European Journal of Medicinal Chemistry 46 (2011) 845–850

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Optimization and *in vitro* toxicity evaluation of G4 PAMAM dendrimer–risperidone complexes

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ARTICLE INFO

Article history:
Received 14 July 2010
Received in revised form
14 November 2010
Accepted 17 December 2010
Available online 22 December 2010

Keywords:
Poly(amidoamine) dendrimers
PAMAM G4
Risperidone
Cytotoxicity

ABSTRACT

Risperidone is an approved antipsychotic drug belonging to the chemical class of benzisoxazole. This drug has low solubility in aqueous medium and poor bioavailability due to extensive first-pass metabolism and high protein binding (>90%). As new strategies to improve treatments efficiency are needed, we have studied cationic G4 PAMAM dendrimers' performance to act as efficient nanocarriers for this therapeutic drug. In this respect, we explored dendrimer–risperidone complexation dependence on solvent, temperature, pH and salt concentration, as well as *in vitro* cytotoxicity measured on L929 cell line and human red blood cells. The best dendrimer–risperidone incorporation was achieved when a mixture of 70:30 and 90:10 v/v chloroform:methanol was used, obtaining 17 and 32 risperidone molecules per dendrimer, respectively. No cytotoxicity on L929 cells was found when dendrimer concentration was below 3×10^{-2} μM and risperidone concentration below 5.1 μM. Also, no significant hemolysis or morphological changes were observed on human red blood cells. Finally, attempting to obtain an efficient drug delivery system for risperidone, incorporation in G4 PAMAM dendrimers was optimized, improving drug solubility with low cytotoxicity.

1. Introduction

The antipsychotic drug risperidone, 3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido[1,2-*a*] pyrimidin-4-one (Risp) belongs to the chemical class of benzisoxazole and is one of the most widely used drugs in autism spectrum disorders (ASD) treatment [1,2]. ASD includes different neurodevelopment disorders that manifest mainly in the earlier years of life [3], affecting language, communication and reciprocal social interaction development [4], with an occurrence of 1 out of 150 individuals [2]. Risp has low solubility in aqueous medium and, when orally administered, exhibits low bioavailability due to extensive first-pass metabolism and high protein binding (>90%) [5]. Moreover, non-targeted delivery usually results in numerous side effects. Since Risp target site is the brain, it is necessary not only to develop a strategy to improve drug bioavailability, by avoiding first-pass metabolism, but also to achieve desired drug concentration at the site of action, thus reducing undesirable side effects [1]. In the last years, strategies to overcome these issues for ASD treatment with chemical therapies have been proposed, particularly with the design of nanostructured drug carrier systems [6]. However, these kinds of carriers (plain, ultradeformable, stealth, pH sensitive liposomes, immunoliposomes, nanoparticles and dendrimers) must be carefully designed and/or chosen because their pharmacokinetics, biodistribution, and tissue selectivity will exclusively depend on the nanocarrier's structure [1,7,8].

In this sense, dendrimers are exceptional polymers presenting important advantages when compared to conventional linear or branched ones such as polyethylene terephthalate or comb polymers, respectively [9,10]. These advantages include: monodispersity [11], controlled size in the range of nanometers units, controlled number of surface groups, and extremely high area/volume ratio. Only intermediate generation (G) dendrimers (3.5–5 G) are suitable drug carriers, with structures open enough to enable the loading and subsequent release of molecules in a controlled fashion [12–14]. In the last years, PAMAM dendrimers were found to be useful in improving the solubility of low aqueous soluble drugs [15,16], so the present work aims at enhancing Risp solubility, by means of PAMAM dendrimers. In this sense, our proposal is the optimization of different solvent, temperature, pH and ionic strength conditions in order to improve Risp complexation with Generation 4 PAMAM dendrimers (DG4) (Fig. 1). *In vitro* releasing profile and drug cytotoxicity, whether complexed or not with DG4, was also analyzed.

