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Evaluation of the toxicity of oligonucleotide/cationic nanoemulsion complexes on Hep G2 cells through MTT assay

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The purpose of this study was to evaluate the toxicity of the oligonucleotide/cationic nanoemulsion complexes on Hep G2 cells through MTT assay. Complexes exhibit droplet size, zeta potential and viscosity of approximately 270 nm, +50 mV, and 1.0 cP. Different parameters which may have an influence on toxicity results obtained by MTT assay, i.e. cells number, concentration of MTT reagent and the addition of Soerensen's glycine buffer were first evaluated. In the optimized conditions (1×10^4 cells and 0.5 mg/mL MTT), the overall results showed that the addition of increasing amounts of complexes (or nanoemulsions) lead to a progressive toxicity on cells attributed to the presence of the cationic lipid stearylamine in the formulations, whatever the medias's pH is. The IC_{50} was approximately 200 μg/ml. Such results open interesting perspectives on the use of these nanoemulsions as oligonucleotide delivery systems for Hep G2 cells.

1. Introduction

Antisense oligonucleotides (ON) are fragments of nucleic acids which present the capacity to specifically hybrid themselves with a complementary sequence of an RNA messenger and inhibit the expression of a determined protein (Hélène and Toulmé 1990). Thus, ON have been investigated in the treatment of diverse diseases, including viral infections and cancer (Pirollo et al. 2003; Stahel and Zangemeister-Wittke 2003). However, the therapeutic application of ON remains limited due to the ON's reduced intracellular penetration and instability in biological fluids (Hélène and Toulmé 1990; Pirollo et al. 2003; Stahel and Zangemeister-Wittke 2003).

The association of ON with nanoemulsions has been considered as a strategy to circumvent these drawbacks (Trimaille et al. 2001; Teixeira et al. 1999, 2003). These systems consist of an oil core stabilized by a mixture of phospholipids and cationic lipids. The presence of the cationic lipid leads to the association of the ON with the nanoemulsions through electrostatic interactions (Teixeira et al. 2001). Recently, Silva et al. reported on the effect of the addition of increasing amounts of the cationic lipid stearylamine and the non-ionic surfactant poloxamer 188 on the properties of nanoemulsions composed of a triglyceride oil core stabilized by egg-lecithin (Silva et al. 2006). The ON were effectively associated with the nanoemulsions, containing different concentrations of stearylamine (>90%), in dictating the formation of an ionic pair in the oil/water interface of nanoemulsions. Teixeira et al. demonstrated that, after intratumoral administration, the formulated complexes lead to an increase in the penetration of the ON in tumor cells (P388/ADR) in ascite (Teixeira et al. 2003). In addition, protection of the enzymatic degradation of the ON through nucleases could be observed when these were carried on the surface of cationic nanoemulsions (Teixeira et al. 2003).

The investigation of the carrier cytotoxicity represents an essential prior condition to studies on genetic transference. Moreover, it is necessary to determine the range of concentration in which the carrier system presents at least 70–80% of the cell viability (Hung et al. 2005). The use of MTT has been widely employed in this assessment (Mosmann 1983). The present study is based on the ability of live cells, under the action of active mitochondria dehydrogenase enzyme, to quantitatively reduce a soluble yellow dye in water, MTT, to a purple formazan product which is insoluble in water, MTT formazan (Mosmann 1983; Plumb et al. 1989). The toxicity of the cationic carrier systems is generally related to the presence of a lipid and/or cationic polymer present in the formulations, which can cause the charge neutralization, cellular distortion, lysis and agglutination, as well as possibly link themselves to the nucleus of the cells and internal membranes and interfere in enzymatic functions (Plumb et al. 1989; Brazeau et al. 1998; Kim et al. 2002; Yoo et al. 2004).

