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Research paper

HIV-1 antiviral behavior of anionic PPI metallo-dendrimers with EDA core

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ABSTRACT

The development of novel strategies to prevent HIV-1 infection is of outstanding relevance. Metal complexes of Cu^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} derived from sulfonated and carboxylated poly(propylene imine) dendrimers with ethylenediamine core were evaluated as tunable antiviral agents against HIV-1. After demonstrating their biocompatibility, specific trends in the antiviral properties were found, related to both the dendritic scaffold (peripheral group, generation) and the bound metal ions (sort, amount). In HEC-1A and VK-2 cell lines, as model of the first barrier against HIV-1 infection, a high preventive inhibitory action was found, which also avoided virus internalization inside cells and inhibited both CCR5 and CXCR4 HIV-1 strains. In peripheral blood mononuclear cells (PBMC), as model of the second barrier, a dual preventive and therapeutic behavior was observed. A rational design of such metallodendrimers opens new avenues for the production of versatile and efficient treatments against HIV-1 infection.

1. Introduction

Due to the raising number of new HIV-1 infections, mainly through sexual transmission [1], an enormous amount of research is being conducted on the pathogenesis, prevention and transmission of this disease. The main objective – the development of a protective vaccine that efficiently controls or eliminates the spread of HIV-1 [2–4] – has not been achieved so far [5–7]. Thus, the basis for controlling the HIV/AIDS epidemic depends on prevention methods and new strategies [8].

A broad range of these new strategies relies on compounds acting on the first steps of HIV-1 infection cycle – i.e. adsorption and fusion of the virus to the cell-, such as topical microbicides [9]. This kind of compounds avoid the interaction between the trimeric protein complex composed of gp120/gp41 on the HIV envelope and the receptors and co-receptors CD4, CXCR5 and CXCR4 and other molecules of the target cell surface [10]. Several compounds interfering in these first steps of the HIV-1 cycle are based on multivalent systems, whose antiviral potency is determined by the amount of active moieties and the scaffold on which these moieties are supported (e.g. polymerized surfactants, micelles, cyclodextrins, dendrimers). Dendrimers have shown promising properties as antiviral agents due to their controlled synthesis and monodisperse architecture and have been mainly functionalized with three types of molecules [11]: carbohydrates [12–19], peptides [20–22] or anionic groups [23]. Dendrimers decorated with anionic moieties interfere in the early stages of viral infection by interacting with the V3 loop of gp120 protein. For example, anionic PAMAM dendrimers have demonstrated to decrease the infection of the cells [24]. Likewise, polylysine dendrimers decorated with carboxylate and sulfonate groups inhibited the entrance of HIV-1 into cells *in vitro*, and in the case of the sulfonated one, acted also on enzymes such as reverse transcriptase and integrase [25,26], as well as sulfonated porphyrins [26]. The SPL7013 polylysine

Abbreviations: HIV-1, Human Immunodeficiency Virus type 1; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; CXCR4, C-X-C chemokine receptor type 4; CCR5, C-C chemokine receptor type 5.

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dendrimer (VivaGel), decorated with naphthalene disulfonate moieties showed microbicide activity in vaginal topic use [27], as well as the anionic carbosilane dendrimers developed in our group, which are effective topical microbicides against HIV-1 [28–33].

The design of efficient anti HIV-1 agents has approached some other strategies involving multivalent systems. Complex dendritic architectures which combine different antiviral moieties have been developed. The inclusion of anionic groups in glycodendritic architectures led to efficient antagonists in *in vitro* assays, where the HIV-1 binding was dependent on both carbohydrates and anionic groups. This is the case of PPI dendrimers with randomly sulfated galactose residues [17,34] or other anionic trisaccharides [17], or polylysine dendrimers with sulfated cellobiose [35]. Glycodendropeptides with up to 16 peptides and 9 mannose moieties appear as an attractive molecular tool for fine-tuning the immune response against viral infections [36]. Another approach is based on the inclusion of metal moieties in the dendritic scaffolds, which clearly increase the range of their applications due to the different properties of metals and modulate the activity of dendritic macromolecules where they are anchored [2,37–42]. This is the case of the copper complexes of anionic carbosilane dendrimers previously synthesized in our group. We demonstrated that the peripheral anchoring of the metal improves the antiviral activity against HIV-1 compared to the dendrimer alone [43].

As starting point of this study, we prepared sulfonated and carboxylated-containing N-donor ligands and their corresponding metal complexes based on Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} [44,45]. These complexes presented a dual preventive-therapeutic behavior when treating HIV-1-infected PBMC, but their activity was limited by the low number of anionic moieties. The development of multivalent systems with higher amount of anionic groups could improve such antiviral properties. With these premises, we generated poly(propyleneimine) dendrimers with an ethylenediamino core and multiple carboxylate or sulfonate groups in the periphery [46]. These systems exhibited a specific pattern on the coordination of metal ions, as we demonstrated by means of UV–Vis and EPR spectroscopy using Cu^{2+} as a probe (Fig. 1). The first equivalent is bound in the core of the dendrimer with a CuN_2O_2 geometry (*I Signal* in EPR spectra), while the following equivalents are distributed in the structure in a precise way, mainly with $CuNO_3$ (*II Signal*) and CuO_4 (*III Signal*) geometries depending on the generation and terminal groups nature. Such control over metal distribution on anionic PPI dendrimers pushed us to evaluate the antiviral behavior of the resultant metallodendrimers and its correlation with properties such as the type and amount of metal ions or the generation of the dendrimer. The study was expanded to evaluate diverse metal ions (M = Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}) at increasing dendrimer:metal (D:M) ratios. Our preliminary *in vitro* results show that these metallodendrimers may act as potential candidates for inhibition of HIV-1 infection.

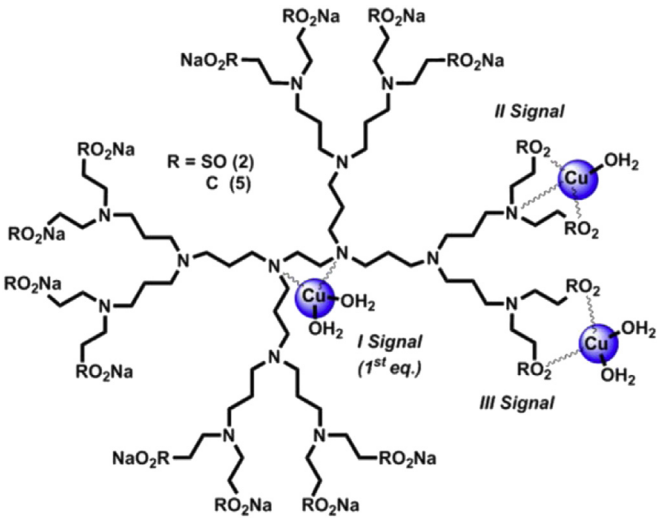

Fig. 1. Metal-coordination ability of the dendrimers. Second generation dendrimer model structure and metal coordination points.

