Article

Towards the Development of a Cream with Antiviral Properties Targeting Both the Influenza A Virus and SARS-CoV-2

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Abstract: Objective: Many severe acute respiratory infections are caused by viral pathogens, and viruses are responsible for a large number of deaths worldwide. Among the most common respiratory viruses are the influenza A virus (IAV) and, more recently, the SARS-CoV-2 that emerged in 2019 and caused the most significant human pandemic of the beginning of the 21st century. Both IAV and SARS-CoV-2 share clinical features and a common transmission route through the emission of viral particles via aerosols and droplets. These penetrate the host after entry from the nose and mouth or an indirect mode of transmission via contact contamination of different media. These facts prompted us to investigate the possibility of designing a soft cream with a virucidal activity targeted against IAV and SARS-CoV-2. Methods: We first investigated the action of chemical compounds known to have antiviral properties such as cyclodextrin, or algae extracts containing sulfated polysaccharides, on cultured cells infected with lentiviral viral particles pseudotyped (VP) with either proteins HA (hemagglutinin) and NA (neuraminidase) from IAV or the G protein from the vesicular stomatitis virus or spike-bearing particles in order to select molecules with antiviral activities in human embryonic kidney (HEK293T) cells. Results: Our results show that some cyclodextrin-containing creams can significantly reduce the stability of HANA- and spike-bearing particles when they are applied prior to challenge with a viral inoculum on skin. Conclusions: We observed some specificities of these creams towards either IAV or SARS-CoV-2, indicating that the neutralization of viral activity is correlated with the mechanism of receptor interaction and entry of these two pathogens.

Keywords: SARS-CoV-2; virucides; cyclodextrins; cell culture; formulation; microbiology

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1. Introduction

Respiratory viruses are responsible for the most common causes of acute respiratory infections in humans. Throughout human history, outbreaks of virally induced respiratory diseases have been reported and have caused severe death tolls. Among the many respiratory viruses, influenza, and more recently coronaviruses, are major concerns for human health and have caused millions of deaths over the last century.

The influenza type A virus pandemic (H1N1 subtype), also referred to as the 'Spanish Flu', was one of the most devastating viral pandemics, with an approximate death toll of about 50 million people worldwide in less than 2 years, between 1918 and 1920 [1]. More recently, the new pulmonary disease (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide since December 2019 and was declared a pandemic by the World Health Organization (WHO) in March 2020 [1].

Influenza viruses belong to the orthomyxovirus family of RNA viruses, which is composed of three groups (A, B and C), with type A being the most virulent [2]. Human-to-human transmission of influenza mainly occurs through the air via respiratory droplets or aerosols, but it can also be contracted via contact with contaminated surfaces. IAV infection causes a spectrum of clinical symptoms, from a benign upper respiratory tract infection to fulminant pneumonia, which mostly strikes the elderly and immunocompromised patients [3]. SARS-CoV-2—in addition to MERS-CoV and SARS-CoV-1—belongs to the beta coronavirus family and is considered to have emerged from bats and spread to humans very recently. The mode of transmission from human to human occurs through multiple routes that mainly include the emission and inhalation of droplets and aerosols, but also indirect contamination via hand contact with surfaces and objects [4]. In addition to the fact that both SARS-CoV-2 and influenza viruses are enveloped and contain a single-stranded RNA genome, they also share several common features such as the transmission route, clinical symptoms, viral shedding and the serial interval of disease. For both pathogens, vaccines are available, and although they have considerably restricted the progression of the COVID-19 pandemic and attenuated the severity of the clinical symptoms, they cannot completely eliminate the spread of and infection with viruses [2].

In order to invade and infect their hosts, both SARS-CoV-2 and influenza need to establish initial contact with the upper respiratory tract through aerosols and droplets that are inhaled through the nose and mouth. Thus, a first line of defense against these pathogens consists of wearing facial masks, and this strategy has been adopted worldwide as a simple and efficient measure for slowing down the progression of the pandemic. However, indirect contamination can also occur through contact transmission of the pathogens from various media on which they can settle. As such, virally contaminated droplets can be deposited on the surface of various objects, such as tissues, coins and bank notes, that can be touched by hand. This, in turn, can contact the mucous membranes of the mouth, nose and eyes that then mediate viral entry to and infection of the patient. As for the influenza H1N1 strain, it has been shown [5] that viruses could persist and remain infectious on stainless steel surfaces for 7 days. Recent experiments with SARS-CoV-2 [6] have also demonstrated the presence of viable virus for up to 72 h, depending on the environmental surface conditions tested. This was confirmed in several general studies [7,8] that showed that the vast majority of respiratory tract viruses, such as coronaviruses, influenza and rhinovirus, could persist on inanimate surfaces for a few days, thus concluding that fomite transmission for influenza and SARS-CoV-2 is likely to occur and form part of the infectious process.

Given that contact transmission is a substantial risk factor in the spreading of both influenza and SARS-CoV-2, this emphasizes the need to employ proper hand hygiene to prevent the transmission of these pathogens, and this was officially recommended by the WHO as one of the first preventative measures to slow down the propagation of SARS-CoV-2 [7,9,10]. Although frequent hand washing can be easily performed, this is not the case for the face, which remains exposed in the absence, or inappropriate wearing, of a facial mask. Furthermore, the frequent use of antiviral disinfectants is a major cause of skin irritations, especially in healthcare workers. As such, the prolonged use of these measures induces various skin reactions, with a prevalence ranging from 43% [11] to 97% [12] among healthcare workers exposed to infected patients.