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doi:10.1016/j.ejmech.2010.12.021

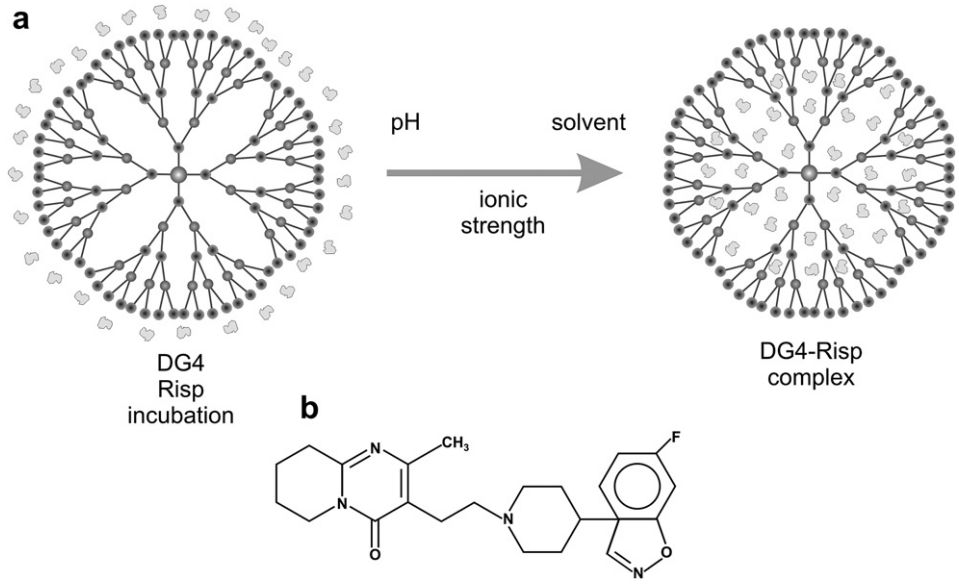

Fig. 1. Schema of Risp complexation with dendrimers PAMAM Generation 4 (DG4) at different solvent, temperature, pH and salt concentration.

2. Experiments

2.1. Materials

Poly(amidoamine) (PAMAM) dendrimer G4 ($-NH_2$) (molecular weight = 14,215 g/mol, 64 amine end groups) (DG4) was purchased from Sigma–Aldrich, Argentina. Risperidone (Risp) 99.0%, was a donation from Jansenn Cilag Laboratory, Argentina. Sodium 3-(4,5-dimethythiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Sigma–Aldrich Argentina. All other reagents used were of analytical grade.

2.2. Preparation of DG4–Risp complex

DG4 was combined with a specific amount of Risp in methanol solution at a 1:250 DG4:Risp molar ratio, and the methanol was immediately evaporated in a Speed Vac SAVANT at 25 °C for 15 min (1010 SAVANT). After evaporation, Risp and PAMAM DG4 were incubated with 1 ml of: i) chloroform:methanol 50:50; ii) chloroform:methanol 70:30; iii) chloroform:methanol 90:10; iv) chloroform; v) HCl solution, pH 4; vi) NaOH solution, pH 8; vii) NaOH solution, pH 9; viii) NaOH solution, pH 10; ix) 1 M NaCl for 48 h at room temperature (20 °C) with continuous stirring. Finally, solvents were completely evaporated in a Speed Vac SAVANT. The obtained solid residues were dissolved in 0.1 ml of 0.5 M Tris buffer, pH 6.8, at room temperature, and centrifuged at 10,000× *g* for 10 min, in order to separate the DG4–Risp complexes (DG4–Risp) (soluble Risp) from the non-incorporated Risp (insoluble). Additionally, to evaluate the temperature effect on Risp complexation, DG4 and Risp were combined in i) chloroform:methanol 70:30 or ii) chloroform:methanol 90:10 at 4 °C for 48 h with continuous stirring, the solvents were evaporated in a Speed Vac SAVANT and solid residues were dissolved in Tris buffer and DG4–Risp separated as stated above.

2.3. Risperidone quantification

The amount of Risp was quantified by measuring the absorbance at 280 nm with a UV–Vis NanoDrop1000, since DG4 do not absorb at this wavelength (see Fig. 2). The calibration curve of Risp in Tris buffer was linear in a concentration range of 0.1–100 μg/ml ($r^2 = 0.9993$).

