



## Research article

## In vitro inhibitory activity against HPV of the monoterpenoid zinc tetra-ascorbo-camphorate

Ralph Sydney Mboumba Bouassa<sup>a,b,c</sup>, Bernard Gombert<sup>d</sup>, Gabin Mwande-Maguene<sup>e</sup>, Aurèle Mannarini<sup>d</sup>, Laurent Bélec<sup>b,c,\*</sup><sup>a</sup> Ecole Doctorale Régionale d'Infectiologie Tropicale de Franceville, BP: 246, Franceville, Gabon<sup>b</sup> Laboratoire de Virologie, Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, 75015, Paris, France<sup>c</sup> Faculté de Médecine Paris Descartes, Université de Paris, Sorbonne Paris Cité, 75006, Paris, France<sup>d</sup> MGB Pharma, 30900, Nîmes, France<sup>e</sup> Faculté de Sciences, Département de Chimie et Biochimie, Université des Sciences et Techniques de Masuku (USTM), BP: 901, Franceville, Gabon

## ARTICLE INFO

## Keywords:

Terpenoid  
Camphor derivatives  
L-ascorbic acid conjugate  
Zn metal  
HPV  
HPV-16  
Pseudovirus  
Inhibition assay

## ABSTRACT

Zinc tetra-ascorbo-camphorate (or drug "C14") is a synthetic monoterpenoid derivative that has potent anti-HIV-1 activity *in vitro*. In this study, we evaluated the *in vitro* antiviral properties of C14 against human papillomavirus (HPV). Inhibition assay of HPV-16-pseudovirus (PsVs) adsorption on COS-7 cells by C14 was used. C14 inhibited HPV-16-PsVs adsorption with IC<sub>50</sub> ranging between 2.9 and 8.3 μM and therapeutic indexes between >410 to >3,300. Pretreatment of COS-7 cells with C14 before addition of HPV-16-PsV was associated with more potent anti-HPV activity than simultaneous deposition on COS-7 of HPV-16-PsV and C14, suggesting that C14 is more effective in preventing HPV attachment to target cells than post-HPV adsorption viral events. Overall, these *in vitro* studies suggest that the monoterpenoid zinc tetra-ascorbo-camphorate molecule may be suitable for further clinical evaluations as potential microbicide or therapeutic drug.

## 1. Introduction

Human papillomavirus (HPV) infection is the most common viral sexually transmitted infection worldwide and high risk-HPV (HR-HPV) genotypes, particularly HPV-16 and HPV-18, are responsible for 5.2% of all cancers worldwide and 7.7% of all cancers in developing countries [1, 2]. According to the World Health Organization (WHO), early 500,000 women will die of cervical cancer annually worldwide from 2025, especially in countries with limited resources such as in sub-Saharan Africa where it constitutes not only a diagnostic but also a therapeutic management challenge [3, 4].

The prophylactic vaccination with Gardasil-9<sup>®</sup> vaccine (Merck & Co. Inc., Kenilworth, NJ, USA) containing virus-like particles from HPV-6 and HPV-11, as well as two α7 (HPV-18 and HPV-45) and five α9 (HPV-16, -31, -33, -52 and -58) high-risk HPV, constitutes one of the main strategies against cervical cancer [5, 6], and is subsidized in underserved and poor countries by Global Alliance for Vaccines and Immunization [7]. Although the benefits of global HPV vaccination program are undeniable [7], prophylactic HPV vaccines do not protect against numerous nonvaccine HPV types associated with several

HPV-related diseases and have low uptake due, in part, to high cost and cold chain storage requirements [8]. Thus, it is increasingly recognized that the best strategy to prevent HPV infections should combine prophylactic vaccination in addition to topical antiviral chemoprophylaxis [9, 10]. Indeed, molecules used as topical broad-spectrum microbicides targeting HPVs could be useful in preventing HPV sexual transmission, in addition to the prophylactic vaccination [9].

Natural products constitute a wide source of antiviral agents, *a priori* devoid of cellular toxicity [11]. Terpenoids, which derive from isoprene (C<sub>5</sub>H<sub>8</sub>) with a great diversity of chemical structure, are abundant plant metabolites, several molecules of which have therapeutic, anti-tumor and anti-inflammatory properties [12, 13, 14, 15, 16]. Furthermore, terpenoids have generated a lot of interest due to their antiviral properties *in vitro* against HIV [15, 16].

We previously demonstrated that the zinc tetra-ascorbo-camphorate molecule (named as "C14" drug), a monoterpenoid synthetic derivative, has a potent anti-HIV-1 *in vitro* activity [17]. The development of anti-HPV molecules would overcome the limitations of the current HPV vaccines [18]. This prompted us to evaluate the inhibitory activity of the C14 molecule against HPV. Herein, we have used pseudovirus (PsVs) of

\* Corresponding author.

E-mail address: [laurent.belec@aphp.fr](mailto:laurent.belec@aphp.fr) (L. Bélec).<https://doi.org/10.1016/j.heliyon.2021.e07232>

Received 11 March 2021; Received in revised form 19 May 2021; Accepted 2 June 2021

2405-8440/© 2021 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

HPV-16, the main oncogenic high-risk HPV worldwide [1], to *in vitro* assess the inhibitory activity of C14 against HPV-16-PsVs adsorption on COS-7 cells.

