Title: Development of water-soluble polyanionic carbosilane dendrimers as novel and highly potent topical anti-HIV-2 microbicides

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ABSTRACT

The development of topical microbicide formulations for vaginal delivery to prevent HIV-2 sexual transmission is urgently needed. Second- and third-generation polyanionic carbosilane dendrimers with silicon atom core and 16 sulfonate (G2-S16), napthylsulfonate (G2-NS16) and sulphate (G3-Sh16) end-groups have showed potent and broad-spectrum anti-HIV-1 activity. However, their antiviral activity against HIV-2 and mode of action has not been probed.

Cytotoxicity, anti-HIV-2, anti-sperm and antimicrobial activities of dendrimers were determined. Analysis of combined effects of triple combinations with tenofovir and raltegravir were performed by using CalcuSyn software. We also assessed the mode of antiviral action on the inhibition of HIV-2 infection through a panel of different *in vitro* antiviral assays: attachment, internalization in PBMCs, inactivation and cell-based fusion. Vaginal irritation and histological analysis in female BALB/c mice were evaluated.

Our results suggest that G2-S16, G2-NS16 and G3-Sh16 exert anti-HIV-2 activity at an early stage of viral replication inactivating the virus, inhibiting cell-to-cell HIV-2 transmission, blocking binding of gp120 to CD4, and HIV-2 entry. Triple combinations with tenofovir and raltegravir increased anti-HIV-2 activity, consistent with synergistic interactions (CI_{wt}: 0.33-0.66). No vaginal irritation was detected in BALB/c mice after two consecutive applications for 2 days with 3% G2-S16.

This work clearly shows that G2-S16, G2-NS16 and G3-Sh16 have high potency against HIV-2 infection. The modes of action confirm their multifactorial and non-specific ability, suggesting that these dendrimers deserve further

studies as potential candidate microbicides to prevent vaginal/rectal HIV-1/HIV-2 transmission in humans.

1. INTRODUCTION

Human immunodeficiency virus (HIV) and other sexually transmitted infections are global threats to public health. Although HIV-1 strains are responsible for most of the global infections, HIV-2 strains are an important cause of disease in West African nations, Portugal, France, and in the United States.[1, 2] Moreover, co-infection with both HIV-1 and HIV-2 occurs in some countries of West Africa where the viruses co-circulate.[3, 4]

Sexual transmission is responsible for the majority of HIV-2 infections due to infected semen or cervico-vaginal secretions containing infected lymphocytes.[5] Ideally, a vaginal/rectal microbicide should have the following features: to be acceptable and affordable, offer maximal and immediate protection, remain in the vagina for a few hours to act against the sexual transmitted diseases (STDs) during and after sexual intercourse, not leak immediately after application, not accumulate to avoid toxicity effects, not affect the normal vaginal flora, and be compatible with male latex condom.[6-8]

Dendrimers are a class of well-defined hyper-branched polymers with a nanoscale globular shape, well-defined functional groups at the periphery, hydrophobic/hydrophilic cavities in the interior and low polydispersity.[9] Dendrimers offer unique opportunities in the synthesis of agents with broad-spectrum anti-HIV-1 activity and activity against Alzheimer's disease, herpes simplex virus (HSV), bacteria, and cancer.[10-14] A group of dendrimers with a carbosilane structure has demonstrated potential against HIV and other pathogens. These dendrimers are particularly suitable for this application due to the simplicity of their synthesis, which allows for large amounts of the polymer to be generated; the ability to obtain a polymer with a defined molecular weight

and number of terminal functions; chemical and biochemical stability; biologically inertness; and the low polarity of the C–Si bond, which imparts hydrophobicity to the carbosilane scaffold.[15-17]

One of the most promising targets of the HIV cycle is the viral entry/fusion process, which is divided into three steps: (i) attachment of gp120 to CD4, (ii) binding to CCR5 and/or CXCR4, and (iii) fusion of the envelope with the cell membrane and releasing of the viral capsid into the cytoplasm of the host cell.[18] Dendrimers containing functionalized groups at their periphery can bind to their target in a multivalent manner, providing a strategy for the development of potent viral entry inhibitors. Although the antiviral activity and the mode of action of carbosilane dendrimers have been studied against HIV-1, their antiviral activity against HIV-2 is currently unknown. Dendrimers may be important to fight HIV-2 infection as few of the currently available antiretroviral drugs work well against this virus and drug resistance is rapidly selected against the drugs that do work.[19]

In order to identify dendrimers with potent anti-HIV-2 activity, we considered several polyanionic carbosilane dendrimers successfully tested for HIV-1 applications with the objective of selecting those compounds with the best results obtained. In previous studies, we have shown that polyanionic carbosilane dendrimers G2-S16, G2-NS16, and G3-Sh16 (generations described as the number of repeating layers of silicon atoms forming the dendrimer, **Fig. 1**) had great anti-HIV-1 activity *in vitro* and *in vivo.*[20-22] G2-S16 and G2-NS16 consists of second-generation carbosilane dendrimers scaffold built from a silicon atom core, which is fully capped on the surface with 16 sulfonate and napthylsulfonate groups, respectively. G3-Sh16 is a sulphate-

terminated generation 3 carbosilane dendrimer, silicon-cored and also with 16 anionic charges at the periphery. The anionic groups are in the form of sodium salts. Here, we have investigated the cytotoxicity, the anti-HIV-2 activity, and the anti-sperm and antimicrobial activities of G2-S16, G2-NS16, and G3-Sh16 dendrimers. Moreover, we researched the anti-HIV-2 activity of combinations of these dendrimers with tenofovir and raltegravir. In order to discover more about the antiviral mechanism of action of these dendrimers, we used several *in vitro* experiments including: attachment and internalization of HIV-2 in PBMCs, HIV-2 inactivation, and cell-based fusion assays. Finally, we show that vaginal application of 3% G2-S16 gel formulation does not cause vaginal irritation or lesions after histological analysis in female BALB/c mice.

2. EXPERIMENTAL SECTION

a. Dendrimers and reagents

Polyanionic carbosilane dendrimers G2-S16 ($C_{112}H_{244}N_8Na_{16}O_{48}S_{16}Si_{13}$; Molecular weight, Mw: 3,717.2 g/mol), G2-NS16 ($C_{184}H_{244}N_{24}Na_{16}O_{56}S_{16}Si_{13}$; Mw: 4,934.0 g/mol) and G3-Sh16 ($C_{256}H_{508}N_{48}Na_{16}O_{64}S_{16}Si_{29}$; Mw: 6,978.4 g/mol) were synthesized as previously reported.[15, 17] 1mM stock solution of dendrimers and subsequent dilutions to obtain μM concentrations were prepared in distilled water. The reagents used as controls for inhibition of viral replication were the peptide HIV fusion inhibitor T-1249 (Trimeris, Inc., Morrisville, NC, USA), tenofovir (TFV; Gilead Sciences, Foster City, CA, USA), and raltegravir (RAL; Merck Sharp & Dohme Corp, Whitehouse Station, NJ, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma-

Aldrich, St. Louis, MO, USA), and serial dilutions to the intermediate concentrations were prepared using distilled water.

b. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy blood donors by a standard Ficoll-Hypaque density gradient (Rafer, Zaragoza, Spain) and cultured following the procedures of Spanish HIV-HGM BioBank.[23, 24] When indicated, PBMCs were stimulated with 2 µg/ml phytohemaglutinin (PHA; Remel, Santa Fe, NM, USA) and 20 U/ml IL-2 (Bachem, Bubendorf, Switzerland) for at least 2 days before the experiments began. The protocol for maintaining the human epithelial TZM-bl and Hela cell lines (National Institute of Health AIDS Research and Reference Reagent Program, NIH-ARRRP) has been described.[25-27]

c. Virus stocks and titration

CCR5- and CXCR4-tropic primary HIV-2 clade A strains were isolated by co-cultivation of PBMCs from infected subjects with PHA-activated PBMCs from healthy individuals.[28] The 50% tissue culture infectious dose ($TCID_{50}$) of viruses was determined in a single round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells as described previously.[29]

The recombinant vaccinia virus vSC50 encodes the full-length env gene from HIV-2SBL/ISY, an X4-tropic infectious molecular clone, which was cloned into the vaccinia (WR) TK gene.[30] Env gene expression is under control of the vaccinia virus P7.5 promoter.[31] Virus stocks were tittered using the Reed & Muench method[32] in Rat2 cells.

d. Cytotoxicity assay

The safety and toxicity profile of G2-S16, G2-NS16 and G3-Sh16 in TZM-bl, Hela, and PBMCs cells was determined by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazoliumbromide; Sigma) cytotoxicity assay according to manufacturer's instructions.

e. Antiviral assay

TZM-bl cells were pretreated with serial dilutions of dendrimers for 1h at 37ºC. Cells were then infected with 200 $TCID_{50}$ of virus-containing primary R5- or X4-HIV-2 isolates for 2h at 37ºC. After 48h, the cells were washed and lysed, and the luciferase activity was measured by using a luciferase assay system kit (Promega, Madrid, Spain) according to the manufacturer's instructions. From the dose-response curve, the 50% cytotoxic concentration (CC_{50}) and the half-maximal inhibitory concentration (IC_{50}) of dendrimers were determined for each strain. The therapeutic index (TI) was determined by the following equation: $TI=CC_{50}/IC_{50}$.[33]

f. Inhibition of HIV-2 attachment and entry to PBMCs

PHA-activated PBMCs were pretreated with dendrimers or controls for 1h. Then, primary HIV-2 isolates were added to PBMCs (the equivalent to 40 ng capsid protein/10^6 cells) and incubated for 2h at 4ºC. Unbound virus was removed by washing with phosphate-buffer saline (PBS; Lonza, Walkersville, MD, USA) three times, the cells were then lysed, and cell-bound viruses were quantified by the capsid p24 content in cell lysates. To measure internalization, the same conditions were used, except that PBMCs were incubated with virus for 2h at 37ºC, washed in acid wash (glycine 50 mM pH 3.2; Sigma) to strip surface-bound viral particles, and cell-internalizated viruses were quantified by the capsid p24 content in cell lysates.

