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Research Article

Combined Therapy for Alzheimer's Disease: Tacrine and PAMAM Dendrimers Co-Administration Reduces the Side Effects of the Drug without Modifying its Activity

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Abstract. Alzheimer's disease has become a public health priority, so an investigation of new therapies is required. Tacrine (TAC) was licensed for treatments; however, its oral administration caused hepatotoxicity, so it is essential to reduce the side effects. PAMAM dendrimer generation 4.0 and 4.5 (DG4.0 and DG4.5) can be used as drug delivery systems and as nanodrugs *per se*. Our work aims to propose a combined therapy based on TAC and PAMAM dendrimer co-administration. TAC and dendrimer interactions were studied by *in vitro* drug release, drug stability, and FTIR. The toxicity profile of co-administration was evaluated in human red blood cells, in Neuro-2a cell culture, and in zebrafish larvae. Also, the anti-acetylcholinesterase activity was studied in cell culture. It was possible to obtain DG4.0-TAC and DG4.5-TAC suspensions, without reducing the drug solubility and stability. FTIR and *in vitro* release studies confirmed that interaction between TAC and DG4.5 was of the electrostatic type. No toxicity effects on human red blood cells were observed, whereas

Highlights

- Proposal of co-administration of TAC with DG4.0 or DG4.5 for Alzheimer's therapy.
- FTIR and release studies confirmed that TAC electrostatically interacts with DG4.5.
- Co-administration reduced the effects of TAC on cell viability and metabolic activity.
- Co-administration reduced the hepatotoxicity of TAC in zebrafish larvae.
- Co-administration maintained the anti-acetylcholinesterase activity of TAC.

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Abbreviations: *AChE*, acetylcholinesterase; *AD*, Alzheimer's disease; *Aβ*, β-amyloid protein; *CV*, crystal violet; *D*, dendrimer; *DG4.0*, generation 4.0 polyamidoamine dendrimer; *DG4.5*, generation 4.5 polyamidoamine dendrimer; *dpf*, days post-fecundation; *DTNB*, 5,5-dithio-bis-(2-nitrobenzoic acid); *FBS*, fetal bovine serum; *FTIR*, Fourier transform infrared spectroscopy; *G*, generation; *hpi*, hours post-incubation; *MTT*, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; *NR*, neutral red; *PBS*, phosphate buffer saline; *TAC*, tacrine.

the co-administration with DG4.5 reduced cytotoxicity of TAC on the Neuro-2a cell line. Moreover, *in vivo* co-administration of both DG4.0-TAC and DG4.5-TAC reduced the morphological and hepatotoxic effects of TAC in zebrafish larvae. The reduction of TAC toxicity was not accompanied by a reduction in its activity since the anti-acetylcholinesterase activity remains when it is co-administrated with dendrimers. In conclusion, the co-administration of TAC with both DG4.0 and DG4.5 is a novel therapy since it was less-toxic, was more biocompatible, and has the same effectiveness than the free drug.

KEY WORDS: tacrine; PAMAM dendrimers; toxicology; alzheimer's disease; combined therapy.

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial neurodegenerative disease characterized by a decrease in cognitive function, memory, and other mental abilities. It is the cause of dementia in 60–70% of cases, so it has become a public health priority [1, 2]. Epidemiological studies showed that the prevalence of AD in people older than 60 years varied between 5 and 7% in most world regions, with a lower prevalence in African regions (2–4%) and a higher prevalence in Latin America (8.5%) [3]. In reports, it has been estimated that 35.6 million people worldwide had AD in 2010, with numbers expected to be 65.7 million in 2030 and 115.4 million in 2050 [3].

The etiology of AD is multifactorial and poorly understood. Several hypotheses tried to explain AD pathogenesis at a molecular level. The most popular ones include the acetylcholine deficiency due to degeneration of cholinergic neurons [4], the progressive accumulation of the β-amyloid protein (Aβ) into toxic oligomers and plaques outside neurons in the brain [2], and the accumulation of twisted strands of the hyperphosphorylated protein TAU into tangles inside neurons [1]. Besides these hypotheses, there is evidence that reactive oxygen species, nitric oxide, and inflammatory mediators might also contribute to the development of AD [5].

The currently approved treatments of AD are based on the inhibition of the acetylcholinesterase (AChE) with donepezil, galantamine, rivastigmine, or tacrine, to restore the cholinergic function, or on the antagonism of *N*-methyl-D-aspartate receptors with memantine, to reduce the excitotoxicity caused by an excess of glutamate [2]. These treatments promote the improvement of the cognitive ability by increasing the neurotransmitters in the brain and slow down the neuronal damage, but none of them stops the advance of AD, which makes the disease fatal [1]. Besides, these drugs are orally administered, which is more convenient for some patients but generates side effects as a result of its action on peripheral tissues [2]. Moreover, the effectiveness of these drugs varies from person to person. These are the reasons why new effective treatments capable of handling with this disease are required.

The main goal of our work is to propose a combined therapy between tacrine and dendrimers of *polyamidoamine* for the treatment of AD. The bases of our hypothesis are explained below.

Tacrine (TAC) was the first AChE inhibitor licensed for the treatment of Alzheimer's disease by the United States Food and Drug Administration in 1993 [6]. It is a robust reversible inhibitor that can penetrate cell membranes in the brain tissue and forms a stable complex with AChE [7]. Indeed, TAC is the most potent and clinically effective AChE inhibitor and has a good intestinal permeability due to its configuration and medium lipophilicity [8, 9]. However, its elimination half-life is short (1.4–3.6 h), and the absolute bioavailability is about 17 ± 13%, due to a high first-pass metabolism [10]. Also, TAC treatments resulted in dose-dependent hepatotoxicity and other peripheral cholinergic side effects [8]. For these, its clinical use has been limited. However, Patocka et al. (2008) proposed that the hepatotoxicity of TAC was probably a result of its reactive metabolite due to extensive first-pass metabolism []. Therefore, it is essential to explore alternative routes of administration to avoid its first-pass metabolism as well as to enhance the bioavailability and brain targeting effect of TAC.

TAC has been widely used in the past and in more recent studies to design hybrid or multi-target compounds in order to combine its potent AChE inhibition effect with other pharmacological properties. As a consequence, several tacrine derivatives are critical, and its hepatotoxicity and cholinergic properties were studied [9, 11]. However, the incorporation of TAC in drug delivery systems that improves its bioavailability and reduces its side effects is a topic that is not profoundly addressed. Besides, other administration routes were not deeply studied either. Two of the possible alternative routes are the transdermal and nasal administration. Transdermal delivery of TAC may minimize the first-pass metabolism, provide fairly constant blood levels for an extended period, and reduce the incidence of gastrointestinal side effects and hepatotoxicity associated with oral administration. On the other hand, intranasal administration of TAC may circumvent the blood-brain barrier, provide a rapid achievement of target drug levels, and avoid the hepatic first-pass effect. Also, intranasal administration offers a simple, practical, noninvasive, convenient, cost-effective, and an alternative route for rapid drug delivery to the brain [12].

Dendrimers, obtained by organic synthesis, are three-dimensional polymers that are composed of a central core and branches with terminal functional groups attached to the core as repetition units. Depending on the chemical type of core and branches, there are different types of dendrimers, including polyamidoamine (PAMAM), phosphodendrimers, polypropylene-imine (PPI), carbosilane, poly-L-lysine (PLL), and triazine. Due to the synthesis method, it is possible to obtain minimal polydispersity, defined surface structure (hydrophilic or lipophilic, charged or neutral), and controlled size in the range of nanometers. Dendrimers of small generations (G) (G 0–3) are asymmetric and open, and those of intermediate generation (G 4–6) are semi-rigid structures capable of interacting with other molecules, while dendrimers of major generation (G 7–10) are rigid spheres with great

steric hindrance on their surface [13]. In this work, we used dendrimers PAMAM G4.0 and G4.5 (DG4.0 and DG4.5, respectively). Both dendrimers have an ethylenediamine core and polyamidoamine branches, and DG4.0 has a positive-charge amine surface at physiological pH, while the DG4.5 has a negative-charge carboxylic surface. Both dendrimers can incorporate drug molecules inside their interior pockets or anchor them to their surface groups.

Dendrimers are used either as delivery systems of biologically active agents [14–16] or as drugs *per se*. Dendrimers have been studied *in vitro* and *in vivo* in animal models for their intrinsic properties to prevent Aβ, PrP, and α-synuclein fibrillation [17, 18]. Studies carried out by Klajnert et al. (2006) have shown the capability of PAMAM DG4.0 to interfere with the formation of β-sheet amyloid fibril structures, which are related to the onset and development of both AD and prion diseases [17]. Also, PAMAM DG4.0 was shown to have an inhibitory effect on AChE activity, as was reported previously [19, 20]. Hence, the importance of considering the PAMAM dendrimers as therapeutic drugs *per se* could be used for the AD treatment.

