In vitro targeting and imaging the translocator protein TSPO 18-kDa through G(4)-PAMAM–FITC labeled dendrimer

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1. Introduction

For optimal therapeutic effect or imaging properties an administered drug or diagnostic agent must safely reach not only its target cell, but often also the appropriate location within that cell. In fact, many targets are localized to intracellular compartments such as the cytosol, endosomes, mitochondria and the nucleus [1]. Over the past decade, research interest in the application of biomedical nanotechnology to the diagnosis and treatment of disease has grown significantly and, while the story of in vivo nanoparticle delivery to date typically ends with cellular internalization, significant opportunities and challenges exist in the programming of specific nanoparticle subcellular interactions and processing. The hallmark of eukaryotic life is membrane compartmentalization, by which specific biochemical processes are localized in intracellular spaces or organelles, and thus in the design of nanoparticle systems, it is reasonable to account for subcellular interactions and specific intracellular targeting, with the aim of improving the therapeutic and/or imaging effect of delivered particles at the cellular site of action [2]. Among the intracellular organelles, mitochondria represent an attractive subcellular target due to its function particularly important for oxidative damage, calcium metabolism and apoptosis. However, the concept of mitochondrial targeting has been a neglected area so far. The translocator protein (TSPO) represents an interesting subcellular target not only to image disease states overexpressing this protein, but also for a selective mitochondrial drug targeting. Recently, we have delivered in vitro and in vivo small molecule imaging agents into cells overexpressing TSPO by using a family of high-affinity conjugable ligands characterized by 2-phenyl-imidazo[1,2-a]pyridine acetamide structure. As an extension, in the present work we studied the possibility to target and image TSPO with dendrimers. These nano-platforms have unique features, in fact, are prepared with a level of control not reachable with most linear polymers, leading to nearly monodisperse, globular macromolecules with a large number of peripheral groups. As a consequence, they are an ideal delivery vehicle candidate for explicit study of the effects of polymer size, charge, composition, and architecture on biologically relevant properties such as lipid bilayer interactions, cytotoxicity, cellular internalization, and subcellular compartments and organelles interactions. Here, we present the synthesis, characterization, cellular internalization, and mitochondrial labeling of a TSPO targeted fourth generation [G(4)-PAMAM] dendrimer nanoparticle labeled with the organic fluorescent dye fluorescein. We comprehensively studied the cellular uptake behavior of these dendrimers, into glioma C6 cell line, under the influence of various endocytosis inhibitors. We found that TSPO targeted-G(4)-PAMAM–FITC dendrimer is quickly taken up by these cells by endocytosis pathways, and moreover specifically targets the mitochondria as evidenced from subcellular fractionation experiments and co-localization studies performed with CAT (Confocal–AFM–TIRF) microscopy.
been a neglected area, and the few examples pertain to bioconjugates and nanoparticulate carriers using mainly lipophilic cations as mitochondrial-specific targeting moieties [2,3].

Discovered in 1977 as an alternative binding site in the kidney for the well known benzodiazepine diazepam, the translocator protein (TSPO), previously known as peripheral-type benzodiazepine receptor, is an 18-kDa high affinity cholesterol- and drug-binding protein found mostly in the OMM as part of a mitochondrial cholesterol transport complex [4–7]. In normal conditions, TSPO is present minimally in the healthy human brain and liver [7–9], vice versa at higher levels in steroid-synthesizing and rapidly proliferating tissues, and its biological role has been mainly linked to mitochondrial function, steroidogenesis and cell proliferation/apoptosis [10,11]. Aberrant TSPO levels have been linked to multiple diseases, including cancer, endocrine disorders, brain injury, neurodegeneration, ischemia-reperfusion injury and inflammatory diseases [12–15]. Thus, for all above mentioned, TSPO has become an extremely attractive subcellular target not only to image disease states overexpressing this protein, but also for a selective mitochondrial drug targeting [16–19].

Investigation of the functions of this protein in vitro and in vivo has been mainly carried out using high-affinity drug ligands, such as isoquinoline carboxamides (e.g. PK11195) and benzodiazepines (e.g. Ro5-4864) [20,21]. PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isooquinoline-carboxamide) and Ro5-4864 (4′-chlorodiazepam) are the two most commonly used TSPO drug ligands because of their high affinity, specificity, and ability to be modified. They have been used to explore TSPO distribution and function in various tissues and pathologies becoming useful in mapping the “peripheral binding site” in almost every tissue examined. More recently, new structurally diverse TSPO drug ligands have been developed and used for functional and imaging studies such as 2-phenylindole-3-acetamides (e.g. FGIN-1) [22], phenoxoylacetamides (e.g. DAA1106) [23,24], imidazo[1,2-a]pyridines (e.g. CLINME) [23,25], and pyrazolopyrimidines (e.g. DPA-713) [23,24]. To our knowledge, none of all the above cited TSPO drug ligands contain organic functions such as amino-, hydroxy- and carboxylic-groups potentially useful for their further conjugation with diagnostic- or with nanovector-imaging agents. In this context, we have recently reported new potent and selective TSPO drug ligands characterized by a 2-phenyl-imidazo[1,2-a]pyridine acetamide structure which, in a versatile manner, can be used as PET tracers or, when containing conjugable functions, as fluorescent probes targeting the mitochondrial-located translocator protein as well [26–28].

More recently, we synthesized the first examples of conjugable imidazopyridinacetamides endowed with high affinity and selectivity for TSPO [26,29]. From the pioneering work on fluorescent TSPO ligands by Kozikowski et al. [30] a wide number of promising candidates have been proposed to potentially replace the expensive and sometime unwieldy radio-labeled TSPO probes. While, only one example of TSPO targeted nanocarrier delivering an imaging agent has been reported so far [31].

Among nanovectors, dendrimers represent one of the emerging nanomaterials which, if not toxic, could be used as promising tools for biomedical purpose [32,33]. These nano-platforms have been successfully employed for a wide range of applications such as drug or gene delivery and as imaging agents as well [34,35]. Dendrimers are prepared with a level of control not reachable with most linear polymers, leading to nearly monodisperse, globular macromolecules with a large number of peripheral groups. As a consequence, they are an ideal delivery vehicle candidate for explicit study of the effects of polymer size, charge, composition, and architecture on biologically relevant properties such as lipid bilayer interactions, cytotoxicity, cellular internalization, and subcellular compartments and organelles interactions [34,35]. Over the last several years, substantial progress has been made toward the use of dendrimers for therapeutic and diagnostic purposes and the majority of studies have been performed on modified poly(amicooaminate) (PAMAM) dendrimer, in part because PAMAM generations 1 through 10 (G1–G10) are relatively easy to synthesize or commercially available featuring a wide number of peripheral groups (4 to 4096), end-group functionality (e.g., amine, carboxylic acid, hydroxyl) and molecular weights (657 to 935,000 g/mol) [36,37].

In this paper we present the synthesis, characterization, cellular internalization, and mitochondria labeling of a TSPO targeted fourth generation (G(4)-PAMAM) dendrimer nanoparticle labeled with the fluorescent dye fluorescein. In particular, the first step was focused on the synthesis of a new potent and selective TSPO drug ligand characterized by a 2-phenyl-imidazo[1,2-a]pyridine acetamide structure and containing a carboxylic-group useful for its further conjugation with G(4)-PAMAM dendrimer nanoparticle. Furthermore, we hybridized TSPO targeted-G(4)-PAMAM dendrimer with the organic dye fluorescein isothiocyanate (FITC) to prepare a new type of TSPO targeted-G(4)-PAMAM-FITC dendrimer. Therefore, we studied the binding and endocytic internalization of cationic G(4)-PAMAM dendrimers in a TSPO over expressing cell line [38], i.e., rat C6 glioma cells. As the toxicity of cationic dendrimers has been extensively documented [33] the cytotoxicity of all dendrimers was first determined in C6 cells using the MTT assay to ensure that non-toxic concentrations could be used in all endocytosis studies. We then comprehensively studied the cellular uptake behavior of these dendrimers, in the above mentioned cell line, under the influence of various endocytosis inhibitors. We found that TSPO-targeted-G(4)-PAMAM–FITC dendrimer is quickly taken up by these cells by endocytosis pathways, and moreover specifically targets the mitochondria as evidenced from subcellular fractionation experiments and co-localization studies performed with CAT (Confocal–AFM–TIRF) microscopy.

