Research Article

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Future Medicinal Chemistry

Combination of G2-S16 dendrimer/dapivirine antiretroviral as a new HIV-1 microbicide

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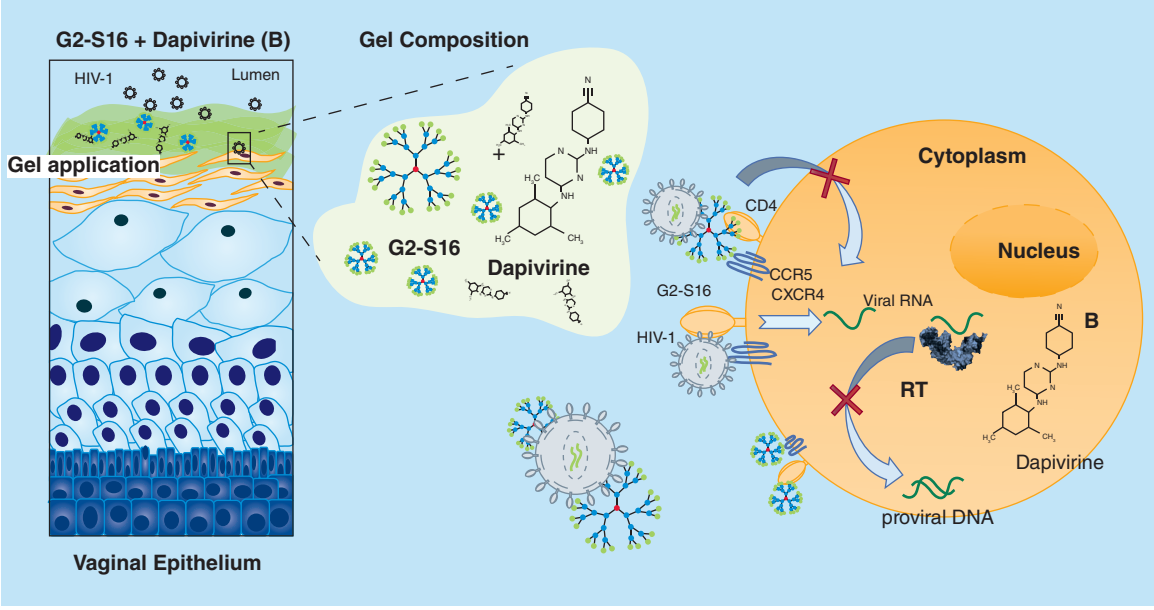
Aim: To research the synergistic activity of G2-S16 dendrimer and dapivirine (DPV) antiretroviral as microbicide candidate to prevent HIV-1 infection. **Materials & methods:** We assess the toxicity of DPV on cell lines by MTT assay, the anti-HIV-1 activity of G2-S16 and DPV alone or combined at several fixed ratios. Finally, their ability to inhibit the bacterial growth *in vitro* was assayed. The analysis of combinatorial effects and the effective concentrations were performed with CalcuSyn software. **Conclusion:** Our results represent the first proof-of-concept study of G2-S16/DPV combination to develop a safe microbicide.

Graphical abstract:



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Keywords: dapivirine • G2-S16 dendrimer • HIV-1 • microbicide

In the last few years, many efforts have been carried out to develop topical microbicides that prevent new viral infections worldwide. In this sense, SPL7013 (VivaGel™) was the first dendrimer candidate to develop a microbicide

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for the prevention of HIV-1 and HSV-2 infection [1,2]. Unfortunately, SPL7013 showed lack of activity and caused vaginal irritation in a clinical trial [2].

Nanotechnology plays an important role in the development of microbicides against infectious diseases such as HIV-1 and HSV-2 sexual infections [3–7]. The specific design of dendrimers with functional end-groups allows the interaction with envelope proteins of several viruses and/or host cell receptors. Therefore, anionic dendrimers interaction with HSV-2/HIV-1 surface proteins could explain the potent susceptibility of HSV-2 or HIV-1 variants to entry inhibitors [8].

