Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Pharmaceutics 319 (2006) 139–146
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Inhibition of malarial topoisomerase II in *Plasmodium falciparum* by antisense nanoparticles

Florian Föger[a], Wilai Noonpakdee[b], Brigitta Loretz[a], Songwut Joojuntr[b], Willi Salvenmoser[c], Marlene Thaler[c], Andreas Bernkop-Schnürch[a,*]

[a] *Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innrain 52, Josef Möller Haus, A-6020 Innsbruck, Austria*
[b] *Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand*
[c] *Institute of Zoology and Limnology, Faculty of Natural Sciences, Leopold-Franzens-University Innsbruck, A-6020 Innsbruck, Austria*

Received 27 September 2005; received in revised form 23 March 2006; accepted 28 March 2006
Available online 25 April 2006

Abstract

New effective antimalarial agents are urgently needed due to increasing drug resistance of *Plasmodium falciparum*. Phosphorothioate antisense oligodeoxynucleotides (ODNs) silencing of malarial topoisomerase II gene have shown to possess promising features as anti malarial agents. In order to improve stability and to increase intracellular penetration, ODNs were complexed with the biodegradable polymer chitosan to form solid nanoparticles with an initial diameter of ~55 nm. The particle zetapotential depended on the chitosan/ODN mass ratio. Nanoparticles with mass ratio of 2:1 displayed a positive surface charge (+15 mV) whereas particles with 1:1 mass ratio were negatively charged (−20 mV). Additionally nanoparticles were found to protect ODNs from nuclease degradation. *P. falciparum* K1 strain was exposed to the chitosan/ODN-nanoparticles for 48 h in order to examine the effects of chitosan/antisense (AS) and chitosan/sense (S) oligodeoxynucleotide nanoparticles on malaria parasite growth. Both negatively and positively charged antisense nanoparticles as well as free antisense ODNs (in a final concentration of 0.5 μM) showed sequence specific inhibition compared with sense sequence controls. However, nanoparticles were much more sequence specific in their antisense effect than free ODNs. Nanoparticles with negative surface charge exhibited a significantly stronger inhibitory effect (~87% inhibition) on the parasite growth in comparison to the positive ones (~74% inhibition) or free ODNs (~68% inhibition). This is the first study demonstrating the susceptibility of *P. falciparum* to antisense nanoparticles.

Keywords: *Plasmodium falciparum*; Malaria; Nanotechnology; Chitosan nanoparticles; Antisense

1. Introduction

Malaria is one of the most prevalent human infectious diseases with up to 500 million infections occuring each year. *Plasmodium falciparum*, the most virulent of the four human malarial parasites, causes 1–3 million deaths each year, most of them among infants and young children in Africa. Both the broad collapse of preventive efforts and the decreased efficacy of current antimalarial drugs account for the global resurgence of malaria (Baird, 2005). New classes of antimalarial drugs are urgently needed due to increased resistance of *P. falciparum* against most currently used antimalarials (Noonpakdee et al., 2003). In this regard, use of antisense (AS) oligodeoxynucleotides (ODNs) offers an interesting alternative to traditional antimalarial drugs. The aim of the antisense approach is to interfere with gene expression by preventing the translation of proteins from mRNA (Lambert et al., 2001). The selectivity and flexibility of the antisense technology makes it an attractive strategy in treating several diseases. Phosphorothioate ODNs have been shown to inhibit HIV in vitro by a sequence specific and non-specific mechanism (Shaw et al., 1991). Similar to these findings several antisense treatments in malaria have demonstrated sequence specific as well as non-sequence specific inhibitory effects (Noonpakdee et al., 2003; Wanidworanun et al., 1999). Presently phosphorothioate ODNs are the most widely used modification of ODNs. Vitravene®, the first FDA-approved antisense drug from Isis, belongs to this type of synthetic nucleic acid. Progress has been made through the design of

* Corresponding author at: Tel.: +43 512 507 5371; fax.: +43 512 507 2933.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.03.034

chemically modified ODNs with improved stability in serum but ineffective transport is still a limiting factor for antisense therapy (Chen et al., 2005). In vivo studies showed that free ODNs disappear from plasma after a few minutes whereas ODNs incorporated into nanoparticles showed a delayed plasma clearance (De Smidt et al., 1991). Additionally, phosphorothioate oligonucleotides still remain a substrate for nucleases. Carrier systems, such as nanoparticles, present a possible approach to improve the delivery properties of antisense ODNs and, furthermore, the complexation of ODNs into nanoparticles, enhances stability against enzymatic degradation (Junghans et al., 2000). Chitosan, a biocompatible polysaccharide has been shown to effectively complex plasmid DNA and to protect DNA from nuclease degradation (Mao et al., 2000). As the malarial parasite divides rapidly after invasion of human erythrocytes, DNA replicating enzymes or their related genes in the parasites offer suitable key targets. DNA topoisomerase II that catalyzes changes in DNA topology, by cleaving and re-ligating both strands of the DNA double helix (Felix, 2001) has been reported to be a target of interest for antisense therapy (Noonpakdee et al., 2003). In this study 30mer antisense ODNs against malarial topoisomerase II gene have been incorporated in chitosan nanoparticles, in order to investigate the feasibility of applying nanotechnology against *P. falciparum*. Antisense and sense chitosan/ODN nanoparticles with negative as well as positive zeta potentials have been developed in order to investigate their release profile, stability against nuclease degradation, toxicity and their antimalarial effects against *P. falciparum* in vitro.