These facts formed the rationale for investigating the design and manufacture of a cream that could be applied to both the face and hands and that would exhibit antiviral activities. Surprisingly, this approach has not been thoroughly investigated since the beginning of the SARS-CoV-2 outbreak, as to our knowledge, there has been only one study published testing the virucidal activity of a cream/lotion on human skin [13]. This is even more surprising if one considers that better face protection would certainly have a significant impact on person-to-person transmission. Therefore, this established the rationale for this work, which was to conceive and produce a daily applicable skin cream with virucidal activity. As highly versatile vehicles, lentivectors are widely used for gene delivery purposes, since they allow for stable integration of transgenes into cell lines and

differentiated cells. Interestingly, they can be engineered to incorporate envelopes from different viral origins that modulate their tropism for recipient cells [14] and authorize their use for serological investigations [15] or the identification of antiviral reagents [16]. In our attempt to compose a cream with viral protective activity, we first tested the ability of different chemical and natural compounds to inactivate lentiviral-based pseudo-particles pseudotyped with the proteins HA (hemagglutinin) and NA (neuraminidase) from influenza or the G protein from the vesicular stomatitis virus in human embryonic kidney (HEK293T) cells. Among these compounds, cyclodextrins have been shown to possess virucidal effects against many viruses cultivated in vitro, including the herpes simplex virus (HSV), respiratory syncytial virus (RSV), dengue virus and Zika virus [17–20]. In addition, sulfated polysaccharides that can be extracted from natural plants or fungi can interfere with several steps in the virus' life cycle without presenting adverse biological effects [21]. Dipotassium glycyrrhizinate (DG), a natural triterpene that can be isolated from the roots of licorice, has been shown to inhibit the replication of hepatitis B virus and human immunodeficiency virus [22,23]. We chose two sulfated polysaccharides from algae extracts: One was furcellaran, a sulfated polysaccharide (carrageenan), which was extracted from the cell wall matrix of red seaweed belonging to the genus *Furcellaria*. The second was an extracellular sulfated polysaccharide (EPS) from the genus *Porphyridium* (*Porphyridium cruentum* extract). The typical antiviral mechanism of most sulfated polysaccharides against enveloped viruses can be explained as follows: These viruses attach to host cells through the interaction between their glycoproteic envelope and the heparan sulfate receptor on the cell surface. The formation of the virus–cell complex primarily relies on ionic interactions between the negatively charged (mostly sulfate) groups in this polysaccharide and the basic amino acids within the glycoprotein [18]. This suggests that the antiviral effect occurs by effectively neutralizing the positively charged sites on the viral envelope glycoproteins, preventing the viral adsorption process. We also tested beta-cyclodextrin (KLEPTOSE®). Cyclodextrins (CDs) are occurring glucose derivatives with a rigid cyclic structure, consisting of (1–4)–linked glucopyranoside units. Beta-cyclodextrin has previously been reported as displaying antiviral activity against a number of enveloped viruses [19]. We also tested calcium D pantetheine (CAD) and DG. We initially tested these compounds in a model of cultured cells challenged with the pseudotyped VLPs containing HA-NA from IAV or the G protein from the vesicular stomatitis virus and expressing a reporter gene in order to assess the virucidal effects of the compounds. In the second part of this study, we used human skin explants derived from donors on which diverse versions of the cream were applied. In this experiment, in addition to HA-VPs, we added lentivectors pseudotyped with the envelope of SARS-CoV-2 into the viral inoculum, and in particular a spike variant, to assay the virucidal activity of our formula on IAV and SARS-CoV-2.

Our results show that some compounds are effective at inactivating VLPs bearing HANA and spike and significantly reduce their infectious potential. Moreover, when incorporated into a cream formulation, these compounds remain as active as in the cell culture. This suggests that they can be developed to be incorporated into a face or a hand cream formulation.

2. Materials and Methods

2.1. Algae Materials and Chemical Molecules Tested

The algae extract (AE) was sourced from the red seaweed *Furcellaria lumbricalis*, located in the Baltic Sea. The Baltic Sea has a salinity of 10 $g \cdot L^{-1}$, very close to physiological serum, which could well explain its affinity for the skin. This extract was obtained from Codif (Saint-Malo, France). Furcellaran is a sulfated polysaccharide that is extracted from the cell wall matrix of red seaweed belonging to the genus *Furcellaria*. The furcellaran was then depolymerized using a patented process. The structure of furcellaran is similar to kappa carrageenan and has been described as a hybrid of kappa–beta carrageenan complex. The essential difference is that kappa carrageenan has one sulfate ester residue per two sugars, whereas furcellaran has one sulfate ester residue per three or four sugar residues. The AE