The purpose of this study was to assess, through the MTT study, the toxicity of complexes formed by a model oligonucleotide (pdT_{16}) and nanoemulsions obtained in the absence (NE) or in the presence of the cationic lipid stearylamine (NE_{SA}) on Hep G2 cells. After the physicochemical characterization of the systems, the effect of

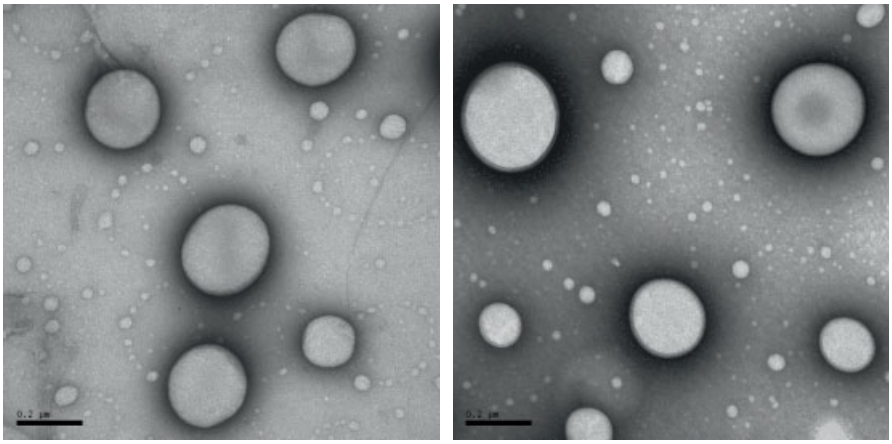

Fig. 1:
TEM micrographs of complexes NE-pdT$_{16}$ and NE$_{SA}$-pdT$_{16}$

some parameters of the MTT assay (i.e. the number of cells, the concentration of the MTT reagent, and the addition of Soerensen's glycine buffer), was evaluated. The toxicity of the formulation was also assessed under optimized conditions.

2. Investigations, results and discussion

In the first stage, the physico-chemical properties of both NE and NE$_{SA}$ were evaluated (Table 1). Regardless of the composition, the formulations presented a droplet size of approximately 200–250 nm and a viscosity of 1.7 to 1.9 cP. These results are in accordance with those reported in previous studies regarding nanoemulsions obtained through the spontaneous emulsification procedure under similar conditions (Martini et al. 2007, 2008). The NE formulation presents a negative zeta potential, which can be attributed to the presence of negatively charged phospholipids in the egg-lecithin (Li and Tian 2002), in contrast to the NE$_{SA}$ formulation, which presents a positive global charge due to the presence of stearylamine cationic lipid.

The amount of ON associated with NE$_{SA}$ was significantly higher ($p < 0.05$) as compared to the control formulation obtained in the absence of stearylamine (Table 1). These results can be attributed to the electrostatic interactions established between the ON pdT$_{16}$ and the stearylamine (Teixeira et al. 2001). This interaction does not influence the ζ-potential due to the excess of positive charges in relation to the phosphate groups present in the pdT$_{16}$. On the other hand, despite the negative global charge of NE the association of pdT$_{16}$ molecules with the nanostructures was observed by means of a probable interaction with the phospholipids from the nanoemulsion interface (Teixeira et al. 1999). The association of the ON does not lead to significant changes in the mean diameter of the oil droplets, remaining similar to the original, for the relation of charges of +2/−, thus corroborating with images from transmission electron microscopy (TEM) (Fig. 1). Spherical droplets with a 200–300 nm size and increased electronic density in the interface, stemming from the interaction of the contrasting agent (uranyl acetate) with nucleic acids could be observed. On the other hand, a significant reduction in the viscosity of the complexes in comparison with the values obtained before the association with the ON could also be observed. Such a result can be attributed to the low concentration of the internal phase (2 mg/mL) of the complexes on this parameter, as previously described (Ishii et al. 1990).

Diverse parameters can influence the results of cytotoxicity determined by the MTT method (Mosmann 1983; Plumb et al. 1989). In this study, the effect of the number of cells (3×10^3 to 30×10^3), as well as the concentration of the MTT reagent (0.5 and 5 mg/mL), on the detected absorbance were investigated (Fig. 2). A linear relation exists between the amount of the cells and the average absorbance under the evaluated experimental conditions, with a coefficient of correlation > 0.98. The results obtained are similar for the two concentrations of the MTT solution tested (0.5 or 5 mg/mL), suggesting that the lower concentration of the reagent is sufficient for the reduction of MTT in MTT formazan through the viable cells. Through these results, the parameters of 1×10^4 cells per well and a concentration of the MTT of the 0.5 mg/mL were selected for further studies.