G2-S16 polyanionic carbosilane dendrimer ($C_{112}H_{244}N_8Na_{16}O_{48}S_{16}Si_{13}$; MW: 3717.2 g mol^{-1}) with 16 sulfonate groups in its periphery and a silicon core is characterized by its short reaction times, easy availability of reagents, high reproducibility and quantitative yields of reaction [9]. This dendrimer exerted a potent anti-HIV activity, great *in vivo* biocompatibility [10–13] revealing no inflammation or irritation in the vaginal mucosa when treated with high doses in female rabbits [10] and BALB/c mice [14]. G2-S16 demonstrated its efficacy both *in vitro* and *in vivo* models against most sexually transmitted viruses, such as HIV-1 and HSV-2 [9,10,15,16] and even retained its ability to halt HIV-1 infection in presence of semen [9]. Moreover, G2-S16 prevents HIV-1 vaginal transmission in human-BLT mouse model at 84% [16] and also protects HSV-2 vaginal transmission in BALB/c mice at 100% [15].

A cure for HIV-1 infection is still elusive and prevention is crucial [17,18]. Dapivirine (DPV), a non-nucleoside reverse transcriptase (RT) inhibitors that binds allosterically to HIV-1 RT and prevents viral replication, is one of the leading drug candidates in the field [19–21]. DPV demonstrated a potent activity against different HIV-1 isolates *in vitro* [22]. In 2012, a ring containing DPV was advanced into Phase III clinical trial. Results showed a 37% of overall protection and this rate increase to 56% in women older than 21 years old [23]. Studies on possible side effects are under way with promising results [21,23,24].

The combination of nonspecific drugs blocking different HIV-1 replication stages or the combination of nonspecific compounds with ARV is a reliable microbicide strategy. These combinatorial approaches acting at both entry and postreplication steps could be considered one of the best strategies to reduce the HIV-1 transmission. Thus, we analyzed the combination of G2-S16/DPV.

Materials & methods

Cell lines & viruses

TZM.bl cells (NIH AIDS Research and Reference Reagent Program), derived from Human epithelial cell line from a cervix carcinoma (HeLa) cell line containing integrated copies of the luciferase and *β-gal* genes under the HIV-1 promoter control, and Vero cells (American Type Culture Collection CCL-81, Manassas, VA, USA), African green monkey kidney cell line, were grown as previously described [10,14,25].

Virus stock of R5-HIV-1_{NLAD8} (laboratory CCR5-tropic isolate) were obtained as described [26]. Briefly, they were produced by transient transfection of the pNL(AD8) plasmid (NIH AIDS Research and Reference Reagent Program) into 293T cells (American Type Culture Collection, VA, USA) by using calcium phosphate method. Viral stocks were centrifuged prior to evaluating the viral titter by using the HIVp24^{gag} ELISA kit (INNOTEST® HIV-antigen mAb; Innogenetics NV, Ghent, Belgium). HSV-2 333 stocks were titrated by plaque assay [1,15]. Finally, viral stocks were stored at -80°C.

Reagents

Polyanionic carbosilane dendrimer G2-S16 was synthesized as previously described by the biodendrimer group of the University of Alcala [14,27]. Stocks of 10 mM and subsequent dilutions were performed with nuclease-free water (Promega, Madrid, Spain). Stocks of DPV (Selleckchem, TX, USA) were prepared from powders as 10 mM solutions in DMSO (Sigma-Aldrich, MO, USA), serial dilutions were prepared in nuclease-free water. Tenofovir disoproxil fumarate (the pharmacological active drug of Tenofovir [TFV]; Selleckchem, TX, USA) was used as inhibition control for HIV-1 replication.

Biocompatibility assay

The toxicity of DPV on TZM.bl and Vero cell lines was determined by the MTT (Sigma-Aldrich) assay following the manufacturer's instructions. Culture medium and DMSO were used as viability and death controls, respectively [15,26].

Inhibitory profile of G2-S16 or DPV against viral infections

TZM.bl cells were seeded in 96-well plates (10^4 cells/well). After 24 h, the cells were treated with G2-S16 or DPV at a range of nontoxic concentrations for 1 h at 37°C before R5-HIV-1_{NLAD8} infection (30 ng of p24/10^6 cells). At 2 h postinfection, cells were washed twice with sterile phosphate-buffered saline and replaced with fresh medium. After 48 h, HIV-1 infection was measured by luciferase quantification (Luciferase Assay System kit, Promega Corporation, WI, USA) of cell lysates [26,28].

