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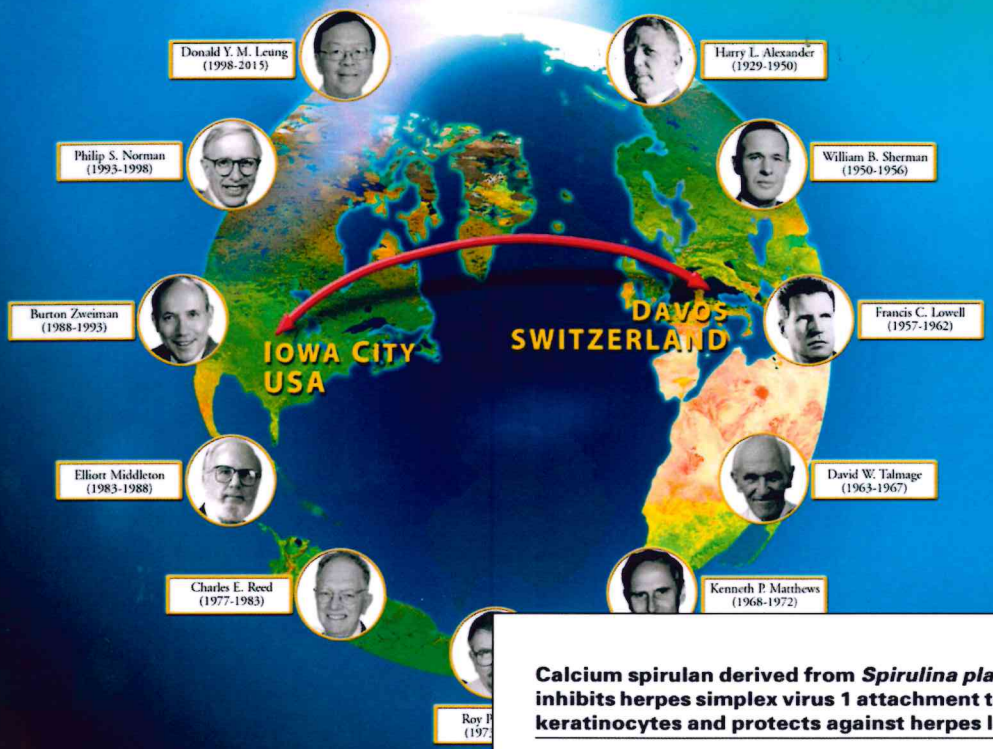

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ELSEVIER

Calcium spirulan derived from *Spirulina platensis* inhibits herpes simplex virus 1 attachment to human keratinocytes and protects against herpes labialis

Julia Mader, MEd,[a,b] Antonio Gallo, PhD,[a] Tim Schommartz, MSc,[a] Wiebke Handke, PhD,[a] Claus-Henning Nagel, PhD,[a] Patrick Günther, RPh,[c] Wolfram Brune, MD,[a,d] Kristian Reich, MD, PhD[b] *Hamburg and Reinbek, Germany*

Background: Chronic infections with herpes simplex virus (HSV) type 1 are highly prevalent in populations worldwide and cause recurrent oral lesions in up to 40% of infected subjects. **Objective:** We investigated the antiviral activity of a defined *Spirulina platensis* microalga extract and of purified calcium spirulan (Ca-SP), a sulfated polysaccharide contained therein. **Methods:** The inhibitory effects of HSV-1 were assessed by using a plaque reduction assay and quantitative PCR in a susceptible mammalian epithelial cell line and confirmed in human keratinocytes. Time-of-addition and attachment experiments and fluorescence detection of the HSV-1 tegument protein VP16 were used to analyze the mechanism of HSV-1 inhibition. Effects of Ca-SP on Kaposi sarcoma–associated herpesvirus/human herpes virus 8 replication and uptake of the ORF45 tegument protein were tested in human retinal pigment epithelial cells. In an observational trial the prophylactic effects of topically applied Ca-SP were compared with those of systemic and topical nucleoside analogues in 198 volunteers with recurrent herpes labialis receiving permanent lip makeup. **Results:** Ca-SP inhibited HSV-1 infection *in vitro* with a potency at least comparable to that of acyclovir by blocking viral attachment and penetration into host cells. Ca-SP also inhibited entry of Kaposi sarcoma–associated herpesvirus/human herpes virus 8. In the clinical model of herpes exacerbation, the prophylactic effect of a Ca-SP and microalgae extract containing cream was superior to that of acyclovir cream. **Conclusion:** These data indicate a potential clinical use of Ca-SP containing *Spirulina* species extract for the prophylactic treatment of herpes labialis and suggest possible activity of Ca-SP against infections caused by other herpesviruses. (J Allergy Clin Immunol 2016;137:197-203.)