g. HIV-2 inactivation

Primary R5- or X4-HIV-2 isolates (the equivalent to 10 ng of the capsid protein) were incubated at 4ºC overnight into wells of a 96-well flat-bottom plate with poly-L-Lysine, to ensure the adherence of viral particles to the bottom of the well. Then, the wells were washed three times with PBS to remove unbound HIV-2 and treated with dendrimers or controls for 1h. PHA-activated PBMCs were then added. After 72h, cells were washed and lysed, and the HIV-2 p24 Gag level in the cell lysates was quantified by using HIVp24^{gag} ELISA kit (Innogenetics, Ghent, Belgium). Cell viability was measured by MTT assay.

h. Cell-to-cell fusion inhibition

Cell-to-cell fusion (CTC) inhibition was tested using a newly developed method with recombinant vaccinia virus expressing HIV-2 ISY env gene (vSC50). Hela cells were transfected using jetPRIME® reagent (Polyplus-transfection SA, Illkirch, France) with the Tat expressing plasmid pcDNA3.1+/Tat101-flag following manufacturer's instructions and infected with the recombinant vaccinia virus. After 3h, Hela were collected and co-cultured with TZM-bl (CD4+, CCR5+, CXCR4+, indicator cells) at 1:1 cell density ratio in the absence or presence of increasing concentrations of dendrimers or controls. The percentage of membrane cell fusion was measured by luciferase activity induced by the Tat protein. The level of syncytium formation was determined by direct microscopic observation.

i. Combination analysis between dendrimers and antiretrovirals

TZM-bl cells were pretreated with different concentrations of dendrimer/antiretroviral (ARV) at a constant fixed ratio for 1h at 37ºC. The cells were then infected with the equivalent to 10 ng of the capsid protein of primary

R5-HIV-2 or X4-HIV-2 strains. 48h post-infection, the cells were washed and lysed and HIV-2 replication was quantified by luciferase activity. The IC_{50} and synergism were determined by using CalcuSyn software (Biosoft, Cambridge, UK). IC_{50} was determined by using the median-effect plot and the dose-reduction index.[34] Combination indices (CIs) were calculated based on the median-effect principle,[35] where CI<0.9 indicates a synergistic effect, 0.9<CI<1.1 indicates an additive effect, and CI>1.1 indicates an antagonism effect.

j. Sperm processing and spermicidal activity

Semen samples were collected by healthy volunteers by masturbation at the laboratory after 2-5 days of sexual abstinence and allowed to liquefy for 30-45 min at room temperature.[36] If completely liquefied, G2-S16 was selected for evaluating the sperm motility at 24h according to previously described method.[37] Only specimens with at least a final motile sperm concentration of $5x10^6$/mL were used. These parameters were evaluated by using the Sperm Class Analyzer software (Microptic S.L., Barcelona, Spain).

k. Antimicrobial assays

G2-S16 was evaluated for its antimicrobial activity against *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 00603, *Lactobacillus plantarum* ATCC 14917, *Pseudomonas aeruginosa* ATCC 28753 and *Staphylococcus aureus* ATCC 29213 microorganisms. The antimicrobial activity of G2-S16 was measured using a broth microdilution test and serial 1:2 dilutions according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines.

l. BALB/c mice vaginal irritation test

Six- to eight-week-old female BALB/c mice (Charles River, Barcelona, Spain) were housed in a specific-pathogen-free animal facility at Centro de Biología Molecular 'Severo Ochoa' for at least one week before the experiments were conducted. All mice were maintained and treated according to protocols approved by the Institutional Animal Care and Research Committee. BALB/c mice were injected subcutaneously with 2 mg of medroxyprogesterone acetate (DepoProvera, Pfizer, New York, NY, USA) five days before treatment. To assess and conduct an easy, low-cost, and a reliable statistical comparison of irritation studies with minimal damage, mice were randomized into 3 groups (n=3 in each group). Forty microliters of 2% hydroxyethylcellulose (HEC; Bohm laboratories, Madrid, Spain) gel containing 3% G2-S16 was carefully applied to the vaginal vault of mice using a stainless steel feeding needle for one day or two consecutive days. On day 7, mice were euthanized and vaginal tissues were excised and fixed in 4% formaldehyde solution (Panreac, Barcelona, Spain) for histology.

m. Histological studies in BALB/c

Formalin-fixed excised vaginal tissues were submitted to the Anatomic Pathology Laboratory (anaPath, Granada, Spain) for embedding, sectioning and evaluation of hematoxylin-and-eosin-stained tissue by an experienced pathologist. Sections were mounted on slides and were subjected to a blind evaluation for epithelial erosion, leukocyte infiltration, thickening of the lamina propria (edema), and vascular congestion. The inflammation scores were assigned by a semiquantitative system.[38, 39]

n. Statistical analysis

The data are presented as mean values and standard deviations (SD). The statistical significance between a treatment group and untreated control was calculated by unpaired *t*-test using GraphPad Prism v5.0 software (GraphPad, San Diego, CA, USA).

3. RESULTS

a. Cell biocompatibility

The biocompatibility of G2-S16, G2-NS16 and G3-Sh16 was evaluated in TZM-bl, Hela, and PBMCs. Cells were treated for 48h with increasing concentrations of dendrimers, which were considered toxic when survival rate were <80%. G2-NS16 and G3-Sh16 were considered non-toxic at 10 μM in PBMCs; G2-S16 was toxic at 50 μM. G2-NS16 and G2-S16 were non-toxic at 100 μM in TZM-bl and Hela cells, whereas G3-Sh16 was non-toxic up to 50 μM (**Fig. 2**). Therefore, the *in vitro* working concentration selected as non-toxic for all types of cells to facilitate a better comparison between compounds was of 10 μM.

b. Anti-HIV-2 activity

Although R5- and X4-HIV-1 viruses are present in body fluids (blood, semen, cervicovaginal and rectal secretions), R5 variants predominate in early stages during the process of sexual transmission, and persist throughout the course of HIV disease.[40] Moreover, late stage R5 HIV-1 variants show more rapid replication and higher cytopathicity relative to early stage R5 variants.[41] Previous studies have demonstrated a similar behavior of the viral coreceptor specificity during the course of HIV-2 infection.[42, 43] Therefore, we analyzed the antiviral activity and the IC_{50} of G2-S16, G2-NS16 and G3-Sh16 against both early- and late-stage R5- and X4-HIV-2 isolates in TZM-bl cells.

The CC_{50} were >100 μM for G2-S16 and G2-NS16 in TZM-bl cells. The CC_{50} for G3-Sh16 was 90 μM in TZM-bl cells (**Table 1**). The concentrations of G2-S16 at which R5- and X4-HIV-2 isolates were inhibited by 50% (IC_{50}) varied between 1.12 and 4.56 μM. The pre-treatment of TZM-bl cells with 10 μM G2-S16 resulted in 73-94% inhibition of infection by primary R5- and X4-HIV-2 isolates (**Fig. 3a**). Pretreatment of TZM-bl cells with G2-NS16 showed a dose dependent inhibition of HIV-2 infection with IC_{50} values ranging from 0.73 to 1.07 μM (**Table 1**). G2-NS16 at 10 μM was the dendrimer with the best inhibition values against the primary R5- and X4-HIV-2 strains (>93%; **Fig. 3b**). G3-Sh16 also showed dose dependent inhibition in HIV-2 infection with IC_{50} values ranging from 0.85 to 2.97 μM (**Table 1**). Maximum percentage of inhibition varied between 82-94% with 10 μM G3-Sh16 (**Fig. 3c**).

The TI of a compound is the ratio between the toxic and the therapeutic dose to measure its relative safety. Although TI values depend on many factors, it is generally considered that a drug has a good safety profile whether its TI >10.[44] The TI values of G2-S16 and G3-Sh16 were in a range of >21.9 to >89.3, and 43.5 to 105.9, respectively (**Table 1**). G2-NS16 showed the highest TI with values that varied between >93.5 and >137.

Summarizing, the three dendrimers were highly active against HIV-2 infection in TZM-bl cells, G2-NS16 being the most potent and broad of them.

c. Virus-cell attachment and viral entry to the susceptible host cells

Viral entry is a process that involves the binding of a virus to the surface of a cell, fusion to the cell membrane, and internalization of the viral genome into the target cells. We evaluated whether G2-S16, G2-NS16 and G3-Sh16 is involved in HIV-2 binding or in entry steps in PHA-activated PBMCs. Virus attachment

was measured at 4ºC, a temperature at which membrane fusion and endocytosis processes are ineffective. Virus internalization was evaluated by incubating the virus-cell mixture at 37ºC, a temperature that allows membrane fusion and viral entry.

Treatment with 10 μM of G2-S16, G2-NS16 and G3-Sh16 significantly decreased the capacity of the primary X4-HIV-2 isolate to bind to PBMCs by 55%, 62%, and 52%, respectively ($p<0.0001$; **Fig. 4a**). This pattern was similar to that observed for the primary R5-HIV-2 particles (58-75% infection inhibition), but the reduction was not significant. As for the internalization of the viruses into PBMCs albeit a reduction was observed for all dendrimers it was only significant for G2-S16 with X4-HIV-2 isolates (45% decrease at 10 μM; $p<0.0001$; **Fig. 4b**). In summary, the three dendrimers bound to target cells and perturbed the ability of the viral envelope to interact with its cell surface receptors. G2-NS16 was the dendrimer that best blocked the virus-cell binding processes.

d. HIV-2 inactivation

The mechanism of inhibition of polyanionic carbosilane dendrimers against HIV-2 could also be associated to a direct viral inactivation, without forgetting the ability of dendrimers to block the gp120/CD4 interaction. Therefore, we evaluated the ability of G2-S16, G2-NS16 and G3-Sh16 to directly inactivate primary HIV-2 isolates before contact with PHA-activated PBMCs.