master solution (10 mg/mL) was prepared in water heated at 70 °C for complete dissolution. The working solutions were prepared rapidly through dilution of the master solution in a warm culture medium. The extracellular sulfated polysaccharide (EPS) extract was developed by Givaudan Active Beauty Marine (Toulouse, France). Red unicellular microalgae from the genus *Porphyridium* (*Porphyridium cruentum* extract) produce an extracellular sulfated polysaccharide (EPS) with acidic characteristics and with potential applications in cosmetics, as an inhibitor of hyaluronidase and having anti-allergic and antiviral properties. Betacyclodextrin (KLEPTOSE®) was developed by Roquette (La madeleine, France). The master solutions for KLEPTOSE® (10 mg/mL), dipotassium glycyrrhizinate (50 mg/mL) and calcium D pantetheine-S-sulfonate (3 mg/mL) were prepared in culture medium. All solutions were filtered through a 0.2 µm-pore-sized filter, except for AE. Hydrapatch® is a unique combination of three polysaccharides: pullulan (repeating maltotriose units), alginate (polymer of D-mannuronic and L-glucuronic units) and hyaluronic acid. Hydrapatch® can regulate the rate and kinetics of absorption of active ingredients and was developed by BASF (Levallois Perret, France). Liposkin® was developed by Lucas Meyer (Massy, France) and is a complex of C12–16 alcohols, hydrogenated lecithin, palmitic acid, phytosphingosine and cholesterol.

2.2. Composition of Formulations for Testing on Skin Explants

Formulations for testing on skin explants were composed as follows:

Formulation a: CD pantetheine 0.7%, KLEPTOSE® 0.1%;

Formulation b: CD pantetheine 0.7%, KLEPTOSE® 0.1%, EPS 5%, AE 2%;

Formulation c: CAD pantetheine 0.7%, KLEPTOSE® 1%, EPS 10%, AE 10%;

Formulation d: CAD pantetheine 0.7%, KLEPTOSE® 1%, EPS 10%, AE 10%, Hydrapatch® 3%;

Formulation b′: CAD pantetheine 0.7%, KLEPTOSE® 0.1%, EPS 5%, AE 2%, Liposkin® 2%;

Formulation c′: CAD pantetheine 0.7%, KLEPTOSE® 1%, EPS 10%, AE 10%, Liposkin® 2%.

For each formulation, 100 mg of cream was prepared.

2.3. Viability and Confluency Assays

Cell viability was measured using the Premix WST-1 Cell Proliferation Assay System (Takara, St. Germain en Laye, France). In brief, 5000 human embryonic kidney (HEK293T) cells (ATCC, Manasas, VA, USA) were plated in a 96-well plate and treated with the different compounds for 48 h. For each condition, the viability was measured in 8 independent dishes (n = 8). The confluency of the treated cells was measured using Inucyte-S3 (Sartorius, Dourdan, France).

2.4. Preparation of Lentiviral Vectors

We first tested the ability of different chemical and natural compounds to inactivate lentiviral-based pseudo-particles pseudotyped with the proteins NAHA (hemagglutinin) and NA neuraminidase) from influenza or the G protein from the vesicular stomatitis virus in HEK293T cells. The lentiviral particles were produced from HEK293T cells plated at 4×10^6 cells in a 10 cm diameter dish. The day after plating, cells were transfected using Jet-Optimus (Polyplus, Illkirch-Graffenstaden, France) with 4 µg of PAX-2 (packaging plasmid; Addgene; #12260), 0.3 µg of pCI-M2 (Addgene; #44170), 1 µg of pNAHA (Addgene; #44169) and 3.7 µg of SWINGY (reporter construct bearing the YFP gene driven by a CMV early promoter derived from GAE) [24]. For VSV-G pseudotyped particles, pNAHA and PCI-M2 were replaced with a phCMV-G plasmid (1 µg). Spike-VPs were produced using HDM-SARS2-spike-del21-D614G (Addgene #158762) as an envelope plasmid (1 µg) and an RFP lentivector (3.7 µg).

Particle-containing supernatants were harvested 48 h after transfection and clarified with 5 min of centrifugation at $500\times g$ before filtration through a 0.45 µm-pore-sized filter.

Next, the particles were ultra-centrifugated at 20,000 rpm in an SW41 rotor (Beckman, Villepinte, France) at 7 °C for 80 min. The sedimented lentivectors were resuspended into cold PBS to obtain a 100×-fold concentration and frozen at −80 °C.

2.5. Titration

To calculate the titer of the G-VP and HA-VP preparations, 2×10^5 HEK293T were transduced using serial dilutions of a lentiviral preparation. The FACS analysis of transduced cells 72 h after transduction allowed for the measurement of the percentage of transduced cells and the calculation of titers as a number of transducing units per mL (TU/mL).

To measure the transduction efficiencies of VPs harvested from the skin, target ACE2-expressing cells were prepared by transfecting 1×10^5 HEK293T cells with 0.75 µg of ACE2 (Addgene; #145033) and 0.25 µg TMPRSS2 (Addgene; #53887) in a 12-well plate. The day after transfection, ACE2-expressing cells were transduced using VPs retrieved from the skin and analyzed 48 h later using FACS. The percentage of cells expressing YFP revealed an efficient transduction with HA-VPs, and the percentage of RFP-expressing cells revealed efficient transduction with spike-VPs.

