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PAMAM Dendrimers as a Carbamazepine Delivery System for Neurodegenerative Diseases: A Biophysical and Nanotoxicological Characterization

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Declaration of interest

None.

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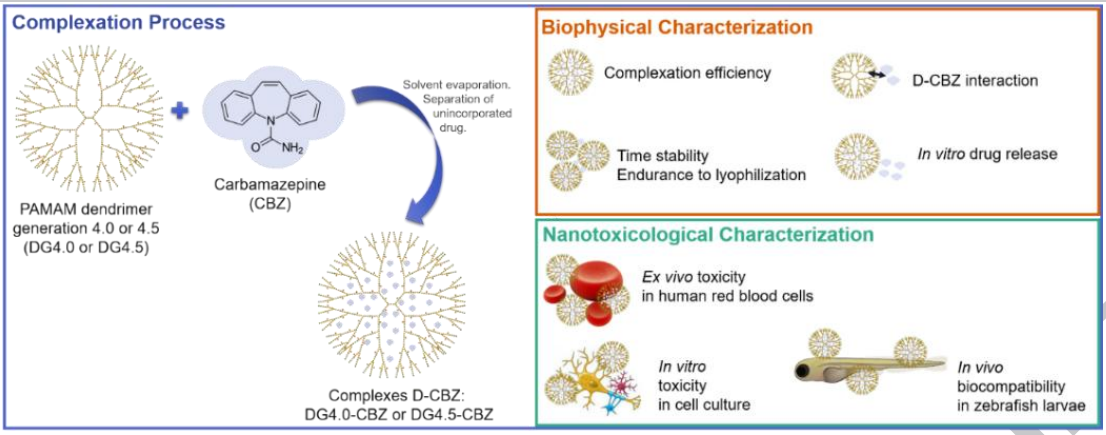
Abstract

Carbamazepine (CBZ) is an antiepileptic drug, which also could be used in the treatment of neurodegenerative diseases, such as the Alzheimer's disease. However, its use has been limited due to its low solubility, inefficient pharmacokinetic profiles, and multiple side effects. PAMAM dendrimers, ethylenediamine core, generation 4.0 (amine terminal groups) and 4.5 (carboxylate terminal groups) (DG4.0 and DG4.5 respectively) are polymers that can increase drug solubility through complexation. Thus, the aim of this work was to obtain and characterize complexes between CBZ and dendrimers. Both DG4.0 and DG4.5 allowed the incorporation of ~20 molecules of CBZ per dendrimer, into their hydrophobic pockets. DG4.0-CBZ and DG4.5-CBZ complexes were found to be stable for 90 days at 37 °C and resistant to a lyophilization process, presenting controlled drug release. Also, the complexes nanotoxicity was tested *ex vivo* (human red blood cells), *in vitro* (N2a cell line), and *in vivo* (zebrafish). No hemolytic effect was observed in the *ex vivo* model. As regards *in vitro* toxicity, the DG4.5-CBZ complexes significantly reduced the toxicity caused by the free drug. Moreover, the DG4.5-CBZ did not cause neurotoxicity or cardiotoxicity in zebrafish larvae. In conclusion, a stable and biocompatible drug delivery system based on the DG4.5 capable of complex the CBZ has been developed. This achievement highlights the advantages of using negatively charged dendrimers for nanomedicine.

Keywords: Carbamazepine, PAMAM dendrimers, Complexation, Toxicology, Neurodegenerative disease, Epilepsy.

Graphical abstract



Highlights

- The PAMAM dendrimers generation 4.0 and 4.5 (DG4.0 and DG4.5) encapsulated around of 20 molecules of carbamazepine (CBZ) per molecule of dendrimer, tripling the solubility of the drug.
- The DG4.0-CBZ and DG4.5-CBZ complexes were stable for 90 days stored at 37 °C and resistant to a lyophilization process.
- Both DG4.0-CBZ and DG4.5-CBZ complexes showed a controlled release of the CBZ under physiological conditions, retaining 40% of the drug after 24 h of dialysis. The interaction between CBZ and dendrimers occurs in their hydrophobic pockets.
- The DG4.0-CBZ and DG4.5-CBZ complexes did not show hemolytic effects. The DG4.5-CBZ complexes reduced the cytotoxicity caused by the free drug in cell culture. The DG4.5-CBZ complexes were biocompatible according to the tests carried out on zebrafish larvae.
- A stable and biocompatible drug delivery system based on the DG4.5 capable of efficiently complex the CBZ has been developed.

Abbreviations

BBB: blood-brain barrier; CBZ: Carbamazepine; CV: crystal violet; DDS: drug delivery system; DG4.0: polyamidoamine dendrimer generation 4.0; DG4.5: polyamidoamine dendrimer generation 4.5; DMSO: dimethylsulfoxide; dpf: days post-fecundation; FBS: fetal bovine serum; FTIR: Fourier Transform Infrared Spectroscopy; hpf: hours post-fecundation; hpi: hours post-incubation; MEM: minimum essential medium; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; NR: neutral red; PAMAM: polyamidoamine; PBS: phosphate buffer saline.

1. Introduction

The protein misfolding and abnormal aggregation of specific proteins are involved in the most prevalent neurodegenerative disorders, including Alzheimer´s disease, amyotrophic lateral sclerosis, Parkinson´s disease, and Huntington´s disease (Guo *et al.*, 2017). The generation of highly diffusible small oligomers, fibrils and large aggregates with amyloid properties are implicated in these abnormal processes (Vidal *et al.*, 2014). Lately, due to the selective degradation of abnormally folded protein by the lysosomal pathway and the disruption of aggregated proteins, the enhancers of natural autophagy process have become attractive as a treatment for these neurodegenerative disorders (Budini *et al.*, 2017; Harris & Rubinsztein, 2012; Hidvegi *et al.*, 2010; Vidal *et al.*, 2014).

More recently, it has been found that carbamazepine (CBZ), an FDA approved anticonvulsant and mood-stabilizing drug has a potent autophagy enhancement effect (Harris & Rubinsztein, 2012; Hidvegi *et al.*, 2010) which reduces the accumulation and toxic effects of aggregation-prone proteins in *in vitro* cell models and protects against neurodegeneration *in vivo* (Li *et al.*, 2013; Williams *et al.*, 2008; L. Zhang *et al.*, 2017). CBZ is traditionally given as a solid dosage or suspensions form by oral administration, but due to its poor solubility in water (120 µg/mL, 500 µM, at 25 °C) (Brewster *et al.*, 1990; Hemenway *et al.*, 2009; Martins *et al.*, 2012), it has a slow and irregular gastrointestinal absorption, leading to an incomplete drug bioavailability (El-Zein *et al.*, 1998; Gavini *et al.*, 2006; Kobayashi *et al.*, 2000). In addition, this reduced solubility does not allow its incorporation in therapeutic dosages in aqueous solutions for intravenous injection (Serralheiro *et al.*, 2014). Also, the CBZ pharmacokinetic is complex because there is no direct relationship between the given oral dose and the concentration of the drug found in plasma (Alrashood, 2016; Jiao *et al.*, 2003). Therefore, it is necessary to use a high oral dose (800-1200 mg/day) to achieve the therapeutic concentration of CBZ, which has been estimated at 0.004-0.012 mg/mL (Kong *et al.*, 2014). Furthermore, CBZ undergoes extensive hepatic metabolism and enzymatic induction that outcome in erratic plasmatic fluctuations and unexpected clearance increments, which demand successive dose adjustments (Serralheiro *et al.*, 2014; Tomson *et al.*, 1987; Yuen *et al.*, 2008).

Taking into account the above considerations, improving the solubility of CBZ may increase the rate of absorption, enhance the oral bioavailability and allow reducing the oral dose needed to accomplish therapeutic concentrations. Therefore, CBZ is a promising candidate to be used into a drug delivery system (DDS) with controlled release properties for the treatment of neurodegenerative diseases. A DDS delivers a

drug to a predetermined specific site (target organ/tissue) and, in this way, they allow to reduce the side effects by decreasing access to other tissues or organs where they do not have the therapeutic effect (Kakkar *et al.*, 2017). The brain is an essential organ from which most of the vital functions are controlled. It is isolated and protected from the external environment by the blood-brain barrier (BBB). The same mechanisms that prevent the entry of harmful foreign substances avoid the access of therapeutic drugs. Hence, it is important to design DDSs that are capable of massively direct the drug to the brain, especially in the treatment of neurodegenerative diseases (Saraiva *et al.*, 2016).

The dendrimers are three-dimensional polymers obtained by organic synthesis that provide unique properties to the DDS field, since they have relevant characteristics such as minimal polydispersity, defined surface structure with globular shape, high water solubility, and very small and controlled size in the range of nanometers (Kalomiraki *et al.*, 2016; Kesharwani *et al.*, 2014; Svenson & Tomalia, 2012; Tomalia *et al.*, 1990). Generation 4.0 and 4.5 PAMAM dendrimers are optimal as DDS since they can incorporate drug molecules into their hydrophobic pockets, which are size- and shape-controlled, or anchor them to their surface groups by ionic or covalent interactions (Markowicz-Piasecka & Mikiciuk-Olasik, 2016). Thus, the complexed drug would acquire the physicochemical properties of the dendrimers, which would significantly increase its solubility in aqueous media and, therefore, modify its pharmacokinetics and biodistribution properties. In previous works, our group demonstrated that the drugs sulfadiazine and risperidone complexed with PAMAM dendrimers increment their solubility in water and the arrival to the brain, generating an increase in the potency of their effect (M. Prieto *et al.*, 2006; M. Prieto *et al.*, 2008; Maria Jimena Prieto *et al.*, 2013; Maria Jimena Prieto *et al.*, 2014; María Jimena Prieto *et al.*, 2011).

For those mentioned above, the present study aims to develop and characterize a novel drug delivery system for CBZ based on PAMAM dendrimers for the treatment of neurodegenerative disorders. This strategy is focused on increasing CBZ solubility, decreasing the dose and frequency of administration reducing side effects and probably improving the cost-effective balance of the therapy.