The pre-treatment of primary X4-HIV-2 with 10 μM of G2-S16, G2-NS16 or G3-Sh16 significantly decreased the infectivity of X4-HIV-2 after 1h of exposure (82-91% reduction; $p<0.0001$). This pattern was similar to that observed for the primary R5-HIV-2 (68-81% reduction), but the decrease was not significant (**Fig. 5**). The cell viability was always over 80%.

To summarize, the pre-treatment with the dendrimers for 1h decreased the infectivity of the HIV-2 particles in a tropism-dependent manner, suggesting that the dendrimers act strongly on the virion. The best results of HIV-2 inactivation were obtained with the G2-NS16 dendrimer.

e. Inhibition of cell-to-cell fusion and syncytium formation

A Tat and luciferase-based fusion assay and a syncytium counting assay were developed and used to assess whether the dendrimers block cell-associated virus entry.

In the presence of a Hela/TZM-bl cell mixture, G2-S16, G2-NS16 and G3-Sh16 efficiently blocked fusion between both cell lines in a dose-dependent manner (**Fig. 6a**). We also observed a significant decrease in the number of syncytia when the cells were pre-treated with the dendrimers, in particular for G2-NS16, which acts like the fusion inhibitor control T-1249 (**Fig. 6a**). Inhibition of HIV-2 CTC fusion requires higher concentrations of each dendrimer than inhibition of HIV-2 cell-free infection. The IC_{50} of G2-S16 for CTC inhibition was 8.4-fold higher than IC_{50} for cell-free virus infection. The IC_{50} of G2-NS16 and G3-Sh16 for CTC inhibition was 4.4-fold higher than IC_{50} for cell-free virus infection (**Fig. 6b**).

In summary, our results indicate that the three dendrimers inhibit cell-associated HIV-2 infection in a dose-dependent manner, in particular G2-NS16, albeit at a higher concentration relative to the inhibition of cell-free infection.

f. Combination of dendrimers and antiretrovirals against HIV-2 infection

The anti-HIV-2 activity of triple drug combinations was assessed in a single cycle assay in TZM-bl cells. For these studies the dendrimers were combined

with TFV, a nucleoside RT inhibitor, and RAL, an integrase inhibitor, at 1:1:1 fixed ratio.

In G2-S16/TFV/RAL, the IC_{50} for G2-S16 decreased against the primary R5- and X4-HIV-2 isolates (IC_{50}: 3.0-60.0 nM) compared with dendrimer treatment alone. A similar result was also observed for the ARVs against the two primary HIV-2 infections (IC_{50}: 60-310 nM for TFV; IC_{50}: 60 nM for RAL) compared with single-drug treatment (**Fig. 7a**). CI was then calculated to determine whether synergistic, additive or antagonistic effects against all primary HIV-2 isolates occurred after this combination. CI calculations showed synergism at 75, 90, and 95% inhibition of R5-HIV-2 (CI: 0.38-0.53) and X4-HIV-2 infection (CI: 0.47-0.61) (**Table 2**).

For G2-NS16/TFV/RAL, the IC_{50} for G2-NS16 dropped against the primary R5- and X4-HIV-2 isolates (IC_{50}: 50.0-60.0 nM) compared with the dendrimers used alone. Reductions were also observed in the IC_{50} for TFV (IC_{50}: 250-610 nM) and RAL (IC_{50}: 100-240 nM) against the primary R5-HIV-2 and X4-HIV-2 infection compared to the drug treatment alone (**Fig. 7b**). The average CI displayed stronger synergy at the calculated EC_{90}, and EC_{95} inhibitory concentrations against R5-HIV-2 infection (CI: 0.24-0.30). CI values indicated a good synergistic inhibitory profile at the three EC_{75}, EC_{90}, and EC_{95} concentrations against X4-HIV-2 infection (CI: 0.46-0.65) (**Table 2**).

With G3-Sh16/TFV/RAL, a reduction in G3-Sh16 concentration was observed (IC_{50}: 10-110 nM) against the primary R5- and X4-HIV-2 infection. The IC_{50} for TFV dropped against R5- and X4-HIV-2 infection (IC_{50}: 260-620 nM) compared with TFV alone; and for RAL decreased against R5- and X4-HIV-2 infection (IC_{50}: 100-360 nM) compared with the drug used alone (**Fig. 7c**). CI

determination showed synergistic interactions at the calculated EC_{75}, EC_{90}, and EC_{95} inhibitory concentrations against R5-HIV-2 infection (CI: 0.31-0.42).This combination exhibited synergism at 90%, and 95% inhibition of X4-HIV-2 (CI: 0.60-0.64) (**Table 2**).

In summary, the strongest synergistic interactions and the highest inhibition of infection in TZM-bl cells were observed with G2-NS16/TFV/RAL, which is consistent with the most potent activity of G2-NS16 alone relative to the other dendrimers.

g. *In vitro* spermicidal activity

To identify whether the topical microbicide candidate G2-S16 is spermostatic or spermicidal, sperm was cultured in the presence or absence of dendrimer and the progressive motility of the sperm was analyzed. No significant changes in sperm progressive motility of G2-S16 (at 10 and 50 μM) at 24h post-treatment were obtained compared with untreated control (**Fig. 8a**).

This result indicates that G2-S16 can be considered safe to be used as topical vaginal microbicide because it did not alter the sperm motility and did not affect other sperm functions. Moreover, this finding is consistent with the results obtained with G2-NS16 and G3-Sh16 in previous studies.[22]

h. Antimicrobial activity

The normal vaginal microbiota contains a wide variety of bacterial species that maintain an acidic pH by hydrogen peroxide and lactic acid production.[45] Alterations in this ecosystem can cause several vaginal infections, such as bacterial vaginosis and *Candida* vaginitis, which represent the majority of these infections among women.[46] A successful microbicide product has to be stable and biocompatible in normal vaginal flora, preventing HIV-2 transmission in this

highly complex microenvironment. Therefore, toxicity of G2-S16 against a composite population of bacteria observed in normal vaginal microbiota was analyzed. No antimicrobial activity of G2-S16 (at 10 and 50 μM) at 24h post-treatment against the list of microorganisms cited in M&M was observed (**Fig. 8b**).

Summarizing, our findings are consistent with the results obtained with G2-NS16 and G3-Sh16 in previous studies.[22] It suggests that G2-S16 is a good potential candidate for the first biological barrier encountered by the viruses because G2-S16 did not have negative effects in the normal vaginal flora.

i. *In vivo* assay of G2-S16 in BALB/c mice model

To evaluate whether microbicide exposure of G2-S16 resulted in toxicity and inflammation of the vaginal mucosa, G2-S16 at 3% was applied intravaginally to BALB/c mice, and pathological examination of the vaginal tissues was performed at 7 days post-application. Histopathological examination indicated that the application of one dose or two doses of 3% of G2-S16 gel-treated BALB/c mice did not induce vaginal irritation or damages in the vaginal mucosa (**Fig. 9**).

4. DISCUSSION

Sexual transmission is the main route of HIV spread throughout the world.[47] In the absence of a prophylactic anti-HIV vaccine, microbicides could offer to women a new strategy to prevent sexually transmitted HIV.[48-50] To identify alternative medicines that provide significant advantage compared to existing therapies, different mechanisms to inhibit the viral lifecycle before the

integrations should be considered, such as the process of receptor-mediated viral entry.

Dendrimers containing several types of functionalized groups at their periphery have shown effective anti-HIV activity as non-specific microbicides.[18, 51, 52] SPL7013 (the active product of VivaGel®), a fourth generation polylysine-based dendrimer with 32 napthylsulfonate groups at the periphery, is the only topical nanomicrobicide that has advanced to human clinical trials for HIV-1/HSV-2 prophylaxis. However, VivaGel® provided low activity against R5-HIV-1 isolates and epithelial injury after 7-14 days of twice-daily administration has been associated with an increased risk for HIV infection.[53] A new water-soluble dendrimers with carbosilane structure, which are characterized by the easy availability of reagents, short reaction times, high reproducibility and quantitative yields of reaction, have been synthesized.[15-17] These polyanionic carbosilane dendrimers are stable compounds and their microbicide capacity against HIV-1 has been previously reported.[22, 54] However, the potential microbicide capacity against HIV-2 infection is still unknown. Here, the anti-HIV-2 activity of G2-S16, G2-NS16 and G3-Sh16 and the mechanism of action were determined.

It has been reported that in heterosexual HIV transmission, R5-tropic HIV variants dominate in the early stages of HIV disease,[55] although X4-tropic HIV variants are also present in body fluids.[56] We compared the half cytotoxic concentration of G2-S16, G2-NS16 and G3-Sh16 when exposed to TZM-bl cells (CC_{50} > 90 μM) with the concentration of these dendrimers at which R5- and X4-HIV-2 infectivity was inhibited by 50% (IC_{50}: 0.73-4.56 μM). Then, the therapeutic index was determined (TI: 21.9 - >137) and used as an indicator of overall efficacy and safety. Despite the fact that TI was high (>10), G2-S16, G2-

NS16 and G3-Sh16 are compounds that should be studied more thoroughly and carefully before passing to clinical trials.

