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Brain and muscle of Wistar rats are the main targets of intravenous dendrimeric sulfadiazine

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ABSTRACT

Cytotoxicity of sulfadiazine (SDZ) complexed with PAMAM dendrimers of fourth generation (SDZ–DG4) determined by MTT assay and LDH leakage, was reduced on *covered* (with mucins) but not on *nude* (without mucins) Caco-2 cell line. SDZ–DG4 adsorption and uptake on *nude* and *covered* Caco-2 cells, determined by flow cytometry and fluorescence confocal microscopy indicated that positively charged DG4 remained electrostatically attracted to the negatively charged mucins macromolecules. Hence, the *in vivo* accession of cationic dendrimers to epithelial cells could partly be impaired by their entrapment into mucins. This fact could account for an *in vivo* decreased cytotoxicity. Besides this finding, when orally administered to Wistar rats, no differences in SDZ biodistribution were found between SDZ–DG4 and free SDZ. However, when intravenously administered at 1.5 mg SDZ per kg body weight, C_{max} for free SDZ was 0.7 ± 0.2 μg/ml vs. 2.7 ± 0.4 μg/ml for SDZ–DG4, whereas AUC_{0-3} for free SDZ was 0.8 ± 0.6 μg/h ml vs. 5.2 ± 2 μg/h ml for SDZ–DG4. SDZ–DG4 initial volume distribution (V_d) was 2.6-fold lower than for free SDZ. Remarkably, 3 h upon SDZ–DG4 administration, SDZ concentration in muscle and in brain were 17- and 10-fold higher, respectively, than those achieved with free SDZ.

1. Introduction

Toxoplasmosis, a zoonotic infection caused by the *Toxoplasma gondii* parasite is calculated to affect one-third of total population of the planet (Tenter et al., 2000; Dubey, 2004). *T. gondii's* eggs, oocysts and cysts resist adverse temperature, desiccation, natural irradiation, chemicals and disinfectants commonly useful in virus and bacteria control (Gajadhar et al., 2006). A recent study places toxoplasmosis as one of the 325 lethal zoonotic infections transmitted at worldwide scale by tap water (Karanis et al., 2007).

T. gondii is the most common cause of congenital neurological defects in humans and is a devastating opportunistic disease in immuno-compromised patients (Hu et al., 2006; Jones et al., 2006; Carruthers and Suzuki, 2007). Mostly as a result of high doses of sulfadiazine (SDZ) that in combination with pyrimethamine are currently employed in conventional treatment, a high percentage of cases presents severe collateral effects, including dermatological and hematological anomalies (Haverkos, 1987; Leport et al., 1988) and renal failure (Hyvernat et al., 2006; de la Prada Alvarez et al., 2007). Because of this, frequently the therapy has to be interrupted. The limited efficacy of the conventional treatment to eradicate the cysts (Couvreur et al., 1991; Araujo et al., 1993; Djurkovic-Djakovic et al., 2000) could probably arise from impaired accession of therapeutic amounts to the intracellular cysts, protected by a complex wall structure. Even today, the lack of tolerable, effective and cheap drugs against *T. gondii* make imperative the search and development of new therapeutic agents (Lavine and Arrizabalaga, 2007). Those agents should take into consideration that the problem of *T. gondii* parasites survival during therapy and subsequent reactivation could be ascribed to the limited anti-bradizoite activity of the drugs, but also to limitations for the drugs to cross the cyst walls and/or to achieve the brain (Petersen et al., 2001). In our working hypothesis, the small size, high surface activity and capability for interacting with biological membranes of dendrimers (D) could make them effective nano-drug delivery system (*nanoDDS*) of anti-toxoplasmic drugs. We have previously prepared complexes of SDZ with cationic (G4) and anionic (G4.5) polyamidoamine (PAMAM) D, at 40- and 10-SDZ molecules per DG4 and DG4.5 molecules, respectively. SDZ–DG4.5 was nontoxic for fibroblasts and macrophages cell cultures up to 33 μM of D concentration, whereas SDZ–DG4 resulted toxic from 3.3 μM. However, 0.03 μM SDZ–DG4 was sufficient to cause a decrease of 60% on the experimental infection of Vero cells with *T. gondii* upon 4 h incubation (Prieto et al., 2006). Remarkably, such SDZ concentration was 10^9-fold lower than free

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SDZ IC_{50} upon 72 h incubation on experimental infected cells (Duval and Leport, 2001).

In this work, the toxicity and adsorption/uptake of G4- and G4.5-PAMAM D and their SDZ–D complexes on fibroblast, macrophages and intestinal epithelial cells *nude* or *covered* with mucins, in culture, were studied. Finally, pharmacokinetics and biodistribution of free SDZ and SDZ–D administered by oral and intravenous route to Wistar rats were determined.

2. Materials and methods

2.1. Materials

Poly(amidoamine) (PAMAM) dendrimer G4 (PAMAM–G4–NH_2) (molecular weight = 14,215 g/mol, 64 amine end groups) (DG4) and PAMAM–G4.5–COOH (molecular weight = 26,258 g/mol, 128 carboxylate end groups) (DG4.5) were purchased from Sigma–Aldrich, Argentina. SDZ 99.0% purity, fluorescein isothiocyanate isomer I (FITC), sodium 3-(4,5-dimethythiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), benzoylated dialysis membrane and porcine stomach mucin type III were also from Sigma–Aldrich, Argentina. Streptomycin/Penicillin/Anfotericin, sodium pyruvate, RPMI 1640 and MEM with non-essential amino acids (MEM-NEAA) were purchased from Invitrogen Corporation. Foetal calf serum was from Bioser Gen SA, Argentina. L-Glutamine, trypsin and EDTA were provided by PAA Laboratories GmbH, Austria. Heparine 25,000 UI/5 ml was from Northia. Tetra-*n*-butylammonium hydrogen sulfate synthesis grade was from Merck. Acetonitrile was HPLC grade from Carlo Erba. Tris buffer and all the other reactive used were analytical grade from Anedra, Argentina.

