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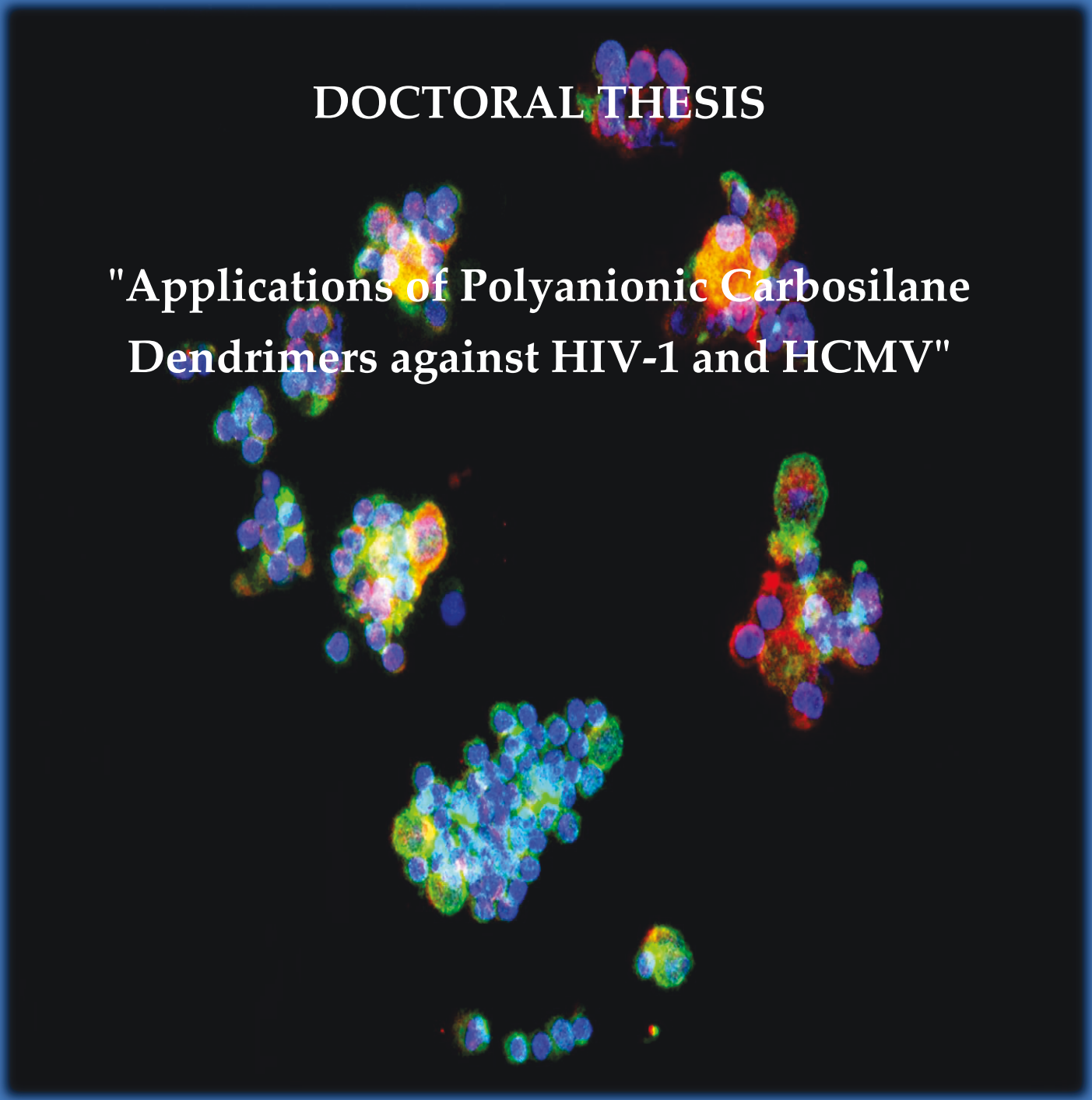
DOCTORAL THESIS

"Applications of Polyanionic Carbosilane Dendrimers against HIV-1 and HCMV"



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Tesis Doctoral

" Applications of Polyanionic Carbosilane Dendrimers against HIV-1 and HCMV"

Memoria presentada por el graduado en Biotecnología

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CERTIFICA QUE,

el trabajo de investigación y la redacción de la Tesis Doctoral titulada: " Applications of Polyanionic Carbosilane Dendrimers against HIV-1 and HCMV" ha sido realizado por Ignacio Rodríguez Relaño, bajo mi dirección. Revisado el trabajo, considero este como satisfactorio y autorizo su presentación y defensa para optar al grado de Doctor con Mención Internacional por la Universidad Autónoma de Madrid.

Y para que quede constancia de ello, firmo el presente documento.

Madrid, 15 de octubre de 2021

Fdo. Dra. MªÁngeles Muñoz Fernández

A mi familia

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List of abbreviations

7AAD	7-Aminoactinomycin D
AIDS	Acquired Immune Deficiency Syndrome
APCs	Antigen Presenting Cells
ARTc	Antiretroviral therapy combination
ATCC	American Type Culture Collection
BBB	Blood-Brain Barrier
BRY	Bryostatin
BSA	Bovine Serum Albumin
CCR	Chemokine (C-C motif) receptor. It can be extended to the rest of abbreviations: CCR2, CCR3 and CCR5
CD	Cluster of differentiation. It can be extended to the rest of abbreviations: CD3, CD4, CD8…
CDC	Center for Disease Control and Prevention
CDV	Cidofovir
CNS	Central nervous system
CTL	Cytolytic T lymphocytes
CXCR4	Chemokine (C-X-C motif) receptor 4
DCs	Dendritic cells
DE	Delayed early gene
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. Coli	*Escherichia coli*
EC_{50}	Half maximal effective concentration
ELISA	Enzyme linked immunosorbent assay

Env	Gene encoding the structural *env* polyprotein of HIV-1
ET	Electron transfer
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGT	Female genital tract
FITC	Fluorescein isothiocyanate.
FRET	Fluorescence resonance energy transfer
FOS	Foscarnet
G0, G1, G2,G3	Generations 0, 1, 2, 3 of dendrimers according to the number of layers with branching units forming the dendrimer
G2-S16	A second-generation sulfonated dendrimer fully capped with 16 sulfonate groups
gag	Group-specific antigen. Gene encoding the structural gag polyprotein of HIV-1
GALT	Gut-associated lymphoid tissue
GCV	Gancivlocir
GM-CSF	Granulocyte/macrophage colony-stimulating factor
gp120	Surface glycoprotein exposed on the surface of HIV-1 envelope
gp41	Transmembrane envelope glycoprotein of the HIV-1
HCMV/ HHV-5	Human Cytomegalovirus/ Human Herpes virus 5
HDACi	Histone deacetylase inhibitors
HIV-1	Human Immunodeficiency Virus Type 1
HSV-1	Herpes Simplex Virus Type 1
IE	Immediate early gene
IL-2	Interleukin 2. It can be extended to the rest of interleukins

LDH	Lactate dehydrogenase
LRA	Latency Reversal Agent
LTR	Long terminal repeat
LTV	Letemovir
M-CSF	Macrophage colony-stimulating factor
MDM	Monocyte-derived-macrophage
mRNA	Messenger ribonucleic acid
MTB	*Mycobacterium tuberculosis*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MVC	Maraviroc
NBs	Nano-belts
Nef	Negative regulatory factor
NIH	National Institutes of Health
NK	Natural killer
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
NWs	Nano-wires
PAMAM	Polyamidoamine dendrimer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCD	Polyanionic carbosilane dendrimer
PCR	Polymerase chain reaction
PKC	Protein Kinase C
PNB	Panabinostat
PPI	Polypropylene-imine dendrimer
PST	Prostatrin
QDs	Quantum dots

RMD	Romidepsin
RNA	Ribonucleic Acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase of HIV-1
RT-qPCR	Real time quantitative polymerase chain reaction
RUNX1	Runt-related transcription factor 1
SD	Standard deviation
STAT5	Signal transducer and activator of transcription 5
STI	Sexually transmitted infection
tat	Trans-activator of transcription
TLR	Toll-like receptor
UL	Unique long region
UNAIDS	United Nations Programme on HIV and AIDS
US	Unique sort region
vif	Viral infectivity factor
VGCV	Valganciclovir
Vpr	Viral protein r
Vpu	Viral protein unique
WHO	World Human Organization

Abstract

Viral outbreaks are one of the main foci nowadays due to the actual Sars-Cov2 pandemic. Therefore, new therapies and treatments are needed to fight, not only the new viral challenges, but also viruses that in several countries have caused a non-stop pandemic since several years ago, as Human Immunodeficiency Virus (VIH) or Human Cytomegalovirus (HCMV). The high cost of present treatments and the lack of routinely tests in these countries urge the necessity to develop new molecules or strategies against these viruses. Foremost, in immunosuppressed individuals, as HIV patients, HCMV infection can lead to dissemination and life-threatening end-organ diseases. The new treatments should be low-cost and capable of avoiding the emerging problem of resistant virus. Nanotechnology is rising as a promising alternative in response to the need of efficient, immediate and cost-effective therapies.

Dendrimers are a group of nanoparticles that have shown promising results against viral infectious diseases. In this work we demonstrated three different applications of Polyanionic Carbosilane Dendrimers. First, we showed the reactivation activity of G3-S16 in a monocytic derived cell line, and the promising results in the combination with the actual Latency Reversal Agents for the "Shock and Kill" approach. Second, we evaluated the ability of G2-S16 dendrimer to inhibit HIV cell to cell transmission in Monocyte Derived Macrophages (MDMs). We co cultured, treated, and infected MDMs with healthy T lymphocytes to measure cell to cell transmission, our results showed that G2-S16 is capable of inhibit HIV infectivity inside MDMs, thus reducing or eliminating reservoir or sanctuaries formation. Third, we demonstrated the potent inhibition activity of G2-S16 and G2-S24P against HCMV infection in human lung fibroblast. We studied the mechanism of action of these dendrimers, showing that in contrast with other viruses, G2-S16 and G2-S24P do not inhibit HCMV attachment in MRC-5. Moreover, we evaluated the combination of both dendrimers with the actual treatment, ganciclovir, the results showed an increased potency and time effect against HCMV infection.

Resumen

Los brotes virales son uno de los focos principales en la actualidad debido a la pandemia de Sars-Cov2. Por tanto, se necesitan nuevas terapias y tratamientos para combatir, no solo los nuevos retos virales, sino también los virus que en varios países han provocado una pandemia continua desde hace varios años, como el Virus de la Inmunodeficiencia Humana o el Citomegalovirus Humano. El alto coste de los tratamientos actuales y la falta de pruebas de rutina en estos países instan la necesidad de desarrollar nuevas estrategias. En primer lugar, en individuos inmunosuprimidos, como pacientes VIH, la infección por CMVH puede conducir a fallos de órganos principales, que pueden ser mortales. Los tratamientos deben ser de bajo costo y capaces de evitar el problema emergente de las resistencias. La nanotecnología está emergiendo como una alternativa prometedora en respuesta a la necesidad de terapias eficientes, inmediatas y rentables. Los dendrímeros son un grupo de nanopartículas que se han mostrado prometedoras contra enfermedades virales. En este trabajo mostramos tres aplicaciones diferentes de los dendrímeros polianiónicos carbosilanos. En primer lugar, mostramos la actividad de reactivación de G3-S16 en una línea celular derivada de monocitos, y los resultados prometedores en la combinación con los agentes de reversión de latencia actuales para la estrategia de "Shock and Kill". En segundo lugar, evaluamos la capacidad del dendrímero G2-S16 en la inhibición de la transmisión célula a célula del VIH en macrófagos derivados de monocitos (MDMs). Co-cultivamos MDMs infectados con linfocitos T para medir la transmisión célula a célula, los resultados mostraron que G2-S16 es capaz de inhibir la infectividad del VIH dentro de MDMs, reduciendo o eliminando la formación de reservorios o santuarios. En tercer lugar, demostramos la actividad inhibitoria de G2-S16 y G2-S24P contra la infección por HCMV en fibroblastos de pulmón humano. Estudiamos el mecanismo de acción de los dendrímeros, demostrando que, a diferencia de otros virus, G2-S16 y G2-S24P no inhiben la unión de HCMV en MRC-5. Además, evaluamos la combinación de dendrímeros con el tratamiento real, ganciclovir, los resultados mostraron una mayor potencia contra la infección por HCMV.

1. Introduction

1.1. Human Immunodeficiency Virus

1.1.1. Epidemiology

The first reported cases of acquired immunodeficiency syndrome (AIDS) were in the United States in 1981, and Human Immunodeficiency Virus (HIV) was first isolated in 1983. Since the start of the epidemic, nearly 80 million people have been infected with HIV-1, and around 32 million people have already died from AIDS-related diseases. Far from reaching the milestone 2020 targets, only 23.3 million people out of the 37.9 million people living with HIV-1 worldwide were accessing antiretroviral therapy (ART) in 2019, according to UNAIDS data. The annual number of AIDS-related deaths is the lowest since the 2004 peak, but there is a prevention crisis, as the number of new HIV-1 infections is not being reduced fast enough. Around 1.7 (1.2-2.2) million people became newly infected with HIV-1 in 2019, with most new infections occurring in South African countries. The objective for 2020 was 500,000 new HIV-1 infections per year, and nowadays, data are tripling the 2020 target milestone.

Even with the decreasing number of new infections each year, patients become chronically infected. The improvement in new treatments against non-AIDS-related deaths decreases the number of deaths, which increases the number of patients living with HIV-1. Thus, it encourages the scientific community to develop new treatments to avoid further infections and eliminate the chronic condition, which is one of the barriers that impede HIV-1 eradication.

HIV-1 sexual transmission infection (STI) is held accountable for around 80% of all infections, roughly half of the affected individuals being women [1, 2]. Sexual transmission is responsible for most HIV-1 infections due to sexual contact with infected cervicovaginal secretions or semen containing HIV-infected cells [3, 4]. Although female genital mucosa is a significant portal for entry of HIV-1 into the body, accounting for the initiation of 40% of global HIV-1 infections, acute events that follow HIV-1 exposure in the female genital tract (FGT) still remain unclear [5]. This difference in HIV-1 transmission and other biological factors likely explains the higher

incidence of STI in women [6]. The fundamental basis for controlling HIV/AIDS epidemic depends on developing new prevention methods and strategies due to the failures in synthesizing an efficient vaccine [7]. But even with these approaches, viral eradication is still far from being reached if efforts are not focused on eliminating viral reservoirs.

1.1.2. Viral structure and cycle

HIV-1 is an RNA virus that belongs to the genus *Lentivirus*, within the family of *Retroviridae*, subfamily Orthoretrovirinae. The genetic material of HIV-1 consists of two identical molecules of single-stranded RNA of 9.2-9.6 kb[8], flanked at both ends by long terminal repeat (LTR) sequences. The 5′ LTR contains the promotor for transcription, and the following genes from 5′ to 3′ are *gag*, *pol,* and *env*. Gag encodes structural proteins, including the outer core membrane p17, capsid protein p24, nucleocapsid p7, and a small nucleic acid stabilizing protein. Pol encodes the enzymes protease, RT, RNase H, and integrase. *Env* encodes for the envelope glycoproteins gp120 (surface) and gp41 (transmembrane) (Figure 1)[9]. Several regulatory proteins, namely Tat, Rev, Nef, Vif, Vpu, and Vpr, are also coded in the HIV-1 genome. The mature HIV-1 particle is sphere-shaped with a diameter of approximately 100 nm. The outer membrane is formed by a lipid bilayer obtained from the human cell, composed of trimers of gp120 protein anchored to the membrane by trimers of the transmembrane protein gp41[10]. This outer membrane surrounds the matrix and the icosahedral capsid, assembled from the p24 protein[11]. The capsid contains the virus's genetic material, along with viral enzymes retrotranscriptase (RT), RNase H, and Integrase.

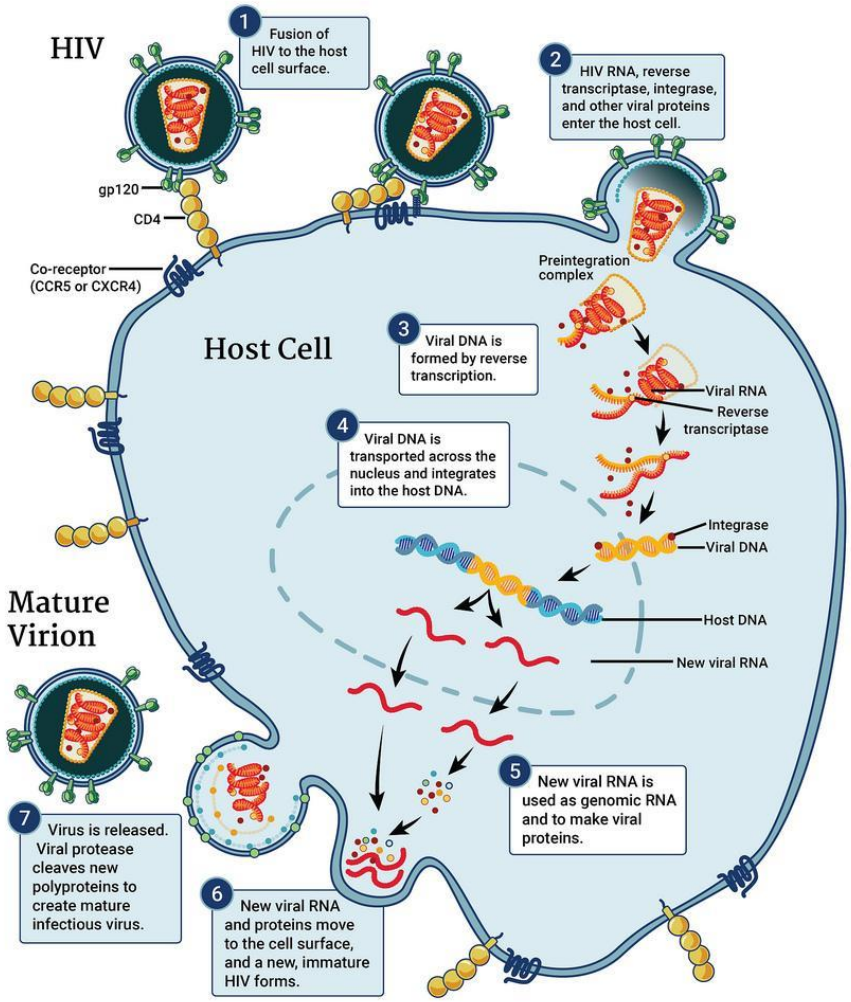

Fig 1. Viral cycle of HIV. Viral particles fuses with the surface of the host cell. A capsid containing the virus's genome and proteins then enters the cell. The shell of the capsid disintegrates, and the HIV protein called reverse transcriptase transcribes the viral RNA into DNA. The viral DNA is transported across the nucleus, where the HIV protein integrase integrates the HIV DNA into the host's DNA. The host's normal transcription machinery transcribes HIV DNA into multiple copies of new HIV RNA. Some of this RNA becomes the genome of a new virus, while the cell uses other copies of the RNA to make new HIV proteins. The new viral RNA and HIV proteins move to the surface of the cell, where a new, immature HIV forms. Finally, the virus is released from the cell, and the HIV protein called protease cleaves newly synthesized polyproteins to create a mature infectious virus image and obtained from NIAID.

Different cell types can be infected by HIV-1, including monocytes, macrophages, dendritic cells, and T cells. A viral cycle takes around 24 h and can be divided into several steps:

Binding and fusion: the gp120 viral protein binds to the CD4 receptor on the surface of host cells [12, 13]. This binding develops conformational changes on gp120 protein, allowing its binding to a co-receptor, CCR5 and CXCR4[14, 15]. This promotes another conformational change that exposes the N-terminal domain of gp41 and subsequent membrane fusion and internalization of the viral capsid.

Retrotranscription: the genetic material contained by the capsid is retrotranscribed to double-stranded DNA by the RT enzyme, and RNA strands are digested to avoid immune detection. However, this process also requires cellular factors (such as dNTPs), whose production is induced during cellular activation and proliferation. Given this requirement, the Retrotranscription is blocked in resting T cells or other non-dividing cell types[16].

Genome integration: the newly synthesized DNA is transported to the cellular nucleus and integrated into the host genome by the action of the integrase enzyme. Other viral proteins are also involved in this process, including Vpr, Vpu, and p17 matrix protein[17-19].

Transcription: the integrated viral genome, known as a provirus, can remain latent in the host cell for long periods before it leads to viral replication. The transcriptional activation depends on the interaction of cellular factors with the regulatory sequences in the viral LTR[20], and the result is the synthesis of a single transcript of mRNA from the proviral DNA.

Translation: the viral mRNA uses the cellular machinery to break the original mRNA into transcripts of different sizes and translate them into viral proteins.

Assembly: once all the viral proteins have been synthesized, they are condensed in a cytosolic region close to the cell membrane, and the virion is assembled. The participation of Vif, Vpu, and the viral protease in this process is required for a successful result[19].

1.1.3.Pathology

After infection, HIV-1 presents active replication in all stages. However, around a month after initial infection, equilibrium begins to be established between replication and control of HIV-1 by the host immune system. In general, rates of HIV-1 clearance are similar among persons, but HIV-1 production by infected cells determines the viral load in the steady state. This points to the clinically latent phase of HIV-1 infection.

The presence of HIV-1 RNA in serum suggests that the immune system cannot control the virus. Increasing levels of serum HIV-1 RNA indicate a loss of equilibrium and emergence from latency to a more rapid progression to AIDS. The absence of a detectable serum HIV-1 RNA suggests a slower progression to clinical AIDS [21].

HIV-1 infection, if not treated, begins to deplete CD4 T cells exponentially, which leads to reduced immune response and AIDS. This reduced immune response opens the door to several opportunistic infections that can attack several organs and tissues. Among these opportunistic infections, *Pneumocystis jiroveci (carinii)* is the main bacteria causing pneumonia in HIV-infected patients [22]. This depressed immune system also allows other viral infections to develop in the host. One of the leading viral co-infections in HIV-1 patients is Human cytomegalovirus (HCMV). In contrast to *Pneumocystis jiroveci*, which only can be found in the lungs, HCMV can spread to several organs, including the gastrointestinal tract, central nervous system, and eyes, increasing morbidity and mortality if not treated properly [23].

Another main agent of opportunistic infections in HIV-1 patients is *Mycobacterium tuberculosis* (MTB); approximately one-third of HIV-infected patients are infected with MTB. The risk for tuberculosis in persons with HIV-1 infection is 20 to 37-fold that of the general population. Patients who are co-infected with HIV-1 and MTB progress to active tuberculosis at a greater rate [24].

1.1.4.Reservoirs

One of the main barriers to the eradication of HIV-1 is the reservoirs and sanctuaries. Reservoirs are defined as a pool of quiescent cells that harbours intact provirus and can

reactivate viral production when ART is interrupted. On the other hand, sanctuaries are defined as anatomic places in which the treatment is not able to reach, as the brain due to the Blood-Brain Barrier (BBB), and there is a continuous low viral particle production [25].