## 2. Material and methods

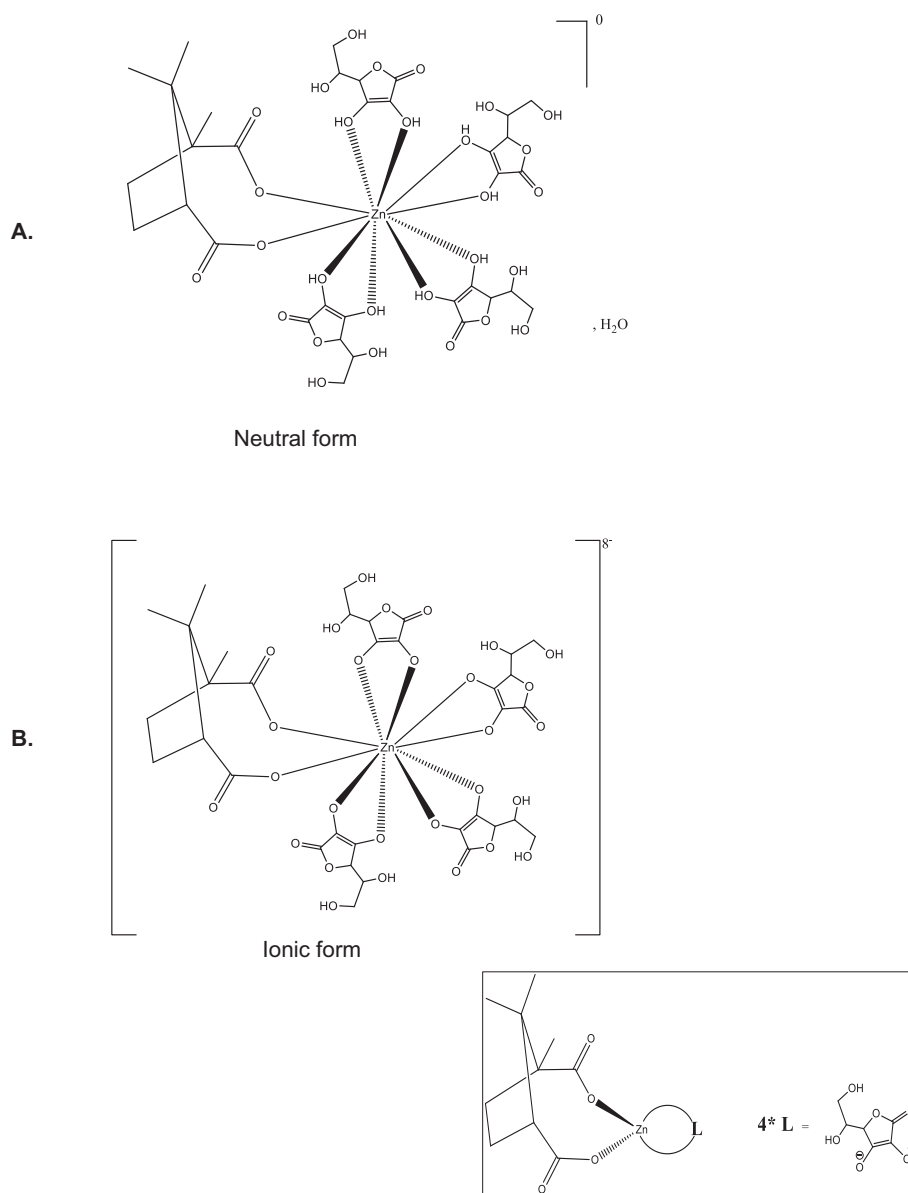
### 2.1. Zinc tetra-ascorbo-camphorate derivative

Based on the chemical interactions of L-ascorbic acid with Zn (II) ions in solid state and in aqueous solutions [19], we have provided Cram's structures of the neutral and ionic form of zinc tetra-ascorbo-camphorate in Figures 1A and B, respectively. Zinc tetra-ascorbo-camphorate is a synthetic compound containing a terpenoid system stably associated to 4 ascorbic acids by a Zn atom. The global formula of the zinc tetra-ascorbo-camphorate is as follows:  $4(C_6H_6O_6)Zn(C_{10}H_{14}O_4)$ . Chemical synthesis of the batch of C14 used in present experiment was previously described in extenso by Saïdi and colleagues [17]. The full detailed synthesis protocol is subjected to a patent and has been verified

and approved by an independent laboratory of organic chemistry (Laboratoire de chimie organique, Institut des Sciences Pharmaceutiques et Biologiques, Université Claude Bernard, Université Lyon 1, Lyon, France), attesting to the high purity of the final compound when synthesized according to this procedure (not shown). For this study, the C14 was diluted in ultra-pure Milli-Q® water (Sigma-Aldrich) and the molarity of the stock solution of C14 used was 0.05155 M.

### 2.2. Production of human papillomavirus pseudovirus

The viral lifecycle of HPV is closely linked to the differentiation process of the stratified squamous epithelium of the mucosa [20]. These particularities make HPV very difficult to be cultured *in vitro* and significantly hinder the assessment of inhibitory activity of a molecule against these viruses. The recent advent of HPV-based pseudoviral particles (PsV) technologies containing a reporter gene has promoted the development of neutralization assay allowing to assess the *in vitro* effect of antiviral candidate or neutralizing antibodies on the early stage of the



**Figure 1.** Cram's structure of the neutral (A) and ionic (B) forms of zinc tetra-ascorbo-camphorate  $4(C_6H_6O_6)Zn(C_{10}H_{14}O_4)$ . The C14 structure comprises a pentacyclic ring including a camphorate terpene [generic formula:  $(C_5H_8)_n$ ] and 4 L-ascorbic acids stably associated with a unique Zn metal. The box depicts a condensed form of the chemical structure of the C14 molecule solubilized in aqueous or physiologic environments providing aqueous complex negatively charged.

HPV infection [20]. These viral pseudoparticles are able to bind and penetrate some cell lines and promote the transcription of reporter gene coding for a reporter protein such as green fluorescent protein (GFP) or luciferase [20]. These reporter proteins will react with their specific substrate and only cells inside which the PsV has penetrate will express the reporter protein. Therefore, this method helps to assess the neutralizing activity of compounds against HPV [20]. In the present study, we have produced PsV for HPV-16 according to the recommendations stated by the papillomavirus vectors production protocol (<https://ccrod.cancer.gov/confluence/display/LCOTF/PseudovirusProduction>) edited by the Laboratory of Cellular Oncology of the Center for Cancer Research (National cancer institute, National Institute of Health, Rockville Pike, Bethesda, Maryland, USA), with minor adaptations, as we fully detailed previously elsewhere [6]. Briefly, the plasmid vector p16sheLL incorporating both L1 and L2 genes for HPV-16 was used to produce HPV-16-PsVs, as previously described [9, 21, 22, 23, 24, 25]. The final PsVs also incorporated the reporter plasmid pGL4.10 [luc2] (Promega, Madison, Wisconsin, USA), encoding the luciferase protein [22]. The plasmid p16sheLL was gently gifted by John Schiller and is available in the Addgene plasmid repository site (Plasmid reference number: # 37320, <http://www.addgene.org/>). COS-7 cells were used to assess the uptake and to titrate the HPV-16-PsVs produced. Briefly, HPV-16-PsVs incorporate a reporter plasmid pGL4.10 [luc2] encoding the luciferase protein. After the infection of COS-7 with this HPV-16-PsVs, cells are washed to remove the unbound PsVs and incubated with the luciferin, the specific substrate of the luciferase. The luminescence generated by the enzymatic activity of the luciferase carried by HPV-16-PsVs on the luciferin substrate is measured by a luminometer. Therefore, adsorption of HPV-16-PsVs by COS-7 cells is associated with luminescence, while lacks of luminescence indicates the absence of cellular uptake. Dilution of PsVs that yielded at least 80% of the positive control luminescence signal on the luminometer (Luminoskan ascent, Thermo Fisher Scientific, SA, USA), after adding 50  $\mu$ L of luciferin substrate and after adjusted with background luminescence, were selected as suitable and therefore selected as the working dilution for neutralization experiments.