2.2. Complexation of SDZ in dendrimers

Complex formation between SDZ and D was carried out according to Prieto et al. (2006). Briefly, DG4 and DG4.5 were combined with SDZ in methanolic solution at 35:1 SDZ:D molar ratio. The mixtures were incubated for 45 h at room temperature (20 °C) and methanol was evaporated in a Speed Vac at 25 °C (1010 SAVANT). The resultant solid residues were dissolved in 0.1 ml of Tris buffer 10 mM pH 7.5 plus NaCl 0.9% p/v (Tris buffer) at room temperature and centrifuged at 10,000 × *g* for 5 min, in order to separate the SDZ–dendrimer (SDZ–D) complexes (soluble SDZ) from non-incorporated, free SDZ (insoluble).

The amount of SDZ complexed to D was quantified by HPLC followed by UV detection as described by Batzias (Batzias et al., 2002), after dilution of SDZ–D in mobile phase.

2.3. Toxicity of SDZ–D complexes

2.3.1. Haemolysis assay and morphological changes on red blood cells

Haemolysis of SDZ–D complexes was assayed as described by (Duncan et al., 1992). Freshly prepared human red blood cells (400 μl) were incubated at 37 °C with 67 μl of both SDZ–D at two concentrations (1×10^{-3} mM SDZ–0.03 μM D and 1 mM SDZ–33 μM D). After 4 or 24 h incubation, samples were centrifuged at 1500 × *g* for 10 min and absorbance of the supernatant at 550 nm was measured in a spectrophotometer Shimadzu UV-160A. Haemolysis was express as percentage of haemoglobin release induced by Triton X-100 (1%, v/v).

Additionally, morphological changes on red blood cells upon incubations were determined by optical microscopy. Briefly, after incubation cells were mounted on a slip, stained with May Grundwald–Giemsa and observed on an Alphaphot-2, YS2 Nikon microscopy.

2.3.2. Cytotoxicity on culture cells

Cytotoxicity of D and SDZ–D, measured as lactate dehydrogenase (LDH) leakage in culture supernatants, was determined on Vero and J-774 cells. Cytotoxicity was also determined on Caco-2 cells, in the presence and absence of mucins by LDH leakage and MTT assay.

Vero and J-774 cells were routinely cultured in RPMI-1640 medium, Caco-2 cells were cultured in MEM-NEAA with 2 mM L-glutamine and 1% pyruvate, both mediums were supplemented with 10% foetal calf serum and 1% Streptomycin/Penicillin/Anfotericin and cells were grown at 37 °C, 5% CO_2 and 95% humidity.

Vero and J-774 cells were seeded at 5×10^4 cells/well in 96-well flat bottom microplates and maintained under cultured conditions. After 24 h, the culture medium was replaced by 100 μl of medium containing SDZ (1×10^{-3} and 1 mM), both D at two concentrations (0.03 and 33 μM) or the respectives SDZ–D (1×10^{-3} mM SDZ–0.03 μM D and 1 mM SDZ–33 μM D). Upon 24 h incubation at 37 °C, supernatants were transferred to fresh tubes, centrifuged 250 × *g* for 4 min and LDH content was measured using lactate dehydrogenase CytoTox Kit (Promega) (Korzeniewski and Callewaert, 1983). LDH concentration was expressed as percentage LDH release relative to treatment with the detergent Triton-X 100 and then percentage of viability was calculated considering the LDH leakage of cells grown in medium.

Caco-2 cells were seeded at 5.10^4 cells/well in a 96-well plate and maintained under culture conditions. After 48 h, medium was replaced with fresh medium without or with 1 and 5 mg/ml of mucins (from a stock solution of 25 mg/ml mucin, 140 mM NaCl and 5 mM KCl pH 2 (Jin et al., 2006)). Upon 15 min of incubation at 37 °C, both D at three concentration levels (0.03, 3.3 and 33 μM) and the respectives SDZ–D (1×10^{-3} mM SDZ–0.03 μM D, 1×10^{-1} mM SDZ–3.3 μM D and 1 mM SDZ–33 μM D) were added. After 24 h incubation at 37 °C, supernatants were transferred to fresh tubes and processed for LDH measurement as previously described. Cells attached to plates were processed for MTT assay, adding 100 μl of 0.5 mg/ml MTT in medium. After 3 h incubation, MTT solution was removed, the insoluble formazan crystals were dissolved with 100 μl of dimethylsulfoxide (DMSO) and absorbance was measured at 570 nm using a microplate reader. Viability of cells was express as percentage of the viability of cells grown in medium.

2.4. Dendrimers–cell interaction

2.4.1. Flow cytometry

D and SDZ–D were labeled with FITC according to (Kolhe et al., 2003). FITC labelling of D resulted an average of four molecules of FITC per DG4 or SDZ–DG4 molecule and 11 molecules of FITC per DG4.5 or SDZ–DG4.5 molecule.

Fibroblasts (Vero cells), macrophages (J-774 cells) and Caco-2 cells were plated in 6-well culture dishes and maintained under culture conditions until 80% confluent. Vero and J-774 medium was replaced by fresh medium containing 0.33 μM of D–FITC, and Caco-2 cells were pre-incubated 15 min with fresh medium without or with 5 mg/ml of mucins, prior to add both D–FITC. All cells were incubated 4 h at 37 °C in the dark; then supernatant was removed, cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. Half of the cells were incubated with trypan blue (0.1%, p/v in PBS) and the remainder with PBS at 37 °C for 10 min. Upon incubation, cells were washed three times with PBS and fixed in 1% formaldehyde solution at 4 °C. Cells were washed and suspended in PBS, and then introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm argon ion laser. Data were analyzed using WinMDI 2.9 software.

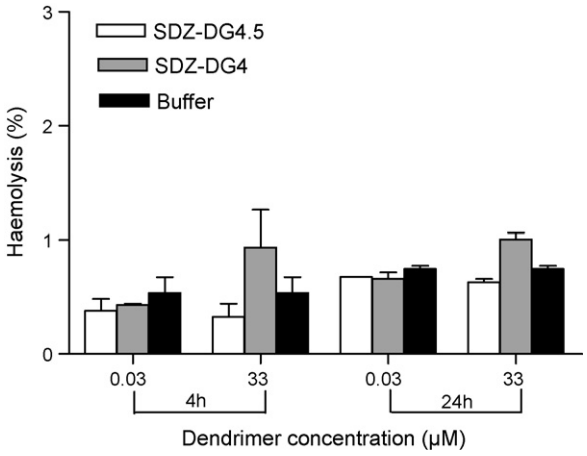

Fig. 1. Haemolysis of SDZ–D complexes as function of concentration and incubation time at 37 °C. Each data point represents the mean ± S.D. ($n = 3$).