In the virucidal and HIV-2 inactivation assays, we observed the capacity to diminish the residual infectivity of HIV-2 particles after 1h of incubation with the dendrimers. These results suggest that diverse mechanisms of HIV-2 inactivation may be involved. The dendrimers can (i) disrupt the integrity of the HIV-2 membrane decreasing the stability of viral RNA,[57] (ii) bind to V3 loop,[20, 58] or (iii) interact and modify by denaturing the two disulfide bonds locate on the HIV-2 gp120 protein (an area implicates in binding to the CD4 receptor).[59] However, additional research is needed to define the mechanism of inactivation occurs. The observations previously obtained by molecular dynamics simulations support this idea because the mechanism of action is associated with electrostatic interactions between HIV gp120 and different functional groups of dendrimers.[20] However, the HIV-2 inactivation has not still been studied and further experiments by molecular modeling should be performed.

The ability to prevent HIV-2 binding to PBMCs but not entry can be explained by the existence of other effective mechanisms of attachment besides the direct HIV-2 fusion with the CD4 cell surface (i.e., through galactosyl-ceramide, ICAM-1, LFA-1 or heparan sulfate),[60] which are beyond the mode of action of the dendrimers. Another possibility is that virus-cell fusion occurs with the endosomal membrane following the endocytic uptake of virus particles.[61] However, all alternatives of HIV-2 entry can probably coexist, which is the reason why G2-S16, G2-NS16 and G3-Sh16 inhibit binding but do not inhibit completely the internalization. Finally, the HIV-2 capsid protein can be altered by the dendrimers during the passage to the cell and this leads to problems in

its disaggregation, most likely the p24 antigen enters the cell but RT, integrase and viral RNA do not. Consequently, HIV-2 particles are not infectious.

The ability of HIV to spread between cells determines its virulence, with direct HIV CTC up to thousand fold more potent and efficient than infection by cell-free virus particles.[62, 63] It is known that CTC protects viruses from humoral immune responses and antiviral treatments, allowing the persistence of residual replication, and the establishment and maintenance of viral reservoirs. Previous studies have demonstrated that HIV-1 CTC is susceptible to ARVs and neutralizing antibodies.[64-67] Here, we have developed a simple and highly effective assay to assess the activity of dendrimers and other drugs in CTC promoted by the HIV-2 envelope. We show that dendrimers can efficiently prevent cell HIV-2 CTC albeit at a higher concentration relative to cell-free infection. These results confirm that cell-to cell fusion is more difficult to inhibit than cell-free HIV infection[62] and, more importantly, indicate that dendrimers can prevent cell-associated HIV-2 infection

ARVs display great potential in the prevention of sexual HIV transmission. However, ARVs-based microbicides could increase the risk of emergence of multidrug-resistant mutants, drug-drug interactions, systematic absorption and adverse side effects associated with high drug concentrations and lifelong therapy.[29, 68, 69] Therefore, to prevent these problems the ideal HIV microbicide should combine different classes of antiretroviral drugs acting in different targets with compounds that act in a non-specific way.[18] Here, we have shown that dendrimer/TFV/RAL combinations at a fixed 1:1:1 ratio have significant synergistic interactions against the primary R5- and X4-HIV-2 isolates. Similar results have been previously obtained for HIV-1 using a combination of these

dendrimers with TFV and/or maraviroc.[25, 70-72] G2-NS16/TFV/RAL was the most potent combination regimen against HIV-2 isolates, which is consistent with the higher anti-HIV-2 potency of the G2-NS16 dendrimer when given alone relative to the other dendrimers.

The safety profile of a microbicide candidate should preserve its activity against HIV infection and other STDs (i.e., HSV-2) and be carefully evaluated before moving the candidate into clinical trials. It is also important to note that an algorithm focused on prevention of HIV infection should address whether the lead topical microbicide candidates are spermicidal. There are some evidences of harm through genital lesions with the spermicide nonoxynol-9 when is vaginally administered for preventing HIV and other STDs.[73] Previously we have shown that G2-NS16 and G3-Sh16 do not induce changes in sperm motility.[22]

In this work, we studied the anti-sperm and antimicrobial activity of G2-S16. The sperm motility and the different bacteria present in normal vaginal flora showed very similar response patterns compared to untreated control, indicating that G2-S16 is not an antimicrobial and spermicidal compound. The results confirm that this class of dendrimers is not spermicidal

Several polyanionic polymers as entry inhibitors (i.e., Ushercell, Carraguard or PRO2000) showed an increased risk of HIV-1 infection because of the disruption of the integrity of mucosal epithelial surface.[74] In the present work, we studied the safety of 3% G2-S16 gel on topical mucosal site using BALB/c mice model. We showed that G2-S16 displayed a good safety profile and did not cause alterations to the vaginal epithelium. For the topical application, the dendrimer establishes a film or a physical barrier to prevent the dissemination of

infected cells from the local mucosa to the regional lymph nodes and acts against the infection once virus has crossed the epithelial barrier.[75]

5. CONCLUSIONS

To summarize, our studies reveal that G2-S16, G2-NS16 and G3-Sh16 are non-specific compounds that inhibit HIV-2 infection acting at different and early steps in the HIV-2 lifecycle. These dendrimers act directly on the virus, block HIV-2 replication at steps prior to the integration of proviral DNA into the infected host cell genome (binding and fusion/entry into target cells), block HIV-2 CTC and are effective at non-cytotoxic concentration easily reachable in the mice model. The use of a combined therapy blocking HIV-2 infection at early steps in the HIV-2 lifecycle is highly effective to stop the infection over existing therapeutic approaches, as they may avoid virus entry into new target cells and accelerate the decay of the latent reservoirs for HIV.

The delivery vehicle for the formulation of the dendrimers is important for a good distribution throughout the vagina or rectum. Therefore, novel routes of administration to those already known (topical gels, intravaginal rings or locally applied solid films and tablets) should be explored to improve the activity, accept ability and adherence of these dendrimers for the prevention of HIV-2 infection and other STDs that enhances HIV. It would be also interesting to test the anti-HIV-2 activity of the microbicide in presence of semen and to study the hypersensitivity, photosensitivity and condom integrity in the presence of the microbicide. Finally, further studies including humanized mice to complement *in vitro* and *in vivo* findings should be considered avoiding high economic losses and time before reaching human clinical trials.

Our data suggests that these dendrimers are promising candidates for future microbicide clinical trials in the field for prevention of HIV-2 infection. The role of these parameters in G2-S16/TFV/RAL, G2-NS16/TFV/RAL and G3-Sh16/TFV/RAL, and other combination activities should be assessed in future studies.

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7. FIGURE AND TABLE LEGENDS

Figure 1. Molecular representation of second- and third-generation dendrimers. (A) G2-S16 with 16 sulfonate end groups; **(B)** G2-NS16 with 16 naphthylsulfonate end groups; **(C)** G3-Sh16 with 16 sulphate end groups. The generation of dendrimers is determined by considering that each generation corresponds to the number of repeating layers of silicon atoms forming the dendrimer. Abbreviations: Mw= Molecular weight

Figure 2. Cytotoxicity associated to polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16. **(A)** TZM-bl, **(B)** Hela and **(C)** PBMCs cells were loaded with increased amounts of dendrimers in a range between 0.01 and 100 µM; or treated with 10 µM dextran (innocuous control) or 10% of DMSO (control of cell death). Histograms represent the percentage of viable

cells as the mean ± SD of at least three independent experiments performed in triplicate (*vs.* NT). Abbreviations: DMSO= Dimethylsulfoxide; NT= Non-treated (medium alone).

Figure 3. Anti-HIV-2 activity and dose-response curve obtained for polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 in TZM-bl cells. TZM-bl cells were pretreated with increased concentrations of **(A)** G2-S16, **(B)** G2-NS16, and **(C)** G3-Sh16 for 1h before HIV-2 infection (concentrations ranged from 0.31 to 20 μM). Equal amounts of virus-containing (2,000 $TCID_{50}$) primary early/late R5- or X4-HIV-2 isolates were used. Luciferase activity was analyzed to 48h post-infection *vs*. control non-treated cells. Data represent the mean ± SD of three independent experiments performed in triplicate.

Figure 4. Effect of polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 on HIV-1 attachment and entry into PBMCs. PHA-activated PBMCs were pretreated with dendrimers (10 μM) or control (5 μM T-1249) for 1h before the infection with primary R5- or X4- HIV-2 isolates. After infection for 2h **(A)** at 4°C (binding) or **(B)** at 37°C (internalization), the cells were washed extensively (with glycine-acid washing for internalization assay) and lysed with 0.1% Triton X-100 buffer. Attachment and internalization levels were quantified by the measure of p24 in cell lysates by p24 ELISA. Results represent the mean ± SD of three independent experiments performed in triplicate. ***: $p<0.0001$ *vs.* untreated control. Abbreviations: PBMCs= Peripheral blood mononuclear cells; PHA= Phytohaemagglutinin.

Figure 5. HIV-2 virus inactivation by polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 prior to infection the PBMCs. Primary R5- and X4- HIV-2 isolates (grey and white bars, respectively) were stuck on poly-L-lysine coated plates and were treated with dendrimers (10 μM) or control (5 μM T-1249) 1h before exposure to PHA-activated PBMCs. After 72h, PBMCs supernatants were collected and p24 levels were measured by p24 capture ELISA. Cell viability was measured by MTT assay (black solid and dashed lines). Data are represented as the mean ± SD of three independent experiments performed in triplicate. ***: $p<0.0001$ *vs.* untreated control. Abbreviations: CI= Control of infection; PBMCs= Peripheral blood mononuclear cells; PHA= Phytohaemagglutinin.