To assess the anti-HSV-2 activity, Vero cells were seeded in 24-well plates (175×10^3 cells/well) and after 24 h, cells were treated with drugs alone or G2-S16/DPV combination for 1 h. Vero cells were infected with HSV-2 333 isolate (150 plaque forming unit/well). After 2 h, the cells were washed with sterile phosphate-buffered saline in order to remove unbound viruses and drugs and replaced with DMEM 2% fetal bovine serum complete medium supplemented with 0.4% IgG. After 48 h of HSV-2 333 infection, a cellular staining with methylene blue was performed for 1 h and viral plaques were counted [15].

G2-S16/DPV combination against HIV-1 infection

Once we obtained dose-response curve for G2-S16 and DPV, we further analyzed the drug interactions at several ratios as previously described [28]. The 50, 75 and 90% effective concentrations (EC_{50}, EC_{75} and EC_{90}, respectively) values were obtained with CalcuSyn software (Biosoft, Cambridge, UK) using the median-effect plot and the dose-reduction index. Moreover, combination index (CIX) was calculated using methods that derive from the law of mass-action principle [29,30]. CIX values <0.9 indicate a synergistic effect, values between 0.9 and 1.1 represent an additive effect, and values CIX>1.1 represent antagonism [28,31].

Susceptibility assays to G2-S16 & DPV on bacteria

A subset of bacterial species from our laboratory collection was used to determine G2-S16 and DPV susceptibility by broth microdilution and by disk diffusion (only for anaerobic bacteria) [32]. Briefly, bacterial isolates were used to a final concentration of 10^5 CFU/ml in Müller–Hinton in a microtiter plate. G2-S16 and DPV were serially diluted. Minimal inhibitory concentrations (MICs) were determined when growth inhibition in a well was observed.

For anerobic bacteria, inhibitory concentrations were manually determined using blank disks (Thermo Scientific, Madrid, Spain) with G2-S16 or DPV (disk diffusion method) in Brucella blood agar with hemin and vitamin k1 (Becton Dickinson GmbH, Madrid, Spain) and incubated at 37°C in anaerobic jar with Oxoid™ AnaeroGen™ sachets (Thermo Scientific).

Statistical analysis

Statistical analyses were performed with GraphPad software Prism v.5.0 (GraphPad Software, CA, USA), including the calculation of the mean, standard deviation, standard error of the mean. p-values were obtained by using Mann–Whitney U nonparametric test. A p-value ≤ 0.05 was considered statistically significant [15,26,28,31].

Results

Biocompatibility assays

The toxicity of G2-S16 was previously determined on TZM.bl cells [15,28] and consequently further assays were performed with nontoxic concentrations. The toxicity of DPV in TZM.bl cell line was studied in order to determine maximum nontoxic concentrations. DPV was considered nontoxic when survival rate was >80%. Results showed that DPV was nontoxic up to 2 μM in TZM.bl cells (Figure 1). We used DMSO as a cell death control.

Inhibitory profile of G2-S16 & DPV against HIV-1 infection

The activity of G2-S16 and DPV against R5-HIV-1_{NLAD8} isolate was studied. We used R5-HIV-1_{NLAD8} isolate because R5-tropic viruses are predominant in the primary HIV-1 infection [12,33]. We observed a dose-dependent behavior for G2-S16 dendrimer. G2-S16 reached inhibition rates >80% at 1 μM and >95% at 5 μM against R5-HIV-1_{NLAD8} isolate (Figure 2A). Moreover, results showed that DPV halted R5-HIV-1_{NLAD8} infection by >95% at 0.5 μM (Figure 2B). Either way, we also analyzed the EC_{50}, EC_{75}, EC_{90}, EC_{95} values (μM) of G2-S16 and DPV to determine their efficacy against HIV-1. G2-S16 showed a low EC_{50} value of 0.032 μM, indicating its suitable activity against R5-HIV-1_{NLAD8} (Table 1). Furthermore, we obtained that all EC_x values for DPV were below 0.6 μM, with an EC_{50} value of 0.205 μM.