2. Materials and methods

2.1. Samples preparation

The previously developed PPI dendrimers with peripheral sulfonate and carboxylate groups [46] were used to prepare metallodendrimers with Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}, at different dendrimer:metal (D:M) ratios (G1, ratios 1:1, 1:3 and 1:5; G2, ratios 1:1, 1:5 and 1:9; and G3, ratios 1:1, 1:9 and 1:17). The stock solutions of dendrimers (*stock D*: 800, 400, 200 and 40 μM) and the different metal salts (*stock M*: 4 mM) were prepared by dissolution of the solids in sterile water, while those of metallodendrimers (*stock MD*) were prepared by mixing the right amounts of *stock D* (800 μM) and *stock M* (Table S1, ESI), and stirring the mixture at room temperature for 4 h. Due to the hygroscopic properties of the isolated metallodendrimers, oily solids difficult to handle, the samples were prepared and maintained in solution. However, the degree of metal coordination to the dendritic macromolecule was also evaluated by nanofiltration of the mixtures against dialysis membranes of 500 g/mol cut-off with water. No free metal ions in the washing solutions were detected by UV–Vis and ^{1}H NMR. Control solutions of the dendrimers (*control D*) and the metal salts (*control M*) were prepared at 182 μM and 3.1 mM, respectively. They were added in different volumes depending on each experiment. For biocompatibility assays, cells were treated with 5 μL of each dendrimer, at final concentrations of 1, 5, 10 and 20 μM. In the case of the metallodendrimers, all experiments were performed with the amounts indicated in Table S1 (ESI), to final concentrations of 10 μM.

2.2. Biomedical assays

2.2.1. Primary cells and cell lines

Blood samples were obtained from healthy anonymous donors from transfusion centers of Madrid (Spain), following national guidelines. Peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll-Hypaque density gradient (Rafer, Zaragoza, Spain) following the current procedures of Spanish HIV BioBank [47]. PBMC were cultured in RPMI-1640 medium (Gibco, Paisley, UK) containing 10% heat-inactivated FBS, 1% (2 mM) L-glutamine and antibiotic cocktail (1% ampicillin, 1% cloxacillin and 0.32% gentamicin; Sigma, St-Louis, MO, USA) at 37 °C in a 5% CO_2 atmosphere. Prior to compounds treatment, PBMC were activated for 48 h with 1 μg/mL of phythemagglutinin (PHA, Remel, Santa Fe, USA) and 60 U/mL of recombinant interleukin-2 (IL-2, Bachem, Bubendorf, Switzerland).

Hec-1A cells (epithelial cell line, derived from human endometrial adenocarcinoma, uterus mucosa carcinoma) and VK-2 (epithelial cell line, derived from human vagina mucosa) were obtained through ATCC. Hec-1A were grown in McCoy's 5A Medium Modified (Biochrom AG©, Berlin, Germany), supplemented with 10% of FBS, 2 mM L-glutamin (ICN Pharmaceuticals, CostaMesa, CA, USA) and antibiotic cocktail (1% penicillin/streptomycin). VK-2/E6E7 were grown in Keratinocyte-Serum Free Medium (GIBCO-BRL 17005-42) with human recombinant EGF (0.1 ng/ml), bovine pituitary extract (0.05 mg/ml, Sigma–Aldrich)

and $CaCl_2$ (0.4 mM).

2.2.2. Cytotoxicity assays

We performed MTT and MTS assays, based on the ability of viable cells to reduce a tetrazolium salt to formazan crystals by using the mitochondrial enzyme succinate dehydrogenase.

MTT assay was performed to test PBMC viability. Cells were seeded in a 96-well plate at a density of cells/well, in complete RPMI-1640 medium (Gibco, Paisley, UK) containing 10% heat-inactivated FBS, 1% (2 mM) L-glutamine and antibiotic cocktail (1% ampicillin, 1% cloxacillin and 0.32% gentamicin; Sigma, St-Louis, MO, USA). After the addition of the adequate amount of the different compounds, cells were incubated for 24 h 37 °C and 5% CO_2. Subsequently, they were centrifuged, the supernatants were removed and it was added 220 μl of the MTT solution (20 μl of 5 mg/ml MTT solution in water (3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazolio bromide) diluted in 200 μl of Opti-MEM). After 4 h of incubation at 37 °C in darkness, the supernatant was decanted and 200 μl DMSO were added to dissolve the formed formazan crystals. Absorbance was measured at 570 nm with a reference of 690 nm in a plate-reader.

MTS assay was performed to test Hec-1A and VK-2 cells viability. The cells were seeded in a 96-wells plate at a density of $3\cdot10^4$ cells/well and incubated for 24 h at 37 °C with 5% CO_2. The cells were then treated with the compounds at appropriate concentrations for 24 h at 37 °C. Afterward, 100 μl of the supernatant were removed and we added 20 μl of MTS (3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxifenil)-2-(4-sulfofenil)-2H-tetrazolio, Promega) solution. For the treatment with metal compounds, it was necessary to eliminate all the supernatant to avoid interferences with the substrate, and incubate in new medium for 2 h at 37 °C in darkness. The absorbance was measured in each well at 490 nm, on a plate reader.

In both MTT and MTS assays, we considered non-toxic concentrations those which revealed viability ≥80%. As controls, we used mock cells (100% viability), DMSO 10% (0% viability) and dextran 5 or 10 μM (complex branched polymer of glucose used as macromolecular control, Sigma–Aldrich).

2.2.3. Infection with viral strains

Two types of viral strains were used for conducting the experiments of infection: X4 HIV-$1_{NL4.3}$ (laboratory isolated strain with X4 tropism, specific of the CXCR4 co-receptor) and R5 HIV-1_{Bal-1} (laboratory isolated strain with R5 tropism, specific of the CCR5 co-receptor). The amount of virus added to the cells was 100 ng (Hec-1A, VK-2) or 10 ng (PBMC) per million cells, and infection was carried out for 2.5 h at 37 °C.