Figure 6. Inhibition of Env/CD4-mediated membrane fusion. The plasmid Tat^+ (pcDNA 3.1/Tat 101-flag) was transfected using the jetPRIME® transfection reagent into Hela-$CD4^-$ cells. The cells were then infected with the rVVenv virus (rVV/ISY). After 3h, TZM-bl (indicator cells) and Hela (effector cells) were co-cultured and treated with dendrimers at a range of concentrations (2.5-50 μM) or T-1249 (1.25-10 μM) for 48h. **(A)** Percentage of fusion was measured by luciferase activity *vs.* cell-to-cell fusion without treatment (NT) and by direct microscopic observation by quantification of the syncytium formation. Data represent the mean ± SD of three independent experiments performed in triplicate. **(B)** The half-maximal inhibitory concentration (IC_{50}) for cell-free and cell-to-cell viral fusion.

Figure 7. The half-maximal inhibitory concentration (IC_{50}) of polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 and antiretrovirals (tenofovir or raltegravir) alone and in combination in TZM-bl cells. Mean 50% effective concentrations (IC_{50}, nM) for **(A)** G2-S16/TFV/RAL, **(B)** G2-NS16/TFV/RAL, and **(C)** G3-Sh16/TFV/RAL at an equimolar ratio (1:1:1) after infection with primary R5- and X4-HIV-2 isolates in TZM-bl cells. The white bars indicate the mean IC_{50} calculated for the compounds when added alone, and the gray bars indicate the mean IC_{50} calculated for the compounds when used in combination. Data are shown as the mean ± SD from three independent experiments performed in duplicate. Abbreviations: TFV= Tenofovir; RAL= Raltegravir

Figure 8. Sperm survival index and antimicrobial activity after 24h of treatment with G2-S16. (A) Sperm were cultured in seminal plasma, in the presence or absence of different concentrations of G2-S16 (10 and 50 μM). The sperm progressive motility was measured by using the Sperm Class Analyzer software. A calculated sperm motility index value <75% was used to indicate sperm toxicity. Data are represented as the mean ± SD of three different donors. **(B)** The determination of the minimum inhibitory concentration (antimicrobial susceptibility) of G2-S16 at 10 and 50 μM, defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation at 35ºC, is calculated by a modified Kirby-Bauer disk diffusion technique.

Figure 9. Effects of 3% G2-S16 on the vaginal mucosa in BALB/c mice model. Mice were inoculated **(A)** once or **(B)** twice for two consecutive days (with an interval of 24h between sessions) intravaginally with 40 μl of 2% HEC containing G2-S16 at 3% (8 mM). The vaginal tract was harvested from the mice at 7 days post-application. Formalin-fixed, paraffin-embedded tissues sections were stained with hematoxylin-eosin for evidence of morphological damage or evidence of inflammation. HEC-treated mice were included as reference control (data no shown). Photographs are representative of all treated mice. Original magnification 100X. **(C)** Histopathological examination of vaginal tissues. Data were calculated as the mean ± SD of the scores estimated at the vaginas of three mice in each group. † Individual score: 0=absence, 1=minimal, 2=mild, 3=moderate, 4=severe irritation. ‡ The cumulative score for epithelial disruption, submucosal leukocyte infiltration, edema and vascular congestion were correlated to human vaginal irritation potential as follows: vaginal irritation index ≤8: Acceptable; 9-10: Borderline; ≥11: Unacceptable. The scoring system was established according to Eckstein et. al[35] and Zhong et. al.[36] Abbreviations: HEC= Hydroxyethylcellulose gel.

Table 1. *In vitro* cytotoxicity and anti-HIV-2 activity of polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 in TZM-bl cells.

† CC_{50}: The cytotoxic concentration of the dendrimers that caused the reduction of viable cells by 50%. Data represents the mean ± SD of three independent experiments performed in triplicate.
‡ IC_{50}: The concentration of the dendrimers that resulted in 50% inhibition in HIV-2 infection. Data represents the mean ± SD of three independent experiments performed in triplicate
¥ TI: Therapeutic index is CC_{50}/IC_{50}

Table 2. Combination indices of three-drug combinations against HIV-2 infection in TZM-bl cells. Computer-simulated combination index at 50, 75, 90 and 95% inhibition of primary R5- and X4-HIV-2 infection *in vitro* in TZM-bl cells. CIs are presented as the mean ± SD from the average of three experiments performed in triplicate.

† CI > 1.1 indicates antagonism (-); 1.1 > CI > 0.9 indicates additive effect (ad) and CI < 0.9 indicates a synergistic effect.
‡ Synergy levels: 0.9 > CI > 0.85: + (sight synergism); 0.85 > CI > 0.7: ++ (moderate synergism); 0.7 > CI > 0.3: +++ (synergism); 0.3 > CI > 0.1: ++++ (strong synergism); CI < 0.1; +++++ (very strong synergism).
¥ Because high degrees of effects are more important to the treatment than the low degrees of effects, the weighted average CI value was assigned as $CI_{wt} = [CI_{50} + 2CI_{75} + 3CI_{90} + 4CI_{95}]/10$, where CI_{50}, CI_{75}, CI_{90}, and CI_{95} are the CI values at 50, 75, 90, and 95% inhibition, respectively.[31, 32]
Abbreviations: CI= Combination index; RAL= Raltegravir; TFV= Tenofovir.

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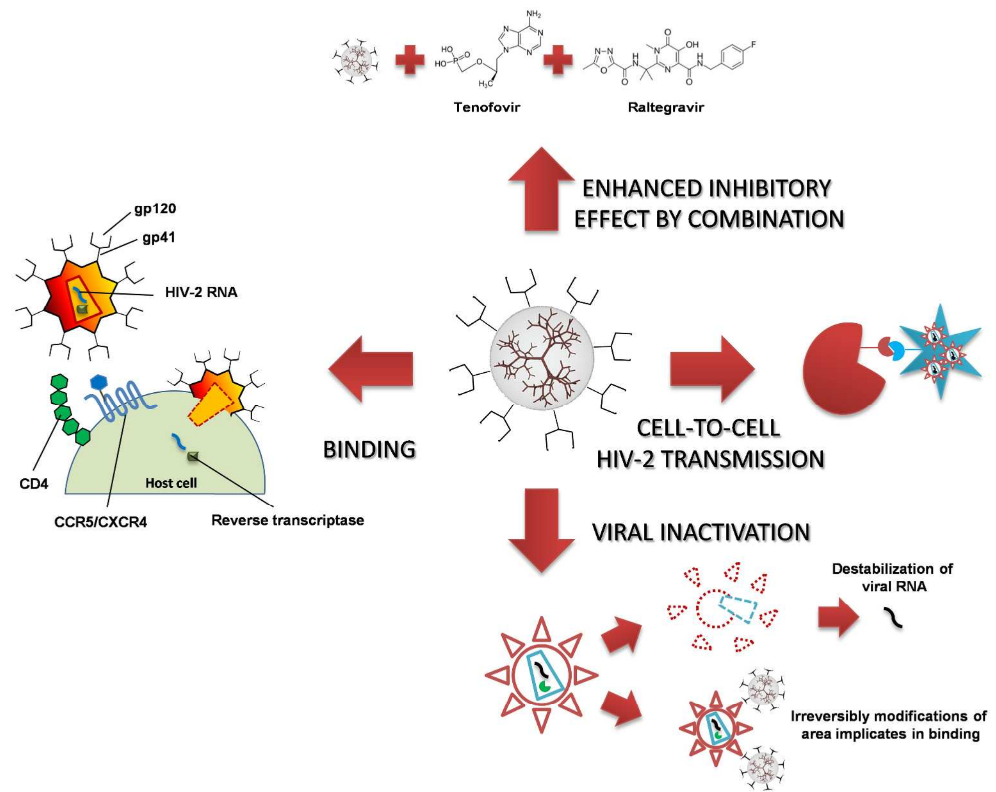
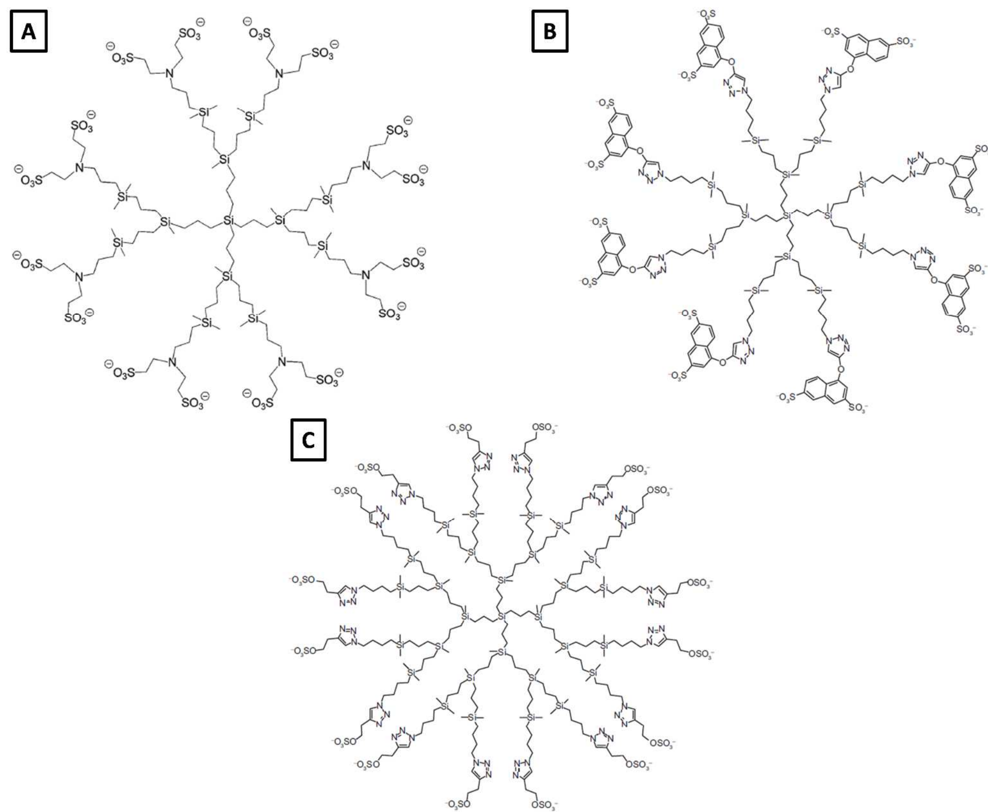


812x645mm (96 x 96 DPI)

Polyanionic carbosilane dendrimers inhibit HIV-2 binding and subsequent internalization, inactivate the virus, block HIV-2 cell-to-cell fusion, and enhance the antiviral activity in combination with tenofovir/raltegravir compared to the compounds alone.