The different mechanism of reservoirs or sanctuaries formation is still in debate, but several approaches arise. Most of them agreed on other HIV-1 integration mechanisms. Pre-Integration Latency refers to silencing mechanisms occurring on LTR circles. A study showed that HIV-1 unintegrated circles adopt an episomal structure, indicating that HIV-1 unintegrated DNA is associated with nucleosomes [26].

Post-integration latency results from several related processes that establish and maintain HIV-1 promoter silence by either epigenetic, transcriptional, and post-transcriptional mechanisms [27]. These post-integration mechanisms could be divided into Nucleosome positioning on the HIV-1 provirus [28] and repressive histone marks on the HIV-1 promoter [29].

Since 1997 most of the research has focused on T CD4 lymphocytes as primary reservoir cells in HIV-1 infection [30] due to the capacity of T CD4 to enter in quiescent state after HIV-1 infection [31]. These cells containing latent proviruses persist in part via homeostatic or antigen-driven proliferation [32]. Most research has focused on the main mechanism of latency in these cells, but several target cells have arisen as reservoirs or sanctuaries in recent years. Among these cells, macrophages, dendritic cells, and microglia are attracting more attention in this field. In this sense, macrophages may play an important role in reservoir and sanctuaries formation. Nowadays, it is well known that HIV-1 viral particles can infected macrophages, or these particles can be phagocytised and "stored" in the inner membrane. In the case that macrophage phagocytises these viral particles, they remain infectious for several days. During these days, macrophages can mobilize to several anatomic parts [33, 34]. They will spread these viral particles to T CD4 by a cell-to-cell infection 10-fold higher than cell-free virus infection [35-37].

There are two main strategies to fight HIV-1 persistence in reservoirs, "shock and kill", which consist of latency reversal agents (LRA) that can induce an active state in the cell, thus activating viral transcription. The infected cell can be recognized either by the immune system or ART. This strategy is promising, but several studies show that only the activation cannot reduce viral reservoir [38] due to immune clearance is not enough after the shock. Thus a combination therapy of LRA and new ART are being studied[39] .

The other strategy to reach a functional cure of HIV-1 is "block and lock", this approach consists in the opposite of the one mentioned above. In this strategy, the goal is to restrict HIV-1 replication by blocking HIV-1 promoters [40]. With this approach, integrated HIV-1 DNA is not entirely eradicated, but viral transcription is absent or low enough that the immune system can clear any occurring viral production [41].

1.1.5. Treatments

Since the first HIV-1 specific antiviral drugs were given as monotherapy in the early 1990s, the standard of HIV-1 care evolved to include the administration of a cocktail or combination of antiretroviral agents (ARVs). Combination antiretroviral therapy dramatically suppresses viral replication and reduces the plasma HIV-1 viral load to below the detection limits resulting in a significant reconstitution of the immune system. During several years trials with early antiretroviral agents helped to establish the principles for effective drug combinations. In the last years, therapies have evolved, with the introduction of newer drugs with greater potency and complex barriers to developing of resistances [42]. There are several groups of antiretroviral drugs classified regarding the viral cycle point targeted.

Reverse transcriptase inhibitors are a group of drugs, which can bind and inhibit the reverse transcriptase enzyme from intercepting the transcription of HIV-1. In this group, there are two types of inhibitors: non-nucleoside reverse transcriptase inhibitors (NNRTIs) [43] and nucleoside reverse transcriptase inhibitors (NRTI) [44]. Examples of this group of drugs include zidovudine, didanosine, abacavir, tenofovir, and combivir.

Protease Inhibitors act in the regulation of HIV-1 protease, which is of high importance for the assembly and production of viral particles. Protease inhibitors effectively block the functioning of protease enzymes in acutely and chronically HIV-infected T CD4 cells. Inhibition of HIV-1 protease enzymes results in the release of immature and non-infectious viral particles [45]. Examples of this group of drugs include lopinavir/ritonavir, indinavir, ritonavir, nelfinavir, and amprenavir.

Fusion Inhibitors are a class of drugs that blocks HIV-1 from entering the TCD4 cells of infected patients. They can inhibit the fusion of viral particles with the CD4 cells [46]. Enfuvirtide is an example of a fusion inhibitor used in HIV-1 treatment.

Chemokine Receptor 5 Antagonist, this group of drugs prevents the infection by blocking the chemokine receptor 5 (CCR5) antagonist receptor present on TCD4 cells. In the absence of vacant CCR5 receptors, HIV-1 fails to enter and infect the cell [47]. Maraviroc is an example of a CCR5 antagonist used in HIV-1 treatment.

Integrase Inhibitors prevent the integration of viral DNA into the host genome of CD4 cells by an integrase enzyme. Blocking integrase prevents HIV-1 from replicating [48]. Raltegravir, elvitegravir, and dolutegravir are some medications in this category.

Even with all these strategies, HIV-1 remains one of the main global infections. Thus, the development of new treatments and approaches are needed to minimize the worldwide implications of the HIV-1 pandemic.

1.2. Human Cytomegalovirus

1.2.1. Epidemiology

Human Cytomegalovirus (HCMV), also known as human herpesvirus-5 (HHV-5), is a member of the β-herpes virus family, widely distributed in human populations.

Intranuclear inclusions typical of cytomegalovirus infections were first noticed in 1881 by German scientists who thought they represented protozoa, but till 1956–1957 viral particles were not isolated and defined by Ender and Weller. Weller named "cytomegalovirus" due to the prominent inclusions observed in the infected cells.

HCMV transmission occurs through person-to-person contact. It can be transmitted transplacentally to neonates or through the breast milk of an infected and shedding mother, by intimate contact, and by transplantation from (or sharing syringes with) an infected individual [49]. This broad mechanism of transmission leads HCMV to a worldwide infection virus.

According to the Centres for Disease Control and Prevention (CDC) and World Health Organization (WHO), CMV can infect people of all ages; over 50% of adults are infected with CMV by 40, and approximately one in three children are infected with CMV by the age of five in the United States [50]. For example, in a study from Finland, the seroprevalence rates were 47% in 10- to 12-year-olds, 68% in 15- to 35-year-olds, and 81% among 36- to 60-year-olds [51]. The HCMV represents an important co-infection in HIV-1 infected patients. HCMV is associated with increased morbidity and mortality [52], specifically in the African population, where the seroprevalence of HCMV infection is near 80% in HIV-1 patients.

1.2.2. Viral structure and cycle

HCMV is a double-stranded DNA (dsDNA) virus that has a 235-kb genome, twice the size of chickenpox-causing varicella-zoster virus and >50% larger than that of the cold sore–causing herpes simplex virus 1 (HSV-1) [53]. The viral genome comprises two unique regions termed unique long (UL) and unique short (US). A pair of inversely repeated sequences flank these two domains. Despite enclosing a much larger genome, the size of the HCMV capsid is similar to that of HSV-1 (as well as those of other herpes viruses) [54]; both have an icosahedral ordered nucleocapsid with triangulation number (T) = 16. Between the outer membrane and the nucleocapsid has located the tegument, a link-layer composed of several different proteins directly delivered into the host cytoplasm, allowing the newly infected virus to adapt to the new cellular environment.

HCMV can infect diverse cell types such as epithelial cells, fibroblasts, lymphocytes, monocytes, and macrophages. This broad tropism is attributed to the envelope proteins that may play roles in recognition, attachment, and fusion. Moreover,

envelope mediators are the key to understand cell-to-cell spread. Entry into different cell types might be associated with effects from various combinations of envelope proteins. Three complexes termed glycoprotein complex I, II, and III (gCI, gCII, and gCIII) are present on the HCMV envelope [55].

During entry, envelope glycoproteins, positioned on the outside of infectious virions, interact with host receptors to mediate fusion of the virion into the cell. Viral tegument proteins bound to the capsid are believed to interact with the host microtubule machinery to transport viral capsids to the nuclear envelope and into the nucleus, where viral transcription, genome replication, and encapsidation occur [56]. Many viral-encoded proteins can regulate cell-signalling pathways and cellular metabolism to support viral replication and immune evasion [57]. The expression of these viral proteins occurs in a sequential cascade of events. It is divided into immediate-early (IE), delayed early (DE), and late (L), each regulating different aspects of the infectious cycle.

Capsids are assembled in the nucleus, and they egress through the nuclear double membrane. Once capsids reach the cytoplasm, the assembly and transport of virions occur via the integration of multiple cellular trafficking pathways [58].

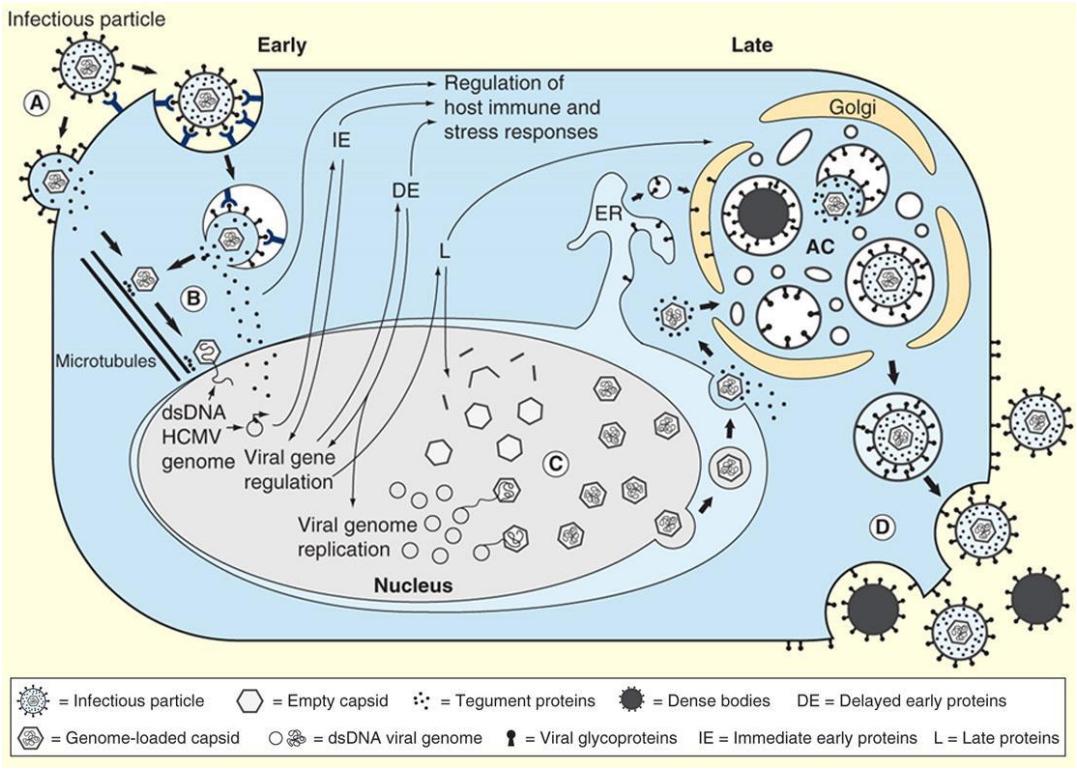

Fig 2. HCMV life cycle. A) Viral particles enter the cell through cellular receptors interaction. Capsid and tegument proteins are targeted to the cytosol. B) Capsid travels to the nucleus and delivers the genome. Tegument proteins regulate host cell responses and initiate the cascade of the expression of viral immediate-early (IE) genes, followed by delayed early (DE) genes, which start viral genome replication, and late (L) genes. C) Late gene expression initiates capsid assembly in the nucleus, then nuclear egress to the cytosol. D) Enveloped infectious particles are released along with non-infectious dense bodies. Image obtained from [58].

Human cytomegalovirus can establish either latent or lytic infection but is dependent on the infected cell. HCMV shows latency in CD34+ hematopoietic progenitor cells (HPCs) and is carried through differentiation in myeloid lineage cells, including CD14+ monocytes [59]. However, in fibroblasts and epithelial cells, HCMV initiates lytic infection by expressing genes in a flowing cascade; IE phase before early gene expression, and finally, late genes are expressed to facilitate virion assemble and release [60].

1.2.3.Pathology

Primary infection of healthy individuals with HCMV is typically asymptomatic, although it can present with mild flu-like symptoms. However, infections in people with compromised or immature immune systems, as transplant recipients, HIV-infected individuals, and congenitally infected infants, can cause significant morbidity and mortality [61]. Studies of the replication rate of HCMV showed that its dynamics are rapid, with a doubling time of viraemia of approximately one day [62]. In this sense, the replication parameters of primary HCMV infection in humans are very similar to those of primary HIV-1 infection. Thus, the reputation of a slowly growing virus is undeserved and is likely derived from the slow evolution of cytopathic effects in fibroblast cell cultures.

The immune system is in constant struggle with HCMV infection. In this sense, HCMV is ready to replicate rapidly if the established immune response becomes impaired.

This usually happens in patients treated with immunosuppressive drugs to prevent graft rejection, in patients with HIV-1 infection, or those with immature immune systems [63]. The main complications in HCMV infection are in the foetus of infected women and bone marrow transplant patients with newly engrafting marrow. The presumed pathogenesis is that HCMV replication stimulates the release of cytokines, which are then toxic to several organs.

Congenital HCMV infections can follow primary maternal infection during pregnancy. About 10% of congenitally infected infants will have clinical symptoms that include microcephaly, hepatosplenomegaly, petechial rashes, neurological abnormalities such as altered tone and seizures, intrauterine growth restriction, and rarely extramedullary haematopoiesis [64], and approximately 30% of infants with symptomatic congenital HCMV will develop neurodevelopmental sequelae [65].

In other of the most affected groups, elderly patients, accumulating decades of chronic immune surveillance for HCMV hiding might produce an excess in mortality. On the one hand, the reduced number of naive T cells might make seropositive people less able to respond to several infections [66]. On the other hand, the increased abundance of activated T cells might mediate inflammatory attacks on bystander cells, increasing in tissue damage, thus affecting several different organs[67].

1.2.4. Treatments

For almost 30 years, ganciclovir (GCV) has been the therapy of choice for HCMV infections. With current standards, this drug exhibits only modest antiviral activity, sometimes insufficient to completely suppress viral replication, and leading to the replication of drug-resistant variants that contribute to disease. While ganciclovir remains the therapy of choice, additional drugs that inhibit different molecular targets, such as letermovir, will be required as highly effective combination therapies are developed not only for the treatment of immunocompromised hosts but also for congenitally infected infants.

In the last decade, several efforts, mainly in the biotech industry and academia, have identified additional highly active lead compounds that have progressed into clinical studies with varying levels of success [61].

Four drugs have been approved for the systemic treatment or prevention of HCMV infections: GCV (and valganciclovir, VGCV), cidofovir (CDV), foscarnet (FOS), and most recently letemovir (LTV) [68]. Of these, GCV, CDV, and FOS are similar and target the viral DNA polymerase and inhibit the synthesis of viral DNA. In contrast, LTV is a small molecule that inhibits the packaging of DNA into the virion and represents a new class of approved drugs to prevent HCMV infections [68].

GCV is like ACV in that it is an acyclic analogue of deoxyguanosine, although it differs in that it has two hydroxyl moieties making it a non-obligate chain terminator

Even with all these approved drugs against HCMV infection, the drug-resistant variants are still a significant problem. In this sense, the development of new strategies to fight HCMV infection is needed. Among these new approaches, nanotechnology could offer new insights against infectious diseases, either by inhibiting the entry of the virus to the host cell or by directly attacking the viral particles.

1.3. Nanotechnology

1.3.1. Introduction

Nanotechnology is defined as the management and restructuring of matter at the order of nanometers to build materials with novel properties and functions [69]. Nanotechnology research can advance in physics, chemistry, engineering, robotics, communications, biology, and medicine**.** The contact of humans with nanoparticles has occurred throughout human history, but it noticeably augmented during the time of the industrial revolution. The concept of ‘‘nanometer’’ was first named by the 1925 Nobel Laureate, Richard Zsigmondy, who introduced the term nanometer for characterizing particle size. He measured dimensions of particles such as gold colloids under the microscope for the first time [69]. The concept of nanomaterials is most precisely described as: “Nanomaterials constitute nanoproducts in the form of

materials containing structural nanoelements which considerably improve or cause qualitatively new physical, chemical, biological, mechanical, and other properties" [70].

Synthetic nano-materials offer multiple types of possibilities due to their shape, size, and properties, such as fluorescence, biocompatibility, magnetism, thermal and electrical conductivity [71].

0D nanomaterials include quantum dots (QDs) which are highly fluorescent semiconductor nanocrystals of size range from 1 to 10 nm. QDs possess broad absorption and narrow emission spectra and are often functionalised with biomolecules. Such complex can probe biocatalytic transformation and recognition events based on fluorescence resonance energy transfer (FRET) or electron transfer (ET). For example, antibody or nucleic acid- functionalised QDs of variable sizes have been explored in the multiplexed analysis of pathogens or DNA [72].

Two external dimensions define the 1D nanomaterials at the nanoscale, and the third one being usually at the micro-scale, including nano-wires (NWs), nano-belts (NBs), nano-fibers, and nano-tubes, which are recognised as a class of most promising materials in energy storage systems [73].

2D nanomaterials got an immense attraction in recent decades due to their unique structures and unusual properties. The revolutionary discovery of graphene changed the known applicability of nanotechnology and increased the possibilities of new strategies in materials science due to graphene versatility. Later, various forms of structurally similar 2D nanomaterials, such as 2D polymers and nanosheets were designed. This boost in material research led to the synthesis of new nanocompounds with several characteristics.

Most of these efforts have focused on biomedical applications. In this area, dendrimers may play a significant role against infectious diseases [74].

1.3.2. Dendrimers

In the last decades, several nano-compounds emerged due to the increasing need for treatments against the uprising infectious diseases. Two of the most promising compounds in the fight against infectious diseases are dendrons and dendrimers.

Dendrimers are a class of polymeric molecules discovered in the 1970–1980s by research teams led by scientists such as Tomalia, Vögtle, Denkewalter, and Newkome [75]. Their name means tree and derives from the Greek word "dendron", which refers to their branched structure. Dendrimers belong to the family of dendritic polymers and stand out due to their controlled synthesis and monodispersity, meaning that they always present the same structure [76]. Their size ranges between 1–10 nm, depending on their generation, and they have a three-dimensional hyperbranched architecture that confers substantial differences compared to linear polymers.

One of these properties is the ability to customize and control the size and shape of the dendrimer through the synthetic process. This enables researchers to fit their design regarding their purpose, for example, by creating complex with drugs, antibodies, or imaging probes in specific positions of the nanoparticle. This capacity offers the possibility of encapsulating drugs and targeting them to the desired tissue, reducing the toxicity and providing greater control over the bio dispersity of the drug [77].

Three main domains describe dendrimers: 1) The core, located in the center of the dendrimer, can comprise one or multiple atoms; 2) the branching units, which are covalently linked to the core, whose repetition leads to a series of concentric layers. The number of these layers is known as the dendritic "generation"; and 3) the functional groups, mainly located on the surface of the dendritic scaffold, and highly responsible for the dendrimer properties [78]. Different combinations in these domains allow several groups of dendrimers, one of the most studied group are the polyamidoamine (PAMAM) dendrimers. PAMAM was one of the first dendrimer families to be fully characterized, synthesized, and commercialized. Due to the numerous possibilities of modifying its core, and surface groups and its highly symmetrical structure, hydrophilic properties, high biocompatibility and non-immunogenic properties, PAMAM dendrimers are extensively used in research on targeted drugs and genetic material delivery [79].

Polypropylene imine dendrimers are probably the oldest dendrimers synthesized by Voegtle et al., 1978 [80]. The surface of PPI dendrimers contains cationic groups that facilitate the interaction between their positive charge and membrane negative charge [81]. Currently, up to five generations of PPI dendrimers have been prepared for applications in drug delivery and a theragnostic [82].

Another big group of dendrimers that have arisen in the last years is carbosilane dendrimers [83]. These compounds consist of a Si skeleton attached to a core that may vary and be surrounded by functional groups. As commented before, the groups in its periphery confer dendrimers their main applicability. Different functional groups are being studied for a variety of applications. In this work, we have focused on sulfonate dendrimers and their biomedical applications. Sulfonate groups have shown several properties against different viral infectious diseases, specifically Polyanionic Carbosilane Dendrimers (PCDs). PCDs are a group of well-defined hyper-branched polymers with a nanoscale globular shape, well-defined functional groups located at the periphery, and internal cavities that can encapsulate guest molecules. The reasonable manufacture cost and controlled synthesis of PCD, their high biocompatibility, solubility, reactivity, low polydispersity, and polyvalency differentiate them from standard linear polymers [84]. PCDs are especially suitable for therapeutic approaches. Their straightforward synthesis allows us to obtain large amounts of polymer, with a defined molecular weight and a specific number of functional terminals to be generated. Moreover, their biochemical stability, biological inertness, and low polarity of the C–Si bond confers dendrimers many capabilities for their use as HIV-1 microbicides [85].

In this thesis, we have worked with G1-S4, with four sulfate groups in the periphery and a Si core, G2-S16 with 16 sulfonate groups in the periphery and a Si core, G2-S16-FITC with 14 sulfonate groups, Si core, and a FITC molecule attached, G2-S24P with 24 sulfonate groups and polyphenolic core, and G3-S16 with 16 sulfate groups in the periphery and a Si core.

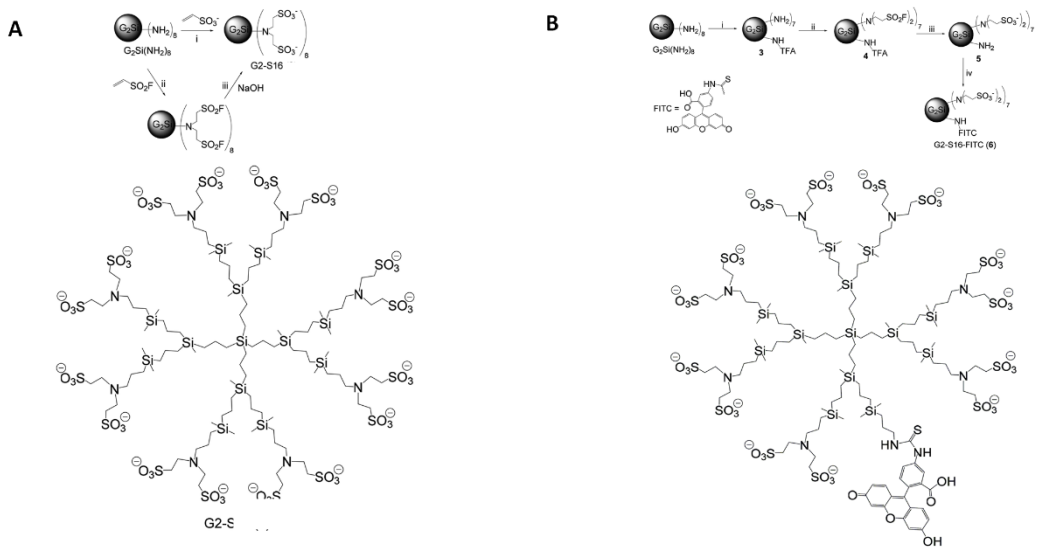

Fig 3. Schematic synthesis route and molecular representation of dendrimers. A) G2-S16 with silicon core and 16 sulfonate end groups and B) G2-S16-FITC with silicon core and 14 sulfonate end groups and FITC molecule were represented. Images adapted from Gutierrez-Ulloa et al. 2020.