2.2.4. Quantification of $p24^{gag}$ antigen through ELISA

The amount of HIV-1 in the different viral isolates was quantified using a commercial immunoassay (ELISA, INNOTEST® HIV antigen mAb, Innogenetics N.V., Zwijndrecht, Belgium), according to the manufacturer's instructions.

2.2.5. Inhibition of HIV infection

Inhibition assays were carried out in Hec-1A, VK-2 and PBMC, previously seeded at a density of $3\cdot10^4$ (VK-2, Hec-1A) or $3\cdot10^5$ (PBMC) cells/well in complete medium.

2.2.5.1. Pre-treatment assay. The cells were pre-treated, immediately after seeding (PBMC) or after 24 h of incubation at 37 °C (VK-2, Hec-1A), for the selected time (30 min, 1 h) depending on the experiment. Subsequently, they were infected with the selected viral isolate for 2.5 h, washed 3 times and incubated at 37 °C and 5% CO_2. The supernatant was collected at 24 or 72 h post-infection, and the production of p24 Ag was quantified by ELISA.

2.2.5.2. Post-treatment assay. Immediately after seeding, PBMC were infected with X4-HIV-$1_{NL4.3}$ strain for 2.5 h. Then, they were repeatedly washed and treated with the compounds at appropriate concentrations. The culture supernatant was collected at 24 or 72 h depending on the experiment, and the production of p24 Ag was quantified by ELISA.

In these assays, different controls were used: untreated infected cells (100% infection); Suramin 10 μM, inhibitor of electrostatic attachment of the V3 region of HIV-1 envelope glycoprotein gp120 to galactosylceramide used as positive control of inhibition (Sigma–Aldrich); T-20 20 μM, Fuzeon, inhibitor of the fusion of HIV-1 with cells by binding to gp41 used as positive control of inhibition (Genetech USA, Inc. And Trimeris Inc.), AMD3100 100 μM, a highly specific antagonist to CXCR4 that blocks T-tropic viral variants and dual-tropic (R5/X4) (Sigma–Aldrich); Tenofovir 0.5 μM (TDF) is a cyclic nucleoside phosphonate analog; dendrimers G2S (**2**) and G2C (**5**) 10 μM (dendrimers controls), and $NiBr_2$, $CoCl_2$, $CuCl_2$ and $ZnSO_4$ 90 μM (metal salts alone controls, Sigma–Aldrich).

2.2.6. Virus internalization assays

Hec-1A cells were seeded in 24-well plates at a density of $1.2\cdot10^5$ (lysates) or $5\cdot10^5$ (cytometry) cells/well. After 1 h pre-treatment with compounds and infection with X4-HIV-$1_{NL4.3}$ strain for 2.5 h, cells were repeatedly washed with PBS, cleaved from the dish with trypsin and centrifuged to obtain the different cellular pellets. Then, the amount of p24 Ag was quantified through two different strategies. In the first of them, cell pellets were lysated with Triton X-100 (0.2% in PBS), centrifuged and the supernatant was isolated to quantify p24 Ag by using ELISA. In the second one, cells were permeabilized by incubation with Cytofix-Cytoperm for 1 h at 4 °C, and after washing with Wash Perm, the samples were incubated with anti-p24 KC57-FITC fluorescent antibody for 1 h, at 4 °C in dark. Again, cells were washed with Wash Perm repeatedly to eliminate all non-specifically-attached antibodies, and they were fixed with *p*-formaldehyde 2%. After washing and suspension in PBS (with 2% FBS), internal fluorescence was measured by FACS (Fluorescence-Activated Cell Sorting) flow cytometry.

3. Results and discussion

Our anionic poly(propyleneimine) dendrimers with an ethylenediamino core showed an appealing metal complexing ability towards divalent metal ions such as Cu^{2+} [46]. They exhibited a specific coordinating pattern and can be exploited as versatile metal carriers. According to the nomenclature used in our previous article, we followed the Meijer's naming system for poly(propylene imine) dendrimers: Core-dendr-termini#, where # indicates the number of terminal groups in the structure; however, dendrimers will be renamed for simplicity. We evaluated sulfonate dendrimers EDA-dendr-$(SO_3Na)_8$ (**1**, generation 1, G1S), EDA-dendr-$(SO_3Na)_{16}$ (**2**, generation 2, G2S) and EDA-dendr-$(SO_3Na)_{32}$ (**3**, generation 3, G3S); and carboxylate dendrimers EDA-dendr-$(CO_2Na)_8$ (**4**, generation 1, G1C), EDA-dendr-$(CO_2Na)_{16}$ (**5**, generation 2, G2C) and EDA-dendr-$(CO_2Na)_{32}$ (**6**, generation 3, G3C). The corresponding metallodendrimers are depicted as **X**-M_n, where X indicates the dendritic scaffold according to the previous numeration (**1**–**6**), M the type of metal ion (Ni, Co, Cu, Zn) and n the number of metal ions in the structure. In this study, the addition of increasing equivalents of the metal ions (M = Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}) to water solutions of polysulfonate (**1**–**3**) or polycarboxylate (**4**–**6**) dendrimers, led to the formation of light colored solutions of metallodendrimers at appropriate concentration (see Materials and methods). These different dendrimer:metal (D:M) ratios are related to different positions of coordination in their structure (G1, ratios 1:1, 1:3 and

1:5; G2, ratios 1:1, 1:5 and 1:9; and G3, ratios 1:1, 1:9 and 1:17). With the aim of discovering if these new metallodendrimers can act as potential microbicides, we studied their ability to inhibit HIV-1 crossing firstly the cervico-vaginal epithelium -modeled in this study by the epithelial cell lines of uterus and vagina Hec-1A and VK-2- and secondly the blood barrier -modeled by the primary PBMC.

3.1. Biocompatibility

The toxicity of the dendrimers and metallodendrimers in Hec-1A and VK-2 epithelial cell lines was evaluated by MTS assay. The MTT assay was performed in PBMC (see Materials and methods).

The toxicity of carboxylate and sulfonate dendrimers was evaluated at increasing concentrations of 1, 5, 10 and 20 μM in different cells (Fig. 2). In VK-2 and Hec-1A epithelial cell lines no signs of toxicity appeared for any dendrimer. In PBMC, the range 10–20 μM revealed proximity to the arbitrary 80% viability cut off point, but the tendencies point to a safe concentration of 10 μM.