2. Materials and Methods

2.1. Materials

Polyamidoamine (PAMAM) dendrimers (D), with ethylenediamine core, generation 4.0 (mol weight = 14,214 g/mol, 64 amine terminal groups) (DG4.0) and PAMAM dendrimers generation 4.5 (mol weight = 26,251 g/mol, 128 carboxylate terminal groups) (DG4.5) were purchased from SIGMA-ALDRICH (MERCK, Argentina) and were used without further purification. Carbamazepine (CBZ), 5H-dibenzo[b,f]azepine-5-carboxamide (mol weight = 236.27 g/mol), was generously donated by Ph.D. Albertina Moglioni from the Chemistry and Drug Metabolism Institute (IQUIMEFA-CONICET) (Buenos Aires, Argentina). Sodium 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was from USB Corporation, cell culture MEM was from HyClone, and antibiotic-antimycotic solution and trypsin were from Gibco, all purchased from Thermo Fisher Scientific, Argentina. Fetal bovine serum was from Internegocios S.A., Argentina. Crystal violet was from Sigma-Aldrich (MERCK, Argentina) and Neutral red was from BioPack (Argentina). All other reagents were of analytical grade and used without further purification.

2.2. CBZ complexation in DG4.0 and DG4.5

Different amounts of CBZ were combined with a constant amount of DG4.0 or DG4.5 in methanolic solution to obtain different D:CBZ molar ratios (1:5, 1:10, 1:25, 1:50, 1:100, 1:150 and 1:200); corresponding to 0.024 mM of dendrimers and 0.12, 0.24, 0.6, 1.2, 2.4, 3.6 or 4.8 mM of CBZ, respectively. The mixtures were incubated for 24 h at 28 °C and, then, methanol was evaporated at 25 °C in a SAVANT ^{TM} SpeedVac Concentrator (Thermo Fisher Scientific, USA). The solid residues obtained were dissolved in 0.1 mL of phosphate buffer saline (PBS 10 mM, pH=7.4) at room temperature and centrifuged at 10,000×g for 5 min, to separate the D-CBZ complexes from the non-soluble unincorporated CBZ **(Figure S1).**

2.3. Drug quantification by UV-Vis spectroscopy

NanoDrop 1000 UV-Vis spectrometer (Thermo Fisher Scientific, USA) was used to quantify the amount of CBZ incorporated in the dendrimer. In PBS (10 mM, pH=7.4), CBZ has its maximum absorbance at λ_{max}= 284 nm. The stock solution of CBZ was obtained in a solution of 10% (v/v) ethanol in PBS. A calibration curve of CBZ at different concentrations in PBS was set determining the absorbance at this wavelength. The calibration curve was linear in a concentration range of 0.41–211 µM (R^2=0.9994). The D-CBZ complexes were dissolved in PBS. Since dendrimers have no absorbance at this wavelength, the absorbance obtained from D-CBZ complexes can be attributed to CBZ **(Figure S2)**. The obtained

absorbance was correlated with the calibration curve, and the amount of CBZ was determined. The total amount of CBZ in the D-CBZ complexes corresponds to both the soluble unincorporated CBZ and the complexed CBZ. The amount of CBZ present in a free CBZ control (performed in the same buffer and conditions) was subtracted from the total amount of CBZ determined for the D-CBZ complexes to calculate the quantity of the complexed CBZ. Since the concentration of dendrimer was known, the results were expressed as the number of moles of CBZ incorporated per mole of dendrimer.

2.4. D-CBZ complexes stability and resistance to lyophilization process

The time stability of free or complexed CBZ was studied after storage at 4, 25 or 37 °C. The amount of CBZ in the suspensions after 1, 7, 14, 21, 28, 35, and 90 days post-complexation were measured as described in Section 2.3. The soluble CBZ present in the control of free drug was used to set the 100% and all sample data was adjusted to this value. Also, the stability of the complexes after the lyophilization process was studied. Lyophilization was performed from frozen suspensions (-80 °C overnight) of CBZ, DG4.0-CBZ and DG4.5-CBZ without additives, in a Freezone 4.5 LABCONCO lyophilizer (LABCONCO Corporation, USA), pre-cooled at -50 ºC maintaining the lyophilization process pressure within the range of $33x10^{-3}$ to $65x10^{-3}$ mbar for 24 h. The amount of CBZ in the suspensions after 1, 7, 14, 21, 28, 35 and 90 days post-reconstituting the lyophilized powder were measured as described in Section 2.3. The soluble CBZ present in the control of free drug was used to set the 100%, and all sample data was adjusted to this value.

2.5. Fourier transform infrared spectroscopy (FTIR)

To study the D-CBZ interaction and the structural changes in the complexes respect to free dendrimers, FTIR spectroscopy was used. FTIR spectra of lyophilized PBS, CBZ, DG4.0, DG4.5, DG4.0-CBZ, and DG4.5-CBZ were measured using an FTIR Nicolet 8700 spectrometer (Thermo Fisher Scientific, USA). Powders were characterized placing into the attachment plate to measure attenuated total reflectance (ATR). After 64 scans in the range of 400 cm^{-1} to 4000 cm^{-1}, the spectrum was obtained with a resolution of 2 cm^{-1}.

2.6. *In vitro* drug release studies

In vitro release of CBZ from D-CBZ complexes was studied against buffer solutions at different pH (2.2, 7.4 and 12.6) by using a *micro-dialysis Eppendorf tube diffusion technique* in which the top internal flap-cover of a 0.5 mL *Eppendorf* tube is replaced with a dialysis membrane (MW cut-off: 12000 from Sigma-Aldrich-Merck, Argentina). This technique was developed *ad hoc* to overcome micro-quantities of the released drug (Igartúa *et al.*, 2015). D-CBZ complexes or free drug were sealed into the micro-dialysis *Eppendorf* tube and incubated in the different buffers under continuous stirring. The CBZ release experimental design consisted of collecting aliquots at pre-determined time intervals (1, 2, 3, 4, 5, 6, 7, 9, 24 and 28 h) from the incubation medium. Each aliquot withdrawn was replaced afterward by an equal volume of fresh medium to maintain the volume constant during the experiment. The assay was repeated six times, and the amount of released CBZ was determined by absorbance at 284 nm, as described in Section 2.3.

2.7. *Ex vivo* toxicity: Hemolysis assay and human red blood cells morphological changes

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of National University of Quilmes (Buenos Aires, Argentina; ethics CE-UNQ No 2/2014). The participant (healthy donor) provided a written informed consent to the experimental protocol before her/his study-participation.

Hemolysis of human red blood cells incubated with CBZ and D-CBZ complexes was assayed as previously described by Prieto *et al.* (2011) and Temprana *et al.* (2017) (María Jimena Prieto *et al.*, 2011; Temprana *et al.*, 2017). Briefly, freshly-prepared human red blood cells from a healthy donor were incubated at 37 °C with CBZ (in the range 0.3 – 30 µM), dendrimers (in the range 0.012 – 1.2 µM), D-CBZ complexes (in the range 0.012– 1.2 µM of D and 0.3 – 30 µM of CBZ), or PBS (10 mM, pH=7.4) as a negative control. After a 4- or 24-h incubation, samples were centrifuged at 2000xg for 10 min, and supernatant absorbance was measured at 414 nm with a UV-Vis NanoDrop1000 spectrophotometer. Hemolysis was expressed as a percentage of the hemoglobin release induced by SDS 2% w/v set as 100% hemolysis. Additionally, morphological changes in red blood cells upon incubations were determined by optical microscopy. Briefly, after incubation, cells were mounted on a slip, stained with Giemsa for 20 min and observed with a Leica DMI 6000 B (Leica Microsystems, USA) microscope.

2.8. *In vitro* toxicity: Cytotoxicity in N2a cell culture

2.8.1. Cell culture and treatment

To study the influence of the proposed delivery systems on cell culture and analyze possible cytotoxic effects, we used the N2a cell line, a fast-growing mouse neuroblastoma cell line that has been widely used to study neuronal differentiation, neurite growth, synaptogenesis and signaling pathway. Also, N2a cells have been used to study neurotoxicity, Alzheimer's disease and asymmetric division of mammalian cell lines (He *et al.*, 2017; Yang *et al.*, 2014). Cytotoxicity upon treatment with CBZ, D or D-CBZ was measured by colorimetric methods as crystal violet (CV) staining, MTT assay, and neutral red (NR) uptake. The cells were seeded at a cell density of 7×10^3 cells/well in a 96-well flat bottom microplate and grown at 37 °C in MEM supplemented with 10% FBS and a 10% antibiotic-antimycotic solution in a humidity chamber with a 5% CO_2 atmosphere. After 24 h, the medium was replaced with 100 µL of 10-fold-serial dilutions (prepared in culture media) of CBZ (in the range $3x10^{-3}$ – 30 µM), dendrimers (in the range $0.12x10^{-3}$ – 1.2 µM), or D-CBZ complexes (in the range $0.12x10^{-3}$ – 1.2 µM of D and 3×10^{-3} – 30 µM of CBZ). After 4 or 24 h of incubation, solutions were removed and replaced by the different reagent solutions according to the implemented method. For each assay, eight technical replicates and three biological replicates were used for each dilution (n=24).

2.8.2. Crystal violet assay

As adherent cells detach from cell culture plates during cell death, determination of attached cells can be used for the indirect quantification of cell death. A straightforward method to detect attached cells involves the crystal violet (CV) dye staining as the colorant binds to proteins and DNA (Kueng *et al.*, 1989). The CV assay is based on the protocol described by Gillies *et al.* (1983) with modifications from Gasparri *et al.* (2011) (Gasparri *et al.*, 2011; Gillies *et al.*, 1986). Following cell exposure of 4 or 24 h to CBZ, D or D-CBZ, the solutions were removed and replaced by 100 µL of CV solution at a final concentration of 0.1% w/v in a 1:4 methanol:water mixture. After 5 min of incubation in a humidity chamber, the CV solution was removed, and the insoluble crystals were dissolved in 100 µL of acetic acid 30% v/v. Absorbance at 530 nm was measured using a Cytation5 microplate reader (BioTek Instruments, USA), and is proportional to attached living cells. The absorbance obtained from cells incubated only with medium was used to set the 100% cell viability, and sample data was adjusted to this value.