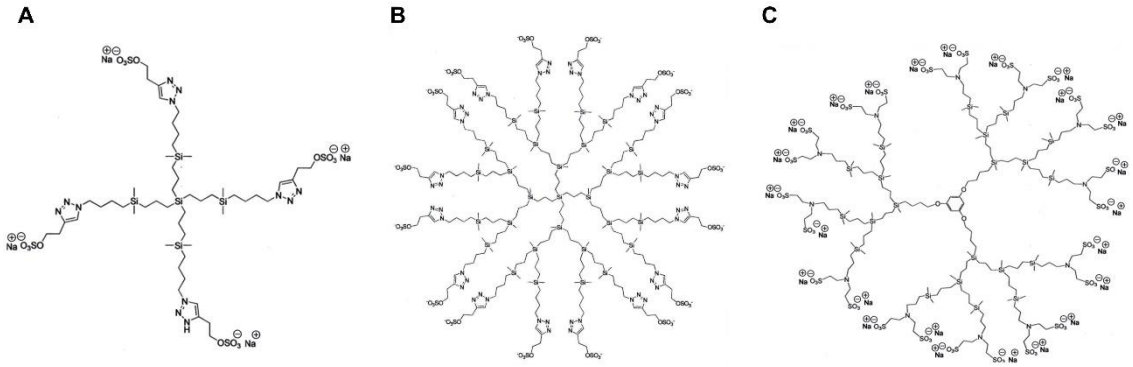

Fig 4. molecular representation of dendrimers. A) G1-S4 with silicon core and 4 sulfate end groups B) G3-S16 with silicon core and 16 sulfonate end groups, and C) G2-S24P with polyphenolic core and 24 sulfonate groups were represented.

1.3.3.Properties and synthesis

The chemistry towards dendrimers and other complex dendritic scaffolds that require structural control of the frameworks has matured since their introduction to the scientific community [86]. Dendrimers and dendrons are typically synthesized using a

cascade of repetitive growth and activation steps. Considering their structural perfection, the successful synthesis of these frameworks relies heavily on robust organic reactions that efficiently proceed at a macromolecular level [86].

There are two main approaches in dendrimer synthesis:

- Divergent Method

  This is a traditional synthesis method, which begins from a core scaffold and involves the attachment of monomer units in a sequential manner around the core. The utilization of commercial reagents at each repetitive step increases the mass of the final product due to generational growth. Each new layer of the monomer gives rise to a new generation of a dendrimer with a terminal surface that can be modified for a specific desired function.

- Convergent Method

  The first dendrimer produced by this method belonged to the polyarylether dendrimer family. This method consists of a reverse synthesis that starts from the surface groups and ends at the inner core. The control over the structural integrity of dendrimers is possible by this process to produce designer-type dendrimers for specific applications. The size and shape of the dendrimer are tuneable with the selection of appropriate dendrons.

Recently a new type of chemical reaction named “Click Chemistry” arise. This reaction utilizes the benefits of two traditional approaches and combines them to produce dendrimers with structural diversity [87]. It is also called the double stage convergent or the combined divergent/convergent approach. Reactions that belong to the family of click reactions must be modular, broad in scope, tolerant to many functional groups, give high yields, leaving inoffensive by-products, and result in products that non-chromatographic procedures can purify.

1.3.4. Biomedical applications

Due to the several properties of dendrimers, their applications in several fields have increased during the last years. As commented before, since its discovery, nanoparticles have been introduced in several industries, their applications vary from farm industries to robotics, but its highlights in biomedicine are remarkable. Nanomedicine plays an important role in establishing new methods for detecting chromosomal rearrangements and mutations for targeted chemotherapeutics or the local delivery of drugs via different types of nano-particle carriers to the lungs or other organs or areas of interest [88]. For example, dendrimer-based drug delivery nanosystems can adopt alternative routes of drug transport across the BBB. Evidence highlights the possibility of the involvement of endocytosis mechanisms mediated by clathrin and caveolin [89] and via specific receptor-mediated pathways driven by brain-targeting ligands conjugated to the dendrimer surface [90].

Another example has attracted attention over the past decades due to their programmability and multifunctionality: DNA nanomaterials. In particular, DNA dendrimer nanostructures have been applied in biosensing, therapeutics, and protein engineering. With the aid of specific recognition probes and inherent signal amplification, DNA dendrimers can achieve ultrasensitive detection of nucleic acids, proteins, cells, and other substances [91].

Dendrimers can play their effect according to different mechanisms of action, generally associated with the multivalency of the branched scaffold. Recent studies drew attention to the plasticity of dendrimer synthesis to produce antibacterial dendritic structures with specific dimensions and surface modifications. In addition, dendrimers have shown the ability to inhibit biofilm formation. The antibiofilm activity has been proved against several bacteria, such as *E. coli* or *P. aeruginosa* [92].

Since high surface-to-volume ratios characterize dendrimers, is it possible the combine and attachment different antiviral compounds to the same structure. This strategy offers the chance of targeting multiple and precise biological sites, limiting the necessity of high doses of antiviral drugs, and reducing side effects on healthy cells

and tissues. Moreover, there are also dendrimers that exhibit antiviral activity to inhibit several viruses in different stages of the infection.

In this sense, one of the most successful applications of dendrimers as antiviral agents is SLP7013. This dendrimer presents a core based on a divalent benzhydrylamineamide of l-lysine, encircled by four l-lysine layers (G4) terminated with 32 amine groups and further functionalized with naphthalene disulfonic acid groups. This dendrimer is commercialised as VivaGel® by Starpharma [93]. This work has been focused on the study of polyanionic carbosilane dendrimers and their applications in biomedicine, specifically against infectious diseases, such as HIV-1 and HCMV.

2. Objectives

The aims of this work are:

1. To evaluate the in vitro activity of Polyanionic Carbosilane Dendrimers in combination with actual Latency Reversal Agents for their application in the "Shock and Kill" approach against HIV-1 reservoirs.
2. To characterize the inhibition activity of G2-S16 dendrimer in the context of macrophage cell to cell transmission against HIV-1 infection to reduce the reservoir or sanctuary formation.
3. To study the properties and mechanism of action of Polyanionic Carbosilane Dendrimers against HCMV infection in vitro.

3. Material & methods & results

3.1. Article " Polyanionic carbosilane dendrimers as a new adjuvant in combination with latency reversal agents for HIV treatment"

The main focus of this work was the study of different PDC in combination with actual LRAs used in studies of HIV-1 reactivation. We already showed the inhibition activity of G1-S4, G2-S16, and G3-S16 against several HIV-1 isolates and laboratory strains. However, we wondered if a combination of LRAs that would reactivate HIV-1 replication, and our dendrimers, that would stop newly formed virions could be possible. To this end, we performed viability studies in J89GFP and THP89GFP to assess the working concentration of each dendrimer in cell culture. Moreover, we analysed the toxicity that the combination of both drugs would cause in the cell lines used. Viability studies showed that either PCDs alone or in combination with LRAs are biocompatible in these cell lines.

The first idea was to study if the combination of LRAs with our PCDs would modify LRAs activity to reduce the actual reservoir size. In this strategy, the LRAs would reactivate HIV-1 latently infected cells, and the newly produced virions would lose their infectivity with the action of PCDs. This feature, with the combination of an immune system enhancer, would help in HIV-1 reservoir clearance. Surprisingly, in this work, we found that G3-S16 dendrimer single treatment could produce reactivation in the THP89GFP cell line. We observed an increased expression of GFP when treated with G3-S16 alone. Nevertheless, this HIV-1 expression was in concordance of actual LRAs, reaching the same levels of reactivation as BRY, PST, PNB, or RMD single treatments. In addition, we observed that the combination of G3-S16 with PNB or RMD increases the basal activity of both drugs alone. These results remark the importance of searching for new molecules in this HIV-1 eradication strategy.

I have participated performing the experiments, analysing the results and composing the figures. I also collaborated interpreting and discussing the data and writing the manuscript in this paper.

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SHORT COMMUNICATION Open Access

Polyanionic carbosilane dendrimers as a new adjuvant in combination with latency reversal agents for HIV treatment

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Abstract

Background: The major obstacle impeding human immunodeficiency virus-1 (HIV-1) eradication in antiretroviral treatment (ART) treated HIV-1 subjects is the establishment of long-lived latently infected resting $CD4^+$ T cells. Due to the fact that no drug has been effective, the search for new drugs and combinations are a priority in the HIV cure. Treatments based on nanotechnology have emerged as an innovative and promising alternative to current and conventional therapies. In this respect, nanotechnology opens up a new door for eliminating latent HIV infection. We studied the role of G1-S4, G2-S16 and G3-S16 polyanionic carbosilane dendrimers in the context of latent HIV-1 persistence. Moreover, we study the efficiency of these dendrimers in combination with latency reversal agents (LRAs) against HIV-1 infection.

Methods: J89GFP lymphocyte and THP89GFP monocyte derived cell lines latently infected with HIV-1 p89GFP were used as an in vitro model of latency for our study. Viability assays by 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) were performed to determine the working concentrations of dendrimers and LRAs. Both cell lines were treated with G1-S4, G2-S16 and G3-S16 either alone or in combination with bryostatin (BRY), romidepsin (RMD) or panobinostat (PNB) for 24 and 48 h. The expression pattern of GFP was measured by flow cytometry and referred as measure of viral reactivation.

Results and discussion: The combination treatment of the dendrimers with the protein kinase C (PKC) agonist did not modify the antilatency activity in J89GFP lymphocyte cell line. Interestingly enough, G3-S16 dendrimer alone and its combination with BRY, RMD or PNB showed a significant increased expression of GFP in the THP89GFP monocyte cell line.

Conclusion: We showed for the first time that nanoparticles, in this case, G3-S16 anionic carbosilan dendrimer may play an important role in new treatments against HIV-1 infection.

Keywords: Nanomedicine, Dendrimers, HIV-1 latency, Latency reversal agents

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Background

Human immunodeficiency virus-1 infection can be treated effectively in the developed world, using new combinations of antiretroviral treatments (cARTs). Despite prolonged cARTs, the persistence of HIV-1 in resting $CD4^+$ T cells reservoirs harbouring transcriptional silence and replication-competent proviruses presents the major hurdle to HIV-1 eradication. These latently-infected cells are a permanent source for virus reactivation and lead to a rebound of the viral load after interruption of cARTs [1, 2].

Various therapeutic interventions to eradicate HIV-1 focus on the stimulation of HIV-1 production from latently infected cells. These interventions involve the use of latency-reversing agents (LRAs) such as prostratin (PST), bryostatin-1,2 (BRY), panobinostat (PNB), and romidepsin (RMD) [3–9]. LRAs reactivate latently-infected cells, whereas the cARTs prevent spreading HIV-1 infection. It has been published that BRY, PNB and RMD present a good reactivation activity both in vitro and ex vivo and have currently been used in HIV-1 eradication clinical trials [10–12]. However, a decrease of the viral reservoir or total eradication of HIV-1 in infected subjects has not been achieved.

There are two major problems in HIV-1 infection: (1) virus persistence in reservoirs in a latent form integrated into the host genome, and virus replication when cells undergo activation; (2) inhibition of current ARV drugs with retrotranscription or with HIV-1 protease. This strategy presents additional troublesome such as the appearance of resistance. Drugs that interfere with ARV entry such as enfurtivide, which inhibits viral and cell membrane fusion, have been postulated as a possible alternative. However, the first cases of resistance have already been described [13].

In the last decade, the nanotechnology has been improved by the development and discovery of a wide range of novel nanoparticles. These new nanotechnology applications are easy to design, develop and synthesize. Dendrimers are promising nanoparticles, described as highly branched tree-like molecules between 1 and 40 nm [14]. We work with G1-S4, G2-S16 and G3-S16 polyanionic carbosilane dendrimers in the context of the HIV-1 infection [15, 16].

Our objective is to study the potential use of our polyanionic carbosilane dendrimers that will be applied in the "shock and kill" therapy increasing the HIV-1 reactivation and avoiding new HIV-1 infections.

Materials and methods

Dendrimers

Polyanionic carbosilane dendrimers G1-S4 with 4 sulfate groups in periphery, G2-S16 with 16 sulfonate groups in the periphery, and G3-S16 with 16 sulfate groups in the periphery were synthesized and analyzed according to methods reported by the Dendrimers for Biomedical Applications Group of University of Alcalá (Madrid, Spain) [15, 16]. Stock solution of dendrimers (10 mM) and subsequent dilutions to working concentrations were prepared in nuclease-free water (Promega, Madrid, Spain). The schematic structures of the polyanionic carbosilane dendrimers are presented in Fig. 1.

Zeta potential

These measurements were done in a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25 °C using disposable Malvern plastic cuvettes (1 ml), by solving 1 mg of dendrimers G1-S4, G2-S16 and G3-S16 in purified water, which was previously filtered through 0.22 μm filter.

Cell lines

J89GFP lymphocyte and THP89GFP monocyte cell lines (kindly donated by Dr. David N Levy, NYU, USA), are derived cell lines latently infected with recombinant

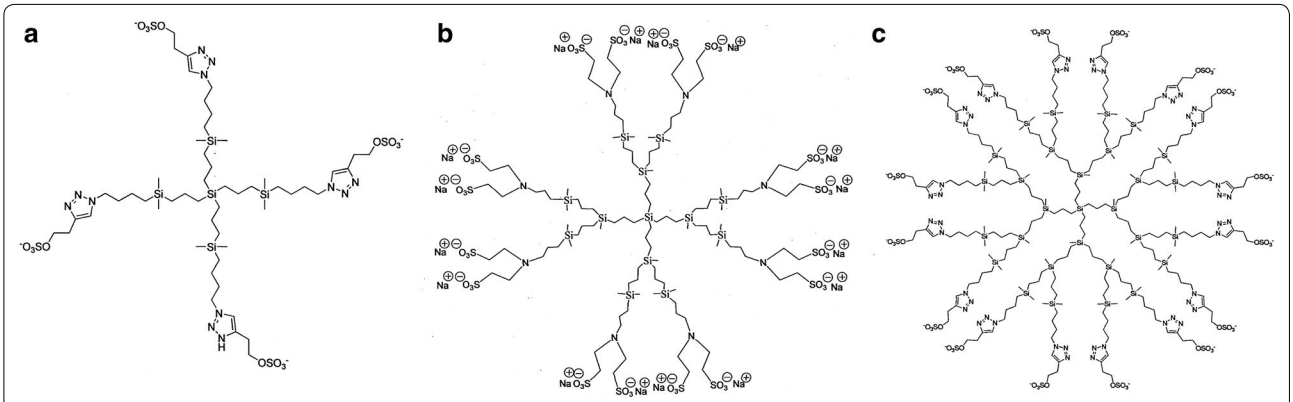

Fig. 1 Molecular representation of dendrimers. **a** G1-S4 with 4 sulfate end groups, **b** G2-S16 with 16 sulfonate end groups and **c** G3-S16 with 16 sulfate end groups were represented. The generation of dendrimers is determined by considering that each generation corresponds to the number of repeating layers of silicon atoms

HIV-1 p89 GFP. J89GFP cell line was maintained in RPMI complemented with 5% FBS, 125 mg/ml ampicillin, 125 mg/ml cloxacillin and 40 mg/ml gentamicin (Normon, Madrid, Spain). THP89GFP cell line was maintained in ultra-low attachment culture plates in DMEM complemented with 5% FBS, 125 mg/ml ampicillin, 125 mg/ml cloxacillin and 40 mg/ml gentamicin (Normon, Madrid, Spain) and were maintained according to the protocol described by Kutsch et al. [17].

Reagents

Bryostatin-1,2 (BRY) and prostratin (PST) were obtained from Sigma-Aldrich (St. Louis, MO, USA), panobinostat (PNB) and romidepsin (RMD) were obtained from Selleck Chemicals (Houston, TX). Drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to prepare stock solutions. DMSO concentration in cell cultures was lower than 0.001%.

Mitochondrial activity assay

The mitochondrial toxicity of compound concentrations was tested by the 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St Louis, USA) according to manufacturer's instructions in G1-S4, G2-S16 and G3-S16 dendrimers for 24 and 48 h and BRY, PST, PNB or RMD for 24 h. Briefly, 6×10^4 of J89GFP or 2.5×10^4 THP89GFP cells were seeded in 96-well plates and treated with the desired compound for 24 or 48 h. After incubation period, culture medium was discarded and 220 µl of a 1:11 MTT/OptiMEM solution was added to the cultured cells. After 3 h, the supernatant was removed, and formazan crystals were dissolved in 200 µl DMSO (Sigma, St. Louis, MO, USA). All points were performed in triplicate. DMSO 10% was used as death cellular control. The concentration range of each compound examined in this study is in agreement with previously published results [18, 19].

Membrane integrity assay

Cell membrane integrity was measured by the lactate deshidrogenase (LDH) assay CytoTox 96® Non-Radioactive Cytotoxicity (Promega, Spain, Madrid) following manufacturer's instructions. Briefly, 3×10^4 cells were seeded in 96-well plates and treated with the desired compounds and their combinations for 24 h and 48 h. After incubation period, cells were lysed in 0.9% Triton X-100 (Promega, Spain, Madrid) for 45 min at 37 °C. After incubation, 50 µl of LDH reagent (Promega, Spain, Madrid) was added for 30 min at room temperature, protected from light absorbance was read in a Berthold Plate Reader at 490 nm. All points were performed by triplicate.

Confocal microscopy

GFP expression and LIVE/DEAD cells were analysed by confocal microscopy. J89GFP and THP89GFP were seeded at a density of 3×10^5 in 24-well culture plates and ultra-low attachment plates, respectively. Subsequently, J89GFP and THP89GFP were treated with BRY, G2-S16 or G3-S16 for 48 h at 37 °C. After incubation, both cell lines were treated with NUCLEAR-ID® Blue/Red cell viability reagent (ENZO, Farmingdale, New York) following manufacturer's instructions and visualized in a Leica TSC SPE confocal microscope. All points were performed in duplicate. DMSO 10% was used as death cellular control.

Latent HIV reactivation

GFP-fluorescence pattern measured by flow cytometry was used to determine viral reactivation in J89GFP and THP89GFP cell lines. J89GFP and THP89GFP cells were seeded at a density of 4×10^5 in 24-well culture plates and ultra-low attachment 24-well culture plates respectively, and after were stimulated with the indicated compounds. At least 30,000 cells were analysed by flow cytometry. The integrated mean fluorescence intensity (iMFI, percentage of GFP expressing cells *MFI) of live cells was used as a measure of HIV-1 reactivation.

Statistics

Statistical analysis was performed using GraphPad software Prism v.5.0 (GraphPad Software, San Diego, CA USA) between two groups (control versus different dosages of compounds or LRA-treated versus combined LRAs and G1-S4, G2-S16 or G3-S16) were assessed by using a paired t-test. ($^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$).

Results and discussion

Biocompatibility of latency reversing agents and dendrimers

We previously described the potent activity of G1-S4, G2-S16 and G3-S16 dendrimers against HIV-1 infection [20–22]. Cell viability of sulfonate G2-S16 or sulfate G1-S4 and G3-S16 dendrimers, of nanoscale between 1 and 20 nm diameter, versatility and multi branching properties were studied on J89GFP lymphocyte and THP89GFP monocyte cell lines by MTT assay. Moreover, we studied by MTT and LDH assays the viability of BRY, PNB, PST and RMD in J89GFP and THP89GFP cell lines. The combinations of LRAs and dendrimers were analyzed by LDH.

J89GFP and THP89GFP cell lines were seeded and treated with G1-S4, G2-S16 or G3-S16 dendrimer in various range of concentrations to determine the maximum non-toxic concentration (Fig. 2). Dendrimers were considered non-toxic when the survival rate was > 80%. Non-treated (NT) cells were used as cell viability control and DMSO 10% was used as death cellular control for MTT assays.

The results of MTT showed that G1-S4 dendrimer was not toxic at 50 μM and G2-S16 at 20 μM in J89GFP cell line and both dendrimers were non-toxic at 50 μM in THP89GFP cell lines during 24 h treatment. G1-S4 and G2-S16 dendrimers did not produce toxicity up to 20 μM in THP89GFP and 10 μM in J89GFP after 48 h. However, G3-S16 maximum non-toxic concentration was only 1 μM in both cell lines. G3-S16 was the dendrimer with the highest toxicity rates due to its major generation and bigger size. G2-S16 dendrimer with sulfonate groups in the surface was less toxic than G1-S4 with sulfate groups in J89GFP cell line after 48 h treatments, showing that not only the generation and size of the dendrimers, but also the functionalizing molecules are determinants for the biocompatibility.

The selection of the LRA concentrations was performed taking into account the maximum non-toxic concentration for each drug previously reported [23, 24]. BRY was non-toxic up to 100 nM, PST up to 20 μM, PNB up to 40 nM, and RMD showed toxicity at 20 nM (Fig. 3).

Combinations of dendrimers with LRAs in the J89GFP cell line showed no toxicity at 24 h. The viability decreased below 80% for PST, RMD and PNB after 48 h treatment analysed by LDH assay. Results indicated that after 48 h dendrimers did not produce cytotoxicity. However, combinations with LRAs reduced viability, probably due to the basal toxicity of LRAs on this cell line (Fig. 4).

In THP89GFP cell line, LRAs treatment for 24 h did not produce a decrease in the cell viability. Similarly, the dendrimers alone or in combination with BRY and PST did not produce toxicity. On the other hand, combination of G1-S4 and G3-S16 with RMD and PNB caused a 20% decrease in viability. No cell toxicity was observed at 48 h following single treatment with either BRY, PST or dendrimers single treatment. However, RMD, PNB and their combinations with dendrimers reduced viability below 80% in THP89GFP cells.

Exposure of J89GFP or THP89GFP cells to either G2-S16 (up to 10 μM) or G3-S16 (up to 1 μM) did not show toxicity following 24 and 48 h of treatment. However, G1-S4 reduced J89GFP cells viability measured by MTT at 48 h.

