Baseline and time-updated factors in preclinical development of anionic dendrimers as successful anti-HIV-1 vaginal microbicides

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Abstract
Although a wide variety of topical microbicides provide promising in vitro and in vivo efficacy, most of them failed to prevent sexual transmission of human immunodeficiency virus type 1 (HIV-1) in human clinical trials. In vitro, ex vivo, and in vivo models must be optimized, considering the knowledge acquired from unsuccessful and successful clinical trials to improve the current gaps and the preclinical development protocols. To date, dendrimers are the only nanotool that has advanced to human clinical trials as topical microbicides to prevent HIV-1 transmission. This fact demonstrates the importance and the potential of these molecules as microbicides. Polyamionic dendrimers

Abbreviations: 7-AAD, 7-aminoactinomycin D; AIDS, acquired immunodeficiency syndrome; API, active pharmaceutical ingredient; ATP, adenosine triphosphate; BLT, marrow-liver-thymus; cART, combined antiretroviral therapy; CC50, compound’s concentration required for the reduction of cell viability by 50%; COVID-19, coronavirus disease 2019; DC, dendritic cell; EC50, half-maximal effective concentration; EC90, 90% effective concentration; EMA, European Medicines Agency; FDA, Food and Drug Administration; FITC, fluorescein isothiocyanate; FIV, feline immunodeficiency virus; GFP, green fluorescent protein; HCV, hepatitis C virus; HEC, hydroxyethylcellulose; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; h-mice, humanized mice; HPMC 4000, hydroxypropylmethylcellulose 4000; HPV, human papillomavirus; HSC, hematopoietic stem cell; HSV-2, herpes simplex virus type 2; hu-HSC, human RAG; hu-PBL, human peripheral blood lymphocyte; hu-Thy/Liv, human fetal thymus-liver tissue; MERS-CoV, Middle East respiratory syndrome coronavirus; M0, macrophage; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NHP, nonhuman primate; NOD, nonobese diabetic; NSG, NOD scid gamma; PAA, poly(alkylideneamine); PAMAM, poly(amidoamine); PBMC, peripheral blood mononuclear cell; PEG 6000, polyethylene glycol 6000; PLL, poly(L-lysine); PPH, phosphorus-containing dendrimer; RSV, respiratory syncytial virus; SARS-CoV-2, respiratory syndrome coronavirus 2; Scid, severe combined immunodeficiency; SHIV, simian/human immunodeficiency virus; SIV, simian immunodeficiency virus; STIs, sexually transmitted infections; T/F, transmitted/founder; TI, therapeutic index; TLR, toll-like receptor; UNAIDS, Joint United Nations Programme on HIV/AIDS; WHO, World Health Organization; WST-1, 4-[3-(4-iodophenyl)-2H-3H-5-tetrazolol]-1-benzene sulfonate; XTT, sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

Ignacio Rodríguez-Izquierdo and Daniel Sepúlveda-Crespo contributed equally to this manuscript.
1 | INTRODUCTION

With almost 40 years of combating the human immunodeficiency virus type 1 (HIV-1) and acquired immunodeficiency syndrome (AIDS) epidemic, HIV-1 remains a significant global public health problem and is the most challenging infectious disease to eliminate. The 2020 Joint United Nations Programme on HIV/AIDS (UNAIDS) report estimates that 38 million people are living with HIV-1 worldwide (UNAIDS, 2020b). Sub-Saharan Africa remains the worst-affected region of HIV-1 infections, where women and girls represent 59% of the HIV-1-infected population (UNAIDS, 2020a). Moreover, the coronavirus disease 2019 (COVID-19) pandemic has raised concerns about the increase in new HIV-1 infections and HIV-associated mortality due to potential effects from disruptions in HIV-1 programs (Drain & Garrett, 2020; El-Sadr & Justman, 2020; Jewell, Mudimu, et al., 2020; Jewell, Smith, et al., 2020). Various factors have contributed to disruptions in HIV care and service during the COVID-19 pandemic: (i) strict quarantine measures, (ii) lack of access to health facilities, (iii) blocking of transport in many cities to prevent the spread of COVID-19 (Guo et al., 2020), (iv) stock out of cART due to the closure of pharmaceutical companies (WHO, 2020), and (v) changing the focus of healthcare personnel to care for COVID-19 patients instead of people living with HIV (Kowalska et al., 2020). The World Health Organization (WHO) has reported that, as of July–April 2020, several countries had interruptions in cART services (over 11 million HIV people affected), had a high risk of cART interruption, or presented a low stock of cART (almost 26 million HIV people) (WHO, 2020). Some mathematical models assessed the impact of COVID-19 on HIV epidemiology, indicating that a 6-month interruption in cART could lead to 500,000 further deaths from HIV-related illnesses (Jewell, Mudimu, et al., 2020) or that HIV-related deaths would increase by 10% over the next 5 years (Hogan et al., 2020).

2 | THE IMPORTANCE OF VAGINAL MICROBICIDES TO PREVENT HIV-1

Sexual transmission is the main route of HIV-1 infection (Davari et al., 2020; LeMessurier et al., 2018).

In the absence of an approved vaccine to prevent HIV-1 transmission (Laher et al., 2020; Medlock et al., 2017), and despite the great efforts made by researchers during recent years (Baden et al., 2020; Dieffenbach & Fauci, 2020;
Pantaleo et al., 2019; Pitisuttithum & Marovich, 2020), combined antiretroviral therapy (cART) remains the primary strategy to treat and control HIV-1 infection (Giacomelli et al., 2019; Phanuphak & Gulick, 2020). However, cART is costly and poses a challenge to apply in low-resource countries (Kahn et al., 2011). Moreover, several concerns, including the long-term toxicity, development of resistance mutations, and drug–drug interactions, have shown a challenge for finding new therapeutic approaches that prevent and control the HIV-1/AIDS epidemic (Back & Marzolini, 2020; Battini & Bollini, 2019; Chawla et al., 2018; Gibas et al., 2019). In this sense, the use of long-lasting, female-controlled, and efficacious topical microbicides among women has emerged as a new strategy to overcome the HIV-1 epidemic in the last years.

A microbicide is a compound formulated as a gel, ointment, cream, insert (suppository, tablets, ovules), film, or ring applied vaginally/rectally to reduce the risk of HIV-1 and other STIs, such as genital herpes (herpes simplex virus type 2 [HSV-2]), human papillomavirus (HPV), hepatitis C virus (HCV), gonorrhea, chlamydia, or syphilis (Coutinho et al., 2017; Garcia & Wray, 2020; Patel & Rohan, 2017). A microbicide must act as a lubricant coat that maintains vaginal defenses, inactivates HIV-1, and prevents other STIs in the presence of vaginal fluids and semen. Moreover, a microbicide must be readily accessible, easy to use, long shelf-life, and compatible with latex. It must also be (among other features) stable at higher temperatures, odorless, colorless, and tasteless (Antimisiaris & Mourtas, 2015). In the infection process, the virus may cross the epithelial barrier through breaches caused by microabrasions, microtrauma, or genital ulcers, or through the thinning and disruption of the multilayer lining by a pre-existing inflammation (Hladik & Doncel, 2010; Thurman & Doncel, 2011). Therefore, the microbicide must also block HIV-1 capture and transmission by dendritic cells (DCs), HIV-1 entry to the host cell, and HIV-1 replication before the integration process (Figure 1).

Several antiretroviral drugs are currently in advanced stages of development as microbicides, either vaginally or rectally (Al-Khouja et al., 2020; Bunge et al., 2020; A. Y. Liu et al., 2019; MTN, 2020; Pleasants et al., 2020). However, there is no commercially available microbicide due to the lack of effectiveness of these products in preclinical and clinical trials for many reasons (Baeten et al., 2020; Musekiwa et al., 2020; Notario-Perez et al., 2017).

**Figure 1** Key factors for microbicides to reduce the risk of HIV-1 transmission. The microbicide must act as a physical barrier against HIV-1 and other sexually transmitted infections (STIs). The microbicide must maintain vaginal defenses and inhibit HIV-1 replication. Once HIV-1 has crossed the epithelial barrier, the microbicide must prevent the HIV-1 uptake by dendritic cells (DCs), HIV-1 binding, fusion, or any process before the integration.
To date, regulatory requirements for approval and licensure for microbicides have not been specified because guidance from the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) date back years ago and are out of date (EMA, 2011; EMA, 2013; USFDA, 2014; WHO, 2009). Following these guidelines, we offer a scientific basis for developing a preventive anti-HIV-1 microbicide (Figure 2). In this way, we provide a more up-to-date approach, considering the problems and novel discoveries involved in such studies. One possible reason for clinical failure may be that preclinical steps with microbicides do not reflect the environment encountered during heterosexual transmission of HIV-1 (Roan & Munch, 2015). Here, we discuss ways to improve preclinical assays (including in vitro, ex vivo, and in vivo models) of active pharmaceutical ingredients (API) as topical microbicides to mimic the real-life conditions of HIV-1 transmission in people. A decreased drug solubility and stability, reduced drug cellular uptake and internalization, lack of adherence, mucus, and tissue penetration, and lack of protection from biological factors like metabolizing enzymes are other reasons for clinical failure (das Neves et al., 2016; Patel & Rohan, 2017). Recent progress in developing better and more promising novel strategies based on nanotechnology opens a new perspective to overcome these drawbacks and improve current systems for dealing with HIV-1 infection (Brako et al., 2017; das Neves et al., 2016; Macchione et al., 2020; Notario-Perez et al., 2017; Sanchez-Rodriguez, Vacas-Cordoba, et al., 2015).