Once established that a dendrimer concentration of 10 μM was non-toxic in Hec-1A, VK-2 and PBMC, we assessed the biocompatibility of the metal derivatives of Ni, Co, Cu, and Zn of the precursor dendrimers, at different dendrimer:metal ratios in the same cells. Biocompatibility values were compared with the metal salts $NiBr_2$, $CoCl_2$, $CuCl_2$ and $ZnSO_4$ at the maximum concentration used in each assay, to analyze the effect of coordination of the metal ions into the dendritic structure.

In Hec-1A cells (Fig. 3A), the anionic dendrimers (0 eq. of M^{2+}) and their metallodendrimers (1–17 eq. of M^{2+}) were biocompatible. For Ni, Co and Cu, the coordination of the metal ions to the dendrimer scaffold significantly increased the biocompatibility of the free metal ions. A clear example was observed when we compared the copper control (84% viability) to **3**-Cu_{17} (98%) or **6**-Cu_{17} (96%), all of them with the same copper concentration of 170 μM (Fig. 3A). Thus, higher amounts of metal ions can be administered when they are coordinated to these dendritic architectures. We also observed a particular generation effect, as second-generation systems presented a higher compatibility than their first and third-generation analogs. Therefore G2 systems were chosen to evaluate their effect as inhibitors of HIV-1 infection. To corroborate the results observed in Hec-1A, the same experiment was performed in VK-2 epithelial cell line with G2 candidates (Fig. S1). In this case, all tested compounds were biocompatible, with the exception of those systems with Zn^{2+} concentration of 90 μM (Zn control and **2**-Zn_9). However, the coordination of metal ions to the dendrimer again enhanced their compatibility in relation to the free metal ions, even in the case of this particular metallodendrimer which was duplicated (from 17% to 34%).

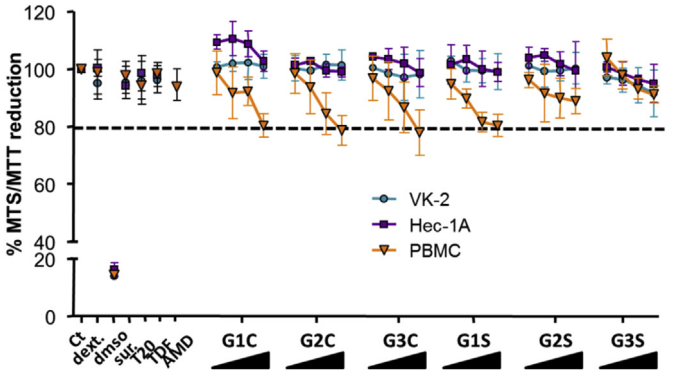

Fig. 2. Dendrimers biocompatibility in different cells. Biocompatibility assay of the carboxylate and sulfonate dendrimers in VK-2 and Hec-1A (MTS assay) and PBMC (MTT assay) at concentrations of 1, 5, 10 and 20 μM. Controls: mock cells, dextran (10 μM), DMSO (10%), Suramin (10 μM), T-20 (20 μM), TDF (0.5 μM) and AMD (100 μM).

After the evaluation of toxicity in epithelial cell lines, biocompatibility was assessed in PBMC as second cell barrier against HIV-1 infection. As Fig. 3B shows, in this primary culture most of tested compounds did not present evidence of toxicity with the exception of those at the maximum Co^{2+} concentration (**3**-Co_{17} and **6**-Co_{17}). Moreover, second generation systems, which were chosen ideal candidates in epithelial cell lines, were also adequate in PBMC for the purpose of the study. Again, the sulfonate derivatives were slightly less toxic that the carboxylate compounds and coordination of the metal to the dendritic scaffold increased the biocompatibility of the final metallodendrimer.

3.2. Inhibition of HIV-1 infection

The ability of the different compounds to inhibit HIV-1 infection in different cell lines and primary cell cultures was evaluated. As established through the biocompatibility tests, metal complexes from second generation candidates G2S (**2**) and G2C (**5**) were analyzed. To discriminate between a putative effect attributed to the non-complexed metal ions and that of the complex itself the inhibitory efficiencies of the complexes and the metal salts $NiBr_2$, $CoCl_2$, $CuCl_2$ and $ZnSO_4$ were compared under the same experimental conditions.

3.2.1. First barrier model: epithelial Hec-1A and VK-2 cells

Although epithelial cells are barely infected by HIV-1, they are the first line of defence against this infection, so it is important to carry out a full study of the inhibitory activity of the compounds in a model of this first barrier, such as Hec-1A and VK-2 cell lines. Indeed, Hec-1A cells have proved to be refractory to cell-free viral infection but capable of sequestering large number of HIV-1 particles on their apical pole and also endocytose a weak proportion of the attached virus and pass it along to underlying cells [48]. An effective antiviral behavior in this barrier reduces the probability of infection of target cells by HIV-1 in subsequent steps.

3.2.1.1. Inhibition of HIV-1 infection. The inhibitory behavior of the dendritic systems was tested at a pre-treatment time of 1 h, to evaluate the preventive effect of the compounds towards HIV-1 infection (Materials and methods). In brief, epithelia cells were pre-treated with the compounds for 1 h and subsequently infected with X4-HIV-$1_{NL4.3}$ isolate for 2.5 h and then the virus was removed. Culture supernatant was collected 24 and 72 h after infection to evaluate the production of p24 antigen. In this experiment, several control samples were used: untreated X4-HIV-$1_{NL4.3}$ infected cells (positive control, 100% infection), Suramin, T-20, TDF and metal controls ($NiBr_2$, $CoCl_2$, $CuCl_2$ and $ZnSO_4$).