2. Materials and methods

Commercial reagent grade chemicals, including methyl acrylate, ethylidenemiamine, ethyl 2-bromocacetate, fluorescein isothiocyanate isomer 1 (FITC), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), triethylamine (TEA), anhydrous tetrahydrofuran (THF), Ethanol 96% (v/v) (EtOH), anhydrous N.N-dimethylformamide (DMF), dimethylsulfoxide (DMSO), and solvents were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise stated, and used without further purification. The TSPO ligand 2-(6,8-dichloro-2-(4-hydroxyphenyl)imidazo[1,2-a]pyridin-3-yl)-N,N-dipropylacetamide (3) was prepared according to synthetic procedures reported elsewhere [26]. Rat C6 glioma cells, from Interlab Cell Line Collection (ICLC, Genova, Italy), were grown in DMEM high glucose medium supplemented with 10% heat-purified FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (Lonza, Italy) in a 5% CO2, humidified atmosphere at 37 °C. Disposable culture flasks and Petri dishes were from Corning. Glassworks (Corning, N.Y. USA). The radioligands [3H]flunitrazepam and [3H]-PK11195 were purchased from PerkinElmer Life Sciences. PK11195, flunitrazepam and 3-(4,5-dimethylthiazol-2)-2,5-diphenyletrazolium bromide (MTT) were purchased from Sigma-Aldrich (Milan, Italy).

2.1. Synthesis of the TSPO ligands 1 and 2

The new TSPO ligand 2-(6,8-dichloro-2-(4-dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-2-yl)phenoxy)acetic acid (1) was prepared by using synthetic methods shown in Scheme 1a starting from the TSPO ligand 3 which, in turn was synthesized according to a previously reported procedure developed in our laboratories [26]. To a stirred solution of 3 (0.30g, 0.71 mmol) anhydrous THF was added, in the order, ethyl 2-bromocacetate (1.57 ml, 14.2 mmol) and K2CO3 (0.49 g, 3.5 mmol). Stirring was continued for additional 24 h at 40°C, then the solvent was evaporated under reduced pressure and the solid residue was dissolved in 25 ml of 10% NaHCO3, extracted with ethyl acetate (3 × 20 ml) and dried (Na2SO4). Evaporation of the solvent gave a residue which was purified by silica gel chromatography [light petroleum ether/ethyl
acetic acid (64/4 v/v) as eluent giving rise to pure compound ethyl 2-[4-(6,8-dichloro-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-f]pyridin-2-yl)]phenoxy]acetate (2). To a solution of ethyl ester (2) (0.10 g, 0.20 mmol) in EtOH (5 ml), NaOH (1 M, 5 ml) was added dropwise. The mixture was stirred at room temperature for 2 h. Then the solvent was evaporated under reduced pressure and the residue was dissolved in 20 ml of water and extracted with ethyl acetate (3 × 20 ml). The cooled water phase was acidified to pH 4 with 0.1 M HCl and the resulting precipitate of the pure acid 1 was collected by filtration and dried under vacuum. Compounds 1–3 were obtained in good yields and were fully characterized by elemental analyses, mass spectrometry, Fourier transform infrared (FT-IR), 1H NMR and UV–Vis spectroscopies. In particular, elemental analyses were carried out with a Eurovector (Milan, Italy) model EA 3000 CHN. Mass spectrometry: electrospray ionization mass spectrometry (ESI-MS) was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MS Trap system Agilent, Palo Alto, CA). FT-IR spectra were carried out on a PerkinElmer 1600 FT-IR spectrometer (Spectrum One). For each spectrum 40 scans from 4000 to 400 cm−1 were performed. 1H NMR spectra were recorded on Varian Mercury 300 MHz instrument. Chemical shifts are given in ppm. 1H chemical shifts were referenced by using the residual protic peak of the solvent as internal reference (2.50 ppm for Dimethyl Sulfoxide-d6, 4.80 ppm for Water-d4, 7.24 ppm for Chloroform-d). UV/Vis spectra were performed on a PerkinElmer Spectrometer Lambda Bio20 spectrometer.

2-(4-((6,8-dichloro-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-f]pyridin-2-yl)]phenoxy)acetic acid 1 (85% yield), IR (KBr): 3436, 1632, 1613 cm−1; 1H NMR (DMSO-d6): 0.6–0.9 (m, 6H, CH3), 1.4–1.6 (m, 4H, CH2), 3.0–3.3 (m, 4H, CH2CON), 4.22 (s, 2H, CH2CON), 4.70 (s, 2H, OCH2CON), 6.99 (d, J = 8.2 Hz, 2H, Ar), 7.52 (d, J = 8.2 Hz, 2H, Ar), 7.62 (s, 1H, Ar), 8.29 (s, 1H, Ar); ESI-MS: calculated for [M + H]+ = 476.1 Found: m/z (%) relative to the base peak) = 476.0 (100) [M + H]+.

Ethyl 2-[(6,8-dichloro-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-f]pyridin-2-yl)]phenoxy]acetate 2 (60% yield), IR (KBr): 1765, 1639 cm−1; 1H NMR (CDCl3): 0.70 (t, 3H, J = 7.4 Hz, CH3), 0.82 (t, 3H, J = 7.4 Hz, CH3), 1.28 (t, 3H, J = 7.1 Hz, CH3), 1.4–1.6 (m, 4H, CH2), 3.06 (t, 2H, J = 7.7 Hz, CH2CON), 3.25 (t, J = 7.7 Hz, 2H, CH2CON), 4.01 (s, 2H, CH2CON), 4.25 (q, 2H, J = 7.4 Hz, CH2OCON), 4.63 (s, 2H, OCH2CON), 6.96 (d, J = 8.5 Hz, 2H, Ar), 7.24 (d, J = 1.4 Hz, 1H, Ar), 7.54 (d, J = 8.5 Hz, 2H, Ar), 8.22 (d, J = 1.4 Hz, 1H, Ar); ESI-MS: calculated for [M + Na]+ = 528.1 Found: m/z (%) relative to the base peak) = 528.0 (100) [M + Na]+.

2.2. Synthesis of dendrimers 4–7

PAMAM dendrimers were synthesized using modifications of the procedure first developed by Tomalia et al. [36,37]. These dendrimers were prepared by divergent synthesis using methanol as solvent. In particular, an ethylenediamine core, and branched units are constructed from both methyl acrylate and ethylenediamine. Each layer is built in two steps: the exhaustive Michael addition reaction and the exhaustive amimation reaction. First, amino groups react with methyl acrylate monomers, and then, ethylenediamine is added. The half-generations of PAMAM dendrimers possess surfaces of methyl ester groups and full-generation surfaces of amino groups. In our studies we used PAMAM dendrimer with generation 4 (32 amine end-group units). Progress during both the alkylation and amimation steps, required to synthesize G(4)-PAMAM dendrimer (4), was monitored by FT-IR, 1H and 13C NMR and mass spectrometry.

As outlined in Scheme 1b, TSPO ligand 1 was coupled to the surface amino groups of dendrimer 4 via a carbodiimide reaction. In particular, compound 1 (32 mmol) was solubilized in 2 ml of deionized water in the presence of TEA (32 mmol) and reacted with EDC (38 mmol) for 15 min. The activated TSPO ligand was added to 2 ml of a water solution of G(4)-PAMAM dendrimer (4) in the presence of TEA (32 mmol) and the reaction was stirred overnight at room temperature. The resulting mixture was transferred into dialysis membrane tubing (Spectra/Por® 3500 MWCO, RC) and purified by three–five subsequent dialyzing procedures against deionized water to remove excess of unreacted compound 1 and coupling agent. The purified suspension was analyzed by size exclusion chromatography (SEC) to confirm the purity of the sample and then freeze-dried giving rise to TSPO targeted-G(4)-PAMAM dendrimer (5) which was stored at 4 °C until further use. Dendrimer 5 was fully characterized as described for dendrimer 4. The number of TSPO ligand 1 molecules conjugated per mole of dendrimer was estimated using both 1H NMR and UV–Vis spectroscopies.

The TSPO targeted-G(4)-PAMAM–FITC dendrimer (6) was synthesized by adding to the dendrimer 5 (50 mg, 0.24 mmol) solution in deionized water, a FITC (6.1 mg, 16 μmol) solution in DMF (4 ml). The solution was stirred overnight at room temperature under N2 in the dark followed by purification in dialysis tubing as described above and lyophilized to give an orange powder. Conjugation of FITC to dendrimer 4 was also carried out as described above giving rise to the known FITC-G(4)-PAMAM dendrimer (7, Scheme 1b). To estimate bound and free FITC, dendrimers 6 and 7 were analyzed by SEC. The presence of the bounded fluorescent dye was also confirmed with UV–Vis, fluorescence and NMR spectroscopies.