2. Materials and methods

2.1. Materials

Phosphorothioate oligodeoxynucleotides (ODNs) were designed to be antisense or sense to sequence within the structural region of *P. falciparum* topoisomerase II gene as listed in Table 1 (Noonpakdee et al., 2003). All phosphorothioate-ODNs were synthesized by VBC Biotech (Vienna, Austria) and were of HPLC grade. Chitosan (middle-viscous, medium molecular mass: 400 kDa; degree of deacetylation: 83–85%) was obtained from Fluka Chemie (Buchs, Switzerland). Giemsa staini was obtained from Fisher (Pittsburg, PA), RPMI 1640 medium, from Gibco, Triton X-100, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and $NaHCO_3$ from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Sample preparation

Chitosan (1% w/v in 6% v/v CH_3COOH, 100 mL) was depolymerised with 10 mL of $NaNO_2$ solution (0.5% w/v) for 1 h. After 1 h agitation, the depolymerised chitosan was precipitated by addition of 4 M NaOH solution to obtain pH 9, filtered and washed twice with acetone (Huang et al., 2004). The solid residue was resuspended in 20 mL 0.1% v/v CH_3COOH, dialysed twice against 4 L double-distilled water (24 h) and lyophilised. Phosphorothioate oligodeoxynucleotides were complexed with depolymerised chitosan with mass ratios (chitosan/ODNs) of 1:1 and 2:1 by mixing aliquots of 100 μg of chitosan, dissolved in 500 μL of 0.025% acetic acid (pH 5.5 adjusted with 1 M NaOH) with solutions of 50 or 100 μg of ODNs, dissolved in 500 μL of double-distilled sterile filtered water. Both solutions were preheated separately to 50 °C with a thermo mixer for 15 min. Then 500 μL of the 0.02% w/v chitosan solution was added to the ODNs solution followed by intensive vortexing for 90 s.

2.3. Size determination of the particles

The hydrodynamic diameters of the nanoparticles were measured by photon correlation spectroscopy (PCS) using a PSS NICOMP™ 380 DLS/ZLS (Santa Barbara, California, USA) with a 7.5 mW laser diode at 635 nm. Size determination of the nanoparticles was carried out in double-distilled water and additionally in RPMI 1640 medium including 10% v/v sterile filtered human serum (incubated for 30 min at 37 °C). Filtration of serum was performed in order to remove cellular components which might influence PCS measurements. The measurements were carried out at room temperature with a scattering angle of 90 °C. To verify the results obtained by PCS, transmission electron microscopy (TEM) of the nanoparticles in absence as well as in presence of 10% v/v sterile filtered human serum was carried out. A drop of approximately 10 μL of each suspension was mounted on pioloform-coated copper grid. Negative staining was carried out with 1% (w/v) uranyl acetate to enhance contrast. For samples with a high density of particles, suspensions were diluted with an equal volume of 1% uranyl acetate and mounted on grids. In addition pure chitosan ODN nanoparticles were mounted without negative stain to avoid artefacts by chemical influence. Specimens were examined with a ZEISS LIBRA 120 digital energy filter transmission electron microscope (EFTEM). Elastic imaging with the in-column omega filter (0 eV) and inelastic imaging with a selected energy loss of 50–130 eV were applied.

Table 1
Sizes and zetapotentials of particles formed by mixing chitosan with 30mer ODNs at 1:1 and 2:1 mass ratios measured as hydrodynamic diameters by PCS

Mass ratio (Chitosan:ODN)	Particle size (nm)		Zetapotential (mV)	
	Gauss number in distilled water	Gauss number in culture medium + 10% serum	Gauss number in distilled water	Gauss number in culture medium + 10% serum
1:1 Antisense	58 ± 25	58 ± 35	−20.7	−2.5
1:1 Sense	51 ± 21	56 ± 32	−19.0	−3.0
2:1 Antisense	55 ± 19	54 ± 39	+14.4	−1.9
2:1 Sense	55 ± 18	56 ± 38	+16.0	−2.0

Microphotography and documentation were performed using a VarioSpeed SSCCD camera (BM-2k-120). For size measurement and also for documentation an analySIS Pro TEM software and Adobe photoshop were used.

2.4. Electrokinetic potential

The zeta potentials of the nanoparticles were measured in double-distilled water and additionally in RPMI 1640 medium including 10% sterile filtered human serum (incubated for 30 min at 37 °C). The zeta potential was analysed by measuring the electrophoretic mobility using a PSS NICOMP™ 380 DLS/ZLS. All measurements were carried out at room temperature.