2.4. In vitro release studies

In vitro release of Risp from DG4–Risp was investigated in PBS buffer using a micro dialysis eppendorf tube diffusion technique, by replacing the top internal flap-cover of a 0.5 ml eppendorf tube with a dialysis membrane. This technique was developed *ad-hoc* to overcome micro quantities of the released drug. DG4–Risp were sealed into the micro dialysis eppendorf tube (MW cut-off: 12000 from Sigma–Aldrich, Argentina) and incubated in PBS buffer under continuous stirring. Risp release experimental design consisted of collecting aliquots at pre-determined time intervals from the incubation medium, and storing them at 4 °C for quantitative analysis. The assay was repeated three times and the amount of released Risp was determined by absorbance at 280 nm as described in Section 2.3. Data analysis was performed adapting GraphPad Prism 5 *t-test* to solve the obtained releasing kinetic profile.

2.5. Cytotoxicity assay

L929 cell viability upon treatment with Risp, DG4 or DG4–Risp, measured as mitochondrial succinate dehydrogenase activity employing a tetrazolium salt (MTT), was determined at 37 °C in MEM medium (Gibco), supplemented with 10% FBS and antibiotic–antimycotic 10% (Gibco) in a 5% CO_2 atmosphere. Cells were seeded at a cell density of 5×10^4 cells/well in a 96-well flat bottom microplate. After 24 h, the culture medium was replaced

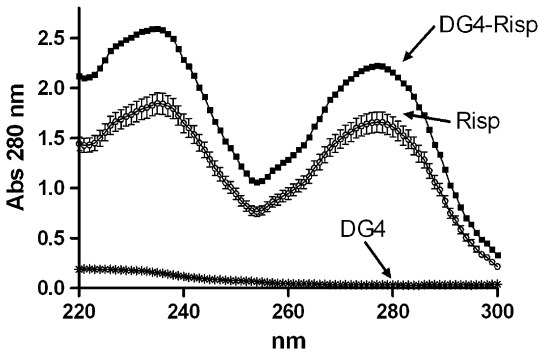

Fig. 2. Absorbance vs wavelength (nm) of DG4, free Risp and DG4–Risp.

with 100 μl of 10-fold-serial dilutions, prepared in culture media, of Risp (in the range 514–0.51 μM), DG4 (in the range 3–3 × 10^{-3} μM), or DG4–Risp (in the range 3–3 × 10^{-3} μM DG4 and 514–0.51 μM Risp). After a 24-h incubation, the solutions containing the different treatments were removed and replaced by MTT at 0.5-mg/ml final concentration. After a 2-h incubation, MTT solution was removed, and the insoluble formazan crystals were dissolved in ethanol 96° (200 μl) measuring absorbance at 570 nm using a Kayto RT-2100C microplate reader.

2.6. Hemolysis assay and human red blood cells morphological changes

Hemolysis of DG4–Risp was assayed as previously described by Duncan et al. [17]. Briefly, freshly-prepared human red blood cells from a healthy donor (100 μl) were incubated at 37 °C with DG4–Risp at two different concentrations (3 μM DG4-514 μM Risp and 3 × 10^{-2} μM DG4-5.14 μM Risp). After a 4-or 24-h incubation, samples were centrifuged at 1500× *g* for 10 min and supernatant absorbance was measured at 414 nm with a UV–Vis NanoDrop1000. Hemolysis was expressed as a percentage of the hemoglobin release induced by SDS (2%, v/v). Additionally, morphological changes on red blood cells upon incubations were determined by optical microscopy. Briefly, after incubation, cells were mounted on a slip, stained with May Grundwald–Giemsa and observed on an Alpha-phot-2, YS 2 Nikon microscope.