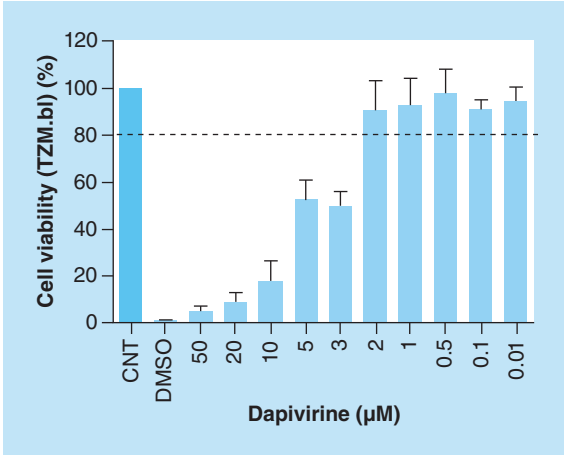

Figure 1. Biocompatibility study of dapivirine on TZM.bl. MTT assay was performed on TZM.bl cells after 48 h of DPV exposure. Cells were treated in range of concentrations between 0.01 µM and 50 µM. Medium alone (CNT) and DMSO (10%) were used as untreated and cell death controls, respectively. Data were represented as mean ± SD of five individual experiments performed in triplicate.
CNT: Untreated control; DMSO: Dimethyl sulfoxide; DPV: Dapivirine.

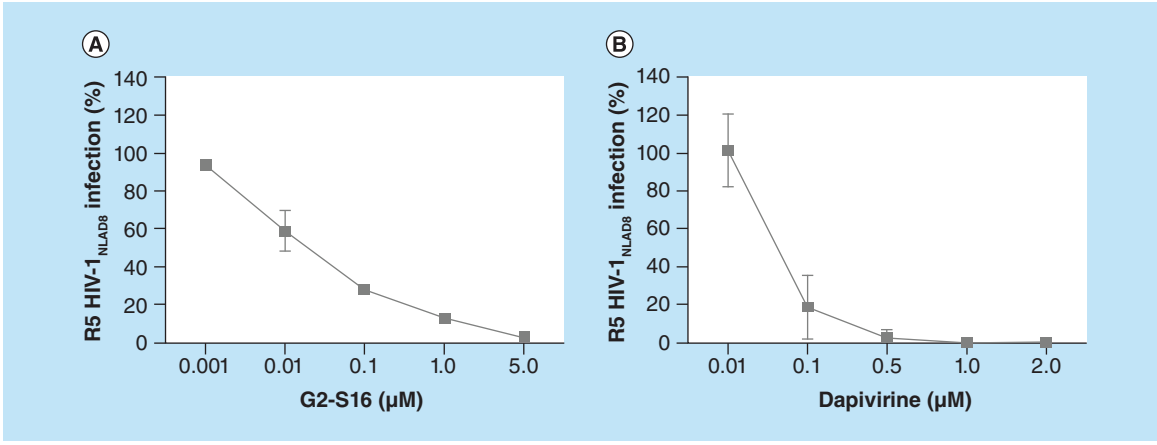

Figure 2. Anti-HIV-1 activity of G2-S16 and dapivirine. TZM.bl cells were treated with **(A)** G2-S16 or **(B)** dapivirinefor 1 h before infection with R5-HIV-1_{NLAD8} isolate for 2 h. Percentage of infection was determined at 48 h postinfection by measuring luciferase activity (versus control of infection). Data represent the mean of three independent experiments performed in triplicate.

Table 1. EC_{50}, EC_{75}, EC_{90}, EC_{95} values (µM) for dapivirine and G2-S16 against R5-HIV-1_{NLAD8} isolate.

Drug	Viral isolate	EC_{50}	EC_{75}	EC_{90}	EC_{95}
DPV	R5-HIV-1_{NLAD8}	0.205	0.297	0.430	0.553
G2-S16		0.032	0.159	0.780	2.299

DPV: Dapivirine; EC: Effcetive concentraion.

Due to the fact that our group showed the potential use of G2-S16 as microbicide against HSV-2 [15], we also studied the anti-HSV-2 activity of the combination G2-S16/DPV (Supplementary Figure 2). As expected, G2-S16 showed higher EC_{50} values against HSV-2 than HIV-1 (Supplementary Figure 1). However, no data were shown for DPV against HSV-2 because DPV showed no activity against this virus.