Nowadays, the preferred route for the administration of dendrimers is the intravenous route. However, they can also be administered by other paths as intraperitoneal, ocular, transdermal, oral, intranasal, pulmonary, intravaginal, and transmucosal [21]. PAMAM dendrimers have been used for the transdermal delivery of bioactive molecules. For example, Yiyun et al. (2007) demonstrated that PAMAM DG5.0 could effectively facilitate skin penetration of ketoprofen and diflunisal [22], and Chauhan et al. (2003) showed that PAMAM DG4.0 increase in 1.6-fold the delivery of indomethacin through transdermal route [23]. Also, a good *in vivo* nasal absorption of insulin and calcitonin using PAMAM DG3.0 was reported in rats, without any membrane damage to the nasal tissues [24], and Katare et al. (2015) demonstrated that PAMAM DG5.0 could improve the delivery of haloperidol after both intraperitoneal and intranasal route [25]. To the best of our knowledge, dendrimers are a unique type of nanoparticles that are suitable for this wide variety of administration routes. In this work, we chose the dendrimers knowing that they could act both as a TAC-delivery system and as a drug *per se*. Also, they can be administered by oral, transdermal, or intranasal routes, which would minimize the first-pass metabolism and provide a rapid achievement of target drug levels to the brain.

For all the above mentioned, the specific aims of this work are to study the interaction between tacrine and dendrimers; the toxicological profile of its co-administration in human red blood cells (*ex vivo*), in cell culture (*in vitro*), and in zebrafish larvae (*in vivo*); and the anti-acetylcholinesterase activity after co-administration.

MATERIALS AND METHODS

Materials

Tacrine (TAC, 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate, mol weight = 234.72 g/mol, CAS N°A3773), and dendrimers of polyamidoamine with ethylenediamine core of generation 4.0 (DG4.0, 64 amine surface groups, mol weight = 14,214 g/mol, CAS N°412,449) and generation 4.5 (DG4.5, 128 carboxylate surface groups, mol weight = 26,251 g/mol, CAS N°470,457) were from Sigma-Aldrich (Merck, Argentina). The dendrimers were purchased in methanol suspension at 10% wt for DG4.0 and 5% wt for DG4.5. The supplier declared a purity > 99% in both cases. These commercial suspensions were stored at 4°C, as recommended by the supplier, and used without additional purification process. Minimum Essential Medium (MEM) was from HyClone, antibiotic-antimycotic solution, and trypsin were from Gibco, all purchased from Thermo Fisher Scientific (Argentina). Fetal bovine serum was from Internegocios S.A., Argentina. MTT (sodium 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) was from USB-Corporation, crystal violet was from Sigma-Aldrich, neutral red was from BioPack, and Giemsa was from BioPurDiagnostics (Argentina). All chemicals used were of analytical grade.

Combination of TAC with Dendrimers

TAC was combined with dendrimers (DG4.0 or DG4.5) in methanol at 1:200 D to TAC molar ratio (0.024:4.8 mM). As controls, suspensions of only TAC or only dendrimers were prepared in methanol. All the samples were mixed for 24 h at 28°C. Then, the methanol was evaporated in a SAVANT SpeedVac Concentrator (Thermo Fisher Scientific, USA) at 25°C. The obtained residues were dissolved in phosphate buffer saline (PBS; 10 mM, pH = 7.4) and centrifuged at 10,000×*g* for 5 min to remove the non-soluble TAC (Fig. S1). In this work, the suspensions obtained after the combination process are called TAC (for drug control without dendrimers), DG4.0 or DG4.5 (for dendrimer control without drug), and DG4.0-TAC or DG4.5-TAC (for dendrimer drug complexes). It is essential to mention that in the obtained suspensions of complexes, there is soluble TAC not incorporated into dendrimers (up to its maximum solubility under these conditions) and TAC interacting with dendrimers (forming the D-TAC complexes).

Quantification of TAC

The quantification of the drug was carried out by monitoring its maximum of UV-Vis absorbance in PBS (10 mM, pH = 7.4). The intensity of absorbance at 240 nm in solutions of TAC of different concentrations was determined by using a UV-Vis spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, USA). The equation of the calibration curve was obtained by linear regression in a concentration range of 0.759–194 μM ($R^2 = 0.9772$) (Fig. S2). Since dendrimers have weak absorbance at this wavelength, the absorbance obtained from the suspensions after the D-TAC combination process can be attributed to TAC. So, the sample absorbances were correlated with the calibration curve, and the total amount of TAC was determined. In order to calculate the complexed TAC, the amount of TAC present in a free drug control was subtracted from the total amount of TAC determined for the D-TAC complexes.

Characterization of TAC-Dendrimers Interactions

Three approaches were used for the characterization of drug-dendrimer interactions.

First, the stability of TAC during storage at 4, 25, and 37°C for 60 days were studied. For this, the amount of TAC in the free drug control and in both D-TAC complexes was determined by UV-Vis absorbance (as we described in section "Quantification of TAC"). The amount of TAC in the suspensions at the initial day of analyses was set as 100%, and all the other results were relativized to this amount. This test was performed in triplicate on samples obtained from different combination processes.

Second, the *in vitro* release profile of TAC from D-TAC complexes was studied by using the microdialysis technique previously described in Igartúa et al. (2015 and 2018) [16, 26]. The release of TAC was studied against the buffer solution (PBS 10 mM, pH = 7.4) at different dilution ratios (1/50 and 1/100). The amount of released TAC through a dialysis membrane (MW cut-off: 12000 from Sigma-Aldrich-Merck, Argentina) was determined by UV-Vis absorbance (as we described in section "Quantification of TAC"). This assay was repeated six times in samples obtained from different combination process.

Third, the specific type of interactions established between TAC and dendrimers was studied by Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were determined from solid-state samples obtained by lyophilization. For this, the aqueous suspensions of TAC, DG4.0, and DG4.5 and the suspensions obtained after the combination process of DG4.0-TAC and DG4.5-TAC were frozen in a freezer at − 80°C overnight. These frozen samples were lyophilized in a LABCONCO lyophilizer Freezone ® 4.5 (LABCONCO Corporation, USA) previously cooled to − 50°C, keeping constant the process pressure at $33–65 \times 10^{-3}$ mbar for 24 h. Solid-state powders were obtained in all cases. The spectra of powders were measured on a Nicolet 8700 FTIR spectrometer (Thermo Fisher Scientific, USA), using the attenuated total reflectance technique. The spectra were obtained after 64 spectrum accumulations in the range of 1250 to 4000 cm^{-1}, with a resolution of 2 cm^{-1}.

Toxicity Profiles of D-TAC Co-Administration

The toxicological profile of free TAC, free dendrimers, and D-TAC co-administration were studied in three different models: human red blood cells (*ex vivo* toxicity), cell culture (*in vitro* toxicity), and zebrafish larvae (*in vivo* toxicity). Human trials demonstrated that the therapeutic concentration of TAC is between 25 and 213 μM [27]. It is important to note that, even at the lowest therapeutic concentrations, TAC showed adverse effects on the hepatic tissue. Considering that dendrimers could act by other routes against the AD progression and that the TAC-dose could be reduced in the proposed co-administration, in this work, a wide range of concentrations was used. For the *in vitro* studies, concentrations between 0.03 and 300 μM of TAC were used, and for the *ex vivo* and *in vivo* studies, concentrations between 0.3 and 30 μM of TAC were selected.

Toxicity in Human Red Blood Cells

For *ex vivo* toxicity, hemolysis and morphological changes of human red blood cells exposed to different formulations were studied. In both assays, the human red blood cells from a healthy donor were incubated at 37°C for 4 and 24 h with TAC (0.3–30 μM), DG4.0 and DG4.5 (0.0018–0.18 μM), DG4.0-TAC and DG4.5-TAC (0.0018–0.18 μM of D with 0.3–30 μM of TAC), and PBS (10 mM, pH = 7.4) as a negative control or SDS 2% *w/v* as a positive control. Hemolysis was determined using UV-Vis absorbance to quantify hemoglobin release, as described previously [16], and was expressed as the percentage of hemoglobin released in each treatment with respect to that released in the positive control. The morphological changes of the cells were determined using Giemsa staining and optical microscopy. For these assays, three technical replicates and three biological replicates were used for each dilution ($n = 9$). Also, this study was carried out following the principles of the Declaration of Helsinki. The participant (healthy donor) gave written informed consent to the experimental protocol before participating in the study.