3. Results and discussion

3.1. Preparation of DG4–Risp complex

At the first stage of this study, incubation of Risp with DG4 in 100% methanol did not allow us to incorporate the drug into DG4, so in order to maximize the number of Risp molecules incorporated per DG4 molecule, Risp and DG4 were co-solubilized in different conditions. In all the assayed conditions, high amounts of Risp were offered to DG4. When solvent polarity effect was evaluated, a higher Risp incorporation was observed as the solvent polarity decreased beyond 50:50 v/v chloroform:methanol. In the case of samples incubated with 70:30 and 90:10 v/v chloroform:methanol, incorporation resulted in 17 and 32 drug molecules per DG4 (Fig. 3). However, when using 100% chloroform, this incorporation efficiency was not reached. Our results also revealed that incubation temperature has an important effect on DG4 drug incorporation. When incubation temperature was lowered to 4 °C, drug molecules incorporated per DG4 were significantly reduced to zero and five for the mixtures 70:30 and 90:10 v/v chloroform:methanol, respectively (data not shown).

On the other hand, incubation at low pH values (pH 4) resulted in an incorporation of only 8 molecules of Risp per DG4, and at high pH values (pH 8–10), no incorporation at all was achieved (Fig. 4). Besides, incubation with 1 M NaCl led to an incorporation of only four molecules of Risp per DG4.

Development of molecular nanostructures with well-defined particle size was evidently of increasing interest in biomedical applications [6,18–20]. Dendrimers, like other delivery systems, offer attractive properties that enable the change of drugs pharmacokinetics and bioavailability. These changes depend not only on the class of dendrimer, but also on the physicochemical nature of the complex the dendrimer forms with the drug. Drugs can be complexed with dendrimers through encapsulation into void spaces (nanoscale container), association with the surface groups (nano-scaffolding) or both [6,21]. The high density of surface groups (one amino group/nm^2 for DG4) combined with the small size (4.5 nm diameter for the DG4 ellipsoids) result in high area/volume ratio [22,23], which can be modified controlling the environmental ionic strength, pH, temperature, etc.

When examining the complexation of DG4 with Risp in different solvents, we found that in high ionic strength (1 M NaCl) few molecules of Risp could be incorporated per DG4 molecule. Welch and Muthukumar [24] determined by simulations methodology that the density profiles of dendritics are tunable from that of dense core to that of a dense shell with salt concentration or pH modification. It has been reported that the nature of the intramolecular density profile and the position of the terminal groups are critical in utilizing dendrimers as drug hosts in controlled release systems [25,26]. Ideally, to incorporate drug, the branches of dendrimers should be highly extended, nevertheless, in the tested ionic strength, this was not the case and DG4 was not capable of incorporating a great number of drug molecules. This finding was consistent with data published by Ma et al., 2007 [26], where salt concentration is related to large changes in DG4 molecular conformation and diminished drug incorporation. The same trend was observed with solvent polarity [27]. It is known that the compact structures of the hydrophobic dendrimers presenting low accessibility to the hydrophobic pockets, are favored by high polarity solvents [28]. For our particular system, hydrophilic dendrimers and Risp, it was found that the best solvents combination was 70:30 and 90:10 v/v chloroform:methanol, rendering 17 and 32 molecules of Risp per DG4 respectively. However, the condition 70:30 presented minimal deviation. In this sense, a small methanol quantity would be necessary to stabilize the primary-surface amines, but a non polar solvent should also be present in this particular case (DG4–Risp) to improve the drug partitioning between the solvent and the dendrimers' inner hydrophobic pocket, which will be wide open to incorporate the drug, but partially close to retain it. However at 100%

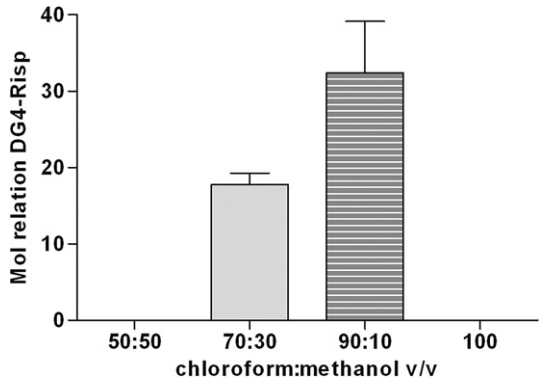

Fig. 3. Mol risperidone per each mol dendrimer at room temperature incubated in different solvent conditions.