Potential z measurements were performed to study the aggregation ratio in aqueous solution. Due to technical

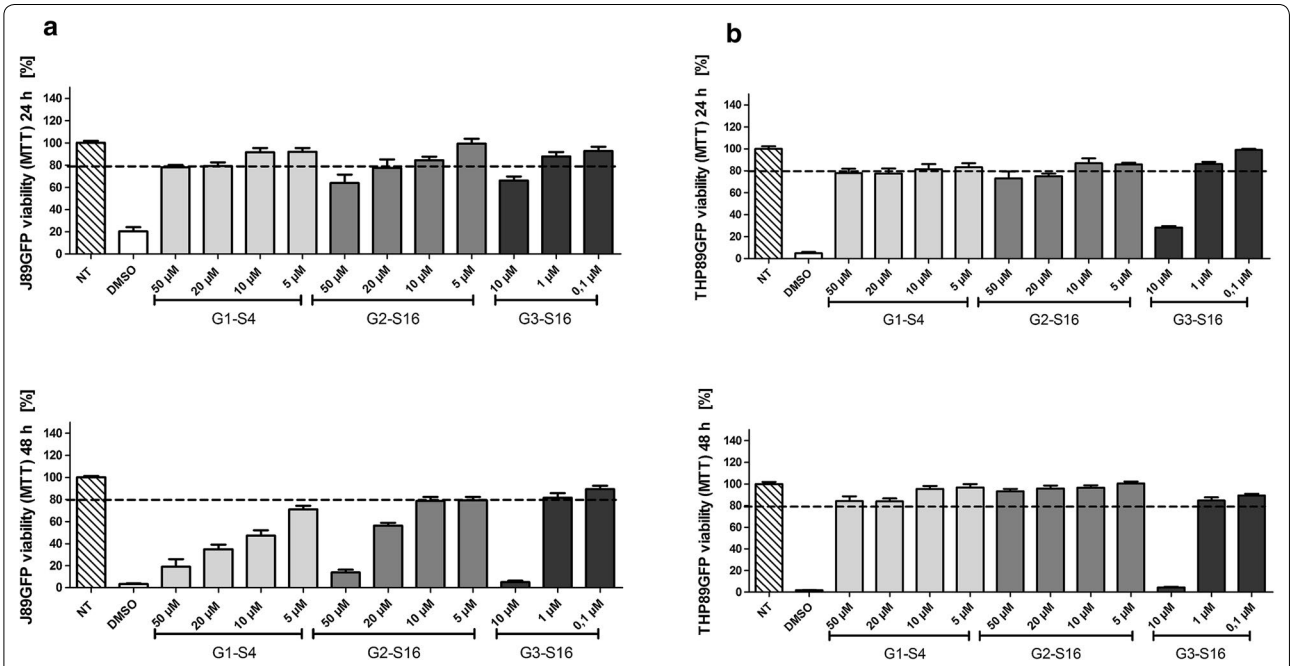

Fig. 2 Mitochondrial activity studies of polyanionic carbosilane dendrimers. MTT assays were performed in **a** J89GFP lymphocyte and **b** THP89GFP monocyte cell lines for 24 h and 48 h. Cell lines were treated with increased concentration range (0.2–50 μM) of G1-S4, G2-S16 or G3-S16 dendrimers. Non-treated (NT) cells were used as cell viability control. DMSO was used as cell death control. *NT* non-treated control, *DMSO* dimethyl sulfoxide. The mean values (mean ± SD) of two or three independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

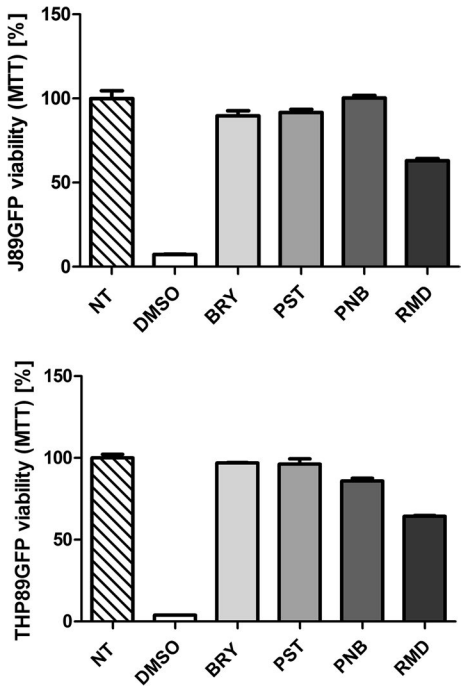

Fig. 3 Mitochondrial activity studies of latency reversal agents. MTT assays were performed on J89GFP lymphocyte and THP89GFP monocyte cell lines during 24 h. Both cell lines were treated with BRY up to 100 nM, PST up to 20 µM, PNB up to 40 nM, and RMD up to 20 nM. Non-treated (NT) cells were used as cell viability control. DMSO was used as cell death control. *NT* non-treated control, *DMSO* dimethyl sulfoxide. The mean values (mean ± SD) of three independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

issues with the small size of G1-S4, potential z for this dendrimer was not available. On the other hand, G2-S16 dendrimer z potential was −74.0 mV showing high long-term stability in aqueous solutions which demonstrate a good biocompatibility in cell culture. G3-S16 showed a higher z potential, −46.2 mV. These results agree with the viability data obtained by MTT and LDH assays, indicating that just 1 µM was the maximum non-toxic concentration for G3-S16, while for G2-S16 was ten times higher, 10 µM.

Reactivation was measured by determining GFP expression in living cells. Confocal microscope images of non-treated, G2-S16, G3-S16, BRY and DMSO 10% were taken to confirm the expression of GFP in live cells (Fig. 5). Results of NT and DMSO 10% in both cell lines represented viability and death control, showed in blue and red respectively. BRY was used as reactivation control, represented in green. In J89GFP cell line results showed that only BRY treatment leads to HIV-1 reactivation. However, in THP89GFP cell line, results indicate that G3-S16 dendrimer promotes GFP expression.

Reactivation profile of LRAs in combinations with dendrimers in latently HIV-1 infected cell lines

Our dendrimers are directed for a possible therapeutic treatment. In this context we have previously shown that these dendrimers inhibit HIV-1 infection and can be used in combination with different antiretrovirals [25–27]. However, we do not know what function the dendrimers have in the presence of LRAs. Therefore, we studied their potential effect in the presence of LRAs. To determine the viral reactivation, the GFP-fluorescence pattern was measured by flow cytometry.

The HIV-1 reactivation effect of BRY, PST, PNB and RMD were analysed as individual drugs or in combination with G1-S4, G2-S16 or G3-S16 dendrimers at various ratios. The concentrations of the dendrimers were based on the non-toxic concentration 10 µM G1-S4, 10 µM G2-S16 and 1 µM G3-S16 previously selected. The selection of LRA concentrations were performed, taking into account the maximum non-toxic concentration of each LRA based on the scientific literature, 100 nM BRY, 20 µM PST, 40 nM PNB, and 20 nM RMD. After 24 h and 48 h of exposure, the reactivation effect was measured by flow cytometry and expressed as GFP (integrated MFI or iMFI) (Fig. 6).

Our results indicate the enhanced GFP expression with the treatment of the LRAs alone in J89GFP cell line at 24 h of exposure. The PST induced the highest response at 55% of EGFP expression. In the case of the dendrimers alone, the GFP expression was not modified in regards of the non-treated control. The combination treatment of LRAs and dendrimers indicate that the antilatency activity of the LRAs was not modified, even in some cases it tends to increase slightly in combination with G1-S4, G2-S16 or G3-S16 dendrimers. At 48 h BRY, PST, RMD or PNB tend to increase the GFP expression in J89GFP cell line. Nevertheless, RMD in combination with G1-S4 showed an increased tendency in the expression of GFP. Neither of the three dendrimers studied alone show any variation in the GFP expression in the lymphocytic derived cell line. The combination of either G1-S4, G2-S16 or G3-S16 with BRY and PST in J89GFP lymphocytic cell line at 48 h did not modify the GFP expression. Our data indicate that the combination treatment of our dendrimers with the PKC agonist did not modify the antilatency activity.

In the THP89GFP, monocytic derived cell line, we obtained different results. The GFP expression in the single LRAs treatment at 24 h was greatly increased in comparison with the J89GFP lymphocytic cell line, reaching 80% of GFP expression with BRY. The LRAs combination with G1-S4 or G2-S16 dendrimer did not show any variation of the GFP expression in regards of treatment of LRAs alone. Surprisingly, in THP89GFP monocytic

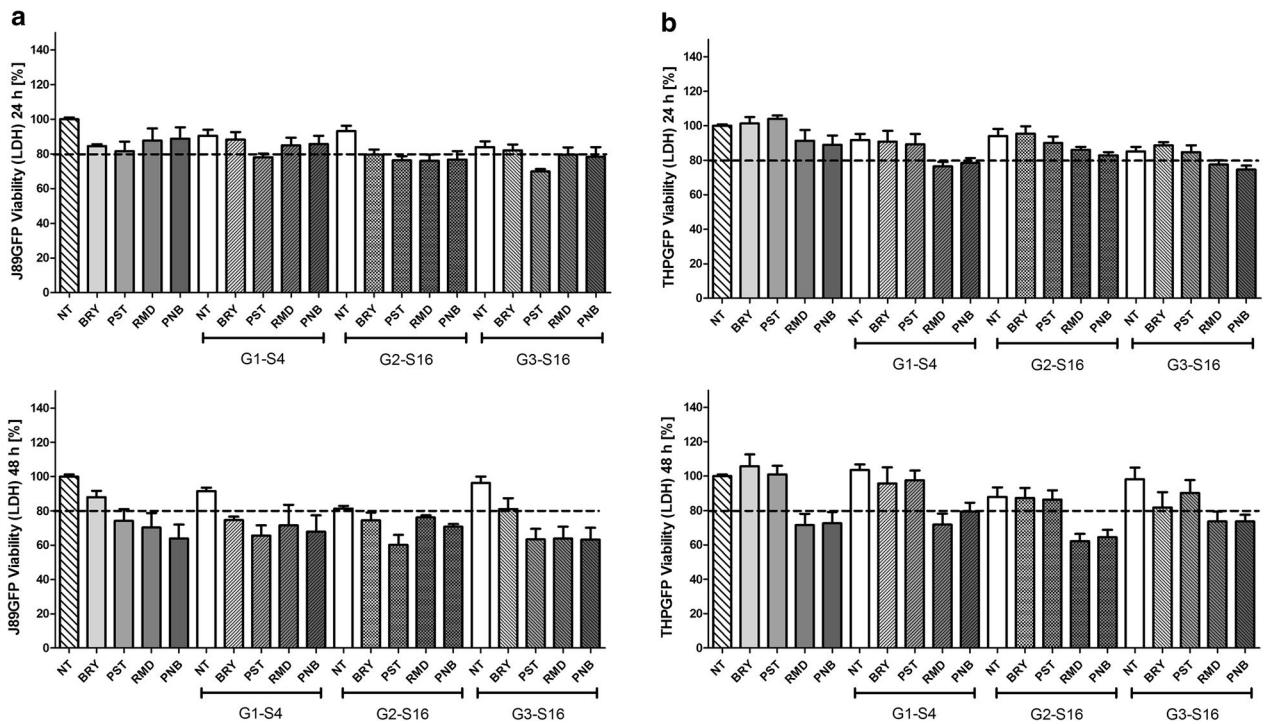

Fig. 4 Membrane integrity studies of carbosilane dendrimers and LRAs combinations. LDH assays were performed on **a** J89GFP lymphocyte and **b** THP89GFP monocyte cell lines during 24 h and 48 h. Cell lines were treated with G1-S4 (10 μM), G2-S16 (10 μM) or G3-S16 (1 μM) dendrimers. Both cell lines were treated with BRY (100 nM), PST (20 μM), PNB (40 nM) or RMD (20 nM). Non-treated (NT) cells were used as cell viability control. *NT* non-treated control, *BRY* bryostatin, *PST* prostratin, *RMD* romidepsin, *PNB* panobinostat. The mean values (mean ± SD) of three independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

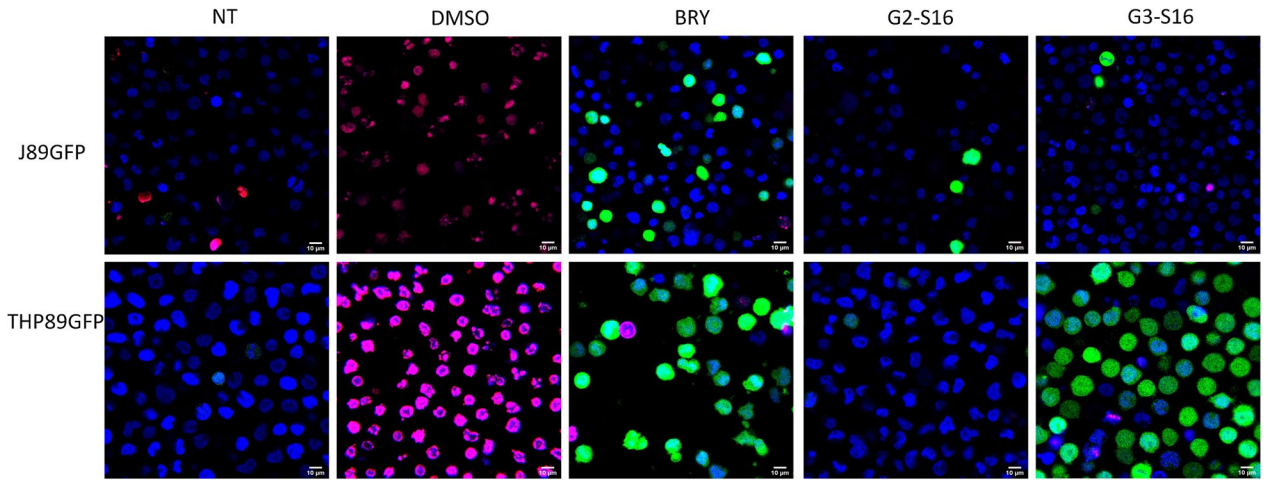

Fig. 5 Confocal microscope images of reactivation and LIVE/DEAD cells. J89GFP or THP89GFP cell lines were treated with BRY (100 nM), G2-S16 (10 μM) or G3-S16 (1 μM) for 48 h. After incubation, cells were stained with NUCLEAR-ID® Blue/Red. Blue: live cells. Green: HIV-1 reactivation. Red: cellular death. Non-treated (NT) cells were used as cell viability control. DMSO was used as cell death control. *NT* non-treated control; *DMSO* dimethyl sulfoxide; *BRY* bryostatin. Representative images of two independent experiments performed in duplicate are shown

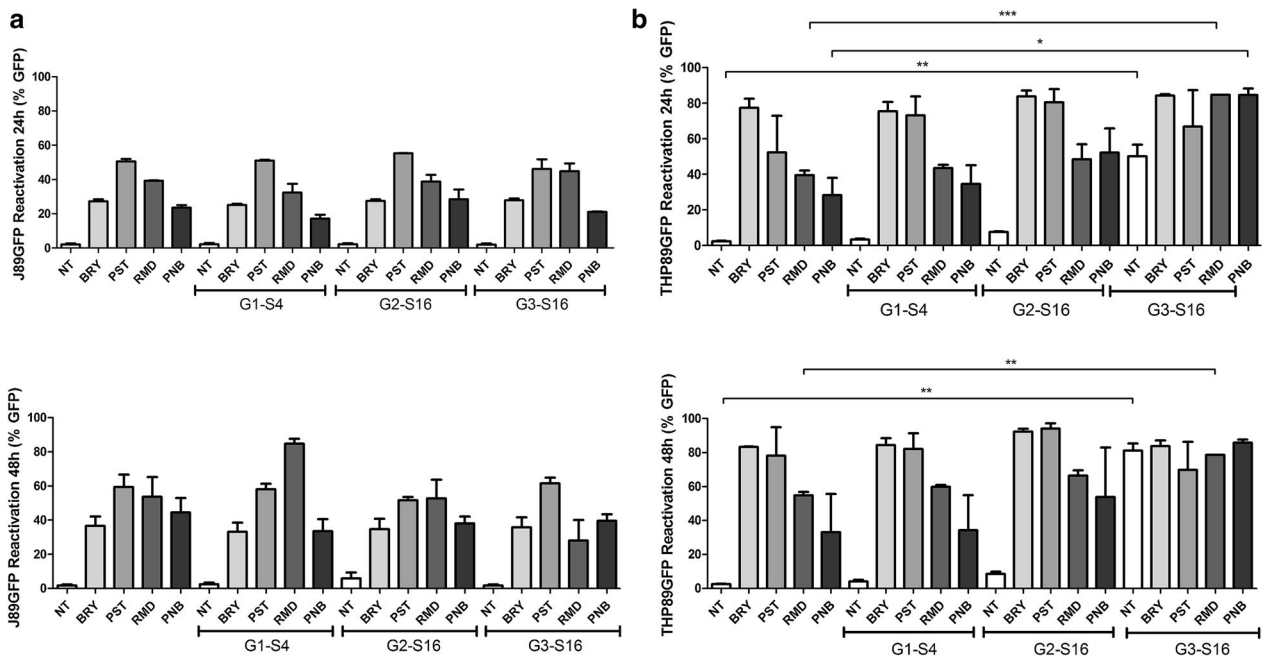

Fig. 6 Reactivation profile of BRY, PST, RMD and PNB in combination with G1-S4 (10 μM), G2-S16 (10 μM), G3-S16 (1 μM) dendrimers. J89GFP (**a**) and THP89GFP (**b**) cell lines were treated with BRY (100 nM), PST (20 μM), RMD (20 nM) and PNB (40 nm), either alone or in combinations with dendrimers for 24 h and 48 h. The integrated mean fluorescence intensity (iMFI, percentage of GFP expressing cells *MFI) of live cells was used as a measure of HIV-1 reactivation. *NT* non-treated control, *BRY* bryostatin, *PST* prostratin, *RMD* romidepsin; *PNB* panobinostat. The mean values (mean ± SD) of two or three independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

cell line, the G3-S16 dendrimer alone treatment showed a significant increased expression of GFP reaching 50%. We hypothesize that high size of G3-S16 in addition to sulfate groups in its periphery activate the monocytes, thus unleashing transcription factors such as NF-Kb or Sp1, forcing the viral reactivation and transcription [28]. In the case of the combinations of LRAs with G3-S16, the results showed an enhanced expression of GFP reaching almost 80% for BRY, RMD and PNB. This result proves a new possible approach for a combination treatment in the "shock and kill" method with dendrimers, which not only inhibit the entry of new replicative viruses, but also help with the reactivation activity of LRAs. Although our results are promising, the mechanism of our dendrimers reactivation and the use of more physiological models such as latently infected primary cells or in vivo models should be studied. Summing up, we demonstrated for the first time that nanoparticles, in this case dendrimers may play an important role in new treatments against HIV-1 infection.

Authors' contributions

MAMF designed the study, developed the methodology, analysed and discussed data and wrote the manuscript. EM designed the study, analysed and discussed data. IR and RJ performed experiments, analyzed the data, composed the figures, interpreted and discussed the data. CP analyzed the data, interpreted and discussed the data. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

3.2. Article "Nanotechnology against human cytomegalovirus in vitro: polyanionic carbosilane dendrimers as antiviral agents"

HCMV HIV-1 co-infection represents significant morbidity in several populations, specifically in African countries. In this sense, we wondered if some of the PCDs that we already showed anti-HIV activity would also be able to act against HCMV. To this end, we performed a screening with several PCDs to study its activity against HCMV. First, after the selection of 3 candidates, we performed viability assays to determine the working concentration, either by LDH and MTT in human lung fibroblast (MRC-5).

Once showed the excellent biocompatibility of dendrimers, we performed inhibition assays against HCMV. These results indicated that G2-S16 and G2-S24P represent two good candidates against HCMV, as they kept high inhibition results. With these inhibition results, we wondered if the mechanism of action of both PCDs would be similar to the observed with HIV-1, thus inhibiting the first step of the infection, the attachment. To this end, we performed several experiments to decipher the mechanism of action of both PCDs. We completed an attachment assay and surprisingly observed that dendrimers are not inhibiting in this step of the infection. These results were not expected, as many dendrimers were used to inhibit the viral attachment to the host cell.

Nevertheless, we also performed virucidal assays to determine if dendrimers were acting directly against HCMV, but results showed that this was not the mechanism of action either. However, to determine the time point of the inhibition of HCMV, we also performed a time of addition assay. We observed that both PCDs promote an early inhibition of the infection and maintain this inhibition for almost four days after the infection. Moreover, we performed a co-treatment of each dendrimer with the election drug for HCMV infection, Ganciclovir. Surprisingly, we observed that both PCDs used to enhance the inhibition activity of ganciclovir, reaching high levels of inhibition, and maintaining it for more time than the actual treatments.

Summing up, we found two PCDs capable of inhibiting HCMV infection but not acting at the first step of the infection. These results promote the use of nanotechnology against several infectious diseases, specifically the use of G2-S16 that, in addition to its

HIV-1 inhibition activity, can inhibit HCMV, becoming a multivalent molecule against sexually transmitted diseases.

I have actively collaborated developing the methodology and performing the experiments. In addition, I have contributed analysing and discussing data, composing figures, and writing the manuscript in this paper.

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Nanotechnology against human cytomegalovirus in vitro: polyanionic carbosilane dendrimers as antiviral agents

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Abstract

Background: Human cytomegalovirus (HCMV) is a worldwide infection, causing different troublesome in immunosupressed patients and very related to Human Immunodeficiency Virus 1 (HIV-1) infection, mainly in developing countries, with a co-infection rate of 80% in Africa. The high cost of present treatments and the lack of routinely tests in these countries urge the necessity to develop new molecules or strategies against HCMV. The new treatments should be low-cost and capable of avoiding the emerging problem of resistant virus. Nanoparticles play an important role in several viral infections. Our main focus is to study the potential activity of polyanionic carbosilane dendrimers (PDC), which are hyperbranched molecules with several sulfonate or sulfate groups in their periphery, against different viruses.

Results: We studied the activity of G1-S4, G2-S16 and G2-S24P PDCs in MRC-5 cell line against HCMV infection by several plaque reduction assays. Our results show that dendrimers present good biocompatibility at the concentrations tested (1–50 μM) for 6 days in cell culture. Interestingly, both G2-S16 and G2-S24P showed a remarked inhibition at 10 μM against HCMV infection. Results on attachment and virucidal assays indicated that the inhibition was not directed to the virus or the virus-cell attachment. However, results of time of addition, showed a longer lasting activity of these dendrimers in comparison to ganciclovir, and the combination of G2-S16 or G2-S24P with ganciclovir increases the HCMV inhibition around 90%.

Conclusions: Nanotechnology, in particular polyanionic carbosilane dendrimers, have proved their potential application against HCMV, being capable of inhibiting the infection by themselves or enhancing the activity of ganciclovir, the actual treatment. These compounds represent a low-cost approach to fight HCMV infections.

Keywords: Polyanionic carbosilane dendrimers, HCMV, Ganciclovir

Introduction

The human herpesvirus 5 or human cytomegalovirus (HCMV) is a widespread pathogen, belonging to betaherpesviridae family. The HCMV genome consists of a double stranded DNA with approximately 230,000 bp. The genome is enclosed by an icosahedral capsid (100–110 nm diameter) and the mature viral particle has a diameter of 150–200 nm [1].