**FIGURE 2**  Updated summary of the main steps of the preclinical microbicide development

| Stage 1: Primary and confirmation assays |
|-----------------------------------------|
| Cytotoxicity assays on different cells (CC_{50}) |
| Efficacy against RS-HIV-1 viruses (EC_{50}) |
| Efficacy against range of HIV-1 clinical subtypes |
| Efficacy against RS transmitted/founder (T/F) viruses |
| Efficacy against X4- and R5/X4-HIV-1 viruses |
| Therapeutic index (TI = EC_{50}/CC_{50}) |

**Endpoint**: If TI > 10 → Secondary assays will be initiated; if TI < 10 → Evaluation of API is terminated

| Stage 2: Secondary assays |
|---------------------------|
| Mechanistic assays |
| Entry inhibition assays: viral inactivation (virucidal), cell protection, entry, attachment, fusion |
| Enzyme inhibition assays: retro-transcriptase, integrase, protease |
| Cell-free and cell-associated virus transmission assays |
| In silico computational methods |
| Transwell dual-chamber and EpIVaginal tissue assays |
| Efficacy with pH transition assays |
| Efficacy in the presence of authentic biological fluids (cervicovaginal, rectal, seminal, seminal plasma) |

**Endpoint**: The most highly ranked API from secondary assays are evaluated in specialized assays

| Stage 3: Specialized assays |
|-----------------------------|
| Efficacy against resistant viruses and HIV reactivation |
| Innate immune system assays |
| Lactobacillus growth inhibition assays |
| Efficacy against other STIs: HIV-2, HSV-2, HPV, hepatitis viruses and other pathogens |
| Spermicidal assays |
| Combination assays |

**Endpoint**: The most highly ranked API from specialized assays are evaluated in Ex vivo assays

| Stage 4: Ex vivo assays |
|------------------------|
| Non-polarized cervicovaginal tissue explants |
| Polarized cervicovaginal tissue explants |
| Colorectal explants |

**Endpoint**: The most highly ranked API from Ex vivo assays are evaluated in In vivo assays

| Stage 5: In vivo assays |
|------------------------|
| General and local toxicological studies: rodents, rabbits |
| HIV-1 challenge |
| Nonhuman primates |
| Humanized mice |
| Pharmacokinetic/pharmacodynamic: absorption, distribution, metabolism, and excretion (ADME) |
| Biodistribution assays |
| Other optional studies: |
| Mutagenicity |
| Reproductive toxicity |
| Carcinogenicity |
| Physical barrier material assays (effect on integrity of condoms) |

**Endpoint**: The most highly ranked API from In vivo assays are evaluated in formulation assays

| Stage 6: Formulation assays |
|-----------------------------|
| Development of dosage forms |
| Semi-solids: gels, creams, ointments |
| Solids: suppositories, tablets, films, rings |
| Physical-chemical characterization: |
| Compatibility with excipients and other drugs |
| Long-term stability |
| Solubility |
| Degradation assays: pH, heat, light, oxidation |

**Endpoint**: The most highly ranked API and the best formulation are evaluated in clinical trials
3 | NANOTECHNOLOGY AND NANOMEDICINE: THE REVOLUTION

Nanotechnology is a multidisciplinary field defined as the intentional design, synthesis, characterization, and applications of materials and devices by controlling their size and shape with length scales in the 1–100 nm range (Bayda et al., 2019; Patra et al., 2018). Nanotechnology is considered an emerging, exponential, and cutting-edge technology with enormous potential in medical applications. Its application to medicine (nanomedicine) takes advantage of that nanomaterials are similar in size to biological structures to open up a wide field of research for the diagnosis and treatment of diseases (Soares et al., 2018), such as cancer (Bahreyni et al., 2020; Khot et al., 2020), Alzheimer (Binda et al., 2020; Zhang et al., 2020) and other neurological disorders (Sim et al., 2020), HIV infection (Macchione et al., 2020; Roy et al., 2015), pulmonary diseases (Doroudian et al., 2020), and currently against COVID-19 (Heinrich et al., 2020; Witika et al., 2020). Examples of nanomaterials used in medicine include liposomes, polymeric micelles, nanoparticles, quantum dots, nanogels, superparamagnetic iron oxide crystals, dendritic structures, carbon nanotubes, and nanoshells (Marchesan & Prato, 2013). Dendritic structures include mainly hyperbranched polymers, dendrigraft polymers, dendrons, and dendrimers (Carlmark et al., 2009; Ma et al., 2016). In particular, the interest in dendrimers has continuously increased over time as promising candidates for many applications in nanomedicine (Malkoch & García-Gallego, 2020).

4 | DENDRIMERS: WHAT AND WHY?

Dendrimers are hyper-branched, well-organized, and nano-sized molecules with a tree-like structure that present a central core, interior layers with repeated units (so-called generations), and functional groups at the periphery (Svenson & Tomalia, 2005; Figure 3a). The size of dendrimers is reached by a controlled synthesis of a stepwise growth of generations, contrary to what happens with classical polymerization processes (Dias et al., 2020). The synthesis of dendrimers can follow either a divergent or convergent approach (Abbasi et al., 2014; Sandoval-Yanez & Castro Rodriguez, 2020). The divergent method comprises the growth of the dendrimer originated from a core outward. This process involves the activation of groups at the periphery and the addition of branching units and is repeated until the desired dendrimer size (or generation) is reached (Mekelburger et al., 1992; Newkome et al., 1985; Newkome et al., 1994; Tomalia et al., 1985) (Figure 3b). The convergent method comprises dendrons’ synthesis through iterative reactions coupling to a multifunctional central core in the last synthesis step (Hawker & Frechet, 1992; Figure 3c). Other less explored strategies for dendrimer synthesis have been reported, such as double exponential growth (Kawaguchi et al., 1995), hypercores and branched monomers growth (Wooley et al., 1991), orthogonal coupling or lego chemistry (Grande et al., 2014; Maraval et al., 2003), click chemistry (Anandan et al., 2019; Anandkumar & Rajakumar, 2017; Deraedt et al., 2015; Juárez-Chávez et al., 2019; Lowe, 2014), and onion peel approach (Bagul et al., 2017; Sharma et al., 2015).

The diameter of dendrimers increases linearly with the generation, reaching sizes averaging 1–20 nm (Kaminskas et al., 2011; Svenson & Tomalia, 2005). An important characteristic that does not follow a fixed rule among researchers in this field is defining the dendrimer’s size. The unquestionable fact is that generations define dendrimers’ size, and the most used nomenclature refers to when each generation corresponds to a layer of branching units (Sepulveda-Crespo, Cena-Diez, et al., 2017; Sepulveda-Crespo, Gomez, et al., 2015). Dendrimers constitute a versatile platform with several intrinsic properties, including nanoscale size and shape, monodispersity (well-defined molecular structure), multivalency (high density of functional end-groups provides many simultaneous interactions with biological membranes and drugs), high biocompatibility, high aqueous and nonpolar solubility, high reactivity, and structural flexibility (core compositions can vary). The main advantages/drawbacks from the synthesis process, physicochemical characterization, and other specific properties from dendrimers have been widely reported and described in detail in excellent seminal reviews (Chis et al., 2020; Dias et al., 2020; Mishra, 2011; Nanjwade et al., 2009; Palmerston Mendes et al., 2017; Sepulveda-Crespo, Cena-Diez, et al., 2017; Sepulveda-Crespo, Gomez, et al., 2015).

Dendrimers offer unique opportunities as biological agents, especially as carriers for chemical drugs or peptides (Hu et al., 2020; Sepulveda-Crespo et al., 2016; Shcharbin et al., 2020), as powerful tool for gene silencing (Dong et al., 2020; Ellert-Miklaszewskia et al., 2019), as antibacterial agents (Abd-El-Aziz et al., 2020; Alfei & Schito, 2020; Mlynarczyk et al., 2020; Sanz Del Olmo et al., 2020), as well as to have intrinsic antiviral activities, such as against HIV-1 (Cena-Diez et al., 2017; Guerrero-Beltran et al., 2019; Rodriguez-Izquierdo et al., 2019; Sepulveda-Crespo, Serramia, et al., 2015; Vacas-Cordoba et al., 2016), HIV-2 (Briz et al., 2015), HSV-2 (Cena-Diez, Vacas-Cordoba, et al., 2016; Guerrero-Beltran et al., 2020; Rodriguez-Izquierdo et al., 2020), HCV (Javadi et al., 2019; San Anselmo et al., 2020; Sepulveda-Crespo,
Jimenez, et al., 2017, HPV (Donalisio et al., 2010), influenza virus (Farabi et al., 2020; Hatano et al., 2014), respiratory syncytial virus (RSV) (Gazumyan et al., 2000), or Middle East respiratory syndrome coronavirus (MERS-CoV) (Kandeel et al., 2020).

For anti-HIV microbicide applications, dendrimers must be nontoxic, nonimmunogenic (unless used in vaccine development), cross biological membranes, stay in tissue or blood circulation during long periods, and reach specific targets (Dias et al., 2020; Nanjwade et al., 2009). The efficacy, membrane permeability, hemolytic and cytotoxic effects of dendrimers depend on their size, core chemistry, and mainly type and charge of the functional groups at the periphery (Duncan & Izzo, 2005). Cationic dendrimers have been shown to interact with the negative charge of biological membranes, causing membrane disintegration, loss of cytoplasmic proteins, cell destabilization, and cell lysis (Duncan & Izzo, 2005; K. Jain et al., 2010; Labieniec-Watala & Watala, 2015; Madaan et al., 2014). Thus, there are many strategies to reduce or mask dendrimers’ charges and overcome these drawbacks for delivery purposes (Janaszewska et al., 2019). Other reports have shown that anionic dendrimers are less toxic than cationic dendrimers (Janaszewska et al., 2019; Jevprasesphant et al., 2003). In addition to the overall advantages of dendrimers, anionic structures have more excellent...
stability than cationic dendrimers and mimic biological receptors or cofactors, constituting ‘authentic’ molecular decoys. In the context of viral infections, anionic dendrimers act as entry inhibitors, blocking HIV replication. These dendrimers nonspecifically interact with HIV envelope proteins and other STIs, preventing electrostatic recognition of the target cell in the early stages of infection. Moreover, they can act against the cationic residues of cell receptors, modifying their structure and avoiding an effective interaction with viruses. In addition to having an antiviral activity per se, anionic dendrimers can trap drugs by encapsulation or charge interactions and deliver drugs within the cell, improving intracellular traffic (Sepulveda-Crespo, Cena-Diez, et al., 2017; Sepulveda-Crespo, Gomez, et al., 2015). Therefore, these dendrimers are excellent molecules as efficient anti-HIV microbicides (Cena-Diez et al., 2019; Maciel et al., 2019; Nandy et al., 2015; Sepulveda-Crespo, Serramia, et al., 2015).

The main dendrimers designed as anti-HIV microbicides and their characteristics (generations, central core, number, and functional end-groups) have been described previously (Sepulveda-Crespo, Cena-Diez, et al., 2017; Sepulveda-Crespo, Gomez, et al., 2015). Amphiphilic dendrimers (S. Han et al., 2012), Boltorn hyperbranched dendritic polymers (Berzi et al., 2012), carboxilane dendrimers (Relano-Rodriguez & Munoz-Fernandez, 2020), glycodendrimers (Ordanini et al., 2016), metalldendrimers (Garcia-Gallego et al., 2015), peptide dendrimers (Bon et al., 2013), phosphorus-containing (PPH) dendrimers (Perez-Apes et al., 2009), poly(alkylideneamine) (PAA) dendrimers (Maciel et al., 2019), poly(amideamine) (PAMAM) dendrimers (Witvrouw et al., 2000), poly(propylene imine) (PPI) dendrimers (Rosa Borges et al., 2010), and poly(ε-lysine) (PLL) dendrimer (Telwatte et al., 2011) are the structures most commonly used as anti-HIV microbicides. The API concentration that results in 50% inhibition in the viral particles are recorded into the viral particles are β-galactosidase (Bartolo et al., 2015), secreted alkaline phosphatase, or chloramphenicol acetyl transferase (Soezi et al., 2015). MT-2, MT4, or CEM-SS cells are also highly permissive for HIV-1 by developing HIV-induced syncytia that can be evaluated (Busso et al., 1991; Fernandez et al., 2019; Nara & Fischinger, 1988; Szucs et al., 1988).