Scheme 1. a) Schematic synthesis of the new TSPO ligands 2 and 1. b) Schematic synthesis of TSPO ligand-G(4)-PAMAM 5. TSPO ligand-G(4)-PAMAM–FITC 6 and G(4)-PAMAM–FITC 7 dendrimers. Symbols: m and f represent the number of estimated amino groups re- placed by TSPO ligand 1 and FITC, respectively (see Tables 1 and 4 for details).
2.3. Determination of TSPO ligand 1 and FITC contents on dendrimers 5–7

The percentage of TSPO ligand 1 covalently linked to the surface of dendrimers 5 (i.e., the conjugation degree) was estimated by 1H NMR and UV spectroscopy. 1H NMR analysis was carried out by comparing the integral values of the appropriate peaks of the ligand moiety, with those of the dendrimer portion. UV analysis of dendrimers 5 was carried out in the range 250–350 nm, in which the carrier does not absorb and DMSO/methanol solution was used as solvent since it allows the solubilization of both the free TSPO ligand 1 and macromolecular conjugates. For dendrimer 5 UV measurements were made comparing its E1 value with that of the pure TSPO ligand at the same maximum absorption (λ = 256 nm) of the latter. In the case of dendrimers 6 and 7, the conjugation degree referred to the fluorescent dye FITC, and was determined by measurements at wavelength of 450 nm corresponding to the maximum absorption at which the TSPO ligand 1 does not absorb. For dendrimers 6 and 7, emission and excitation spectra were recorded by using a Fluorolog 3 spectrofluorimeter (HORIBA Jobin-Yvon), equipped with double grating excitation and emission monochromators. Since the emission spectrum from a turbid solution can have a peak at twice the excitation wavelength, due to 2nd-order transmission through the emission monochromator, bandpass excitation filters were used to remove unwanted wavelengths from the excitation beam. In particular, emission spectra of dendrimers 6 and 7 were performed by exciting sample at 350 or 375 nm and using a Schott RG830 longpass optical filter before the emission monochromator, to avoid the interference from scattered excitation light from the higher-order transmission of monochromators.

2.4. Morphological characterization of dendrimers 4–7

The molecular size and the polydispersity index (PDI) of dendrimers 4–7 were measured using photon correlation spectrosopy (PCS, Nanosizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) after dilution in deionized water or PBS. The same instrument was also used to measure the zeta potential by laser Doppler velocimetry after dilution of dendrimers suspension with 1 mM KCl. The average molecular weights of dendrimers 4–7 were determined by SEC analysis, performed with a Waters Associates (Milford, MA) Model 1515 HPLC isocratic pump, an ultrahydrogel 500 (Waters, 7.8 × 300 mm, 5 μm) column, a UV–Vis detector Waters 2487 or RID detector Waters2414 (Breeze Software to analyze the chromatographic data). The system was calibrated with standards of dextran. The morphological examination of dendrimers 4–6 was assessed by atomic force microscopy (AFM). The AFM images were obtained using aXE-100 (Park Systems, Suwon, Korea) in noncontact mode and using a PPP-NCHR cantilever with 330 kHz resonant frequency. Scan speed was between 0.5 and 1.5 Hz using the adaptive scan mode function set in the software. All the images are 256 × 256 data points. For AFM visualization, a drop of diluted polymer suspension in deionized water or DMSO (1 mg/ml) was drop casted or spin-coated, respectively, on mica; then, after a period of evaporation under vacuum, the sample was processed directly on the AFM scanner. To get high resolution images of polymer aggregates on mica, typically 125 μm long PPP-NCHR silicon cantilevers with a resonance frequency of about 270 kHz and a nominal force constant around 42 N/m were used. The tip to sample distance was kept constant using the amplitude feedback function in attractive forces regimen to prevent the damage of the NPs.

2.5. Receptor binding assays

The ability of compounds 1 and 2 to bind with high affinity and selectivity the translocator protein was assessed by in vitro receptor binding assays. These experiments were carried out as previously reported by Denora et al. [28] for Central Benzodiazepine Receptor (CBR) and translocator protein 18-kDa (TSPO) receptor.

2.6. Evaluation of the cytotoxicity and stability of dendrimers 4–7

The growth inhibition activity of all synthesized dendrimers (4–7) was evaluated as previously described, according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program [18,39]. C6 gloma cells were seeded in 96-well microtiter plates on day 0 at a density of 5 × 10^4 cells/well in 200 μl of medium. The cell concentration was adjusted according to the cell population doubling time. Test agents were then added on day 1 in seven dilutions ranging from 1 mg/ml to 0.001 mg/ml. The number of living cells was assessed by quantitative colorimetric MTT assay after 72 h of incubation at 37 °C in a humidified atmosphere with 5% CO2. Untreated cells were used as positive control and cells incubated with a 2% (w/v) SDS solution were used as negative control. The absorbance of the individual well was measured by microplate reader at 570 nm (Wallac Victor3, 1420 Multitlab Counter, PerkinElmer). Each test was performed in triplicate in three separate experiments. The results are expressed as IC50 which is the concentration necessary for 50% inhibition. The IC50 values for each tested dendrimer are calculated from concentration–response curves using non-linear multipurpose curve-fitting program SigmaPlot 9.0.

To determine conjugate stability in the presence of cell culture medium and cells a previously reported procedure was used [40]. Briefly, dendrimers 5–7 (0.01 mg/ml) were incubated in complete culture medium for 4 h at 37 °C. Then, 1 ml of the medium was withdrawn and dried under vacuum. The residue was re-dissolved in 1 ml of PBS and analyzed by SEC as described above.

2.7. Cellular uptake in the presence and absence of endocytosis inhibitors, extracellular binding, exocytosis and competition studies of dendrimers 6 and 7

Cells were seeded on day 0 in 6 well plates at a density of 1 × 10^5 cells/well in 1 ml of medium. Cellular uptake of dendrimers 6 and 7 was determined in the presence and absence of endocytosis inhibitors. In particular, various well characterized inhibiting drugs (Siga-Aldrich) and potassium depletion buffer were selected for their ability to inhibit specific steps in the endocytosis pathway and were added to the cell culture media at the indicated concentrations 45 min prior to addition of dendrimers. After consultation of the literature, a suitable concentration range was chosen for each inhibitor and tested for efficacy as well as cytotoxicity [41]. In fact, potential short-term cytotoxicity (24 h) of endocytosis inhibitors was assessed in C6 cells (96 well plates, 5000 cells/well) to ensure cell viability during uptake assays (2 h). Chemical inhibitors were prepared at a range of concentrations known to reduce endocytosis [genistein (20–400 μM), phenylarsine oxide (PAO, 0.1–50 μM), methyl-6-(β-cyclodextrin [MβCD, 5–100 mM], dynasore (5–100 μM), and wortmannin (50 nM)] [41,42]. For potassium depletion, cells were washed twice with potassium depletion buffer (140 mmol/l NaCl, 20 mmol/l HEPES, 1 mmol/l CaCl2, 1 mmol/l MgCl2, 1 mg/ml d-glucose, pH 7.4), then incubated for 15 min with hypotonic buffer (1:1 potassium depletion buffer and distilled water) at 37 °C, and finally washed again three times with potassium-free buffer. Cytotoxicity of the inhibitors was assessed by colorimetric MTT assay as above described. Hence, inhibitors were used at concentrations that showed a minimum of 85% cell viability during the 24 h assay period (Table 2). In particular, as endocytosis inhibitors genistein was used at 100 μM, PAO at 1 μM, MβCD at 10 mM, dynasore at 50 μM, wortmannin at 50 nM, and potassium depletion buffer with the above described composition.