2.6. Treatment of Particles and Living Skin Explants with Reagents

2.6.1. Treatment of Viral Particles with Compounds

To evaluate the antiviral activity of compounds in a transduction assay, reagents were added at the given concentration into the medium of cultivated HEK293T cells plated in a 96-well plate (2.5×10^4 per dish). After 3 h of culture, diluted viral particles (40 µL) were added to obtain a final volume of 140 µL. The viral dilutions were performed in a reagent containing PBS to avoid modification of reagent concentrations in the final transduction medium. Then, 72 h later, the transduced cells were trypsinized, and the means of fluorescence (MFI) were analyzed using FACS.

2.6.2. Treatment of Skin with Compound-Containing Creams

The skin experiments were performed using NativeSkin® kits (Genoskin, Toulouse, France). This system consists of round skin biopsies prepared from surgical residues from healthy adult volunteers. Written informed consent was obtained from the donors, in full respect of the Declaration of Helsinki and Article L.1243-4 of the French Public Health Code. This method does not require any prior authorization from an ethics committee for sampling and using surgical waste.

The skin explants were cultivated in a 12-well plate format and embedded in a nourishing medium that kept them alive for several days. In this system, the skin surface was exposed and equipped with a silicon ring, allowing for the deposit of a liquid droplet that was maintained on the surface of the skin until evaporation. To evaluate the virucidal effect of creams, NativeSkin® systems were cultivated at 37 °C for 24 h before administration of reagent-containing mixtures. The formulations were added using a polypropylene micropestle (Sigma Aldrich, St. Quentin Fallavier, France) previously immersed in the cream preparation and used to spread the mixture on the skin surfaces. An excess of cream was avoided, and complete absorption of cream by the skin explants was ensured for all tests. We estimated that this treatment administered close to 2 mg of cream per cm^2.

After the formulation treatment, the skin explants were left for 3 h at room temperature before being deposited at the center of a silicon ring of a 50 µL inoculum containing HA-VPs and spike-VPs. After 30 min at room temperature, VLP-containing droplets were harvested from the skin surfaces. Next, the titration of retrieved particles was performed on ACE2-HEK293T cells.

2.7. Statistical Analysis

The results were expressed as mean ± SEM and analyzed using GraphPad Prism 8.4.3 software. Student's *t*-tests were used to compare data sets.

3. Results

3.1. Exploring Compounds That Have the Potential to Prevent Viral Infections

In our attempt to compose a cream with viral protective activity, we tested several compound candidates that were previously identified as natural broad-spectrum antivirals [17–19,21]. Hence, our study first focused on the test of one beta-cyclodextrin, KLEPTOSE® (K), and two sulfated polysaccharides, one being furcellaran, which is similar to kappa carrageenan and has been described as a hybrid of a kappa–beta carrageenan complex from an algae (AE), and one being an exopolysaccharide (EPS) from *Porphyridium cruentum* extract combined with dipotassium glycyrrhizinate (DG) and calcium D pantetheine (CAD). In essence, our study explored different associations of these reagents, whose protective effect against viral infection was measured in HEK293T cells used to titer the residual viral activity. Thus, we first investigated the potential toxicity of each candidate compound when tested on growing HEK293T cells. For this purpose, HEK293T cells were cultivated with increasing concentrations of calcium D pantetheine (CAD), dipotassium glycyrrhizinate (DG), KLEPTOSE® (K), algae extract (AE) and one exopolysaccharide (EPS). After 48 h of culture in serum-containing medium, the cell viability and confluency were measured for each compound.

We next sought to identify whether the five compound candidates had an effect on viral infectivity, and we developed a method for measuring this. For this purpose, we chose a transduction assay where compounds were added into a transduction medium, and efficiencies of infection were monitored after 3 d (Figure 1A). To optimize this assay, we chose to exploit lentiviral vectors that can be engineered to transfer a reporter transgene such as the yellow fluorescent protein (YFP), which facilitates the measurement of infection efficiency. Furthermore, these defective lentiviral particles can be pseudotyped using diverse fusogens, including envelopes from the vesicular stomatitis virus (VSV-G) or the influenza virus (HANA) (Figure 1B). This experimental design allowed us to investigate the antiviral properties of compounds using two different modes for cell entry. Two viral batches of VSV-G-pseudotyped particles (G-VPs) and HANA-pseudotyped particles (HA-VPs) were generated, purified and titrated using human permissive HEK293T cells. A range of low viral non-saturating doses of pseudoparticles corresponding to a multiplicity of infection (MOI) of 3.8 and 0.33, respectively, were applied (Figure 1C). Human HEK293T target cells permissive for both pseudotypes were next transduced with G-VPs and HA-VPs diluted in either phosphate-buffered saline (PBS) for the control or a selection of the compounds of interest at the concentration previously defined. In this assay, we applied pseudoparticles at low doses and verified that that the corresponding multiplicities of infection (number of theoretical particles per target cell: 3.8 for VSVG and 0.33 for HANA) were non-saturating, as illustrated in Figure 1C, in order to detect and measure the effects of tested compounds. The results are expressed as the means of fluorescence measured in recipient cells 3 d after transduction and are plotted in Figure 1D. We noted that most compounds have an impact on the YFP-transfer, with the highest effect obtained with CAD, which significantly inhibited both G-VP and HA-VP transductions (60% and 66% inhibition, respectively). Interestingly, the addition of AE, KLEPTOSE® and, to a lesser extent, EPS in the cell medium also decreased transduction for both pseudotypes, with a marked inhibition for HA-VPs. This test also illustrates some differences between the pseudotypes with a specific inhibition of HA-VP-mediated transduction using dipotassium glycyrrhizinate (DG), which did not affect G-VP transduction in this assay.