Subsequent evaluations must be conducted using several laboratory-adapted R5-HIV-1 isolates and the most geographically prevalent HIV-1 clinical subtypes (A, B, and C). It is also essential to use R5 transmitted/founder (T/F) viruses because around 80% of heterosexual transmissions are established from this single HIV-1 variant (Joseph et al., 2015). Currently, a collection of full-length T/F HIV-1 infectious molecular clones are available (Parrish et al., 2013). Once primary infection occurs (R5-HIV-1), X4-HIV viruses emerge late in the infection, and both HIV-1 variants are present in sexual fluids. Therefore, API must be active against laboratory-adapted and clinical viruses with different tropism, CXCR4, and dual (R5/X4)-tropic viruses.

6 | IN VITRO PRECLINICAL STUDIES

6.1 | Screening: selecting the best model

To optimize the in vitro algorithm with many API with potential as topical microbicides, the cell line(s) and HIV-1 variant(s) must be carefully selected.

Initial screening with a large number of API must be performed using permissive cells to HIV-1 infection: epithelial cell lines from the human vagina (Vk2/E6E7), ectocervix (Ect1/E6E7), endocervix (End1/E6E7), uterus (HEC-1A, SiHa, CaSki) (Gali, Delezay, et al., 2010) or rectum (Caco-2, HT-29, SW1463) (Mukhopadhyya et al., 2016), peripheral blood mononuclear cells (PBMCs), CD4+ T lymphocytes and cells from mononuclear phagocyte lineage (monocytes, macrophages [MØs], and DCs; Coutinho et al., 2017). As R5-HIV-1 viruses predominate during the early stages of HIV sexual transmission, primary and established human cells that express CCR5 co-receptor should be used. TZM.bl reporter cell-based assay with a readout of HIV-1 infection through luminescence is considered the gold-standard assay (Roan & Munch, 2015). Another single-round infectivity assay with the enhanced green fluorescent protein (GFP) gene is used. The infectivity is measured by quantifying the number of GFP expressing cells by flow cytometry (Relano-Rodriguez et al., 2019). Other single-round infection systems with reporter genes incorporated into the viral particles are β-galactosidase (Bartolo et al., 2018), secreted alkaline phosphatase, or chloramphenicol acetyl transferase (Soezi et al., 2015). MT-2, MT4, or CEM-SS cells are also highly permissive for HIV-1 by developing HIV-induced syncytia that can be evaluated (Busso et al., 1991; Fernandez et al., 2019; Nara & Fischinger, 1988; Szucs et al., 1988).
6.2 Primary assays: cytotoxicity and efficacy

It is crucial to define the working concentration of API that can suppress HIV transmission in cell culture. Several standard assays are available to measure metabolic markers that estimate the number of viable cells in culture: tetrazolium reduction (MTT, MTS, XTT, or WST-1), resazurin reduction, cell-permeable protease detection, ATP detection, or bioluminescent nonlytic real-time detection (Riss et al., 2004). Possible highly active API can be not toxic to mitochondria with these colorimetric cell viability assays but could affect other enzymatic pathways. Therefore, additional Vybrant and Live/Dead or 7-aminoactinomycin D (7-AAD) cytotoxicity assays to eliminate false active API should be performed (C. S. Lackman-Smith et al., 2010; Lecoeur et al., 2001). Vybrant and Live/Dead assay measures plasma membrane integrity and intracellular esterase activity, whereas 7-AAD penetrates the damaged cell membranes, stains the DNA, and can be measured by flow cytometry.

Primary assays include a measurement of viability (CC50; cytotoxic concentration of API that causes the reduction of the viable cells by 50%) and efficacy (EC50) with appropriate viruses and cells to generate the therapeutic index (TI). The TI is the ratio between API amount that causes toxicity and therapeutic effect (TI = CC50/EC50). API evaluation is concluded if TI fails to meet the cut-off TI > 10 (Muller & Milton, 2012). EC90 (90% inhibition) can also be calculated to select a rational dose for pharmacokinetic/pharmacodynamic assays.

6.3 Secondary assays

APIs identified as the most effective and not toxic in the initial screening are selected to evaluate their unknown mechanism of anti-HIV-1 action. Mechanistic assays should be included, such as virucidal activity, entry, attachment, fusion, reverse transcription, integration, proteolytic processing, maturation, and/or budding. In silico computational methods, including molecular docking, molecular dynamics, and free energy calculations, should also be performed (Guerrero-Beltran et al., 2018; Nandy et al., 2015). Cell lines enabling multi-round infections like activated PBMCs isolated from healthy individuals are required to evaluate the activity of API targeting late stages of replication. Other cells supporting HIV-1 infection, such as U373-MAGI-CCR5E cells, B-THP-1/DC-SIGN cells, H9 cells, A3R5 cells, and C8166 cells, can also be used to evaluate the activity of API. Focal immunoassay, quantification of viral antigen (p24), or reverse transcriptase activity are used to titrate HIV-1 (McMahon et al., 2009). Cervicovaginal epithelial cells do not express CD4 and CCR5/CXCR4 receptors. However, the prevalent infectious forms of HIV-1 present in semen and cervicovaginal secretions can cross the epithelium and infect the target cells. Since cell-associated HIV-1 transmission is more efficient than the cell-free virus, cell-associated HIV-1 transmission inhibition assays, including CD4-dependent and CD4-independent assays, should be performed (Gupta et al., 2013). For further studies of cell-free and cell-associated HIV-1 transmission, a dual-chamber culture system as a sensitive model of a tight epithelial monolayer mimicking human vagina epithelium is used. The model evaluates API permeability and transport across the epithelial cell monolayer by measuring transepithelial electrical resistance. In this assay, API must cross the monolayer to the lower tissue culture chamber with PBMCs protecting against HIV-1 infection (Gali, Arien, et al., 2010; Mesquita et al., 2009). The organotypic 3D EpiVaginal tissue model (Cena-Diez et al., 2017; Ugaonkar et al., 2015) is a validated in vitro method used to evaluate the toxicity, absorption, and permeability of API into and through the tissue. Vaginal organoids can be probably used soon (just as it happens with other organs [Lancaster & Huch, 2019; Simian & Bissell, 2017; Takebe & Wells, 2019]) to mimic structural, functional and dynamic characteristics of vagina, to test anti-HIV-1 drugs and their interactions with stem cells, and even so to research vaginal microbiome (Ali, Syed, Jamaluddin, et al., 2020; Ali, Syed, & Tanwar, 2020).

The healthy female genital tract’s pH is acidic (pH: 4.0–6.0), whereas the pH of normal semen is slightly alkaline (pH: 7.2–8.0). During heterosexual transmission, the acidic vaginal pH is neutralized by semen, providing an environment that facilitates bacterial vaginosis and yeast infections (Sabatte et al., 2011). On the other hand, a study has shown that the presence of semen reduces the sensitivity of HIV by diverse microbicides (Zirafi et al., 2014). Therefore, it is critically important to verify the biological activity of API over a wide range of pH and in the presence of natural simulated semen/semenal plasma (final concentration of 12.5–25% [Cena-Diez, Garcia-Broncano, et al., 2016; Garcia-Broncano et al., 2017]), rectal and cervicovaginal fluids to mimic the effects of the introduction of semen to the vagina. Since the microbicide will be applied before HIV-1 infection and heterogeneity between biological samples, adjusting the viral load or diluting the biological matrix material is needed.
6.4 Specialized assays

A microbicide should also be effective against several resistant HIV-1 viruses, not interfere with normal flora in the vagina or immune system, and prevent other STIs.

Resistant viruses pre-existing in the vaginal/rectal fluids or tissues can cross the microbicidal barrier due to the presence of defined mutations that causes a loss of antiviral susceptibility. In this sense, assays to evaluate API’s antiviral activity against resistant and multidrug-resistant viruses should be included: viruses are passaged in the presence of increasing concentrations of API, with a starting concentration that allows virus replication at a low level (Arien et al., 2016; Rodriguez-Izquierdo et al., 2019). On the other hand, latently infected resting CD4+ T cells are the most source of HIV-1 reactivation, leading to a rebound of the viral load if treatment is interrupted. Moreover, the non-suppressive regimens can provide the emergence of drug-resistance mutations, being the major obstacle to HIV-1 eradication. Therefore, “shock and kill” assays to increase HIV-1 reactivation and that, at the same time, avoid new HIV-1 infections should be performed (Relano-Rodriguez et al., 2019).

The innate immune system constitutes the first defense against viral infection, especially after disrupting the mucosal epithelium during sexual intercourse. The cervicovaginal epithelial cells, DCs, MØs, and lymphocytes are vital in early HIV recognition. Therefore, several assays evaluating cytokines, toll-like receptor (TLR) expression, and differentiation, maturation, and activation of the primary immune cells localized in the vaginal mucosa should be performed (Martin-Moreno et al., 2019). Given the importance of maintaining the normal flora in the vagina and its low pH through lactic acid production, it is essential to determine the impact of API on the microbiome (Donahue Carlson et al., 2017; Klatt et al., 2017; Taneva et al., 2018) and the efficient growth H2O2-producing Lactobacilli sp. (Petrova et al., 2013). It is also essential to evaluate API’s ability to inhibit other STIs, such as HIV-2, HSV-2, HPV, hepatitis viruses, and other pathogens associated with vaginosis or candidiasis (chlamydia, gonorrhea, or syphilis; Briz et al., 2015; Cena-Diez, Vacas-Cordoba, et al., 2016; Guerrero-Beltran et al., 2017; Sepulveda-Crespo, Jimenez, et al., 2017; Telwatte et al., 2011).

The API activity may extend as a spermicidal candidate because it would provide a women-controlled method to prevent or delay unwanted pregnancies. The spermicidal potential of API should be evaluated through some parameters, such as the minimum effective spermicidal concentration, sperm motility and viability, sperm membrane permeability and intracellular pH, induction of sperm cell apoptosis, changes at sperm membrane and sperm mitochondrial transmembrane, and topographical imaging of membrane domains over the sperm head (Cena-Diez et al., 2019; Chakraborty et al., 2014; R. K. Jain et al., 2010).