### 2.3. HPV L1 & L2-based pseudovirus inhibition assay

Serial dilutions of the stock solution of C14 diluted to tenth covering an appropriate concentration range (from 0.0001 to 5,000  $\mu$ g/mL at working concentrations of 5,000, 500, 50, 5, 0.5, 0.05, 0.005, 0.0005 and 0.0001  $\mu$ g/mL) were subjected to HPV-16-PsVs-based papillomavirus inhibition assay. Briefly, COS-7 cells were pre-plated ( $10^4$  cells/well) in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific), supplemented with 10% Fetal Bovine Serum (FBS, Dominique DUTSCHER SAS, France), 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco, USA) and 50  $\mu$ g/mL of Fungizone™ Amphotericin B (Thermo Fisher Scientific) in 96-well plates and incubated 24 h in 5% CO<sub>2</sub> at 37 °C. In the first experimentation, COS-7 cells were preincubated during 3 h in set of C14 dilutions (C14 diluted in non-supplemented DMEM). After the preincubation time, the dilutions of C14 were removed and replaced by HPV-16-PsVs diluted in non-supplemented DMEM. In the second experimentation, the C14 stock solution and HPV-16-PsVs stock were mixed, in the same tube, with non-supplemented DMEM, until reaching the desire concentration for C14 and HPV-16-PsVs into the inoculum (100  $\mu$ L). Infected cells were grown overnight at 37 °C and then fed with 100  $\mu$ L of supplemented DMEM. After an additional 24 h of growth at 37 °C, the medium was removed, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) and 100  $\mu$ L of Pierce™ Firefly Luciferase One-Step Glow Assay solution (Thermo Fisher Scientific) was added to each and plate was incubated in the dark, for 15 min. Cells lysates were harvested and transferred to a LumiNunc™ 96-well white microplates and the luciferase enzyme activity was measured as described above. A well containing only the pGL4.10 [luc2] (1  $\mu$ g) served as negative control (Full luminescent signal) and a background luminescence control well was

constituted by only COS-7 cells (no PsV or reporter plasmid). The inhibition of the luminescence signal over 80% was considered as effective inhibition of the PsV transduction of the COS-7 cells. A pool of sera from individuals vaccinated by the Gardasil-9® vaccine (Merck & Co. Inc.) constituted the positive control for the HPV-16-PsVs-based inhibition assay (luminescence signal inhibition of more 80%). All experiments were performed in triplicate.

### 2.4. Cytotoxicity assay

The cytotoxicity of the C14 derivative against COS-7 cells was assessed using MTT assay (Sigma-Aldrich), as previously described [26]. The percentage survival was obtained using the following formula: Survival (%) = live cell number (test)/live cell number (control) x 100. The cytotoxic concentration 50 (CC<sub>50</sub>) corresponding to the C14 concentration ( $\mu$ M) that causes 50% cytotoxicity on COS-7 cells and inhibitory concentration 50 (IC<sub>50</sub>), corresponding to the C14 concentration ( $\mu$ M) that induces 50% inhibition of HPV-16-PsVs activity in luciferase assay in COS-7 cells, were calculated using a dose–response–inhibition analysis on GraphPad Prism v5.04 software (GraphPad Software, San Diego, CA, USA). Then the therapeutic indexes (TI = CC<sub>50</sub>/IC<sub>50</sub>) were calculated.

## 3. Results

### 3.1. High concentrations of C14 are not toxic in vitro

Therefore, the intrinsic toxicity of C14 derivate concentrations up to 5,000  $\mu$ g/mL was evaluated by using a colorimetric cell viability assay. COS-7 cells were exposed to serial five-fold dilutions of C14 stock solution for 24 h. The viability index corresponding to the ratio of viable cells after the treatment on the fraction of viable mock-exposed cells was calculated. Cells treated by a solution of PBS-azide 0.1% were used as a positive control for toxicity (data not shown). The viability index corresponding to the ratio of viable cells after the treatment on the fraction of viable mock-exposed cells, was calculated. As shown in Figure 2, C14 demonstrated viability indexes from 0.8 to 1.1 at all concentrations tested, which indicated that it was largely non-toxic. Estimated CC<sub>50</sub> was >0.00515 M of C14.

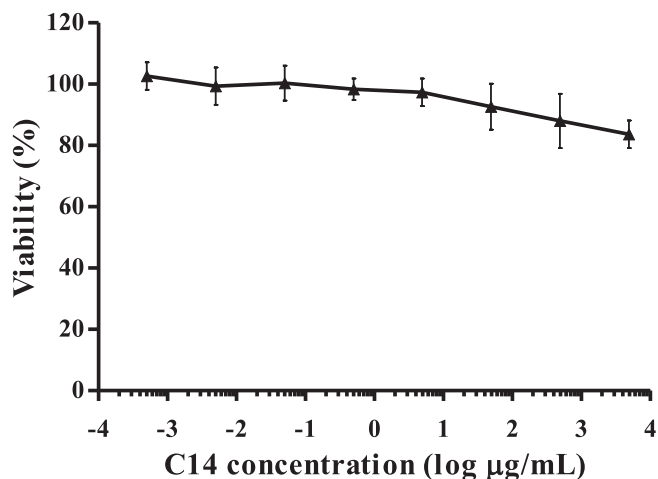
### 3.2. HPV-16-PsVs adsorption inhibition by C14 in COS-7 cells

Figure 3 depicts the inhibition curve of HPV-16-PsVs adsorption by serial dilutions of C14 added at the surface of COS-7 cells during 3 h before adding the PsVs. Estimated IC<sub>50</sub> was 2.8  $\mu$ g/mL (2.9  $\mu$ M) of C14 (95% confidence interval [CI]: [1.5–5.2]), and TIs ranged from >960 to >3,330.