Hence, dendrimers 6 (0.015 mg/ml, 1 μM in terms of FITC) or 7 (0.010 mg/ml, 1 μM in terms of FITC) were added on day 1 to the wells in the presence and absence of endocytosis inhibitors and incubated further for 2 h at 37 °C in a humidified atmosphere with 5% CO2. At determined intervals of time the medium was withdrawn and cells were washed three times with cold PBS and lysed by adding 1 ml/well...
of a 50% DMSO/50% Ethanol solution. Then, 200 μl of the resulting solution was analyzed by microplate reader (Wallac Victor3, 1420 Multilabel Counter, PerkinElmer) with an excitation and emission wavelength set at 485 and 535 nm, respectively. To determine external binding of dendrimers 6 and 7 we have used a slightly modified previously reported procedure [40]. Briefly, before the study cells were incubated at 4 °C for 25 min and tested dendrimers were dissolved in complete medium and equilibrated at 4 °C. Experiment was started by addition of complete media containing dendrimers 6 (0.015 mg/ml, 1 μM in terms of FITC) or 7 (0.010 mg/ml, 1 μM in terms of FITC) in each well. The cells were incubated for times between 0 and 120 min at 4 °C, and after the incubation period, analyzed as described above for uptake studies. In the exocytosis experiments, cells were incubated for 2 h with dendrimers 6 or 7 at 37 °C as described above. Following the 2 h “loading” period, cells were rinsed with PBS and immediately incubated for 0–60 min (i.e., exocytosis phase) with medium only. Further experimental details have been described in detail elsewhere [40]. Moreover, to detect competition of dendrimer 6 with parent compound 1, C6 cells were treated with TSPO ligand 1 at increasing concentration (10, 20 and 30 μM) for 45 min prior to replacement with dendrimer 6 (0.015 mg/ml, 1 μM in terms of FITC) for 2 h at 37 °C. Dendrimer 7 (0.010 mg/ml, 1 μM in terms of FITC) was used as reference since it is not a TSPO targeted dendrimer.

2.8. Cellular fractionation

C6 glioma cells were fractioned into nuclear (N), mitochondrial (M), lysosomal (L), microsomal (Mic) and soluble (S) fraction by means of differential centrifugation using a protocol adapted from Seib et al. [43]. In brief, C6 cells were exposed to dendrimers 6 and 7 (0.015 and 0.010 mg/ml, respectively; 1 μM in terms of FITC) for 24 h before extracellular content was washed away with cold PBS. This was followed by washing with PBS twice and homogenization buffer once (250 mM sucrose, 10 mM HEPES, 1 mM EDTA pH 7.4 and a protease cocktail containing aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (1 mg/ml) and PMSF (1 mM)), C6 cells were harvested, re-suspended in homogenization buffer at 2.5-fold wet cell weight and homogenized by 7 passes in a cell cracker (clearance 6 μm). Next, the diluted homogenate was centrifuged at 1500 × g for 2 min at 4 °C and the resulting pellet was washed with homogenization buffer and centrifuged again to obtain a nuclear fraction. The supernatant of the later centrifugation was centrifuged at 3000 × g for 15 min at 4 °C. The pellet was washed with homogenization buffer and subjected to a further 15 min spin at 3000 × g. The resulting pellet was denoted the mitochondrial fraction. The combined supernatant was further centrifuged in sequence, first at 22,000 × g for 10 min to obtain the lysosomal pellet, then at 100,000 × g for 30 min to obtain the microosomal pellet and the soluble fraction. Each fraction was diluted with PBS and the FITC fluorescence was measured on a microplate reader (Wallac Victor3, 1420 Multilabel Counter, PerkinElmer) with an excitation and emission wavelength set at 485 and 535 nm, respectively.

2.9. Live cell fluorescence microscopy

These experiments were carried out as previously reported by Laquintana et al., see reference [18] for details. In particular, uptake of FITC-conjugated dendrimers 6 and 7 (0.015 and 0.010 mg/ml, respectively; 1 μM in terms of FITC) into rat C6 glioma cells was imaged 24–30 h after the treatment, whereas mitochondrial morphology of control cells was also studied at the designated times after incubation for 15–30 min at 37 °C in a 5% CO2 atmosphere with 25 nM MitoTracker Red CMXRos (Molecular Probes) used as a mitochondrial marker.


CAT microscopy is a combination of an advanced scanning probe microscope (Bioscope Catalyst, Bruker Inc. USA), a confocal microscope
(LSM 700, Zeiss, Germany), and a total internal reflection fluorescence microscope (Laser TIRF 3, Zeiss, Germany). Devices are mounted on an inverted microscope. Their combined use provides a topographic and spectroscopic imaging, and nano-scale adhesion forces and elastic forces mapping of the sample. In particular, in the present work we have used only confocal microscopy for fixed and living cells investigation.

2.10.1. Confocal imaging

Laser scanning confocal microscopy was performed on a Zeiss LSM700 (Zeiss, Germany) confocal microscope equipped with an Axio Observer Z1 (Zeiss, Germany) inverted microscope using a ×100, 1.46 numerical aperture oil immersion lens for imaging. Laser beams with 359 nm, 488 nm, and 542 nm excitation wavelengths were used for DAPI, FITC, and Phalloidin–TRITC imaging, respectively. We have acquired confocal images for rat C6 glioma cells, which are about 10-μm thick, 15 z-stacks of typically 0.5 μm thick slices were taken, each slice being the individual average of three laser scans. Single confocal sections, z-stack images, and image rendering of confocal data files were processed using ZEN software (Zeiss, Germany).

In the case of fixed cells, the cells (10⁶ cells/well) were grown on glass bottom dishes (WillCo-Dish®) for 24 h and then exposed to dendrimer 6 or 7 at concentration of 0.015 and 0.010 mg/mL, respectively (1 μM in terms of FITC). Following the incubation period of 12 h, cells were placed on ice and washed three times with ice cold PBS. Cells were then fixed for 20 min on ice with 3% w/v paraformaldehyde in PBS. Following three PBS washes, cells were incubated for 30 min at 37 °C with 25 nM MitoTracker Red CMXRos (Molecular Probes), 1 μg/ml DAPI (Molecular Probes), or with 1 μg/ml Phalloidin–TRITC (Sigma) used as mitochondrial, nuclear and F-actin markers, respectively. After the staining with the appropriate marker, cells were imaged.

For live cell imaging, 100,000 cells/well were seeded in glass bottom dishes (WillCo-Dish®) and treated as described above, but the fixation steps were omitted. In particular, cells were incubated for 30 min at 37 °C with 25 nM MitoTracker Red CMXRos (Molecular Probes), or with 25 nM LysoTracker Red (Molecular Probes) used as mitochondrial, nuclear and lysosomal markers, respectively. All washing steps were performed at 25 °C. Finally, PBS lacking phenol was added to the dish and cells were subsequently visualized for a maximum of 30 min.

2.10.2. Co-localization analysis

We have estimated the different intracellular localizations of dendrimers 6 and 7 into lysosomes and mitochondrial structures through co-localization analysis. This is a powerful tool for the demonstration of spatial and temporal overlapping in the distribution patterns of fluorescent probes. Co-localization analysis shares the following steps:

- Definition of a threshold intensity value. This is a crucial operation: it is necessary to remove the background signal to separate features of interest from the rest of the image in each channels. We have established it by software for each image working individually on each channel.
- Definition of an area of analysis. The parameter of co-localization is normalized to the selected area therefore we have chosen same value of area for analysis of all images acquired; this allows to compare directly the value of co-localization obtained.
- Choice of parameter of co-localization. Several coefficients are used to estimate co-localization; we have estimated the co-localization through overlapping coefficient according to Manders et al. [44].

\[
 r = \frac{\sum S_{1i} - \sum S_{ii}}{\sqrt{\sum S_{1i}^2 - \sum S_{ii}^2}} \quad 0 \leq r \leq 1
\]

where \(S_1\) and \(S_2\) represent signal intensity of pixels in the channel 1 and in the channel 2. A major advantage of this coefficient is that it is not sensitive to the typical fluorescent image, such as differences in intensities between the components of an image caused by different labeling with fluorochromes, such as photo-bleaching or different settings of the amplifiers. We have estimated the coefficient \(r\) as average value of the \(r\) calculated in 10 regions of the same area of each confocal image. Co-localization analysis has been performed by using Software ZEN 2010 (ZEISS).

2.11. Statistic

Statistical significance was assigned to \(p < 0.001 (**), p < 0.05 (**), p < 0.01 (*)\) and calculated using a one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc tests (GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, CA). Where indicated, standard error of the mean (SEM) for data points has been calculated and the number of experiments is given (n).