Preclinical microbicide development also includes combination prevention strategies. Drugs must be combined in a correct molar ratio to avoid chemical incompatibilities, drug–drug interactions, or competitiveness on the same target. Combination assays enhance efficacy with lower concentrations, decrease toxicity and underlying side effects, and reduce the probability or delay of the development of resistance. It is essential to evaluate synergistic, additive, or antagonistic properties of the combination assays using any software packages: CalcuSyn, Compusyn, MacSynergy II, MixLow R package, ... (Foucquier & Guedj, 2015). At this point, API identified as highly active with a defined mechanism of action are advanced for further ex vivo assays.

6.5 Summary of in vitro assays performed with dendrimers

The first conclusion is that cytotoxicity and efficacy assays were the first and most repeated studies (see Table 1). A few dendrimers were not evaluated for toxicity or presented in the manuscript (Clayton et al., 2011; Domenech et al., 2010; Garcia-Vallejo et al., 2013; Morales-Serna et al., 2010). Few cell lines in most cytotoxicity studies were used. A single cellular model was used in most cases, and the best models were not selected according to the guidelines discussed in this manuscript. It may be due to either unavailability in labs or the aim to obtain preliminary results to optimize other more potent dendrimers. Ideally, preliminary studies should select vaginal (or rectal) epithelial cell lines and a primary cell model or an immortalized cell line that resembles this cell type (Chonco et al., 2012; Dezzutti et al., 2004; Sanchez-Rodriguez, Diaz, et al., 2015). Efficacy tests were aimed at inhibition assays against different HIV-1 isolates or binding and kinetic studies in the presence of recombinant proteins. Overall, very few studies showed the values of CC50 and EC50, and consequently TI values. This parameter must be mandatory for any research that seeks to demonstrate the anti-HIV activity of dendrimers (and any other drug candidates).
**TABLE 1** Primary in vitro assays performed with dendrimers as vaginal microbicides against HIV-1

| In vitro assays | Cytotoxicity | Efficacy against R5-transmitted/RS-HIV-1 | Efficacy against X4-HIV-1 and/or R5/X4-HIV-1 | Transwell dual-chamber/EpiVaginal tissue assays | In silico computational assays | Mechanistic assays | Efficacy in the presence of authentic biological fluids | Efficacy against resistant viruses/HIV-1 reactivation | Efficacy against other STIs | Spermicidal assays | Innate immune system assays | Vaginal normal flora assays | Combination assays | References |
|----------------|-------------|------------------------------------------|---------------------------------------------|-----------------------------------------------|---------------------------------|-------------------|------------------------------------------------|------------------------------------------------|---------------------------|----------------|-----------------------------|-----------------------------|----------------|------------------|
| BH30muMan      | Boltron     |                                          |                                              |                                               |                                 |                   |                                                 |                                                 |                           |                |                             |                             |                | (Arce et al., 2003; Tabarani et al., 2006) |
| Dendron12      | Boltron     |                                          |                                              |                                               |                                 |                   |                                                 |                                                 |                           |                |                             |                             |                | (Berzi et al., 2012; Sattin et al., 2010) |
| BH30PSGal      | Boltron     |                                          |                                              |                                               |                                 |                   |                                                 |                                                 |                           |                |                             |                             |                | (Morales-Serna et al., 2010) |
| G2-S16         | Carbosilane |                                          |                                              |                                               |                                 |                   |                                                 |                                                 |                           |                |                             |                             |                | (Arnáiz et al., 2014; Cena-Diez, Garcia-Broncano et al., 2016; Cena-Diez et al., 2017; Cena-Diez et al., 2019; Chonco et al., 2012; Guerrero-Beltran et al., 2018; Gutierrez-Ulloa et al., 2020; Jaramillo-Ruiz et al., 2016; Martin-Moreno et al., 2019; Relano-Rodriguez et al., 2019; Relano-Rodriguez & Munoz Fernandez, 2020; Rodriguez-Izquierdo et al., 2020; Sepulveda-Crespo Jimenez et al., 2014; Sepulveda-Crespo et al., 2017; SEPULVEDA-CRESPO ET AL., 2017; SEPULVEDA-CRESPO ET AL., 2014) |
| Dendrimer Classification | In vitro assays | Cytotoxicity | Efficacy against R5 transmitted/ founder | Efficacy against R5 X4 HIV-1 and/or | Transwell dual-chamber/ EpiVaginal tissue assays | Efficacy in the presence of authentic biological fluids | Efficacy against HIV-1 resistant viruses/ HIV-1 reactivation | Efficacy against other STIs | Spemcidal system assays | Innate immune system assays | Vaginal normal flora assays | Combination assays | References |
|--------------------------|----------------|-------------|----------------------------------------|-----------------------------------|-----------------------------------------------|-------------------------------------------------|-------------------------------------------------|------------------|------------------|------------------|------------------|------------------|-------------------|
| G2-STE16 Carbosilane     |                |             |                                        |                                   |                                               |                                                 |                                                 |                  |                  |                  |                  |                  |                   |
|                          |                |             |                                        |                                   |                                               |                                                 |                                                 |                  |                  |                  |                  |                  | Sepulveda-Crespo, Sanchez-Rodriguez, et al., 2015; Sepulveda-Crespo, Serramí, et al., 2015 |
| G2-CTE16 Carbosilane     |                |             |                                        |                                   |                                               |                                                 |                                                 |                  |                  |                  |                  |                  |                   |
|                          |                |             |                                        |                                   |                                               |                                                 |                                                 |                  |                  |                  |                  |                  | Galán et al., 2014; Sanchez-Rodriguez, Diaz, et al., 2015; Sepulveda-Crespo, Jimenez, et al., 2017; Sepulveda-Crespo, Sanchez-Rodriguez, et al., 2015 |
| G1-NS16 Carbosilane      |                |             |                                        |                                   |                                               |                                                 |                                                 |                  |                  |                  |                  |                  |                   |
|                          |                |             |                                        |                                   |                                               |                                                 |                                                 |                  |                  |                  |                  |                  | Arnáz et al., 2014; Briz et al., 2015; Cordoba et al., 2013; Vacas-Cordoba et al., 2014; Vacas-Cordoba et al., 2016; Vacas Cordoba et al., 2013 |
| Dendrimer          | Classification | In vitro assays | Cytotoxicity | Efficacy against R5-transmitted/ founder HIV-1 | Efficacy against X4-HIV-1 and/or R5/X4 HIV-1 | Mechanistic assays | Transwell dual-chamber/ Epivaginal tissue assays | Efficacy in the presence of authentic biological fluids | Efficacy against HIV-1 resistant viruses/ HIV-1 reactivation | Efficacy against other STIs | Spemmicidal system assays | Vaginal normal flora assays | Combination assays |
|-------------------|----------------|----------------|--------------|-----------------------------------------------|---------------------------------------------|--------------------|-----------------------------------------------|------------------------------------------------|------------------------------------------------|-------------------------|-------------------------|--------------------------|-------------------------|
| G2-Sh16           | Carbosilane    |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| G0-Sh4            | Carbosilane    |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| G2-S24P           | Carbosilane    |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| G2-S8T            | Carbosilane    |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| G2-C18PEG         | CPEGC          |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| NPAg-G2-C18PEG    | CPEGC          |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| [G1]-CO2Na        | GATG           |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| Bol13.4           | Glycodendrimer |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |
| ROD3.5.6          | Glycodendrimer |                |              |                                               |                                             |                    |                                               |                                                 |                                                 |                         |                         |                         |                         |

References:
- Arnbiz et al., 2014;
- Cordoba et al., 2013;
- Herrera-Broncano et al., 2017;
- Relano-Rodriguez et al., 2019; Vacas Cordoba et al., 2013, 2014, 2016.
- Cena-Diaz et al., 2019;
- Guerrero-Beltran et al., 2018;
- Relano-Rodriguez et al., 2019.
- Sepulveda-Crespo et al., 2018;
- Sepulveda-Crespo, Jimenez, et al., 2017;
- Sepulveda-Crespo et al., 2014;
- Sepulveda-Crespo, Sanchez-Rodriguez, et al., 2015.
- Varga et al., 2014.
| Dendrimer                  | Classification       | Cytotoxicity   | Efficacy against R5-transmitted/ foundered HIV-1 | Efficacy against X4-HIV-1 and/or R5/X4-HIV-1 | Transwell dual-chamber/ EpiVaginal tissue assays | Efficacy in the presence of authentic biological fluids | Efficacy against HIV-1 resistant viruses/ HIV-1 reactivation | Efficacy against other STIs | Spermicidal system assays | Innate immune system assays | Vaginal normal flora assays | Combination assays | References                  |
|---------------------------|----------------------|---------------|-------------------------------------------------|---------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------------------------|-----------------------------------------------------------------|
| ROD3.7.6                  | Glycodendrimer       |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Ordanini et al., 2015; Ordanini et al., 2016)                     |
| G1-C8                     | PAA                  |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Maciel et al., 2019)                                               |
| G1-S8                     | PAA                  |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Maciel et al., 2019)                                               |
| Lewis<sup>5</sup> -PAMAM  | PAMAM                |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Garcia-Vallejo et al., 2013)                                      |
| Sulfo-6                   | PAMAM                |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Clayton et al., 2011)                                              |
| SPL2923                   | PAMAM                |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Hanson et al., 2005; Witvrouw et al., 2000)                       |
| SPL6195                   | PAMAM                |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Hanson et al., 2005; Witvrouw et al., 2000)                       |
| SPL7304                   | PAMAM                |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (McCarthy et al., 2005)                                             |
| SB105-A10                 | Peptide dendrimer    |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Donalsko et al., 2010; Lagannini et al., 2011)                   |
| Trp(5a-5f) and modifications | Peptide dendrimer   |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Martinez-Gualda et al., 2017, 2019, 2020, Rivero-Buceta et al., 2015) |
| SCSLD3                    | PLL                  |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (S. Han et al., 2012)                                               |
| PLLDG3-PSCel              | PLL                  |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Shuqin Han et al., 2010)                                           |
| SPL7013                   | PLL                  |               |                                                 |                                             |                                                 |                                                 |                                                 |                             |                             |                             |                             |                             | (Barnes et al., 2008; Dezzutti)                                    | (Continues)
| Dendrimer       | Classification | References                                                                 |
|-----------------|----------------|----------------------------------------------------------------------------|
| SPL7115         | PLL            | Tyssen et al., 2010 (Tyssen et al., 2010)                                 |
| PPH-3d-G1       | PPH            | Perez-Anes et al., 2010 (Perez-Anes et al., 2010)                         |
| PPH-Sc-Gc’1     | PPH            | Perez-Anes et al., 2009 (Perez-Anes et al., 2009)                         |
| PSGal64mer      | PPI            | Kensinger, Catalone, et al., 2004; Kensinger, Yowler, et al., 2004 (Kensinger, Catalone, et al., 2004; Kensinger, Yowler, et al., 2004) |
| MVC-G8T         | PPI            | Rosa Borges et al., 2010 (Rosa Borges et al., 2010)                       |
| MVC-3SL         | PPI            | Rosa Borges et al., 2010 (Rosa Borges et al., 2010)                       |
| SPL7320         | PPI            | McCarthy et al., 2005 (McCarthy et al., 2005)                             |
| Metallodendrimer G2S | PPI          | Garcia-Gallego et al., 2015 (Garcia-Gallego et al., 2015)                 |
| Metallodendrimer G2C | PPI          | Garcia-Gallego et al., 2015 (Garcia-Gallego et al., 2015)                 |
| Viol36          | Viologen       | Asaftei et al., 2012; Asaftei & De Clercq, 2010 (Asaftei et al., 2012; Asaftei & De Clercq, 2010) |
| Dendrimer | Classification | Cytotoxicity | Efficacy against R5 transmitted/ founder HIV-1 | Efficacy against X4-HIV-1 and/or R5/X4-HIV-1 | Transwell dual-chamber/ EpiVaginal tissue assays | Efficacy in the presence of authentic biological fluids | Efficacy against HIV-1 resistant viruses/ HIV-1 reactivation | Efficacy against other STIs | Spermicidal assays | Innate immune system assays | Vaginal normal flora assays | Combination assays | References |
|-----------|----------------|-------------|-----------------------------------------------|-------------------------------------------|-----------------------------------------------|------------------------------------------------|------------------------------------------------|--------------------------------|------------------|---------------------------|------------------|----------------|----------------|
| Viol7     | Viologen       | ✓           | [Green shading]                               | [Gray shading]                            | [Gray shading]                                | [Gray shading]                                  | [Gray shading]                                  | [Gray shading]                                  | [Gray shading]                  | [Gray shading]                  | [Gray shading]                  | [Gray shading]                  | (Asaftei & De Clercq, 2010; Asaftei et al., 2012) |