The HCMV infects and replicates in a wide variety of cells, including epithelial cells of gland and mucosal tissue, smooth muscle cells, fibroblasts, macrophages, dendritic cells, hepatocytes and vascular endothelial cells [2]. This broad cell tropism causes systemic spread in the human body, and inter-host spread.

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In immunocompetent individuals, HCMV infection is asymptomatic in most cases or yield minor symptoms. However, asymptomatic individuals are able to spread the HCMV by several body fluids [1]. Foremost, in immunosupressed individuals, as AIDS patients, elders or transplant recipients, HCMV infection can lead to dissemination and life-threatening end-organ diseases [3]. Moreover, congenital HCMV (cCMV) infections can cause severe clinical deficiencies in the development of the foetus and can produce an impact of 0.7 in newborns, being the pathogen of congenital transmission more common in the worldwide [4]. Congenital infections are usually caused by a primary infection of the mother during pregnancy with an intrauterine transmission rate of 40–50%.

The HCMV represents an important co-infection in HIV-1 infected patients. HCMV is associated with increased morbidity and mortality [5], specifically in African population, where the seroprevalence of HCMV infection is near 80% in HIV-1 patients. Data obtained from patients in the South Africa indicate that in severely HCMV patients, ganciclovir therapy could be life-saving. However, in this country, the access to common treatments is not always available as a consequence of their high cost. Moreover, no routinely tests for HCMV infection are performed in most developing countries [6]. Due to these factors, the discovery of new treatments is essential, since new resistances are emerging in current treatments [7–9].

Our group has focused on the study of different polyanionic carbosilane dendrimers (PCDs). These dendrimers have shown their ability to prevent or even to eliminate the transmission of several sexual infectious diseases such as HIV-1, Human Herpes Virus (HSV-1), (HSV-2) or Hepatitis C Virus (HCV) [10–14]. In last decades the nanotechnology has achieved many goals in different fields, from basic application to biomedicine, emerging new applications and treatments [15–18]. Dendrimers are hyperbranched nanoparticles, consisting of a silicon or polyphenolic core, surrounded by tree-like branches in which generations are defined by the number of repeated layers of the branches units. In the periphery of branches, several functional groups are located, which can be modified, allowing dendrimers to develop several functionalities.

Different groups have shown that the probability of HCMV infection increases in HIV-1 infected patients due to different protein and cofactor interactions, and vice versa [18–20]. In those cases, a low-cost treatment capable of inhibiting one, or even both infections, could drastically reduce the great incidence of both diseases in different developing countries. Altogether, along with the high prevalence of HCMV and HIV-1 co-infection in several countries, the developing of a cost-effective treatment that would be capable of diminishing both infections is of remarked importance. In our work, we present G2-S16 and G2-S24P PCDs, which are capable of reducing HCMV infection showing good biocompatibility. Although the mechanism of action of G2-S16 or G2-S24P dendrimer against HCMV infection has not been deciphered yet, the promising results, along with the cost-effective production of these nanocompounds, position those PCDs as good antiviral agents against HCMV.

Our objective is to demonstrate that nanoparticles, specifically PCDs with sulfonate or sulfate groups in their periphery, are capable of inhibiting HCMV infection *in vitro.*

Materials and methods

Dendrimers

PCDs G1-S4 with silicon core and 4 sulfate groups in periphery, G2-S16 with silicon core and 16 sulfonate groups in the periphery, and G2-S24P with a polyphenolic core and 24 sulfonate groups in the periphery. All dendrimers ranged between 1 and 20 nm being bigger as the generation of the dendrimers increases. PCDs were synthesized and analyzed according to methods reported by the Dendrimers for Biomedical Applications Group of University of Alcalá (Madrid, Spain) [21–23]. NMR spectroscopy data confirmed the identity of compounds: G1-S4 1H NMR (DMSO-d6): 7.85 (s, 4H, NCHCN), 4.26 (s, 8H, SiCH2CH2CH2CH2N), 3.91 (s, 8H, CCH-2CH2OSO32), 2.85 (s, 8H, CCH2CH2OSO32), 1.77 (s, 8H, SiCH2CH2CH2CH2N), 1.25 (s, 16H, SiCH2CH2), 0.50 (s, 24H, SiCH2), 20.10 (s, 24H,Si(CH3)2). 13 C{1H} NMR (DMSO-d6): 143.1 (NCHCN), 121.8 (NCHCN), 64.3 (CCH2CH2OSO32), 48.3 (SiCH2CH2CH2CH2N), 33.1 (SiCH2CH2CH2CH2N), 25.5 (CCH2CH2OSO32) 19.9–13.8 (SiCH2CH2 y SiCH2), 23.8 (Si(CH3)2). 29Si NMR (DMSO-d6): 1.7 (Si(CH3)2) G2-S16 (1H-NMR (D2O): − 0.21 (br. s, 12 H, SiMe), − 0.16 (s, 48 H, SiMe2), 0.28 (m, 16 H, SiCH2CH2CH2N), 0.43 (m, 48 H, SiCH2CH2CH2Si), 1.21–1.31 (m, 40 H, SiCH-2CH2CH2Si and SiCH2CH2CH2N), 2.28 (m, 16 H, CH2CHCH2N), 2.76 (m, 32 H, NCH2CH2SO3), 2.86 (m, 32 H, NCH2CH2SO3). 13 C-NMR{1H} (D2O): − 4.4 (SiMe), − 3.5 (SiMe2), 12.4 (SiCH2CH2CH2N), 17.7–18.5 (CH2), 20.1 (CH2), 46.7 (NCH2CH2SO3), 48.0 (NCH2CH2SO3), 56.9 (CH2CH2CH2N). 29Si-NMR (D2O): G0-Si not observed, 1.1 (G1-Si), 2.1 (G2-Si)) and G2-S24P 1H-NMR (D2O): − 0.18 (br. s, 99 H, SiMe), 0.28 (m, 30 H, SiCH2), 0.41 (m, 72 H, SiCH2), 1.05–1.20 (m, 66H, CH2CH2CH2), 1.60 (m, 2 H, OCH2CH2), 2.27 (m, 24 H, CH2CHCH2N), 2.75 (m, 48 H, NCH2CH2SO3), 2.85 (m, 48 H, NCH2CH2SO3), 3.42 (m, 2 H, OCH2), 5.64 (br. s, 3 H, C6H3).

13 C-NMR{1H} (D2O): − 4.6 (SiMe),−3.4 (SiMe2), 12.7 (SiCH2CH2CH2N), 18.5 (CH2), 20.0 (CH2), 20.7 (CH2), 22.4 (CH2), 47.1 (NCH2CH2SO3), 47.8 (NCH2CH2SO3), 57.2 (CH2CH2CH2N), 66.9 (OCH2), 93.6 (C6H3, CH), 160.6 (i-C6H3). 15 N-NMR (D2O): − 343.2. 29Si-NMR (D2O): 1.7 (only one peak was observed) (Additional file 1). Stock solution of dendrimers (5 mM) and subsequent dilutions to work concentrations were prepared in nuclease-free water (Promega, Madrid, Spain). The schematic structures of PDCs are presented in Fig. 1. NMR spectroscopy data NMR spectra were recorded on a Varian Unity VXR-300 (300.13 (1H), 75.47 (13 C) MHz) or on a Bruker AV400 (400.13 (1H), 100.60 (13 C), 79.49 (29Si) MHz).

Cells and viruses

Human lung fibroblast cells (MRC-5) (ATCC CCL-171) were cultured in Dulbeco's Modified Essential Medium (DMEM) supplemented with 10% Heat- inactivated Fetal Bovine Serum (FBS) and 1% antibiotics cocktail containing 125 mg/ml ampicilin, 125 mg/ml cloxaciclin and 40 mg/ml gentamicin (Normon, Madrid, Spain). The viral strain $HCMV_{AD-169}$ (ATCC VR-538) was expanded and titrated in MRC-5 cell line by plaque assay with serial dilutions. Stock aliquots at 3.5×10^6 PFU/mL were prepared by ultracentrifugation and stored at − 80 °C.

Mitochondrial activity assay

The mitochondrial toxicity of G1-S4, G2-S16 and G2-S24P PCDs was tested by the 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, USA) according to manufacturer's instructions. Briefly, 1×10^5 cells/well of MRC-5 cells were seeded in 96-well plates and treated with the desired compounds for 6 days in a concentration range (1–50 μM). After incubation, culture medium was discarded and 220 μl of a 1:11 MTT (5 mg/ml)/OptiMEM solution was added to cultured MRC-5 cells. After 3 h, the supernatant was removed, and formazan crystals were dissolved in 200 μl DMSO (Sigma, St. Louis, MO, USA) absorbance was read in a Berthold Plate Reader at 570 nm. All points were performed in triplicate. DMSO 10% was used as death cellular control. Non-treated cells were used as viability control.

Membrane integrity assay

Cellular toxicity was measured by the lactate deshidrogenase (LDH) assay CytoTox 96® Non-Radioactive Cytotoxicity (Promega, Spain, Madrid) following manufacturer's instructions. Briefly, 1×10^5 MRC-5 cells were seeded in 96-well plates and treated with the desired compounds for 6 days in a concentration range (1–50 μM). After the incubation period, MRC-5 cells were lysed in 0.9% Triton X-100 (Promega, Spain, Madrid) for 45 min at 37 °C and 50 μl of LDH reagent (Promega, Spain, Madrid) was added for 30 min at room temperature, protected from light. Absorbance was read in a Berthold Plate Reader at 490 nm. All points were performed by triplicate. Non-treated MRC-5 cells were used as viability control.

Inhibition assay

The potential activity of PCDs against $HCMV_{AD-169}$ infection was measured by plaque reduction assays. Briefly, 7.5×10^4 cells/well of MRC-5 cells were seeded in 24-well plates and incubated at 37 °C for 24 h. After incubation, MRC-5 cells were treated with G1-S4, G2-S16 or G2-S24P for 1 h at 37 °C and infected with 60 PFU of $HCMV_{AD-169}$ for 2 h at 37 °C, inoculum was discarded and DMEM supplemented with 2% FBS was added. MRC-5 cells were incubated for 6 days at 37 °C, MRC-5 cells need 6 days after the virus was added to develop cytopathic effects by HCMV. After that supernatant was discarded and MRC-5 cells were dyed with Methylene Blue (Sigma, St. Louis, MO, USA) for plaque counting. Percentage of inhibition is given as the reduction of the plaques formed with dendrimers treatments in comparison with the infection control.

Attachment assay

To study if the inhibition of PCDs is directed to block the virus-membrane interaction, membrane attachment studies were performed by plaque reduction assays. Briefly, 7.5×10^4 MRC-5 cells/well were seeded in 24-well plates and incubated at 37 °C for 24 h. After incubation, MRC-5 cells were pre-cooled at 4 °C for 15 min and treated with G1-S4, G2-S16 or G2-S24P for 1 h at 4 °C and infected with 60 PFU of $HCMV_{AD-169}$ for 2 h at 4 °C, inoculum was discarded and DMEM supplemented with 2% FBS was added. MRC-5 cells were incubated for 6 days at 37 °C, supernatant was discarded and MRC-5 cells were dyed with Methylene Blue for plaque counting. Percentage of inhibition is given as the reduction of the plaques formed with dendrimers treatments with regard to the infection control.

Virucidal activity

To study the direct interaction between dendrimers and virus, virucidal activity by plaque reduction assays were performed. Briefly, 7.5×10^4 MRC-5 cells/well were seeded in 24-well plates and incubated at 37 °C for 24 h. We combine G2-S16 or G2-S24P at 10 μM with 60 PFU/ml of MRC-5 cell free $HCMV_{AD-169}$ for 2 h at 37 °C. After incubation, the eppendorfs were centrifugated at 12,000 rpm for 1 h at 4 °C, supernatant

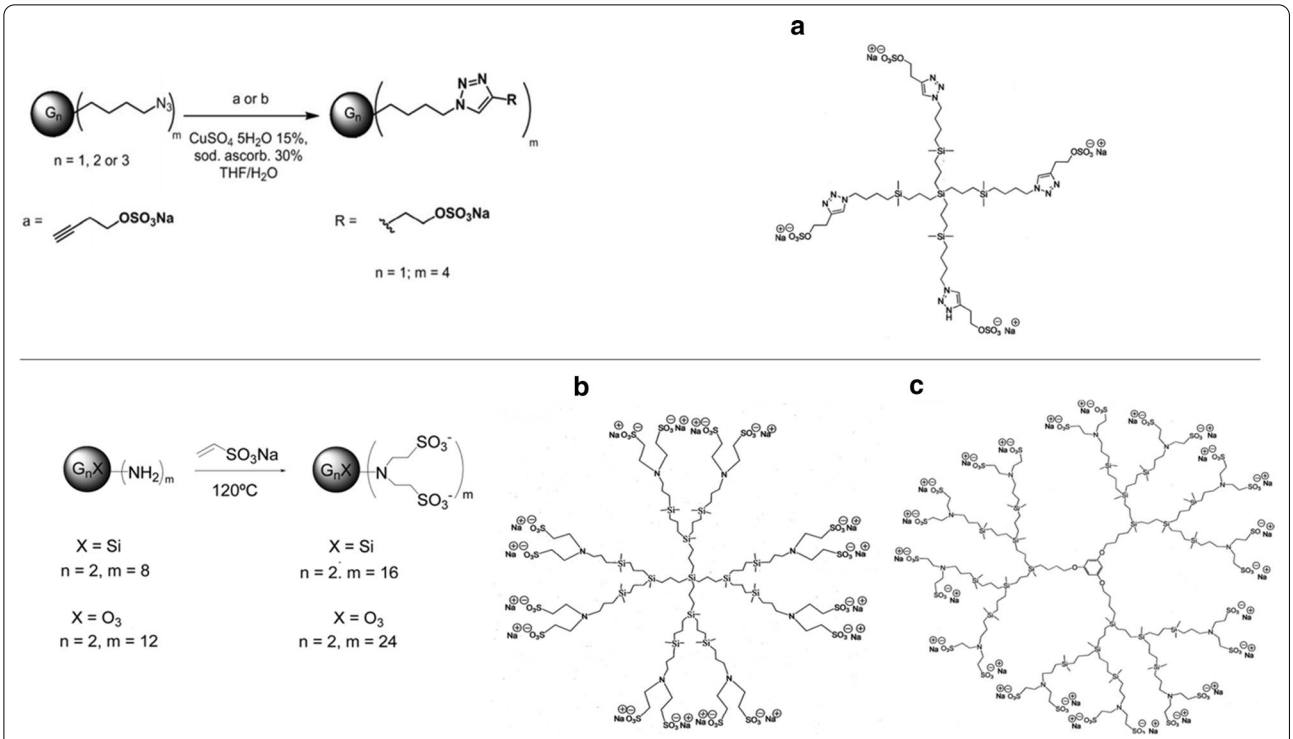

Fig. 1 Schematic synthesis route and molecular representation of dendrimers. **a** G1-S4 with silicon core and 4 sulfate end groups, **b** G2-S16 with silicon core and 16 sulfonate end groups and **c** G2-S24P with polyphenolic core and 24 sulfonate end groups were represented. The generation of dendrimers is determined by considering that each generation corresponds to the number of repeating layers of silicon atoms

was discarded, rinsed with PBS and centrifugated at 12,000 rpm for 1 h at 4 ºC to eliminate the compound. Finally, supernatant was replaced with fresh DMEM supplemented with 2% FBS and added to MRC-5 cells. Triton X-100 at 0.1% was used as positive control of disrupting $HCMV_{AD-169}$ membrane. $HCMV_{AD-169}$ infection was revealed as described before.

Time of addition

To specify the inhibition check point of dendrimers in the viral cycle, the activity of G2-S16 and G2-S24P dendrimers was studied at different time points post-infection by plaque reduction assays. Briefly, 7.5×10^4 MRC-5 cells/well were seeded in 24-well plates and incubated at 37 ºC for 24 h. After incubation, MRC-5 cells were infected with 60 PFU of $HCMV_{AD-169}$ and treated at 0, 24, 48, 72 and 96 h post-infection, either with G2-S16, G2-S24P, ganciclovir or a combination of PCDs with ganciclovir. In all conditions, MRC-5 cells were incubated for 6 days after infection at 37 ºC, and $HCMV_{AD-169}$ infection was revealed as described before. Percentage of inhibition is given as the reduction of the plaques formed with G2-S16 dendrimer, ganciclovir or G2-S16/ganciclovir treatments with regard to the infection control.

Confocal microscopy

To confirm the results obtained by plaque reduction assays, we performed confocal microscopy in MRC-5 cells. Briefly, 7.5×10^4 MRC-5 cells were plated and incubated for 24 h in 24-well plates, in which was previously added a 12 mm cover-glass per well pre-treated with Collagen IV (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After incubation, MRC-5 cells were treated with G2-S16 for 1 h at 37 ºC and infected with 60 PFU of $HCMV_{AD-169}$ for 2 h at 37 ºC, inoculum was discarded and fresh DMEM, supplemented with 10% FBS, was added. MRC-5 cells were incubated for 6 days, then supernatant was discarded and cells were fixed in 4% paraformaldehide (PFA; Panreac, Barcelona, Spain) for 10 min, washed 3 times in PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. After incubation MRC-5 cells were washed 3 times in PBS, blocked with 1% bovine serum albumin (BSA Sigma-Aldrich, St. Louis, MO, USA) and 0.1% Triton X-100 in PBS for 30 min and incubated with Cytomegalovirus pp65 Antibody, ALEXA FLUOR® 488 Conjugated (bioNova cientifica S.L) and phalloidin ALEXA FLUOR® 555 Conjugated antibody for 1 h, MRC-5 cells were washed 3 times in PBS and dyed with DAPI (Sigma-Aldrich, St. Louis, MO, USA) 10 min. Finally, the cover glasses were mounted in

slides and analyzed in a Leica TSC SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence was analyzed using ImageJ software (National Institute of Health, USA).

Statistics

Statistical analysis was performed using GraphPad software Prism v.5.0 (GraphPad Software, San Diego, CA USA) and results were assessed by using a paired *t*-test between two groups (control versus different dosages of compounds or treated versus ganciclovir and G2-S16 or G2-S24P) ($^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$). Time of addition assays were analyzed by a 2 ways ANOVA between 2 groups (ganciclovir versus combinations with G2-S16 or G2-S24P).

Result and discussion

Biocompatibility of dendrimers in human lung fibroblast cell line

The analysis of biocompatibility of G1-S4 dendrimer with 4 sulfate groups in periphery, G2-S16 dendrimer with 16 sulfonate groups in periphery and G2-S24P dendrimer with a polyphenolic core and 24 sulfonate groups in periphery was performed by studying the mitochondrial activity (MTT) and cell membrane integrity (LDH) of treatments at increasing concentrations (1, 5, 10, 20, 35, 50 µM) for 6 days in MRC-5 cell culture (Fig. 2). The MTT results in MRC-5 cells show that G1-S4, G2-S16 and G2-S24P PCDs at more than 1 µM cause a dose-dependent reduction in mitochondrial activity. In contrast with MTT results, that shows a specific state of mitochondrial activity and is not categorical of cell death [24]. LDH results, which refers to cell membrane integrity, show different results, indicating that treatments with G1-S4, G2-S16 and G2-S24P PCDs for 6 days do not cause any damage in MRC-5 cells membranes, even the tendency was similar.

G1-S4, G2-S16 and G2-S24P PCDs inhibition against $HCMV_{AD-169}$ infection

Previously published reports of PCDs against HIV-1 [25], HSV-2 [11, 12] or even HCV [10] propose PCDs as alternative agents against several viral infections [26]. The studies of the potential activity of G1-S4, G2-S16 and G2-S24P PCDs against $HCMV_{AD-169}$ infection was performed by plaque reduction assays in MRC-5 cells (Fig. 3). The results represent the percentage of infection in regard of the infection control. Results show that G1-S4 dendrimer at all concentrations tested do not cause any reduction in the number of plaques. In contrast G2-S16 and G2-S24P dendrimers show a significant reduction of the $HCMV_{AD-169}$ infection in comparison with the infection control. Both second generation dendrimers at 5 µM cause approximately 50% of inhibition, reaching the maximum inhibition for G2-S24P. However, G2-S16 dendrimer at a concentration of 10 µM leads to 80% of plaque reduction in MRC-5 cells, showing a dose-dependent mechanism of action.

Confocal microscopy of $HCMV_{AD-169}$ infection in MRC-5 cell line

In order to confirm the inhibitory activity of G2-S16 against $HCMV_{AD}$-169, previously observed by plaque reduction assays in the MRC-5 cell line, confocal microscopy images were taken of treatments with G2-S16 dendrimer.

The fibroblast-like morphology of MRC-5 cells was visualized by interaction with actin filaments, through a red phalloidin stain, $HCMV_{AD}$-169 infection was visualized thanks to the labelling with an antibody against the viral protein integument pp65 in green colour and cell nuclei were marked in blue by DAPI staining. The results indicate that treatments with G2-S16 dendrimer, compared to control of infection, not only reduce notably the number of plaques as shown in Fig. 4, but also the size of the plaques itself decreases in about 40% (*data not shown*). We hypothesize that this decreasing in the plaque size is probably meaning, either that dendrimer is not allowing a normal replication of the virus or that the new transcribed virus is not able to infect the nearest cells, as we can observe viral protein in the centre of the plaque with G2-S16 dendrimer treatment, but not the same spreading as control of $HCMV_{AD}$-169 infection.

Inhibition of virus-cell membrane attachment

To study if the mechanism of action of dendrimers against $HCMV_{AD-169}$ infection was directed to block the interaction of the virus at the cell membrane, attachment assays were performed. In order to observe this interaction, the cell membrane was forced at 4 °C to be less permeable either to virus and dendrimer. Previously data published in our group with those PCDs against HSV-1 and HSV-2 infection, indicated that G1-S4 and G2-S16 dendrimers inhibition mechanism was directed to inhibit virus-cell membrane interaction [10]. In contrast, in our results (Fig. 5) $HCMV_{AD-169}$ infection was not reduced in this assay at any PCDs concentration, leading to the hypothesis that in this case, G1-S4, G2-S16 and G2-S24P PCDs are not inhibiting the $HCMV_{AD-169}$ entry to the MRC-5 cells. There is no inhibition at the cell membrane level. In fact, these results indicate that the inhibition observed in previous assays is occurring in an intracellular level, and that PCDs do not have an unspecific mechanism of action, even more, the behaviour depends on the viral cycle and the host cell.