2.4.2. Confocal microscopy

Caco-2 cells were seeded in 24-well plates with rounded coverslips on the bottom. Upon 48 h incubation at 37 °C, the medium was removed and replaced with fresh medium without or with 5 mg/ml of mucins. Upon 15 min at 37 °C, both D–FITC were added at 0.33 μM final concentration. After 4 h incubation, cells were washed with PBS, fixed with methanol for 10 min and the emission of FITC was monitored with a confocal laser microscopy Olympus FV300 with an Ar 488 nm laser.

2.5. Pharmacokinetics and biodistribution

Wistar rats (180–250 g body weight) received a single dose of both SDZ–D or free SDZ (dissolved in Tris buffer), by the oral, or the intravenous (i.v.) route as a bolus via the lateral tail vein. Three groups of seven rats each received i.v. 0.14 μmol D–1.5 mg SDZ as SDZ–DG4, 0.2 μmol D–0.5 mg SDZ as SDZ–DG4.5 and 1.5 mg of free SDZ. Three groups of five rats were fasted for 12 h and then received orally 0.8 μmol D–9.3 mg SDZ as SDZ–DG4, 1.2 μmol D–3.1 mg SDZ as SDZ–DG4.5 and 7.8 mg of free SDZ. All doses were expressed per kilogram body weight.

Blood samples were collected from the retroorbital sinus at predeterminate times on heparinized tubes. Plasma was obtained by blood centrifugation at 2000 × *g* for 5 min and was stored at −20 °C for SDZ extraction and quantization. Animals were kept in separate metabolic cages during the experiment, for urine collection. Immediately after sacrifice liver, kidney, muscle and brain were collected, washed, weighed and stored at −80 °C.

SDZ was extracted from plasma and tissues, and quantified with slight modifications as described by Batzias (Batzias et al., 2002), by a liquid–liquid extraction followed by HPLC separation and UV detection. Briefly, 0.1 ml of plasma was extracted by the addition of 0.2 ml of acetronitrile. After 5 min of centrifugation at 2000 × *g*, supernatant was transferred to fresh tubes, and mixed with 40 μl of phosphate buffer pH 6.8 and 1 ml of dichloromethane. Upon 10 min of centrifugation at 2000 × *g*, 1 ml of the bottom phase was filtered trough a 0.22 μm nylon membrane and evaporated in a Speed Vac. Finally, the residues were reconstituted with 0.1 ml of mobile phase and 10 μl was injected to the column.

A portion of solid tissue was thawed and homogenized with a potter. 500 mg of tissues were extracted by the addition of 1 ml of acetonitrile. The process was completed following the same steps as described for plasma using fivefold more solvent volume, and finally 10 μl was injected to the column.

Quantization of SDZ in plasma and tissues was based on the peak area–concentration response of HPLC calibration curves performed on a Gibson HPLC instrument, equipped with a reverse phase Luna 5 μm, C18 column (250 mm × 4.6 mm i.d., Phenomenex). The mobile phase used was acetonitrile–phosphate buffer (20:80, v/v) containing 20 mM potassium dihydrogenphosphate, 10 mM disodium hydrogenphosphate and 2.5 mM tetrabutylammonium hydrogen sulfate, as competing base, pH adjusted to 3.5 with phosphoric acid, filtered through 0.22 μm and degassed by vacuum. Elution was performed isocratically at a flow rate of 0.9 ml/min, detection was set at 270 nm and the peak area was determined by Gilson Unipoint software 2.3.

A five-point calibration curve ranging from 0.06 to 60 μg/ml SDZ was prepared by successive dilution of SDZ standard solution in Tris buffer with the mobile phase. Quantization of SDZ in plasma and tissues was carried out by reference to corresponding standard curves and multiplying by the appropriate recovery factor. Recovery factors were calculated from the peak area resulted from plasma or liver spiked with SDZ at two levels 9.6 and 35 μg/ml.

2.6. Pharmacokinetic and statistical analysis

The plasma pharmacokinetic parameters were calculated using GraphPad Prism® 4.00 (Graphpad Software Corporation). Pharmacokinetic data were express as mean ± S.D. and analyzed for statistical significance by one-way ANOVA, followed by Bonferroni's test.

3. Results

3.1. Toxicity

3.1.1. Haemolysis and morphological changes of red blood cells

We had previously shown the absence of toxicity up to 33 μM for SDZ–DG4.5 and 3.3 μM for SDZ–DG4, on fibroblasts and macrophages upon 4 h incubation (Prieto et al., 2006). Haemolysis caused by cationic and anionic PAMAM D is reported to be generation and concentration-dependent, but in general nonhaemolysis is found at less than 1 mg/ml (70 μM for DG4) (Malik et al., 2000), meanwhile cationic PAMAM G4 produces red blood cell aggregation from 10 μM (Domanski et al., 2004).

In this case, prior to SDZ–D i.v. administration, haemolytic activity and possible induction of morphological changes on red blood cells were surveyed upon 4 and 24 h incubation at 0.03 and 33 μM. Our results indicated that, independently of incubation time and

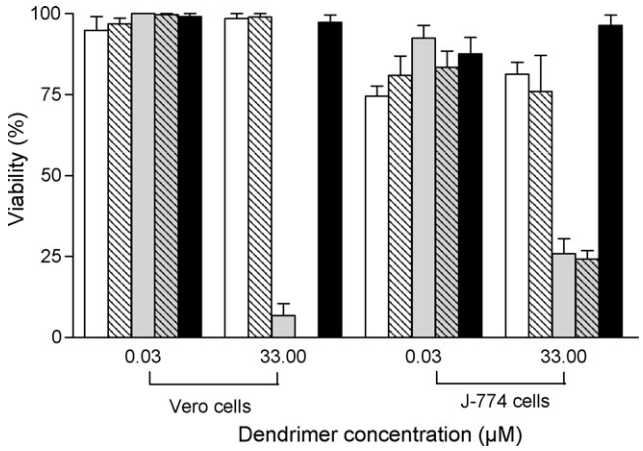

Fig. 2. Cytotoxicity of D (□ DG4.5, ▭ DG4), SDZ–D (▨ SDZ–DG4.5, ▩ SDZ–DG4) and SDZ (■) on Vero and J-774 cells upon 24 h incubation, measure by LDH leakage.