Note: The assays carried out are represented with a green shading; not carried out with a gray shading.

Abbreviations: CPEGC, citric acid-polyethylene glycol-citric acid; GATG, gallic acid-triethylene glycol; PAA, poly(alkylideneamine); PAMAM, poly(amidoamine); PLL, poly(L-lysine); PPH, phosphorus-containing dendrimers; PPI, poly(propylene imine).
Another aspect to be taken into account are studies to elucidate the mechanism of action of dendrimers. All dendrimers inhibit HIV-1 infection, particularly at the viral entry-level. Some studies have performed additional assays and inhibited HIV-1 entry, retro-transcriptase, and integration processes (McCarthy et al., 2005; Witvrouw et al., 2000) or inhibited dimerization of HIV-1 capsid protein (Domenech et al., 2010). It is possible to elucidate the potential binding sites of dendrimers to virus proteins or cell receptors, thus preventing the virus's binding from hosting cells and subsequent infection with computational models (Guerrero-Beltran et al., 2018; Nandy et al., 2015; Sanchez-Rodriguez, Diaz, et al., 2015).

There are a few dendrimers that continue to advance in in vitro assays to reach clinical trials. Those that do not advance are either because the expected results are not obtained or because the authors prefer to optimize the synthesis of other potential dendrimers, as is the case of tryptophan dendrimers (Martinez-Gualda et al., 2017, 2019, 2020; Rivero-Buceta et al., 2015). The importance of conducting further assays has been demonstrated over the years, especially with efficacy studies in the presence of cervicovaginal fluids or semen or innate immunity tasks. The most advanced dendrimers are G2-STE16, G2Sh16, and SPL7013, although none reaches the projection of G2-S16 dendrimer with all the in vitro assays of the algorithm presented in this manuscript and very encouraging results. SPL7013 showed excellent results in each of the in vitro assays carried out. Still, some crucial studies are lacking, such as testing its efficacy against several resistant viruses, T/F viruses, and demonstrating that SPL7013 does not alter innate immunity.

7 | EX VIVO PRECLINICAL STUDIES

Due to the sexual transmission being the primary mode of HIV-1 infection, human tissue explants from cervicovaginal and colorectal samples must be incorporated into preclinical protocols to eliminate potential discrepancies with in vitro models (Arien et al., 2012) (see Table 2). Tissue explants models evaluate API’s efficacy against different HIV-1 isolates, toxicity (histology), cytokine changes, and viral evolution, thus understanding the basic mechanisms of HIV-1 transmission, pathogenesis, and antimicrobial factors (Anderson et al., 2010; Dezzutti, 2015; Fernandez-Romero et al., 2015). Inflammation processes and mucosal response to API can be evaluated using polychromatic flow cytometry and multiplex cytokine assays (Merbah et al., 2011; Richardson-Harman et al., 2009).

The system of cervicovaginal tissue is considered the gold standard model for microbicide development. Several explants systems have been described with polarized or nonpolarized exposure of virus and API (Dezzutti, 2015). The nonpolarized tissues are submerged in a medium containing HIV-1, creating a worst-case scenario where other STIs could damage epithelium, such as HSV-2, HPV, other viruses, bacteria, or fungi (Rollenhagen et al., 2014; Tugizov et al., 2013). This model determines the effective API’s concentration, irritation, and the API’s ability to block HIV-1 infection of the immune cells that migrate out from tissue. In the polarized tissues, HIV-1 and API are applied directly to the epithelium, mimicking humans’ transmission. Cell viability and tissue permeability need to be continuously monitored due to this model is deteriorating quickly.

There are other explant models where target cells are activated before HIV-1 infection to start symptoms of inflammation. However, these tissues also deteriorate after 48 h of culture (Cummins Jr. et al., 2007). Some studies use 3D human-made organotypic ectocervical-vaginal tissues to evaluate API toxicity (Ayehunie et al., 2006). However, these models lack the entire epithelial thickness and the correct balance of the immune system’s cells. Therefore, novel models of reconstructed endocervical and ectocervical tissues have been developed (Ayehunie et al., 2006; Bouschbacher et al., 2008; Stoddard et al., 2009).

Cervicovaginal explants are in constant evolution and provide additional information. However, the effective concentration of API may be challenging to determine due to the heterogeneity of mucosal tissue and different HIV-1 replication rates in the mucosal tissue (Dezzutti et al., 2017). These explants show several limitations; thus, new protocols will be needed to overcome these weaknesses and exploit the strengths (see Table 3; Anderson et al., 2010; Dezzutti, 2015; Grivel & Margolis, 2009).

Due to several heterosexual and homosexual transmissions occurring across the colorectal epithelial lining, colorectal explants are used as a model to study API toxicity and efficacy (Ham et al., 2015; Mukhopadhyya et al., 2016; Scott et al., 2016). However, the majority of the problems encountered with cervicovaginal explants apply to colorectal explants. Finally, as in the case of in vitro studies, semen, cervicovaginal secretions, and normal flora must be included to study their API activity effects (Introini et al., 2017; Nahui Palomino et al., 2017).
7.1 Summary of ex vivo assays performed with dendrimers

Of all the dendrimers presented in this manuscript with in vitro potential anti-HIV-1 activity as microbicides, only two were tested for ex vivo activity in explants against HIV-1 (see Table 4).

Dendron 12 inhibited HIV-1 infection of human cervical tissues (Berzi et al., 2012), whereas SPL7013 prevented HIV-1 infection in human cervical explants and human colorectal explant culture (Abner et al., 2005; Cummins Jr. et al., 2007). Not even the most advanced dendrimer in in vitro studies (G2-S16) was tested ex vivo. This intermediate step could save time and costs before going through in vivo studies. However, ex vivo validation has not been mandatory to transfer any candidate for a microbicide to the clinic. On the other hand, there are many varieties of explant models that require the standardization of protocols. The concerns generated by the explants’ limitations and weaknesses, which have not been resolved to date, can be the main reasons for the absence of ex vivo assays with dendrimers.

Moreover, the possible emergence of vaginal organoids and their probable easy maintenance in vitro may rule out ex vivo systems if these drawbacks are not resolved. In this regard, the solution could be to develop an engineered human tissue as a gold standard model to study the inflammatory and inhibitory processes derived from HIV-1 infection. In other words, a single explant model that can recapitulate mucosal cell diversity, immune response, and inter-donor variation (Herrera, 2019).

8 IN VIVO PRECLINICAL STUDIES

Another challenge in preclinical microbicide testing is the employment of a correct animal model. The absence of a validated animal model is the major obstacle for selecting and evaluating API that advances human clinical trials. Before testing in animals or collecting tissue (and cells) from animals, the study must be approved by the Institute Animal Ethics Committee, considering that the protocols for conducting experiments and regulatory requirements vary according to the local governing body. This review discusses the two most commonly used animal models for HIV-1 research: nonhuman primates (NHPs) and humanized mice (h-mice).

8.1 HIV-1 challenge: nonhuman primates

NHPs are the first animal model to study the pathogenesis and transmission of HIV-1. NHPs, particularly Asian macaques, have been widely used for HIV-1 microbicide testing. Their genital tract anatomy, immunology, and physiology show remarkable similarities to human structures (Thippeshappa et al., 2020). NHPs have a high susceptibility to simian immunodeficiency virus (SIV, e.g., SIVmac239, SIVmac251) and engineered simian/human immunodeficiency virus chimeras (SHIV, e.g., SHIV89.6P, SHIV162P3, SHIVKU1, SHIVAD8, SHIVBAL, SHIV1157) capable of replicating and causing macaques’ disease (Hatzioannou et al., 2009; Veazey & Lackner, 2017). The most common and accepted NHPs are the rhesus macaque (Macaca mulatta), the pig-tailed macaque (Macaca nemestrina), and the cynomolgus macaque, also

| Characteristics          | VK2/E6E7 | EpiVaginal | Vaginal explant |
|--------------------------|----------|------------|-----------------|
| Model                    | Cell line| Vaginal    | Tissue          |
| Assay                    | In vitro | In vitro   | Ex vivo         |
| Real conditions          | No       | Closely    | Yes             |
| Maintenance              | Months   | Weeks      | Weeks           |
| Reproducibility          | High     | High       | Variable        |
| Homogeneity              | High     | Lower      | Lower           |
| Tissue structure         | No       | Similar to native human vaginal tissue | Complete |
| Relevance to humans      | Moderate | High       | High            |
| Commerciality            | Yes      | Yes        | No              |

Note: Herein a vaginal cell line (VK2/E6E7), a commercial vaginal model (EpiVaginal), and ex vivo tissue explants are compared.
known as long-tailed or crab-eating macaque (*Macaca fascicularis*) (see Table 5 for analyzing their main characteristic for HIV-1 research).