When PsVs was added simultaneously with serial dilutions of C14, the inhibition curve was displaced on the right, with estimated IC<sub>50</sub> reaching 8.1  $\mu$ g/mL (8.3  $\mu$ M) of C14 (95% CI: [6.1–12.1]), and TIs ranged from >410 to >820.

## 4. Discussion

We herein evaluated the in vitro inhibitory activity of the monoterpene C14 molecule against the major oncogenic high-risk HPV-16, using an inhibition assay of HPV-16-PsV adsorption on COS-7 cells. C14 exhibited potent inhibition of HPV-16-PsVs adsorption on COS-7 cells, with IC<sub>50</sub> ranging between 2.9 and 8.3  $\mu$ M and TIs between >410 to >3,300. Pre-treatment of COS-7 by C14 before adding HPV-16-PsVs was associated with more potent anti-HPV activity than simultaneous deposition on COS-7 of HPV-16-PsVs and C14, suggesting that C14 would act in early stages of the infection by preventing the attachment of HPV on binding sites in target cells, rather than acting directly on the viral post adsorption events. Our observations complement and extend the previous demonstration of in vitro efficacy of C14 against against both R5- and X4- HIV-1 [17]. Taken together, the monoterpene zinc

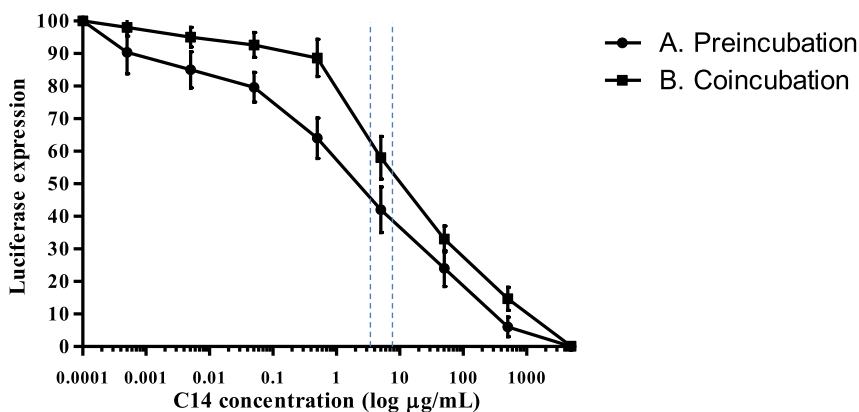


**Figure 2.** Evaluation of C14 toxicity on COS-7 cells. COS-7 cells were cultured with increased concentrations of C14 for 24 h. After washing, culture viability was determined by using the MTT-cytotoxicity assay according to the manufacturer's instructions. The values given are the mean viability  $\pm 1$  standard deviation of COS-7 cells, expressed in percentage. Means  $\pm$  SD are representative of 3 independent experiments.

tetra-ascorbo-camphorate molecule may be suitable for further testing as a broad-spectrum drug candidate to prevent as microbicide male-to-female heterosexual acquisition of HIV-1 or HPV, as well as to be used in the cure of HPV-associated lesions.

The zinc tetra-ascorbo-camphorate molecule belongs to the heterogenous family of terpenoids, which is abundant, diverse in nature, extracted from various plants or obtained by chemical synthesis, and which comprises several characterized therapeutic agents having a large array of pharmacological properties such as anticancer, analgesic, anti-inflammatory, immunomodulatory, and antiviral activities [11, 15, 16]. Antiviral activities of terpenoids have been reported for a wide variety of RNA and DNA viruses, including HIV [11, 16, 17, 27, 28], influenza virus A/WSN/33 (H1N1) [29], or other influenza viruses [15], hepatitis A virus [15, 30], hepatitis B virus [12, 15, 31, 32], hepatitis C virus [15, 33], vesicular stomatitis virus [15], human enterovirus 71 [15], orthopoxviruses [34] pathogenic flaviviruses [35] including dengue virus [36], *Herpesviridae* family viruses [varicella-zoster virus, herpes simplex virus type (HSV) type 1 (HSV-1), Epstein-Barr virus, cytomegalovirus] [15, 31, 33, 37, 38] and emerging pathogenic SARS-coronaviruses [15, 39].

Our observations report, to our knowledge for the first time, on the antiviral activity of a terpenoid against HPV. This may simply be due to



**Figure 3.** Evaluation of C14 inhibitory activity on HPV-16-PsVs adsorption on COS-7: (A) HPV-16-PsVs was added in culture medium after 3 h preincubation with serial C14 dilutions; (B) HPV-16-PsVs and C14 dilutions were added simultaneously. Pooled sera from individuals having received three doses of Gardasil-9® vaccine (Merck & Co. Inc.) used as positive control showed luminescence signal inhibition above 80% (not shown). The anti-HPV-16-PsVs  $IC_{50}$  values (shown as a vertical dotted line) were estimated using the luciferase inhibitory assay in COS-7 cells by the GraphPad Prism v5.04 software (GraphPad Software). Means  $\pm$  SD are representative of 3 independent experiments. Therapeutic indexes ( $TI = CC_{50}/IC_{50}$ ) were between  $>410$  to  $>3,330$ .

the technical difficulties of *in vitro* HPV inhibition tests. Indeed, at this time, HPVs cannot be cultured *in vitro*, nor replicate in a conventional monolayer cell cultures [40, 41]. However, the production of papillomavirus-based gene transfer vectors, known as of HPV L1&L2 based-PsVs, using conventional monolayer cell lines allows the assessment of the initial phases (entry and assembly) of the HPV replication cycle [41].