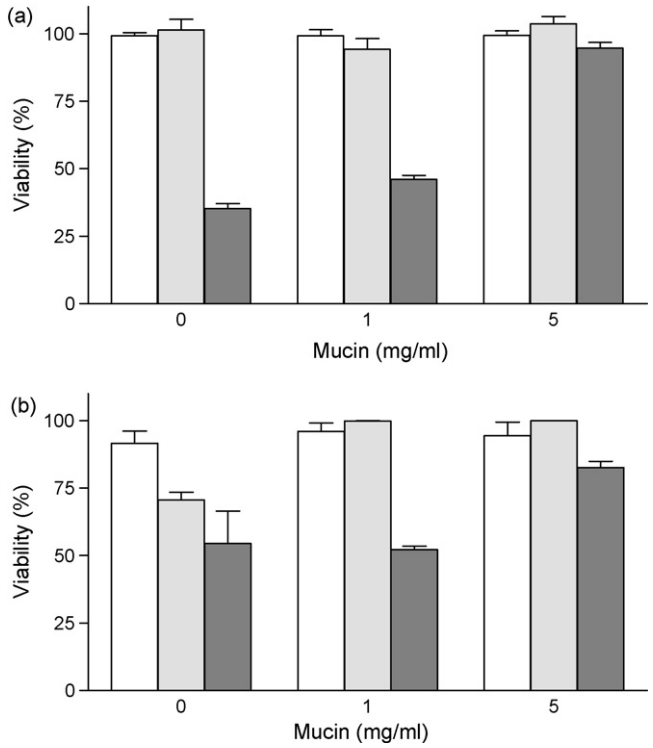

Fig. 3. Cytotoxicity of DG4 (□ 0.03, ▨ 3.3 and ■ 33 μM) on Caco-2 cells as function of mucins concentration, measure by: (a) MTT assay and (b) LDH leakage. Each data point represents the mean ± S.D. ($n = 3$).

D type, nor significant haemolysis, neither morphological changes were observed for both SDZ–D tested concentrations, as compared with red cells incubated in isotonic buffer (Fig. 1). However, slight aggregation of red blood cells was observed after 24 h incubation with 33 μM of SDZ–DG4.

3.1.2. Cytotoxicity

The effect of D and SDZ–D on cell membrane integrity of fibroblasts (Vero cells) and of macrophages (J-774 cells) was determined by LDH leakage. Neither DG4.5 nor SDZ–DG4.5-induced LDH leakage on Vero cells upon 24 h incubation, but produced a 25% LDH basal release on J-774 cells, at the two tested concentrations (Fig. 2). On the other hand, DG4 and SDZ–DG4 did not induce release of LDH at 0.03 μM, but caused 100% and 75% leakage of LDH on Vero and J-774 cells, respectively, upon 24 h incubation at 33 μM (Fig. 2).

Cytotoxicity of D and SDZ–D was also determined on the human intestinal adenocarcinoma cell line Caco-2, in the absence (*nude* Caco-2 cells) and presence of mucins (*covered* Caco-2 cells) by MTT assay and LDH leakage.

Jin et al. (2006) proposes that 5 mg/ml is a suitable mucin concentration to proportionate a physiological environment for a Caco-2 cell monolayer. In this work, we formerly screened the effect of 3, 5 or 10 mg/ml mucins onto Caco-2 cells upon 24 h incubation, to find that mucins themselves did not cause toxicity, both by MTT and LDH leakage (data not shown). We performed then, toxicity test for SDZ–D, in the absence or presence of low- and high-mucin concentration (1 and 5 mg/ml, respectively). The results showed that DG4.5 and SDZ–DG4.5 did not reduce viability of Caco-2 cells by MTT assay or by LDH leakage over the tested concentrations, independently of the presence of mucins (data not shown). On the other hand, DG4 and SDZ–DG4 did not reduce the viability, mea-

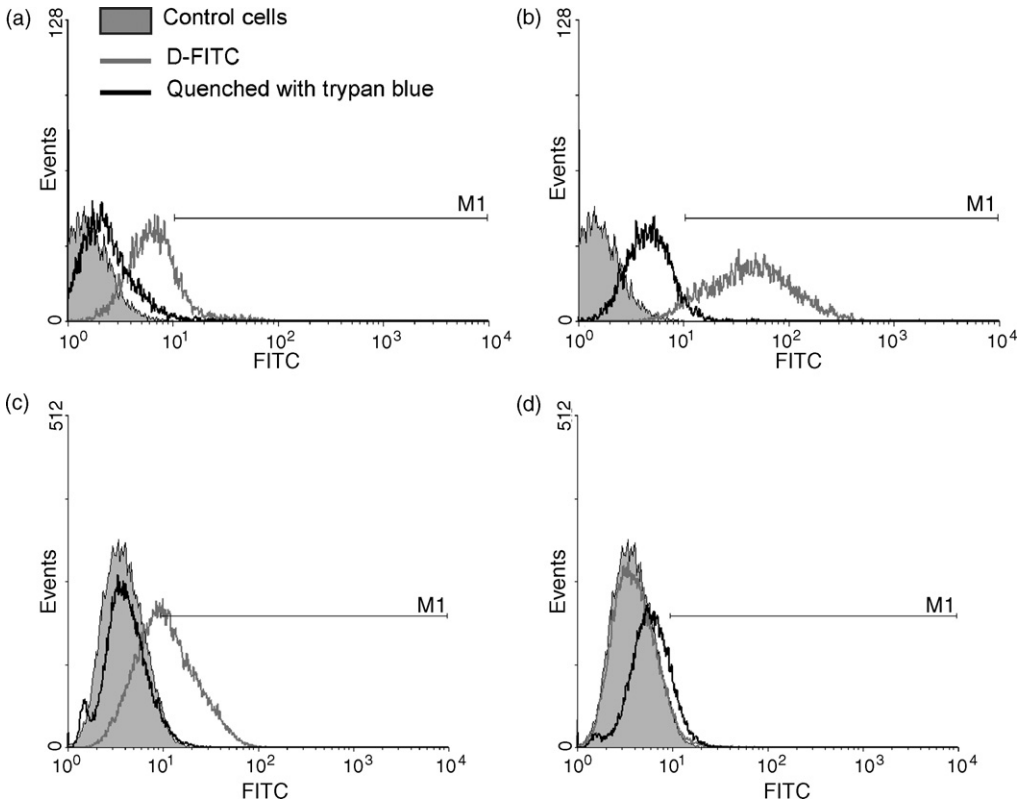

Fig. 4. Flow cytometry histograms of *nude* Caco-2 cells (a and b) and *covered* Caco-2 cells with 5 mg/ml of mucins (c and d) upon 4 h incubation with DG4.5-FITC (a and c) or DG4-FITC (b and d), without trypan blue (D–FITC) or quenched with trypan blue. The marker M1 defines the region of FITC fluorescence. The data presented are the mean fluorescent signals for 10,000 cells.

