications. Nat. Rev. Drug Discov. 1, 503–514.

Rabinovich-Guilatt, L., Couvreur, P., Lambert, G., Dubernet, C., 2004. Cationic vectors in ocular drug delivery. J. Drug Target. 12, 623–633.

Ramasamy, R., Kanagaratnam, R., Misiura, K., Rebowski, G., Amerakoon, R., Stec, W.J., 1996. Anti-sense oligodeoxynucleoside phosphorothioates nonspecifically inhibit invasion of red blood cells by malaria parasites. Biochem. Biophys. Res. Commun. 218, 930–933.

Rapaport, E., Misiura, K., Agrawal, S., Zamecnik, P., 1992. Antimalarial activities of oligodeoxynucleotide phosphorothioates in chloroquine-resistant *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. 89, 8577–8580.

Roland, I., Piel, G., Delattre, L., Evrard, B., 2003. Systematic characterization of oil-in-water emulsions for formulation design. Int. J. Pharm. 263, 85–94.

Sakurai, F., Nishioka, T., Saito, H., Baba, T., Okuda, A., Matsumoto, O., Taga, T., Yamashita, F., Takakura, Y., Hashida, M., 2001a. Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. Gene Ther. 8, 677–686.

Sakurai, F., Nishioka, T., Yamashita, F., Takakura, Y., Hashida, M., 2001b. Effects of erythrocytes and serum proteins on lung accumulation of lipoplexes containing cholesterol or DOPE as a helper lipid in the single-pass rat lung perfusion system. Eur. J. Pharm. Biopharm. 52, 165–172.

Senior, J.H., Trimble, K.R., Maskiewicz, R., 1991. Interaction of positively-charged liposomes with blood: implications for their application in vivo. Biochim. Biophys. Acta 1070, 173–179.

Stein, C.A., 2001. The experimental use of antisense oligonucleotides: a guide for the perplexed. J. Clin. Invest. 108, 641–644.

Tang, F., Hughes, J.A., 1999. Synthesis of a single-tailed cationic lipid and investigation of its transfection. J. Control. Release 62, 345–358.

Teixeira, H., Dubernet, C., Chacun, H., Rabinovich, L., Boutet, V., Deverre, J.R., Benita, S., Couvreur, P., 2003. Cationic emulsions improves the delivery of oligonucleotides to leukemic P388/ADR cells in ascite. J. Control. Release 89, 473–482.

Teixeira, H., Dubernet, C., Puisieux, F., Benita, S., Couvreur, P., 1999. Submicron cationic emulsions as a new delivery system for oligonucleotides. Pharm. Res. 16, 30–36.

Teixeira, H., Dubernet, C., Rosilio, V., Laigle, A., Deverre, J.R., Scherman, D., Benita, S., Couvreur, P., 2001. Factors influencing the oligonucleotides release from O-W submicron cationic emulsions. J. Control. Release 70, 243–255.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Trimaille, T., Chaix, C., Delair, T., Pichot, C., Teixeira, H., Dubernet, C., Couvreur, P., 2001. Interfacial deposition of functionalized copolymers onto nanoemulsions produced by the solvent displacement method. Colloid Polym. Sci. 279, 784–792.

Trimaille, T., Chaix, C., Pichot, C., Delair, T., 2003. Polymer functionalized submicrometric emulsions as potential synthetic DNA vectors. J. Colloid Interf. Sci. 258, 135–145.
Van Damme, M.P., Tiglias, J., Nemat, N., Preston, B.N., 1994. Determination of the charge content at the surface of cells using a colloid titration technique. Anal. Biochem. 223, 62–70.
Wanidworanun, C., Nagel, R.L., Shear, H.L., 1999. Antisense oligonucleotides targeting malarial aldolase inhibit the asexual erythrocytic stages of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 102, 91–101.
WHO, 2008. Guidelines for Treatment of Malaria. World Health Organization, Geneva.