Key words: *Herpes simplex virus 1, Kaposi sarcoma–associated herpesvirus/human herpes virus 8, Spirulina microalgae, calcium spirulan, keratinocytes, herpes labialis, Kaposi sarcoma*

Infections with herpes simplex virus (HSV) type 1 are highly prevalent in populations worldwide and frequently associated with chronic exacerbations. Sixty percent to 90% of the world's population are seropositive, and 20% to 40% of infected subjects have recurrent symptoms.[1] The primary infection, which is often asymptomatic and affects mainly the orofacial region, leads to a latent viral infection of the sensory neurons. Reactivation is triggered by endogenous and exogenous stimuli, such as emotional stress, UV light exposure, immunosuppression, or mechanical injuries, and can occur with or without symptoms. Herpes labialis, the most common form of symptomatic HSV-1 recurrence, is usually mild and self-limited but can cause considerable distress in subjects with frequent exacerbations. Eczema herpeticum, the symptomatic infection of larger skin areas usually affected by atopic dermatitis; herpes simplex keratitis; and herpes encephalitis are rare but severe complications of HSV-1 infection.[2,3]

Spirulina platensis (*Arthrospira platensis*) is a filamentous blue-green microalga naturally occurring in rivers and lakes with high salt content in the subtropical and tropical regions of Central America, Southeast Asia, Africa, and Australia. It is used as a dietary supplement,[4] and various rodent models suggest protective effects in experimentally induced damage of different organ systems, including the liver, heart, and kidney.[5-7] More recently, dietary *S platensis* was found to reduce UV-induced skin inflammation and carcinogenesis in wild-type and genetically susceptible mice.[8] Bioactivity-directed fractionation of an *S platensis* hot water extract found to inhibit HSV-1 infection[9] led to identification of an antiviral sulfated polysaccharide, which was named calcium spirulan (Ca-SP).[10] From experiments in Vero cells, a lineage originally isolated from African green monkey kidney epithelial cells, it was concluded that Ca-SP blocks the penetration of HSV-1 into host cells.[10,11] Further structural analysis revealed Ca-SP to be composed of rhamnose, 3-O-methylrhamnose, 2,3-di-O-methylrhamnose, 3-O-methylxylose, uronic acids, and sulfate ester.[12] The chelation of calcium ions with sulfate groups is considered indispensable to the antiviral effects.

Here we establish the HSV-1 antiviral effects of a *Spirulina platensis* microalgae extract (SPME) and purified Ca-SP in human keratinocytes and show that inhibition of viral attachment is the

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Abbreviations used
ACV: Acyclovir
Ca-SP: Calcium spirulan
CHX: Cycloheximide
HSV: Herpes simplex virus
IC_{50}: Half-maximal inhibitory concentration
KSHV/HHV-8: Kaposi sarcoma–associated herpesvirus/human herpes virus 8
MOI: Multiplicity of infection
pfu: Plaque-forming units
PMU: Permanent lip makeup
PRA: Plaque reduction assay
SPME: *Spirulina platensis* microalgae extract
$TCID_{50}$: 50% Tissue culture infective dose

likely underlying mechanism. We extend the spectrum of antiviral effects of Ca-SP to Kaposi sarcoma–associated herpesvirus/human herpes virus 8 (KSHV/HHV-8), which is the cause of cutaneous Kaposi sarcoma, a disease accessible to topical therapy. Finally, we show the prophylactic effects of a newly developed cream containing Ca-SP and SPME in a clinical model of HSV reactivation, indicating potential clinical relevance.

METHODS

Cell culture

HaCaT (CLS 300493), Vero (ATCC CCL-81), and retinal pigment epithelial (RPE-1; ATCC CRL-4000) cells were used as host cells. HSV-1 strain 17+, provided by Roger Everett (Medical Research Council, Center for Virus Research, Glasgow, United Kingdom), was propagated and titrated by using a plaque assay on Vero or HaCaT cells, according to standard procedures.[13] A lytically replicating KSHV/HHV-8[14] was grown and titrated on RPE-1 cells by using the 50% tissue culture infective dose ($TCID_{50}$) method, as previously described.[15] Culture conditions and viability tests are described in the Methods section in this article's Online Repository at www.jacionline.org.