sured by MTT at 0.03 and 3.3 μM, but produced 25% leakage of LDH in *nude* Caco-2 cells upon 24 h incubation at 3.3 μM; however, 1 mg/ml of mucins was sufficient to impair the LDH leakage (Fig. 3a and b). Likewise, Caco-2 viability was significantly reduced (70% measured by MTT and 50% by LDH leakage), upon incubation with DG4 at 33 μM either both in *nude* and in *covered* with 1 mg/ml of mucins. However, on *covered* Caco-2 cells with 5 mg/ml of mucins DG4 did no reduce viability by MTT, and only 15% leakage of LDH was registered (Fig. 3a and b).

3.2. SDZ–D adsorption and uptake by flow cytometry and confocal microscopy

Trypan blue is a quencher of FITC fluorescence that is excluded from viable cells. In this study, it was employed as described by Jevprasesphant et al. (2004) to distinguish between D–FITC that is bound to the cell surface and D–FITC that has been internalized by different cell types.

Quenching the fluorescence of DG4.5-FITC bound to cell surface with trypan blue produced a 50 ± 16, 75 ± 6 and 30 ± 18% reduction of J-774, Vero and *nude* Caco-2 fluorescent cells (this last showed in Fig. 4a), respectively. Hence, after 4 h incubation, 50% of the DG4.5-FITC was captured by J-774 cells while a 25% and 70%, of DG4.5-FITC was captured by Vero and *nude* Caco-2 cells, respectively. Quenching the fluorescence of DG4-FITC bound to cell surface with trypan blue resulted in 75 ± 15% fluorescence reduction on the three cell types, indicating that 25% of the DG4-FITC was captured by all cells (Fig. 4b).

On the other hand, the fluorescence of DG4.5-FITC incubated with 5 mg/ml mucins *covered* Caco-2 cells disappeared after quenching with trypan blue, meaning that DG4.5 was all adsorbed on the cells surface (Fig. 4c). *Covered* Caco-2 cells incubated with DG4-FITC showed no fluorescence at all even in the absence of trypan blue (Fig. 4d).

Nude Caco-2 cells showed both surface fluorescence and internal points of fluorescence upon 4 h incubation with DG4.5-FITC, as revealed by 0.5 μm sequential images taken along the *z*-axis by confocal microscopy (Fig. 5a). The same pattern of fluorescence was observed on *covered* Caco-2 cells (Fig. 5b). On the other hand, the endocytic points observed upon 4 h incubation of *nude* Caco-2 cells with DG4-FITC (Fig. 5c), where absent after incubation with *covered* Caco-2 cells (Fig. 5d).