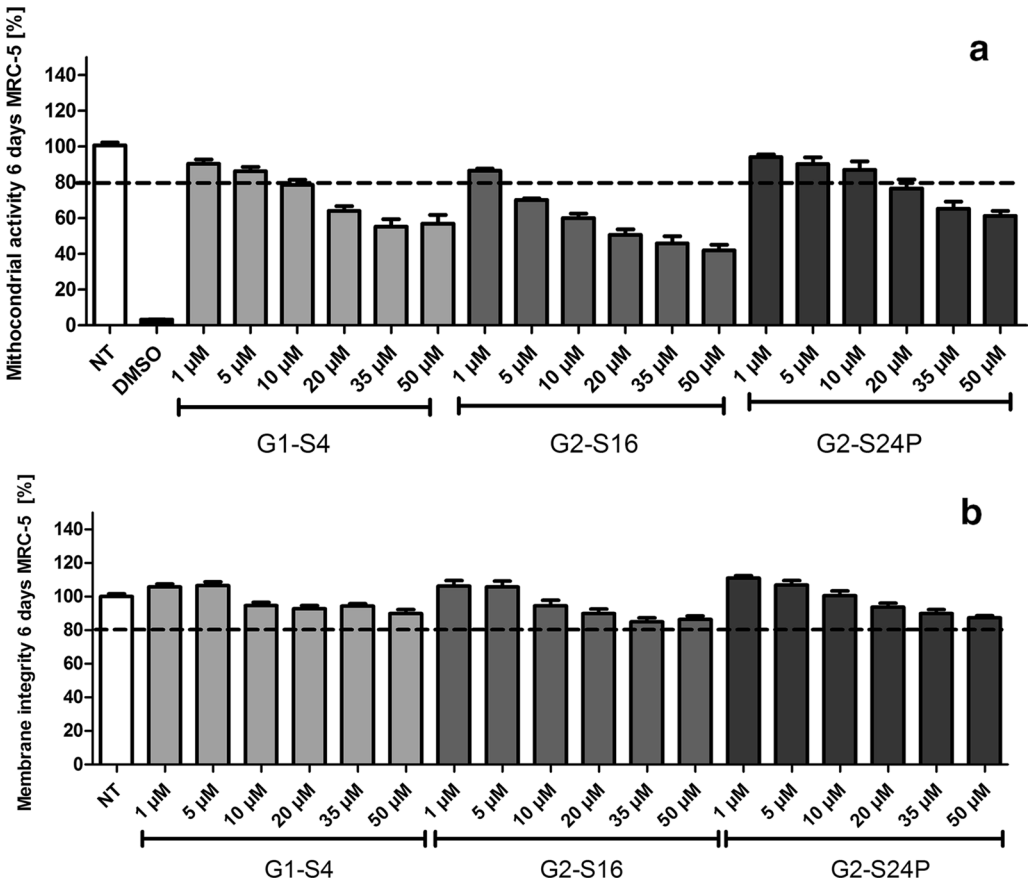

Fig. 2 Biocompatibility studies of polyanionic carbosilane dendrimers (PCDs). **a** MTT and **b** LDH assays were performed in MRC-5 cells for 6 days treatments. MRC-5 cells were treated with increased concentration range (1 μM – 50 μM) of G1-S4, G2-S16 or G2-S24P PCD. Non-treated (NT) MRC-5 cells were used as cell viability control. DMSO was used as cell death control. NT, non-treated control; DMSO, Dimethyl sulfoxide. The mean values (mean ± SD) of at least three independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

Virucidal activity of G2-S16 and G2-S24P dendrimers against $HCMV_{AD-169}$

Antiviral activity of PCDs has already been described, specifically direct interaction of G2-S16 dendrimer against the viral glycoprotein 120 (gp120) in HIV-1 infection [13], leading to a disruption on viral membrane, reducing the viral infection. To study the direct interaction between G1-S4, G2-S16 and G2-S24P PCDs against cell-free $HCMV_{AD-169}$, virucidal assays were performed. The results (Fig. 6) show that direct contact of PCDs with $HCMV_{AD-169}$ do not cause any disruption on virus membrane, leading to a complete infection in G2-S16 and G2-S24P dendrimers treatments in comparison with Triton X-100.

Time-of-addition of G2-S16 and G2-S24P dendrimers during $HCMV_{AD-169}$ infection

A time of addition assay was performed to study the mode of action of G2-S16 and G2-S24P dendrimers either alone, or in combination with ganciclovir during a viral infection cycle in MRC-5 cells (Fig. 7). Ganciclovir results, measured by plaque reduction assay, show that it maintains its inhibitory activity if added 48 h post $HCMV_{AD-169}$ infection. After that time point, the $HCMV_{AD-169}$ inhibition is reduced to 40% at 72 h and 20% at 96 h. However, we obtained different results with dendrimers single treatment, in the case of G2-S16, inhibition activity is maintained longer than we observed with ganciclovir. During the first stages of the $HCMV_{AD-169}$ cycle, G2-S16 at 10 μM is able to maintain 70–80% of $HCMV_{AD-169}$ inhibition activity, interestingly at the 48 h time point, ganciclovir and G2-S16 reach the same inhibition rate, but in contrast of the election drug, G2-S16 inhibition activity last longer, reaching 55–60% of $HCMV_{AD-169}$ inhibition even if treated 96 h post-infection. We also performed a combination of G2-S16 dendrimer and ganciclovir and we observe that the plaque number at almost all time points is greatly reduced, specifically, for 72 h post infection all points show an $HCMV_{AD-169}$ inhibition of almost 90–95% of

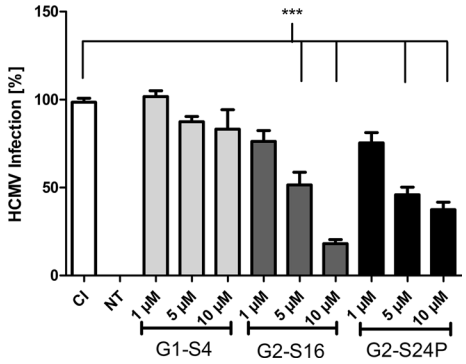

Fig. 3 Inhibition activity of dendrimers against $HCMV_{AD-169}$. Plaque reduction assays were performed in MRC-5 cells for 6 days treatments. MRC-5 cells were pre-treated for 1 h at 37 ºC with increased concentrations of G1-S4, G2-S16 and G2-S24P PCDs (1, 5 and 10 μM) and infected with 60 PFU at 37 ºC. Plaques were counted after 6 days with a methylene blue dye. Percentage of infection is represented as the number of plaques in treatments in regard of the control of infection. CI, Control of Infection; NT, non-treated control. The mean values (mean ± SD) of at least four independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

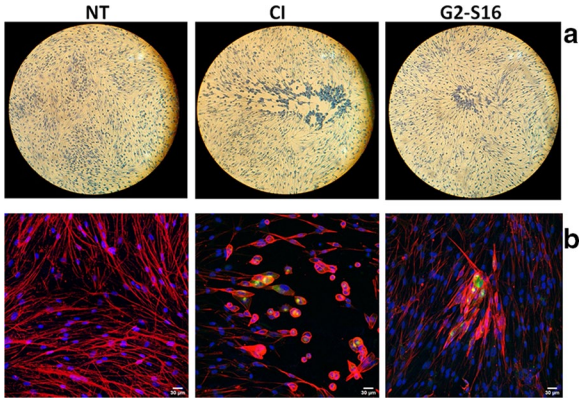

Fig. 4 Representative images of $HCMV_{AD-169}$ plaques in treatments with G2-S16 dendrimer. **a** Optical and **b** confocal representative images of MRC-5 cells. Briefly, MRC-5 cells were plated and treated with G2-S16 dendrimer (10 μM) for 1 h and infected with 60 PFU for 2 h at 37 ºC. After 6 days incubation **a** MRC-5 cells were dyed with methylene blue and images were taken on a DMIL Leica microscope at 4X augment. **b** MRC-5 cells were treated with HCMVpp65 ALEXA FLUOR® 488 Antibody Conjugated for viral particles visualization, phalloidin ALEXA FLUOR® 555 Conjugated and DAPI for membrane and nucleus localization. NT: non-treated; CI: control of infection

the infection, and this combination reduces its inhibitory activity at 96 h, with almost 80% of $HCMV_{AD-169}$ inhibition.

In the other hand, G2-S24P dendrimer report similar behaviour but with lower $HCMV_{AD-169}$ inhibition rate than G2-S16, in this case, at 24 h post infection G2-S24P dendrimer reaches 60% inhibition and maintains this activity for 72 h, at this time point the activity is similar to ganciclovir inhibition rate. Surprisingly, the combination of G2-S24P and ganciclovir shows an increased inhibition, reaching 90–95% for the first 48 h post infection, and decreases to 70–80% at 72 h and 96 h.

This increased inhibition rate is probably due to the combination of different mechanisms of action. Ganciclovir is a well know synthetic nucleoside analogue of guanine, related to acyclovir but has greater activity against HCMV and acts as DNA polymerase inhibitor [27].

In this respect, results on attachment, virucidal activity and time of addition, that compares G2-S16 and G2-S24P dendrimers with ganciclovir behaviour, may set an approximate mechanism of action of these dendrimers. However specific mechanism of action against $HCMV_{AD-169}$ has not been deciphered yet. Both dendrimers show activity against $HCMV_{AD-169}$ infection. We hypothesize, based on MTT assays (Fig. 2), that G2-S16 and G2-S24P dendrimers decrease mitochondrial activity, either by direct inhibition of mitochondria without causing global cell toxicity, or modifying other pathways in cell metabolism that may be covering mitochondrial activity in the MTT results. This reduction in mitochondrial activity is probably one of the key factors that induces the reduction of $HCMV_{AD-169}$ infection, as this virus is known to depend on this pathway for production and assembly of viral proteins [28, 29].

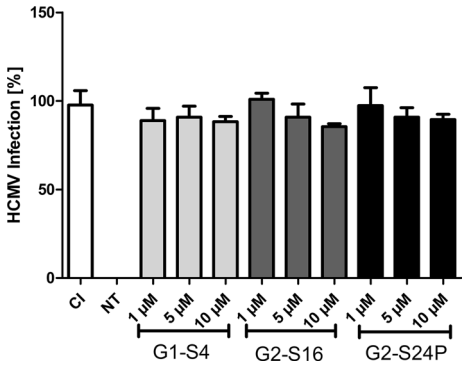

Fig. 5 Activity of PCDs at cell membrane-virus attachment. Plaque reduction assays were performed in MRC-5 cells for 6 days treatments. MRC-5 cells were pre-cooled at 4 ºC and pre-treated for 1 h at 4 ºC with increased concentrations of G1-S4, G2-S16 and G2-S24P PCDs (1, 5 and 10 μM) and infected with 60 PFU at 4 ºC. Plaques were counted after 6 days with a methylene blue dye. Percentage of infection is represented as the number of plaques in treatments in regard of the control of $HCMV_{AD-169}$ infection. CI: Control of Infection NT = non-treated control. The mean values (mean ± SD) of at least four independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

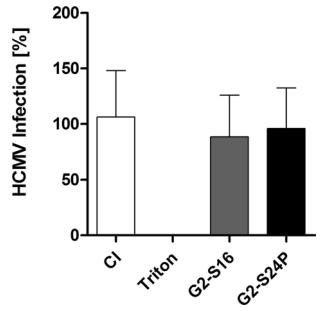

Fig. 6 Virucidal activity of G2-S16 or G2-S24P dendrimers against $HCMV_{AD-169}$. Plaque reduction assays were performed in MRC-5 cells for 6 days treatments. G2-S16 or G2-S24P dendrimer at 10 µM was incubated with 60 PFU for 2 h at 37 ºC. By several centrifugations at 12,000 RPM virus was pelleted, resuspended in fresh DMEM and added to MRC-5 cells for plaque reduction assays. Plaques were counted after 6 days with a methylene blue dye. Percentage of $HCMV_{AD-169}$ infection is represented as the number of plaques in treatments in regard of the control of infection. Triton X-100 was used as a positive control of virus membrane disruption. CI: Control of Infection, NT: non-treated control. The mean values (mean ± SD) of at least four independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

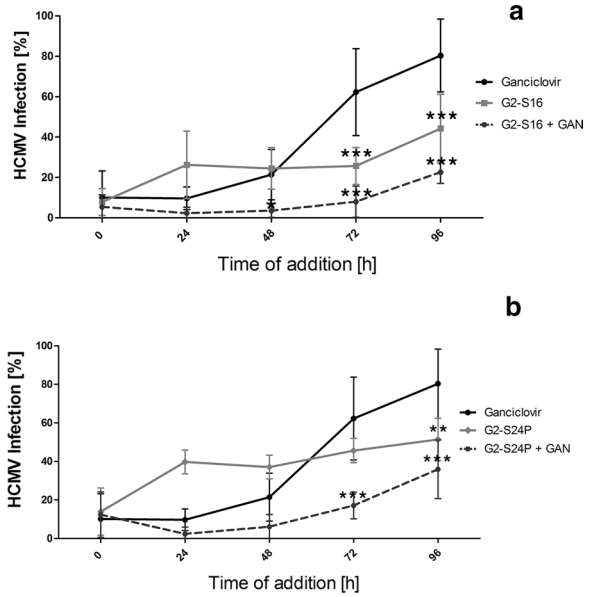

Fig. 7 Time of addition of G2-S16 or G2-S24P dendrimer and their combinations with ganciclovir. Plaque reduction assays were performed in MRC-5 cells for 6 days. MRC-5 cells were seeded, infected with 60 PFU and treated at different time points (0, 24, 48, 72 and 96 h post-infection) with either **a** ganciclovir, G2-S16 dendrimer and G2-S16 dendrimer + ganciclovir combination **b** ganciclovir, G2-S24P dendrimer, and G2-S24P + ganciclovir. Plaques were counted after 6 days with a methylene blue dye. Percentage of $HCMV_{AD-169}$ infection is represented as the number of plaques in treatments in regard of the control of $HCMV_{AD-169}$ infection. CI: Control of infection, NT: non-treated control. The mean values (mean ± SD) of at least four independent experiments are shown (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$)

Conclusions

Summing up, treatments against HCMV infection started to fail in last years, as new resistances appeared. In addition, common treatments as ganciclovir or valganciclovir are not available in developing countries such as African continent, due to their high cost and no accessibility of HCMV tests. Thereby, the spread of HCMV infection in those countries is not reduced. Due to this facts, antiviral dendrimers could be a new approach in several fields. Its main role would be as a treatment or prophylaxis in countries with little accessibility to commercial antivirals, but also as a result of the good biocompatibility showed, it could be used in the field of transplant recipients. Dendrimer could eliminate the virus before the organ reach final host, or even so in congenital infections as HCMV is very present in vertical transmission. In either case G2-S16 or G2-S24P are presented as potent tools against HCMV either alone or in combination with the actual treatments. However, further studies should be performed to decipher the specific mechanism of action to develop new compounds for specific targets, for example in order to stop virus from crossing the placenta or to eliminate it in blood transfusions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12951-021-00809-4.

Additional file 1. Nuclear Magnetic Resonance spectra of G1-S4 (DMSO), G2-S16 (D_2O) and G2-S24P (D_2O).

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Authors' contributions
MAM-F designed the study, analysed and discussed data and wrote the manuscript. JLJ discussed and interpreted data. IR-R developed the methodology, analysed, discussed data and composed figures. MSE-B and VM-C performed experiments, analyzed the data, composed the figures and interpreted the data. RG-R provided the dendrimers. All authors read and approved the final manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's note

3.3. Article " G2-S16 Polyanionic Carbosilane Dendrimer Can Reduce HIV-1 Reservoir Formation by Inhibiting Macrophage Cell to Cell Transmission"

In these last years, several studies proved the high importance of macrophages in HIV-1 infection. These cells have been unattended in HIV-1 infection for so many years because, in the first publications, macrophages were not considered a target cell for HIV-1. Nevertheless, several studies have highlighted their importance in HIV-1 spreading and reservoir formation. In this sense, and with the addition of the previous knowledge of G2-S16, we wondered if this dendrimer would inhibit HIV-1 infection inside macrophages, with the endpoint of inhibiting HIV-1 spread through several organs and tissues, thus reducing the reservoir and sanctuaries formation.

In this work, we first isolated PBMCs from healthy donors. After PBMCs purification, we isolated monocytes and T lymphocytes from the same buffy coat. T lymphocytes were frozen at -80°C for later use. We first differentiated monocytes into MDMs. After differentiation, MDMs were treated with G2-S16, infected with HIV-1, and co-cultured with T lymphocytes. These assays measured HIV-1 infectivity either in MDMs alone or in the T lymphocytes after co-culture. For analysing the HIV-1 quantity, we performed p24 ELISA, and for HIV-1 infectivity, we titrated either supernatants or cell pellets by TZM.bl. In addition, we performed confocal microscopy to localize either HIV-1 viral particles or G2-S16 dendrimer.

Surprisingly enough, results showed that G2-S16 could inhibit HIV-1 spread from infected MDMs to healthy T lymphocytes by almost 80%. In the results, we observed that the reduction of infectivity was not directly related to p24 protein, thus probably meaning that viral particles do not get disrupted totally, but they cannot infect new cells after being phagocytised by MDMs and treated with G2-S16 dendrimer.

With these promising results, G2-S16 position as a new candidate in the fight against HIV-1, inhibiting HIV-1 infection in the first steps of the infection and reducing the reservoirs or sanctuaries. These results enhance the position of G2-S16 as a new molecule capable of reaching clinical trials.

I have actively participated designing and performing the experiments, analysing and discussing the data, and writing the manuscript in this work.

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Article

G2-S16 Polyanionic Carbosilane Dendrimer Can Reduce HIV-1 Reservoir Formation by Inhibiting Macrophage Cell to Cell Transmission

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Abstract: Human immunodeficiency virus (HIV-1) is still a major problem, not only in developing countries but is also re-emerging in several developed countries, thus the development of new compounds able to inhibit the virus, either for prophylaxis or treatment, is still needed. Nanotechnology has provided the science community with several new tools for biomedical applications. G2-S16 is a polyanionic carbosilane dendrimer capable of inhibiting HIV-1 in vitro and in vivo by interacting directly with viral particles. One of the main barriers for HIV-1 eradication is the reservoirs created in primoinfection. These reservoirs, mainly in T cells, are untargetable by actual drugs or immune system. Thus, one approach is inhibiting HIV-1 from reaching these reservoir cells. In this context, macrophages play a main role as they can deliver viral particles to T cells establishing reservoirs. We showed that G2-S16 dendrimer is capable of inhibiting the infection from infected macrophages to healthy T CD4/CD8 lymphocytes by eliminating HIV-1 infectivity inside macrophages, so they are not able to carry infectious particles to other body locations, thus preventing the reservoirs from forming.

Keywords: macrophages; HIV reservoir; nanotechnology; dendrimers

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1. Introduction

HIV-1 has become one of the main pandemics in modern times. The Joint United Nations Program on HIV/AIDS (UNAIDS) concluded that there are 1.7 million new infections and 37.9 million people living with HIV-1 infection [1]. After the implementation of combination antiretroviral therapy (ART), the pathological outcome of HIV-1 infection has improved substantially; however, a functional cure for HIV-1 has not been achieved. For this reason, finding a more accessible and long-lasting prevention/therapeutic approach against HIV-1 infection is one of the biggest current challenges of the biomedical community.

In this sense, multiples approaches are being studied to fight against HIV-1, from the elimination of the pathogen during different stages of infection, to prophylaxis, as well as eliminating the focus of latency. Including all this, nanotechnology has the capability to provide great advances and in particular, the use of dendrimers should be highlighted [2–4]. Dendrimers are actively developing, with promising anti-HIV-1 activity demonstrated in vitro. In particular, carbosilane dendrimers have shown great efficacy as microbicides, specifically G2-S16 [5]. This second-generation dendrimer with a silica core and 16 sulfonate

end groups has demonstrated in previous assays that it is capable of destabilizing the GP120-CD4 complex by blocking HIV-1 entry and cell-to-cell fusion [6–8] (reviewed in [9]).

Based on the mechanism of action of this dendrimer against HIV-1 both in vitro and in vivo, we studied the possibility that our G2-S16 dendrimer was able to inhibit the HIV-1 within macrophages. Macrophages play a major role as potential viral reservoirs, not only because they appear to have greater resistance to cytopathic effects than T cells [10,11], but they also are capable of containing competent viruses for weeks in lymphoid organs, preventing the interaction of ART. Thus, during the acute phase of HIV-1 infection, macrophages establish primary infection but perivascular macrophages deliver the virus to different organs, including the brain [12,13], establishing itself as an immune sanctuary [14].

They also undergo viral accumulation in cellular compartments connected to the surface, inaccessible to neutralizing antibodies [15]. It should be noted that the virus is internalized in exosomes or microvesicles to facilitate and improve spread, establishing itself as mechanisms of immune evasion. Therefore, HIV-1-infected macrophages can cause viral rebound after discontinuation of ART.

In addition to acting as HIV-1 viral reservoirs, infected macrophages can transmit HIV-1 to T CD4/CD8 cells through cell-to-cell contact, leading to a 10-fold higher rate of infection than free cell virus [16–18]. Although activated T CD4/CD8 cells are the main target cells of the virus, both the location and function of macrophages make it possible to generate a continuous infection of T cells through continuous cell–cell interactions [19,20]. In this way, we investigated if G2-S16 dendrimer could prevent the spread of HIV-1 when macrophages perform their function as an antigen-presenting cell for CD4 / CD8 T lymphocytes.

2. Results and Discussion

2.1. Biocompatibility of G2-S16 Dendrimer

Previous published work has demonstrated the biocompatibility of G2-S16 dendrimer in several cell lines, as well as human primary cell cultures. The maximum non-toxic concentrations for almost all cell lines (HEC-1A, HeLa, VK2/E6E7, Ect1/E6/E7, End1/E6E7, and TZM.bl) and primary human cells (PBMCs, CD4+ T lymphocytes, Treg, monocytes, MDMs, and MDDCs) were up to 20 μM [21–23]. However, as described in our previous work, we chose 10 μM as this was the maximum inhibitory concentration obtained in several studies in vitro [5–7].

2.2. G2-S16 Dendrimer Internalizes into Monocyte-Derived Macrophages and Is Stable for 48 h

In order to study the internalization of G2-S16 dendrimer into MDMs, fluorescence images of confocal microscopy were taken (Figure 1). G2-S16-FITC dendrimer was incubated with MDMs for 2 h, washed with glycine (pH 3) to eliminate the non- internalized G2-S16 dendrimer, and analyzed by confocal microscopy at different times post incubation. Results showed that G2-S16 dendrimer is able to internalize into MDMs at relatively short times and it remains inside the cell for at least 72 h. We observed high fluorescence intensity for the first 4, 6, and 24 h, indicating high absorption of G2-S16 dendrimer. At 48 h post-treatment, the fluorescence intensity began to diminish, but we still observed a high quantity of G2-S16 dendrimer inside the cells. As reported in the scientific literature, viral particles are able to resist inside macrophages, maintaining the ability to infect new T lymphocytes [24–26]. In our results, we observed at 72 h post-treatment that fluorescence intensity decays to low levels but is still observable using confocal microscopy. However, the G2-S16-FITC molecule stability in G2-S16 dendrimer is not as high as its sulfonate groups, thus meaning that this reduced fluorescence may be caused by loss of the G2-S16-FITC molecule but not the G2-S16 dendrimer structure or functional groups. This result indicates that G2-S16 dendrimer is capable of internalizing into MDMs, being stable for at least 48 h. Thus, this indicates that in the main window of HIV-1 spreading from macrophages to other tissues or circulating cells, our G2-S16 dendrimer is still inside the cell and probably maintaining its antiviral activity.