2.5. Oligonucleotide loading and release

The amount of ODNs complexed in the chitosan nanoparticles was determined by agarose gel-electrophoresis and by HPLC. Electrophoresis was performed in 2% agarose gel in 1 × Tris/acetic acid/EDTA buffer (pH 7.4) containing 500 μg/L of ethidium bromide at 30 V for 1 h. Gels were visualized under UV light. In order to evaluate the amount of released ODNs under physiologically buffered solution, nanoparticles were complexed with chitosan in the mass ratios 1:1 and 2:1 as described above and then diluted 1:1 with 2 × phosphate buffer saline (PBS) (pH 7.4). Nanoparticles were incubated at 37 °C and 400 rpm with a thermomixer. After 0, 0.5, 1, 2, 6, 24, 48 h, samples were removed, centrifuged at 29,700 × *g* for 60 min (Sigma 3–18 K centrifuge) and 30 μL of the supernatant was analyzed by a weak-base anion exchange HPLC assay, using a PRP-X600 Anion Exchange 4.6 × 100 HPLC column (Hamilton, Reno, Nevada, USA). A two-eluent system, with eluent A consisting of 80:20 100 mM Tris, pH 8.0:acetonitrile and eluent B consisting of 80:20 100 mM Tris, 2.5 M LiCl, pH 8.0:acetonitrile was used. A linear gradient from 100% eluent A to 100% eluent B in 15 min at a flow rate of 2 mL/min was performed. The amounts of ODNs were determined by measuring absorbance at 260 nm.

2.6. Protection of ODNs against digestion in plasma

Digestion of oligonucleotides was determined by incubating 50 μL of free or complexed ODNs in RPMI 1640 medium containing heat or non-heat inactivated human plasma at a final concentration of 20% at 37 °C and 400 rpm (Eppendorf Thermomixer comfort). After 0, 0.5, 2, 4 and 8 h, digestion was terminated with 50 μL of 0.5 M EDTA per 200 μL of samples. The chitosan/ODN particles were then dissolved by the addition of 100 μL of 5 M sodium chloride and incubation for 24 h. The amount of non-degraded ODNs were analysed by HPLC as described.

2.7. Evaluation of red blood cell lysis

Red blood cell lysis test was performed as described previously (Guggi et al., 2004). In brief, blood was obtained from male human (blood group 0+) and erythrocytes were collected by centrifuging (1500 × *g*, 5 min, 10 °C; SIGMA 3–16 K centrifuge) and washing in a washing solution (17.5 g sorbitol and 0.8 g NaCl in 100 mL double distilled water) for four times. A 2% v/v erythrocyte solution was prepared by resuspending the final cell pellet in an appropriate volume of washing solution. Aliquots of 400 μL of the erythrocyte solution were transferred into each well of a 24-well plate. Then 50 μL of the antisense nanoparticles with mass ratio of 1:1 and 2:1, free ODNs or corresponding controls were added and the suspensions incubated for 3 h at 37 °C. The final concentration of ODNs was 0.5 μM. After incubation, the samples were centrifuged and the supernatants (400 μL) analyzed for haemoglobin release by measuring the absorbance at 570 nm (UV-1202 SHIMADZU spectrophotometer). A 50 μL aliquot of washing solution was used as negative control, and positive control (100% haemoglobin release) was a Triton X-100 detergent (5% v/v) lysed solution. Results were expressed as the amounts of haemoglobin released caused by the test compounds as percent of the total amount. Furthermore the same experiments were repeated in the presence of 10% human plasma.

2.8. Growth inhibition of P. falciparum

P. falciparum K1 strain isolated in 1979 from an infected individual in Kanchanburi province, Thailand, was maintained in human erythrocytes in RPMI 1640 medium supplemented with 10% human serum, 25 mM Hepes, 32 mM $NaHCO_3$ under continuous culture using the candle-jar method of Trager and Jensen (1976). The parasites were synchronized to the ring stage by repeated sorbitol treatment (Lambros and Vanderberg, 1979). A 200 μL aliquot of 2% v/v cell suspension with 2% parasetemia was pre-exposed to 25 μL of the medium, containing the test compounds or serum-free culture medium for negative control, in 96-well culture plates. All ODN containing test compounds have been evaluated in final ODN concentrations of 0.5 and 1 μM. Additionally, free chitosan in a final concentration of 0.001% w/v was tested, in order to exclude an unspecific inhibitory effect. After 48 h incubation at 37 °C under candle jar condition, supernatant from each well was removed. One drop of the residue was used to prepare thin blood smears on microscopic glass slides and stained with Giemsa. Parasitemia (the number of parasites per 100 red blood cells), parasite stages and morphology of the cultures were determined by microscopic examination by counting of 5000 erythrocytes under oil immersion. Results were expressed as the percent reduction of parasite growth as compared to the control receiving serum-free medium alone without ODNs. All results presented were the average of at least three independent experiments in triplicate. Statistical significance between average percentage reduction in parasite growth compared with control was conducted using Student's *t*-test.