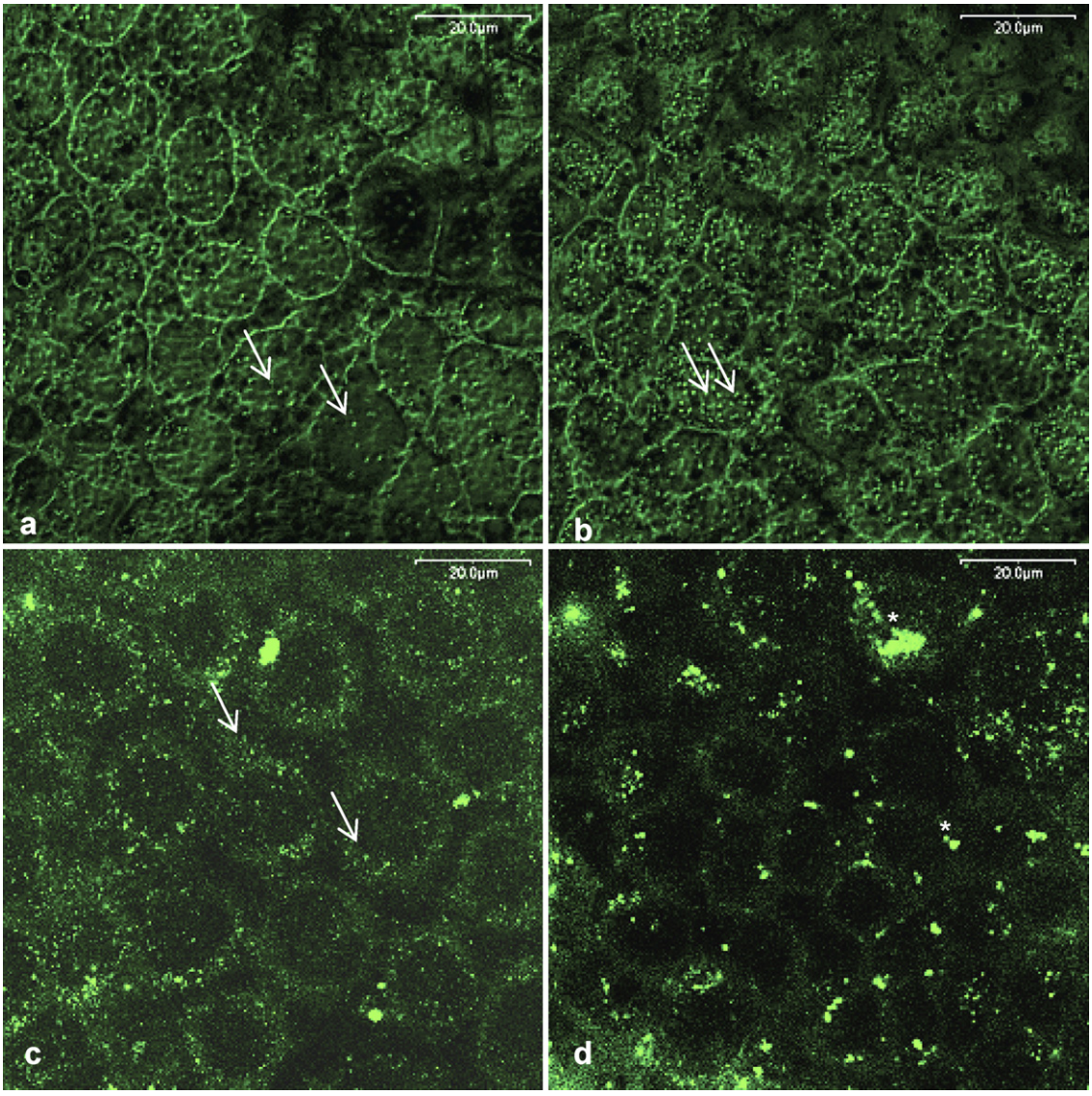

Fig. 5. Confocal scanning microscopy images of *nude* Caco-2 cells (a and c) and *covered* Caco-2 cells with 5 mg/ml of mucins (b and d) upon 4 h incubation with DG4.5-FITC (a and b) or DG4-FITC (c and d), *z*-axis deep 2.5 μm. Arrows show points of fluorescence, *shows DG4-FITC and mucins aggregates.

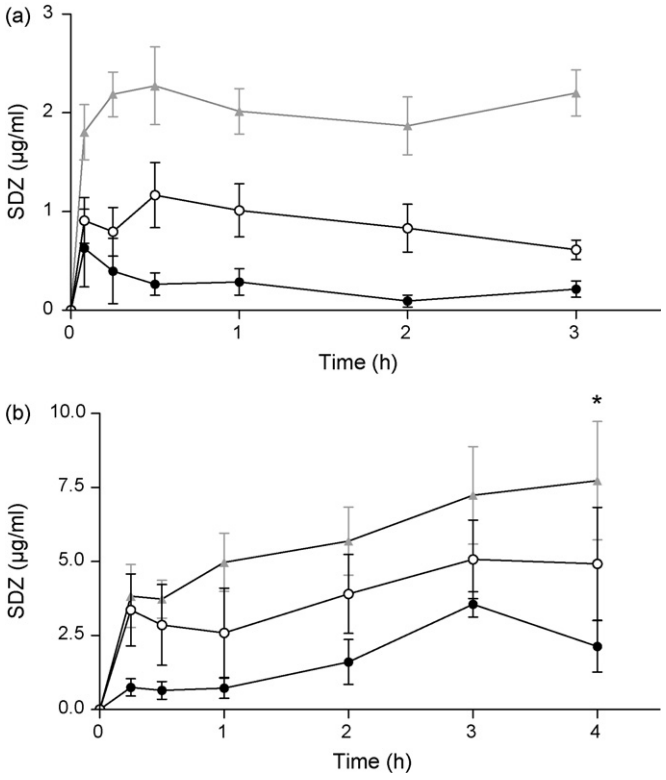

Fig. 6. Plasma SDZ concentration vs. time profile upon SDZ, SDZ–DG4 and SDZ–DG4.5 administration: (a) i.v. bolus and (b) oral. Each data point represents the mean ± S.D. ($n = 7$). $^{*}p < 0.01$.

No differences between adsorption and uptake of D–FITC and their respectives SDZ–D–FITC were observed by flow cytometry or confocal fluorescence microscopy.

3.3. Pharmacokinetics and biodistribution

Retention time of SDZ was 7.05 ± 0.12 min, and calibration curves resulted linear in the concentration range of 0.06–60 μg/ml, with a correlation coefficient of 0.9900. The extraction recovery was 20% and 10% with coefficient of variation of 10% and 15%, for plasma and liver, respectively. Doses of both SDZ–D were selected with the aim of administering similar number of moles of DG4 and DG4.5.

Though SDZ–DG4 provoked slight aggregation at 33 μM after 24 h incubation, there was not significant haemolysis or morphological changes. For the i.v. route, the administrations were nearly 3.5 μM D, nearly one order of concentration below those observed to cause no haemolysis.

SDZ concentration vs. time profile in plasma, after i.v. administration as single bolus, is shown in Fig. 6a. Along the three first hours, the plasma concentration of SDZ was significantly higher for both SDZ–D than for free SDZ. As a consequence, maximum plasma concentration (C_{max}) was 4- and 2.3-fold higher for SDZ–DG4 (2.7 ± 0.4 μg/ml) and SDZ–DG4.5 (1.6 ± 0.8 μg/ml) respectively, than for free SDZ (0.7 ± 0.2 μg/ml). Area under the curve from 0 to 3 h (AUC_{0-3}) was 5.2 ± 2 and 2.5 ± 1.3 μg/h ml, for both SDZ–D, resulting 6.3- and 3-fold higher than the corresponding of free SDZ (0.8 ± 0.6 μg/h ml). Initial volume distribution (V_d) was 2.6- and 4.4-fold lower for SDZ–DG4 and SDZ–DG4.5, respectively, than for free SDZ.

SDZ concentration in muscle was 17- and 7-fold higher for SDZ–DG4 and SDZ–DG4.5 respectively, than that achieved with

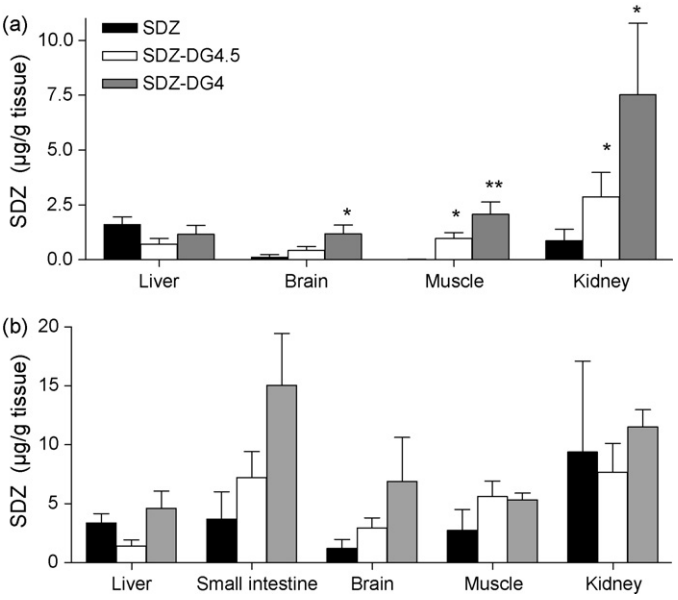

Fig. 7. SDZ concentration in tissues: (a) 3 h after i.v. administration and (b) 4 h after oral administration. $^{*}p < 0.01$; $^{**}p < 0.001$.