2.8.3. MTT assay

MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan product by the succinate dehydrogenase enzyme within the cell mitochondria. The formazan product is impermeable to the cell membranes, and therefore it accumulates inside healthy cells (Fotakis & Timbrell, 2006). The MTT assay is based on the protocol described for the first time by Mosmann (1983) (Mosmann, 1983). Briefly, after 4 or 24 h of incubation with CBZ, D or D-CBZ, solutions were removed and replaced by 100 µL of MTT at a final concentration of 0.5 mg/mL. After 2 h of incubation, the MTT solution was removed, and the insoluble formazan crystals were dissolved in 100 µL of DMSO; absorbance at 570 nm was measured by using a Cytation5 microplate reader. Cells incubated only with medium were used to set the 100% metabolic activity, and sample data was adjusted to this value.

2.8.4. Neutral red uptake

Living cells take up the neutral red (NR) dye, which concentrates within the lysosomes of living cells (Fotakis & Timbrell, 2006). The NR assay is based on the protocol described by Borenfreund and Puerner (1984) (Borenfreund & Puerner, 1985). Briefly, following exposure of 4 or 24 h to CBZ, D or D-CBZ, the solutions were removed, and replaced by 100 µL of NR solution at a final concentration of 50 µg/mL in serum-free medium. After 2 h of incubation, the NR solution was removed and cells were incubated with 100 µL of 1% formaldehyde – 1% sodium carbonate mixture, to remove unincorporated dye and simultaneously promote adhesion of the cells to the substratum. The formaldehyde solution was removed, and cells were washed with 100 µL of saline solution (NaCl 0.9% w/v). Then, 100 µL of a mixture of 1% acetic acid – 50% ethanol was added to each well followed by gentle shaking for 10 min so that complete extract of NR was achieved. The absorbance at 540 nm was measured by using a Cytation5 microplate reader. Cells incubated only with medium were used to set the 100% NR uptake, and sample data was adjusted to this value.

2.9. *In vivo* biocompatibility: Zebrafish larvae

2.9.1. Animals

Adult zebrafish (*Danio rerio*) were maintained at 28.0 ± 1.0 °C in aquaria with a 14 h light − 10 h dark cycle, as described previously (Feas *et al.*, 2017; Igartúa *et al.*, 2015). Fishes were fed with dry flakes (TetraMin

PRO®) three times a day and nauplius larvae of Artemia once a day. The water in the aquarium was aerated and maintained at pH 7.0 - 8.0. In this study, embryos refer to zebrafish before hatching (0-3 day post-fecundation - dpf), while larvae refer to post-hatching animals (over 3 dpf) **(Figure S3).** Embryos were obtained from natural mating and were reared in E3 medium (NaCl 5 mM, KCl 0.17 mM, $CaCl_2$ 0.33 mM and $MgSO_4$ 0.33 mM in deionized water and 50 ppb methylene blue as a fungicide). Selected fertilized eggs in good condition were used for further treatment. The characteristics of the eggs were determined with a Nikon SMZ800 stereomicroscope (Nikon, USA.

2.9.2. Compliance with ethical standards

All zebrafish procedures were performed in strict accordance with the National Institute of Health guidelines for animal care and maintenance. The study protocols were approved by the Institutional Animal Care Committee of the National University of Quilmes (CE-UNQ 2/2014) (Buenos Aires, Argentina) and Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) (CICUAL-UNQ 013-15 and 014-15).

2.9.3. Treatment

For the treatment, three non-hatched zebrafish embryos at 1 dpf were placed in each well of a 96-well plate containing E3 medium and incubated for additional 4 days at 28 °C. At 5 dpf, the medium was replaced by 250 µL of 10-fold-serial dilutions prepared in the E3 medium of CBZ (0.3-30 µM) or D-CBZ complexes (0.012-1.2 µM D and 0.3-30 µM CBZ).

2.9.4. Viability

Viability was studied at 1, 4, 24 and 48 hours post-incubation (hpi) with a stereomicroscope. It was considered that the larvae were dead when no heartbeat was observed. Viability was expressed as a percentage of the live larvae respect to the total of larvae per treatment (n=72).

2.9.5. Neurotoxicity

The spontaneous movement was studied in a multichannel ADC system (WMicrotracker, Designplus SRL, Argentina) as previously described in Igartúa *et al.* (2015) (Igartúa *et al.*, 2015). Activity events were

recorded for 15 min at 1, 4, 24 and 48 hpi, at room temperature. The spontaneous movement was expressed as percentage respect to the movement in the non-treated larvae control. Changes in spontaneous locomotor activity events could reflect the neurotoxicity of the different treatments (Maria Jimena Prieto *et al.*, 2012; Selderslaghs *et al.*, 2013), as well as a morphological or lethal effect. For each assay, eight technical replicates and three biological replicates were used for each dilution (n=24).

2.9.6. Cardiotoxicity

The heart rate of zebrafish was assessed at 48 hpi (7 dpf). Control and experimental zebrafish larvae were individually transferred to a slide with sodium carboxymethylcellulose and placed under a trinocular stereomicroscope. The heart rate was determined by counting the number of beats every 15 s and expressed as beats per minute (bpm). Experiments were performed three times on eight larvae per group (n=24). The difference between the heart rates of control and treated larvae was regarded as cardiotoxicity (Berghmans *et al.*, 2008).

2.9.7. Morphological Changes

The larvae were photographed after 48 hpi (7 dpf) to determine morphological changes. Several morphological alterations as the bent spine, jaw malformation, opaque head region, small head, opaque liver, opaque yolk sac, yolk not depleted, uninflated swim bladder, edema and tail malformation were observed. Fish were scored based on the degree of morphological anomalies [0=no visible toxic effects; 1=minor, one to two morphological anomalies; 2=moderate, three to four effects; 3=severe, more than four minor toxic effects; and 4=dead] (Adaptation from (Yang *et al.*, 2014)). The mean toxicity score for each treatment was determined by the score of individual larvae. Experiments were performed in triplicate on eight larvae per group for each dilution (n=24).

2.10. Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Graph Pad Prism v6.0 software. ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test or by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-tests were used

depending on the obtained data. The different statistical analyses used are detailed within the figures´ captions. Differences were considered to be significant only when $p<0.05$.

3. Results and discussion

3.1. CBZ complexation to obtain DG4.0-CBZ and DG4.5-CBZ complexes

Dendrimers may increase the solubility and stability of bioactive compounds, and protect them from the biological environment. Solubilization of drugs mediated by dendrimers depends on the type, generation, size, core and functional groups of the dendrimer (Jain *et al.*, 2010) and also depends on pH, temperature, and hydrophobic or electrostatic interaction affinity between the drug and the dendrimer (Kolhe *et al.*, 2003). The PAMAM dendrimers generation 4.0 (DG4.0) and generation 4.5 (DG4.5) can encapsulate drug molecules into their hydrophobic pockets or anchor them on their external functional groups. The complexed drug would acquire the physicochemical properties of the dendrimers, which can significantly increase its solubility in an aqueous media and provide a controlled-release profile (D'emanuele & Attwood, 2005). In this sense, the drug carbamazepine (CBZ) is poorly soluble in water (120 µg/mL, 500 µM, at 25 °C), so complexation ability of different amounts of CBZ in DG4.0 and DG4.5 was studied to estimate the maximum number of molecules that can be incorporated into the dendrimers molecules **(Figure 1).** The studied molar ratios were 5, 10, 25, 50, 100, 150, and 200 moles of CBZ per mole of DG4.0 or DG4.5. When the molar ratio was higher than 10, the soluble CBZ present in D-CBZ formulations was always significantly higher than the CBZ present in the control of the free drug. This result confirms the interaction of CBZ with both DG4.0 and DG4.5. As no significant differences were observed between DG4.0 and DG4.5 dendrimers, we hypothesize that the drug-dendrimer interaction is through complexation inside the hydrophobic pockets of dendrimers, which are chemically identical and have similar size. At molar ratios greater than 50, the dendrimers saturation was achieved, and higher combination ratios were not associated with higher amounts of complexed CBZ. However, particularly at a molar ratio of 150, a tendency to a more considerable amount of complexed CBZ with DG4.5 was observed, so this molar ratio was used to confirm the complexation and to study the reproducibility of the protocol **(Table 1).** By complexing with dendrimers, the solubility of CBZ was significantly increased in aqueous media ($p<0.0001$)

by almost three times more. At this ratio, an average of 21±7 and 24±11 moles of CBZ per mole of DG4.0 and DG4.5, respectively, were calculated.

Figure 1. Optimization of D:CBZ molar ratio. Different amounts of CBZ were combined with a constant quantity of DG4.0 or DG4.5 in methanolic solution to obtain different D:CBZ molar ratios. **(A)** Concentration of soluble CBZ after complexation process. Results are shown as mean ± SD. Significant differences respect to free CBZ were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (**p<0.01, ****p< 0.0001). **(B)** Moles of CBZ per mole of DG4.0 or DG4.5 obtained after the complexation process. Data are shown as mean ± SD.

Table 1. D-CBZ complexation at 1:150 D:CBZ molar ratio (n=16).

	Soluble CBZ (μM)	CBZ moles per D mole
CBZ	322.2 ± 52.72	-
DG4.0-CBZ	834.8 ± 187.2 ****	21 ± 7
DG4.5-CBZ	898.7 ± 279.4 ****	24 ± 11

Results are shown as the mean ± SD. Significant differences respect to CBZ were analyzed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (****p< 0.0001). No statistical difference between CBZ moles per D mole was observed by T-test when DG4.0-CBZ or DG4.5-CBZ was compared.

3.2. DG4.0-CBZ and DG4.5-CBZ stability and resistance to lyophilization

The stability of the DG4.0-CBZ and DG4.5-CBZ complexes was evaluated at various conditions of temperature (4, 25 and 37 °C) keeping in the dark up to 90 days, and after lyophilization process, as can be seen in **Figure 2**. The formulations were found to be more stable at 37 °C as concluded by observing the physical appearance and by quantifying the soluble CBZ, which were significantly higher in DG4.0-CBZ and DG4.5-CBZ respect to free CBZ during the analyzed period of 90 days post-complexation (p<0.0001). The formulations were found to be stable at 25 °C only for seven days post-complexation and were non-stable at 4 °C. The release and crystallization of non-soluble CBZ were minimum at 37 °C and maximum at 4 °C. The observed results may be due to the shrinking of the dendrimer structure, which would lead to a decrease in the cavity entrapping the drug molecules, as was previously described (Bhadra *et al.*, 2003). Furthermore, as far as we know, the resistance to lyophilization was not tested for drug complexed in DG4.0 and DG4.5. We observed that the formulations were stable after the lyophilization process without additives. In all of the formulations, the concentration of CBZ was reduced by a ~25%, but the DG4.0-CBZ and DG4.5-CBZ complexes remained stable at 25 °C until 60 days post-lyophilization. The increase in the

stability of lyophilized respect to non-lyophilized complexes at 25 °C could be because during the lyophilization process the drug-dendrimer interactions are maximized.