3. Results

3.1. Particle size and surface charge

The hydrodynamic diameters of antisense and sense ODNs complexed with chitosan at mass ratio of 1:1 and 2:1 were

measured by PCS (Table 1). The measurements were carried out in double distilled sterile filtered water at room temperature. Chitosan was complexed with 30mer antisense and sense ODNs in the form of spherical particles with mean diameters of ~51–58 nm. Different amounts of chitosan did not noticeably affect the mean diameters of the particles. Also the sequence of the ODNs showed no influence on particle size. In addition, TEM of antisense nanoparticles of chitosan/ODN was performed to verify the results obtained by the PCS measurement and to determine the structure of the nanoparticles (Fig. 1A). The diameters of the antisense nanoparticles with 1:1 and 2:1 mass ratios ranged between ~30 and ~90 nm, which was in good agreement with the PCS measurements. After incubation in serum, particle size analysed by PCS did not change significantly (Table 1). This observation could be verified by TEM of nanoparticles in presence of human serum (Fig. 1B). No agglomeration between nanoparticles and serum components could be identified.

In addition, the surface charges of the nanoparticles were determined in double distilled water and in RPMI 1640 medium containing 10% human plasma. In distilled water, the antisense and sense nanoparticles with mass ratio of 1:1 were negatively charged. The zeta potential of the 1:1 antisense and sense nanoparticles was −20.7 and −19.0 mV, respectively. At the mass ratios of 2:1 of chitosan/ODN, nanoparticles were positively charged, +14.4 mV for antisense and +16.0 mV for sense nanoparticles.

On the other hand, after incubation of the nanoparticles in RPMI 1640 medium containing 10% human serum for 30 min at 37 °C, all particles showed slightly negative zeta potentials, ranging from −1.9 to −3.0 mV. Absorption of serum proteins on the charged nanoparticles and a shielding effect of ions from the culture medium could account for the decrease of the surface charge.

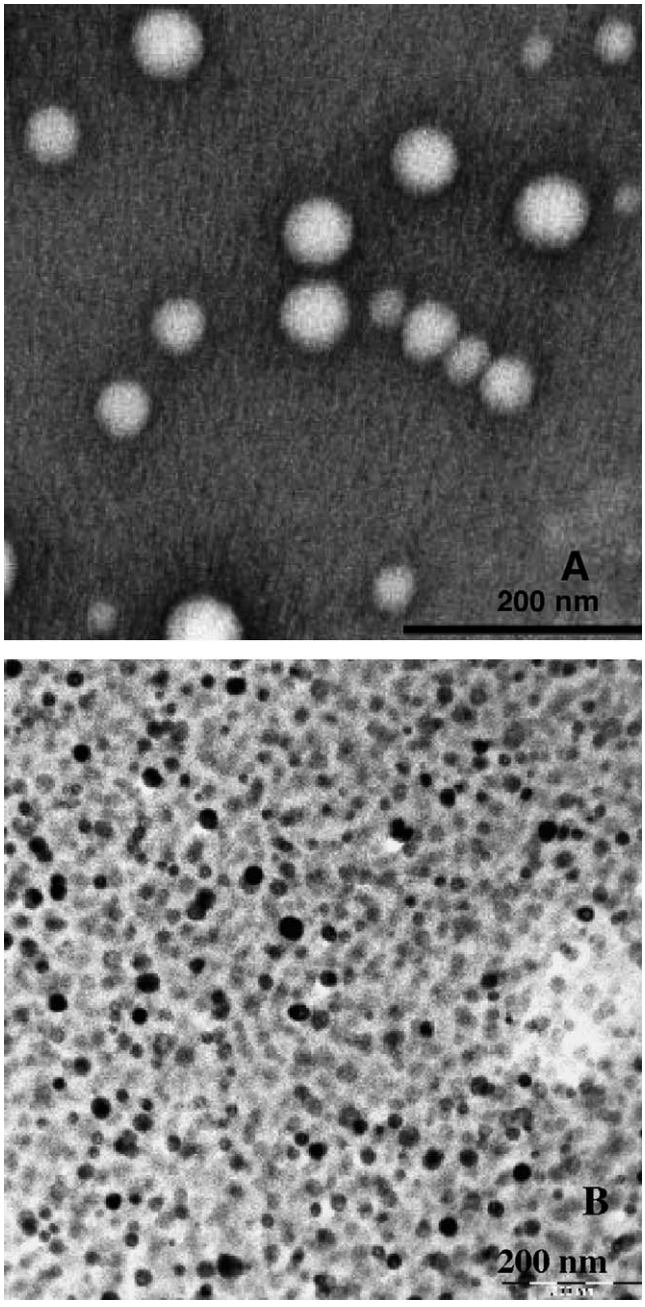

Fig. 1. Transmission electron micrographs of 2:1 nanoparticles in absence (A) (elastic imaging with 0 eV) and presence of 10% v/v human serum (B) (inelastic imaging with an energy loss of 50 eV).