G2-S16/DPV combination against R5-HIV-1_{NLAD8} infection

We study the potential combinatorial profile of G2-S16 with DPV against R5-HIV-1_{NLAD8}. G2-S16 acts in the first stages of HIV-1 infection and DPV, a non-nucleoside RT inhibitor, blocks the RT of HIV-1. The combination of these two drugs acting at different points of HIV-1 viral cycle could result in a more powerful microbicide. We obtained inhibitions by 70% at 0.05 µM of DPV (ratios 1:5 and 1:10) for G2-S16/DPV combination (Figure 3). A potent activity against R5-HIV-1_{NLAD8} isolate at maximum concentration tested was observed showing inhibition rates of >95%.

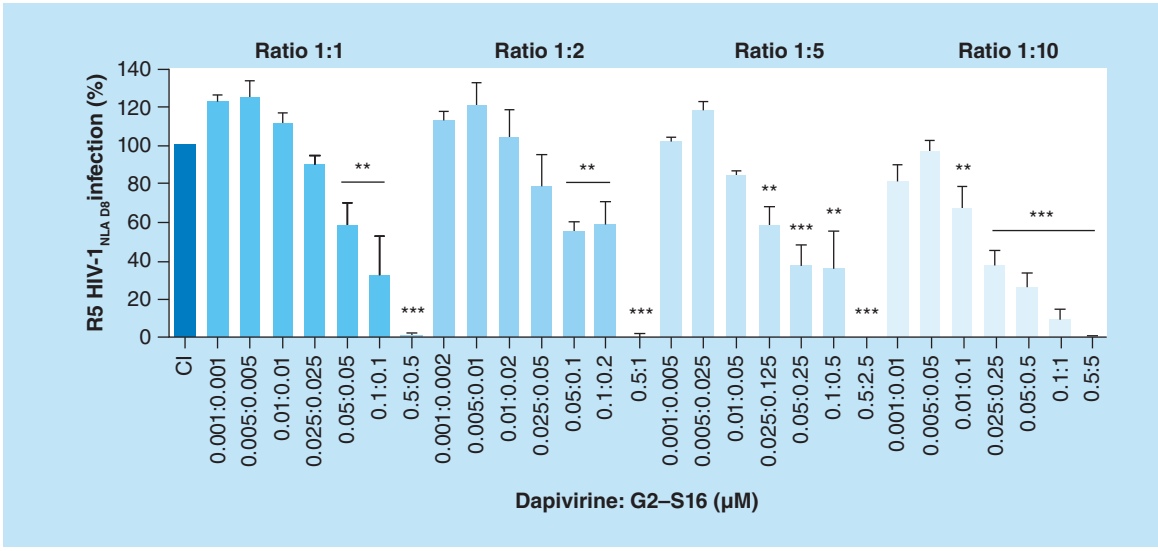

Figure 3. Activity profile of G2-S16/dapivirine combinations against R5-HIV-1_{NLAD8} isolate. TZM.bl cells were treated with G2-S16/dapivirine at several fixed ratios for 1 h before R5-HIV-1_{NLAD8} infection. Percentage of infection was measured by luciferase activity at 48 h postinfection vs CI. The data are represented as the mean ± SD of three individual experiments performed in triplicate.
**$p < 0.001$; ***$p < 0.0001$ vs CI.
CI: Control of infection.

Table 2. EC_{50}, EC_{75}, EC_{90}, EC_{95} values (µM) for G2-S16 and dapivirine combinations against R5-HIV-1_{NLAD8} isolate in TZM.bl cells.

Combination	Ratio	EC_{50}	EC_{75}	EC_{90}	EC_{95}
DPV + G2-S16	1:1	0.083	0.115	0.159	0.199
DPV + G2-S16	1:2	0.096	0.140	0.206	0.266
DPV + G2-S16	1:5	0.178	0.297	0.495	0.702
DPV + G2-S16	1:10	0.079	0.235	0.699	1.467

DPV: Dapivirine; EC: Effcetive concentraion.

Table 3. Combination index for combinations of G2-S16 and dapivirine against R5-HIV-1_{NLAD8} isolate in TZM.bl cells.