At 24 h, sulfonate dendrimer (**2**) and carboxylate dendrimer (**5**) in their sodium salt forms presented an inhibition of 50% and 30%, respectively, increasing 10% their effectiveness at 72 h (Fig. 4A). In metal complexes, the inhibitory effect diminished by increasing the metal ratio, probably because the anionic groups became less available for their inhibitory action (Fig. S2). At 24 h, carboxylate 1:1 complexes had an inhibition of 80% (Co and Zn) and 90% (Cu) (Fig. 4B), while the sulfonate complexes showed activities of 40% (Co) and 60% (Cu and Zn). Nickel derivatives even decreased the inhibitory capacity of the dendrimer itself, so they were discarded for the following studies. After 72 h, Ni and Co complexes increased their HIV-1 inhibitory ability, reaching a level of 95–100% for Co systems. This tendency was kept in Cu and Zn sulfonate complexes, whereas the analogous carboxylate systems slightly decreased their activity. In all cases, the HIV-1 inhibition of the

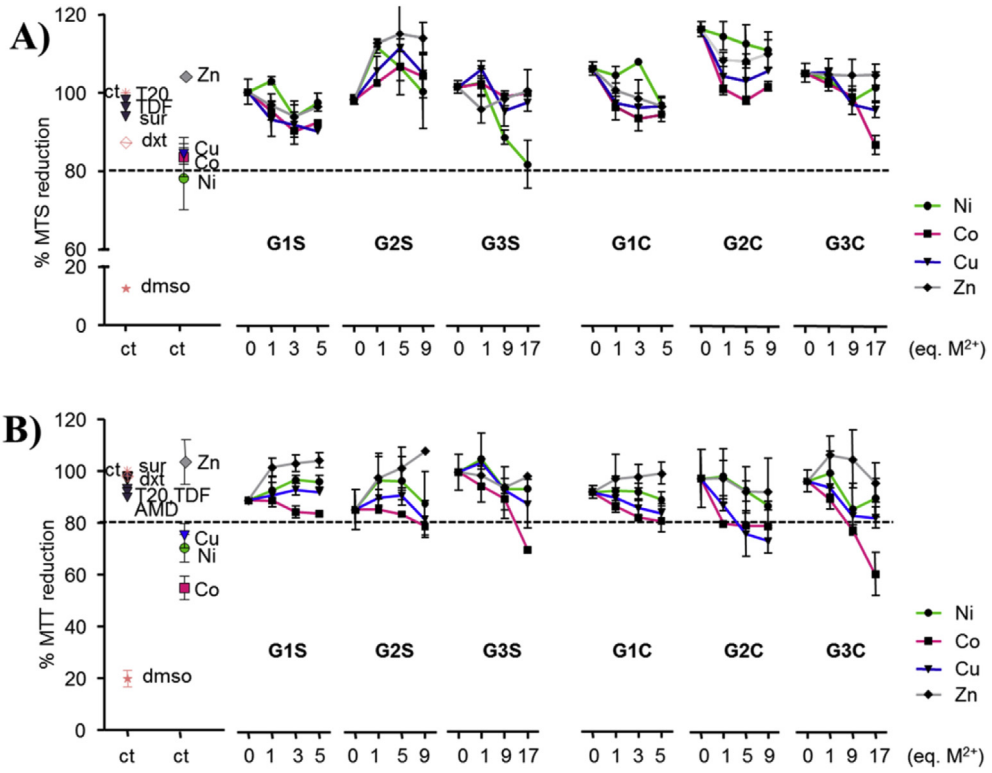

Fig. 3. Metallodendrimers biocompatibility in different cells. Biocompatibility assays in A) Hec-1A cells (MTS assay) and B) PBMC (MTT assay) of the anionic dendrimers (10 μM) with metals at different D:M ratios. Controls: mock cells, dextran (10 μM), DMSO (10%), Suramin (10 μM), T-20 (20 μM), TDF (0.5 μM), AMD (100 μM) and the corresponding metal controls at 170 μM.

metallodendrimers was significantly higher than that of the metal and commercial controls.

Pre-treatment time was optimized with a comparative study at 30 min and 1 h, through the standard protocol (Materials and methods), with a final time of treatment of 24 h (Fig. S3). A 30 min pre-treatment was enough for the dendrimers to exert their maximum HIV-1 inhibitory activity (around 50%), which was maintained over time. However, a longer treatment with the metallodendrimers produced a higher HIV-1 inhibition rate. Thus, the optimal pre-treatment time was established in 1 h.

A third experiment was performed through the standard protocol, in this case in VK-2 cells, to check if the behavior of our compounds was consistent in other epithelial cell line. The test was carried out with a set of selected compounds that showed significant HIV-1 inhibition in Hec-1A cells (Fig. S4). A similar effect was observed in both systems: dendrimers presented similar or slightly higher levels of inhibition in this cell line and the metal complexes presented the previously observed trend, although at lower levels.

3.2.1.2. HIV-1 strain and co-receptor specificity. The HIV-1 infection is triggered when the protein complex gp120-gp41 on the viral surface is attached to the CD4 cell receptor [49]. This contact induces a conformational change on gp120 which subsequently binds to a cellular co-receptor and triggers the final fusion between the viral and cellular membrane. Different HIV-1 strains use the main co-receptors CCR5, CXCR4 or both, which exert a different role in the infection process. While CCR5 co-receptor is the main responsible of HIV-1 infection in the acute phase, the course of the disease leads to HIV-1 strains which bind CXCR4 and are related to a faster disease progression [50]. In the particular case of Hec-1A cells, Bélec et al. demonstrated that R5- and X4-HIV-1 differentially use these cells to ensure their own spread [48]. Therefore, we used R5-HIV-1_{Bal} and X4-HIV-$1_{NL4.3}$ strains with the aim of establishing a possible specificity of the compounds by one, another or both virus strains and co-receptors (Fig. 5).

The dendritic macromolecules exhibited higher inhibition of X4-HIV-$1_{NL4.3}$ strain, except in the case of nickel compounds, which did not present any significant effect on this strain. In addition, the trend observed on X4-HIV-$1_{NL4.3}$ was consistent on R5-HIV-1_{Bal}, decreasing the inhibition when increasing the amount of metal in the dendritic structure. The combination of polyanionic end-groups and metal centers in the same dendrimer enhances the antiviral activity, particularly when not all anionic groups are bound to metal ions. The increase on the amount of metal ions produces the saturation of the peripheral anionic groups, which become less available for their inhibitory action, and consequently the total inhibitory effect decreases. Therefore, it is very important to optimize the amount of metal ion in the coordination with the dendrimer to balance the benefits associated to a high biocompatibility and possible disadvantages associated to reduced inhibitory capacity by overload the terminal anionic groups of the dendrimer with metal ions.