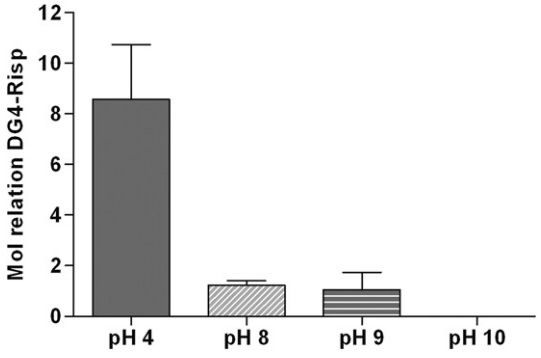

Fig. 4. Mol risperidone per each mol dendrimer at room temperature incubated in different pH.

chloroform no drug incorporation was achieved, since the drug partitions better in the highly non polar solvent than in the hydrophobic DG4 pocket, and as the solvent is non polar, DG4 compactation is expected and no drug entrapment in the hydrophobic DG4 inner can be achieved.

Finally, cationic DG4 are weak bases capable of protonating the tertiary amines of their branching points (6.85 p*K*a) under physiologic pH [29]. They exhibit open conformations at low pHs, due to the electrostatic repulsion between the primary superficial amines, which force branches to move away from each other. At pHs higher than 9, the branches get closer again as a consequence of the hydrogen bonds between the tertiary amines of the interior and the primary amines of the surface, resulting in a compact structure [30]. According to the results obtained in this work, we can conclude that the amount of Risp incorporated to DG4 is inversely proportional to pH values, which is also consistent with the literature.

Milhem et al., 2000 [31] reported that at low temperatures a high amount of ibuprofen was incorporated into PAMAM dendrimers; though according to Ma et al. [26], temperature had no significant effect. In our experimental conditions it was found that lowering temperature negatively affected the number of Risp molecules that could be hosted in DG4, leading us to infer that the temperature of maximum incorporation efficiency is intimately related to the physical chemistry and geometry of both, the host and the hosted molecules.

3.2. *In vitro* release

The stability of DG4–Risp was examined by determining the *in vitro* Risp release against PBS buffer. Fig. 5 shows that almost 100% of free Risp was released in 106 h; however, DG4–Risp retained around 40% of Risp after the same period of time. These results are somewhat consistent with those presented by Kolhe et al. [32], where 85% of the acidic drug ibuprofen was retained in these DG4–Risp in deionized water after 9 h, but no available data is near the 106 h release schedule studied in this work. Hence, we were able to obtain DG4–Risp which resulted structurally stable for a longer period in buffer.

3.3. Cytotoxicity

3.3.1. Culture cells

We measured the effect of DG4, Risp and DG4–Risp on L929 cells viability by the MTT assay. Free Risp significantly reduced cell viability after a 24-h incubation when concentrations above 5.1 μM were used. On the other hand, DG4 presented low cytotoxicity in concentrations below 3 μM, since cell viability decreased only 10% at the maximum DG4 concentration tested (3 μM), which is in accordance with data previously published for the same dendrimer, on Vero and J774 cell lines [7]. No cell viability reduction was observed with DG4–Risp when dendrimer concentration was below 3×10^{-2} μM and Risp concentration was below 5.1 μM (3×10^{-2} μM DG4-5.1 μM Risp), but this was not the case when concentration was raised to 3 μM DG4-514 μM Risp, or 3×10^{-1}μM DG4-51.4 μM Risp, since cell viability was significantly reduced in 95 and 40%, respectively (Fig. 6).