3.2. Oligonucleotide loading and release

The amounts of ODNs complexed in the chitosan nanoparticles were determined by agarose gel-electrophoresis (Fig. 2). At mass ratio of 1:1, as well as 2:1, ODNs were complexed and bound to the nanoparticles. In the HPLC assay ~4% of unbound ODNs were found in solution for the 1:1 nanoparticles whereas no unbound ODNs were detected for the 2:1 particles. In order to determine the stability of the particles under physiological pH and salt conditions, the release of ODNs was investigated in PBS (pH 7.4) at 37 °C (Fig. 3). The 2:1 particles showed a minor release of ODNs. After 48 h, 6% of unbound ODNs were detected. Nanoparticles with 1:1 mass ratio showed a faster release. A 48% of uncomplexed ODNs were detected after 48 h.

3.3. Protection of ODNs against digestion in plasma

The protective effect of chitosan against degradation of oligonucleotides in non-heat inactivated human plasma was investigated. Antisense nanoparticles as well as free ODNs were

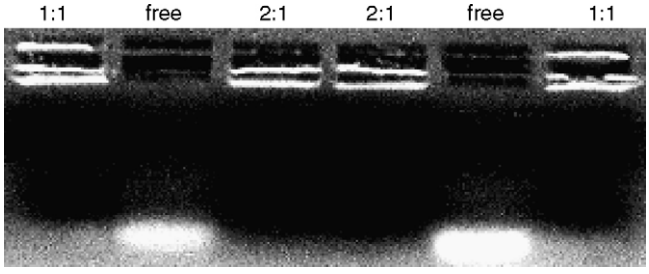

Fig. 2. Determination of the ODN content of the complexes. Gel electrophoresis of mixtures of chitosan with antisense and sense oligonucleotides in the mass ratios 1:1, free ODNs and 2:1 from the left line on. (agarose gel pH 7.4).

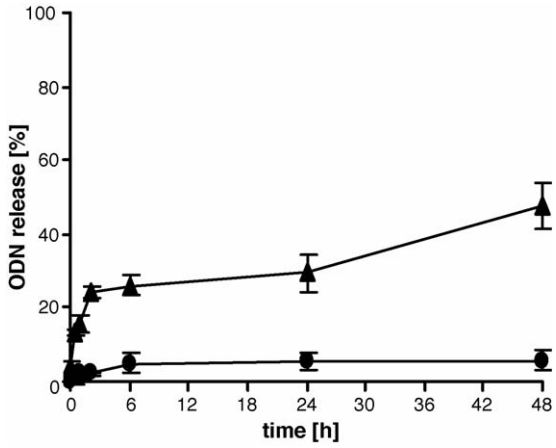

Fig. 3. Release of ODNs from particles in PBS. Particles in the mass ratio 1:1 (triangles) and 2:1 (balls).

incubated in RPMI 1640 culture medium containing human plasma in a final concentration of 20% at 37 °C. The amounts of undegraded ODNs were measured for 8 h (Fig. 4). Intact oligonucleotides were analysed by HPLC. After an initial phase of rapid degradation (∼17% in 0.5 h), digestion of unbound ODNs was decreased. Significant protection of ODNs degradation was demonstrated with both types of chitosan nanoparticles, but particularly for the 2:1 nanoparticles. After 2 h incubation, ∼99% of oligonucleotides remained intact for the 2:1 and ∼96% for the 1:1 nanoparticles. The small amounts of uncomplexed ODNs of the 1:1 nanoparticles, as visualized in the agarose gel, might have been degraded, explaining the small difference between the two mass ratios. In addition stability of ODNs and nanoparticles in heat inactivated plasma was evaluated and no degradation for all test compounds was detected still after 24 h (data not shown).

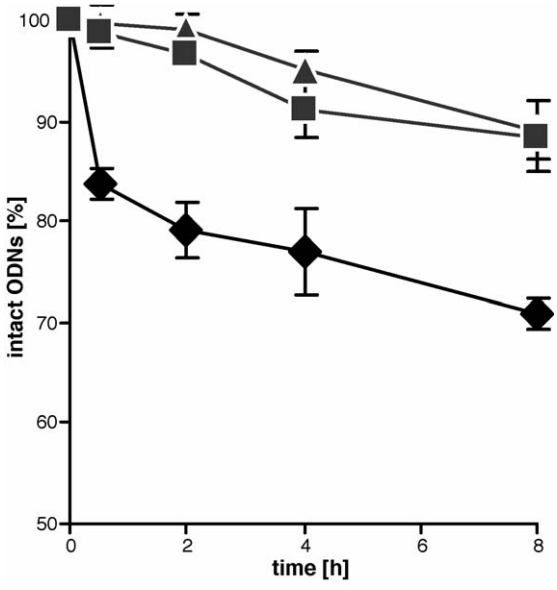

Fig. 4. Protection of oligonucleotides against degradation in cell culture medium containing not heat inactivated human plasma. Free ODNs (diamonds), nanoparticles in the 1:1 (squares) and 2:1 (triangles) mass ratio.