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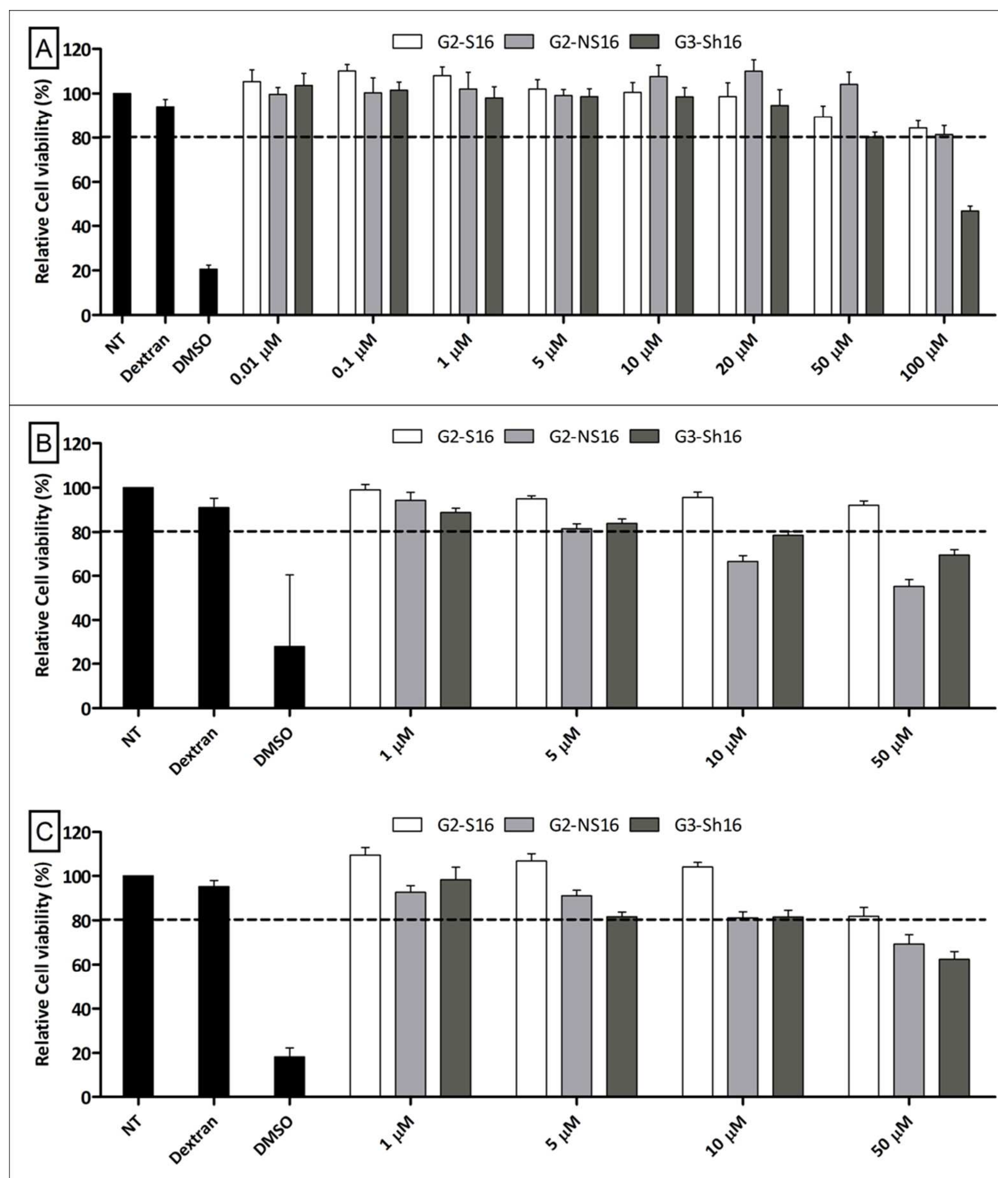

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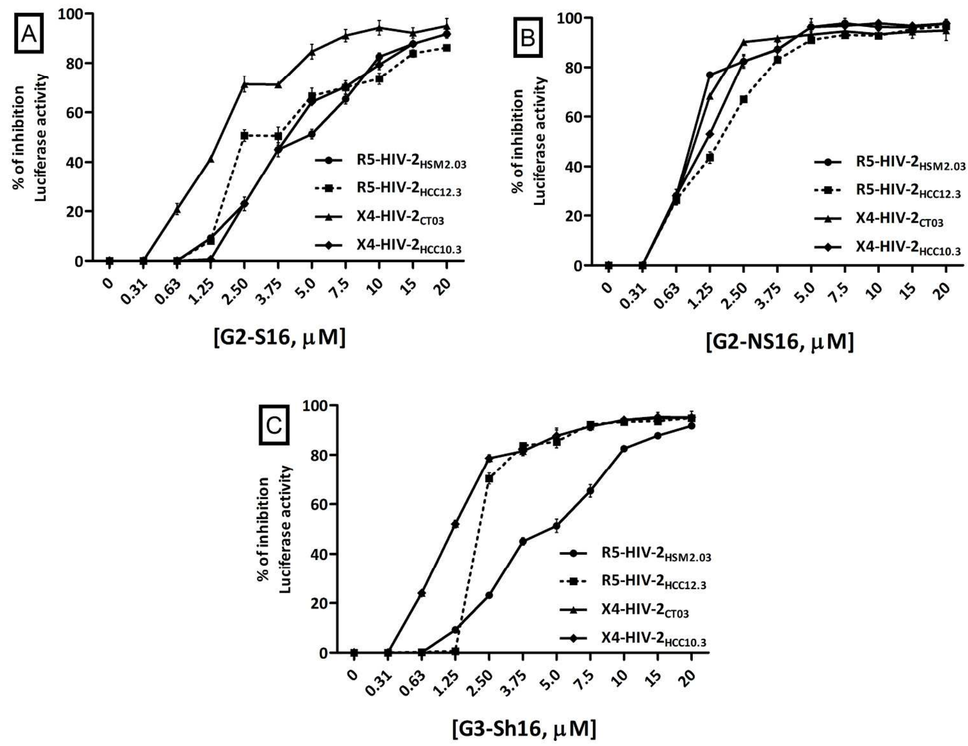

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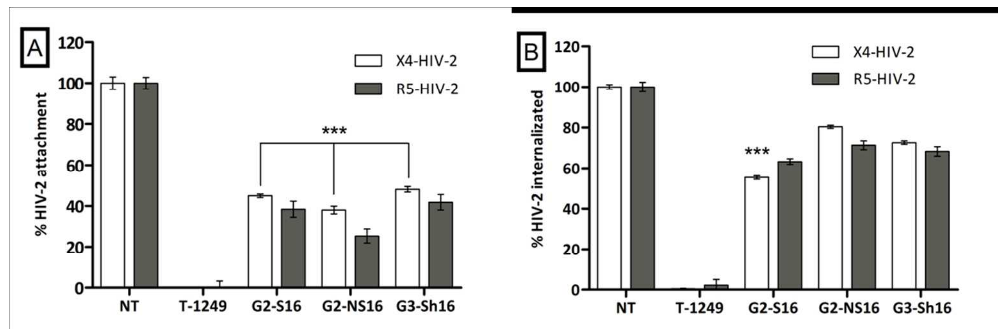