Table 1: Physico-chemical properties of nanoemulsions and complexes

	Size (nm)	ζ-potential (mV)	Viscosity (cP)	Association (%)
NE	216 ± 4	−32.8 ± 0.1	1.78 ± 0.15	−
NE/pdT$_{16}$	221 ± 2	−33.1 ± 0.8	1.03 ± 0.02	66.1 ± 2.2
NE$_{SA}$	248 ± 5	+55.4 ± 2.3	1.90 ± 0.19	−
NE$_{SA}$/pdT$_{16}$	272 ± 8	+51.7 ± 3.8	1.02 ± 0.08	95.9 ± 0.4

[a] Complexes (nanoemulsions/pdT$_{16}$) were obtained at a charge ratio of +2/−

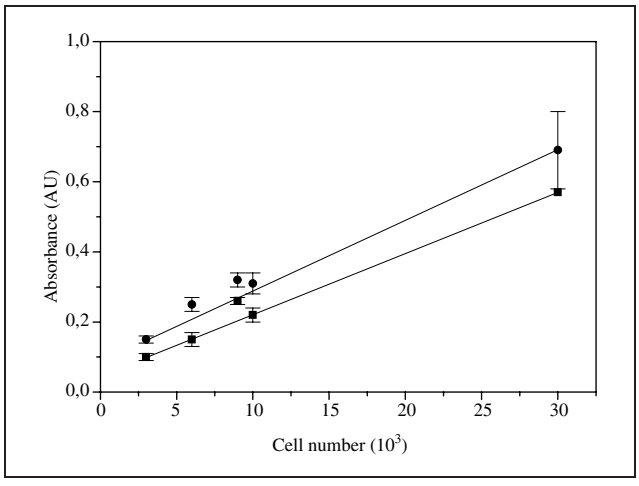

Fig. 2: Effect of the Hep G2 cell number and the concentration of MTT reagent on absorbance. Hep G2 cells (3×10^3 to 30×10^3) were incubated during 24 hours. MTT reagent was added at 0.5 mg/ml (●) or 5 mg/ml (■)