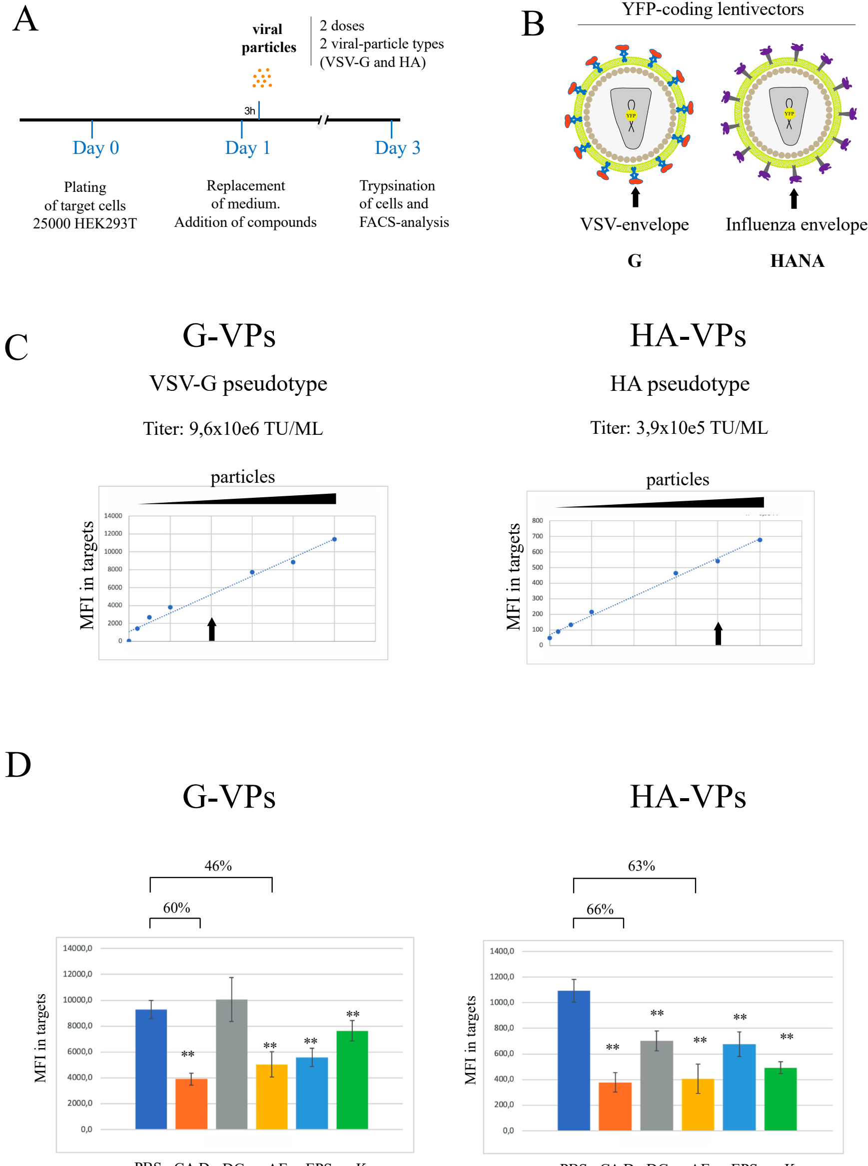


Figure 1. Identification of compounds inhibiting infection in a cell culture transduction assay: (**A**) principle of the transduction assay. HEK293T-target cells plated at d 0. At d 1, the culture media were replaced and supplemented with compounds before infection with two doses of viral particles (VP). At d 3, the means of fluorescence in transduced cells (MFI) were measured using FACS. (**B**) Scheme of VPs used: G-VPs and HA-VPs consist of YFP-encoding lentiviral vectors pseudotyped with VSV-G and HANA, respectively. (**C**) Characterization of particle batches. Graphs represent the dynamic of transduction efficiencies measured for both VP types. Increasing amounts of particles led to a linear increase in means of fluorescence (MFI) in target HEK293T cells. Titers of VP preparations are indicated. Arrows show the doses used in the assay. (**D**) Transduction assays using VPs-G and VPs-HA in HEK293T target cells treated with different reagents: Phosphate-buffered saline (PBS), CAD pantetheine sulfonate (CAD), dipotassium glycyrrhizate (DG), one algae extract containing a hybrid of kappa–beta carrageenan complex (AE), one extracellular sulfated polysaccharide (EPS) extract from the genus *Porphyridium* (*Porphyridium cruentum* extract) and KLEPTOSE® (K). MFI of target cells 48 h after transduction are given for each treatment. Bars indicate percentages of transduction inhibition measured for cells treated with CAD pantetheine sulfonate (CAD) and AE. Statistical analysis is expressed vs. phosphate-buffered saline (PBS) (mean ± SEM; $n = 3$; ** $p < 0.01$, with Student's t-test).