2.3. G2-S16 Dendrimer Internalized into Monocyte-Derived Macrophages Eliminates HIV-1 Infectivity

To study if G2-S16 dendrimer maintains its ability to inhibit HIV-1 internalized by MDMs, we analyzed the remaining virus in supernatants (SN) and cell pellets (PE) obtained on inhibition assays by titration on TZM-bl cells. Briefly, TZM.bl cells were seeded, incubated for 24 h, and treated with 100 µL of supernatants or cell pellets for 48 h. Viral infectivity on TZM.bl was measured by luciferase assay. Our result (Figure 2) showed that G2-S16 dendrimer is capable of inhibiting almost 85–90% of the internalized HIV-1 viral particles. We observed a reduction of more than 80% in supernatants, meaning that a very reduced number of viral particles are released to extracellular medium with G2-S16 dendrimer treatment. In addition, the results obtained when cell pellets were analyzed showed the same reduction, reaching almost 85%. Thus, this means that G2-S16 dendrimer is not only able to inhibit HIV-1 release to extracellular media but is also capable of practically eliminating the infectivity of the remaining viral particles inside MDMs.

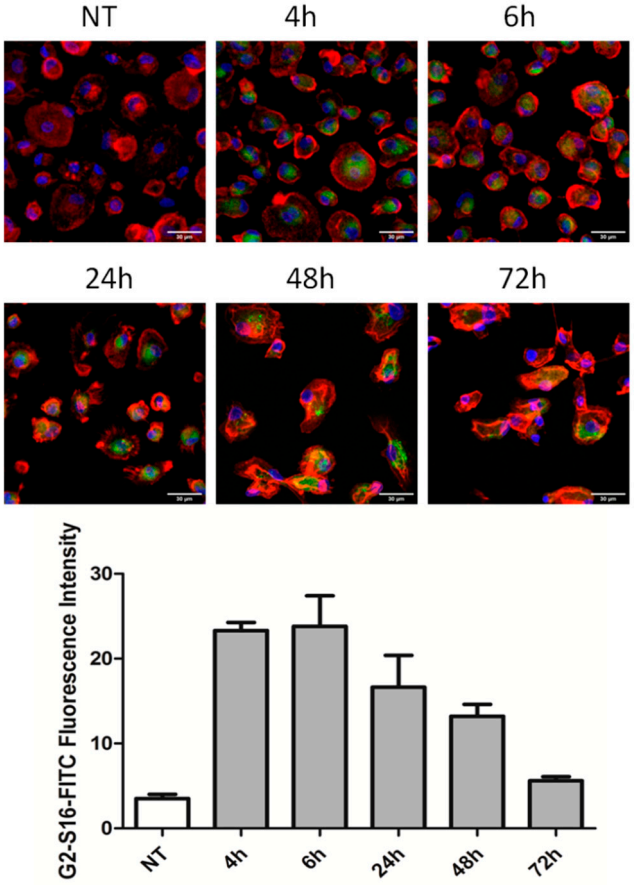

Figure 1. Representative confocal images of G2-S16 internalization in MDMs. Human monocytes were isolated and differentiated to MDMs. After differentiation, MDMs were treated with G2-S16-FITC for 2 h and washed with glycine (pH 3) to detach all non-internalized dendrimer in the cell membrane and washed 3 times with PBS. Confocal images were taken at 4 h, 24 h, 48 h, and 72 h after treatment. FITC fluorescence was measured and is represented in the bars. Red: cell membrane. Blue: cell nuclei. Green: G2-S16-FITC dendrimer. Representative images of 3 independent experiments are shown.

Figure 1. Representative confocal images of G2-S16 internalization in MDMs. Human monocytes were isolated and differentiated to MDMs. After differentiation, MDMs were treated with G2-S16-FITC for 2 h and washed with glycine (pH 3) to detach all non-internalized dendrimer in the cell membrane and washed 3 times with PBS. Confocal images were taken at 4 h, 24 h, 48 h, and 72 h after treatment. FITC fluorescence was measured and is represented in the bars. Red: cell membrane. Blue: cell nuclei. Green: G2-S16-FITC dendrimer. Representative images of 3 independent experiments are shown.

2.3. G2-S16 Dendrimer Internalized into Monocyte-Derived Macrophages Eliminates HIV-1 Infectivity

To study if G2-S16 dendrimer maintains its ability to inhibit HIV-1 internalized by MDMs, we analyzed the remaining virus in supernatants (SN) and cell pellets (PE) obtained on inhibition assays by titration on TZM-bl cells. Briefly, TZM.bl cells were seeded, incubated for 24 h, and treated with 100 µL of supernatants or cell pellets for 48 h. Viral infectivity on TZM.bl was measured by luciferase assay. Our result (Figure 2) showed that G2-S16 dendrimer is capable of inhibiting almost 85–90% of the internalized HIV-1 viral particles. We observed a reduction of more than 80% in supernatants, meaning that a very reduced number of viral particles are released to extracellular medium with G2-S16 dendrimer

showed the same reduction, reaching almost 85%. Thus, this means that G2-S16 dendrimer is not only able to inhibit HIV-1 release to extracellular media but is also capable of practically eliminating the infectivity of the remaining viral particles inside MDMs.

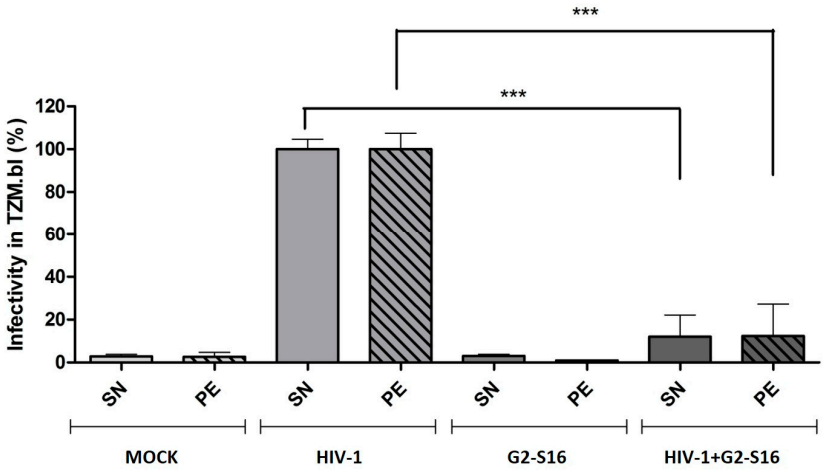

Figure 2. G2-S16 inhibits HIV-1 inside MDMs. Monocytes were isolated and differentiated to MDMs, after differentiation cells were treated for 2 h with G2-S16 dendrimer, washed with glycine (pH 3) to detach non-internalized dendrimer, and washed with PBS. MDMs were then infected with 6 ng/µL of HIV-1 per 1×10^6 cells for 2 h, inoculum was discarded, and cells incubated at 37 °C for 3 days. Supernatants and cell pellets were separated by centrifugation and titrated by TZM.bl infection. Mock cells were used as non-treated control. The mean values (mean + SD) of three independent experiments are shown (*** $p < 0.001$).

Figure 2. Monocytes derived Macrophages Do Not Spread HIV-1 Infection to CD4/CD8 Lymphocytes after G2-S16 Dendrimer Treatment

Infected macrophages are one of the main factors in HIV-1 spreading to different tissues and organs [27]. This is due to macrophages' ability to carry the virus without losing infectivity for long times, leading to a higher infectivity when they contact with CD4/CD8 T lymphocytes due to the cellular synapses [28,29]. As commented before, these synapses protect viruses from antiviral molecules and immune defenses present in the extracellular medium and are not capable of internalizing into the cell [15]. To study if G2-S16 dendrimer is able to inhibit cell-to-cell infection of T lymphocytes from MDMs, we performed cocultures of MDMs infected and G2-S16 dendrimer treated with T lymphocytes obtained from the same buffy coat. We first performed confocal images to determine if HIV-1 particles were able to be released from MDMs and reach CD4/CD8 T cells. In our results (Figure 3), we found that MDMs treated with G2-S16 dendrimer (green) and HIV-1 infected present very low viral particles (white), in contrast with HIV-1-infected MDMs, which present high fluorescence intensity, meaning that they preserve viral particles in the internal membrane. We observed that most remaining viral particles co-localize with G2-S16 dendrimer, thus confirming our previously published work describing that G2-S16 dendrimer is able to form complex with viral gp120 [7,30].

To confirm that the viral particles could not reach CD4/CD8 T cells, lymphocytes were isolated from MDMs by several washes, verified by microscopy that T cells were isolated correctly, and analyzed by two different techniques. First, Elisa enzyme immunoassay was performed to determine the amount of p24 protein present in TCD4/CD8 lymphocytes. Our results show that treatment with G2-S16 reduced the amount of p24 protein, both for supernatants and pellets. In fact, p24 protein values in the pellets did not exceed 10%, which shows that G2-S16 dendrimer significantly reduces the quantity of virus that reaches T cells (Figure 4).

In addition, in order to verify the infectivity of HIV-1 in these T cells, a titration in TZM.bl was performed (Figure 5). In this analysis, we observed that T lymphocytes treated with G2-S16 dendrimer carried just 20% of the infective viral particles that reached them from MDMs. Thus, this means that G2-S16 dendrimer is capable of not only minimizing the number of infectious viral particles that reach T CD4/CD8 cells, but also eliminating 80% of their infectivity in vitro.

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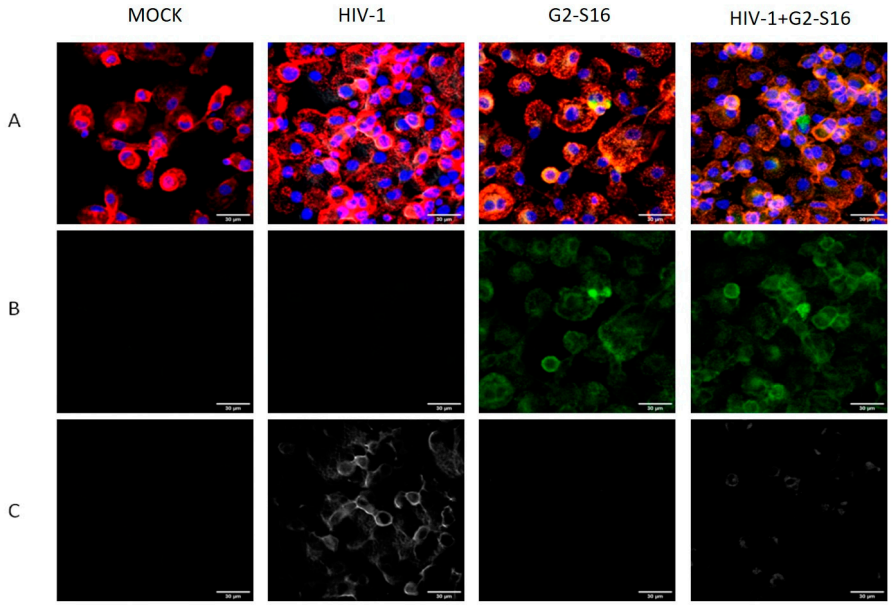

Figure 3. Representative confocal images of HIV-1 infection in MDMs and T cell co-cultures. Human monocytes and T lymphocytes were isolated from the same buffy coat. Monocytes were differentiated to MDMs. After differentiation, MDMs were treated with G2-S16-FITC for 2 h and washed with glycine (pH 3) to detach all non-internalized dendrimer in the cell membrane and washed 3 times with PBS. After washes, MDMs were infected with 6 ng/µL per 1×10^6 cells of HIV-1 and co-cultured with T lymphocytes for 48 h. Supernatant was gently discarded and cells were mounted to perform confocal microscopy. Confocal images were taken 48 h after co-culture with T cells. (**A**) Merge (**B**) G2-S16-FITC (**C**) HIV-1 P24. Red: cell membrane. Blue: cell nuclei. Green: G2-S16/G2-S16-FITC dendrimer. White: HIV-1 p24 protein Scale bar: 30µm. Representative images of 3 independent experiments are shown.

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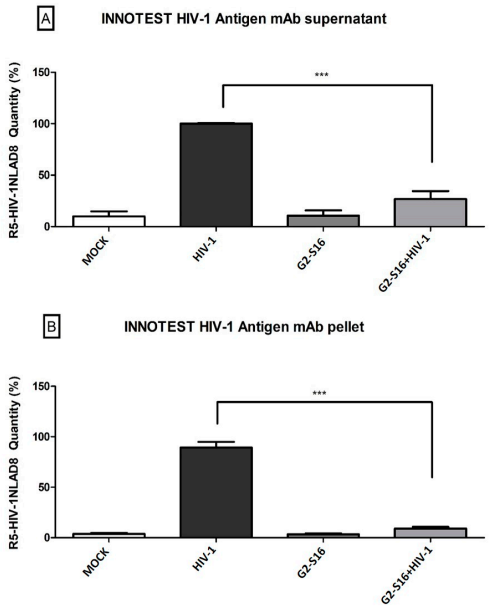

Figure 4. G2-S16 decreases the HIV-1 amount reaching T cells from infected MDMs. MDMs treated with G2-S16 and infected with 6 ng/µL of HIV-1 per 1×10^6 cells for 2 h were co-cultured with isolated T CD4/CD8 for 48 h. After incubation with T cells, (**A**) supernatant and (**B**) pellets were isolated and titrated by HIV-1 p24 enzyme immunoassay (Elisa kit INNOTEST HIV-1 Antigen mAb). Mock cells were used as the non-treated control. The mean values (mean ± SD) of three independent experiments are shown (*** $p < 0.001$).

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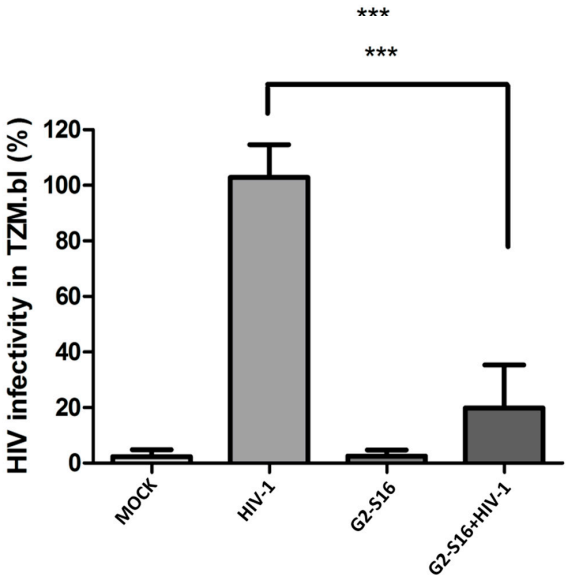

Figure 5. G2-S16 inhibits infection of T cells from infected MDMs. MDMs treated with G2-S16 and infected with 6 ng/µL of HIV-1 per 1×10^6 cells for 2 h were co-cultured with isolated T cells for 48 h. After incubation, T cell foci were isolated and HIV-1 infectivity was titrated by infection of TZM.bl. Mock cells were used as the non-treated control. The mean values (mean ± SD) of three independent experiments are shown (*** $p < 0.001$).

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3. Materials and Methods

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3.1. Reagents

G2-S16 and G2-S16-FITC anionic carbosilane dendrimers (Figure 6) with 16 functional groups sulfonates and silicon nucleus ($C_{112}H_{244}N_8Na_{16}O_{48}S_{16}Si_{13}$; Mw = 3717.15 g/mol) were synthesized and analyzed according to the methods of the University Dendrimer Biomedical Applications group of University of Alcala (UAH) [31] (Madrid, Spain). A 5 mM stock solution of G2-S16 dendrimer and subsequent dilutions to obtain µM concentrations were prepared in nuclease-free water (Promega, Madrid, Spain).

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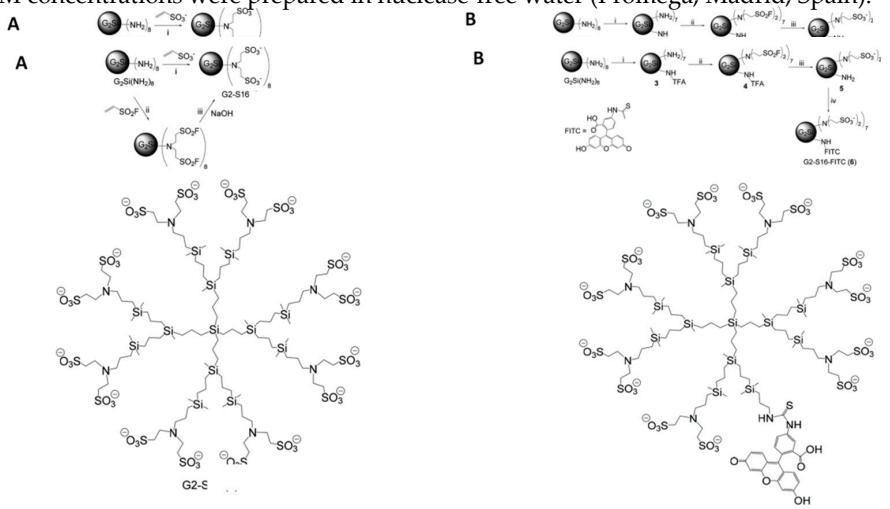

Figure 6. Schematic synthesis route and molecular representation of dendrimers. (**A**) G2-S16 with silicon core and 16 sulfonate end groups and (**B**) G2-S16-FITC with silicon core and 14 sulfonate end groups and FITC molecule were represented. The generation of dendrimers is determined by considering that each generation corresponds to the number of repeating layers of silicon atoms. Images adapted from Gutierrez-Ulloa et al. 2020.

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3.2. Cell Culture and Viral Strains

The TZM.bl cell line (ATCC, Manassas, NA, USA) derived from a human endometrial carcinoma was cultured in DMEM complemented with 5% FBS, 125 mg/mL ampicillin, 125 mg/mL cloxacillin, and 40 mg/mL gentamicin (Normon, Madrid, Spain).

Viral stocks of the CCR5-tropic R5-HIV-1NLAD8 laboratory strain were obtained by transient transfection of pNLAD8 plasmid (NIH AIDS Research and Reference Reagent Program [ARRRP]) in the HEK-293T cell line (ATCC, Manassas, VA, USA). Stocks were clarified before the evaluation of viral titter by HIVp24gag enzyme-linked immunosorbent assay (ELISA kit INNOTEST; Innogenetics, Ghent, Belgium).

3.3. Primary Cell Cultures, Purification, and Differentiation

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from anonymized healthy blood donors (Transfusion Centre of Madrid) following national guidelines. PBMCs were isolated by a standard Ficoll-Hypaque density gradient (Rafer, Madrid, Spain) and cultured following the procedures of Spanish HIV HGM BioBank [32]. Monocytes were purified using immune-magnetic antiCD14 microbeads (Miltenyi, Madrid, Spain), seeded at 1×10^6 cells/mL, and cultured for 7 consecutive days in RPMI-1640 medium supplemented with 10% FBS, 1% l-glutamine, and 10 ng/mL of rhGM-CSF (Immunotools) to generate monocyte-derived macrophages (MDMs).

CD4/CD8 T cells were purified from PBMCs using immunomagnetic anti-Pan T microbeads (Pan T Cell Isolation Kit, human MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany). Purified T cells were frozen at −80 °C for later use. T cells were seeded at 5×10^6 cells/mL in RPMI 1640 medium (Biochrom AG, Berlin, Germany) with 10% fetal bovine serum (FBS; Gibco), 1% l-glutamine (Lonza, Walkersville, MD, USA), antibiotic cocktail (125 mg/mL ampicillin, 125 mg/mL cloxacillin, 40 mg/mL gentamicin (Normon, Madrid, Spain), and 60 U/mL of IL-2.

3.4. Immuno Fluorescence and Confocal Images

First, 0.5×10^6 monocyte cells isolated and purified from PBMCs were incubated in 24-well plates for maturation to MDMs. After 7 consecutive days of incubation, MDMs were treated with G2-S16-FITC for 2 h at 37 °C. After incubation, medium was discarded, cells were rinsed with pH 3 glycine to detach non-internalized dendrimers, and fresh medium was added to cell cultures and incubated for 2, 4, 6, 24, 48, and 72 h. Then, supernatant was discarded, and cells were fixed in 4% paraformaldehyde (PFA; Panreac, Barcelona, Spain) for 10 min, washed 3 times in PBS, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. After incubation, cells were washed 3 times in PBS, blocked with 1% bovine serum albumin (BSA Sigma-Aldrich, St. Louis, MO, USA) and 0.1%Triton X-100 in PBS for 30 min, and incubated with phalloidin ALEXA FLUOR® 555 Conjugated antibody for 1 h. MDMs cells were washed 3 times in PBS and stained with 4′,6-diamidino-2- phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Finally, cover glasses were mounted in slides and analyzed in a Leica TSC SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence was analyzed using ImageJ software (National Institute of Health, Maryland, NA, USA).

3.5. Inhibition Assay

The potential activity of G2-S16 dendrimer against R5-HIV-1_{NLAD8} infection was measured by infectivity of HIV-1. For MDMs maturation, 1×10^6 cells/well were seeded in 12-well plates and incubated at 37 °C for 7 days. After incubation, MDMs were treated with G2-S16 dendrimer for 1 h at 37 °C, washed with acid glycine, and infected with 6 ng/μL of R5-HIV-1_{NLAD8} for 2 h at 37 °C. Inoculum was discarded and RPMI-1640 medium supplemented with 10% FBS, 1% l-glutamine. The MDMs were incubated for 3 days. Then, the supernatants were collected, and cells were scraped in a solution of PBS (1% BSA, 2 mM EDTA) on ice. To quantify HIV-1 infectious viral particles, supernatants and pellets were analyzed by titration on TZM.bl cells.

3.6. Inhibition Assay Cell–Cell

After MDMs maturation, cells were treated with a mix 9/1 of G2-S16/G2-S16-FITC dendrimer for 2 h and infected with HIV-1 for 3 days. T CD4/CD8 cells isolated as described before were added to the culture at 1×10^6 cells/mL and incubated for 48 h. After incubation, medium was collected in tubes and T CD4/CD8 cells were isolated by several washes with PBS and added to the tubes. Tubes were centrifuged at 1500 RPM for 10 min. Either cell pellets or supernatants were titrated for HIV-1 infectivity in the TZM.bl cell line.

3.7. Infectivity of HIV-1

The infectivity of the R5-HIV-1NLAD8 was measured by luciferase activity assay following the manufacturer's protocol (Promega Corporation, WI, USA). Briefly, TZM.bl cells were seeded in 96-well plates at 1×10^4 cell/well for 24 h. After incubation, medium was discarded and replaced with 100 µL of supernatants or cell pellets for 48 h. TZM.bl cells were lysed, and luciferase activity was measured at 570/650 nm in a BioTek Synergy™ 4 Hybrid Microplate Reader.