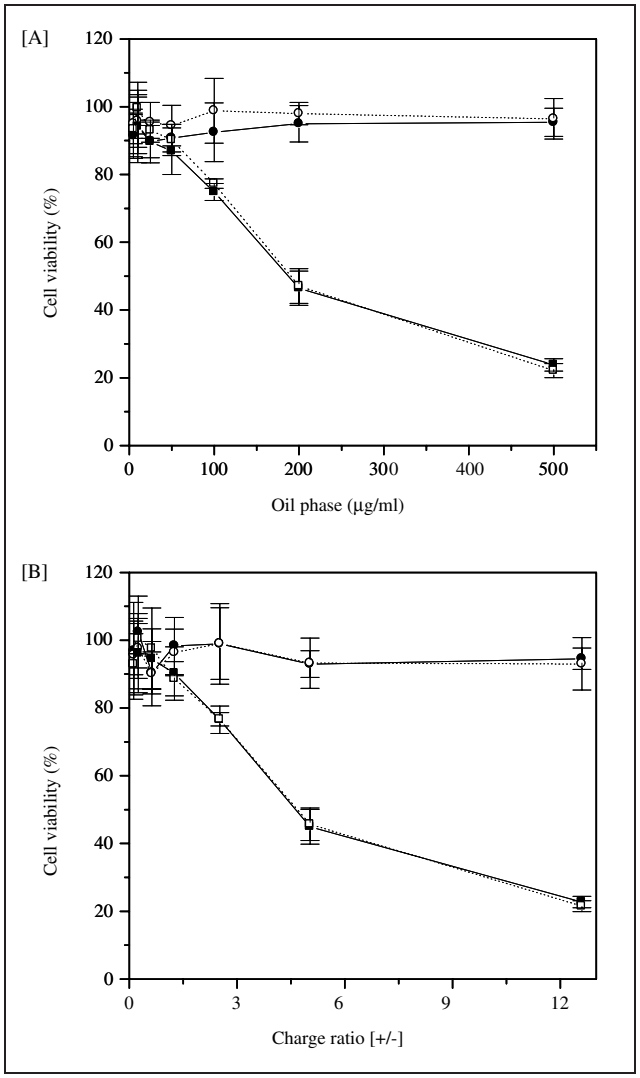

Fig. 3: Hep G2 cell viability after incubation for 24 hours with increasing amounts of NE (●, ○), NE_{SA} (■, □) [A] or NE-pdT_{16} (●, ○) and NE_{SA}-pdT16 (■, □) [B]. Solid and open symbols represent values obtained before and after the addition of Soerensen's glycine buffer, respectively

Fig. 3A presents the effect of the NE and NE_{SA} on the viability of the Hep G2 cells. Regardless of the amount added, the viability is greater than 90% for the NE obtained in the absence of the cationic lipid. This result can be attributed to the biocompatibility of the components used in this formulation: medium-chain triglycerides, egg-lecithin, and poloxamer 188. In contrast, the toxicity of the NE_{SA} formulation increased progressively with the incubated quantity, reaching nearly 20% of the cell viability for the largest concentration of the internal phase tested (500 μg/mL). These results are in accordance with previous works which reported the toxicity of the carrier systems containing one-tailed cationic lipids, such as stearylamine, in different cell lines (Senior et al. 1991; Weyenberg et al. 2007). Nevertheless, the IC_{50} value for the NE_{SA} formulation obtained in this study (200 μg/mL) was higher than that described by Fraga and collaborators (∼140 μg/ml), for a formulation of similar qualitative and quantitative composition, obtained in the absence of poloxamer 188. The lesser toxicity of the formulations within the poloxamer 188 (a amphiphilic non-ionic polymer) may be related to the steric barrier formed at the interface of the nanoemulsions, reducing the interaction of the nanoemulsions with the cells, or even, to the formation of a more stable interface blocking the migration of the colloidal structure and direct interaction with the cell membranes, as previously described (Senior et al. 1991; Korner et al. 1994). The toxicity of the NE-pdT_{16} and NE_{SA}-pdT_{16} was similar to that observed for the NE e NE_{SA} nanoemulsions, indicating that the positive charge of the complexes is the determining factor of the toxicity in Hep G2 cells (Fig. 3B). The addition of increasing amounts of NE_{SA}-pdT_{16} leads to the reduction of cellular viability in up to 80% in the higher charge ratio. In fact, the cationic lipids are capable of interacting electrostatically with the negatively charged cellular membranes, which can interfere in their functions through different mechanisms (Gao and Huang 1991; Lasch et al. 1995; Brazeau et al. 1998).

In a final stage, the effect of the addition of Soerensen's glycine buffer on the results obtained was evaluated since, according to Plumb et al. (1989), the MTT formazan absorption spectrum can vary according to the pH (Fig. 3). Stearylamine presents a primary amine group capable of altering the pH of the medium. Nevertheless, results obtained for both formulations remained similar, before and after the addition of Soerensen's glycine buffer, thus demonstrating that, in the final pH of the study, the MTT absorption spectrum remained unchanged.

In conclusion, this study allowed for the determination of experimental conditions to assess the toxicity of cationic nanoemulsions used as ON carriers in Hep G2 cells, by means of the MTT method. Under optimized conditions, it was possible to determine the concentration of the internal phase of the NE_{SA}, which were capable of guaranteeing the viability of the Hep G2 cells at approximately 80% (∼100 μg/mL). These results open interesting perspectives regarding the use of cationic nanoemulsions as ON delivery systems for Hep G2 cells.

3. Experimental

3.1. Materials

Egg-lecithin (Lipoid E-80®) and medium chain triglycerides were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Poloxamer 188, stearylamine, and glycerol were obtained from Sigma (São Paulo, Brazil). Oligonucleotide (pdT_{16}) was purchased from Biogen (São Paulo, Brazil). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from USB Corporation (Cleveland, USA).