3.2. Identification of Cream Formulations Conferring a Protective Effect against Viral Infection

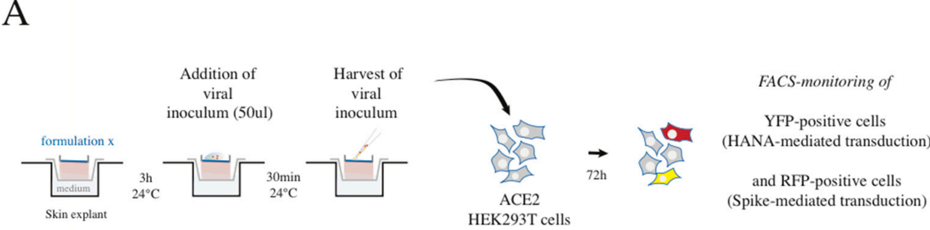
From these data, we next decided to extend our remit and formulate creams in which the active compounds were mixed at different concentrations. To test these formulations, we used the skin explant model, NativeSkin®, a patented and standardized technology that maintains human skin biopsies alive for up to 7 d to enable efficacy and safety testing. In our test, the stratum corneum of the skin explants were first treated through the homogenous application of the cream formulations on the surface, and then its exposure to a viral inoculum containing lentivectors that express fluorescent proteins was measured. After a short period of incubation (30 min) to mimic the natural exposure of human skin to a viral droplet, the inoculum was retrieved from the skin surface, and its infectivity was measured using titration on human cells.

In this experiment, in addition to HA-VPs, we added lentivectors pseudotyped with the envelope of SARS-CoV-2 into the viral inoculum, and in particular, a spike variant identified as an optimal envelope for neutralization assay [25]. The HA-VPs encode YFP, and the spike-VPs were engineered to package a lentiviral construct expressing the red fluorescent protein (RFP). This allows the same FACS assay to be used to measure how the creams affect both HA-VPs and spike-VPs through monitoring the fluorescence of YFP and RFP in the cell population used for titration of the retrieved inoculum. This experimental framework is summarized and depicted in Figure 2A. Briefly, viral inoculum retrieved from treated skins was next used to infect new permissive cells. Should a particular cream affect viral integrity or interfere with a given viral envelope, the transduction abilities of a viral inoculum exposed to the treated skin should be decreased. Thus, our final readout is the level of YFP and RFP into these target cells, with any decrease in fluorescence revealing a potential antiviral effect.

As a positive control, we verified that such an assay could detect the antiviral effect of a hydroalcoholic solution (HS) that destroyed more than 90% of VPs. We also checked for the absence of conflict between HA-VP transduction and spike-VP transduction through measurement of a non-biased proportion of double-positive (YFP + RFP) transduced cells, indicating that the same cell can be transduced with both pseudotypes.

In total, four creams were created containing a combination of cyclodextrins (calcium D pantetheine sulfonate and KLEPTOSE®), as in our previous tests, we identified them as potential antiviral compounds. The addition of AE and EPS to the formulation was also evaluated, since both of these reagents could exhibit a potential antiviral activity. The results are summarized in Figure 2B, and they show the transduction efficiencies of HA-VPs and spike-VPs retrieved from untreated skin (NT), skin treated with a placebo cream (Pl) or with the four different formulations that are labeled from (a) to (d). We observed that treatment of skin explants with a cream containing calcium D pantetheine sulfonate combined with KLEPTOSE® (formulation a) inhibited the viral transduction of HA-VPs by 27% but had no significant effect on spike-VP-mediated transduction. The further addition of AE and EPS at a low concentration did not affect the magnitude of inhibition (formulation b). However, we obtained a significant effect when the formulations with a higher concentration of KLEPTOSE® (1%) and hygroscopic compounds (AE 10% and EPS 10%) were tested, as they decreased the amount of active HA-VPs measured in the retrieved inoculum by about 2-fold (Figure 2B; formulation c and d). This effect was even further increased to 59% inhibition of HA-VPs following the addition of Hydrapatch®. Hydrapatch® is an active ingredient that is a unique combination of three polysaccharides that regulate the rate and kinetics of absorption of active ingredients. The idea was to use it to help maintain the antiviral ingredients in the stratum corneum of the skin explants and prevent their penetration, to improve their reaction with the viral particles.

Likewise, formulations c and d, which are inhibitory to HA-VPs, did not affect the spike-containing viral particles. This suggests that these mixtures have a specific effect on the stability or the entry mechanism of particles using the HA envelope.