Combination	Ratio	CIX values at			
		EC_{50}	EC_{75}	EC_{90}	EC_{95}
DPV + G2-S16	1:1	2.95 ± 1.12† - - -	1.11 ± 0.42-	0.57 ± 0.24‡+++	0.45 ± 0.21+++
DPV + G2-S16	1:2	3.19 ± 1.22- - -	1.12 ± 0.42-	0.50 ± 0.20+++	0.36 ± 0.16+++
DPV + G2-S16	1:5	5.65 ± 1.94-	2.06 ± 0.69-	0.86 ± 0.31+	0.56 ± 0.21+++
DPV + G2-S16	1:10	2.47 ± 1.03- - -	1.56 ± 0.59- - -	1.06 ± 0.45 ad	0.90 ± 0.44 ad

Combinatory indexes are represented as the mean ± SD of three independent experiments performed in triplicate.
†Antagonism levels: 1.1 < CIX < 1.2: - (slight); 1.2 < CIX < 1.45: - - (moderate); 1.45 < CIX < 3.3: - - - (antagonism); 3.3 < CIX < 10: - - - - (strong); CIX > 10: - - - - - (very strong).
‡Synergy levels: 0.9 < CIX < 1.1: additive effect ad; 0.85 < CIX < 0.9 + (slight synergism); 0.7 < CIX < 0.85 ++ (moderate synergism); 0.3 < CIX < 0.7 +++ (synergism); 0.1 < CIX < 0.3 ++++ (potent synergism); 0.1 > CIX +++++ (very strong synergism).
CIX: Combinatory index; DPV: Dapivirine; EC: Effcetive concentraion.

We further study the EC_x values and the synergistic or antagonistic profiles. A reduction of EC_{90} values when compared G2-S16 and G2-S16/DPV at all ratios tested was observed. The decrease of these values was even higher at lower ratios (Table 2). Moreover, data clearly revealed synergistic or additive effects at EC_{90} and EC_{95} values (Table 3). Lower values of CIX were obtained at ratios 1:1 and 1:2 and no reduction of EC_{50} values were observed for the G2-S16/DPV combinations.

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Table 4. Effect of G2-S16 and dapivirine on bacterial growth.

Bacterial species	G2-S16‡	DPV§
Shigellasonei	>	>
Shigellaflexineri 2b	>	>
Shigellaflexineri 2a	>	>
Shigellaboydil	>	>
Citrobacteryoungae	>	>
Citrobacterfreundii	>	>
Enterobacteraerogenes	>	>
Enterobacter cloacae	>	>
Enterbacterintermedius	>	>
Lactobacillus acidofillus	>	>
Klebsiellapneumoniae	>	>
Klebsiellaoxytoca	>	>
Yersinia enterocolotica	>	>
Hafniaalvei	>	>
Salmonella typhimirium	>	>
Serratiamarcescens	>	>
Bacilussubtilis	>	>
Escherichia coli	>	>
Pseudomonas aeuruginosa PAO1	>	>
Pseudomonas aeuruginosa PAK	>	728.6
Enterococusfaecalis	>	>
Mycobacterium smegmatis	>	>
Mycobacterium chelonae	>	364.3
Mycobacterium fortuitum	>	364.3
Bacteroidefragilis†	>	>
Bacteroideovatus†	>	>
Clostridium subterminale 3435	>	>
Clostridium tertium†	>	>
Clostridium innocuum†	>	>

†Disk diffusion method.
‡G2-S16, minimal inhibitory concentration >250 μM in all cases.
§DPV, minimal inhibitory concentration for most bacterial species was >728.6 μM.
DPV: Dapivirine.

In vitro susceptibility of G2-S16 & DPV on bacteria

In order to study the impact on bacterial flora of G2-S16 and DPV we used an array of bacterial species typically found in gut/vagina (and some pathogenic bacteria) to determine the MIC. We did not observe growth inhibition in any of the 29 bacterial species tested in presence of G2-S16 dendrimer (Table 4), including *Lactobacillus acidofillus*, which is abundant specie in the intestinal tract and vagina. Bacterial cultures grew normally, even at the maximum concentration used (250 μM).