In relation to the type of metal ion, we observed that Co complexes seem to be more active in X4-HIV-$1_{NL4.3}$, those of Ni present little activity in R5-HIV-1_{Bal}, and Cu and Zn seem to act in both of them although more effective in X4-strain. This trend was not previously observed in assays with metal complexes from generation zero [44,45] and could be related to the dendritic structure. It has been shown that Hec-1A cells lack of CCR5 co-receptor, but do express CXCR4 co-receptor and the alternative viral receptor GalCer [48]. However, this cell line was capable of concentrating and sequestering significant amounts of both R5- and X4-strains at their apical pole, although X4-HIV strains were bound more efficiently due to the higher affinity of the X4-gp120 to the cell-surface

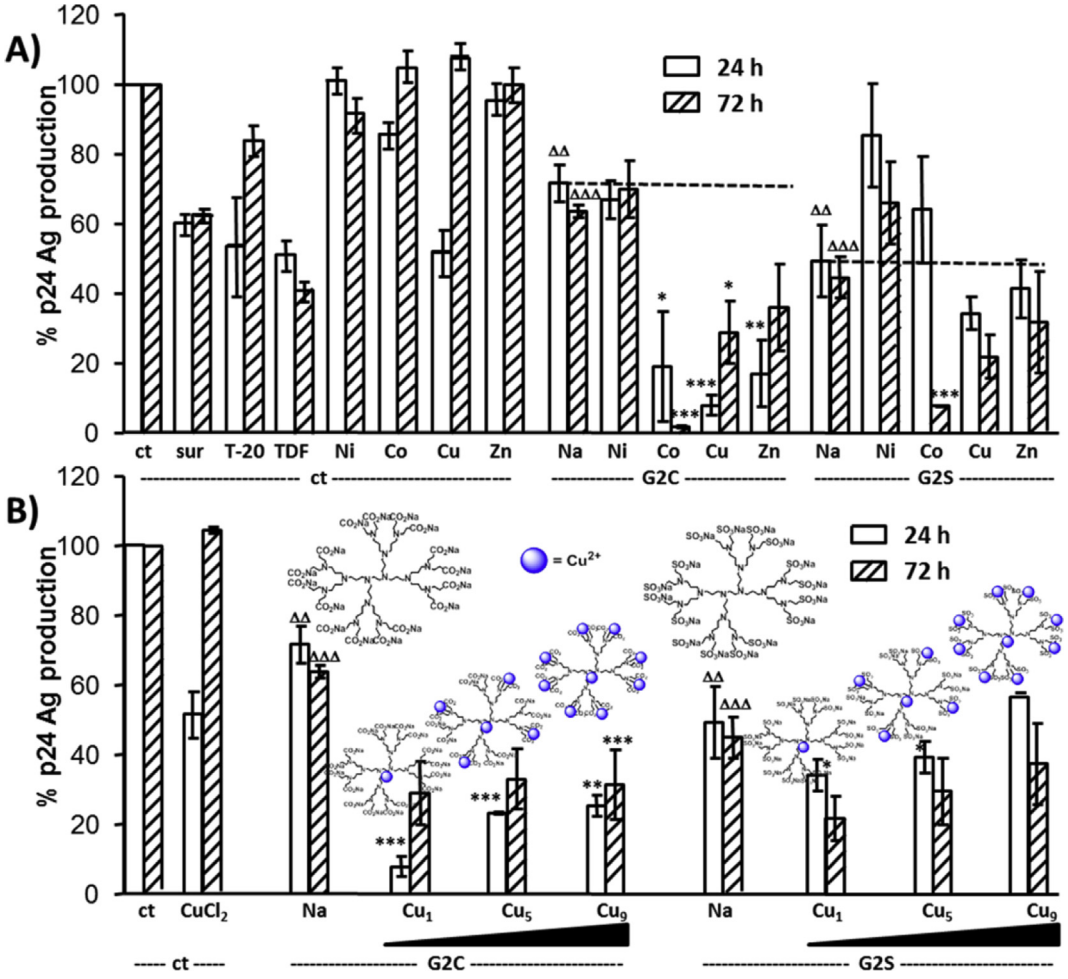

Fig. 4. Inhibition of HIV-1 infection in Hec-1A after pre-treatment with the compounds. Hec-1A cells were pre-treated with the compounds (10 μM) for 1 h and subsequently infected with X4-HIV-$1_{NL4.3}$ isolate for 2.5 h and then the virus was removed. The supernatants were collected 24 and 72 h later measuring p24 Ag by ELISA. A) Treatment with dendrimer:metal ratio of 1:1. B) Treatment with dendrimer:metal ratio of 1:1, 1:5 and 1:9 when M = Cu^{2+}. The results for M = Ni^{2+}, Co^{2+} and Zn^{2+} are depicted in Supporting Information. Controls: untreated cells, Suramin (10 μM), T-20 (20 μM), TDF (0.5 μM) and metal controls (90 μM). The results are the average of 2 experiments. Statistical comparison was performed in relationship with the sodium salt dendrimer [$p < 0.01$ (***), <0.05 (**) and <0.1(*)], and it was also compared to untreated cells [$p < 0.01$ ($^{\Delta\Delta\Delta}$), $p < 0.05$ ($^{\Delta\Delta}$)].

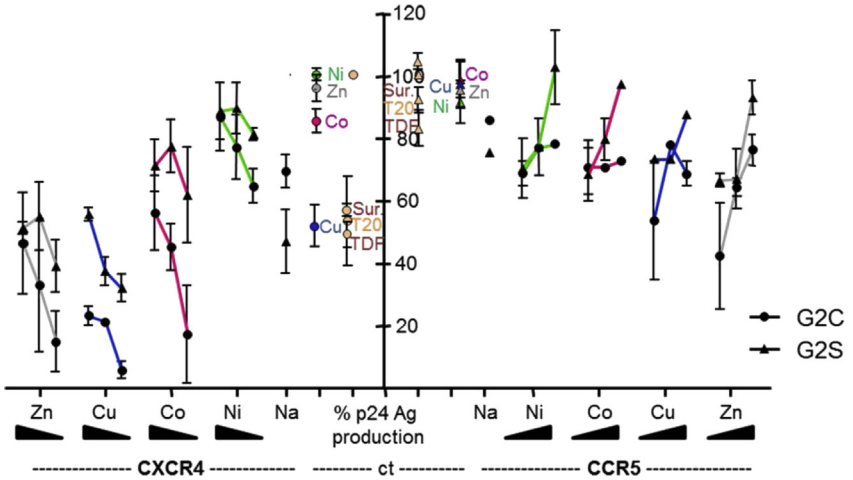

Fig. 5. Comparative inhibition of HIV-1 infection with X4-HIV-1 or R5-HIV-1 strains in Hec-1A after pre-treatment with the compounds. Hec-1A cells were pre-treated with the compounds (10 μM) for 1 h and subsequently infected with X4-HIV-$1_{NL4.3}$ (left) or R5-HIV-1_{Bal} (right) isolate for 2.5 h and then the virus was removed. The supernatants were collected 24 h later measuring p24 Ag by ELISA. Controls: untreated cells, Suramin (10 μM), T-20 (20 μM), TDF (0.5 μM) and metal controls (90 μM). The results are the average of two independent experiments.