Toxicity in Neuro-2a Cell Culture

For *in vitro* toxicity, the Neuro-2a cell line was selected, which is a fast-growing mouse neuroblastoma line that has been used to study Alzheimer's disease [28]. In this line, the effects of TAC, D, and D-TAC on viability (by crystal violet staining), metabolic activity (by MTT assay), and membrane state (by neutral red uptake) were studied.

In the three assays, the cells were seeded in a 96-well flat-bottom microplate at a density of 10×10^3 cells/well and grown at 37°C in MEM supplemented with a 10% antibiotic-antimycotic solution and 10% FBS in a humidity chamber with a 5% CO_2 atmosphere. After 24 h of growth, the media were replaced with 100 μL of 10-fold-serial dilutions of TAC (0.03–300 μM), DG4.0 or DG4.5 (0.00018–1.8 μM), and DG4.0-TAC or DG4.5-TAC (0.00018–1.8 μM of D with 0.03–300 μM of TAC), all prepared in culture media. After 4 or 24 h of incubation, the treatments were removed and replaced by the different reagent solutions according to the used colorimetric method.

For viability studies, crystal violet staining was used, as we described in Igartúa et al. (2018) [16]. For metabolic activity, the protocol of MTT assay described by Mosmann (1983) [29] was selected. For the membrane state studies, the method of neutral red uptake developed by Borenfreund and Puerner (1985) [30] was implemented. In all assays, the effects were expressed as percentage respect to the negative control, which are cells treated only with PBS and had 100% viability, metabolic activity, and neutral red uptake. For each assay, eight technical replicates and three biological replicates were used for each dilution ($n = 24$).

Toxicity in Zebrafish Larvae

For *in vivo* toxicity, zebrafish larvae were used as a high-through model that allows studying the general toxicity (viability and morphological changes) and the organ-specific toxicity (neuro-, cardio-, and hepatotoxicity) of the D-TAC co-administration. The zebrafish protocols were carried out in strict accordance with the National Institute of Health guidelines for animal care and maintenance and were approved by the Institutional Ethics Committees of the National University of Quilmes, Buenos Aires, Argentina (CE-UNQ 2/2014, CICUAL-UNQ 013-15 and 014-15).

Adult zebrafish (*Danio rerio)* were maintained and used to obtain embryos by natural mating, as described previously [16, 31]. In this study, embryos refer to zebrafish before hatching (0–3 days post-fecundation—dpf), while larvae refer to post-hatching animals (over 3 dpf) (Fig. S3). For treatments, three embryos at 1 dpf were placed in each well of a 96-well plate containing E3 medium and maintained for 4 days at 28°C. At 5 dpf, the medium was replaced by 250 μL of 10-fold-serial dilutions of TAC (0.3–30 μM) and DG4.0-TAC or DG4.5-TAC (0.0018–0.18 μM of dendrimers with 0.3–30 μM of TAC), all prepared in E3 medium. As a negative control, larvae incubated only with E3 medium were used. At 1, 4, 24, and 48-h post-incubation (hpi), the viability and the spontaneous movement (neurotoxicity) were studied. At 48 hpi, the heart rate (cardiotoxicity), the morphological changes, and the hepatic damage (hepatotoxicity) were analyzed. For each assay, eight technical replicates and three biological replicates were used for each dilution ($n = 24$).

For viability studies, larvae were observed with a stereomicroscope, and when no heartbeat was observed, it was considered that the larvae were dead. Viability was expressed as a percentage of the live larvae respect to the total of larvae per treatment.

For neurotoxicity studies, changes in the locomotor activity of larvae were studied, because it could reflect the cerebral damage, as well as a morphological or lethal effect, caused by different treatments. The spontaneous movement was determined in a multichannel analog to digital converter system (WMicrotracker, Designplus SRL, Argentina) as we previously described [16] and was expressed as the percentage of respect to the movement in the negative control.

For cardiotoxicity studies, changes in the heart rate of larvae were studied, because it could reflect the heart damage, as well as a morphological o lethal effect, caused by different treatments. The heart rate of larvae was measured by counting the number of beats every 15 s in a stereomicroscope and was expressed as the percentage of respect the heart rate in the negative control.

For morphological changes analyses, the larvae were photographed and scored based on the degree of malformations (Fig. S4). The score of individual larvae determined the mean toxicity score for each treatment.

For hepatotoxicity studies, the livers of the control and treated larvae were analyzed by using the method validated by He et al. [32]. The liver is situated posterior to the pericardium and anterior to the gut and could be observed when the larva is in the dorsolateral position. Healthy zebrafish liver is clear, whereas it became darker after treatment with hepatotoxic drugs, indicating degeneration and/or necrosis (Fig. S4) [32]. Hepatotoxicity was expressed as a percentage of larvae with necrotic liver respect to the total of larvae per treatment.

Anti-Acetylcholinesterase Activity of D-TAC Co-Administration

The Neuro-2a cells, which express acetylcholinesterase enzyme (AChE), were used as an *in vitro* model to study the anti-AChE activity of the free TAC, free dendrimers, and D-TAC co-administration. To measure its activity, Ellman's method, with some modifications, was used [33, 34]. First, the cells were seeded in a 96-well flat-bottom microplate at a density of 15×10^3 cells/well and grown at 37°C in MEM supplemented with a 10% antibiotic-antimycotic solution and 10% FBS in a humidity chamber with a 5% CO_2 atmosphere. After 24 h of growth, the media were replaced with 100 μL of dilutions prepared in culture media of TAC (0.3–100 μM), DG4.0 or DG4.5 (0.018–0.6 μM), and DG4.0-TAC or DG4.5-TAC (0.018–0.6 μM of D with 0.3–100 μM of TAC). After 4 h of incubation, the treatments were removed, and the cells were washed twice with PBS. Then, 200 μL of the reaction mixture (freshly prepared from 160 μL of 0.075 M acetylthiocholine iodide, 800 μL of 0.01 M 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), and 24 mL of buffer phosphate 0.1 M pH = 8,0) were added to each well. The AChE activity was determined through the conversion of acetyl-thiocholine to thiocholine, a molecule that reacts with DTNB to produce the yellow 5-thio-2-dithionitrobenzoic acid. The increment of absorbance of this yellow compound was measured at 414 nm every 5 min per 120 min, keeping the plate under agitation at 37°C. The AChE activity was calculated through the linear regression of the absorbance data as a function of time. The absorbance in control wells (without cells), due to non-enzymatic hydrolysis, was subtracted to the other wells. The results were expressed as a percentage of activity with respect to the positive control (cell without inhibitory treatment).

Statistics

All results were expressed as the mean ± the standard deviation (SD) of the data obtained. Statistical analysis was performed using the Graph Pad Prism v6.0 program. Depending on the experimental design of each assay, *T* test and one-way or two-way ANOVA test followed by corresponding multiple comparisons post-tests were used. In all cases, the differences were considered to be significant only when the *p* value was less than 0.05. The different degrees of significance obtained were represented with asterisks, as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. The abbreviation *ns* was used to express not significant differences. The particular statistical analysis used in each trial is detailed in the supplementary material.

RESULTS

Combination of TAC and Dendrimers to Obtain DG4.0-TAC and DG4.5-TAC Complexes

The combination of TAC with DG4.0 or DG4.5 was studied at a 1:200 D to TAC molar ratio. By using this high molar ratio, we could ensure that each molecule of dendrimer has 200 molecules of TAC available, assuring the drug-dendrimer interaction. Also, to study the reproducibility of the combination method, 60 replicates were performed (Fig. 1).