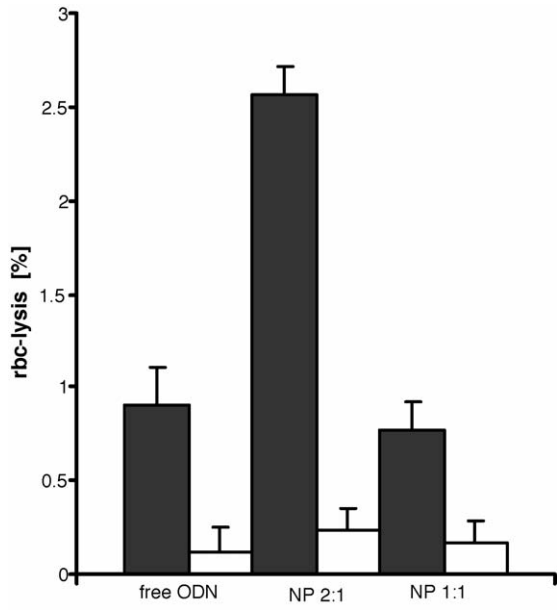

Fig. 5. Red blood cell (RBC) lysis in absence (black bars) and presence (white bars) of plasma.

3.4. Evaluation of red blood cell lysis

In this study haemolysis experiments were performed to investigate interactions of nanoparticles, with positive as well as with negative surface charge, with the negatively charged red blood cell membrane. The membrane damaging properties of the test compounds were determined by the quantification of released haemoglobin. Results are shown in Fig. 5. After 3 h of incubation, nanoparticles with positive zeta potential showed a higher membrane damaging effect causing a significantly higher haemoglobin release (2.6%) as compared to nanoparticles with negative surface charge (0.8%) or unbound ODNs (0.9%).

Furthermore the membrane damaging properties of the test compounds in the presence of 10% human plasma were investigated. Haemoglobin release was significantly lower for all test compounds (∼0.2%) in the presence of human plasma.

3.5. Growth inhibition of P. falciparum

The antimalarial effects of antisense nanoparticles with negative as well as positive zeta potential and free antisense ODNs were evaluated. *P. falciparum*, synchronized to ring stage, was exposed for 48 h to the samples. In control assays, parasites proceeded through their full life cycle from ring forms to trophozoite and schizont forms and invasion of red cells to produce the next generation of daughter rings. The antimalarial activity of the test compounds is presented in Fig. 6. All antisense phosphorothioate oligonucleotide-containing samples in a final concentration of 0.5 μM significantly reduced parasite growth compared to the sense sequence-containing control samples or with medium alone, suggesting sequence specific inhibition (Table 2). Highest inhibition of *P. falciparum*

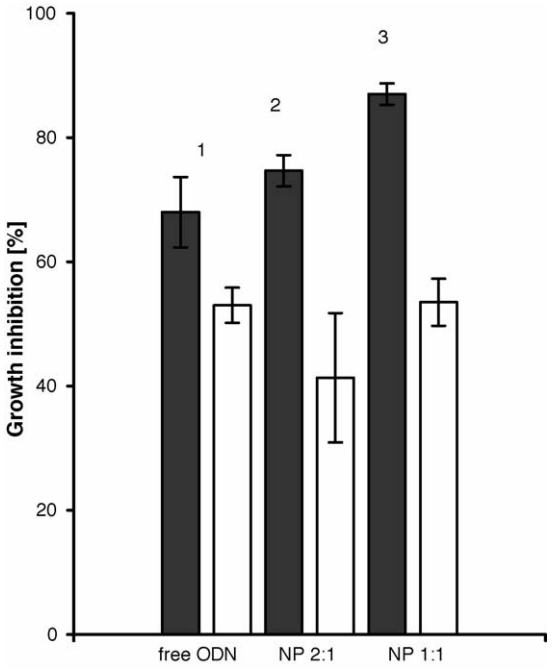

Fig. 6. Growth inhibition of *P. falciparum* with antisense (black bars) and sense (white bars) ODNs. Free ODNs, nanoparticles in the mass ratio 2:1 and 1:1 from the left line on. Indicated values are means ± S.D. of three experiments, each with triplicate samples; 1: differs from sense ODNs, $p < 0.015$; 2: differs from 2:1 sense complex, $p < 0.006$; 3: differs from 1:1 sense complex, $p < 0.0002$.

growth, approximately 87% reduction, was achieved with antisense nanoparticles with negative surface charge. Sense ODN nanoparticles with the same mass ratio reduced parasite growth by 53%. This difference clearly demonstrated sequence specific inhibition.

Antisense nanoparticles with the 2:1 mass ratio reduced parasite growth by ∼75% compared to 41% inhibition by sense nanoparticles, demonstrating that complete neutralization of the negative charge by complexation with positively charged chitosan did not prevent sequence specific inhibition of parasites. Free antisense ODNs reduced *P. falciparum* growth by 68% compared to 53% by sense oligonucleotides demonstrating lower significance of sequence-specific inhibition. In order to exclude an effect of pure chitosan on parasite growth, polymer without ODNs was added to the culture, but displayed no inhibition (data not shown). At a concentration of 1 μM, all ODNs-containing samples reduced parasite growth independent of the sequence (data not shown).