Figure 2. Stability of D-CBZ complexes during storage and resistance to the lyophilization process. The time stability of free or complexed CBZ was studied after storage at 4, 25 or 37 °C, and after lyophilization. **(A)** Percentage of CBZ (respect to initial concentration) at different times after complexation and storage at 4, 25 or 37 °C. **(B)** Concentration of CBZ before and after the lyophilization process. **(C)** Percentage of CBZ (respect to initial concentration) at different times after lyophilization and storage at 25 °C. **(A-C)** In all of the cases, results are shown as the mean ± SD. Significant differences respect to CBZ were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*p<0.05; **p<0.01; ***p<0.001; ****p< 0.0001) **(Table S1)**.

3.3. D-CBZ interaction studied by Fourier transform infrared spectroscopy (FTIR) and *in vitro* release

As both DG4.0 and DG4.5 dendrimers encapsulated a similar amount of CBZ and presented similar profiles of stability over the time, we hypothesize that the drug-dendrimer interaction occurs into the hydrophobic pockets. These are chemically identical in both dendrimers whereas the terminal groups are different (–NH_3^+ for DG4.0 and –COO^- for DG4.5 at physiological pH). To confirm this hypothesis, the interaction between CBZ and dendrimers was studied using FTIR and *in vitro* release studies.

Infrared spectroscopy is a well-established nondestructive method for highly sensitive and selective concentration determination and identification of chemical species. FTIR works by exposing the sample to infrared radiation, and the infrared region of the spectrum is absorbed by the sample, so that each sample would have a characteristic set of absorption bands. The specific absorption of the substance in the “fingerprint” region enables the recognition of various chemical species and even of the structural isomers. FTIR spectrum of solid CBZ **(Figure 3A)** showed all the specific absorption bands of the drug (Milović *et al.*, 2012; Wu *et al.*, 2011), including the N-H stretching band at 3460 cm^{-1}, the –CO-R- vibration at 1670 cm^{-1}, and the –C=C-, -C=O and N-H deformation vibrations at 1590 cm^{-1}. FTIR spectra of lyophilized PBS (**Figure 3B and C)** was included as an internal control of our assay because the D-CBZ formulations were dissolved in this buffer before lyophilization and the buffer´s salts would remain in solid formulations. In this control, it was found that the absorption bands at 528, 548, 668, 861, 949, 1074, 1163, 2341 and 2359 cm^{-1} correspond to the vibrations of phosphate groups of buffer´s salts.

FTIR spectrum of DG4.0 **(Figure 3B)** showed the –CH and –NH stretching vibrations of the dendrimer core at 2917 y 2849 cm^{-1}; the –NH deformation vibration of the terminal groups at 1651 cm^{-1}; and the C-C stretching bonds inside the core at 1557 cm^{-1} (Kolhe *et al.*, 2003; Popescu *et al.*, 2006; Prajapati *et al.*, 2009; M. Prieto *et al.*, 2006). In the FTIR spectrum of DG4.0-CBZ, the disappearance of the absorption bands at 2917 and 2849 cm^{-1} and the displacement of another band from 1651 to 1692 cm^{-1} were observed. Since the first two signals correspond to internal groups of DG4.0 and the third to the terminal amine groups, CBZ molecules could be interacting both with the hydrophobic pockets of the DG4.0 and with their terminal amine groups.

On the other hand, FTIR spectrum of DG4.5 **(Figure 3C)** showed the –NH flexion vibration of amides of dendrimer core at 3299 cm^{-1} and at 1642 cm^{-1}; the –CH and –NH stretching vibrations of the dendrimer core at 2915 y 2849 cm^{-1}; the -C=O stretching vibration of –COOH terminal groups at 1730 cm^{-1}; and the –COO symmetric vibration of terminal group at 1393 cm^{-1} (M. Prieto *et al.*, 2006; Maria Jimena Prieto *et al.*, 2014). In the FTIR spectrum of DG4.5-CBZ, the disappearance of the absorption bands at 2915, 2849 and 1730 cm^{-1} and the displacement of the bands to 3332 and 1652 cm^{-1} were observed. As these signals correspond to the internal and terminal groups of the DG4.5, the CBZ molecules could be interacting both with the hydrophobic pockets of the DG4.5 and with their terminal carboxylic groups.

It is important to remember that in the D-CBZ formulations obtained from the complexation protocol there is the free CBZ (up to the limit of saturation) and the CBZ complexed in the dendrimers (drug solubilized by complexation into the hydrophobic pockets of the dendrimers). When these D-CBZ formulations are lyophilized, the interactions between the CBZ (both free and complexed) and the dendrimers are maximized, due to the evaporation of the solvent.

As all the absorption bands corresponding to the groups of the hydrophobic pockets change in the D-CBZ formulations concerning the free dendrimers, we can confirm our hypothesis that the drug interacts with the hydrophobic pockets of both nanocarriers. As there are also some changes in the signals corresponding to the terminal groups of both DG4.0 and DG4.5, it is not possible to rule out that the CBZ interaction with them due to the proximity during the lyophilization process.

Figure 3. Interaction between CBZ and DG4.0 or DG4.5 by FTIR analysis. (A) FTIR spectrum of CBZ powder; **(B)** FTIR spectra of lyophilized DG4.0, DG4.0-CBZ, and PBS control**; (C)** FTIR spectra of lyophilized DG4.5, DG4.5-CBZ, and PBS control. **(B-C)** Shifts or disappearance of the maximums observed in the spectrum of dendrimers respect to

D-CBZ complexes are included into the study because the complexes and dendrimers were lyophilized from buffer suspension, and phosphate salts have a strong signal in FTIR.

The strength and stability of the drug-dendrimer complex were also studied using *in vitro* release assays of CBZ from DG4.0-CBZ and DG4.5-CBZ against PBS at physiological pH 7.4 **(Figure 4)** and extreme pHs of 2.2 and 12.6 **(Figure S4)**. CBZ is a molecule with hydrophobic rings and a polar region due to the presence of an amide group. Under non-catalyzing conditions and at extreme basic pHs, CBZ can form negatively charged amide (pKa of amide group 13.9) (Röhricht *et al.*, 2009). In all the pHs used in this work, the CBZ can be found as an uncharged molecule. Moreover, DG4.0 present primary amino groups as terminal ends with pKa 8-10 and internal tertiary amines with pKa 4-6; while DG4.5 present carboxylic acids as terminal ends with pKa 4-5 and internal tertiary amines with pKa 3-6 (Devarakonda *et al.*, 2007; Maiti *et al.*, 2005; Maiti *et al.*, 2004). Therefore, both dendrimers are macromolecules whose degree of ionization and the conformational state will depend on the pH.

At acid pH (2.2) **(Figure S4 A)** the internal tertiary amines of both dendrimers would be protonated; this excessive positive charge would lead to an extended conformation, preventing the formation of hydrophobic pockets inside the dendrimers. Moreover, it was previously reported that at pH<4.0, the dendrimer size increased almost 30-40% (Maiti *et al.*, 2005; Maiti *et al.*, 2004). These structural changes lead to the complete release of the CBZ from D-CBZ complexes.

At basic pH (12.6) **(Figure S4 B)** the internal tertiary amines of both dendrimers would be deprotonated, and the end groups $-NH_2$ of the DG4.0 would also be deprotonated; this absence of charges would give rise to a compact globular conformation (Maria Jimena Prieto *et al.*, 2013). However, Maiti *et al*. (2005) found that at pH>12.0 and in the presence of a solvent such as water, the size of the dendrimer was increased by almost 10-15% (Maiti *et al.*, 2005). The same conformational change can be expected for the DG4.5 considering that their terminal groups $-COO^-$ would be negatively charged. Under these conditions, CBZ was completely released from both DG4.0-CBZ and DG4.5-CBZ complexes.

At physiological pH (7.4) **(Figure 4)** the internal tertiary amines of both dendrimers would be deprotonated forming hydrophobic pockets, while the end groups would have positive charges $-NH_3^+$ for DG4.0 and negative charges $-COO^-$ for DG4.5. As it was previously described, at neutral pH, there was no significant change in the dendrimer size (Maiti *et al.*, 2005; Maiti *et al.*, 2004). Under these conditions, the CBZ molecules could interact hydrophobically or by hydrogen bonding with the core of the dendrimers, which

results in a controlled release over time. After 2 h of dialysis, the free CBZ was released by 70%, while the D-CBZ by 45% ($p<0.001$). Subsequently, after 24 h of dialysis, the free CBZ was completely released (100%), while the D-CBZ complexes still retained 40% of the drug ($p<0.0001$). Consistent with our results, the drug retention capacity of both dendrimers under physiological conditions had previously been observed for the drug risperidone (Maria Jimena Prieto *et al.*, 2014; María Jimena Prieto *et al.*, 2011) and ibuprofen (Kolhe *et al.*, 2003). Moreover, it was also found that the interaction between dendrimers and drugs is strongly dependent on pH (Beezer *et al.*, 2003; Devarakonda *et al.*, 2007).

Figure 4. *In vitro* release of CBZ from D-CBZ complexes in physiological pH 7.4. The *in vitro* release of CBZ from D-CBZ complexes was studied against buffer solution with pH 7.4 by using a *micro-dialysis Eppendorf tube diffusion* technique developed *ad hoc*. Results are shown as mean ± SD. Significant differences respect to CBZ were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p< 0.0001$) **(Table S2)**.

3.4. *Ex vivo* toxicity: Hemolysis assay and human red blood cells morphological changes

Red blood cell hemolysis and morphological changes study are simple methods widely used to analyze polymer-membrane interaction and *ex vivo* toxicity (Duncan & Izzo, 2005; Temprana *et al.*, 2017). Despite hemolysis caused by cationic and anionic dendrimers is reported to be generation- and concentration-dependent, non-hemolysis is found at low dendrimer concentration (<1 mg/mL) (Asthana *et al.*, 2005; Bhadra *et al.*, 2003; Malik *et al.*, 2000; María Jimena Prieto *et al.*, 2011). However, it is of importance to test the hemolytic effect of the complexes as it has been reported that CBZ can induce hemolytic anemia (Sobotka *et al.*, 1990; Stroink *et al.*, 1984; Yamamoto *et al.*, 2007). In this work, we evaluated the hemolytic effects **(Figure 5)** and possible morphological changes **(Figure S5)** after 4- and 24-h human red blood cells incubation with free CBZ, dendrimers and D-CBZ complexes. No significant hemolysis or morphological changes were observed when compared with red blood cells incubated with isotonic PBS buffer (negative control) at any incubation time.