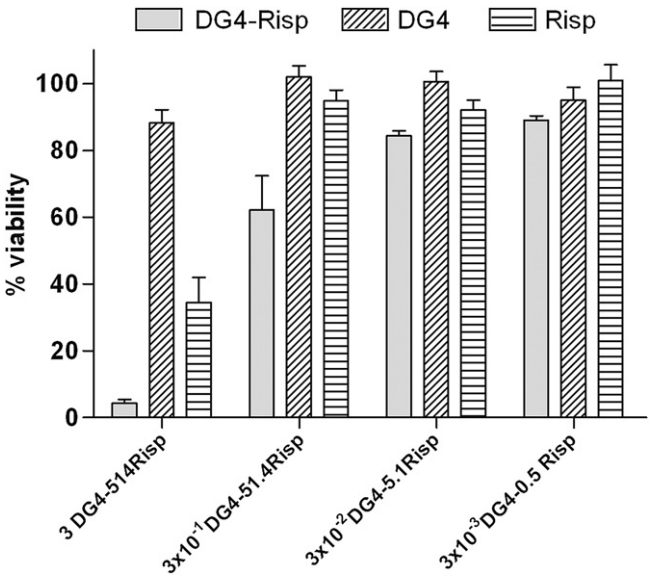

Fig. 6. Cytotoxicity of DG4, DG4-Risp and free Risp on L929 cells upon 24 h incubation by MTT assay. Concentration expressed as μM ($n = 5$).

3.3.2. Hemolysis and morphological changes of red blood cells

Hemolysis caused by cationic and anionic PAMAM dendrimers is reported to be generation and concentration dependent but, in general, nonhemolysis is found at low dendrimer concentration [8,33]. However, hemolysis and possible morphological changes on red blood cells should be tested before an i.v. administration. In this work, we evaluated these effects *in vitro* after human red blood cells 4 and 24-h incubation with 3 μM DG4-514 μM Risp, or 3×10^{-2} μM DG4-5.14 μM Risp. Independently of the incubation time and the complex concentration, no significant hemolysis (Fig. 7) or

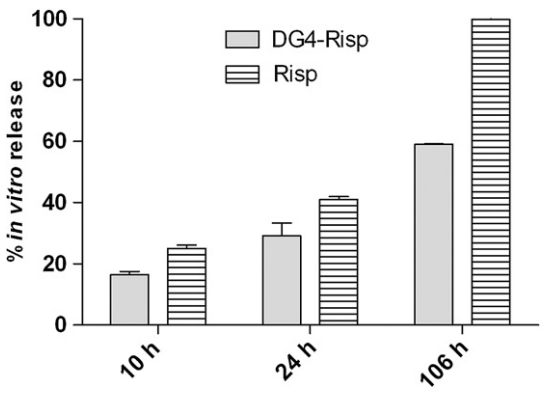

Fig. 5. *In vitro* release of Risp against PBS buffer through micro dialysis technique after 100-fold dilution ($n = 3$).

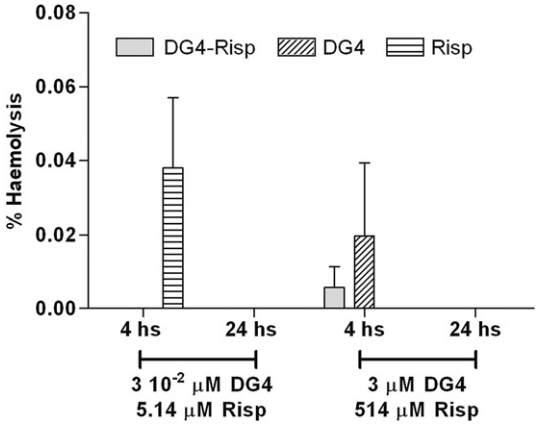

Fig. 7. Hemolysis (%) as function of concentration DG4–Risp, free Risp and DG4, and incubation time ($n = 3$).