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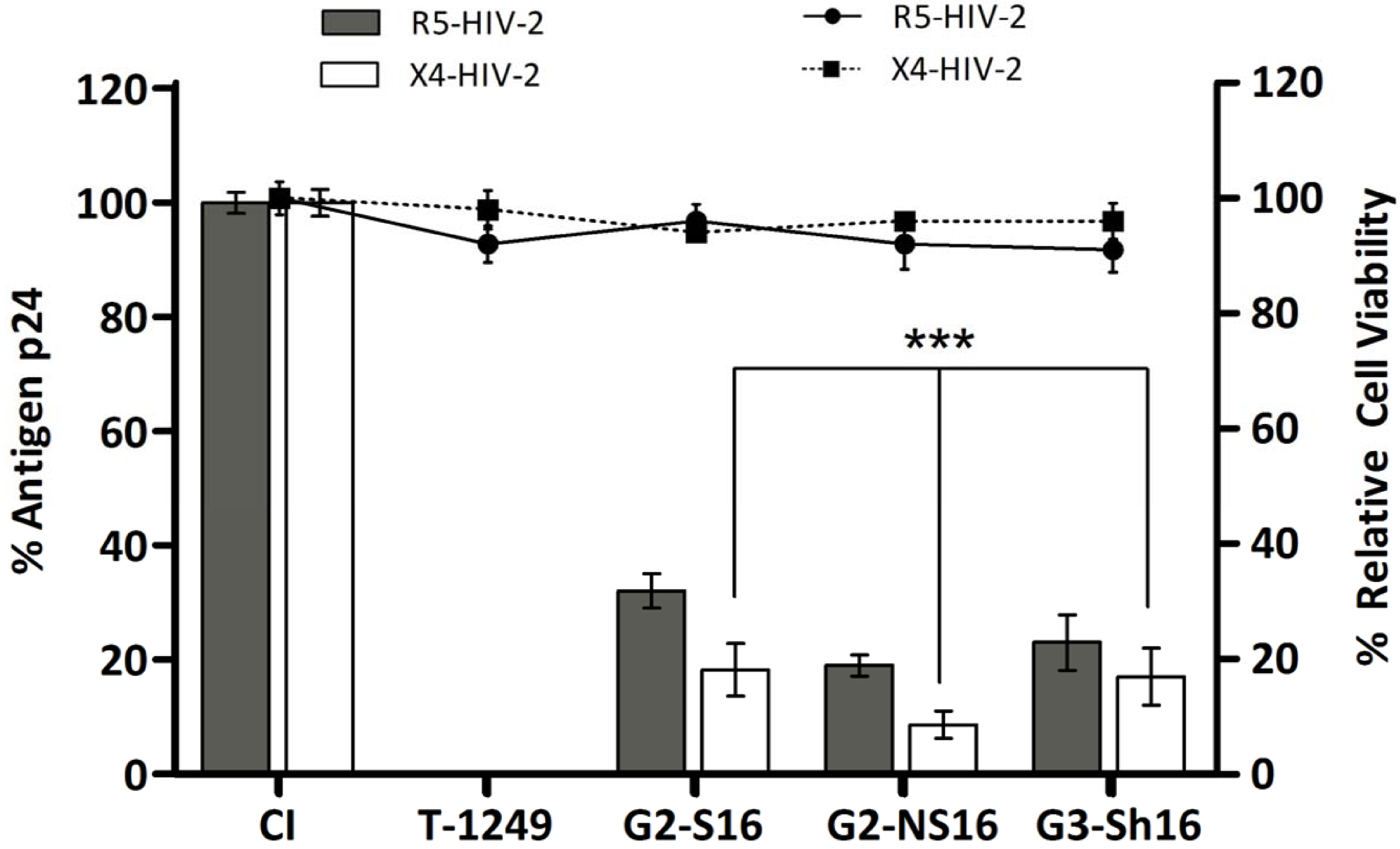
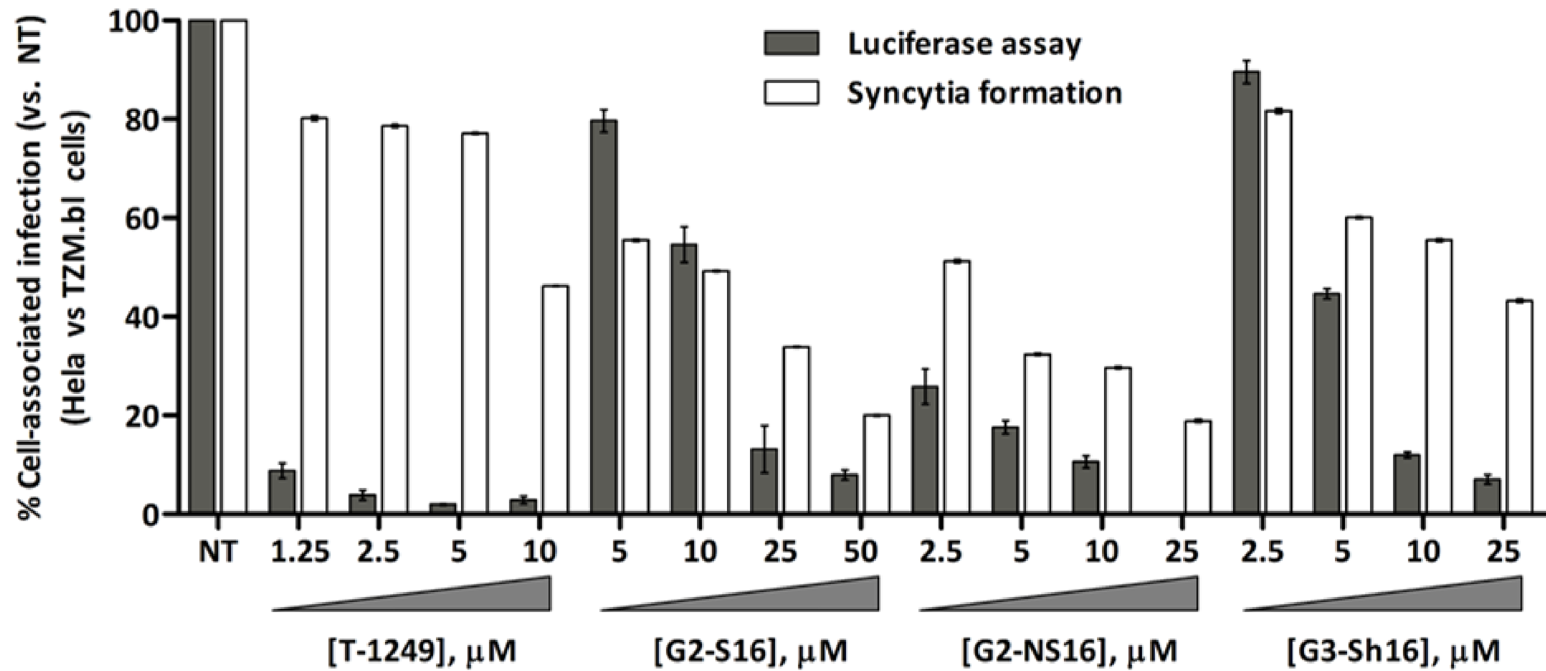
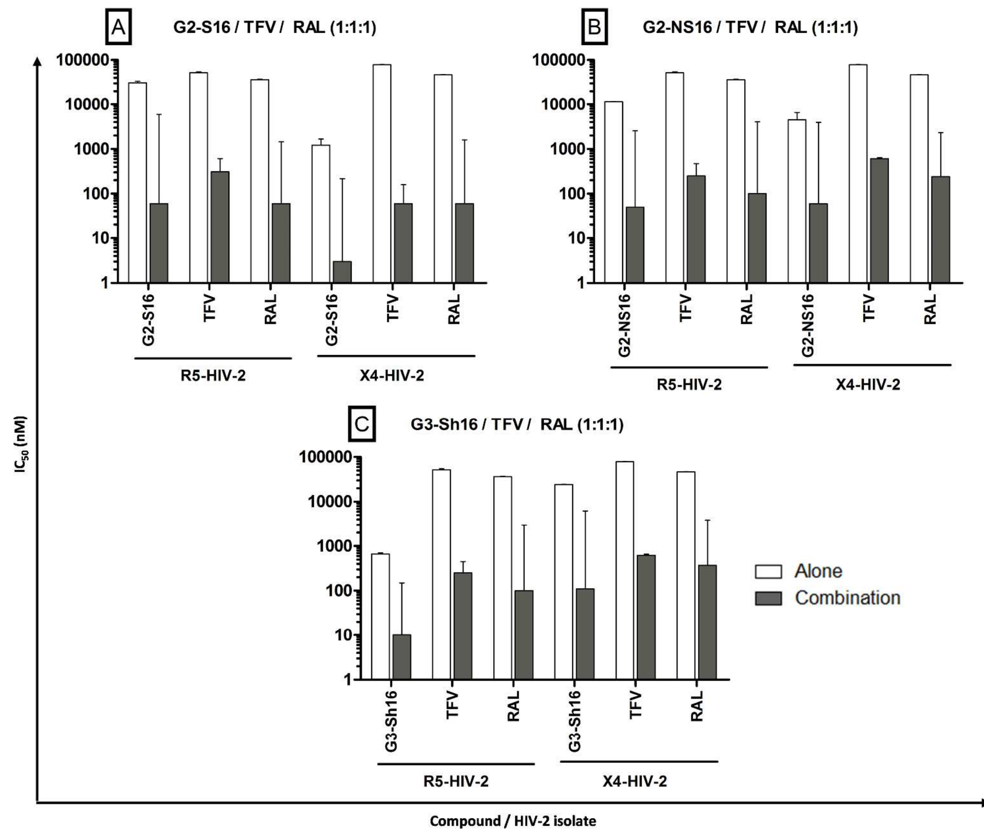

HIV-2 Inactivation
Cell Viability
R5-HIV-2
X4-HIV-2
R5-HIV-2
X4-HIV-2
% Antigen p24
% Relative Cell Viability
120
100
80
60
40
20
0
***
CI
T-1249
G2-S16
G2-NS16
G3-Sh16

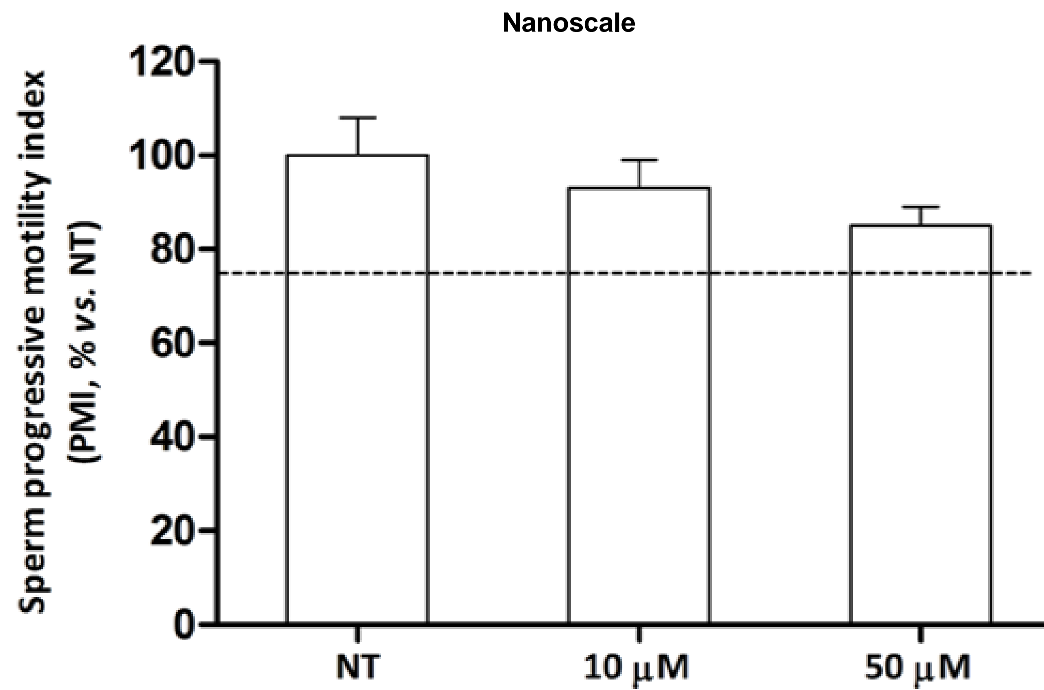


B

Dendrimers	Inhibition of cell-free viral fusion IC_{50} (nM)	Inhibition of cell-to-cell viral fusion IC_{50} (nM)	Ratio (cell-to-cell/cell-free)
G2-S16	2.97	13.21	8.4
G2-NS16	0.88	7.34	4.4
G3-Sh16	1.33	5.92	4.4