Although for DPV most of the species showed no growth inhibition (maximum concentration of 240 μg/ml), for the opportunistic bacteria *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Pseudomonas aeruginosa* an MIC was determined (728.6, 364.3 and 728.6 μM, respectively).

Discussion

Our study represents a proof-of-concept to determine whether the G2-S16/DPV combination could be considered as a new potential microbicide. We determined the EC_{50} values of individual compounds against R5-HIV-1_{NLAD8} and HSV-2 333 isolates. We demonstrated a synergistic or additive profile at EC_{90} values, showing their powerful effect of this G2-S16/DPV combination. Interestingly enough, G2-S16 retained their activity against HSV-2 isolate in presence of DPV (Supplementary Figure 2). Interestingly enough, the combination G2-S16/DPV could

be considered as a new and interesting microbicide approach with the *in vitro* and *in vivo* results previously demonstrated.

On the other hand, many *in vitro* candidates for a topical microbicide failed in human clinical trials due to the impact on vaginal or rectal fluid and microbiota, mucosal immune-crosstalk or possible epithelial injury and inflammatory intercourses [31]. However, we showed that, *in vitro*, either G2-S16 or DPV had no impact on bacterial species present in intestinal and vagina flora. For G2-S16 in particular, the concentration used was 50-fold higher than the one inhibiting R5-HIV-1_{NLAD8} and no growth inhibition was detected, suggesting no disruptions in the vaginal/intestinal ecology.

Further studies should be focused on analyze whether G2-S16/DPV combination is safe on BALB/c mice vaginal application or not. In that case, the formulation as vaginal ring should be the first option to take into consideration as good acceptability, long-term delivery and high adherence observed previously in clinical trials with DPV [22,23,34,35].

Summarizing, our results strongly support that G2-S16/DPV combination is highly effective to prevent HIV-1 infection by interfering in the viral cycle at different points. The synergistic or additive effects obtained resulted in lower doses employed, thus minimizing systemic exposure and toxic side effects. Taking these data with those previously obtained for G2-S16 and DPV *in vivo* models or in clinical trials, resulted in a new approach to reduced infections worldwide. Interestingly enough, although DPV does not affect the replication of HSV-2 infection, G2-S16 maintains the protection of HSV-2 vaginal transmission. G2-S16/DPV combination could be a good dual microbicide against HIV-1 and HSV-2 sexual infection, but further assays are required to assess its *in vivo* safety and efficacy.

Conclusion

G2-S16 in combination with DPV is highly effective to prevent HIV-1 infection by interfering in the viral cycle at different points. The synergistic or additive effects obtained resulted in lower doses employed, thus minimizing systemic exposure and toxic side effects. We proved that G2-S16 and DPV did not inhibit bacterial growth *in vitro*, thus maintaining normal vaginal microbiota. Taking these data with those previously obtained for G2-S16 and DPV in *in vivo* models or in clinical trials, resulted in a promising new approach to 'getting zero' infections worldwide. Moreover, although DPV does not affect the replication of HSV-2, G2-S16 maintains the protection of HSV-2 vaginal transmission. Therefore, G2-S16/DPV combination should be consider a new strategy to develop a safe and powerful topical microbicide.

Future perspective

Future perspective is in clinical trials to demonstrate that G2-S16 is safe and effective, to show that DPV can be used as the first microbicide which protects women against HIV-1 infection. As the DPV alone does not inhibit HIV-1 infection in 100%, clinical trials should be carried out with the combination of G2-S16/DPV.

Summary points

- MTT assays showed the non-toxic effects of G2-S16 and dapivirine (DPV) on TZM.bl and Vero cell lines.
- G2-S16 and DPV exerted high anti-HIV activity, inhibiting the viral cycle at different points.
- G2-S16 and DPV displayed a synergistic or additive profile against R5-HIV-1 isolate.
- G2-S16 and DPV did not inhibit bacterial growth, maintaining the normal microbiota.
- DPV did not interfere in the anti-HSV-2 efficacy of G2-S16 dendrimer.
- The combinations G2-S16/DPV could be effective to prevent HSV-2 infections.

Supplementary data

See online at: www.future-science/doi/suppl/10.4155/fmc-2018-0539

Financial & competing interests disclosure

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Papers of special note have been highlighted as: • of interest; •• of considerable interest

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