The mechanism of action of C14 against HPV-16 is yet unknown. It was previously demonstrated that C14 harbors potent HIV-1 entry inhibition activity and/or targets pre-integrative step of viral cycle, indicating that C14 inhibits the initial stages of the HIV replication cycle [17]. In our hands, C14 prevented more efficiently HPV attachment on target cells than HPV post-adsorption events, also suggesting that the inhibitory action of C14 against HPV-16 depends more on the initial stages of viral attachment and penetration into its target cell rather than on later stages of the replication cycle. While further work is needed to determine precisely the molecular mechanism of anti-HPV activity of C14, it is possible to make some assumptions. In the zinc tetra-ascorbo-camphorate molecule, the combination of the monoterpene with 4 L-ascorbic acids stabilized by a Zn metal was used to create a new biological terpenoid. Similar strategy of conjugation to create new bioactive terpenoids was previously reported for pentacyclic triterpenoid conjugated with L-ascorbic acid causing increased potent antiviral activity against influenza virus, likely because disruption of the interaction of influenza hemagglutinin (HA) with the sialic acid receptor and thus of the attachment of viruses to host cells secondary to structurally stoichiometric effect of the novel conjugated multi terpenoids complex [42, 43, 44]. Similarly, the conjugation of the monoterpene ascorbate of the C14 complex with 4 L-ascorbic acid molecules could have resulted in a spatial configuration capable to alter the first step of host cell infection. In addition, the camphor, an abundant monoterpene with a bicyclic framework structure, and camphor derivatives, possess broad antiviral activity [45, 46, 47]. Interestingly, camphor derivatives have demonstrated antiviral activity *in vitro* against orthopoxviruses belonging to the *Poxviridae* family [34], which is close to the *Papoviridae* family to which HPV belongs. Otherwise, L-ascorbic acid is well known to have anti-inflammatory properties and has demonstrated numerous effects on the immune system [48], and may have indirect anti-viral properties [49, 50]. Furthermore, the four ascorbic acid groups with high polarity [51] likely allow the C14 molecule to be solubilized in aqueous or physiologic environments, providing  $Zn^{2+}$ -camphorate or hydrated species of zinc-camphorate, which are highly reactive with proteins, as previously described [51, 52]. In this study inhibition assay, aqueous derivative of C14 molecule could interact with the L1 and L2 proteins of HPV-16-PsVs and influence their ability to bind to cells. Such hypothesis could support our findings showing that the pre-treatment of COS-7 before adding HPV-16-PsVs was associated with more potent

anti-HPV activity than simultaneous deposition on COS-7 of HPV-16-PsVs and C14.

The risk of HPV infection can be reduced by complementary or even synergistic strategies combining the use of microbicidal molecules and prophylactic vaccines for primary prevention, and early detection and treatment of high risk-HPV-associated lesions [18]. We previously reported the anti-HIV-1 activity of zinc tetra-ascorbo-camphorate molecules on both R5- and X4- HIV-1 infection of primary mucosal target cells and on HIV-1 transfer from dendritic cells to T cells [17]. We herein demonstrated a significant reduction in HPV-16-PsVs infection associated with high TIs when applying C14 3 h before HPV-16-PsV challenge, with IC<sub>50</sub> relatively closed to that previously observed against HIV-1 (ranging from 0.02 to 1.3 μM) [17]. This property of high inhibition of the adsorption of HPV-16-PsVs when the C14 is applied in anticipation of exposure to PsVs could be particularly useful in the development of C14 as potential microbicide molecule, to prevent the acquisition of HPV in sexually exposed women. In addition to its antiviral properties against HIV and HPV, C14 has shown inhibitory activity against HSV type 2 (HSV-2) [53]. Thus, the antiviral activity of C14 against the reference strain HSV-2-MS (ATCC® VR-540™) and acyclovir-sensitive clinical HSV-2 and HSV-1 isolates was evaluated using plaque reduction assay on Vero (ATCC: CCL81) and human fibroblast MRC5 cells [53]. Anti-HSV action was further approached by attachment (target cell exposed to the virus in the presence or absence of C14) and penetration (viruses adsorbed on pre-chilled cells) assays. C14 inhibited both HSV-2 and HSV-1 replication with IC<sub>50</sub> ranging between 7.3 and 15.9 μM and selectivity indexes between 170 to 2,500. The simultaneous treatment was more efficient than the post-infection treatment, suggesting that a direct inactivation of viral particles or inhibition of virus replication at the initial phases of the viral replication cycle could be involved. Finally, the cytotoxicity for host primary cells of a non-specific antiviral compound is a major issue. For example, the nonoxynol-9, a non-specific surfactant, which destroys HIV-1 particles *in vitro*, caused lesions in the vaginal epithelium *in vivo* and increased the probability of being infected with HIV-1 [54]. In addition, the C14 molecule was found to be largely non-cytotoxic *in vitro* at high concentrations against primary mucosal target cells (macrophages, dendritic cells and T cells) and lack of significant inflammation and adverse changes *in vivo* could be observed in New Zealand White rabbit cervicovaginal tissue integrity after repeated exposure during 10 days to formulations containing C14 [17]. In total, all these observations allow us to hypothesize that the monoterpene zinc tetra-ascorbo-camphorate molecule could be used as a potential broad-spectrum microbicide to prevent male-to-female heterosexual acquisition of HIV-1 or HPV. Furthermore, tackling HIV, HSV-2, and HPV with a single strategy may improve anti-HIV efficacy, since HSV-2 and HPV are cofactors of HIV sexual transmission [18], and combining HIV protection with HSV and HPV protection may be beneficial [18]. Such strategies for the use of non-antiretroviral molecules as microbicides with broad-spectrum antiviral activities against HIV, HPV and HSV-2 are already very advanced in clinical trials [10, 18, 55, 56]. This is the case with carrageenan which is highly studied for use as a microbicidal gel that can be applied before sex to prevent sexually transmitted viral infections such as HIV, HSV-2 and HPV [10]. Carrageenan is an extract of red algae which has demonstrated antiviral properties *in vitro* and *in vivo* against HPV (IC<sub>50</sub>: 1–20 ng/mL) [9, 10], HIV (IC<sub>50</sub>: 3 μg/mL) and HSV-2 (IC<sub>50</sub>: 0.9–3.6 μg/mL) with high TIs [10]. Similar to carrageenan, C14 has also demonstrated antiviral properties *in vitro* against HPV (IC<sub>50</sub>: 2.8 μg/mL), HIV (IC<sub>50</sub>: 1 μM) [17], and HSV-2 (IC<sub>50</sub>: 7.3–15.9 μM) [53] with high TI. Finally, the C14 molecule could also potentially be used in the treatment of diseases associated with HPV and HSV-2.