Table 2
Phosphorothioate oligodeoxynucleotides specific for the translation initiation sites and internal coding regions of *P. falciparum* topoisomerase II gene

Oligomers	Sequence	Nucleotide numbers
AS	ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT	163-134
S	AGA ATC GTA TGG TTC AAA AGA ATA TTA CAT	Sense

4. Discussion

Generally intracellular uptake of ODN nanoparticles occur via an endocytic–phagocytic process (Fattal et al., 1998). However erythrocyte membrane of infected red blood cells does not show any endocytic properties (Haldar and Uyetake, 1992; Pouvelle et al., 1994). But it is widely recognized that the intracellular malaria parasite induces in the host red blood cell membrane new permeation pathways that are absent from the membrane of the uninfected erythrocytes (Go et al., 2004). Macromolecules like dextrans, protein A and IgG2a antibody were shown to gain access to the parasite through permeation pathways induced by the malaria parasite (Pouvelle, 1991). Using a variety of fluorescent latex spheres, Goodyer et al. determined that macromolecules up to 50–80 nm in diameter access intracellular parasites (Goodyer et al., 1997). In this regard, we developed ODN-chitosan nanoparticles with mean diameters of ∼55 nm. To evaluate the impact of charge, nanoparticles with negative as well as with positive surface charge have been designed, depending on the mass ratio. On the surfaces of the 1:1 mass ratio nanoparticles an excess of ODNs are bound by electrostatic interactions, whereas particles with 2:1 mass ratio an excess of non-neutralised chitosan produced positive charged surfaces.

Stability of nanoparticles under physiological conditions is an important factor influencing the release profile of incorporated ODNs. More easily dissociated complexes mediate a faster onset of action. Köping-Höggard et al. (2004) reported that higher gene expression in vitro as well as in vivo is achieved with less stable chitosan-plasmid DNA complexes (Köping-Höggard et al., 2004). As *P. falciparum* develops from ring to mature trophozoite and schizont stages within 36 h, and antisense ODNs against malarial topoisomerase II were expected to arrest the maturation of throphozoite form (Noonpakdee et al., 2003), thus antisense nanoparticles with a rapid onset of action are required. This notion was supported by the results from this study demonstrating higher inhibition was achieved with nanoparticles displaying a faster release profile compared to more stable chitosan-complexes. However, complexation of ODNs with higher amounts of chitosan leading to more stable particles did not prevent inhibition of parasites. Even after 48 h particles with the 2:1 mass ratio remained stable at pH 7.4 and under physiological salt conditions. However, chitosan has been shown to be degraded in vitro as well as in vivo by enzymes, such as lysozyme and chitosanase, into oligomers (Huang et al., 2004), which assures on the one hand its biocompatibility and on the other hand accelerates drug release from chitosan nanoparticles. PfCHT1, a chitinase gene, has been identified in *P. falciparum* genome (Tsai et al., 2001), but no report is currently available indicating if chitinase is expressed during parasite blood stages. PfCHT1 is reported to be essential for intracellular trafficking and secretion and to be necessary for ookinetes to invade the mosquito midgut (Tsai et al., 2001).

In this study nanoparticles clearly demonstrate a more pronounced sequence specific antisense effect as compared to free ODNs. This could be due to the sustained release of ODNs

from chitosan nanoparticles leading to a relatively lower initial concentration. This contention is in good accordance with previous studies (Noonpakdee et al., 2003; Rapaport et al., 1992; Barker et al., 1998) showing that oligonucleotides inhibit cellular gene expression in a sequence specific manner at low concentrations. At high concentrations (1 μM and more) both sense and antisense ODNs inhibit growth of parasites in a non-specific manner by the polyanionic properties of oligonucleotides which interfere with the merozoite invasion into red blood cells (Noonpakdee et al., 2003; Barker et al., 1998) in a manner similar to that observed with dextran sulphate (Dalton et al., 1991; Kanagaratnam et al., 1998). An additional explanation for the high percent of inhibition induced by sense nanoparticles might be partly attributed to induced perturbations in the intracellular metabolic activity caused by the complexes (Lambert et al., 1998). In the present study an inhibitory effect of free chitosan could be ruled out, however its intracellular uptake might be different than nanoparticles uptake. Even if non-toxic, some complexes, irrespective of their DNA content, are able to modify intracellular signalisation pathways (Filion and Phillips, 1997). However, antisense-nanoparticles presented in this study clearly demonstrate a significant higher inhibition than in comparison with sense-nanoparticles.

Furthermore nanoparticles demonstrated effective protection of oligonucleotides against nuclease degradation that is a major requirement for in vivo use of antisense technology. Another requirement for in vivo use of nanoparticles against malaria is the proof that they do not harm red blood cells. The erythrocyte membrane contains anionic glycoproteins which can interact with protonated amino groups of chitosan. This process induces membrane curvature, leading to rupture and haemoglobin release (Carreno-Gomez and Duncan, 1997). Nanoparticles with positive surface charge showed a higher membrane damaging effect than compared to negatively charged particles. However, in the presence of plasma, haemoglobin release was markedly reduced. The lower membrane damaging effect in the presence of plasma might be explained by absorption of negatively charged plasma proteins on the surface of charged particles, and this shielding effect of plasma suggests that these chitosan nanoparticles may not harm erythrocytes under in vivo conditions. As far as we know this study is the first dealing with nanotechnology against malaria, demonstrating the susceptibility of human malaria parasite, *P. falciparum*, to antisense nanoparticles.