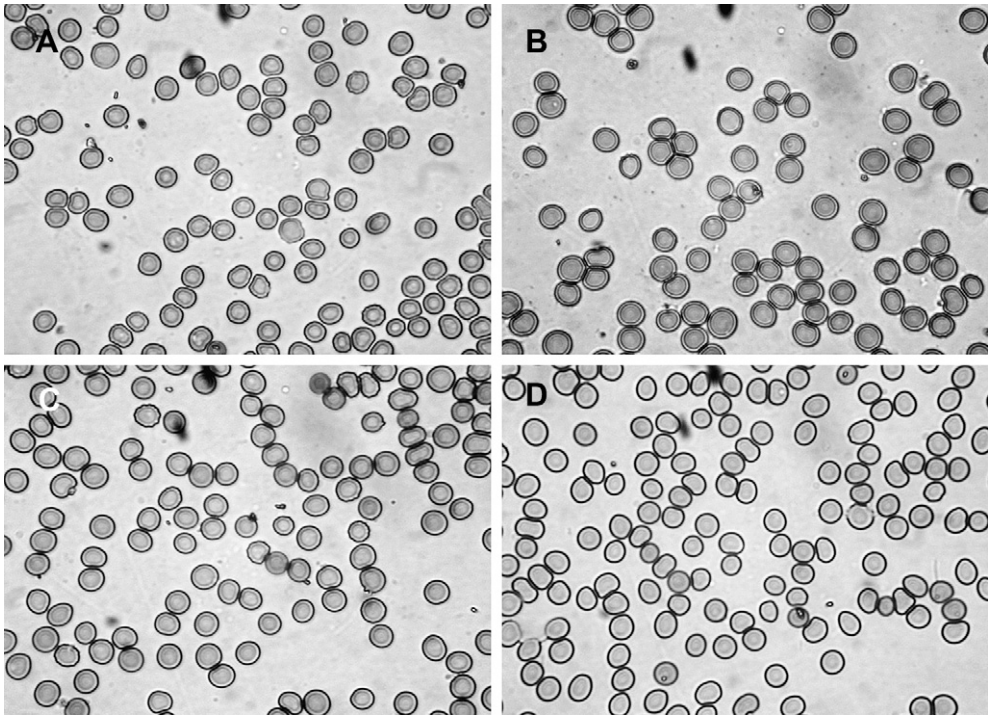

Fig. 8. Red blood cells microscopy images, incubated in: a) Buffer PBS, b) Risp 514 μM, c) DG4 3 μM and d) DG4–Risp complexes: 3 μM DG4, 514 μM Risp (40×) ($n = 3$).

morphological changes were observed, when compared with red blood cells incubated with isotonic PBS buffer (Fig. 8).

High dendrimer area/volume ratio confers a special capacity to establish surface interactions with cell membranes [22,23]. However, complexation with Risp could modify the internal volume, and hence the size and density of surface groups, leading to an alteration of the interaction with cells [14,34]. For this reason, Risp, DG4 and DG4–Risp cytotoxicity needed to be evaluated.

Previous reports on systems involving dendrimers show that cytotoxicity is concentration dependent [35–37]. We found a great decrease in cellular viability caused by the incubation with free Risp. This was expected because cell toxicity induced by Risp should be enhanced when DG4-complexed, since drug delivery is mediated by dendrimer complexation delivering Risp intracellularly [7,8].

Nevertheless, none of the tested concentrations produced hemolysis or significant morphological changes on human red blood cells when incubated 24 h at the highest concentration assayed (3 μM DG4-514 μM Risp). This phenomenon has already been observed in previous work [8], suggesting that the interaction of the complexes with cell lines do not always correlate with that observed for red blood cells, and is particular for each system. Malik et al. [33], found that cationic DG4 produces hemolysis in concentrations higher than 1 mg/ml (30 times the concentration used in this work); however, the hybrids with PEG are not hemolytics [38]. Following this idea, structure surface modification of DG4 can solve possible problems with system toxicity [39–41].

4. Conclusion

Incorporation of Risp molecules into DG4 was studied, obtaining the best solvents mixtures, temperature, pH and ionic strength conditions for efficient Risp complexation with low cytotoxicity.

The best conditions encountered for drug incorporation were: low solvent polarity mixture, pH c.a. 7, room temperature and low ionic strength. DG4–Risp showed no cell viability reduction at concentrations below 3×10^{-2} μM DG4 and 5.1 μM Risp, and resulted structurally stable for a period of 106 h in buffer. Independently of the incubation time and the complex concentration, no significant hemolysis or morphological changes were observed on red blood cells, when compared with those incubated with isotonic buffer PBS.

The results obtained so far contribute with crucial knowledge to efficiently incorporate Risp into DG4, needed to begin *in vivo* studies of biodistribution and therapeutic effects of this particular delivery nanodevice in a controlled released drug design.

Acknowledgements

This research was supported by a grant from Universidad Nacional de Quilmes, Silvia del Valle Alonso is a member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). M.J. Prieto and C.F. Temprana acknowledge fellowships from CONICET, Argentina. We also thank Jansenn Cilag Laboratory Argentina for its collaborations with the drug Risperidone as well as UNQ and MINCyT grants.

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