Our study obviously has many limitations. Concerning HPVs, we only evaluated the adsorption inhibition of HPV-16-PsVs on COS-7 cells, and these first observations should be verified with other oncogenic HPV genotypes. The molecular action mechanism of C14 against HPV-16 remains unknown. Further analytical works such as liquid chromatography coupled with mass spectrometry (LC-MS) could be helpful to fully

understand the molecular interactions of C14 with HPV-16 viral particle and what happens to C14 after having interacted with the viral particle. In addition, the antiviral activity of monoterpene unit and ascorbic acid, which are the component units of C14, should be examined. Furthermore, our study clearly lacks the demonstration in an animal model that the antiviral activity of C14 molecule *in vitro* is also observed *in vivo*, as for example using the HPV-PsVs vaginal challenge in mice [10, 24, 56].

## 5. Conclusion

Together, our results demonstrate that the C14 is a promising microbicide molecule that should be advanced for further *in vivo* evaluation and clinical testing.

## Declarations

### Author contribution statement

Ralph Sydney Mboumba Bouassa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Bernard Gombert; Gabin Mwande-Maguene; Aurèle Mannarini: Conceived and designed the experiments; Performed the experiments.

Laurent Bélec: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

### Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Data availability statement

Data will be made available on request.

### Declaration of interests statement

The authors declare the following conflict of interests: Ralph Sydney Mboumba Bouassa, Gabin Mwande-Maguene and Laurent Bélec report no conflicts of interest. Bernard Gombert, the chief executive officer of MGB Pharma, Nîmes, France, gave the C14 molecule for the study. Aurèle Mannarini is chemist adviser for MGB Pharma, and discussed the interpretation of the results. Bernard Gombert and Aurèle Mannarini did not play a role in the study design, data collection and analysis, as well as decision to publish.

### Additional information

No additional information is available for this paper.

## Acknowledgements

The authors are grateful to MGB Pharma, Nîmes, France, for providing for the C14 molecule for the study. Ralph-Sydney Mboumba Bouassa was PhD student of the *Ecole Doctorale en Infectiologie Tropicale*, Franceville, Gabon, benefiting of a scholarship from the Gabonese Government and was holder of merit from the Agence Universitaire de la Francophonie. The authors thank Pr Antoine Touze, Université de Tours, Tours, France, for his invaluable advice and technical assistance.

## References

- [1] M.E. Scheurer, G. Tortolero-Luna, K. Adler-Storthz, Human papillomavirus infection: biology, epidemiology, and prevention, *Int. J. Gynecol. Canc.* 15 (5) (2005 Sep-Oct) 727–746.
- [2] D.M. Parkin, F. Bray, Chapter 2: the burden of HPV-related cancers, *Vaccine* 24 (Suppl 3:S3) (2006 Aug 31) 11–25.