heparan sulfate. In Hec-1A cells, our metallodendrimers could interact with both the CXCR4 co-receptor and the gp120 of the HIV-1 through their negative charges and metal ions. A higher inhibition is thus expected for X4-HIV strains, which is in agreement with the experimental results. Nevertheless, further experiments are required to confirm the interaction with the co-receptors and/or the virus proteins. Despite the mild inhibition observed for R5-HIV-1_{Bal}, the target strain when designing a microbicide, significant results were obtained for X4-HIV-$1_{NL4.3}$. This is also a relevant fact for our purpose, as Hec-1A present a highlighted role as X4 virus reservoir [48].

3.2.1.3. HIV-1 internalization in Hec-1A cells. HIV-1 internalization process in epithelial cells allows, on the one hand, the triggering of the infection, and on the other, the transmission to other cells. Hec-1A cells have the capacity to endocytose a weak proportion of the attached virus and pass it along the underlying cells [48]. The infectivity of the virus recovered from the basolateral pole is reduced 25–50%. Compounds which prevent this process paralyze HIV-1 infection at the level of the first barrier, with the great interest that it entails. To corroborate the inhibitory action of the dendrimers in the first step of the replicative cycle of HIV-1, we performed a comparative study of HIV-1 internalization in Hec-1A cells (Materials and methods). The assay was initiated by the standard inhibition protocol in Hec-1A cells. After 24 h of HIV-1 infection, the amount of p24 Ag was quantified by A) lysating cells with Triton X-100 and performing p24 Ag ELISA in the culture supernatant, or B) attaching a fluorescent antibody anti-p24 KC57-FITC in permeabilized cells and measuring their internal fluorescence through FACS flow cytometry.

The results of both assays supported our previous HIV-1 inhibition studies, showing that the dendritic macromolecules avoided the internalization of HIV-1 into the cells (Fig. 6). The sodium salt forms of dendrimers G2S and G2C presented levels of HIV-1 inhibition between 20 and 30%, which increased up to 55% with some metal derivatives (Fig. 6). Again, the inclusion of metal ions in the dendritic structure increased its effectiveness, as it can be deduced from the comparison with controls metal for each one of the techniques. In addition, certain compounds presented an efficacy higher than commercial drugs Suramin and T-20.

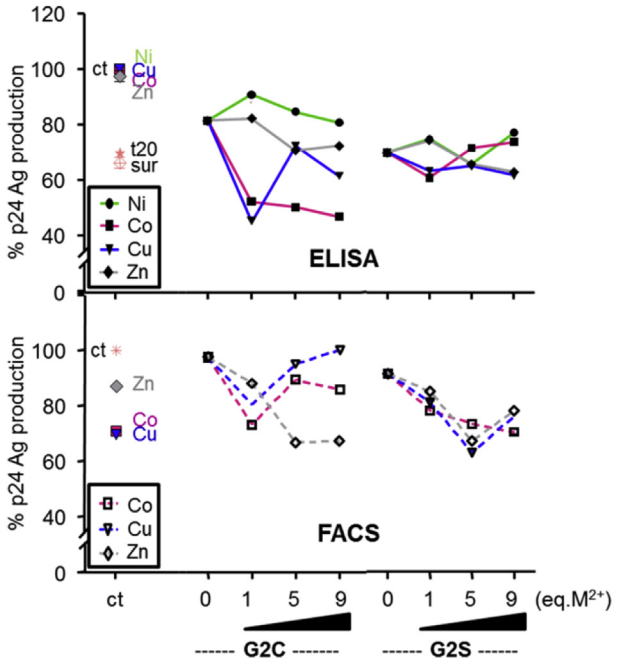

Fig. 6. The dendritic macromolecules avoid HIV-1 internalization inside Hec-1A cells. Inhibition of HIV-1 internalization (% p24 Ag production) in Hec-1A after 1 h pre-treatment with the compounds (10 μM). Comparison of results obtained by ELISA and FACS. Controls: mock cells, Suramin (10 μM), T-20 (20 μM) and metal controls (90 μM). HIV-1 infection was carried out with X4-HIV-$1_{NL4.3}$ strain. The graph shows the results from one independent experiment.

3.2.2. Second barrier model: PBMC

Once we demonstrated the ability of the compounds to inhibit the entry of HIV-1 in VK-2 and Hec-1A epithelial cell lines, we evaluated their action in PBMC, as model of the second barrier against HIV infection. Two different studies were conducted to study the preventive behavior (pre-treatment assay) and the therapeutic properties (post-treatment assay), whose protocol can be seen in the Materials and methods section. The pre-treatment assay was performed treating the cells with the compounds for 1 h and then infecting them with X4-HIV-$1_{NL4.3}$ strain for 2.5 h. In the post-treatment assay PBMC were first infected with X4-HIV-$1_{NL4.3}$ strain and subsequently treated with the compounds for 24 h. In both cases, after the removal of the X4-HIV-$1_{NL4.3}$ strain, the culture supernatant was collected 24 h later to quantify the amount of p24 Ag by ELISA.

Sodium salt dendrimers G2S and G2C produced X4-HIV-$1_{NL4.3}$ inhibition of 20–30% in PBMC, both at pre- and post-treatment (Fig. 7 and Fig. S6). Pre-treatment with carboxylate metal complexes increased the inhibition rate, especially for copper (50%) and zinc (35%) systems, while for the sulfonate complexes the differences were not significant. Again, the trend observed in epithelial cells appeared, where the X4-HIV-$1_{NL4.3}$ inhibition decreased by increasing the concentration of metal, due to the reduced availability of anionic groups to exert their action (Fig. S6). The most successful inhibition rates obtained in the pre-treatment assay were similar to some commercial controls like Suramin and T-20. However, in post-treatment assays the trend was reversed in the case of carboxylate compounds, with a higher inhibition rate in metallodendrimers at the maximum concentration of metal (Fig. S6). This result is consistent with the higher strength of the COO-M interaction [46] which holds the metal ions together until the internalization into the cell, and their subsequent release to exert a therapeutic effect. In all cases, we observed an additive effect between the activity of the dendrimer and the free metal ion.