3. Results and discussion

3.1. Synthesis of the TSPO ligands 1 and 2

In order to synthesize a new TSPO targeted dendrimer capable to image in live cells the mitochondrial translocator protein 18-kDa, the first step of this work was focused on the synthesis of a high selective TSPO ligand characterized by organic functions, such as the carboxylic group, that allow for its further conjugation. We have recently reported new potent and selective TSPO drug ligands characterized by 2-phenyl-imidazol-1,2-[l]pyridine acetamide structure containing conjugatable functions [28-29]. Based on these findings, we have decided to introduce, with a well known synthetic procedure shown in Scheme 1, a carboxylic group on the potent and selective TSPO ligand 3 which, in turn was synthesized according to a previously reported procedure developed in our laboratories [26]. In particular, the new TSPO ligand 2-(4-(6,8-dichloro-3-(2-(dipropylamino)-2-oxoethyl)imidazol-1,2-lyphenox)acetic acid (1) was prepared by hydrolysis of the corresponding ethyl ester TSPO ligand, compound ethyl 2-(4-(6,8-dichloro-3-(2-(dipropylamino)-2-oxoethyl)imidazol-1,2-lyphenox)acetate (2), in aqueous 1 M NaOH/ethanol (1:1, v:v) solution. In turn, compound 2 was synthesized by reacting the TSPO ligand 3 with ethyl 2-bromacetate in anhydrous conditions. Compounds 1-3 were obtained in good yields and were fully characterized by elemental analyses, mass spectrometry, Fourier transform infrared (FT-IR), 1H NMR and UV–Vis spectroscopies as reported in the Experimental Section.

3.2. Affinity and selectivity of compounds 1 and 2 toward TSPO

Once synthesized the new TSPO ligands 1 and 2 characterized by a carboxylic and an ethyl ester group, respectively, the obvious following step was to evaluate the affinity and selectivity of those compounds toward TSPO. Besides, the selectivity of compounds 1 and 2 toward TSPO was evaluated by measuring the affinities of those compounds for the central-type benzodiazepine receptor (CBR). In fact, TSPOs were discovered as peripheral-type binding sites for the benzodiazepine diazepam, which represent a ligand for CBR. A great number of TSPO ligands are usually characterized by high affinity not just for TSPOs, but also for CBR, hence they are not considered selective TSPO ligands. Therefore, we evaluated the binding of compounds 1 and 2 to the TSPO and CBR receptors by measuring their ability to inhibit [3H]-flunitrazepam and [3H]-PK11195 binding to membrane preparations arising from the rat cerebral cortex. Their effects were compared with those of unlabelled flunitrazepam and PK11195. In these studies flunitrazepam was used as a selective ligand for CBR, while PK11195 as a selective ligand for TSPO. The inhibitory concentration (IC₅₀) values determined are listed in Table 3 together with that of the starting TSPO

Vis
ligand 3, PK11195 and flunitrazepam. As can be seen, compounds 1, 2 and 3 displayed high affinity for TSPO (2.12, 3.47, 2.74 nM, respectively), even better than that of PK11195 (4.27 nM), which was used for comparison. Moreover, none of the tested TSPO ligands was better than that of PK11195 (4.27 nM), which was used for comparison. The obtained values were in agreement with the theoretical ones. The TSPO targeted-G(4)-PAMAM dendrimer (4) was synthesized using the carboxylic acid 1 as TSPO binding moiety. In particular, TSPO ligand 1 (Scheme 1) was activated with EDC in water in the presence of TEA, and added to a solution of G(4)-PAMAM 4 in the presence of TEA. As reported in Table 1, an average of 16 units of TSPO ligand 1 was attached by reacting 1 mol of dendrimer 4 with 32 mol of the activated carboxylic acid 1. Excess of free TSPO ligand 1 and coupling agent were removed by extensive dialysis against deionized water using membranes with a molecular weight cutoff (MWCO) of 3.5 kDa. The resulting purified suspension was freeze-dried and the purity of conjugates was confirmed by SEC analysis and NMR spectrometry. Following characterization of the TSPO targeted-G(4)-PAMAM dendrimer 5 with FT-IR, UV, NMR spectra and SEC analysis, the dendrimer was further functionalized by reacting it with the well known organic dye FITC in DMF overnight to give an average of 6 fluorescein units per dendrimer 6 (Scheme 1, Table 1). The DMF was removed in vacuo and excess dye was removed by dialysis (MWCO = 3.5 kDa) in water. UV–VIS and fluorescence were employed to characterize the optical imaging agent. As reported on Table 5, attaching the dye to the dendrimer produced a compound with an absorbance maximum at 467 nm (versus 494 nm for free dye) and a slight-green emission at 518 nm (versus 521 nm for free dye). A control FITC–G(4)-PAMAM dendrimer 7, the agent absent of the targeting moiety, was synthesized similarly to the targeting agent by reacting G(4)-

Table 3

Affinities of compounds 1–3 for CBR and TSPO from rat cerebral cortex.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>CBB&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2.12</td>
<td>&gt;10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>3.47</td>
<td>&gt;10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>2.74</td>
<td>&gt;10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>PK11195</td>
<td>4.27</td>
<td>&gt;10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are means of three separate experiments performed in duplicate which differed by less than 10%.}

<sup>b</sup> PK11195 a selective ligand for TSPO 18-kDa was used for comparison.

<sup>c</sup> Flunitrazepam a selective ligand for CBR was used for comparison.

<sup>d</sup> Measured in PBS.

Section. Dendrimer 4 was characterized in terms of molecular weight, polydispersity and number of amino end groups (Table 1). The obtained values were in agreement with the theoretical ones. The TSPO targeted-G(4)-PAMAM dendrimer (5) was synthesized using the carboxylic acid 1 as TSPO binding moiety. In particular, TSPO ligand 1 (Scheme 1) was activated with EDC in water in the presence of TEA, and added to a solution of G(4)-PAMAM 4 in the presence of TEA. As reported in Table 1, an average of 16 units of TSPO ligand 1 was attached by reacting 1 mol of dendrimer 4 with 32 mol of the activated carboxylic acid 1. Excess of free TSPO ligand 1 and coupling agent were removed by extensive dialysis against deionized water using membranes with a molecular weight cutoff (MWCO) of 3.5 kDa. The resulting purified suspension was freeze-dried and the purity of conjugates was confirmed by SEC analysis and NMR spectrometry. Following characterization of the TSPO targeted-G(4)-PAMAM dendrimer 5 with FT-IR, UV, NMR spectra and SEC analysis, the dendrimer was further functionalized by reacting it with the well known organic dye FITC in DMF overnight to give an average of 6 fluorescein units per dendrimer 6 (Scheme 1, Table 1). The DMF was removed in vacuo and excess dye was removed by dialysis (MWCO = 3.5 kDa) in water. UV–VIS and fluorescence were employed to characterize the optical imaging agent. As reported on Table 5, attaching the dye to the dendrimer produced a compound with an absorbance maximum at 467 nm (versus 494 nm for free dye) and a slight-green emission at 518 nm (versus 521 nm for free dye). A control FITC–G(4)-PAMAM dendrimer 7, the agent absent of the targeting moiety, was synthesized similarly to the targeting agent by reacting G(4)-

Table 4

Conjugation degree of dendrimers 5, 6 and 7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>E&lt;sup&gt;1%b&lt;/sup&gt;</th>
<th>SD (g/g)</th>
<th>SD (mol/mol)</th>
<th>SD %</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;, Ex</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;, Em</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(UV)</td>
<td>(NMR)</td>
<td>(NMR)</td>
<td>(nm)</td>
<td>(nm)</td>
</tr>
<tr>
<td>1</td>
<td>897.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>401.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>467</td>
<td>518</td>
</tr>
<tr>
<td>6</td>
<td>13.5 ± 0.026&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.0%</td>
<td>15.0%</td>
<td>462</td>
<td>523</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by UV analysis in H<sub>2</sub>O at 256 nm wavelength (5) or at 464 nm (6,7).

<sup>b</sup> Refers to the substitution degree of TSPO ligand 1.

<sup>c</sup> Refers to the substitution degree of FITC dye.

<sup>d</sup> Percentage of amino end groups replaced by TEA.

<sup>e</sup> Percentage of amino end groups replaced by FITC.