- [3] World Health Organization, Projections of Mortality and Causes of Death, 2015 and 2030, 2015. Available at: [http://www.who.int/healthinfo/global\\_burden\\_disease/projections/en/](http://www.who.int/healthinfo/global_burden_disease/projections/en/). (Accessed 23 August 2020).
- [4] R.S. Mboumba Bouassa, T. Prazuck, T. Lethu, M.A. Jenabian, J.F. Meye, L. Bélec, Cervical cancer in sub-Saharan Africa: a preventable noncommunicable disease, *Expert Rev. Anti Infect. Ther.* 15 (6) (2017 Jun) 613–627.
- [5] D.M. Harper, L.R. DeMars, HPV vaccines - a review of the first decade, *Gynecol. Oncol.* 146 (1) (2017 Jul) 196–204.
- [6] R.S. Mboumba Bouassa, H. Péré, C. Gubavu, T. Prazuck, M.A. Jenabian, D. Veyer, J.F. Meye, A. Touzé, L. Bélec, Serum and cervicovaginal IgG immune responses against  $\alpha 7$  and  $\alpha 9$  HPV in non-vaccinated women at risk for cervical cancer: implication for catch-up prophylactic HPV vaccination, *PLoS One* 15 (5) (2020 May 18), e0233084.
- [7] Gavi, The Vaccine Alliance. Gavi Progress Report, 2018 (last access 21st August 2020), <https://www.gavi.org/sites/default/files/publications/progress-reports/Gavi-Progress-Report-2018.pdf>.
- [8] D.R. Lowy, J.T. Schiller, Reducing HPV-associated cancer globally, *Canc. Prev. Res.* 5 (1) (2012 Jan) 18–23.
- [9] C.B. Buck, C.D. Thompson, J.N. Roberts, M. Müller, D.R. Lowy, J.T. Schiller, Carrageenan is a potent inhibitor of papillomavirus infection, *PLoS Pathog.* 2 (7) (2006 Jul) e69.
- [10] A. Rodríguez, K. Kleinbeck, O. Mizenina, L. Kizima, K. Levendosky, N. Jean-Pierre, G. Villegas, B.E. Ford, M.L. Cooney, N. Teleshova, M. Robbiani, B.C. Herold, T. Zydowsky, J.A. Fernández Romero, In vitro and in vivo evaluation of two carrageenan-based formulations to prevent HPV acquisition, *Antivir. Res.* 108 (2014 Aug) 88–93.
- [11] D. Yu, S.L. Morris-Natschke, K.H. Lee, New developments in natural products-based anti-AIDS research, *Med. Res. Rev.* 27 (1) (2007 Jan) 108–132.
- [12] M.H. Yan, P. Cheng, Z.Y. Jiang, Y.B. Ma, X.M. Zhang, F.X. Zhang, L.M. Yang, Y.T. Zheng, J.J. Chen, A.-D. Periglauces, Anti-HBV and HIV-1 alkaloid from *Pericampylus glaucus*, *J. Nat. Prod.* 71 (2008) 760–763.
- [13] R.Y. Kuo, K. Qian, L. Susan, M. Natschke, K.H. Lee, Plant-derived triterpenoids and analogues as antitumor and anti-HIV agents, *Nat. Prod. Res.* 26 (2009) 1321–1344.
- [14] R. Kiyama, Estrogenic terpenes and terpenoids: pathways, functions and applications, *Eur. J. Pharmacol.* 815 (2017) 405–415.
- [15] S. Xiao, Z. Tian, Y. Wang, L. Si, L. Zhang, D. Zhou, Recent progress in the antiviral activity and mechanism study of pentacyclic triterpenoids and their derivatives, *Med. Res. Rev.* 38 (3) (2018 May) 951–976.
- [16] R. Kaur, P. Sharma, G.K. Gupta, F. Ntie-Kang, D. Kumar, Structure-activity-relationship and mechanistic insights for anti-HIV natural products, *Molecules* 25 (9) (2020 Apr 29) 2070.
- [17] H. Saïdi, M.A. Jenabian, B. Gombert, C. Charpentier, A. Mannarini, L. Bélec, Pre-clinical development as microbicide of zinc tetra-ascorbo-camphorate, a novel terpenoid derivative: potent in vitro inhibitory activity against both R5- and X4-tropic HIV-1 strains without significant in vivo mucosal toxicity, *AIDS Res. Ther.* 5 (2008 Jun 3) 10.
- [18] J.A. Fernández-Romero, C. Deal, B.C. Herold, J. Schiller, D. Patton, T. Zydowsky, J. Romano, C.D. Petro, M. Narasimhan, Multipurpose prevention technologies: the future of HIV and STI protection, *Trends Microbiol.* 23 (7) (2015 Jul) 429–436.
- [19] H.A. Tajmir-Riahi, Coordination chemistry of vitamin C. Part II. Interaction of L-ascorbic acid with Zn(II), Cd(II), Hg(II), and Mn(II) ions in the solid state and in aqueous solution, *J. Inorg. Biochem.* 42 (1) (April 1991) 47–55.
- [20] R.S. Mboumba Bouassa, H. Péré, M.A. Jenabian, D. Veyer, J.F. Meye, A. Touzé, L. Bélec, Natural and vaccine-induced B cell-derived systemic and mucosal humoral immunity to human papillomavirus, *Expert Rev. Anti Infect. Ther.* 18 (6) (2020 Jun) 579–607.
- [21] C.B. Buck, C.D. Thompson, Production of papillomavirus-based gene transfer vectors, *Curr. Protoc. Cell Biol.* (2007 Dec). Chapter 26:Unit 26.1.
- [22] E. Draper, S.L. Bissett, R. Howell-Jones, D. Edwards, G. Munslow, K. Soldan, et al., Neutralization of non-vaccine human papillomavirus pseudoviruses from the A7 and A9 species groups by bivalent HPV vaccine sera, *Vaccine* 29 (47) (2011 Nov 3) 8585–8590.
- [23] K. Kondo, Y. Ishii, H. Ochi, T. Matsumoto, H. Yoshikawa, T. Kanda, Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region, *Virology* 358 (2) (2007 Feb 20) 266–272.
- [24] J.N. Roberts, C.B. Buck, C.D. Thompson, R. Kines, M. Bernardo, P.L. Choyke, et al., Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan, *Nat. Med.* 13 (7) (2007 Jul) 857–861.
- [25] K. Kondo, H. Ochi, T. Matsumoto, H. Yoshikawa, T. Kanda, Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitopes, *J. Med. Virol.* 80 (5) (2008 May) 841–846.
- [26] H. Saïdi, N. Nasreddine, M.A. Jenabian, M. Lecerf, D. Schols, C. Krief, J. Balzarini, L. Bélec, Differential in vitro inhibitory activity against HIV-1 of alpha-(1-3)- and alpha-(1-6)-D-mannose specific plant lectins: implication for microbicide development, *J. Transl. Med.* 5 (2007) 28.
- [27] H. Hasegawa, S. Matsumiya, M. Uchiyama, T. Kurokawa, Y. Inouye, R. Kasai, S. Ishibashi, K. Yamasaki, Inhibitory effect of some triterpenoid saponins on glucose transport in tumor cells and its application to in vitro cytotoxic and antiviral activities, *Planta Med.* 60 (3) (1994 Jun) 240–243.
- [28] I.-C. Sun, Y. Kashiwada, S.L. Morris-Natschke, K.H. Lee, Plant-derived terpenoids and analogues as anti-HIV agents, *Curr. Top. Med. Chem.* 3 (2) (2003) 155–169.
- [29] W. Song, L. Si, S. Ji, H. Wang, X-m Fang, L-y Yu, R-y Li, L-n Liang, D. Zhou, M. Ye, Antiviral triterpenoid saponins from the roots of *Glycyrrhiza uralensis*, *J. Nat. Prod.* 77 (7) (2014 Jul 25) 1632–1643.
- [30] J.M. Crance, F. Leveque, E. Biziagos, H. van Cuyck-Gandre, A. Jouan, R. Deloince, Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication in vitro, *Antivir. Res.* 23 (1994) 63–76.
- [31] R. Pompei, O. Flore, M.A. Marccialis, A. Pani, B. Lodo, Glycyrrhizic acid inhibits virus growth and inactivates virus particles, *Nature* 281 (5733) (1979 Oct 25) 689–690.
- [32] H. Sato, W. Goto, J. Yamamura, M. Kurokawa, S. Kageyama, T. Takahara, A. Watanabe, K. Shiraki, Therapeutic basis of glycyrrhizin on chronic hepatitis B, *Antivir. Res.* 30 (1996) 171–177.
- [33] U.A. Ashfaq, M.S. Masoud, Z. Nawaz, S. Riazuddin, Glycyrrhizin as antiviral agent against hepatitis C virus, *J. Transl. Med.* 9 (2011 Jul 18) 112.
- [34] A.S. Sokolova, O.I. Yarovaya, N.I. Bormotov, L.N. Shishkina, N.F. Salakhutdinov, Synthesis and antiviral activity of camphor-based 1,3-thiazolidin-4-one and thiazole derivatives as Orthopoxvirus-reproduction inhibitors, *Medchemcomm* 9 (10) (2018 Sep 19) 1746–1753.
- [35] J.M. Crance, N. Scaramozzino, A. Jouan, D. Garin, Interferon, ribavirin, 6-azauridine and glycyrrhizin: antiviral compounds active against pathogenic flaviviruses, *Antivir. Res.* 58 (1) (2003 Mar) 73–79.
- [36] L.A. Baltina, Y.T. Tasi, S.H. Huang, H.C. Lai, L.A. Baltina, S.F. Petrova, M.S. Yunusov, C.W. Lin, Glycyrrhizic acid derivatives as Dengue virus inhibitors, *Bioorg. Med. Chem. Lett* 29 (20) (2019 Oct 15) 126645.
- [37] C.M. Simões, M. Amoros, L. Girre, Mechanism of antiviral activity of triterpenoid saponins, *Phytother Res.* 13 (4) (1999 Jun) 323–328.
- [38] T. Sekizawa, K. Yanagi, Y. Itoyama, Glycyrrhizin increases survival of mice with herpes simplex encephalitis, *Acta Virol.* 45 (2001) 51–54.
- [39] G. Hoever, L. Baltina, M. Michaelis, R. Kondratenko, L. Baltina, G.A. Tolstikov, H.W. Doerr, J. Cinatl Jr., Antiviral activity of glycyrrhizic acid derivatives against SARS-coronavirus, *J. Med. Chem.* 48 (2005) 1256–1259.
- [40] J. Doorbar, The papillomavirus life cycle, *J. Clin. Virol.* 32 (Suppl 1) (2005) S7–S15.
- [41] C.B. Buck, D.V. Pastrana, D.R. Lowy, J.T. Schiller, Efficient intracellular assembly of papillomaviral vectors, *J. Virol.* 78 (2004) 751–757.
- [42] H. Wang, R. Xu, Y. Shi, L. Si, P. Jiao, Z. Fan, X. Han, X. Wu, X. Zhou, F. Yu, Y. Zhang, L. Zhang, L. Zhang, D. Zhou, S. Xiao, Design, synthesis and biological evaluation of novel L-ascorbic acid-conjugated pentacyclic triterpene derivatives as potential influenza virus entry inhibitors, *Eur. J. Med. Chem.* 110 (2016) 376–388.
- [43] S. Xiao, L. Si, Z. Tian, P. Jiao, Z. Fan, K. Meng, X. Zhou, H. Wang, R. Xu, X. Han, G. Fu, Y. Zhang, L. Zhang, D. Zhou, Pentacyclic triterpenes grafted on CD cores to interfere with influenza virus entry: a dramatic multivalent effect, *Biomaterials* 78 (2016 Feb) 74–85.
- [44] A.S. Sokolova, C.O. Yarovaya, C.A. Shernyukov, C.E. Pokrovsky, C.A. Pokrovsky, V.A. Lavrinenko, V.V. Zarubaev, T.S. Tretiak, P.M. Anfimov, O.I. Kiselev, A.B. Beklemishev, N.F. Salakhutdinov, New quaternary ammonium camphor derivatives and their antiviral activity, genotoxic effects and cytotoxicity, *Bioorg. Med. Chem.* 21 (21) (2013 Nov 1) 6690–6698.
- [45] T.F. Kubiga, S.H. Alves, R. Weiblen, L.T. Lovato, In vitro inhibition of the bovine viral diarrhoea virus by the essential oil of *Ocimum basilicum* (basil) and monoterpenes, *Braz. J. Microbiol.* 45 (1) (2014 Apr 11) 209–214.
- [46] V.V. Zarubaev, A.V. Garshina, T.S. Tretiak, V.A. Fedorova, A.A. Shtro, A.S. Sokolova, O.I. Yarovaya, N.F. Salakhutdinov, Broad range of inhibiting action of novel camphor-based compound with anti-hemagglutinin activity against influenza viruses in vitro and in vivo, *Antivir. Res.* 120 (2015 Aug) 126–133.
- [47] A.C. Carr, S. Maggini, Vitamin C and immune function nutrients, *Nutrients* 9 (2017) 1211.
- [48] H. Hemilä, Vitamin C and infections, *Nutrients* 9 (4) (2017 Mar 29) 339.
- [49] H. Hemilä, E. Chalker, Vitamin C as a possible therapy for COVID-19, *Infect. Chemother.* 52 (2) (2020 Jun) 222–223.
- [50] V. Kuellmer, Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley & Sons, New York, NY, 2001. Ascorbic Acid. Online Posting Date: Apr 16, 2001.
- [51] A. Krężel, W. Maret, The biological inorganic chemistry of zinc ions, *Arch. Biochem. Biophys.* 611 (2016 Dec 1) 3–19.
- [52] E. Selimović, S. Jeremić, B. Ličina, T. Soldatović, Kinetics, DFT study and antibacterial activity of zinc(II) and copper(II) terpyridine complexes, *J. Mex. Chem. Soc* 62 (1) (2018) 1.
- [53] R.S. Mboumba Bouassa, B. Gombert, A. Manarini, L. Bélec, In vitro inhibitory activity against HSV and HPV of the monoterpenoid zinc tetra-ascorbo-camphorate, in: Abstract Presented at 7th World Conference on Pharmaceutical Science and Drug Manufacturing, Flora Creek Deluxe Hotel Apartments, Dubai, United Arab Emirates, 2020. Available at: <https://bioleagues.com/downloads/book/Pharma-Dubai-UAE.pdf> (last access 23th August 2020).
- [54] L. Van Damme, G. Ramjee, M. Alary, B. Vuylsteke, V. Chandeying, H. Rees, P. Sirivongrangson, L. Mukenge-Tshibaka, V. Etiëgne-Traoré, C. Uaeowitchai,