4. Conclusions

We have demonstrated that PPI dendrimers with EDA core and carboxylate and sulfonate groups in the periphery were biocompatible up to a concentration of 20 μM in Hec-1A and VK-2 epithelial cell lines and PBMC. Likewise, the metal complexes of Ni, Co, Cu and Zn prepared from them at different dendrimer:metal ratios were also biocompatible. The capacity of these dendrimers to decrease metal ions toxicity by means of their coordination into the dendritic scaffold is a highly valuable property for drug delivery agents.

In Hec-1A and VK-2 epithelial cell lines, as model of the first barrier against HIV-1 infection, pre-treatment with some second generation systems produced high viral inhibition rates (up to 90% at 24 h, around 100% at 72 h). For all metals, the inhibition rate decreased by increasing the ratio of metal ion, due to the saturation of the peripheral anionic groups. The most successful inhibition was achieved with metallodendrimers **2**-M_1 and **5**-M_1, where there is a real cooperative effect between free anionic groups and the metal atom towards the interaction with viral and cellular proteins. This behavior has been previously detected in different metal complexes like sulfonated and carboxylated porphyrins and

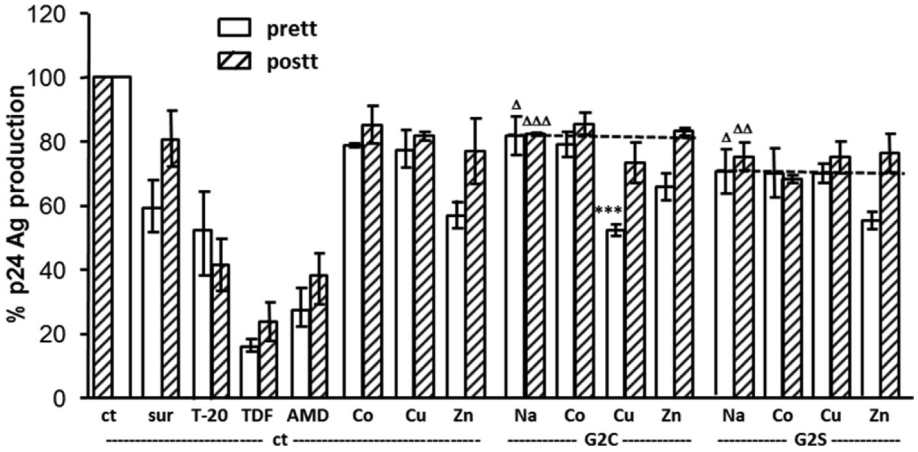

Fig. 7. The dendritic macromolecules inhibit HIV-1 infection in PBMC in pre and post-treatment. PBMC were treated with the compounds (10 μM, dendrimer:metal ratio of 1:1) for 1 h and then infected with X4-HIV-$1_{NL4.3}$ strain for 2.5 h (pre-treatment), or first infected with X4-HIV-$1_{NL4.3}$ strain and subsequently treated with the compounds for 24 h (post-treatment). 24 h after the removal of the X4-HIV-$1_{NL4.3}$ strain, the supernatants were collected and measured p24 Ag by ELISA. Controls: mock cells, Suramin (10 μM), T-20 (20 μM), TDF (0.5 μM), AMD (100 μM) and metal controls (90 μM). The results are the average of two independent experiments. The statistical comparison was carried out with respect to the sodium salt dendrimer [$p < 0.01$ (***), <0.05 (**) and <0.1 (*)], while it was compared with mock cells [$p < 0.01$ ($^{\Delta\Delta\Delta}$), $p < 0.1$ ($^{\Delta}$)].

phtalocianins and their metal complexes [26] or platinum complexes with sulfonated triazines [51], where the combination of anionic groups and metal centers in a same molecule enhances the antiviral activity, especially when the anionic groups are not directly bound to the metal ion. Moreover, anionic PPI dendrimers also prevented the internalization of HIV-1 into cells and the interaction of metal ion in the dendritic structure potentiated the inhibitory activity prominently. Contrary as previously observed with analogous metal complexes [39,40], these metallodendrimers presented certain viral strain specificity, correlated to the type of metal ion. This fact, together with the possibility of controlling the amount and type of metal ions in the dendritic structure opens new avenues for the design of personalized antiviral treatments.

In PBMC, as model of the second barrier against HIV-1 infection, both pre-treatment and post-treatment with our dendrimers and their metal complexes produced HIV-1 inhibition rates in the order of the commercial inhibitors (Suramin and T-20), or even higher in the case of 1:1 of Cu and Zn ratio. Again a decrease in HIV-1 inhibition was observed by increasing the amount of metal, trend that was reversal in post-treatment with carboxylate systems, probably by the greater interaction with metal atoms that allows a better internalization within cells.

Similarly to the analogous metal complexes [43–45], these metallodendrimers point to a dual behavior, preventing HIV-1 binding to the cell, through the blockade of the proteins of membrane of the virus and the cell through the anionic groups, interaction that is modulated by the metal center, and on the other hand, acting on subsequent steps of the viral replicative cycle. Nevertheless, our results demonstrate that the presence of a poly(-propyleneimine) scaffold with an ethylenediamino core drastically modifies the properties of the final metallodendrimers when compared to analogous carbosilane macromolecules [43]. Despite their lower biocompatibility, comparable treatment conditions of Hec-1A and PBMC reveal a significant inhibition of PPI metallodendrimers using smaller concentrations and lower metal doses.

Overall, we demonstrated that their antiviral properties are both modulated by the type of dendritic scaffold (backbone, peripheral group, generation) and the metal ion bounded to it (type, amount), and also their time and dose dependence. These preliminary results present our metallodendrimers as potential candidates as inhibitors of HIV-1 infection, with controllable properties. A possible topical application of these compounds would inhibit the HIV-1 infection at the epithelial level (both vaginal and uterine stages), reducing also the internalization of the virus in these cell types, in agreement with the pre-treatment experiments carried out in VK-2 and Hec-1A cells. In the case that some viral particles or HIV-1 infected cells reached the sub-epithelium, these compounds could inhibit T-cells infection, as well as reduce the damage of previously infected cells, as observed in pre and post-treatment assays in PBMC. By combining the properties of the dendritic scaffold and those of the metal moieties, these metallodendrimers open new avenues for the design of tunable and efficient treatments against HIV-1 infection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.05.026.

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