Figure 5. *Ex vivo* toxicity studied by hemolysis in human red blood cells. Hemolysis after 4- or 24-h treatments with CBZ, dendrimers or D-CBZ complexes. Hemolysis is expressed as a percentage of the hemoglobin release induced by the SDS positive control (100% hemolysis). Results are shown as mean ± SD. Significant differences

respect to PBS negative control was performed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. There were no significant differences for any of the formulations at the tested concentrations.

3.5. *In vitro* toxicity: Cytotoxicity in N2a cell culture

Regardless of the extensive pharmaceutical and biomedical applications of dendrimers, it is reported that dendrimer cytotoxicity might restrict their use as drug delivery systems (Jain *et al.*, 2010; María Jimena Prieto *et al.*, 2011). Thus, it is imperative to evaluate the cytotoxicity of dendrimers themselves and the obtained complexes. The cytotoxicity of the dendrimers is dependent on the kind of the core, but mainly on the nature of its surface groups (Duncan & Izzo, 2005). Also, the cytotoxicity is found to be concentration- and time-dependent in different cell lines (Duncan & Izzo, 2005; Jain *et al.*, 2010). DG4.0 are cationic dendrimers at physiological pH that can cause more cytotoxic effects than negative dendrimers DG4.5. For example, on Vero and J774 cell lines, DG4.0 presented cytotoxicity at 3 µM concentration after 24 h (María Jimena Prieto *et al.*, 2011) whereas DG4.5 presented no cytotoxicity up to 33 µM (M. Prieto *et al.*, 2006). The observations reported are due to cationic dendrimers´ ability to interact with the negatively charged plasma membrane, generating destabilization and cell membrane nanoholes, thus resulting in cell lysis (Jain *et al.*, 2010).

While most chemical compounds cause cellular damage through interaction with specific biomolecules, nanoparticles can cause toxicity through a combination of different mechanisms such as inducing ROS, genotoxicity, morphological modifications and immunological effects at the same time (Joris *et al.*, 2013). So, it is important to study the effect of dendrimers and complexes in cell culture by several methods simultaneously (Fotakis & Timbrell, 2006). In the present work, we studied the effects of CBZ, dendrimers, and D-CBZ complexes in N2a cell line after 4- and 24-h treatments on viability by the crystal violet (CV) staining; on cellular metabolic activity by the MTT assay; and on lysosomal activity by the neutral red (NR) uptake **(Figure 6 and S6)**. After 4 h **(Figure S6),** no reduction in N2a cell viability (CV staining) or effects on metabolic activity (MTT assay) were observed for any of the treatments and the concentrations tested. However, 1.2 µM of DG4.0 caused a reduction of ~20% in the uptake of NR, which might be related to the toxic effect of cationic dendrimers on cells membranes, particularly in the lysosomal membrane. After 24-h treatments **(Figure 6)**, results of the three assays were not in accordance, which can be explained by the fact that each test analyzes different parameters involved in cellular toxicity. The MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria, whereas the NR assay is a colorimetric

assay measuring the uptake of the dye by functional lysosomes, and CV staining measured the uptake of the dye by attached and viable cells (Fotakis & Timbrell, 2006). The CV staining revealed that 0.03 and 0.003 µM of CBZ reduced cell viability by 40% and 30% respectively. The DG4.5-CBZ complex prevented this cytotoxic effect, although the DG4.0-CBZ did not. Regarding the metabolic activity studied by the MTT assay, none of the formulations in the evaluated concentrations produced cytotoxic effects. However, cytotoxicity measured as reduction of NR uptake was observed at the higher concentration of free dendrimers and D-CBZ complexes. This effect on lysosomes produced by dendrimers was previously described (Mukherjee *et al.*, 2010; J. Zhang *et al.*, 2016). As expected, the toxic effect was more significant for DG4.0 than for DG4.5 and it was reflected in shorter times.

In conclusion, the use of different concentrations, treatment-times, and methods to study the toxic effects in the N2a cells, allowed to demonstrate that the DG4.5-CBZ complexes presented reduced toxicity concerning the other formulation (DG4.0-CBZ) or the free CBZ.

Figure 6. *In vitro* toxicity studied in N2a cell culture by colorimetric methods. Cytotoxicity measured as viability by the crystal violet (CV) staining, metabolic activity by the MTT assay, and lysosomal activity by the neutral red (NR) uptake at 24 hours post-incubation (hpi) with CBZ, dendrimers or D-CBZ complexes. All data are expressed as the percentage respect to non-treated cells control, which was considered as 100% of viability, 100% metabolic activity or 100% neutral red uptake. Results are shown as the mean ± SD. Statistics were performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significantly different respect to the control (**p<0.01; ***p<0.001; ****p< 0.0001).

3.6. *In vivo* biocompatibility: Zebrafish larvae

While the term “toxicity” is referring to the non-specific and undesired effects of a drug or nanoparticle, the term “biocompatibility” is wider and could be referring to the ability to be in contact with a living system without producing an adverse effect (Vert *et al.*, 2012). Hence, it is critical to study the effect of the obtained complexes in a living organism. Particularly, in this work, we chose to work with Zebrafish (*Danio rerio*) which is an animal model that offers whole-animal information, which cannot be obtained from *in vitro* studies. Furthermore, we used zebrafish larvae as a rapid, high-throughput, cost-effective model to provide a more comprehensive and predictive screening of biocompatibility of the free CBZ and D-CBZ complexes. The effect on the viability **(Figure 7A)**, spontaneous swimming activity (**Figure 7B),** morphological

development **(Figure 7C**), and heart rate (**Figure 7D)** of zebrafish larvae after treatment with CBZ or D-CBZ were studied. Neither free CBZ nor DG4.5-CBZ complexes produced mortality, malformations or changes in heart rate or spontaneous movement at the evaluated concentrations. On the other hand, when high concentrations of the DG4.0-CBZ complex (30 µM CBZ encapsulated in 1.2 µM of DG4.0) were administered, only 68 and 57% of viability was observed after 4- and 24-h treatments, respectively. At 48-h treatments, the viability remained at 57%. In addition, at this dose, significant morphological changes were also observed **(Figure 8)** such as uninflated swim bladder, opaque yolk sac, and yolk not depleted. The tendency in the reduction of the movement and the heartbeat are directly related to the lethal effect of the DG4.0 and not with neurological or cardiological effects indeed. In non-lethal doses, complexes did not cause any other effects. Results are consistent with those obtained by Calienni *et al*. (2017), in which free DG4.0 were lethal for zebrafish larvae in concentrations higher than 0.5 µM (Calienni *et al.*, 2017).

Figure 7. ***In vivo*** **toxicity studied in zebrafish larvae. (A)** Viability of zebrafish larvae at 7 dpf after a 48-h treatment with CBZ or D-CBZ complexes. Viability is expressed as the percentage of the live larvae respect to the total of larvae, and data are shown as mean ± SD. Statistics were performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significantly different respect to CBZ (***p<0.001). **(B)** Spontaneous movement in zebrafish larvae after 1-, 4-, 24- and 48-h treatment with CBZ or D-CBZ complexes. Spontaneous movement is expressed as the percentage respect to movement in the non-treated larvae control, and results are shown as mean ± SD. None significant differences respect to CBZ was found after analysis by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. **(C)** Morphological changes scored based on the degree of anomalies of zebrafish larvae at 7 dpf after 48-h treatment with CBZ or D-CBZ complexes. Statistics were performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significantly different respect to CBZ (****p<0.0001). **(D)** Heart rate of zebrafish larvae at 7 dpf after 48-h treatment with CBZ or D-CBZ. Heart rate is expressed as the percentage of heartbeat in treated larvae respect to the heartbeat in the non-treated larvae control, and data are shown as mean ± SD. Significant differences respect to CBZ was performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (****p<0.0001).

Figure 8. ***In vivo*** **morphological changes studied in zebrafish larvae**. Representative photographs of live 7-dpf zebrafish larvae after a 48-h treatment with 30 µM of CBZ or D-CBZ complexes. Significant morphological changes are indicated with arrows.

Taking into account these results to those previously obtained *ex vivo* and *in vitro*, we can conclude that the DG4.5-CBZ complexes were non-toxic and biocompatible in the concentrations tested. These complexes are presented as an alternative to the delivery of CBZ, a water-insoluble drug which has been approved for the treatment of epilepsy and shows the potential for the development of new therapies against neurodegenerative diseases.

4. Conclusions and perspectives

A stable drug delivery system based on the PAMAM dendrimer generation 4.5 capable of efficiently complex the antiepileptic drug CBZ has been developed. Not only the drug solubility was increased by using DG4.5 but also a controlled release profile was achieved observing that a 40% of the CBZ was retained after 28-h dialysis. Moreover, drug cytotoxicity was reduced when CBZ was complexed within this dendrimer. Furthermore, the complex DG4.5-CBZ did not have a hemolytic activity or induced morphological changes in human red blood cells and presented high *in vivo* biocompatibility as no neurotoxicity, cardiotoxicity or malformations were observed in the zebrafish model.

Considering all mentioned above, the DG4.5-CBZ could be incorporated into clinical trials, leading to an improvement of the already established treatment of epilepsy. Moreover, this work could be considered a starting point to study new treatments for other neurodegenerative diseases. In line with this idea, further experiments will be carried out to explore the ability of the DG4.5-CBZ complexes in reducing abnormal aggregation of β-amyloid proteins in cell culture and mammalian models. Finally, our work highlights the advantage of using negatively charged PAMAM dendrimers when designing stable and non-toxic drug delivery systems.