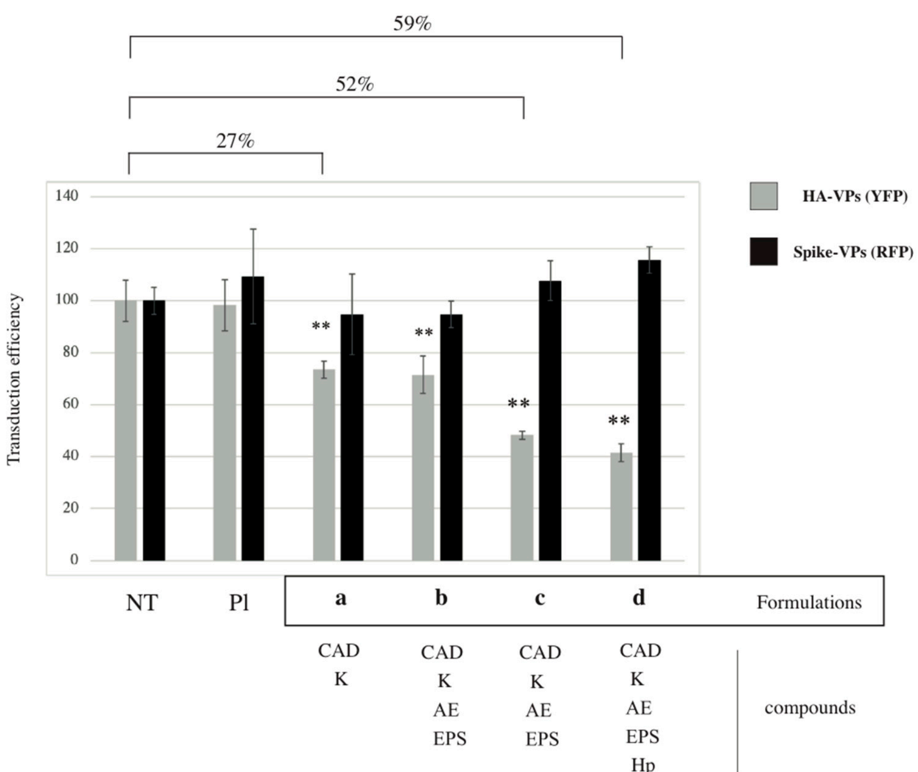


Figure 2. Identification of formulations conferring an antiviral protection to living skin: (**A**) Framework of the living skin assay. Skin explants were cultivated at 37 °C and treated with formulated creams before the addition of a viral inoculum on the skin surface. The viral solutions contained both HA-VPs and spike-VPs. After 30 min of exposure, VP solutions were retrieved from skin surfaces and titered on HEK293T cells expressing ACE2. (**B**) Effect of cream mixture on viral stability. Transduction efficiency measured for non-treated skin (NT) was fixed at 100%. Placebo (Pl) and compound-containing mixtures are indicated. CAD pantetheine sulfonate (CAD), dipotassium glycyrrhizate (DG), AE, EPS, KLEPTOSE® (K) and Hydrapatch® (Hp) concentrations are provided in the Materials and Methods Section. (+) indicates higher concentrations for K, AE and EPS. Results are shown as the infection efficiencies measured for VPs-HA (in grey) or spike-VPs (in black). Analyses were performed 48 h after transduction with harvested inoculum (n = 3). Statistical analysis is expressed vs. NT (mean ± SEM; n = 3; ** $p < 0.01$, with Student's t-test).

3.3. Supplementation of Creams with Liposkin® Decreased the Infectious Capacities of Spike-VPs Retrieved from Treated Skin

Natural emulsifier may considerably improve the efficacy of active ingredients as essential oils or cosmetic products [26,27]. In our effort to develop a protective cream, we tested whether the addition of an emulsifying compound could enhance the antiviral

properties of our candidates. Liposkin® (LIPS) contains hydrogenated lecithin, a natural emulsifier that has also skin-hydrating properties. Though the cream formulations containing LIPS did not show relevant antiviral activity on HA-VPs, we surprisingly observed that the spike-VPs were sensitive to skin treated with LIPS. These results are depicted in Figure 3 and illustrate the transduction efficiencies of spike-VPs after they have been collected from skin surfaces. In all formulations in which LIPS was added (formulations b′ and c′), as well as the LIPS–placebo (Pl′), we noted that the infectious capacity of spike-VPs was decreased by almost 40%. This value was not increased significantly for creams supplemented with the active compounds CAD, K, AE or EPS, suggesting that the action of LIPS does not synergize with other antiviral compounds.