free SDZ. Remarkably, SDZ–DG4 administration resulted in 10-fold higher SDZ concentration in brain (Fig. 7a). SDZ concentration in kidney and elimination were higher for both SDZ–D, 15 and 5.7 more SDZ was found in urine after SDZ–DG4 and SDZ–DG4.5 administration, than for free SDZ (Fig. 8a).

Fig. 6b shows SDZ concentration vs. time profile in plasma upon oral administration. Along the first four hours, the plasma concentration of SDZ was significantly higher for SDZ–DG4 with

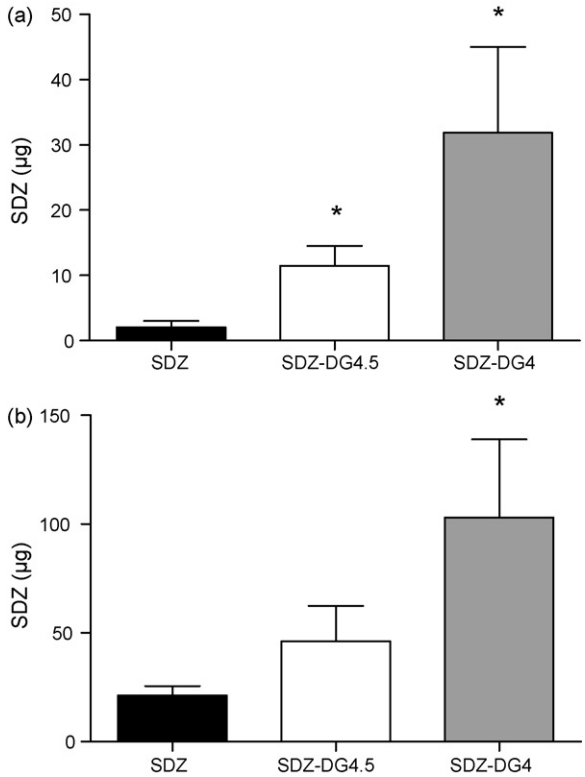

Fig. 8. Amount of SDZ eliminated in urine: (a) after i.v. administration and (b) after oral administration. $^{*}p < 0.01$.

C_{max} of 7.4 ± 4.3 μg/ml, whereas C_{max} for SDZ–DG4.5 and free SDZ were similar (2.3 ± 1.8 and 4.3 ± 2.8 μg/ml, respectively). C_{max} was achieved at 3 h post-administration in all cases. However, C_{max} for SDZ–DG4 was 3.2 and it's AUC_{0-4} (22 ± 12 μg/h ml) 3.5-fold higher than those for free SDZ. Remarkably, doses threefold lower of SDZ as SDZ–DG4.5 rendered AUC_{0-4} (10.5 ± 8.2 μg/h ml) similar to free SDZ (6.2 ± 4.7 μg/h ml).

There were no significant differences in the accumulated SDZ concentration in the analyzed body organs (Fig. 7b), although SDZ–DG4.5 was administered at doses threefold lower. It was found nearly fivefold higher SDZ amount in urine of animals that received SDZ–DG4 as compared to the other groups (Fig. 8b).

4. Discussion

Ideally, delivery systems should solve the problem of accession to intracellular targets, enabling the carried drugs to cross-anatomical and phenomenological barriers. Frequently toxicity is associated to high drug doses, that in order to overcome those barriers, have to be administered. Delivery of SDZ for toxoplasmosis treatment should require of *nanoDDS* with increased selectivity for target tissues, capable of crossing plasma membrane, modifying intracellular transit of SDZ and/or impairing its metabolization to toxic derivatives. When examining the role of D as *nanoDDS* for SDZ, we found that it was possible to modify pharmacokinetics and biodistribution of SDZ according to the D type and the administration route.

The high density of surface groups (one amino group/nm^2 for PAMAM G4 and 1.6 carboxylate groups/nm^2 for PAMAM G4.5) combined with small size (4.5 and 5 nm diameters for the DG4 ellipsoids and DG4.5 spheres, respectively) resulted in high area/volume ratio of D, and this confers D an unusual capacity to establish surface interactions with cell membranes (Fischer et al., 2003; Mecke et al., 2004). PAMAM D do not only interact with membrane lipids (Domanski et al., 2004), but also modify the conformation of membrane proteins (Klajnert and Bryszewska, 2002). Complexation of SDZ with D could modify the internal volume and hence the size and density of surface groups, and such surface modifications are known to alter interaction between D and cells (Han et al., 2005; Withers and Aston, 2006). Because of this, first toxicity and adsorption/uptake on different cell types both of D and SDZ–D were determined. It was found that the effect of each D was cell line-dependent, but no differences between the cytotoxic effects caused by the SDZ–D or identical concentrations of D were recorded.

It was observed that chemical nature of D conditioned the cytotoxicity – measured as LDH release – and that this was related to surface activity – measured as adsorption on Vero, J-774 and *nude* Caco-2 cells. Both D were internalized by endo/phagocytic mechanisms. However, DG4 experienced higher adsorption, and caused massive, concentration-dependent, LDH leakage.