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Declaration of interest

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Figure Legends

Figure 1. Optimization of D:CBZ molar ratio. Different amounts of CBZ were combined with a constant quantity of DG4.0 or DG4.5 in methanolic solution to obtain different D:CBZ molar ratios. **(A)** Concentration of soluble CBZ after complexation process. Results are shown as mean ± SD. Significant differences respect to free CBZ were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (**p<0.01, ****p< 0.0001). **(B)** Moles of CBZ per mole of DG4.0 or DG4.5 obtained after the complexation process. Data are shown as mean ± SD.

Figure 2. Stability of D-CBZ complexes during storage and resistance to the lyophilization process. The time stability of free or complexed CBZ was studied after storage at 4, 25 or 37 °C, and after lyophilization. **(A)** Percentage of CBZ (respect to initial concentration) at different times after complexation and storage at 4, 25 or 37 °C. **(B)** Concentration of CBZ before and after the lyophilization process. **(C)** Percentage of CBZ (respect to initial concentration) at different times after lyophilization and storage at 25 °C. **(A-C)** In all of the cases, results are shown as the mean ± SD. Significant differences respect to CBZ were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*p<0.05; **p<0.01; ***p<0.001; ****p< 0.0001) **(Table S1)**.

Figure 3. Interaction between CBZ and DG4.0 or DG4.5 by FTIR analysis. (A) FTIR spectrum of CBZ powder; **(B)** FTIR spectra of lyophilized DG4.0, DG4.0-CBZ, and PBS control**; (C)** FTIR spectra of lyophilized DG4.5, DG4.5-CBZ, and PBS control. **(B-C)** Shifts or disappearance of the maximums observed in the spectrum of dendrimers respect to D-CBZ complexes are included into the study because the complexes and dendrimers were lyophilized from buffer suspension, and phosphate salts have a strong signal in FTIR.

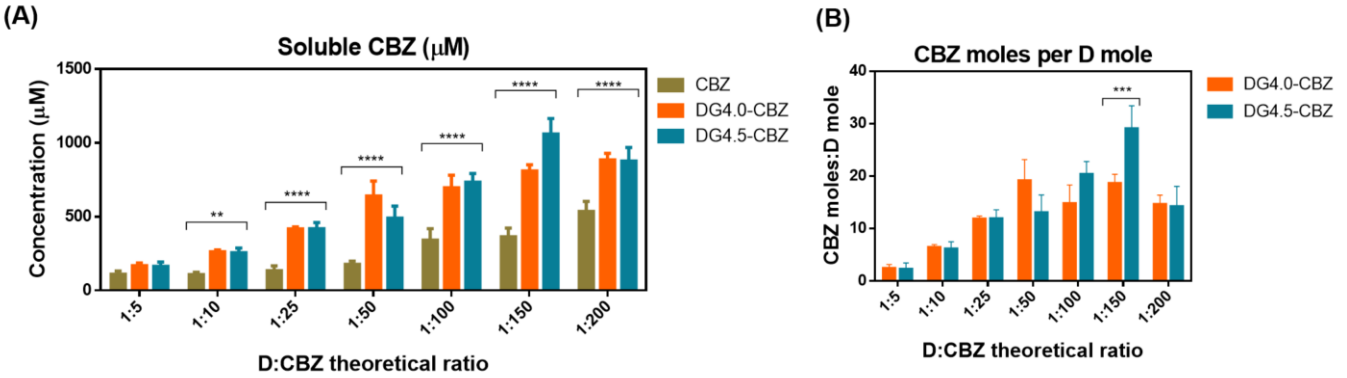
Figure 4. *In vitro* release of CBZ from D-CBZ complexes in physiological pH 7.4. The *in vitro* release of CBZ from D-CBZ complexes was studied against buffer solution with pH 7.4 by using a *micro-dialysis Eppendorf tube diffusion* technique developed *ad hoc*. Results are shown as mean ± SD. Significant differences respect to CBZ were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*p<0.05; **p<0.01; ***p<0.001; ****p< 0.0001) **(Table S2)**.

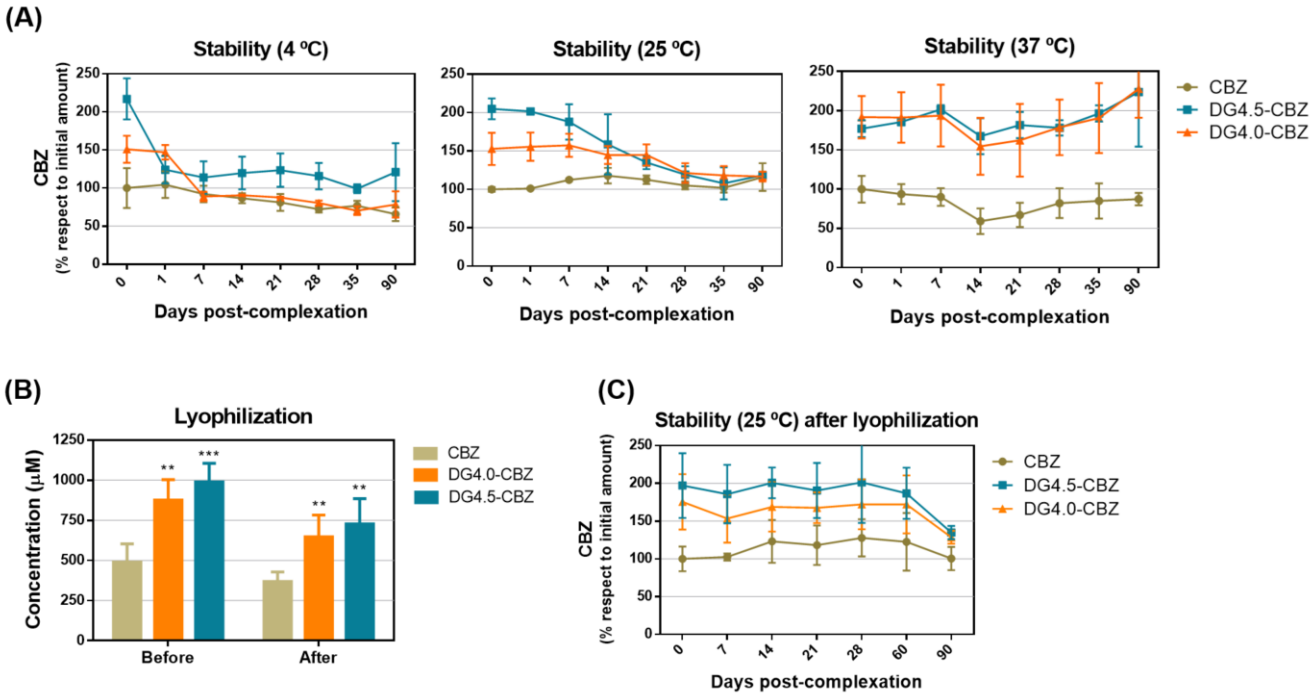
Figure 5. *Ex vivo* toxicity studied by hemolysis in human red blood cells. Hemolysis after 4- or 24-h treatments with CBZ, dendrimers or D-CBZ complexes. Hemolysis is expressed as a percentage of the hemoglobin release induced by the SDS positive control (100% hemolysis). Results are shown as mean ± SD. Significant differences respect to PBS negative control was performed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. There were no significant differences for any of the formulations at the tested concentrations.

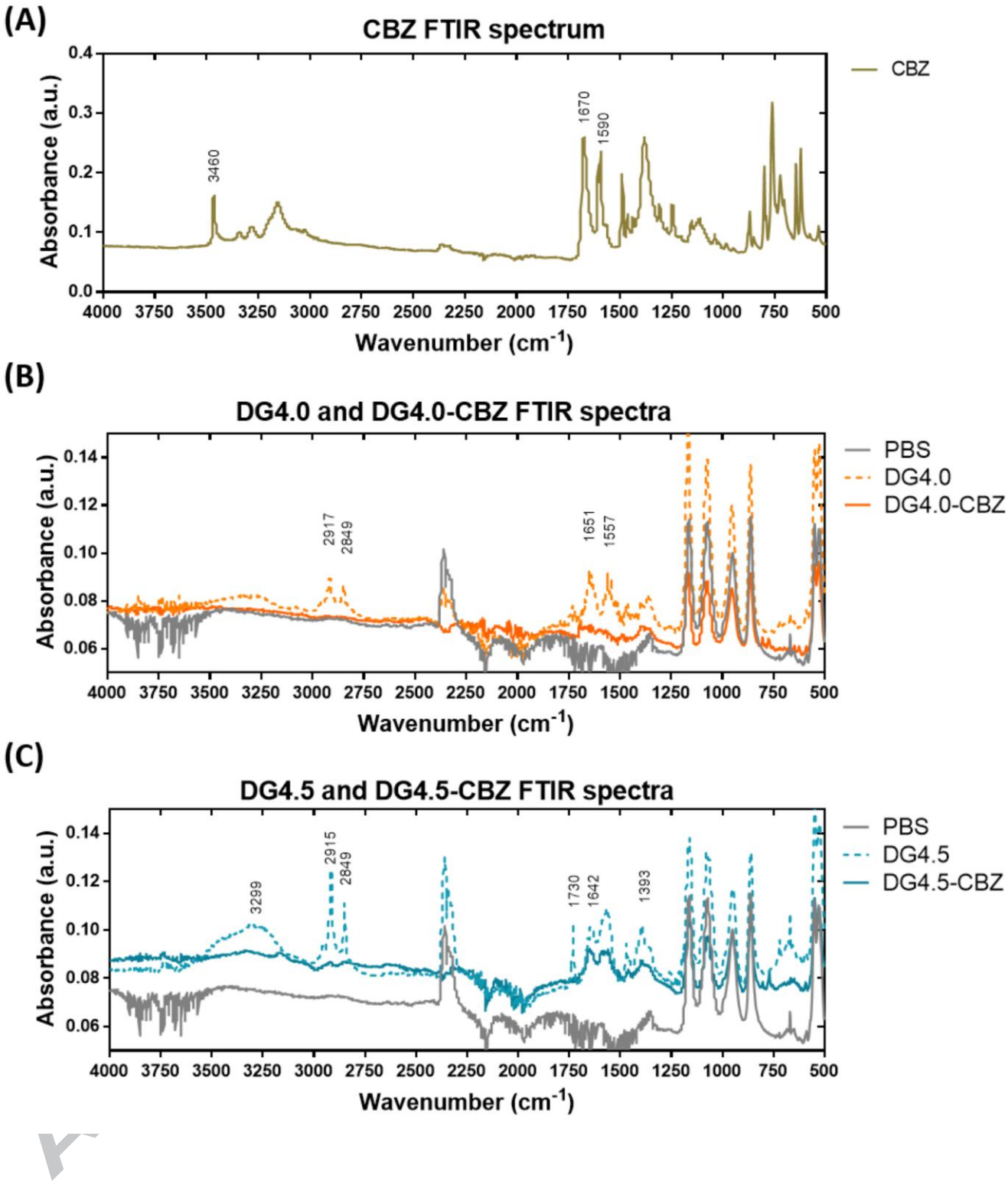
Figure 6. *In vitro* toxicity studied in N2a cell culture by colorimetric methods. Cytotoxicity measured as viability by the crystal violet (CV) staining, metabolic activity by the MTT assay, and lysosomal activity by the neutral red (NR) uptake at 24 hours post-incubation (hpi) with CBZ, dendrimers or D-CBZ complexes. All data are expressed as the percentage respect to non-treated cells control, which was considered as 100% of viability, 100% metabolic activity or 100% neutral red uptake. Results are shown as the mean ± SD. Statistics were performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significantly different respect to the control (**p<0.01; ***p<0.001; ****p< 0.0001).