The TAC control of the combination process (without dendrimers) resulted in the solubilization of 3878 ± 513 μM of the drug, while DG4.0-TAC resulted in 4050 ± 438 μM and DG4.5-TAC in 4435 ± 444 μM of TAC. Statistical analyses showed that soluble TAC was significantly higher only when DG4.5 was present. It is important to note that these values correspond to the averages of all the replications made of the

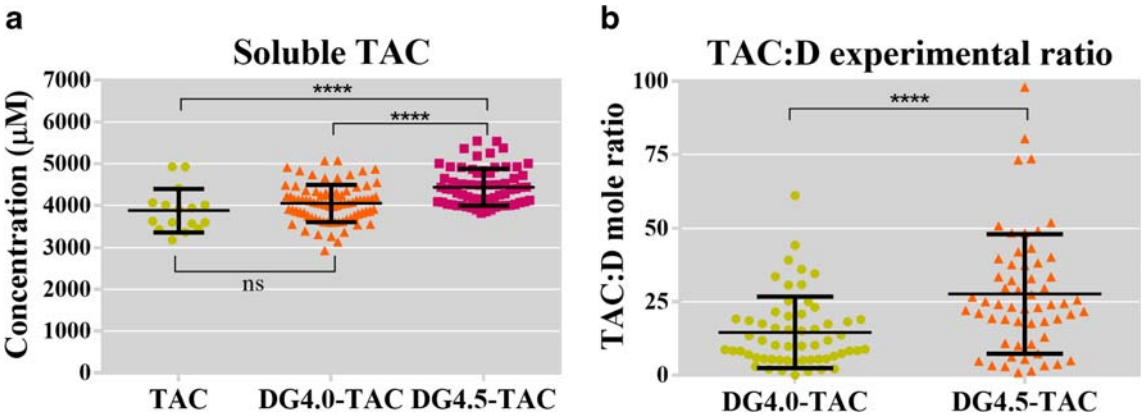

Fig. 1. Combination of TAC and dendrimers to obtain D-TAC complexes. **a** Concentration of soluble TAC and **b** mole of TAC per mol of dendrimer, both after the combination process

complexation process. However, within each replicate, there was always a trend towards higher solubility of the drug in the presence of both dendrimers.

The total amounts of TAC obtained after the combination process corresponds to both the soluble unincorporated TAC and the complexed TAC (in D-TAC complexes). The amount of TAC present in a free TAC control of each replication was subtracted from the total amount of TAC in the D-TAC combinations to calculate the complexed TAC. Since the concentration of dendrimer was known (24 μM), the results can be expressed as the number of moles of TAC per mole of dendrimers. An average of 15 and 28 mol of TAC per mole of DG4.0 and DG4.5, respectively, was calculated. In both cases, a large dispersion was observed in the results, so that the amount of TAC was quantified before each trial. These unexpected results can not be validated through other groups since, and as far as we know, this is the first work where the interaction between the TAC and the dendrimers was studied.

Characterization of TAC-Dendrimers Interactions

Three approaches were used for the characterization of drug-dendrimer interactions: stability throughout the storage time was studied, the *in vitro* release profile of the drug from complexes was determined, and the spectra obtained by FTIR spectroscopy were analyzed.

The stability of the TAC in aqueous suspension in the presence of both dendrimers was compared with the stability of the free drug. For this, the amount of soluble drug was quantified during 60 days of storage at 4, 25, or 37°C (Fig. 2). Both complexes (DG4.0-TAC and DG4.5-TAC) were kept the initial amount of TAC in suspension during the entire analysis time without presenting changes in absorbance spectra or at the macroscopic level. These results mean that there were no chemical nor physical changes in the suspensions.

The strength of the drug-dendrimer interaction was studied by the *in vitro* release assay of TAC from DG4.0-TAC and DG4.5-TAC against buffer solution at a dilution ratio of 1/100 (Fig. 3) and 1/50 (Fig. S5). The use of two different dilution factors made it possible to predict the force of differential interaction between the two types of dendrimers and the same drug molecule. However, a complete release of the TAC from both complexes (release profile similar to the free drug control) was observed when using both dilution factors. This means that, if there are some interactions between the drug and dendrimers, they are weak and do not resist a dilution process.

The specific type of drug-dendrimer interaction was studied by FTIR. For this, the spectra of lyophilized TAC, DG4.0, DG4.0-TAC, DG4.5, and DG4.5-TAC were determined (Fig. 4).

FTIR spectrum of solid TAC showed the $–NH_2$ vibration bands at 3329 and 1272 cm^{-1}, the –CH stretching vibration bands at 2918 and 2821 cm^{-1}, and the aromatic ring vibration between 1700 and 1400 cm^{-1}. FTIR spectrum of cationic dendrimer (DG4.0) showed the –CH and –NH stretching vibrations of the core at 2917 y 2849 cm^{-1}, the –NH deformation vibration of the amine terminal groups at 1651 cm^{-1}, and the C–C stretching bonds inside the dendrimer core at 1557 cm^{-1}. In the FTIR spectrum of DG4.0-TAC, the disappearance of the bands at 2917 and 2849 cm^{-1} was observed. FTIR spectrum of anionic dendrimer (DG4.5) showed the –NH flexion vibration of amides of core at 3240 and 1642 cm^{-1}; the –CH and –NH stretching vibrations also of the core at 2915 and 2849 cm^{-1}; the –C=O stretching and the –COO symmetric vibrations of carboxylic terminal groups at 1730 and 1393 cm^{-1}, respectively. In the FTIR spectrum of DG4.5-TAC, the disappearance of the bands at 2915, 2849, and 1730 cm^{-1} and the displacement of the band at 1393 cm^{-1} were observed. Also, the maximum at 1272 cm^{-1} corresponding to the $–NH_2$ vibration of the TAC disappears in the DG4.5-TAC.

Toxicity Profiles of D-TAC Co-Administration

The toxicity of TAC treatment and D-TAC co-administration was studied in human red blood cells (*ex vivo* toxicity), cell culture (*in vitro* toxicity), and zebrafish larvae (*in vivo* toxicity), to determine a complete toxicological profile.

In the first place, the possible hemolytic effect (Fig. S6) and morphological changes (Fig. 5) in human red blood cells were studied after treatments of 4 and 24 h with free TAC or dendrimers and with D-TAC co-administration. No significant hemolysis or morphological changes were observed for any treatment when compared with red blood cells incubated with isotonic buffer (negative control) at any incubation time.

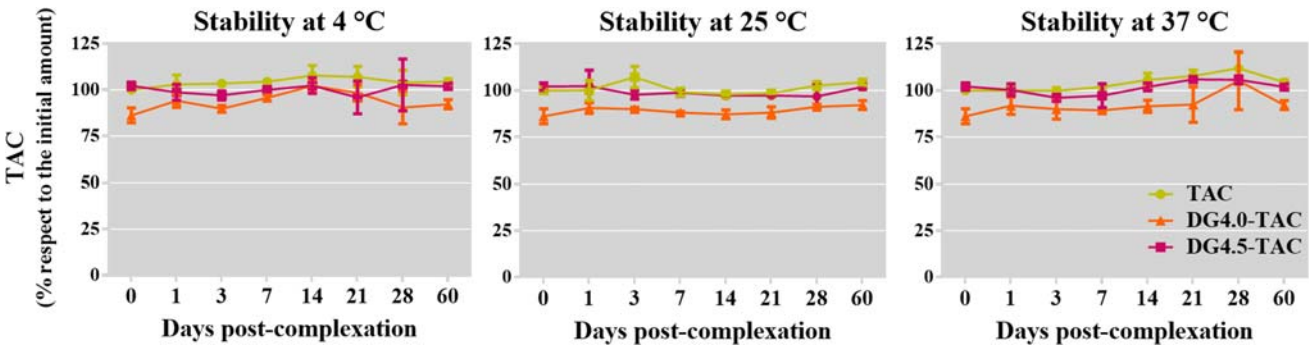

Fig. 2. Stability of TAC in D-TAC complexes storage at 4, 25, or 37°C for 60 days

These results suggest that treatment could be applied intravenously, as well as transdermally or intranasally.

In the second place, the possible cytotoxic effect in Neuro-2a cell culture was studied after treatments of 4 and 24 h with free TAC or dendrimers and with D-TAC co-administration. These effects were analyzed in function of the viability measured by crystal violet (CV) staining, the metabolic activity measured by MTT assay, and the state of the cellular membranes measured by the neutral red (NR) uptake. The three colorimetric methods that we used are different each other because the CV staining measured the dye-uptake by attached and viable cells, while the MTT assay is based on the enzymatic conversion of MTT to an insoluble purple formazan by succinate dehydrogenase enzyme within the cell mitochondria, and the NR assay is measuring the dye-uptake by functional lysosomes of cells with membranes in good state [35].