Rhesus macaques are the most used NHPs for microbicide testing due to their availability. SIV infections result in high viral loads, a progressive loss of CD4$^+$ T cells, and a disease progression rate similar to that observed in HIV-1-infected humans. Rhesus macaques are used in microbicide studies for topical and systemic distribution and to evaluate API efficacy (Calenda et al., 2017; McBride et al., 2017; Zhao et al., 2017). Pig-tailed macaques have a vaginal flora similar to humans and are more susceptible to the same STIs. Viral loads and CD4$^+$ T cell depletion rates are slightly more vulnerable to SIV transmission, and disease progress more rapidly than rhesus macaques. Pig-tailed macaques are commonly used to study immunology, pathogenesis, toxicity, and efficacy of API (Cole et al., 2010; Moss et al., 2012). Cynomolgus macaques are smaller and less expensive than rhesus macaques, but researchers employ them less. The viral load and CD4$^+$ T cell turnover are less pathogenic than those observed in rhesus or pig-tailed macaques. The smaller size of the vagina of cynomolgus macaques makes vaginal biopsies more complex, being only useful for efficacy studies (Bouchemal et al., 2015; Murphy et al., 2014; Murphy et al., 2018).

NHP models have important limitations due to high costs, not being readily available, limited supply for large-scale screening of many API, more complicated for international transport, and the inability to employ HIV-1 and drug-resistant HIV-1 isolates challenge studies (Garcia-Tellez et al., 2016). Therefore, it is unclear whether NHP models precisely predict what would occur in humans, though our knowledge from HIV-1 infection or pathogenesis has, to a large extent, been possible due to macaque investigation models.

### 8.2 HIV-1 challenge: humanized mice (h-mice)

Small-model animals (rodents, rabbits, and cats) can be used to evaluate potential microbicide candidates rapidly. Feline immunodeficiency virus (FIV) served as a surrogate model for HIV-1 infection in humans due to similarities in TABLE 3 Advantages, limitations, and potential solutions to improve HIV-1 transmission research on cultures from human mucosal explants. (Modified from Anderson et al. (2010), Dezzutti (2015), and Grivel and Margolis (2009))

| Advantages | Disadvantages | Possible solutions |
|------------|---------------|--------------------|
| Real tissue architecture | • Lack of physiological variables | • Add the desired parameters, such as semen or cervical mucus |
| Presence of lymphocyte subtypes, dendritic cells, and other immune cells | • Donor variability | • Use tissue as fresh as possible |
| | • Integrity of tissue deteriorates after 3 weeks | • Check cellular status and viability |
| | • Physiological conditions deteriorate in culture | |
| Measurement of HIV-1 infection and replication without activation | • Not mimic in vivo systemic conditions | • Ectocervix must be a more transparent color |
| | • Tissue explants collected using surgical methods must differentiate ectocervix versus endocervix (ectocervix contains fewer HIV-1 target cells) | • Mucus produced by the endocervix could be used for further studies as a delivery method for HIV-1 physiological infection |
| | • Endocervix produces mucus in culture | |
| Presence of the immune system | • Absence of immune cells migration or recruitment | • Implement immune-histological analysis of tissue to identify types of HIV-1 target cells avoiding data interpretation |
| | • Disparate number, localization, or types of HIV-1 target immune cells | |
| Response to exogenous hormones and spectrum of cytokine release | • Donor status of hormones and innate immunity | • Test the natural state of donor tissue |
| | • Evaluation of cytokine and hormone response in tissue could be difficult | • Digest tissue and analyze isolated cells |
| Tissue infection by other sexually transmitted infections | • Absence of microbiome to study bacterial interactions | • Include conditions and protocols for microbiome efficacy |
| Use of antibiotic and controlled parameters | • Amphotericin B inhibits HIV-1 infection | • Use amphotericin-free antibiotic cocktail |
| Dendrimer | Classification | Ex vivo and in vivo assays | Cervicovaginal/colorectal explants | HIV-1 challenge in vivo: NHP | HIV-1 challenge in vivo: humanized mice | Other STIs challenge in vivo | In vivo toxicological studies | Biodistribution: in vivo studies | Other in vivo studies | References |
|-----------|----------------|---------------------------|-------------------------------------|-----------------------------|----------------------------------------|-----------------------------|-------------------------------|-------------------------------|---------------------------|-------------|
| Dendron12 | Bolaoni         |                           |                                     |                             |                                        |                             |                                |                               |                           | (Berzi et al., 2012) |
| G2-S16    | Carbosilane     |                           |                                     |                             |                                        |                             |                                |                               |                           | (Briz et al., 2015; Cena-Diez et al., 2017, 2019; Cena-Diez, Vacas-Cordoba, et al., 2016; Chonco et al., 2012; Guerrero-Beltran et al., 2020; Relano-Rodriguez & Munoz-Fernandez, 2020; Rodriguez-Izquierdo et al., 2020; Sepulveda-Crespo, Serramia, et al., 2015) |
| G2-STE16  | Carbosilane     |                           |                                     |                             |                                        |                             |                                |                               |                           | (Galán et al., 2014; Sanchez-Rodriguez, Diaz, et al., 2015) |
| G2-CTE16  | Carbosilane     |                           |                                     |                             |                                        |                             |                                |                               |                           | (Galán et al., 2014; Sanchez-Rodriguez, Diaz, et al., 2015) |
| G1-NS16   | Carbosilane     |                           |                                     |                             |                                        |                             |                                |                               |                           | (Vacas Cordoba et al., 2013) |
| G2-Sh16   | Carbosilane     |                           |                                     |                             |                                        |                             |                                |                               |                           | (Vacas Cordoba et al., 2013) |
| G0-Sh4    | Carbosilane     |                           |                                     |                             |                                        |                             |                                |                               |                           | (Cena-Diez et al., 2019; Cena-Diez, Vacas-Cordoba, et al., 2016) |
| G1-C8     | PAA             |                           |                                     |                             |                                        |                             |                                |                               |                           | (Maciel et al., 2019) |
| G1-S8     | PAA             |                           |                                     |                             |                                        |                             |                                |                               |                           | (Maciel et al., 2019) |
| SPL7013   | PLL             |                           |                                     |                             |                                        |                             |                                |                               |                           | (Abner et al., 2005; Bernstein et al., 2003; Cummins Jr. et al., 2007; Jiang et al., 2005; McCarthy et al., 2005; Patton et al., 2006) |

Note: The assays carried out are represented with a green shading; not carried out with a gray shading.

Abbreviations: PAA, poly(alkylideneamine); PLL, poly(L-lysine).
the pathogenesis with HIV-1. However, cats are not susceptible to HIV-1. They are not used because FIV lacks specific accessory genes present in HIV-1, uses CD134 rather than CD4 as a primary receptor, and can infect B cells and CD8+ T-cells (Hatziioannou & Evans, 2012). Mice, rats, and rabbits are relatively inexpensive and easy to handle. However, cells from these animals do not provide essential cofactors to support robust HIV-1 replication. Although these small-animal models offer advantages in terms of high reproductive rates, low maintenance costs, and the ability to conduct studies using inbred, genetically identical animals, they are distantly related to humans. Therefore, h-mice are the best small-animal models for HIV/AIDS research and recently to evaluate HIV-1 latent reservoirs and persistence (Deruaz & Tager, 2017; Flerin et al., 2019; Llewellyn et al., 2019; Marsden, 2020; Schmitt & Akkina, 2018). H-mice are genetically immunocompromised mice transplanted with human hematopoietic stem cells (HSCs), lymphoid tissue, or peripheral blood lymphocytes cells obtaining an identity, metabolic system, and functionality similar to humans (Ibeh et al., 2016; Marsden & Zack, 2017; Masse-Ranson et al., 2018; Victor Garcia, 2016; Weichseldorfer et al., 2020). Several h-mice models include the severe combined immunodeficiency (scid) mice with adult human peripheral blood lymphocytes (hu-PBLs) or human fetal thymus-liver tissue (hu-Thy/Liv), the human RAG (hu-HSC), and the bone marrow-liver-thymus (BLT) models. Hu-PBLs-SCID and hu-Thy/Liv-SCID mice models can be infected with HIV-1 by intraperitoneal injection or into the human implants to assess the efficacy of drugs and monoclonal antibodies against HIV-1 infection (Safrit et al., 1993; Stoddart et al., 2014; Yoshida et al., 2003). However, these mice models cannot be used for studying mucosal transmission (Hatziioannou & Evans, 2012).

H-BLT mice and h-RAG (Rag2−/−γc−/−) mice models have been developed to test microbicides against HIV-1 (Deruaz & Luster, 2013). BLT mice are generated from nonobese diabetic (NOD) scid or NOD scid gamma (NSG) mice, implanted with fetal thymus-liver cells, and transplanted with human CD34+ HSCs from the same donor. This model mimics T cell development in humans because human T cells develop within the human thymus. More importantly, BLT mice can be infected with HIV-1 topically and are widely used (Denton et al., 2008, 2011; Destache et al., 2016; Gallay et al., 2017; Hur et al., 2012). Despite the significant advantages of the BLT mice model, several limitations must be addressed (see Table 6; Lavender et al., 2018). H-RAG mice model engrafted with CD34+ HSCs is a model that increases tolerance to transplanted human HSCs and reduces graft-versus-host-disease (Lavender et al., 2013). This model does not develop tumors like mice with the mutation scid, and enhances human immune cells dissemination in the peripheral blood, liver, spleen, and vagina (Veselinovic et al., 2012). However, humoral responses to HIV-1 are not detectable, and secondary lymphoid tissues development is limited (Berges et al., 2006; Nochi et al., 2013).

8.3 Other in vivo studies

Before introducing the microbicidal candidate to clinical trials, safety and pharmacokinetic (absorption, distribution, metabolism, and excretion; Fu et al., 2020; Huang et al., 2020; Pandey et al., 2020) assays should be evaluated to estimate the margin of safety and risks.

Effects on vaginal tissue at several dose levels (single and repeated doses) and two different animal species (one rodent and one nonrodent) should be evaluated. Signs of inflammation, damage to the vaginal mucosa, or alteration of epithelial cells should be assessed following the scoring system described previously by Eckstein et al. (1969). There is

| Characteristics | Rhesus macaques | Pigtail macaques | Cynomolgus macaques |
|-----------------|-----------------|-----------------|---------------------|
| Microbicidal studies | Most widely used | Widely used | Limited |
| Vaginal | Yes | Yes | No |
| Rectal | Yes | Yes | Yes |
| Disease progression | Similar to human | Rapid progression | Less pathogenic |
| Viral load | High | Slightly higher | Lower |
| CD4+ T depletion | Progressive | Rapid | Rapid |
| Vaginal anatomy | Similar to human | Similar to human | Vaginal vault smaller |
| Breeding time | Seasonal | Year-round | Seasonal |
| Availability | Wide | Limited | Wide |
no established standard intravaginal administration. However, it is recommended to assess vaginal toxicity and irritation at two and 24 h after application, 5 or 7 days after daily application, or even after 14 days with two consecutive intravaginal administrations (Amaral et al., 2006; Dhondt et al., 2005; Fields et al., 2014; Nuttall et al., 2008).