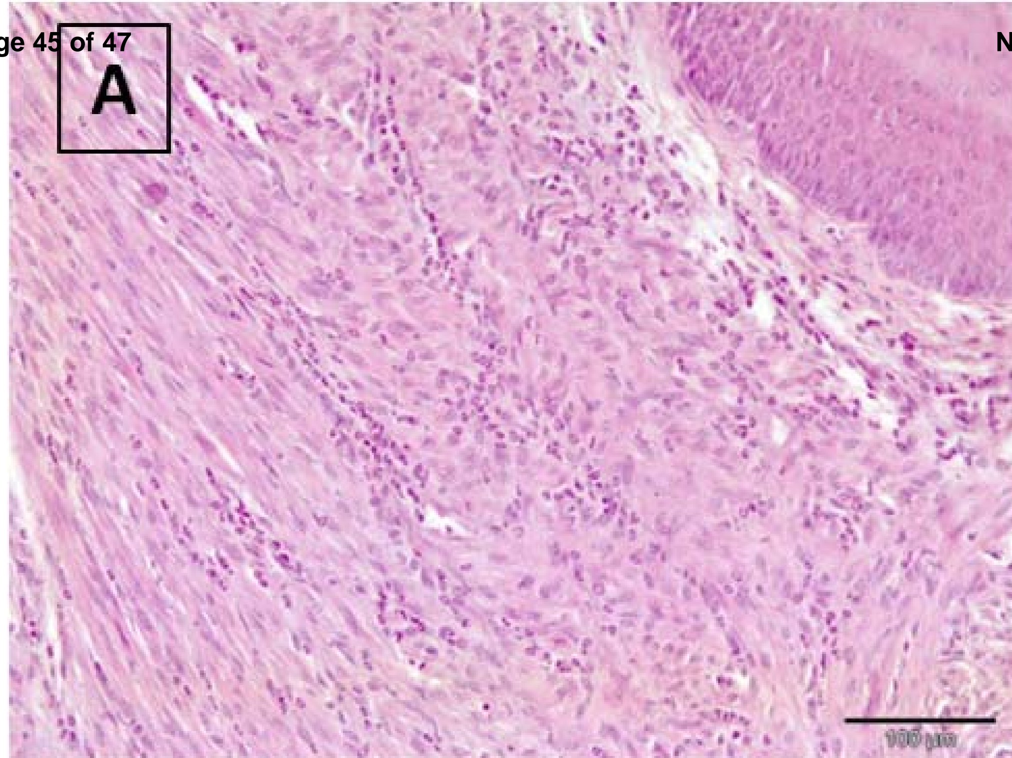


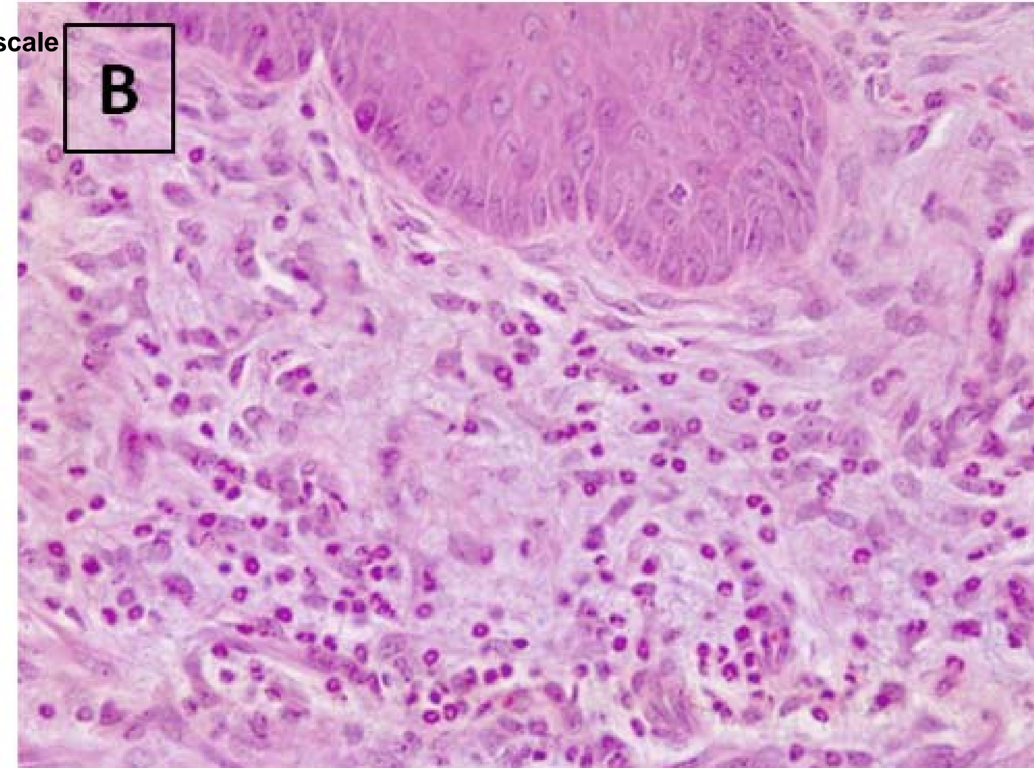
850x716mm (96 x 96 DPI)



B

Bacteria	NT, [mg/mL]	G2-S16 10 μM, [mg/mL]	G2-S16 50 μM, [mg/mL]
Candida albicans ATCC 10231	3.717	3.717	3.717
Enterococcus faecalis ATCC 29212	3.717	3.717	3.717
Escherichia coli ATCC 25922	3.717	3.717	3.717
Klebsiella pneumonia ATCC 00603	3.717	3.717	3.717
Lactobacillus plantarum ATCC 14917	3.717	3.717	3.717
Pseudomonas aeruginosa ATCC 28753	3.717	3.717	3.717
Staphylococcus aureus ATCC 29213	3.717	3.717	3.717





C

Lesion	Vehicle	1 dose of 3% G2-S16	2 doses of 3% G2-S16
Epithelial cell disruption †	0.0 ± 0.0	0.3 ± 0.6	0.7 ± 0.6
Leukocyte infiltration †	0.0 ± 0.0	0.7 ± 1.2	1.3 ± 1.2
Vascular congestion †	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Edema †	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Vaginal irritation index ‡	0.0 ± 0.0	1.0 ± 1.7	2.0 ± 1.7

Dendrimer	HIV-2	CC_{50} (μM) †	IC_{50} (μM) ‡	TI ¥
G2-S16	**Early R5-HSM2.03**	>100	4.56	>21.9
	Late R5-HCC12.3		2.46	>40.7
	Early X4-CT03		1.12	>89.3
	Late X4-HCC10.3		3.72	>26.9
G2-NS16	**Early R5-HSM2.03**	>100	0.73	>137.0
	Late R5-HCC12.3		1.07	>93.5
	Early X4-CT03		0.81	>123.5
	Late X4-HCC10.3		0.88	>113.6
G3-Sh16	**Early R5-HSM2.03**	90	1.56	57.7
	Late R5-HCC12.3		2.07	43.5
	Early X4-CT03		0.85	105.9
	Late X4-HCC10.3		0.85	105.9

Triple Combination (Combination ratios)	HIV-2	CI values at inhibition of: †				CI_{wt}-values ¥
		50%	75%	90%	95%	
G2-S16 + TFV + RAL (1:1:1)	R5	0.66 ± 0.04 ‡ +++	0.53 ± 0.01 +++	0.43 ± 0.01 +++	0.38 ± 0.02 +++	0.45 ± 0.02 +++
	X4	0.73 ± 0.05 ++	0.61 ± 0.01 +++	0.52 ± 0.03 +++	0.47 ± 0.05 +++	0.54 ± 0.03 +++
G2-NS16 + TFV + RAL (1:1:1)	R5	0.63 ± 0.09 +++	0.42 ± 0.02 +++	0.30 ± 0.01 ++++	0.24 ± 0.03 ++++	0.33 ± 0.04 +++
	X4	0.84 ± 0.02 ++	0.65 ± 0.05 +++	0.52 ± 0.08 +++	0.46 ± 0.09 +++	0.55 ± 0.06 +++
G3-Sh16 + TFV + RAL (1:1:1)	R5	0.51 ± 0.05 +++	0.42 ± 0.05 +++	0.35 ± 0.02 +++	0.31 ± 0.01 +++	0.36 ± 0.02 +++
	X4	0.84 ± 0.03 ++	0.73 ± 0.02 +++	0.64 ± 0.05 +++	0.60 ± 0.06 +++	0.66 ± 0.04 +++