It is well documented that the inherent toxicity of 10 μM PAMAM G4 on *nude* Caco-2 cells impairs the measurement of D permeability (El-Sayed et al., 2002). However, we found that if Caco-2 cells were covered with mucins, toxicity of DG4 and its SDZ complex was reduced, even up to 33 μM D. The higher the mucin concentration covering Caco-2 cells, the less cytotoxic DG4 resulted. Besides, it was shown by flow cytometry on *covered* Caco-2 cells, that once mucins were retired, fluorescence of DG4.5-FITC remained adsorbed on cell surfaces, while fluorescence of DG4-FITC disappeared. These results were coincident with fluorescence confocal images taken along the *z*-axis on *covered* Caco-2 cells. Both surface adsorption and endocytic points upon incubation with DG4-FITC were observed; however, the whole fluorescence was practically absent once mucins were retired. For DG4.5-FITC on the other hand, both surface fluorescence and endocytic points were also observed, but those images experienced practically no changes when the 5 mg/ml mucins was retired. Taken together those results should indicate that positively charged DG4 remained electrostatically attracted to the negative charged mucins macromolecules. On the contrary, and due to the absence of electrostatic attraction, DG4.5 could diffuse across mucins to get the cell surface, where it should be taken up. Hence, the *in vivo* accession to epithelial layer for cationic D could partly be impaired by its entrapment into mucins. This fact could account for an *in vivo* decreased cytotoxicity: not surprisingly *in vitro* previously reported toxicities for G4 in the absence of mucins (El-Sayed et al., 2002; Jevprasesphant et al., 2003; Kitchens et al., 2006; Kolhatkar et al., 2007; Pisal et al., 2008) could *in vivo*, be substantially lower. The absorption of SDZ–D and therefore of SDZ, should be dependent on the interactions between D and mucins.

In second place, the pharmacokinetics and biodistribution of both D–SDZ upon i.v. and oral administration to healthy rats was determined.

Oral administration of SDZ–D was not done with the purpose of increasing absorption of SDZ, since this is known to be fast and extensively absorbed (70–100%) (Association, 1990; Montvale, 1995; Oradell, 2005a,b) but to investigate if once complexed, SDZ could be absorbed by the gastrointestinal tract, and to further determine its pharmacokinetic profile a well as biodistribution. Hence, surprisingly after oral administration, SDZ–DG4 rendered higher plasma concentrations than free SDZ at the same dose; likewise, a threefold lower dose of SDZ as SDZ–DG4.5 rendered the same pharmacokinetic profile than free SDZ. Previous studies have found a low generation window (2.5–3.5) for anionic PAMAM D, capable when in high concentrations (1 mM) of sequestering Ca^{2+} from the tight junctions, therefore increasing the paracellular transit (Wiwattanapatapee et al., 2000; El-Sayed et al., 2003). By flow cytometry and confocal microscopy it was shown that DG4.5 should not be retained by mucins and that G4 on the contrary, could be retained in the bed of anionic macromolecules. In spite of these data were insufficient to explain the high SDZ–D AUC, both DG4.5 and DG4 could favor the absorption of SDZ across the mucosa, each D mediating a different mechanism. No differences in biodistribution were found between SDZ–D and free SDZ.

After i.v. administration on the other hand, AUC for SDZ–DG4 and also for SDZ–DG4.5 were higher than for free SDZ. It was also found an increased delivery to muscle and to brain for SDZ–DG4 as compared to free SDZ.

An explanation to the increased AUC_{0-3} of SDZ–D ($AUC_{SDZ-DG4}/AUC_{SDZ}$ for the i.v. route was nearly six, whereas $AUC_{SDZ-DG4}/AUC_{SDZ}$ for the oral route was nearly three) could be that SDZ–D associated to plasma proteins in higher degree than free SDZ. The association should impair the extravasation of SDZ–D to peripheral tissues, where hydroxylation and part of SDZ acetylations occur. Even when studies by intravital microscopy indicate that DG 0–4 rapidly extravasate to peripheral tissues (El-Sayed et al., 2001), it is important to note that in this technique the plasma is retired and replaced by aqueous buffer. The association to plasma proteins is known to diminish the V_d and to increase the residence time of drugs in the vascular compartment; probably the same could explain the behavior of SDZ–D in circulation. To this respect, for instance, a strong electrostatic interaction between cationic PAMAM D with negatively charged domains and between anionic PAMAM with positive domains of albumin has recently been described (Klajnert and Bryszewska, 2003).

Once in circulation, SDZ is detoxified to a *N*-acetylated product without antiparasitic activity (Leone et al., 1987), by the arylamine *N*-acetyltransferases (NAT) 1 and NAT 2. In humans and other mammals, NAT 2, is presented in polymorphic forms (Weber and Hein,

1985; Hein, 1988; Vatsis et al., 1995) that are responsible for the fast, intermediate and low acetylator phenotypes. A cytochrome P450 isoform found in hepatic microsomes (CYP2C8/9) mediates the SDZ hydroxylation (Winter and Unadkat, 2005). In humans this 4-hydroxylated metabolite is toxic, because it conduces to a nitrose electrophilic intermediate that forms covalent bonds with proteins (Shear and Spielberg, 1985; Rieder et al., 1988). Slow acetylators produce higher amount of hydroxylated toxic products, up to 12% the SDZ dose (Vree et al., 1995). Parental SDZ presents maximal antiparasitic activity, which is consumed by acetylation and hydroxylation; as we mentioned before, alterations in enzymatic phenotypes contribute to its toxicity. The lower V_d for SDZ–DG4 suggested that opportunities for SDZ metabolization in tissues by the two NAT as well as by CYP2C8/9, could also be reduced. Hence, the generation of hydroxylated toxic products, and the damage caused by phenotypic variations on detoxifying mechanisms for SDZ such as those occurring in slow acetylators, could be minimized.

Although SDZ–D modified absorption and pharmacokinetic of SDZ, SDZ biodistribution remained unchanged when complexes were given by the oral route. This could be ascribed to partial complex dissociation occurring along the transit or during the absorption across the gastrointestinal route. This could be reflected in the fact that the $AUC_{SDZ-DG4}/AUC_{SDZ}$ for the i.v. route was twofold higher than for the oral route, indicating a decreased capacity of D for increasing the time vs. SDZ plasma concentration product. We have no current explanation for the increased delivery to muscle and brain observed after i.v. administration of SDZ–DG4. However, it can be concluded that differences in SDZ–D biodistribution upon i.v. or oral administration were owed to the higher amounts of circulating SDZ–D, that could only be achieved by i.v. route. Hence, i.v. injected SDZ–DG4, could be used as a tool to increase the SDZ selectivity for two main infected tissues, brain and muscle, in the *in vivo* infection.

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