After treatment of 4 h (Fig. S7), a significant reduction of Neuro-2a cell viability (CV staining) was observed in treatment with 300 μM of TAC. The co-administration of the same concentration of TAC with 1.8 μM of DG4.0 or DG4.5 reduced this cytotoxic effect, incrementing the viability of cells. Also, the administration of 300 μM of free TAC caused an increment of the metabolic activity that could be related to the cytotoxic effect on the cell viability mediated by this drug. The increment in the metabolic activity was not observed in the co-administration of D-TAC treatments. Nevertheless, the treatment with 300 μM of free TAC significantly reduced the NR uptake, which might be related to an effect at the membrane level, and the co-administration with dendrimer cannot reverse these cytotoxic results. Indeed, the administration of 1.8 μM of DG4.0 or DG4.5 alone (without drug) also reduces the NR uptake, which might be related to the toxic effect of dendrimers on cell membranes, particularly in the lysosomal membrane. In summary, the co-administration of TAC with dendrimers reduced the cytotoxicity of the drug at cell viability and at metabolic activity levels, but it could not reduce the effects at the membranes. This could lead to long-term toxic effects, so we also studied the cytotoxicity after treatments of 24 h (Fig. 6). In these cases, the treatment with 300 μM of free TAC caused a significant reduction in cell viability, metabolic activity, and NR uptake. The co-administration of TAC with DG4.0 or DG4.5 could not reverse these effects, and the co-administration with DG4.0 even increased the cytotoxicity at lower TAC concentrations. While this may be related to a higher uptake of the drug by target neuronal cells, further studies are required to ensure that co-administration would not cause these toxic effects in other tissues. Beyond the toxic effects of 300 μM TAC treatment, the administration of concentrations equal to or less than 30 μM of free drug or co-administration with DG4.5 did not cause toxic effects measured by any of the three methods in the two incubation times studied.

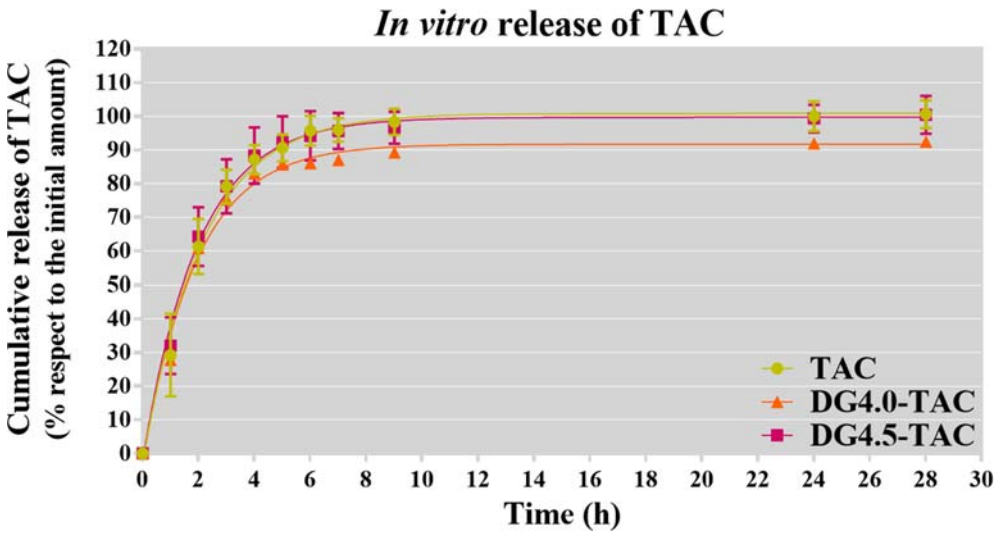

Fig. 3. *In vitro* release of TAC from D-TAC complexes at 1/100 dilution ratio

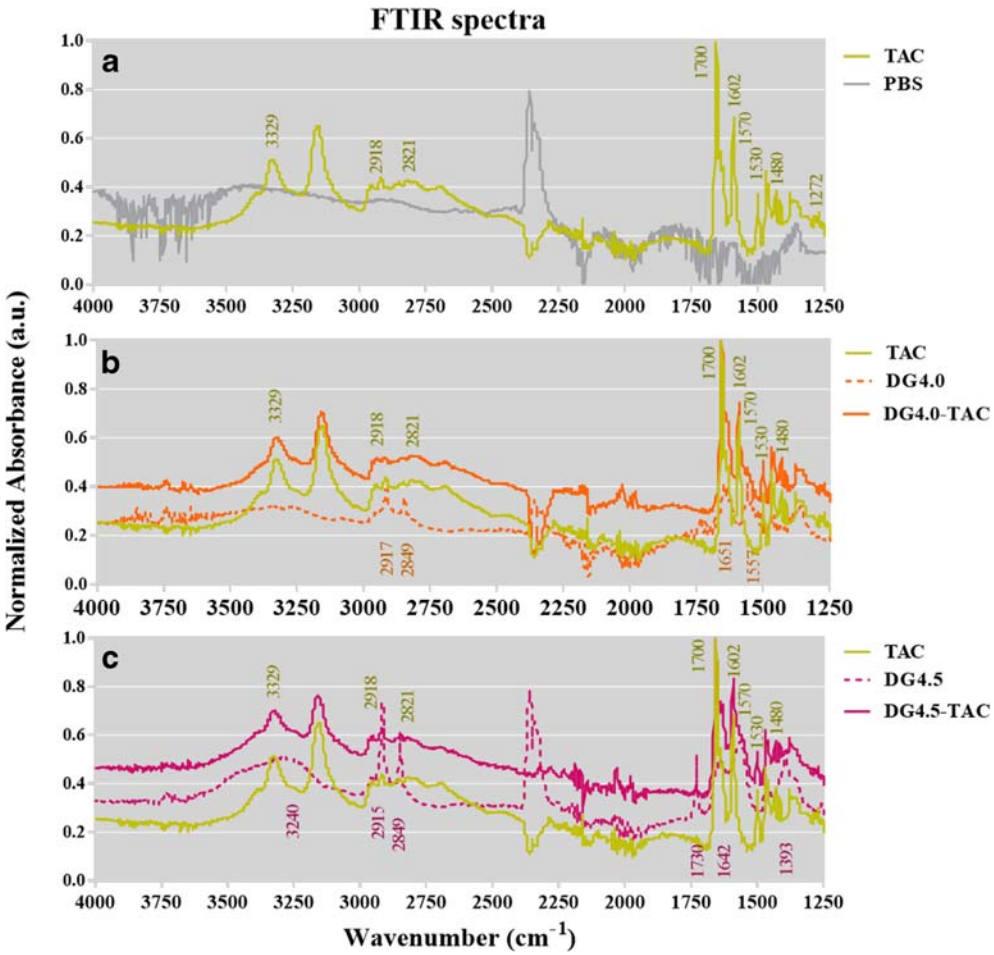

Fig. 4. Interaction of TAC and dendrimers studied by FTIR. FTIR spectra of **a** TAC and PBS, **b** TAC, DG4.0, and DG4.0-TAC, and **c** TAC, DG4.5, and DG4.5-TAC were obtained from lyophilized powders

In the third place, the possible *in vivo* toxicity effects, with relevance in hepatotoxic ones, were determined in zebrafish larvae. Specifically, the effects of the viability, spontaneous swimming activity, heart rate, morphological development, and liver state of zebrafish larvae were studied after treatment with TAC or D-TAC (Fig. 7 and Fig. S8). As far as we know, this is the first work where TAC biocompatibility and toxicity were studied in this animal model.

Concerning the viability, no formulation produced lethal effects up to treatments of 1, 4, or 24 h. But after 48 h, the administration of 30 μM of TAC and the co-administration of 30 μM of drug with 0.18 μM of dendrimers caused the mortality of 20% of the larvae. However, none of the treatments, in any of the tested concentrations, modified the swimming activity of zebrafish larvae after 1, 4, 24, and 48 h of treatment, which could be related to neurotoxic effects (Fig. S9). Regarding cardiotoxicity, the administration of 30 μM of free or complexed TAC significantly reduced the heart rate compared to the non-treated control, showing toxic effects at the cardiac level. Moreover, significant morphological changes (Fig. S8) were observed in larvae after treatments, such as bent column, mandibular malformation, necrosed head region, head malformation, necrosed liver, necrosed yolk, yolk not depleted, deflated swim bladder, and tail malformation. The treatment with 30 μM of TAC scores at an average of 1.8 in the degree of malformations, whereas the treatments with dendrimers score at an average of 1.3, being 4 the maximum degree of malformations. Despite not wholly reducing these anomalies, the co-administration resulted in minor effects. In addition, the malformations observed after co-administration with dendrimers were not lethal, while those observed in free TAC treatment were lethal and included necrosis in several tissues. On the other hand, as concerning hepatotoxicity, 66.7% of larvae treated with 30 μM of TAC presented necrotic (opaque) liver, while only 46.7% of those treated with DG4.5-TAC presented these effects. Surprisingly, treatment with DG4.0-TAC only caused hepatotoxicity in 26.7% of the larvae. This means that the co-administration of TAC with dendrimers has a lesser hepatic effect.

Activity of D-TAC Co-Administration

Finally, with the aim of the study, if the co-administration of TAC with dendrimers modify the anti-acetylcholinesterase (AChE) activity of the drug, and if the dendrimers have an effect by itself, the activity of the AChE expressed in Neuro-2a cell line was measured (Fig. 8).