- S.S. Karim, B. Mäse, J. Perriens, M. Laga, COL-1492 Study Group, Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial, *Lancet* 360 (9338) (2002 Sep 28) 971–977.
- [55] L. Kizima, A. Rodríguez, J. Kenney, N. Derby, O. Mizenina, R. Menon, S. Seidor, S. Zhang, K. Levendosky, N. Jean-Pierre, P. Pugach, G. Villegas, B.E. Ford, A. Gettie, J. Blanchard, M. Piatak Jr., J.D. Lifson, G. Paglini, N. Teleshova, T.M. Zydowsky, M. Robbiani, J.A. Fernández-Romero, A potent combination microbicide that targets SHIV-RT, HSV-2 and HPV, *PLoS One* 9 (4) (2014 Apr 16), e94547.
- [56] N. Derby, M. Lal, M. Aravantinou, L. Kizima, P. Barnable, A. Rodriguez, M. Lai, A. Wesenberg, S. Ugaonkar, K. Levendosky, O. Mizenina, K. Kleinbeck, J.D. Lifson, M.M. Peet, Z. Lloyd, M. Benson, W. Heneine, B.R. O'Keefe, M. Robbiani, E. Martinelli, B. Grasperge, J. Blanchard, A. Gettie, N. Teleshova, J.A. Fernández-Romero, T.M. Zydowsky, Griffithsin carrageenan fast dissolving inserts prevent SHIV HSV-2 and HPV infections in vivo, *Nat. Commun.* 9 (1) (2018 Sep 24) 3881.