Fig. 1. SEC elution profiles of dendrimers 4, 5, 6 and 7.
PAMAM dendrimer 4 with FITC (Scheme 1). The FITC-G(4)-PAMAM dendrimer 7 was also purified by dialysis in deionized water to remove unreacted FITC. Dendrimer 7 was characterized as for dendrimer 6 by UV–Vis and fluorescence spectroscopies and, as reported in Table 4, it has an absorbance maximum at 462 nm and an emission at 523 nm. The chemical structures of dendrimers were determined by FT-IR, UV and $^1$H NMR spectra. Thus, the FT-IR spectra of dendrimers 4, 5, 6, and 7 show the typical bands of a poly(amideamine) structure between 3400 and 3200 and 1650 cm$^{-1}$ attributable to the amino- and amide carbonyl-group, respectively. The $^1$H NMR spectra of conjugates 5 and 6 show signals attributable to the methylene protons of PAMAM together with the signals of compound 1. In particular, the signals attributable to protons of the alkyamine chain and the aromatic ones of 1 are clearly detected. The average molecular weights of synthesized dendrimers 4, 5, 6, and 7 were determined by SEC analysis and showed molecular weight (Mw) of 7025, 15,600, 15,530, and 12,130 Da, respectively as summarized in Table 1. The polydispersity index (PDI) Mw/Mn of all conjugates was in the range 1.02–1.08 indicating a narrow dispersion of molecular weights. Typical SEC profiles of TSPO targeted G(4)-PAMAM dendrimer are shown in Fig. 1. All the SEC profiles exhibit a broad unimodal molecular weight distribution and it can be seen that the conjugates were eluted slightly first than the unbound TSPO ligand 1. The SEC profiles, monitored with a refractive index detector and at dual UV wavelengths of 230 and 260 nm for G(4)-PAMAM 4 and dendrimers 5, 6, and 7, were almost overlapped. It should be also mentioned that the TSPO ligand 1 eluted at 18.2 min as very narrow peak, and free unbound compound 1 was not evidenced by SEC analysis in all the purified dendrimers. Hence, further evidence proving covalent conjugation of TSPO ligand 1 to dendrimers 5 and 6 is based on the absence of unbound carboxylic acid 1.

The percentage of amino end groups replaced by TSPO ligand 1 and/or FITC units on the dendrimer (i.e., the conjugation degree) was estimated by $^1$H NMR and UV spectroscopy. It was shown from proton NMR spectra of the G(4)-PAMAM 4 and TSPO ligand 1 that the aromatic proton signals are exclusively from the carboxylic acids attached (6.5–8.5 ppm) to the dendrimer, while the PAMAM methylenes are the only signal observed from 2.0 to 3.6 ppm and the amides are found from 7.8 to 8.5 ppm (Fig. 2). By calculation there are approximately 484 methylene protons in a G(4)-PAMAM dendrimer and 6 aromatic protons per TSPO ligand 1 molecule. To determine the average number of TSPO ligand 1 per dendrimer, the integral from 6.5 to 8.5 ppm was calibrated to 6 and the resulting integral from 2.0 to 3.6 ppm was divided into 484. As shown in Table 4, for dendrimer 5 we find 23.7% of amino end groups replaced by TSPO ligands 1. Considering that each G(4)-PAMAM dendrimer 4 has 32 amino end groups, for dendrimer 5 the number of linked TSPO ligand 1 is equal to 7.6 units (Table 1). UV analysis of conjugates was carried out in the range 250–350 nm, in which the carrier does not adsorb and THF was used as solvent since it allows the solubilisation of both the free TSPO ligand 1 and macromolecular conjugates. For conjugates 5, 6 and 7 UV measurements were made comparing their E1% values with that of the pure TSPO ligand at the same absorption maximum ($\lambda = 260$ nm) of the latter. In the case of compounds 6 and 7, the conjugation degree referred to the fluorescent probe was determined by measurements at wavelength of 464 nm corresponding to the absorption maximum at which the TSPO ligand 1 does not adsorb. The maximum excitation and emission wavelength recorded for dendrimers 6 and 7 are also reported in Table 4. In each case, the results obtained with the three analytical procedures and listed in Table 4 were in fair agreement and comparable.

3.4. Physicochemical and morphological characterization of dendrimers 4–7

Table 5 summarizes the physicochemical properties of the different synthesized dendrimers 4, 5, 6 and 7. For the starting G(4)-PAMAM dendrimer 4, around 4.28 ± 0.99 nm average particle size ($d_{mean}$ (nm), number PSD) was found, which is in agreement with that previously reported [37]. As expected, when dendrimers are functionalized with either the TSPO ligand 1 or with the organic dye FITC otherwise with both of them, an increase of the hydrodynamic diameter was observed (Fig. 3A). In particular, for dendrimers 5, 6 and 7 we have found average
the higher zeta potential value of +16.5 mV (Table 5). Whereas, when adsorptive endocytosis [2]. Moreover, previously studies have widely demonstrated that positive charged polymers, such as PAMAM dendrimers, that amine-terminated PAMAM dendrimers are protonated and bear a positive charge at physiological pH (Fig. 38). This feature allows dendrimers first to adhere on the negatively charged cell membranes by electrostatic interactions, and then to be transported into the cytosol by adsorptive endocytosis [2]. Moreover, previously studies have widely demonstrated that positive charged polymers, such as PAMAM dendrimers, could also allow the endosomal escape through several different mechanisms, the most popular model of which is the osmolytic or so-called “proton-sponge” effect for polymeric particles [2]. It is interesting to note that dendrimer 4, taken as control, presented the higher zeta potential value of +16.5 mV (Table 5). Whereas, when functionalized the zeta potential lowered to 13.8 and 11.4 mV for dendrimers 5 and 6, respectively. When just FITC is introduced on the surface of G(4)-PAMAM dendrimer, as for dendrimer 7, the measured zeta potential value is of 12.7 mV. The morphological appearance of the dendrimers 4, 5, 6 and 7 was examined by AFM by spreading on mica, with spin-coating procedure, a drop of dendrimer suspension in DMSO (1 mg/ml). The AFM images of dendrimers 4-7 showed a uniform and flat surface. In some cases, a size increase was found, especially for dendrimers 6 and 7, which probably indicates a self-assembly of dendrimers due to the introduction of compound 1 and/or FITC, and also probably because the slow solvent evaporation induces to concentrate the dendrimer suspension, which in turn forces the dendrimer monomers to aggregate on the mica surface. Their shape appears almost spherical probably because this arrangement reduces the surface energy with the polar substrate. In particular, Fig. 4a and b shows dendrimer 4 and 6 are narrowly distributed as proved by the PDI values (0.030 and 0.084. These results are in agreement with those obtained in deionized water for dendrimer 6. While, for dendrimer 5 was dispersed in deionized water and deposited on mica, AFM images (Fig. 5) evidenced larger and more structured fractal aggregates. Their origin could be related to the use of a more polar solvent (Dielectric Constant at 25 °C; water = 78.5; DMSO = 46.7) that induces the formation of well-organized fractal aggregates already present in water; these aggregates show very high mechanical stability that allows AFM imaging on the mica surface after solvent evaporation. Fig. 4d and e shows 20 μm × 20 μm phase and topography images of dendrimer 6, respectively. AFM images of dendrimer 7 (data not shown) evidenced the aggregation propensity of dendrimers on mica where very thick nanoparticle islands can be easily found. This is because the conjugation of FITC decreases, as evidenced by zeta potential measurements, the surface charge density of PAMAM dendrimer and also the electrical attraction between PAMAM dendrimer (positive charge) and mica surface (negative charge.). Thus, the dendrimer branches can easily interpenetrate other dendrimer molecules, establishing intermolecular interactions causing plate and more flat like films. Definitely, AFM measurements are in good agreement with PCS data indicating that the dendrimers on glass keep intact their spherical morphology and dimension, even if some aggregates are also noted.

3.5. Evaluation of the cytotoxicity and stability of dendrimers in C6 glioma cells

The effects of dendrimers 4, 5, 6 and 7 on the viability of C6 glioma cells were studied. As shown in Fig. 6, all the tested dendrimers displayed cytotoxicity, after 72 h of incubation, that was concentration dependant. In particular, dendrimer 4 was the most effective, as shown by its IC₅₀ value of 0.0065 mg/ml. It is interesting to note that dendrimers 5, 6, and 7 (IC₅₀ values of 0.15, 0.39 and 0.011 mg/ml, respectively) are less cytotoxic than dendrimer 4, suggesting that the cytotoxicity is proportional to the positive charge expressed by the nanoparticle. These results were in agreement with those observed by F. P. Seib et al. [40]. Hence, for all subsequent experiments dendrimers 6 and 7 were used at concentrations of 0.015 and 0.010 mg/ml, respectively, and bright-field microscopy confirmed the absence of obvious signs of cellular toxicity at this concentration.