After treatments of 4 h, the administration of 100 and 30 μM of TAC significantly reduced the activity of AChE to 20.8 and 34.8%, respectively, while the co-administration with both DG4.0 and DG4.5 in the same concentrations,

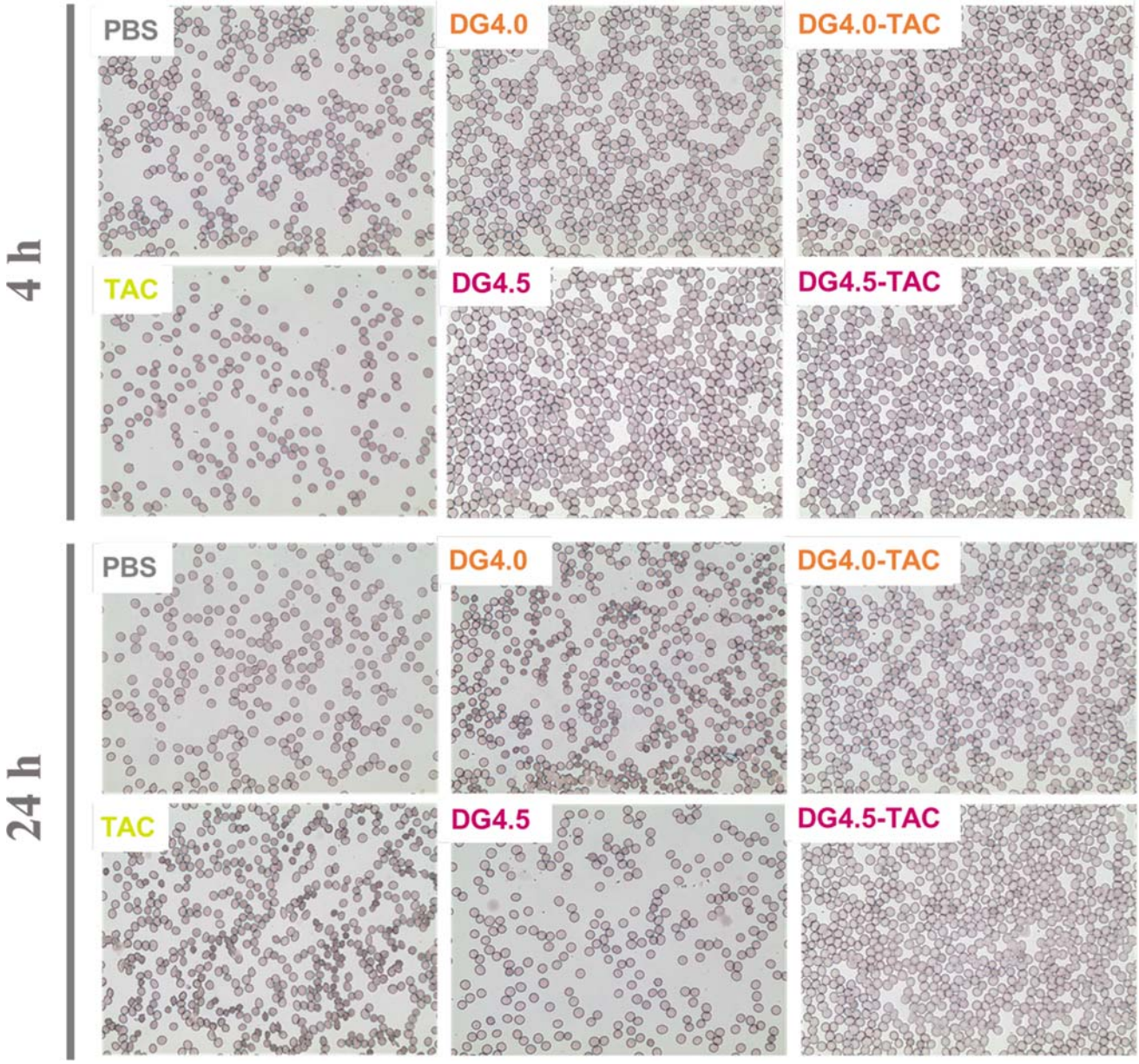

Fig. 5. Morphological analysis of human red blood cells after treatments of 4 or 24 h with TAC (30 μM), dendrimers (0.18 μM), or D-TAC complexes (0.18 μM of dendrimers with 30 μM of TAC)

maintained the positive effect of this drug (20.4 and 35.5%, respectively). Moreover, the higher concentrations of free dendrimers (0.18 and 0.6 μM) significantly reduced the AChE activity to 75%, confirming the hypothesis that they can act as a nanodrugs *per se*.

DISCUSSION

Dendrimers can interact in several ways with organic compounds, which could result in the increment of the solubility or stability of these bioactive drugs. The drug-dendrimer interaction may depend on the dendrimer type and generation, and the pH and temperature conditions. Also, the interaction strength depends on hydrophobic or electrostatic interaction affinity between the dendrimer and the drug. The PAMAM dendrimers generation 4.0 (DG4.0) has positive-charge amine terminal groups ($-NH3^{+}$), whereas the dendrimer generation 4.5 (DG4.5) has negative-charge carboxylic terminal ones ($-COO^{-}$). Both dendrimers have chemically identical internal cavities. These properties make possible the encapsulation of poorly soluble drugs through hydrophobic interactions and the anchorage of ionic drugs through electrostatic interactions. In this sense, tacrine (TAC) is a relatively strong base with a pKa value of 9.85 [36] and has a relatively high water-solubility of 0.25 ± 0.02 mg/mL [37]. Hence, TAC is protonated at physiological pH and could interact electrostatically with the carboxyl-terminal groups of DG4.5 or could be encapsulated into the internal cavities of both dendrimers.

When the combination process of TAC with dendrimers was carried out, the solubility of TAC in control without dendrimers was similar to those obtained in the presence of DG4.0, whereas the presence of DG4.5 lead to a significant

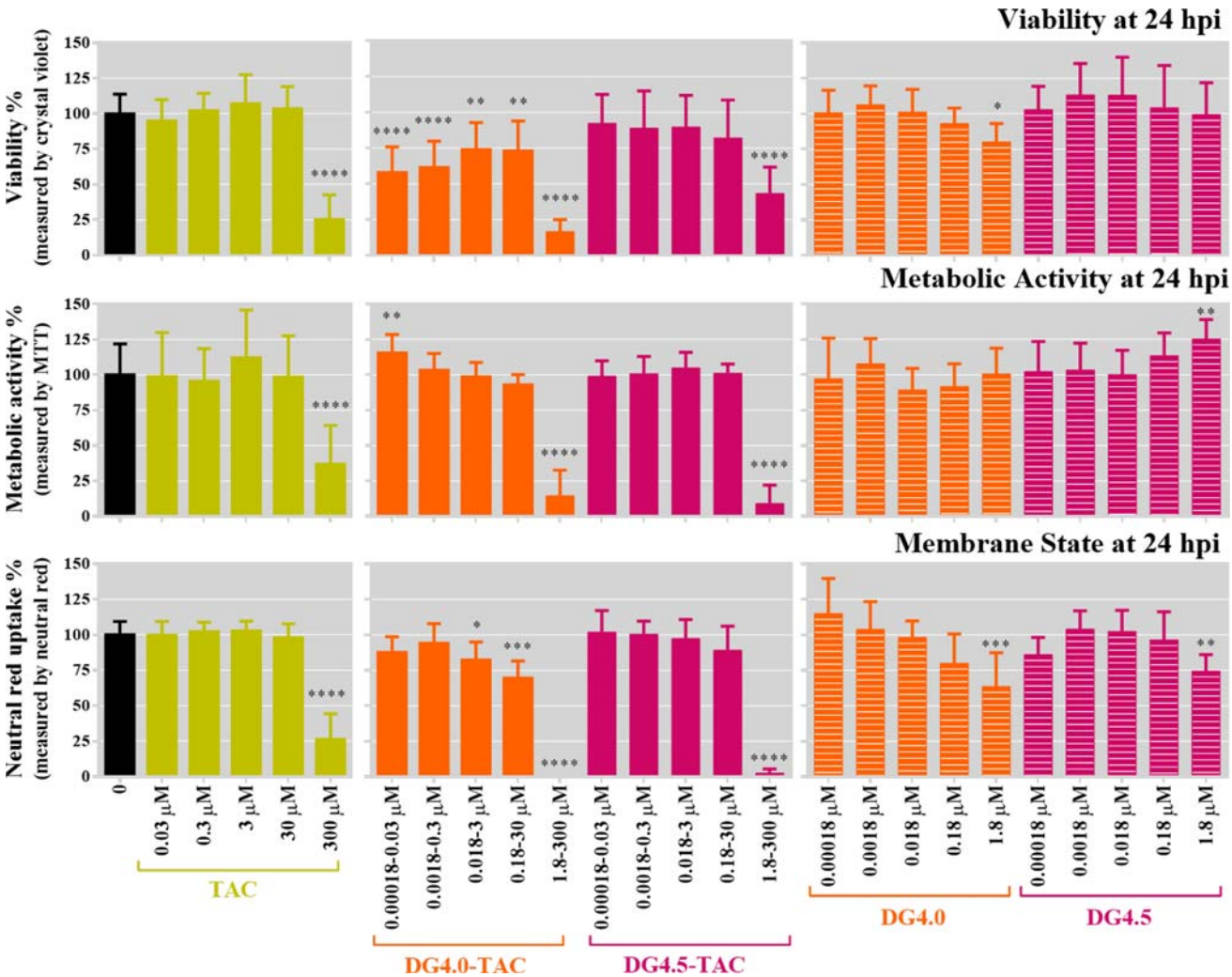

Fig. 6. Cytotoxicity of TAC, D, and D-TAC complexes in Neuro-2a cell culture after 24 h of treatment