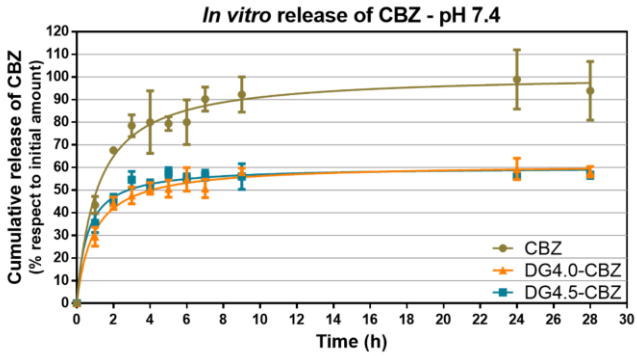
Figure 7. ***In vivo*** **toxicity studied in zebrafish larvae. (A)** Viability of zebrafish larvae at 7 dpf after a 48-h treatment with CBZ or D-CBZ complexes. Viability is expressed as the percentage of the live larvae respect to the total of larvae, and data are shown as mean ± SD. Statistics were performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significantly different respect to CBZ (***p<0.001). **(B)** Spontaneous movement in zebrafish larvae after 1-, 4-, 24- and 48-h treatment with CBZ or D-CBZ complexes. Spontaneous movement is expressed as the percentage respect to movement in the non-treated larvae control, and results are shown as mean ± SD. None significant differences respect to CBZ was found after analysis by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. **(C)** Morphological changes scored based on the degree of anomalies of zebrafish larvae at 7 dpf after 48-h treatment with CBZ or D-CBZ complexes. Statistics were performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significantly different respect to CBZ (****p<0.0001). **(D)** Heart rate of zebrafish larvae at 7 dpf after 48-h treatment with CBZ or D-CBZ. Heart rate is expressed as the percentage of heartbeat in treated larvae respect to the heartbeat in the non-treated larvae control, and data are shown as mean ± SD. Significant differences respect to CBZ was performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (****p<0.0001).

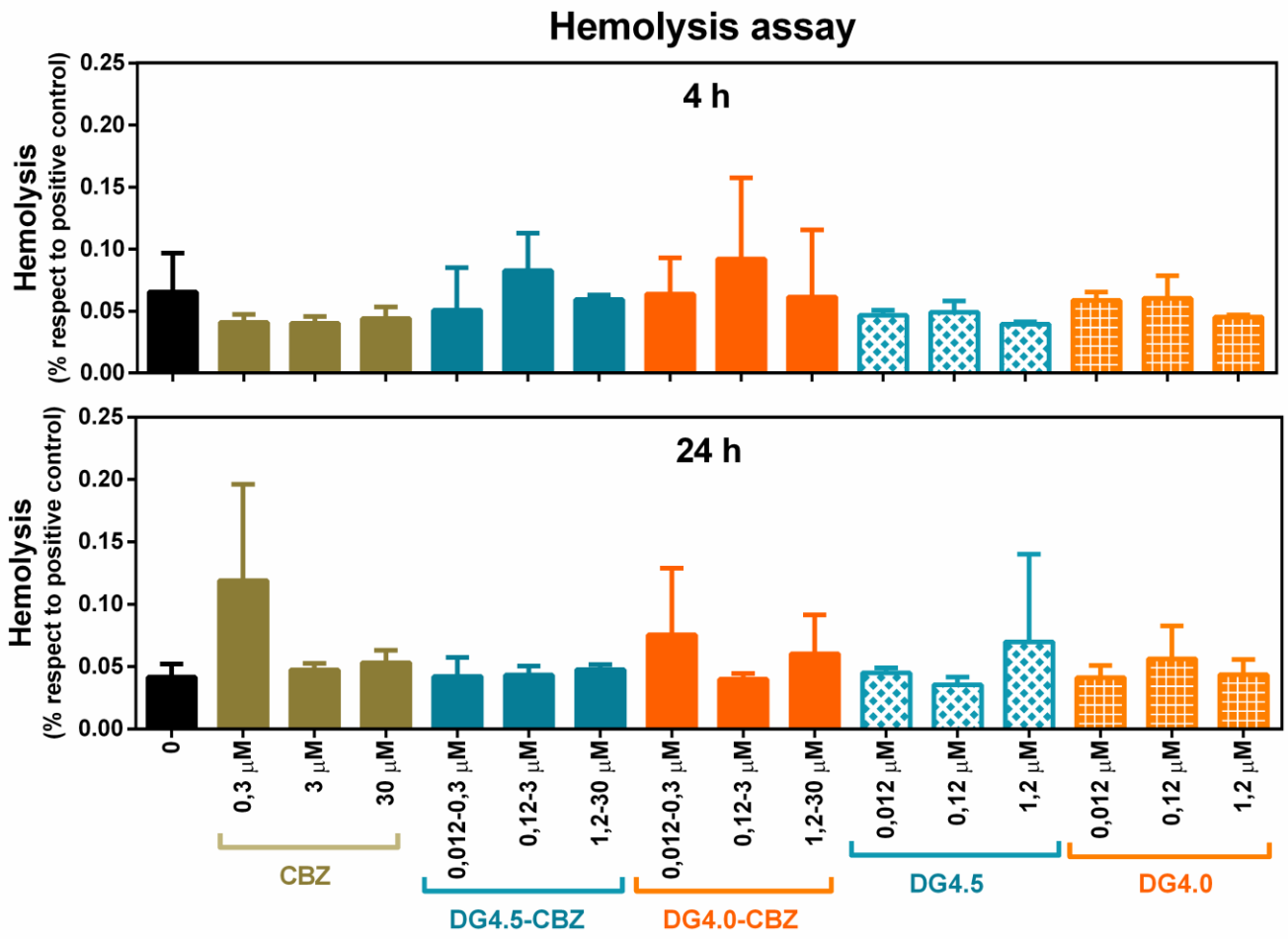
Figure 8. ***In vivo*** **morphological changes studied in zebrafish larvae**. Representative photographs of live 7-dpf zebrafish larvae after a 48-h treatment with 30 µM of CBZ or D-CBZ complexes. Significant morphological changes are indicated with arrows.