Acknowledgments

We are grateful to Prof. Bernd M. Rode from the ASEA-UNINET who supported this work. Also the Austrian Nano-Initiative co-financed this work as part of the Nano-Health project (No. 0200), the sub-project NANO-N-0204 being financed by the Austrian FWF (Fonds zur Förderung der Wissenschaftlichen Forschung). We also thank Prof. Prapon Wilairat for his kind assistance in the preparation of the manuscript.

References

Baird, K.J., 2005. Effectiveness of antimalarial drugs. N. Engl. J. Med. 135, 1565–1577.

Barker, R.H., Metelev, V., Coakley, A., Zamecnik, P., 1998. *Plasmodium falciparum*: effect of chemical structure on efficacy and specificity of antisense oligonucleotides against malaria in vitro. Exp. Parasit. 88, 51–59.

Carreno-Gomez, B., Duncan, R., 1997. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. Int. J. Pharm. 148, 231–240.

Chen, X., Dudgeon, N., Shen, L., Wang, J.H., 2005. Chemical modification of gene silencing oligonucleotides for drug discovery and development. Drug Discov. Today 10, 587–593.

Dalton, J.P., Hudson, D., Adams, J.H., Miller, L.H., 1991. Blocking of the receptor-mediated invasion of erythrocytes by *Plasmodium knowlesi* malaria with sulphated polysaccharides and glycosaminoglycans. Eur. J. Biochem. 195, 789–794.

De Smidt, P.C., Le Doan, T., de Falco, S., van Berkel, T.J.C., 1991. Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. Nucl. Acids Res. 19, 4695–4700.

Fattal, E., et al., 1998. Biodegradable polyalkylcyanoacrylate nanoparticles for the delivery of oligonucleotides. J. Control. Rel. 53, 137–143.

Felix, C.A., 2001. Leukemias related to treatment with DNA topoisomerase II inhibitors. Med. Pediatr. Oncol. 36, 525–535.

Filion, M.C., Phillips, N.C., 1997. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. Biochim. Biophys. Acta 1329, 345–356.

Go, M.L., Liu, M., Wilairat, P., Rosenthal, J., Saliba, K.J., Kirk, K., 2004. Antiplasmodial chalcones inhibit sorbitol-induced hemolysis of *Plasmodium falciparum*-infected erythrocytes. Antimicrob. Agents Chemother. 48, 3241–3245.

Goodyer, I.D., et al., 1997. Characterization of macromolecular transport pathways in malaria-infected erythrocytes. Mol. Biochem. Parasit. 87, 13–28.

Guggi, D., et al., 2004. Comparative evaluation of cytotoxicity of a glucosamine-TBA conjugate and a chitosan-TBA conjugate. Int. J. Pharm. 278, 353–360.

Haldar, K., Uyetake, L., 1992. The movement of fluorescent endocytic tracers in *Plasmodium falciparum* infected erythrocytes. Mol. Biochem. Parasitol. 50, 161–177.

Huang, M., Khor, E., Lim, L.Y., 2004. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharm. Res. 21, 344–353.

Junghans, M., Kreuter, J., Zimmer, A., 2000. Antisense delivery using protamine-oligonucleotide particles. Nucl. Acids Res. 28, E45.

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.

Köping-Höggard, M., et al., 2004. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. Gene Ther. 11, 1441–1452.

Lambert, G., Fattal, E., Brehier, A., Feger, J., Couvreur, P., 1998. Effect of polyisobutylcyanoacrylate nanoparticles and Lipofectin® loaded with oligonucleotides on cell viability and PKCα neosynthesis in HepG2 cells. Biochimie 80, 969–976.

Lambert, G., Fattal, E., Couvreur, P., 2001. Nanoparticulate systems for the delivery of antisense oligonucleotides. Adv. Drug Deliv. Rev. 47, 99–112.

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

Mao, H.Q., et al., 2000. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J. Control. Rel. 70, 399–421.

Noonpakdee, W., Pothikasikorn, J., Nimitsantiwog, 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.

Pouvelle, B., 1991. Direct access to serum macromolecules by intraerythrocytic malaria parasites. Nature 353, 73–75.

Pouvelle, B., Gormley, J.A., Taraschi, T.F., 1994. Characterization of trafficking pathways and membrane genesis in malaria-infected erythrocytes. Mol. Biochem. Parasitol. 66, 83–96.

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

Shaw, J.P., et al., 1991. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. Nucl. Acids Res. 19, 747–750.

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

Tsai, Y.L., et al., 2001. Disruption of *Plasmodium falciparum* chitinase markedly impairs parasite invasion of mosquito midgut. Infect. Immun. 69, 4048–4054.

Wanidworanun, C., Nagel, R.L., Shear, H.L., 1999. Antisense oligonucleotides targeting malarial aldolase inhibit the asexual erythrocytic stages of *Plasmodium falciparum*. Mol. Biochem. Parasit. 102, 91–101.