increase in the concentration of the drug **(**Fig. 1**)**. Also, the DG4.5 presented a higher loading capability for TAC that DG4.0. Anyhow, the DG4.0 does not affect the solubility of TAC since a similar amount of drug respect to the free drug control was obtained after the DG4.0-TAC combination, allowing a correct combination of the treatments. Altogether, these results suggest that the interaction between TAC and DG4.5 occurs by electrostatic interactions and that there is no interaction of TAC with internal pockets of both dendrimers at this pH condition.

To confirm these hypotheses, we characterized the interaction between TAC and dendrimers throughout the study of the stability over time **(**Fig. 2**)**, the release profile of the drug from the complexes (Fig. 3) and the spectra obtained by FTIR of drug-dendrimer complexes (Fig. 4).

The stability assay showed that both complexes (DG4.0-TAC and DG4.5-TAC) were able to keep the TAC in suspension during 60 days at 4, 25, or 37°C, without presenting chemical nor physical changes in the drug suspensions. The *in vitro* release assay showed that the drug-dendrimer interactions are weak and do not resist a dilution process since both D-TAC complexes presented the same drug-release profile than the free drug control. This was expected, given that TAC is ionized and soluble in water, and will tend to be released. Our results are in agreement with those obtained by Wilson et al. (2010), in which they studied the release of TAC from chitosan nanoparticles [10]. Moreover, our results showed a similar release profile of free and complexed TAC, while the results presented by Vihola et al. (2002) showed an increased release of TAC from PVCL particles respect to free TAC [38].

Infrared spectroscopy is a nondestructive well-established method for the identification of chemical species with high sensitivity and selectivity and for determination of the specific type of molecules interaction. In this work, FTIR was used to analyze the drug-dendrimers interaction through the comparison of free dendrimer or free drug spectra with D-TAC complexes spectra. FTIR spectra of solid TAC, DG4.0, and DG4.5 showed all the specific absorption bands defined for each one [39, 40]. When DG4.5 and DG4.5-TAC spectra were compared, it could be observed that some absorption bands disappeared and others displaced. As two bands correspond to groups in the pockets of the dendrimers and the other two corresponds to carboxylic terminal groups, TAC molecules could be interacting both with the hydrophobic pockets of the DG4.5 and with their terminal carboxylic groups. However, the encapsulation process was not expected, due to TAC is a hydrophilic drug. So, it is essential to take into consideration that the samples were lyophilized before FTIR analysis, and during this process of solvent evaporation, the interaction between dendrimers and

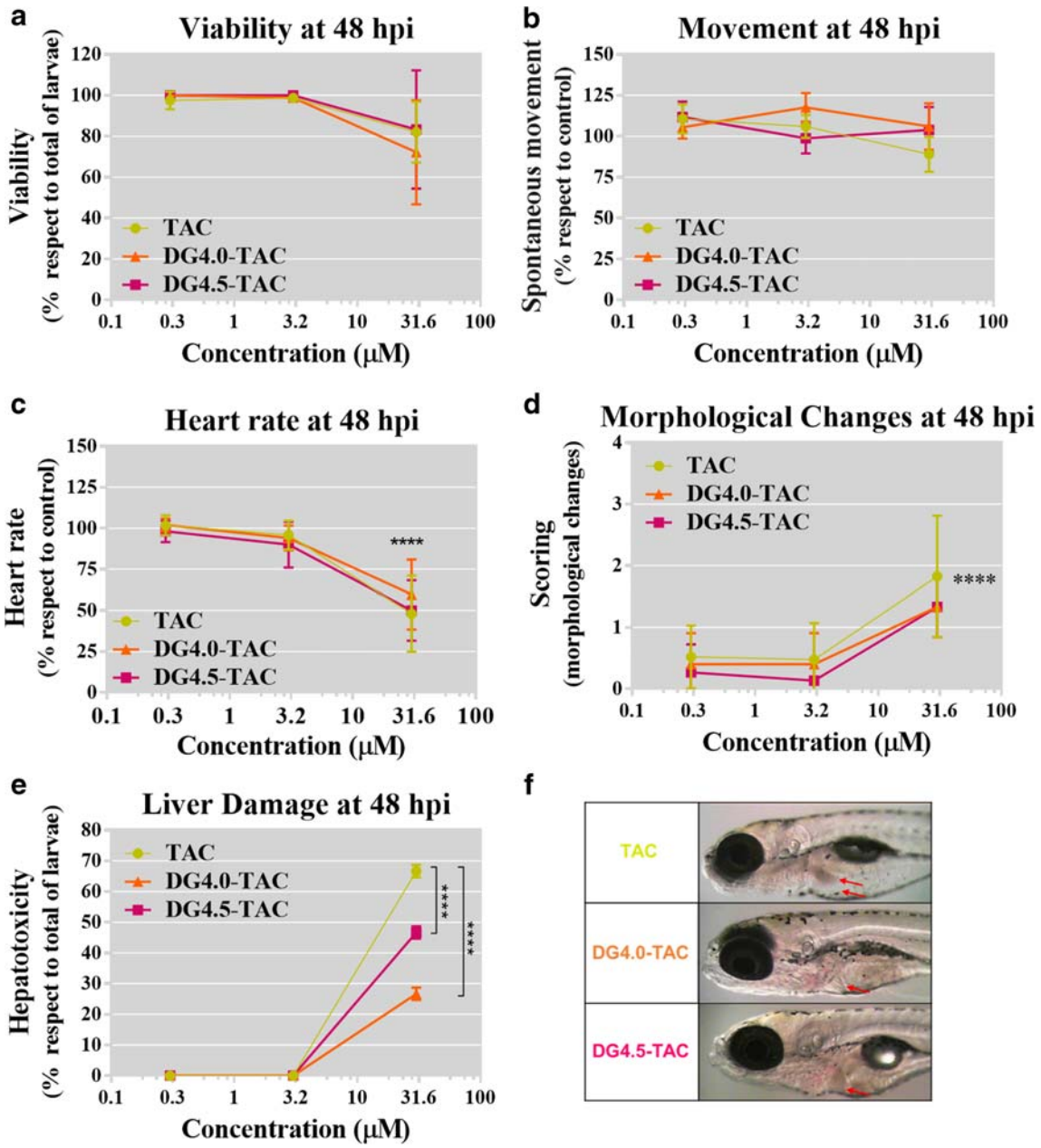

Fig. 7. Toxicity of TAC and D-TAC complexes in zebrafish larvae after 48 h of treatment. Larvae were exposed to TAC (0.3–30 μM) or D-TAC complexes (0.0018–0.18 μM of D with 0.3–30 μM of TAC), and different endpoints were evaluated: **a** viability, **b** spontaneous movement as measured of neurotoxicity, **c** heart rate as measured of cardiotoxicity, **d** morphological changes, and **e** liver damage as measured of hepatotoxicity. In **f** some examples of hepatic state are presented

between dendrimers and TAC could be maximized. Even, Maiti et al. (2004 and 2005) reported that dendrimers could suffer a process of back folding in conditions of poor solvent, which could explain the changes in absorption bands of the groups of internal pockets [41, 42]. However, the maximum corresponding to the amine group of TAC also disappears in the DG4.5-TAC spectrum, which confirms the electrostatic interaction between the drug and carboxylic terminal groups of DG4.5 dendrimer. On the other hand, when DG4.0 and DG4.0-TAC spectra were compared, it could be observed that only the bands that correspond to the core groups of the dendrimers disappeared. Regardless, the maxima corresponding to the amine groups of TAC was not affected. These results confirmed that TAC molecules did not interact with DG4.0.