3.2. Nanoemulsion preparation

Formulations were prepared by means of the spontaneous emulsification procedure as previously reported by Martini (2008). In such a procedure, the components of the oil (MCT, egg-lecithin, and stearylamine) and aqueous (glycerol and poloxamer 188) phase were dissolved in ethanol and water, respectively. The ethanol phase was slowly added to the water phase under moderate magnetic stirring which immediately turned into a milky phase as a consequence of the emulsion formation. Part of the solvents was then removed under reduced pressure below 50 °C until reaching the desired final volume. The ethanol/water ratio was 30/60 to prepare 10 ml of final formulation. The bulk pH of the nanoemulsions was recorded using a pH-meter B474 (Micronal, Brazil) in recently prepared nanoemulsions and after having been adjusted to about 7.0. The final composition

Table 2: Final composition of nanoemulsions (%, w/w)

	NE	NE_{SA}
Lipoid E-80	2.00	2.00
Stearylamine	–	0.05
MCT	8.00	8.00
Poloxamer 188	1.68	1.68
Glycerol	2.25	2.25
Water q.s.	100.00	100.00

(%) of the formulations was described in Table 2. The formulations obtained in the absence or in the presence of stearylamine were codified as NE and NE_{SA}, respectively.

3.3. Complexation of ON with nanoemulsions

Complexation of ON with nanoemulsions was performed at the end of the manufacturing process. NE or NE_{SA} were added to water solutions of ON and incubated for 30 minutes at room temperature. Free ON was determined in the ultrafiltrate obtained after centrifugation through a porous membrane 30.000 Da (Ultrafree MC Millipore, USA). The amount of ON in the ultrafiltrate was measured by determining the optical density at 260 nm (Hewlett-Packard 8452 spectrophotometer, USA). The final concentration of the oil phase of nanoemulsions in the adsorption media was of 2 mg/ml. The association efficiency (%) was estimated by the difference between the added and the detected amount of ON in the clear ultrafiltrate.

3.4. Nanoemulsion characterization

The mean droplet size and ζ-potential of the nanoemulsions were determined through photon correlation spectroscopy (PCS) and electrophoretic mobility, respectively (3000HS Zetasizer, Malvern Instruments, England). The samples were adequately diluted in water for size determinations or in 1 mM NaCl for ζ-potential measurements. The viscosity of the nanoemulsions was evaluated by capillary viscosimetry. Each nanoemulsion were poured into a filling tube and transferred to the capillary tube (viscosimeter constant, $k = 0.0212$) by gentle suction. The time was recorded, in seconds, for the liquid to flow from the upper to the lower mark in the capillary tube. Morphological examination of nanoemulsions was performed by means of TEM. Formulations were diluted at a 1 : 10 ratio, obtaining an oil phase concentration equal to 1% (v/v). Specimens were prepared by mixing samples with one droplet of 2% (w/v) uranyl acetate solution. The samples were then adsorbed to the 200 mesh formvar-coated copper grids, left to dry, and examined by TEM (JEM-1200 ExII, Jeol, Japan).

3.5. Cytotoxicity assay

The toxicity of the nanoemulsions and the complexes was evaluated in human hepatome Hep G2 cells through MTT assay according to Mosmann (1983). The cells were cultivated in a culture medium (DMEM) containing 10% calf fetal serum. In a first step, the effect of the number of cells (3×10^3 to 30×10^3), the concentration of the MTT reagent (0.5 and 5 mg/mL), and the addition of Soerensen's glycine buffer on the detected absorbance were tested. Under optimized conditions, all formulations were incubated with cells (1×10^4) for 24 h. The medium was removed; MTT solution (0.5 mg/mL) was added and maintained under incubation for 4 h. After this time interval, 100 μL of DMSO was added to dissolve the blue formazan crystals. The optic density was measured at 570 nm using a plate reader (Hantos, Alemanha). Cells incubated in the culture medium (DMEM) or with Triton X-100 (2%, w/v) were used as a control of 100% and 0% of cell viability, respectively. The cytotoxicity was evaluated by the IC_{50}, i.e., the formulation concentration needed to inhibit cell growth by 50%.

3.6. Statistical analysis

Results were expressed as mean ± standard deviation of three independent experiments and were analyzed using the Student t-test with a $p < 0.05$ significance.

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