API should be labeled with any probes to determine whether API crosses the epithelial barrier. Biodistribution studies must label API with fluorophores (fluorescein isothiocyanate [FITC]) to analyze the compound across the animal organism (das Neves et al., 2014; Henry et al., 2016). If API does not cross the epithelial barrier, only toxicological assays will be carried out. If API crosses the epithelial barrier, a battery of six assays of safety and activity that cannot be studied in humans should be included: mutagenicity, carcinogenicity, one (and two)-generation reproduction toxicity, neurotoxicity, embryotoxicity, and genetic studies (Lard-Whiteford et al., 2004). Mutagenicity and genetic studies evaluate chromosomal and structural DNA changes, such as duplications, insertions, or translocations (da Silva Dantas et al., 2020; Nakamura et al., 2020; Prado-Ochoa et al., 2020), and identify gene mutations (Gao et al., 2020; Marcelino et al., 2020; Park et al., 2020). Carcinogenicity studies evaluate tumors’ development after 18 in mice or hamsters or 24 months in rats (Dekant et al., 2020; Prado-Ochoa et al., 2020; Saleh et al., 2020). Reproduction toxicity studies analyze the morphology and motility of male sperm, parturition, the number of live and dead pups, and the sex of the puppies. The same procedure is repeated to obtain the offspring of the second generation (Ganiger et al., 2007; Q. Liu et al., 2018; Montagnini et al., 2018; Wang et al., 2019). Neurotoxicity studies evaluate neuropathological lesions and neurological dysfunctions (loss of memory, sensoric defects, and learning and memory dysfunctions) after 28 or 90 days of administration (Ishtiaq et al., 2021; Xu et al., 2021). Embryotoxicity studies evaluate embryofetal effects (hemorrhagic bullae, malformations, deformities, and mortality) between the 8th and 14th day of pregnancy (Carvalho et al., 2020; Maziero et al., 2020). In addition to these studies, compatibility with condoms, hypersensitivity, and photosensitivity studies should also be evaluated.

8.4 | Summary of in vivo assays performed with dendrimers

All dendrimers that reach this in vivo step were toxicologically tested to evaluate inflammation and vaginal irritation parameters after a short or prolonged vaginal exposure (see Table 4). G1-C8 and G1-S8 dendrimers showed no damage or alteration into the vaginal epithelium after seven consecutive days of exposure in a BALB/c mouse model (Maciel et al., 2019). G2-STE16 and G2-CTE16 in a female BALB/c mouse model (Galán et al., 2014; Sanchez-Rodriguez, Diaz, et al., 2015), and G2-Sh16 and G1-NS16 in CD1(ICR) mice (Vacas Cordoba et al., 2013), did not show irritation or vaginal lesions after two and 24 h vaginal application. G0-Sh4, like G2-S16 dendrimer, did not generate alteration of epithelial cells, inflammation, or damage to the vaginal mucosa after 7 days with daily application in BALB/c mice.
Remarkably, G0-Sh4 halted the same mouse model’s HSV-2 infection both vaginally and rectally (Cena-Diez et al., 2019; Cena-Diez, Vacas-Cordoba, et al., 2016). The G2-S16 is the dendrimer with more toxicological studies vaginally. G2-S16 did not show toxicity and vaginal irritation after daily intravaginal administration during five consecutive days in New Zealand White rabbits (Chonco et al., 2012). Moreover, one or two successive doses of G2-S16 after 7 days postapplication did not disrupt epithelial cells nor produce damage in the vaginal mucosa in BALB/c mice (Briz et al., 2015; Sepulveda-Crespo, Serramia, et al., 2015), maintaining intact vaginal microbiome (Guerrero-Beltran et al., 2020). And more importantly, G2-S16 dendrimer prevented HIV-1 vaginal transmission in BLT-mice and HSV-2 in BALB/c female mice (Cena-Diez et al., 2019; Cena-Diez, Vacas-Cordoba, et al., 2016; Rodriguez-Izquierdo et al., 2020; Sepulveda-Crespo, Serramia, et al., 2015). G2-S16 dendrimer is the only dendrimer with vaginal

**FIGURE 4** Summary of main in vivo assays performed with polyanionic carbosilane dendrimers as vaginal microbicides

**TABLE 7** Advantages and limitations of vaginal dosage forms for anti-HIV-1 vaginal microbicides. (Modified from Antimisiaris and Mourtas (2015), Garg et al. (2010), and Rohan et al. (2014))

| Formulation | Advantages | Disadvantages |
|-------------|------------|---------------|
| Gel         | Self-controlled | Applicator required |
| Cream       | Self-controlled | Not uniformity of API distribution |
| Ointment    | Self-controlled | Administration with frequency |
|             | Lubricant effects | Low side-effects |
|             | Low side-effects | Low systemic absorption |
|             | Low side-effects | Low cost |
|             | Low side-effects | Combination system |
|             | Absorption in the vaginal epithelium is dependent on local hydration |
|             | Not uniformity of API distribution |
| Tablet      | Self-controlled | Vaginal irritation due to contact with solids |
| Suppository | No applicator is required | High cost |
| Film        | No applicator is required | Uncomfortable placement |
|             | Rapid or sustained release | Not uniformity of API distribution |
|             | Rapid or sustained release | Difficult sustained release |
|             | Combination system | Complex manufacturing |
FIGURE 5  Key preclinical steps in the development of a vaginal microbicide against HIV-1 infection to lead to clinical trials. API, active pharmaceutical ingredient; DCs, dendritic cells; EC50, half-maximal effective concentration; EC90, 90% effective concentration; HCV, hepatitis C virus; HSV, herpes simplex virus; LDH, lactate dehydrogenase; MΦ, macrophages; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TI, therapeutic index.
biodistribution studies in BALB/c mice and teratogenic studies in a zebrafish model. G2-S16 did not cross the vaginal barrier even after 20 h of vaginal application and did not show mortality, sublethal, or teratogenic effects in any dose and at 96 h after intravaginal application in the zebrafish embryos (Cena-Diez et al., 2017; for a detailed summary, see Figure 4).

SPL7013 is another of the most advanced dendrimers in in vivo assays. SPL7013 was not toxic vaginally and rectally after a 4-day repeat dose in a female pig-tailed macaque model (Patton et al., 2006) and did not irritate 5-day repeat dose intravaginal administration in rabbits (Bernstein et al., 2003). More importantly, SPL7013 prevented vaginal transmission in pig-tailed macaques after cell-free SHIV89 infection (Jiang et al., 2005; McCarthy et al., 2005) and HSV-2 infection in female Swiss Webster mice (Bernstein et al., 2003). The lack of more relevant vaginal toxicological studies in different conditions, doses and times, could be the leading cause of failure in clinical trials as vaginal microbicide: poor acceptability, modifications in the microbiome, inflammation, and epithelial damage (Carballo-Diequez et al., 2012; Cohen et al., 2011; McGowan et al., 2011; Moscicki et al., 2012; Pellett Madan et al., 2015).

**9 | VAGINAL FORMULATIONS: DEVELOPMENT AND CHARACTERIZATION**

Microbicide formulation or delivery system plays a key role because API must be stable in the vehicle, reach the right activity site, distribute throughout the vaginal compartment, and remain there for a sufficient time. Liquid formulations (e.g., suspensions or solutions) are inappropriate due to their short retention and contact time in the vaginal surface to exert their activity. Therefore, appropriate formulations for vaginal delivery of microbicides should include semi-solid (gels, creams, ointments) or solid (tablets, films, suppositories, rings) dosage forms (see Table 7).

The gel is a suspension of inorganic or organic particles interpenetrated by an aqueous or nonaqueous liquid (Antimisiaris & Mourtas, 2015; Garg et al., 2010; Rohan et al., 2014). Its high lubrication capacity and wide acceptance contribute to it being the most widely studied formulation as vaginal microbicide (Al-Khouja et al., 2020; Bunge et al., 2018; Delany-Moretlwe et al., 2018; Halwes et al., 2016). The tablets are polymers that gel in the presence of vaginal fluids and overcome stability concerns related to gels (Antimisiaris & Mourtas, 2015; Garg et al., 2010; Rohan et al., 2014). Tablets protect the vagina instantly (fast-dissolving tablets) or in a controlled and sustained manner to promote better adherence and less use (Clark et al., 2014; McConville et al., 2013; McConville et al., 2016). Fast-dissolving films are water-soluble polymers that release the API to dissolve in the vaginal mucosa. Although vaginal films do not require an applicator to use, their possible influence during sexual intercourse is a disadvantage (Antimisiaris & Mourtas, 2015; Garg et al., 2010; Rohan et al., 2014). However, their formulation as a vaginal microbicide has been studied (Notario-Perez et al., 2019; Notario-Perez et al., 2020; Patki et al., 2020). Despite the microbicide formulation’s progress, and most vaginal drugs have been in the form of gels, vaginal rings have gained acceptance in the last years due to a controlled release for a long time (Bunge et al., 2020; Katz et al., 2020; Roberts et al., 2020; Thurman et al., 2019; Vincent et al., 2018). Recently, there has been a growing interest in alternative dosage forms such as nanogels (das Neves & Sarmento, 2015; Destache et al., 2016; Koavarova et al., 2015; Lai & He, 2016) or electrospun fibers (Tyo et al., 2017; Tyo et al., 2019).

Physicochemical properties of vehicles should be evaluated: type of polymer used (Carbopol 934, hydroxypropylmethylcellulose 4000 [HPMC 4000], hydroxyethylcellulose [HEC], polyethylene glycol 6000 [PEG 6000]), dose volume, viscosity, yields stress, pH, osmolality, appearance, odor, and impurities. Vehicles must maintain the acidic pH of the vagina once in contact with semen, be bioadhesive to hold the vaginal surface in the long term, and have a relatively thick viscosity (El-Enin et al., 2020; Nelson, 2018). The API solubility and compatibility with formulation excipients and other drugs and long-term stability should also be evaluated in preformulation research. Other studies, such as API capacity across a wide range of pH or degradation assays (acid/base, heat, light, oxidative stress), should also be evaluated. Therefore, assessing the best API dosage form (API monodispersity, dose volume, product dimensions, or impurities) is mandatory.