So far, we showed that it is possible to combine in aqueous solution the TAC and both types of dendrimers, without reducing the solubility and stability of the drug and that the solubility can even be increased in the presence of DG4.5. Furthermore, we showed that TAC did anchor to terminal carboxylic groups of DG4.5 through electrostatic interactions. These interactions were weak and did not modify the release profile of the drug for its bioavailability. Therefore, TAC and dendrimers can be combined in aqueous solutions to perform co-administration, without modifying the solubility, stability, and release profile of the drug.

Then, we wonder if co-administration with dendrimers could reduce the side effects of TAC, considering that TAC was removed

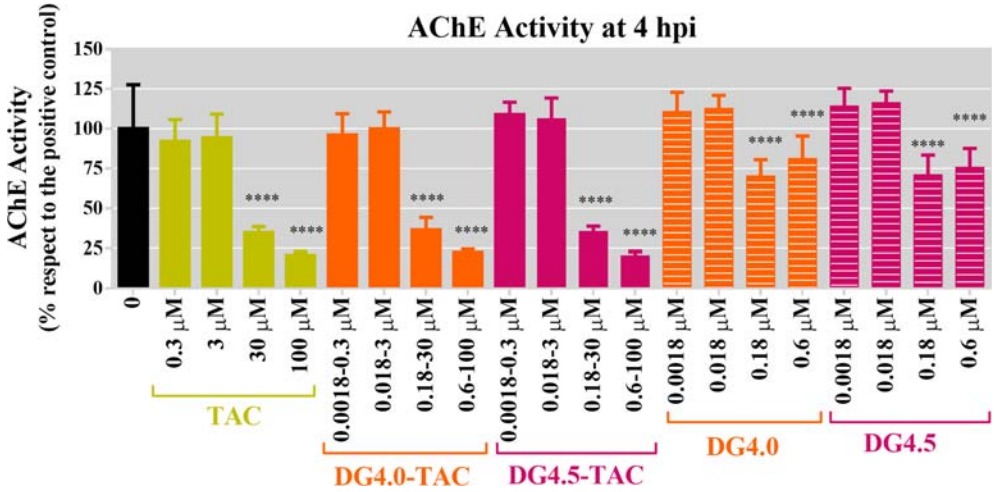

Fig. 8. Inhibition of TAC, D, and D-TAC over the acetylcholinesterase activity of Neuro-2a cells

from the USA market due to it is hepatotoxicity. For the study of the toxicological profile, we used three models: human red blood cells (Fig. 5), cell culture (Fig. 6), and zebrafish larvae (Fig. 7).

The study of hemolysis and morphological changes in red blood cells are methods widely used to analyze the interaction of compounds with membranes and to predict the toxicity after intravenous administration. The treatments with free TAC and the co-administration with dendrimers did not produce significant effects when compared with red blood cells incubated with isotonic buffer (negative control) at any incubation time. These results suggest that treatment could be applied intravenously, as well as transdermally or intranasally.

The study of toxicity in cell culture is imperative to determine the possible effect of the free drug, free dendrimers, and the obtained complexes with TAC. Also, it is essential to consider that while most drugs cause cellular damage through interaction with specific biomolecules, nanoparticles can cause toxicity by a combination of complex mechanisms [35]. So, the study of cytotoxicity must be performed through the combination of methods that analyze different endpoints. In the present work, we studied the effects on viability measured by CV staining, on metabolic activity measured by MTT assay and on the membrane state measured by the NR uptake. After treatment of 4 h, the co-administration of TAC with dendrimers reduced the toxic effect at the viability and metabolic activity levels but did not reduce the effects on membranes state. Indeed, the administration of free dendrimers also reduced the NR uptake, which might be related to the toxic effect of dendrimers on cell membranes, particularly in the lysosomal membrane. This effect on lysosomes produced by dendrimers was previously described [43]. After treatment of 24 h, the toxicity that free TAC caused on viability, metabolic activity, and membrane state cannot be reduced by the co-administration with dendrimers. While this may be related to a higher uptake of the drug by target neuronal cells, further studies are required to ensure that co-administration would not cause these toxic effects in other tissues. Beyond the toxic effects of 300-μM tacrine treatment, the administration of concentrations equal to or less than 30 μM of free drug or co-administration with DG4.5 does not cause toxic effects measured by any of the three methods in the two incubation-times studied. In this sense, the adjustment of the dose will be critical about the implementation of the proposed co-administration. In conclusion, the use of different methods to study the toxic effects of the proposed co-administration in the Neuro-2a cells, allowed to demonstrate that the DG4.5-TAC complexes presented reduced toxicity concerning the other formulation (DG4.0-TAC) or the free TAC.

The study of *in vivo* toxicity was carried out using zebrafish larvae. We chose this animal model because it offers information about whole-animal and organ-specific toxicity, which cannot be obtained from *in vitro* studies. Also, assays with larvae are rapid, high-throughput, and cost-effective. In addition, zebrafish liver is fully formed and functioning by 3 days post-fertilization (dpf), which permits the study of hepatotoxic effects. As far as we know, this is the first work where TAC biocompatibility and toxicity are studied in zebrafish larvae. Our results showed that TAC reduced de viability of zebrafish in about 20% and caused cardiotoxicity, effects that co-administration with dendrimers cannot reverse. It could be considered that this cardiotoxic effect is only due to the effect of free TAC, given that it was previously reported that PAMAM dendrimers do not have toxic effects on zebrafish larvae at tested concentrations. Notably, it was reported by our group that DG4.0 was toxic for zebrafish larvae in concentrations higher than 0.5 μM, while DG4.5 was not toxic up to 20 μM [44]. On the other hand, the co-administration of TAC with dendrimers resulted in fewer morphological abnormalities and significantly reduced the hepatotoxicity of the free TAC, which could mean benefit with respect to the actual treatment. Again, we want to highlight that the administration in sub-lethal doses of free or complex TAC produced no toxic effect in zebrafish larvae.

Finally, we wonder if the administration of TAC with dendrimers could modify its therapeutic action, that of inhibiting the enzyme acetylcholinesterase (AChE). For determining this, the activity of the AChE expressed in the Neuro-2a cell line was measured (Fig. 8). Our results showed that the administration of free TAC, free dendrimers, and D-TAC complexes inhibited the AChE activity. It is important to note that co-administration of TAC with both DG4.0 and DG4.5 presented the same therapeutical activity than free drug, resulting in a suitable combination for new treatments. Moreover, both dendrimers showed also to inhibit the enzyme. For DG4.0, this result is in concordance with those presented by Klajnert et al. (2004) and Shcharbin et al. (2006) [19, 20]. Notably, this work is the first to report the anti-AChE activity of DG4.5. These results reflect the advantage

of using dendrimers as drug delivery systems because they also have therapeutical activity on their own.

CONCLUSIONS

Regarding the necessity of new effective treatments capable of handling with the Alzheimer's disease, we proposed a combined therapy based on the tacrine and PAMAM dendrimers co-administration by non-traditional routes (transdermal or nasal), to avoid the toxicity caused by the extensive first-pass metabolism of the drug. In this work, we showed a method to obtain DG4.0-TAC and DG4.5-TAC suspensions, without reducing the solubility and stability of TAC, and studied the effect of this co-administration *ex vivo*, *in vitro*, and *in vivo*. No hemolytic effect was observed in human red blood cells. The *in vitro* cytotoxic-effects over Neuro-2a viability and metabolic activity caused by the free drug was reduced by treatment with DG4.5-TAC. The *in vivo* hepatotoxicity consequence of TAC treatment was reduced by co-administration of both DG4.0-TAC and DG4.5-TAC. Also, the TAC remains therapeutically active when it is administered with dendrimers. In conclusion, the combination of TAC with DG4.0 or DG4.5 was less-toxic, more-biocompatible, and same-effective than free TAC, in the concentrations tested. As a perspective of our work, further experiments will be carried out to explore the ability of the DG4.0-TAC and DG4.5-TAC complexes in delivering the drug through intranasal or transdermal route. Considering all mentioned above, the co-administration of TAC with dendrimers could be incorporated into clinical trials, leading to an improvement of current treatment. Moreover, this work could be considered a starting point to study new treatments based on the combination of dendrimers with already approved drugs by non-traditional routes of administration (transdermal or intranasal). These new treatments could deal with the disease confronting more than one of the etiological pathways.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest All authors declare that they have no conflict of interest.

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