3.8. Determination of HIV-1 Quantity

In order to determine the R5-HIV-1_{NLAD8} quantity, each supernatant and cell pellet was analyzed by enzyme immunoassay for the quantitative detection of HIV-1 p24 antigen (INNOTEST HIV Antigen mAb, FUJIREBIO, Ghent, Belgium) following the manufacturer's instructions. Supernatants and pellets were incubated in a 96-well plate coated with p24 antigen, and the absorbance was measured by the presence of viral protein HIV-1 p24 at 450 nm in a microplate reader BioTek Synergy™ 4 Hybrid Microplate Reader (BioTek, Winooski, NA, USA).

3.9. Statistical Analysis

Statistical analysis of the quantity and infectivity of HIV-1 was performed with GraphPad Prism v5.0 software (GraphPad, CA, USA) using the nonparametric unpaired *t*-test. Differences were considered significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All data were obtained from three independent experiments performed in triplicate.

4. Conclusions

In conclusion, our results indicates that G2-S16 dendrimer can act in different stages of HIV-1 infection. The main objective of G2-S16 dendrimer is its use as a microbicide, creating a physical barrier that protects the vaginal mucosa from being disrupted [33,34] and inhibiting several viral infections [35–38]. Thus, these results exacerbate the applicability of this G2-S16 dendrimer, as it is capable of inhibiting macrophages from spreading to other target cells, minimizing viral charge [39], as well as reducing viral reservoirs and sanctuaries due to the ability of macrophages to deliver viral particles to different anatomic targets. These results along with other results obtained by our group ease the way of G2-S16 to clinical trials.

Author Contributions: M.Á.M.-F. designed the study, analyzed and discussed data, and wrote the manuscript. I.R.-R. developed the methodology, analyzed and discussed data, and composed figures. M.d.l.S.E.-B. and V.M.-C. performed experiments, analyzed the data, composed the figures, and interpreted the data. R.G.-R. discussed and interpreted data. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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4. Discussion

4.1. Biocompatibility of dendrimers

One of the first steps in the development of new antiviral molecules is the cytotoxicity test. This kind of assay would show the working concentration of each compound regarding a specific cell line. Several experiments are related to toxicity in cells, i.e., 7-Aminoactinomycin D (7AAD). 7AAD is a fluorescent chemical compound with a strong affinity for GC-rich regions from the double-stranded DNA and is used in fluorescence microscopy and flow cytometry. However, cells with compromised membranes will stain with 7-AAD, while live cells with intact cell membranes remain dark [94]. Tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is one of the most used viability assays. MTT assay is used to measure cellular metabolic activity to indicate cell viability, proliferation, and cytotoxicity. This colorimetric assay is based on reducing MTT to formazan crystals by metabolically active cells[95]. Moreover, the other primary viability assay consists of the measurement of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme that is found in all cells. LDH is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cells undergoing apoptosis, necrosis, and other forms of cellular damage [96].

All of the experiments, as mentioned earlier, are included in the called viability assays, but each one of them measures different parameters in cell culture. For example, MTT measures the mitochondrial activity of the cells. In most cases, the reduction in the mitochondrial activity would mean that the cell is undergoing its normal respiration, thus suggesting that this mechanism is impaired by some of the compounds tested. However, it is well known that mitochondrial activity may vary according to the metabolic moment of the cell. So, a reduction in this activity is not always unconditional of cell death. Moreover, this assay is also dependent on the compound tested, as some of them may interfere with the reaction [97]. The sensibility of the MTT assay also is of high relevance, as it can detect minimum changes in mitochondrial activity. In contrast, LDH assay is more categorical of cell death. If the cell membrane

releases LDH, it is damaged, and eventually, the cell will reach the death. This also means that LDH is not as accurate as MTT but is more cutting with the results. In this sense, 7AAD works with the same mechanism of LDH. If the cell membrane is compromised, 7AAD will intercalate into DNA and be analysed either by flow cytometry or confocal microscopy.

With all this information in mind, we have performed several viability assays to determine the working concentrations of each dendrimer in each cell used in this thesis. Because every compound is toxic regarding the concentration, we observe that in J89GFP and THP89GFP cell lines, most PCD are nontoxic at a concentration of 1 to 10 μM in the MTT results, but as commented before, we can find different results according to the assay used. In the LDH results, we observed in contrast that most dendrimers exhibit increasing viability with the same concentrations. When measured by MTT viability is rounding 80%, in LDH results, we observed that viability is around 90-95% [98]. As another confirmation method, when measuring reactivation in these cell lines, 7AAD assay was performed routinely (data not shown) and confirmed the same results. Thus, when analysing the biocompatibility of different compounds, more than one assay is needed to verify the results. This point is of particular importance when measuring nanoparticle toxicity, as regarding their size, they can interact with several organelles in cell metabolism.

J89GFP and THP89GFP cell lines are both cell lines derived from lymphocytes and monocytes, respectively, and we observed that the working concentration of PCD was 10 μM for G1-S4, 10 μM for G2-S16, and 1 μM for G3-S16 for 48 h of treatment. Probably the increasing size of G3-S16 is causing the toxicity at higher concentrations. However, we still observe more biocompatibility in the LDH results, meaning that maybe, with the confirmation of another toxicity assay targeting other cellular metabolites, we could increase the working concentration of G3-S16.

However, in the study of the interaction of nanoparticles and HCMV [23], the cell line MRC-5 was used. These cells are derived from human lung fibroblasts. In contrast with the monocyte and lymphocyte cells used in the previous study, MRC-5 cells are adherent cells and tend to form a well-defined monolayer. This feature and the

capability of HCMV to actively replicate in these cells make them the ideal to study the HCMV cell cycle and, in this work, the inhibition that the nanoparticles can cause. In contrast to the previous toxicity assays, which were 48 h due to the experiment timing, in this case, the toxicity will be measured six days after the treatment with the compounds. This is because HCMV infection takes this time to show cytopathic effects in MRC-5. In this case, the results of mitochondrial activity showed an increase in toxicity in the case of G2-S16, being just 1 μM for the 6 days treatment in lung fibroblast. Nevertheless G1-S4 still showed 10 μM working concentration, and G2-S24P showed also viable at 10 μM. However, in this study, we also performed LDH assays to confirm compounds' viability. As indicated before, these results exhibit an increasing working concentration for these dendrimers, reaching almost 50 μM maximum nontoxic concentration for all of them after 6 days after treatment. With this disparity in the results, we hypothesize that in human lung fibroblast, the dendrimers used in this study may diminish the mitochondrial activity, but not resulting in cell death as shown in LDH results after 6 days, as if the treatment with dendrimers would have caused any senescence or apoptosis it would have been noted before 6 days in cell culture [99].

Nanoparticle toxicity, either *in vitro* or *in vivo*, is well known. In the last years, several studies have arisen to address the correct use of nanotechnology regarding the toxicity it may cause [100-102]. These studies remark the importance of the viability assays in the first step of the road to the use of nanoparticles in any industry, especially in biomedical applications, as the requirements for the success in the first steps of development of a compound are the toxicity assays.

Therefore, we showed the excellent biocompatibility of polyanionic carbosilane dendrimers in different cell lines of remarked importance, as for lymphocytes, monocytes, and lung fibroblasts.

4.2. G3-S16 can promote HIV-1 reactivation

The battle against HIV-1 has changed in the last years due to cART. Nowadays, the main foci to eradicate HIV-1 infection are the reservoirs or sanctuaries. As commented

before, to address this issue, several treatments are being studied, in particular the two main strategies, “shock and kill” and “block and lock”. In this thesis, the focus is on the “shock and kill” approach.

HIV-1 “shock and kill” strategies are based on the use of latency-reversing agents (LRA) to induce the production of HIV-1 proteins in latently infected cells and render these cells susceptible to virus-specific cytolytic T lymphocytes (CTL) or cART. Several compounds are in studies for this approach. The most studied are Protein Kinase C (PKC) agonist and histone deacetylase inhibitors (HDACi). Both groups of molecules are characterized to activate HIV-1 transcription, and some of these promising molecules are actually on clinical trials. Romidepsin (RMD) is a well-characterized HDACi approved for the treatment of cutaneous T-cell lymphoma. Several studies have demonstrated that it can induce HIV-1 gene expression in latently infected CD4+ T cells from HIV-1 individuals on suppressive antiretroviral therapy [103]. Nevertheless, clinical trials with LRAs have demonstrated that activation of viral gene expression is possible in vivo, but there is limited or no clearance of the reactivated cells [104].

This expression of HIV-1 in latently infected cells is the first step in the “shock and kill” method and is the most characterized one. However, this is not sufficient to eliminate the reservoir. In the second part of the strategy, the immune system plays a major role in recognizing and eliminating these infected cells. However, multiple studies have demonstrated that activation of viral transcription alone is insufficient to induce cell death, and some LRAs may counteract cell death by promoting cell survival [105]. Actually, are several new compounds being developed to address this issue, i.e., the BDZ Alprazolam functions as an inhibitor of the transcription factor RUNX1, which negatively regulates HIV-1 transcription. Moreover, Alprazolam potentiates the activation of STAT5 and its recruitment to the viral promoter. The activation of STAT5 in cytotoxic T cells may enable immune activation [106].

We previously showed that the HIV-1 inhibition activity of G3-S16 [107, 108] can eliminate HIV-1 infection in PBMCs with an EC_{50} of 0.632 μM. Moreover, we demonstrated in this work that G3-S16 can reactivate HIV-1 in monocyte-derived cell

lines THP89GFP. This reactivation activity reached 60% at 24 h, which is similar as showed with bryostatin (BRY), prostatin (PST), panobinostat (PNB), or RMD, the main LRAs in studies. This reactivation showed at low concentrations of dendrimer increases at 48 h post-treatment, reaching almost 80%. This 80% reactivation is probably the maximum for the cell line model, compared with other LRAs at 48 h. Of the three dendrimers studied, we observed that only G3-S16 produced reactivation. However, G2-S16 dendrimer did not activate HIV-1 production, even though the two have the same number of functional groups and relatively the same size. Nevertheless, G1-S4, even being a sulfate dendrimer as G3-S16, did not produce any reactivation either. The combination of size and number of sulfonated groups in G3-S16 dendrimer is the key to understanding the action mechanism.

Moreover, we performed combinations of the LRAs mentioned above with G1-S4, G2-S16, and G3-S16 to study if dendrimers would modify LRAs activity. These results showed that PCDs, not only does not modify LRAs activity, but G3-S16 increased the basal activity of both HDACi used in this study. We observed that this increase in reactivation is not noticeable when G3-S16 is in combination with PKC agonist. This could be due to both stimuli are so strong that may collapse cell metabolism as we observed increased toxicity in LDH results, or that the mechanism of action of G3-S16 is similar to PKC agonist, activating cell membrane receptor, so that they may compete.

This data, along with the known ability of G3-S16 to inhibit HIV-1, remark the pathway for a novel LRA that can eliminate the newly produced virions. The combination of an immune system enhancer finally achieves a curative strategy for HIV-1 infection.

4.3. G2-S16 is capable of inhibit HIV-1 macrophage cell to cell transmission

HIV-1 sexual infection begins when the viral particle passes through the mucosa and epithelial layer. This disruption in the epithelial layer would recruit several cells to defence; primary defence cells are T CD4 and macrophages, both target cells of HIV-1

virions. In this thesis, we will address the specific role of macrophages in HIV-1 infection.

The "Trojan Horse" paradigm posits that HIV-1-infected macrophages traffic the virus by the cell-to-cell transmission into the central nervous system (CNS) across the blood-brain barrier, which normally excludes most pathogens and host proteins, or to several organs [109]. This kind of transmission is usually 10-fold more effective than free viral particles and there are no drugs that inhibit that kind of transmission. Furthermore, ART achieves little if any reduction of HIV-1 replication in anatomic reservoirs such as the lymph node, spleen, GALT, CNS, and genital tissues [110]. In this sense, a drug that can inhibit this interaction would reduce the reservoir formation, specifically the sanctuaries formed in CNS.

In our group, we already showed the ability of G2-S16 to inhibit HIV-1 infection by interactions with GP120 [111], but in several experiments, we observed a second mechanism of action of this dendrimer. Moreover, we have proved that G2-S16 does not interfere with immune system cells [112]. Keeping in mind the ability of macrophages to phagocytize viral particles, we wondered if G2-S16 dendrimer would behave similarly in MDMs. To this end, we isolated monocytes and T lymphocytes from buffy coats, T lymphocytes were frozen at -80 °C for later use. We first cultured and differentiated monocytes into monocyte-derived macrophages (MDMs). To study the behaviour of G2-S16 in MDMs, we treated cells with G2-S16-FITC at different time points and performed confocal images to localize dendrimer inside MDMs. We observed that G2-S16 dendrimer is easily internalized into MDMs, at 4 h after treatment G2-S16 is found all inside macrophages. After 48 h post-treatment, we observed that the fluorescence levels start to decay, probably due to the loss of fluorescence of FITC molecule, and even after 72 h we still observe G2-S16 dendrimer inside the cell. This decay in fluorescence does not mean that G2-S16 is losing its activity, as FITC molecule is less stable than the dendrimer structure in cell culture.

We now know that G2-S16 is stable inside macrophages, at least for 48 h, so we wondered if this dendrimer would maintain its inhibitory ability inside MDMs. Thus, we treated MDMs with G2-S16, infected cells for 2 h and co-cultured with T

lymphocytes isolated from the same buffy coat. The infection was incubated for 48 h, and we analysed HIV-1 infection by different techniques. We titrated HIV-1 infectivity in TZM.bl and quantified p24 protein by ELISA. Our results showed that HIV-1 infectivity is reduced more than 80% in TZM.bl, meaning that only 20% of virions with the treatment of G2-S16 remained infectious. However, in the ELISA results, we observed that p24 protein was not reduced similarly, probably meaning that G2-S16 is inhibiting infections but without disrupting viral p24. Moreover, when analysed supernatants or cell pellets individually, we observed that in supernatant result p24 protein remains at 20%, in contrast, in cell pellets we observed a reduction of almost 95%. These results probably suggest that the remaining infected MDMs are releasing HIV-1 particles to the culture medium but, these particles are not infective, so they cannot transcribe p24 protein inside T lymphocytes.

We observed that G2-S16 could reduce HIV-1 infection in T Lymphocytes after MDMs phagocytised viral particles. In contrast with most ART that cannot inhibit cell to cell transmission, G2-S16 exhibits the ability to reduce the infectivity of virions before reaching T Lymphocytes. Within all the previous results of G2-S16 as a microbicide, either in vitro or in vivo [85], and the addition of this new feature, this dendrimer could become one promising molecule against HIV-1 infection.

We already showed that G2-S16 could inhibit viral attachment when treated before infection. The mechanism of action of G2-S16 dendrimer may be critical to determine its success in clinical trials. Computational modelling assays showed that G2-S16 dendrimer disrupts the union of the virus to the host cell. Still, it could do so by blocking residues on both the viral gp120 and the CD4 cellular receptor [113]. In addition, in this work, we showed that this dendrimer could inhibit this bond inside MDMs after infection.

As commented before, the main barrier for HIV-1 eradication is the sanctuaries and reservoirs formed during the first infection, mainly due to macrophage spread. Thus, a compound that could prevent the formation of this latently reservoir would make a point in the fight against HIV-1.

4.4. Mechanism of action of PCD against HCMV infection

As mentioned in the introduction, HCMV is a common co-infection with HIV-1, especially in developing countries. Actual treatments usually don't reach these countries the way they should, mainly due to the cost, but also influenced by a lousy adherence or lack of supplies. Thus, new cost-effective treatments are needed, highlighted with the actual viral pandemic, several compounds are being developed. In this field, compounds that could fight against several viral diseases are remarkable. As we have shown the inhibition activity of several PCDs against HIV-1 or HSV, we wondered if these effective antiviral particles could inhibit HCMV infection. To this end, we performed several inhibition experiments with three PCDs against HCMV in the MRC-5 cell line.

In the inhibition experiment, we first treated cells for 1 h with G1-S4, G2-S16, and G2-S24P and infected them with HCMV. HCMV is known to produce a slow cytopathic effect in fibroblast cell lines, so for every experiment with HCMV, the incubation after infection was six days. During this time, HCMV produced plaques of infection in the MRC-5 cell line, and these plaques were stained with Methylene Blue and counted to quantify HCMV infection. We observed that G1-S4 did not reduce the infectivity of HCMV at any concentration used. However, G2-S16 and G2-S24P showed a reduction of HCMV infection, G2-S16 at 5 μM reached 50% of inhibition and G2-S24P showed 50-60% reduction at 10 μM. Surprisingly, G2-S16 at 10 μM reached 80% of HCMV inhibition. In addition to this reduction in the number of plaques, we also observed that these plaques were reduced in size compared to the infection control. To this end, we also performed confocal images of HCMV infection with G2-S16. The results shown that the infection plaque in control is almost three times bigger than that treated with G2-S16.

With these results, we wondered if the mechanism of action of G2-S16 and G2-S24P would be similar against HIV-1 than against HCMV. As commented before, most dendrimers inhibit the first step of infection. In HIV-1, G2-S16 dendrimer can inhibit the fusion of HIV-1 to cell target. So, we performed an attachment assay to study if the

mechanism of action would be similar. To this end, before PCDs treatment and infection we pre-cooled cell cultures at 4°C to increase the rigidity of the cell membrane, so it would be less permeable to either dendrimers or viral particles. After treatment and infection at 4°C, cells were cultured for 6 days at 37°C, stained and plaques were counted to assess the membrane attachment infectivity. Surprisingly, we observed that no reduction was noticed in any of the treatments performed, thus meaning that these dendrimers are not inhibiting HCMV infection at the attachment to cell membrane. However, HCMV is well known for its versatility in the receptors needed for entry into the host cell. As an example, there is no treatment directed to this point in the viral cycle. In contrast with several viruses that different treatments are directed to membrane attachment, as maraviroc against HIV-1 infection, which specifically blocks CCR5.

To study the activity of these dendrimers directly against HCMV, we performed virucidal assays. We incubated HCMV viral particles with either G2-S16, G2-S24P or triton X-100 as control of disrupting viral membrane. After 2 h incubation, the remaining viral particles were added to MRC-5 cultures and incubated for 6 days. Then cells were stained to analyse the infectivity of HCMV viral particles after direct contact with dendrimers. In this experiment, we observed the same results as membrane attachment assay: no reduction in HCMV infectivity, almost the same number of plaques were counted in treatments and control of infection, thus meaning that G2-S16 and G2-S24p are not inhibiting membrane attachment to MRC-5, neither disrupting directly HCMV viral particles.

Therefore, we showed that G2-S16 and G2-S24P could reduce HCMV infection in MRC-5, but the exact mechanism of action was not deciphered. However, we wondered how much time the antiviral effect of these dendrimers would last in infected cell culture. To this end, we performed time of addition assays, we infected MRC-5 cell cultures and treated them with G2-S16 or G2-S24P at different time points after infection. Moreover, we completed the experiments with ganciclovir, the actual election treatment, and the combination of both drugs. The results obtained showed the good antiviral effect of both dendrimers single treatment during the viral cycle of

HCMV. We observed that G2-S16 maintained its inhibitory activity for 72 h reaching 80% HCMV reduction. In contrast, G2-S24P could only reduce 60% of the infection just 24 h after infection. After this time, this molecule lost the antiviral effect showed in pre-treatments assays. As commented before, we used Ganciclovir as a negative control of infection. Even being the election treatment for HCMV, this compound reduces its ability to inhibit HCMV 48 h after infection, showing a poor inhibition compared with G2-S16. Surprisingly, we observed that the combination of either G2-S16 or G2-S24P with ganciclovir, increases in a remarkable manner the antiviral activity against HCMV, in G2-S16/ganciclovir combination, we observed an inhibition of almost 100 % at 24 and 48 h, and it was reduced to 90% at 72h. We observed that the single treatment with G2-S24P did not reduce the infection more than 60% in the 24 h after infection. However, in the combination of G2-S24P with ganciclovir, we obtained different results. We observed that the inhibition of this combination reached 90% if treated even after 48 h of HCMV infection, this activity is reduced to 75% at 72 h, and finally, at 4 days after infection, the treatment would reduce 70% of the infection.

These promising results promote the use of nanotechnology, specifically PCDs, against several infectious diseases, being versatile, easily synthesised. Compared to some existing treatments, this paper shows that these molecules could be an exciting tool against viral infectious diseases.

5. Conclusions

1. G1-S4 and G2-S16 showed good biocompatibility in J89GFP and THP89GFP, either alone or in combination with BRY, PST, PNB and RMD. This combination allows de dual activity of LRAs at reactivating HIV-1 latent infection and PCDs at inhibiting the new infections.
2. G3-S16 dendrimer not only showed good biocompatibility, but also showed reactivation in THP89GFP cell line either alone or increasing the reactivation of PNB and RMD in the combination.

3. In the context of HIV-1 infection, G2-S16 has showed the ability to inhibit macrophage cell to cell transmission to T lymphocytes, thus being capable of reduce reservoir or sanctuaries formation.
4. G1-S4, G2-S16 and G2-S24P showed high biocompatibility in human lung fibroblast for six days treatments. Moreover, G2-S16 and G2-S24P presented HCMV inhibition, the mechanism of action of these dendrimers is not at the attachment level, neither against viral particles.
5. The inhibition activity of G2-S16 and G2-S24P against HCMV last longer, as compared with the actual treatment, ganciclovir. Moreover, the combination of these PCDs with ganciclovir, increases the inhibition of HCMV, not only in potency, but also in time.

6. Conclusiones

1. G1-S4 y G2-S16 mostraron una buena biocompatibilidad en J89GFP y THP89GFP, ya sea solos o en combinación con BRY, PST, PNB y RMD. Esta combinación permite la actividad dual de los LRA para reactivar la infección latente por VIH-1 y los PCD para inhibir las nuevas infecciones.
2. El dendrímero G3-S16 no solo mostró buena biocompatibilidad, sino que también mostró reactivación en la línea celular THP89GFP ya sea solo o aumentando la reactivación de PNB y RMD en la combinación.
3. En el contexto de la infección por VIH-1, G2-S16 ha demostrado la capacidad de inhibir la transmisión de célula a célula de macrófagos a linfocitos T, pudiendo así reducir la formación de reservorios o santuarios.
4. G1-S4, G2-S16 y G2-S24P mostraron una alta biocompatibilidad en fibroblasto de pulmón humano durante tratamientos de seis días. Además, G2-S16 y G2-S24P presentaron inhibición del HCMV, el mecanismo de acción de estos dendrímeros no está a nivel de adhesión, ni contra partículas virales.
5. La actividad inhibidora de G2-S16 y G2-S24P contra HCMV dura más tiempo, en comparación con el tratamiento actual, ganciclovir. Además, la combinación de estos PCD con ganciclovir aumenta la inhibición del CMVH, no solo en potencia, sino también en tiempo.

7. References

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