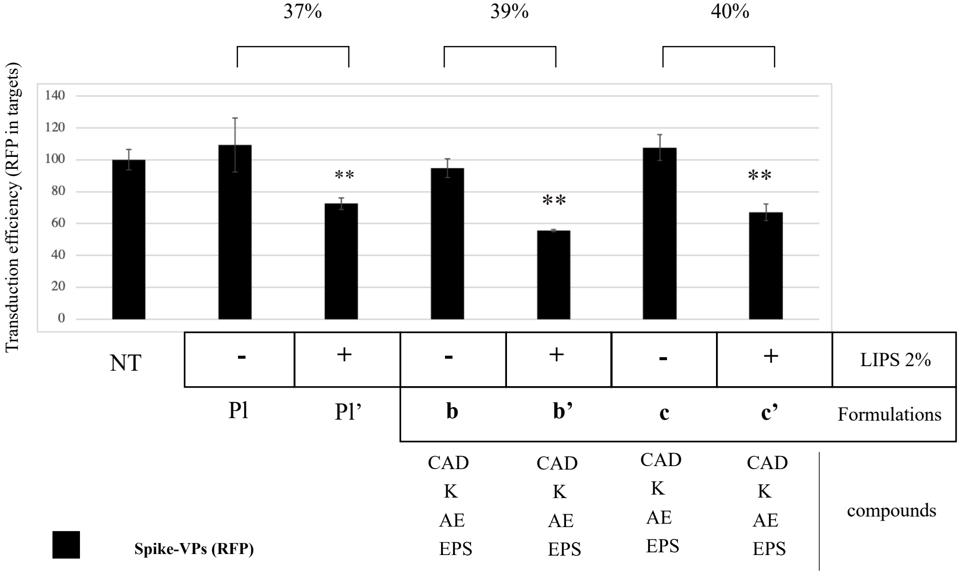


Figure 3. Treatment of skin with Liposkin® decreases the infectivity of exposed SARS-CoV-2 VPs. Cream formulations supplemented with Liposkin® (LIPS) were tested for their capacity to affect the infectivity of spike-VPs. Placebo (Pl) and formulations b and c were supplemented with Liposkin® (LIPS): these new mixtures are noted as Pl′, b′ and c′, respectively. The results show transduction efficiencies achieved with spike-VPs in reporting ACE2-HEK293T (RFP signal) after retrieving of inoculum from treated skin. Compounds in formulation are noted. (+) indicates a 10-fold higher concentration of KLEPTOSE® (K) AE and EPS. Bars indicate the level of transduction inhibition observed for conditions supplemented with Liposkin® (LIPS) as compared with the non-supplemented condition. (n = 3). Statistical analysis is expressed vs. NT (mean ± SEM; n = 3; ** $p < 0.01$, with Student's t-test).

4. Discussion

Both IAV and SARS-CoV-2 have been shown to be remarkably resistant on these surfaces and can remain transmissible and infectious for more than 3 days. As such, this is a major concern for the health professionals who are highly exposed in their working environment. In this context, the development of virucidal creams that can be applied to the hands and face without the adverse effects seen with an HS solution could constitute an efficient first line of defense against viral transmission.

This established a framework for our work in order to develop and identify cream formulations that confer skin protection against the transmission of respiratory viruses. For this purpose, we first focused on the use of previously described antiviral compounds, including cyclodextrin-derived reagents that interfere with the entry of a variety of viruses and limit their transmission 18 Ferreira, 2023 [18,20]. To measure the antiviral effect of these reagents and how they can affect entry of IAV and SARS-CoV-2, we chose to use reporter-coding lentivectors pseudotyped with a HANA envelope or with the spike envelope from SARS-CoV-2. Those defective particles exploit the same entry route and cellular receptors

as the parental virus but are not pathogenic to humans and can be manipulated with minimal safety requirements (BSL2 facilities). We first showed that transduction with these lentivectors encoding fluorescent proteins can be accurately monitored and quantified with fluorescence-activated cell sorting (FACS) analysis (Figure 1). Furthermore, these lentivectors can be pseudotyped with a broad range of viral envelopes, and they provide a safe and rapid method for the evaluation of viral stability. Therefore, this technology could also be effectively adapted to the study of numerous enveloped viruses, including novel emerging strains of SARS-CoV-2.

Initial toxicity assays were performed, which indicated the optimal concentrations at which the compounds should be used to guarantee the appropriate balance between activity and cell viability. Then, our data first indicated that the addition of calcium D pantetheine sulfonate and KLEPTOSE® had the effect of reducing the viral infectivity of both HANA and VSVg pseudoparticles (Figure 1D). This effect was confirmed when the two compounds were added to the cream formulation; those molecules remained active and limited the entry of HA-VPs, especially when the KLEPTOSE® concentration was raised to 1% (Figure 2B; formulations c and d). However, it remains to be determined how these compounds interact with particles on the skin surface. One possibility would be that the exposure of VPs to cyclodextrins could sequester cholesterol from the viral particles, which may, in turn, disrupt lipid rafts and alter the fusogenic properties of the viral envelope. This most probably disorganizes the integrity of HA-VPs, as previously described [28,29]. At the same time, the same compounds only slightly affect the infectivity of the spike-VPs, suggesting that the SARS-CoV-2 envelope is resistant to these compounds. On the other hand, we noted that LIPS, an emulsifying agent used in cosmetic creams to create an oil–water interface on the skin and that can optimize the activity of reactive biomolecules, specifically altered the capacity of spike-VPs to infect new recipient cells after exposure to LIPS-treated skin (Figure 3). This surprising result showed that HANA and spike particles, though both enveloped, do not exhibit the same biological properties nor the same resistance to external reagents, even if those reagents are not specifically designed to target their respective viral envelope.

In addition to the identification of cream formulations that can serve as a base for future developments, our results show the first proofs of concept of a strong experimental design that can be used to test lotions or creams on human skin and assess their interactions with human pathogens.

In this work, we have assayed the potential anti-viral activities of several creams on human skin explants derived from donors. Our results showed different anti-viral specificities of these creams towards either IAV or SARS-CoV-2 envelopes, indicating that viral activity is correlated with receptor interaction and entry of these two pathogens.

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Institutional Review Board Statement: The study was performed on biopsies, obtained from surgical residues after written informed consent from the donor, in full respect of the Declaration of Helsinki and article L.1243-4 of the French Public Health Code. The latter does not require any prior authorization by an ethics committee for sampling and using surgical residues.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: P.M. and T.O. declare a conflict of interest. This work was part of collaborative work between CIRI/INSERM/CNRS/ENS/UDL and LVMH Recherche. The remaining authors are employed by LVMH Recherche.

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