The stability of dendrimers 5, 6 and 7 was evaluated in complete culture medium and in the presence of cells in order to avoid during cellular uptake measurements the potential artifact due to the free fluorophore, and further, to quantify the amount of free TSPO ligand 1 originated from the hydrolysis of the amide linkage between the amino end group of the dendrimer moiety and the carboxylic group of 1. After a 4 h incubation with cells in culture medium the free FITC measured for dendrimer 6 and 7 was 0.6 and 1.2% of the total, respectively. Moreover, after the same period of incubation, the free TSPO ligand 1 measured for dendrimer 5 and 6 was 0.2 and 0.3% of the total. The
impact of such contaminants was considered to not interfere with the following uptake experiments.

3.6. Endocytic uptake, extracellular binding and exocytosis of dendrimers 6 and 7 in C6 glioma cells

The cellular association of both FITC labeled dendrimers 6 and 7 at 37 °C was linear over the incubation time as shown in Fig. 7a and c, respectively. When the experiments were performed at 4 °C, a not significant extracellular binding was observed for dendrimer 6 and 7. In particular, in the case of dendrimers 6 and 7 an extracellular binding fluorescence of about 14 and 15% of the total was measured, respectively. Not surprising, the cell association profile of the tested dendrimers resembled that previously observed for G(4)-PAMAM dendrimer functionalized with the organic dye Oregon Green [40]. Moreover, no discernible exocytosis over time of dendrimers 6 and 7 was observed (Fig. 7c and d, respectively).

3.7. Cellular uptake in the presence of endocytosis inhibitors and competition studies of dendrimers 6 and 7

Endocytosis can be classified in two broad categories: phagocytosis and pinocytosis. Phagocytosis is typically restricted to specialized...
cells, such as macrophages, while pinocytosis occurs in all cell types. The study of different pinocytic pathways is still an evolving field and no current classification system is completely satisfactory. Currently, pinocytosis is subdivided into macrophinocytosis, clathrin-dependent (CDE) and clathrin-independent (CIE) endocytosis. Macrophinocytosis is a nonselective endocytic mechanism for internalizing suspended macromolecules while, CDE is the well characterized endocytic pathway and represent the preferred mechanism for internalizing nanoparticles up to 200 nm in size [41]. On the contrary, CIE mechanism has not been completely elucidated and its current sub-classification is based on the role of dynamin and several small GTPases [41]. These include uptake from lipid rafts in caveolae or via flotillin-dependent pathways. A number of endocytic pathways have been demonstrated to be involved in the uptake of nanoparticle and this is highly dependent on cell type, the nature of the nanoparticle and the particle size [40,41,45]. Much of the data on the cellular uptake of nanoparticles comes from studies using chemical inhibitors of endocytosis. However, the precise routes of PAMAM dendrimer internalization are still undefined, hence we explored the cellular uptake of dendrimers 6 and 7 in the presence of well known endocytosis inhibitors at concentrations tested for efficacy as well as cytotoxicity. In fact, potential short-term cytotoxicity (24 h) of endocytosis inhibitors was assessed in C6 cells to ensure cell viability during uptake assays (2 h). In particular, inhibitors were used at concentrations that showed a minimum of 85% cell viability during the 24 h assay period (Table 2). In this study, as endocytosis inhibitors we used genistein (100 μM), PAO (1 μM), MJ/CD (10 mM), dynasore (50 μM), wortmannin (50 nM), and potassium depletion buffer (for the composition see Experimental Section). In particular, PAO and potassium depletion buffer are evaluated as inhibitors of CDE. MJ/CD and genistein are evaluated as inhibitors of CIE, while, dynasore and wortmannin as inhibitors of dynamin-dependent endocytosis and macrophinocytosis, respectively. As shown in Fig. 8, comparison between PAO- and MJ/CD-treated and untreated C6 cells incubated with dendrimer 6 for 2 h at 37 °C exhibited a significant reduction in the cell-associated fluorescence of 42 and 24%, respectively. A marginal reduction in cell-associated fluorescence was observed following potassium depletion buffer, genistein, and wortmannin. While, no significant changes were observed in dynasore treated cells. Results obtained for dendrimer 6 parallel to those observed for 7 (data not shown). As previously reported and from the results presented here, PAMAM dendrimers 6 and 7 internalization was in part due to CDE (42% inhibition in the presence of PAO, 21% inhibition in the presence of potassium depletion buffer), and also to cholesterol-dependent (24% inhibition in the presence of MJ/CD) mechanism [40].

In vitro competition was performed to quantify interaction of dendrimers 6 and 7 with TSPO in the presence and absence of increasing concentrations (10, 20 and 30 μM) of the agonist TSPO ligand 1. Preloading with 1 for 45 min prior to addition of dendrimer 6 (0.015 mg/ml, 1 μM) of the TSPO agonist 1 (Fig. 9). This indicates that the TSPO targeted-G(4)-PAMAM-FITC dendrimer 6 functionally competes for localization in the same plane as the parent TSPO ligand 1. As expected, in the case of dendrimer 7, the mean fluorescence intensity did not change with increasing concentrations of TSPO ligand 1 (Fig. 9) indicating the absence of competition between the TSPO ligand 1 and the non-TSPO targeted dendrimer 7.

![Fig. 7. Endocytic uptake, extracellular binding and exocytosis of FITC labeled dendrimers 6 and 7 in C6 glioma cells. Panel (a) endocytosis and panel (b) exocytosis of TSPO targeted-G(4)-PAMAM-FITC dendrimer 6. Panel (c) endocytosis and panel (d) exocytosis of G(4)-PAMAM-FITC dendrimer 7. Plot symbols for panel (a, c): amount of internalized dendrimer (•) estimated by subtracting 4 °C values from 37 °C uptake values. Data represents mean ± SEM, n = 3.](image)

![Fig. 8. Effect of endocytosis inhibitors on accumulation of dendrimer 6 (37 °C; 2h). Results are expressed relative to cell-associated fluorescence of dendrimer 6, used as control, in absence of inhibitor. Statistical values p < 0.001 (**), p < 0.05 (*), and p < 0.01 (*) were estimated using one-way ANOVA and Bonferroni post hoc test. Data represents mean ± SEM, n = 3.](image)
3.8. Intracellular fate and co-localization of dendrimers 6 and 7 in C6 glioma cells

Two procedures are frequently used to quantify intracellular trafficking of polymer therapeutics or diagnostics, specifically the subcellular fractionation and confocal microscopy. To date, most studies investigating the intracellular trafficking of polymers have used confocal microscopy. Such fluorescence microscopy techniques have major limitations including: the optical resolution of the microscope, ability to investigate a limited number of cells in the population and not the least, the properties of the fluorescent probe. The latter can be subject to photo-bleaching, concentration and pH-dependent fluorescence quenching and the probe itself can influence the cellular uptake mechanisms and subsequent trafficking of the conjugate. While, subcellular fractionation is challenging (labour intensive, requires large number of cells, requires many control experiments to verify organelle integrity) and the use of differential centrifugation to monitor the intracellular trafficking of polymer therapeutics is still a very important tool. Therefore, in our study, in order to gain more and accurate information about the cellular trafficking and co-localization of dendrimers 6 and 7, we have used both subcellular fractionation and confocal microscopy. In particular, in the case of cellular fractionation, C6 glioma cells were fractioned into nuclear (N), mitochondrial (M), lysosomal (L), microsomal (Mic) and soluble (S) fractions by means of differential centrifugation using a protocol adapted from Seib et al. [43]. C6 cells were exposed to dendrimers 6 and 7 (0.015 and 0.010 mg/ml, respectively; 1 μM in terms of FITC) for 24 h before the fractionation procedure.

Then, we quantified the amount of FITC that was bound to the cell membrane, and subsequently internalized into the cytoplasm or translocated to the mitochondria using the above mentioned standard procedure. As shown in Fig. 10, when C6 cells were exposed for 24 h to TSPO targeted-G(4)-PAMAM–FITC dendrimer 6, the mean fluorescence recovered in the mitochondrial fraction (39%). Fluorescence was also detected in the lysosomal (31%) fraction and to a lesser extent in the nuclear (4%), microsomal (7%) and soluble fraction (2%). In contrast, G(4)-PAMAM–FITC dendrimer 7, which is not a TSPO targeted system, showed a different distribution (Fig. 10). In fact, after the incubation period, FITC detected in the mitochondrial fraction (21%) was significantly lower than that seen for dendrimer 6. This evidence confirms that the TSPO moiety is able to recognize the mitochondrial translocator protein. The overall recoveries for dendrimers 6 and 7 were 83 and 72% of the total, respectively.