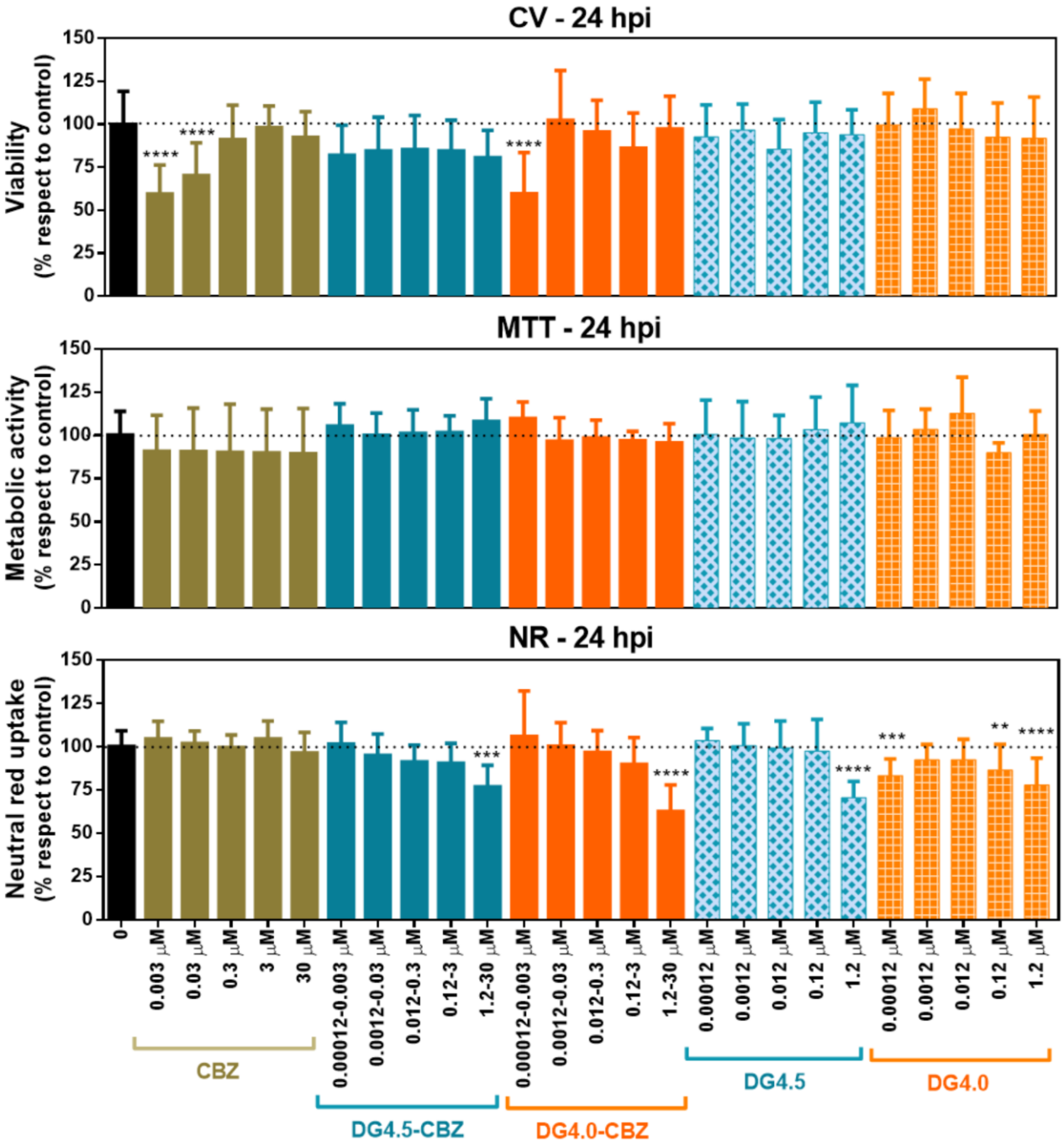


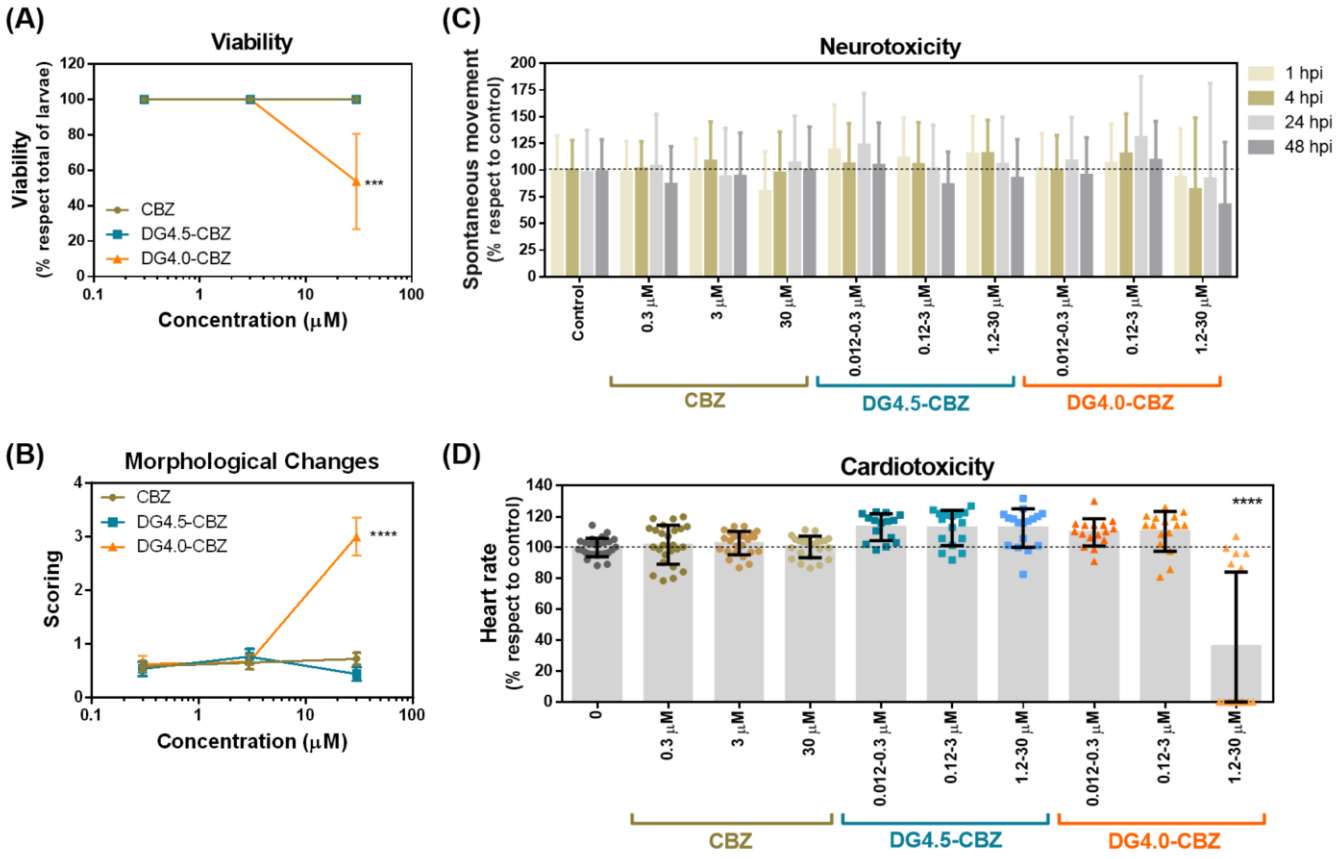



(A)
CBZ FTIR spectrum
Absorbance (a.u.)
Wavenumber (cm-1)
3460
1670
1590
CBZ
(B)
DG4.0 and DG4.0-CBZ FTIR spectra
Absorbance (a.u.)
Wavenumber (cm-1)
2917
2849
1651
1557
PBS
DG4.0
DG4.0-CBZ
(C)
DG4.5 and DG4.5-CBZ FTIR spectra
Absorbance (a.u.)
Wavenumber (cm-1)
3299
2915
2849
1730
1642
1393
PBS
DG4.5
DG4.5-CBZ






CV - 24 hpi
Viability (% respect to control)
MTT - 24 hpi
Metabolic activity (% respect to control)
NR - 24 hpi
Neutral red uptake (% respect to control)
0
0.003 µM
0.03 µM
0.3 µM
3 µM
30 µM
0.00012-0.003 µM
0.0012-0.03 µM
0.012-0.3 µM
0.12-3 µM
1.2-30 µM
0.00012-0.003 µM
0.0012-0.03 µM
0.012-0.3 µM
0.12-3 µM
1.2-30 µM
0.00012 µM
0.0012 µM
0.012 µM
0.12 µM
1.2 µM
0.00012 µM
0.0012 µM
0.012 µM
0.12 µM
1.2 µM
CBZ
DG4.5-CBZ
DG4.0-CBZ
DG4.5
DG4.0



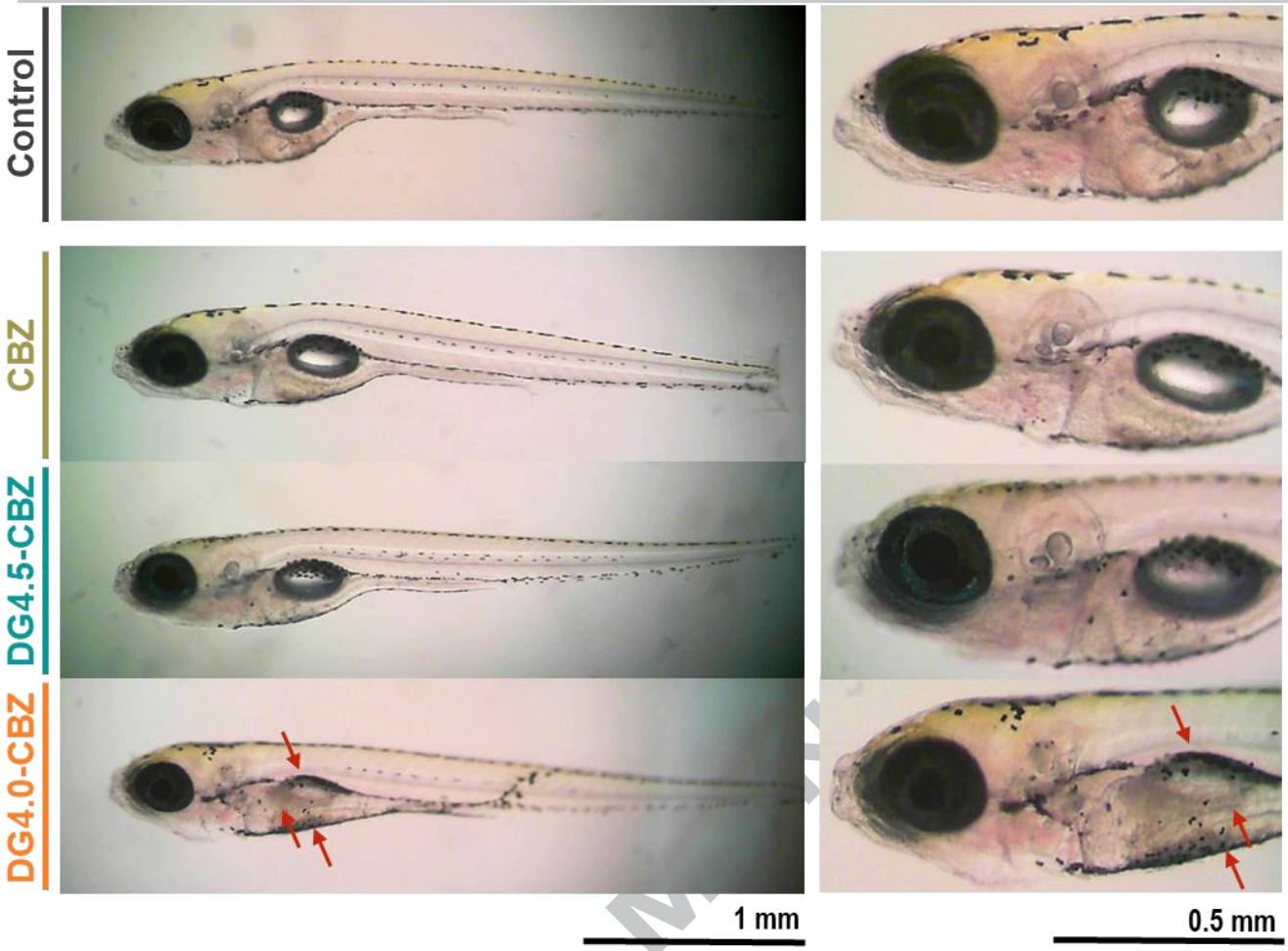


Table 1. D-CBZ complexation at 1:150 D:CBZ molar ratio (n=16).

	Soluble CBZ (μM)	CBZ moles per D mole
CBZ	322.2 ± 52.72	-
DG4.0-CBZ	834.8 ± 187.2 ****	21 ± 7
DG4.5-CBZ	898.7 ± 279.4 ****	24 ± 11

Results are shown as the mean ± SD. Significant differences respect to CBZ were analyzed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (****p< 0.0001). No statistical difference between CBZ moles per D mole was observed by T-test when DG4.0-CBZ or DG4.5-CBZ was compared.