SPL7013 has been formulated in three different gel prototypes containing a mucoadhesive carbopol gel (VivaGel®). SPL7013 and carbopol were tested for buffering capacity, pH, osmolality, and viscosity. The prototypes differed only in propylene glycol and glycerin (Bernstein et al., 2003; Mumper et al., 2009; Telwatte et al., 2011). The rest of the carboxylate and PAA dendrimers tested in in vivo assays were formulated in a universal HEC gel with encouraging results (Briz et al., 2015; Cena-Diez, Vacas-Cordoba, et al., 2016; Maciel et al., 2019; Sanchez-Rodriguez, Diaz, et al., 2015).
**BOX 1  List of drugs based on dendrimers as anti-HIV-1 microbicides**

**Boltorn**

- *BH30sucMan*: G3 with 2,2-bis(methylol)propionic acid (bis-MPA) as a core and 32 mannoses at the periphery
- *Dendron12*: G3 with 3-azidopropanoic acid as a core and four pseudotrimannoside groups at the periphery
- *BH3OPSGal*: G3 with bis-MPA as a core and functionalized with 32 β-Galceramide groups at the periphery

**Carbosilane**

- *G2-S16*: G1 with silicon as a core and 16 sulfonate groups at the periphery
- *G2-STE16*: G2 with silicon as a core and 16 sulfonate groups at the periphery
- *G2-CTE16*: G2 with silicon as a core and 16 carboxylate groups at the periphery
- *G1-NS16*: G1 with silicon as a core and 16 naphthylsulfonate groups at the periphery
- *G2-Sh16*: G1 with silicon as a core and 16 sulfate groups at the periphery
- *G2-S24P*: G2 with a polyphenolic core and 24 sulfonate groups at the periphery
- *G2-S8T*: G2 with a triazole core and eight sulfonate groups at the periphery

**Citric acid-polyethylene glycol-citric acid (CPEGC)**

- *G2-C18PEG*: G2 with polyethylene glycol as core and 18 carboxylate groups
- *NPAg-G2-C18PEG*: Nanoconjugated silver G2 dendrimer with polyethylene glycol as core and 18 carboxylate groups

**Gallic acid-triethylene glycol (GATG)**

- *[G1]-CO2Na*: G1 with a GATG core and nine benzoate groups at the periphery

**Glycodendrimers**

- *Bol13.4*: G1 with pentaerythritol as a core and six pseudodimmanoside groups at the periphery
- *ROD3.5.6*: G0 with an aromatic rod-like core and six pseudodisaccharide groups at the periphery
- *ROD3.7.6*: G0 with an aromatic rod-like core and two pseudodisaccharide groups at the periphery

**Poly(alkylideneamine) (PAA)**

- *G1-C8*: G1 with 1,6-diaminohexane/hexamethylenediamine as a core and eight carboxylate groups at the periphery
- *G1-S8*: G1 with 1,6-diaminohexane/hexamethylenediamine as a core and eight sulfonate groups at the periphery

**Poly(amidoamine) (PAMAM)**

- *LewisX*: G5 with ethylenediamine as a core and 14–16 glycan groups at the periphery
- *Sulfo-6*: G2 with ethylenediamine as a core and 16 sialic acid groups or 11 sulfate groups at the periphery
- *SPL2923*: G4 with ammonia as a core and 24 1-(carboxymethoxy) naphthalene-3,6-disulfonate groups at the periphery
- *SPL6195*: G4 with ethylenediamine as a core and 32 benzene dicarboxylate groups at the periphery
- *SPL7304*: G4 with benzhydrlyamine amide as a core and 32 1-(carboxymethoxy) naphthalene-3,6-disulfonate groups at the periphery
Peptide dendrimers

- **SB105-A10**: G1 with benzhydrolamine amide as a core and four sequence peptide chain (ASLRVRRIKK) at the periphery
- **Trp(5a-5f) and modifications**: G1 with carboxylic acid with an aminetriester as a branching unit and 9–18 tryptophans at the periphery

Poly(L-lysine) (PLL)

- **SPL7013 (VivaGel®)**: G4 with benzhydrolamine amide as a core and 32 1-(carboxymethoxy) naphthalene-3,6-disulfonate groups at the periphery
- **SCSLD3**: G3 with stearalamide as a core and 32 cellobioses at the periphery. Degree of sulfation: 2.3
- **PLDG3-PSCel**: G3 with benzhydrolamine amide as a core and 24 cellobioses at the periphery. Degree of sulfation: 1.9
- **SPL7115**: G2 with benzhydrolamine amide as a core and 8 1-(carboxymethoxy) naphthalene-3,6-disulfonate groups at the periphery

Phosphorus-containing (PPH) dendrimers

- **PPH-3d-G1**: G1 with cyclotriposphazene as a core and 12 galactosylceramide, N-hexadecylaminolactitol groups at the periphery
- **PPH-5c-Gc’1**: G1 with cyclotriposphazene as a core and 12 phosphonic acid moiety and lateral alkyl chain at the periphery

Poly(propylene imine) (PPI)

- **PSGal64mer**: G5 with 1,4-diaminobutane as a core and 44 galactoses and 2 sulfate groups at the periphery
- **MVC-GBT**: G5 with 1,4-diaminobutane as a core and 46 globotrioses at the periphery
- **MVC-3SL**: G5 with 1,4-diaminobutane as a core and 28 3’ sialyllactoses at the periphery
- **SPL7320**: G4 with benzhydrolamine amide as a core and 32 1-(carboxymethoxy) naphthalene-3,6-disulfonate groups at the periphery
- **MD-G2S**: G2 metallodendrimer with ethylenediamine as a core and 16 sulfonate groups at the periphery
- **MD-G2C**: G2 metallodendrimer with ethylenediamine as a core and 16 carboxylate groups at the periphery

Viologen

- **Viol36**: G1 with a benzyl as a core and 6 ethyl groups at the periphery
- **Viol7**: G1 with a benzyl as a core and 6 thymine groups at the periphery

Sepulveda-Crespo, Serramia, et al., 2015. However, not all gel’s physicochemical properties have currently been evaluated.

10 | CONCLUSIONS AND FUTURE PERSPECTIVES

A better knowledge based on the sexual needs of women living with HIV will lead to better approaches to prevent HIV-1 transmission. In this sense, topical vaginal microbicides address these issues, considering that funding in this field has increased over the years. Different formulations have been developed to protect against HIV infection but should be used appropriately. The great advantage of topically applied microbicides is to avoid negotiation with the partner. A microbicide should prevent transmission of HIV-1 and other STIs in the vaginal mucosa and systemically in the case of injuries.
An exhaustive preclinical evaluation of API as a microbicide candidate is vital to save costs and time before reaching clinical trials. Preclinical steps start optimizing API’s structure–activity relationship using in vitro, ex vivo, and in vivo assays and developing a suitable vaginal delivery system. However, candidates can fail due to an inadequate optimization of their structure or an incomplete preclinical characterization. Considering the correct optimization of API structure, new models, assays, and endpoints that mimic the vagina and the penis’ environmental conditions during sexual intercourse have been developed and incorporated into preclinical steps.

This algorithm (see Figure 5) identifies potential compounds as vaginal microbicides. The algorithm designed must also consider the risk of HIV-1 transmission rectally. Microbicide candidates, such as anionic dendrimers that inhibit viral entry, are primary compounds. pH transition assays with acidic, essential, and seminal plasma, different cytotoxicity assays to check if API damages the mitochondria or the integrity of the plasma membrane, assays with T/F viruses are some of the assays that have been updated in this preclinical algorithm. Remarkably, combination assays with different mechanisms of action, such as the HIV-1 entry (anionic dendrimers) and other processes before integration, are highly recommended.

There is no perfect model that fully recapitulates how humans become HIV-infected or how the dendrimer would act in humans. Currently, h-mice are excellent models for evaluating mucosal HIV transmission in vivo due to engrafted parts of the human immune system. However, their short lifespan and low sample volumes that can be obtained are their main limitations. A better understanding of the human and mouse immune systems will lead to better h-mice models.

Despite its good results in the different in vitro, ex vivo, and in vivo assays, the anionic SPL7013 dendrimer failed in the first clinical trials due to signs of inflammation and epithelial damage (Carballo-Dieguez et al., 2012; Cohen et al., 2011; McGowan et al., 2011; Moscicki et al., 2012; Pellett Madan et al., 2015). However, it is intended to take advantage of its enormous benefits as a molecule and is currently being used against bacterial vaginosis (Chavoustie et al., 2020; Waldbaum et al., 2020). Remarkably, Starpharma wants to use SPL7013 as a COVID-19 nasal spray after showing significant activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19 (Starpharma, 2021). This news represents more than enough reasons to continue believing in anionic dendrimers as potential candidates as vaginal microbicides against HIV-1 and other viruses. However, they must go through extensive preclinical assays before reaching clinical trials (Box 1).

ACKNOWLEDGMENT
This work has been (partially) funded by the RD16/0025/0019 projects as part of Acción Estratégica en Salud, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (2013–2016) and cofinanced by Instituto de Salud Carlos III (ISCIII) and Fondo Europeo de Desarrollo Regional (FEDER), RETIC PT17/0015/0042, Fondo de Investigacion Sanitaria (FIS) (grant no. PI16/01863) and EPIICAL project. COST CA17140 Cancer Nanomedicine-“From the Bench to Bedside.” This work has also been supported by the Ministry of Economy and Competitiveness #CGL2013-40564-R and Gordon and Betty Moore Foundation grant no. 5334. This work was also funded by research grants from ISCIII (grant numbers PI20CIII/00004, and RD16CIII/0002/0002) to Salvador Resino. The study was also funded by the Centro de Investigación Biomédica en Red en Enfermedades Infecciosas (CB21/13/0044). DS-C is a “Sara Borrell” researcher from ISCIII (grant no. CD20CIII/00001).

CONFLICT OF INTEREST
The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS
Ignacio Rodríguez-Izquierdo: Data curation (equal); investigation (equal); writing – original draft (equal). Daniel Sepúlveda-Crespo: Data curation (equal); formal analysis (lead); investigation (equal); methodology (lead); writing – original draft (equal). Jose Maria Lasso: Supervision (equal); validation (equal); writing – review and editing (equal). Salvador Resino: Supervision (equal); validation (equal); visualization (equal); writing – review and editing (equal). Ma Ángeles Muñoz-Fernández: Conceptualization (lead); funding acquisition (lead); investigation (equal); project administration (lead); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study.
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**How to cite this article:** Rodriguez-Izquierdo, I., Sepúlveda-Crespo, D., Lasso, J. M., Resino, S., & Muñoz-Fernández, M. Á. (2022). Baseline and time-updated factors in preclinical development of anionic dendrimers as successful anti-HIV-1 vaginal microbicides. *WIREs Nanomedicine and Nanobiotechnology*, 14(3), e1774. https://doi.org/10.1002/wnan.1774