In order to confirm the evidences outlined by cellular fractionation, cellular co-localization studies of fluorescent dendrimers 6 and 7 were initially screened by epifluorescence microscopy in live cells. In particular, C6 glioma cells were incubated with FITC-labeled dendrimers 6 and 7 (0.015 and 0.010 mg/ml, respectively; 1 μM in terms of FITC) and imaged 24–30 h after the treatment. Before the image acquisition, cells were incubated for 15–30 min at 37 °C in a 5% CO2 atmosphere with 25 nM MitoTracker Red CMXRos used as a mitochondrial marker. As shown in Fig. 11 dendrimer 6 enters the cell and binds the mitochondria. In fact, the overlay of the red (MitoTracker Red) and green fluorescence (FITC moiety of 6) converges to yellow, indicating that the majority of TSPO targeted-G(4)-PAMAM–FITC 6 co-localizes in the mitochondria. In contrast, the case of dendrimers 7 co-localization in the mitochondria was not evident as for that observed for the TSPO targeted-G(4)-PAMAM–FITC dendrimer 6 (Fig. 11). These findings support the results evidenced with the cellular fractionation.

For that concerning confocal microscopy, we have used an innovative Confocal–AFM–TIRF (CAT) microscope. In particular, CAT microscopy is a combination of an advanced scanning probe microscope, a confocal microscope and a total internal reflection fluorescence microscope. Devices are mounted on an inverted microscope. Their combined use

**Fig. 10.** Subcellular fraction of C6 cells after incubation with dendrimers 6 and 7. Key: microsomal (Mic), soluble (S), lysosomal (L), mitochondrial (M) and nuclear (N) fractions. Statistical values p < 0.001 (**), and p < 0.05 (*) were estimated using one-way ANOVA and Bonferroni post hoc test. Data represents mean ± SEM, n = 3.

**Fig. 11.** Morphological analysis of the mitochondrial network structure in C6 glioma cells imaged with TSPO targeted-G(4)-PAMAM–FITC dendrimer 6 or with G(4)-PAMAM–FITC dendrimer 7 (green) and MitoTracker Red (red), after an incubation period of 24 h. Overlay: when the red and green fluorescence converges to yellow, indicates co-localization.
provides a topographic and spectroscopic imaging, and nano-scale adhesion forces and elastic forces mapping of the sample. However, for our aim, in the present work we have used fixed and live cell confocal microscopy. Fluorescence microscopy of C6 cells incubated with dendrimers 6 and 7 clearly demonstrated that cell-fixation dramatically altered subcellular distribution of both FITC-labeled dendrimers (data not shown). Although such methodology has been used previously to study nanoparticles intracellular trafficking its usefulness is limited [43]. Hence, in the present study we have used fixed cells, stained with DAPI and Phalloidin, in order to gain preliminary information about the intracellular distribution of dendrimers 6 and 7. As shown in Fig. 12, clearly both FITC labeled dendrimers are able to enter into C6 cells with no apparent surface binding on the cellular membrane. Only live cell imaging gives an accurate picture of the intracellular location of dendrimers 6 and 7. Therefore, to further explore whether dendrimer 6 was targeting the mitochondria, a co-incubation experiment was performed. This was accomplished by first incubating C6 cells with dendrimers 6 or 7 as described above, and then with MitoTracker Red or LysoTracker Red, for 30 min at 37 °C, in order to label mitochondria or lysosomes, respectively. Fig. 13 panel a displays co-registration of TSPO targeted-G(4)-PAMAM–FITC dendrimer 6 and MitoTracker Red with yellow (arrows) indicating areas of overlap while red and green indicate areas where dendrimer 6 (green) and the mitochondrial marker (red) are not coincident. It is encouraging to see that most of the green converge to yellow, indicating that the majority of dendrimer 6 is in the same location of the cell as the MitoTracker Red. Moreover, dendrimer 6 is co-localized and it is not labeling other parts of the cell, such as the nucleus. In contrast, as shown in Fig. 13 panel a, the fluorescence of G(4)-PAMAM–FITC dendrimer 7 does not overlap with that of the mitochondrial marker. This evidence, in agreement with the results acquired by epifluorescence microscopy (Fig. 11), confirms our hypothesis that the dendrimer with the TSPO binding ligand 1 produces bright localized cellular fluorescence due to its ability to pass through the cellular membrane and bind to the target protein TSPO. Further,

![Fig. 12. Representative single confocal slices of fixed C6 cells imaged with (a) i) DAPI; ii) Phalloidin; iii) TSPO targeted-G(4)-PAMAM–FITC 6 (1 μM in FITC); iv) overlay; (b) i) DAPI; ii) Phalloidin; iii) G(4)-PAMAM–FITC 7 (1 μM in FITC); iv) overlay, after an incubation period of 12 h. Bar: 10 μm.](image1)

![Fig. 13. Representative single confocal slices of live C6 cells imaged with (a) TSPO targeted-G(4)-PAMAM–FITC 6 or G(4)-PAMAM–FITC 7 (1 μM in FITC) and MitoTracker Red (b) TSPO targeted-G(4)-PAMAM–FITC 6 or G(4)-PAMAM–FITC 7 (1 μM in FITC) and LysoTracker Red, after an incubation period of 12 h. Overlay: when the red and green fluorescence converges to yellow, indicates co-localization (yellow arrow). Bar: 10 μm.](image2)
the same colocalization study was conducted in the presence of the lysosomal marker LysoTracker. Fig. 13 panel b, display co-registration of dendrimers 6 or 7 and LysoTracker Red with yellow indicating areas of overlap, while red and green indicate areas where dendrimers (green) and the lysosomal marker (red) are not coincident. As expected, both dendrimers are to some extent co-localized in lysosomes. Moreover, to quantify the amount of co-localization of dendrimers 6 and 7, a computational method was used. In particular, all image pixels are displayed on the scatter diagram, in which the two channels of the image are compared with one another. Pixels of both channels having identical positions in the image can be regarded as a pair. Accordingly, each pixel pair has two intensities, one for each channel (Fig. 14). The intensities of the two channels are represented by the axes of the scatter graph. In the scatter region 3 are presented the co-localizing pixels. Results evidenced that for TSPO targeted-G(4)-PAMAM dendrimer 6 and for G(4)-PAMAM dendrimer 7 the co-localizing pixels are equal to 47 and 20% of the total, respectively. It is interesting to note that results obtained using subcellular fractionation and confocal microscopy are completely in agreement.

4. Conclusion

In this work we have developed a TSPO targeted nano-platform imaging agent, i.e., dendrimer 6, which was synthesized using a G(4)-PAMAM dendrimer as backbone, compound 2-4-(6,8-dichloro-3-(2-dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-2-yl)phenoxy)acetic acid as high-affinity conjugable TSPO ligand (compound 1), and FITC as organic fluorophore. After a comprehensive physicochemical and morphological characterization, that pointed out the monodispersity and the spherical shape of synthesized dendrimer with a hydrodynamic diameter of about 24 nm, the ability of this TSPO targeted-G(4)-PAMAM dendrimer imaging agent to be internalized into C6 glioma cells and target a specific mitochondrial protein was assessed. In particular, endocytosis experiments evidenced that dendrimers studied here were quickly internalized by pinocytosis and no significant exocytosis was observed. We then broadly studied the ability of the TSPO targeted dendrimer to specifically target the mitochondria with two procedures frequently used to quantitate intracellular trafficking of polymer therapeutics or diagnostics, precisely the subcellular fractionation and confocal microscopy, and with competition studies. In fact, when dendrimer 6 was incubated in the presence of TSPO ligand 1, the mean measured fluorescence decreased of about 20% highlighting competition toward mitochondrial protein. It is interesting to note that results obtained using subcellular fractionation and confocal microscopy are completely in agreement and evidenced that TSPO targeted-G(4)-PAMAM-FITC dendrimer co-localizes to the mitochondrial fraction and to the mitochondria, respectively.

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Fig. 14. Representative co-localization analysis in a double tagged C6 glioma cell (upper panel confocal microscopy image; green: TSPO targeted-G(4)-PAMAM dendrimer 6 tagged with FITC 1 µM; red: mitochondrion tagged with MitoTracker Red). All image pixels are displayed on the scatter diagram (lower panel), in which the two channels of the image are compared with one another. Pixels of both channels having identical positions in the image can be regarded as a pair. Accordingly, each pixel pair has two intensities, one for each channel. The intensities of the two channels are represented by the axes of the scatter graph. In the scatter region 3 are presented the co-localizing pixels.