Microalgae and inhibitory compounds

SPME was generated by using a patented extruder-based method (Ocean Pharma GmbH, Reinbek, Germany), as described in the Methods section in this article's Online Repository. Ca-SP was extracted by using a modified 3-step method[12] and finally provided as lyophilized powder dissolved in H_2O (10 mg/mL; IGV, Potsdam, Germany). Purity of the obtained Ca-SP was between 94% and greater than 98%, as determined by using HPLC/gel permeation chromatography and refractive index analysis. Acyclovir (ACV), heparin sodium salt, and foscarnet were obtained from Sigma (Taufkirchen, Germany) and dissolved in H_2O.

Cell-culture assays for antiviral effects

The inhibitory effects of HSV-1 were tested in Vero cells by using a plaque reduction assay (PRA), essentially as described.[13] Inhibitory effects in HaCaT cells were analyzed by using the same conditions, except that plaques were counted 72 hours after infection. Inhibitory effects on KSHV/HHV-8 were tested by using a $TCID_{50}$ reduction assay in RPE-1 cells. As a second method, HSV-1 and KSHV/HHV-8 infection was quantified by using quantitative PCR. Details are provided in the Methods section in this article's Online Repository.

Cell-culture assays for mechanisms of HSV-1 and KSHV/HHV-8 inhibition

In time-of-addition experiments Ca-SP, SPME, ACV, and heparin (2× half-maximal inhibitory concentration [IC_{50}]) were added to Vero and HaCaT cells at different time points from 2 hours before infection to 24 hours after infection, and plaque-forming units (pfu) were determined.

A modified preadsorption and postadsorption assay was performed to further dissect effects on HSV-1 attachment and penetration.[16] Briefly, compounds were added exclusively either before attachment or during the postattachment phase, and their effects on HSV-1–induced plaque formation were determined 72 hours after infection, as previously described.

Finally, HaCaT cells were incubated with Ca-SP, ACV, and heparin, followed by exposure to HSV-1 at 4°C. Nonattached virus was removed by means of extensive washing, and cells were incubated at 37°C in the presence of cycloheximide (CHX; Sigma). Cells were either harvested for flow cytometry 3.5 hours after infection or processed for immunofluorescence staining 5 hours after infection. In both assays VP16 was detected by using a rabbit polyclonal anti-VP16 antibody (Sigma), followed by an Alexa Fluor 488–coupled anti-rabbit antibody (Invitrogen, Darmstadt, Germany). A similar approach was used to further investigate the effects of Ca-SP on KSHV/HHV-8 attachment. Briefly, RPE-1 cells were incubated with test substances, as described for HSV-1, and infected with KSHV/HHV-8. The tegument protein ORF45[17,18] was detected by using a mouse anti-ORF45 mAb (Santa Cruz, Heidelberg, Germany). Details of the culture conditions and analysis of VP16 and ORF45 are provided in the Methods section in this article's Online Repository.

Clinical model of herpes reactivation

Subjects with a history of herpes labialis undergoing permanent lip makeup (PMU), a cosmetic tattoo of the lip lining, have a high risk of herpes exacerbation. Therefore cosmetic institutes offering PMU frequently ask susceptible clients to use either topical or systemic prophylactic antiviral treatment before and after the procedure. Thirty-five cosmetic institutes in Germany offering PMU participated in this observational trial. Adult subjects (>18 y years) with previous herpes labialis who planned to have PMU were asked to either follow the standard scheme of the institute or apply a cream containing SPME and Ca-SP (Spirularin HS; Ocean Pharma) for prophylaxis of herpes labialis. Standard prophylaxis included topical ACV cream (50 mg/g), systemic ACV (400 mg 3 times daily or 200 mg 5 times daily), or systemic valacyclovir (1 g twice daily). Tablets were usually started 2 to 3 days before PMU and continued up to 1 week thereafter. Topical treatments were used twice daily and started 1 week before PMU and continued for up to 2 weeks thereafter. Medical history and herpes activation were assessed by questionnaire before and 1 month after PMU. Clinical observation followed the Declaration of Helsinki protocol.

Statistical analysis

Reoccurrence rates of herpes labialis after PMU were first compared with an overall χ^2 test ($P < .0001$), followed by the Marascuilo *post hoc* multiple proportion comparison test for comparisons between different treatment modalities by using R-3.0.1 software (http://cran.r-project.org/bin/windows/base/old/3.0.1/).

RESULTS

Determination of the HSV-1 antiviral activity of Ca-SP and SPME in human keratinocytes

Neither Ca-SP nor extracts of *S platensis* are standardized preparations. Therefore we first assessed the antiviral potential of our purified Ca-SP and the newly prepared SPME in comparison with ACV in a standard PRA commonly used to investigate HSV-1–inhibitory substances. When added 2 hours before infection of Vero cells, Ca-SP strongly inhibited HSV-1 infection with an IC_{50} of 0.04 μg/mL, which is comparable with ACV (Fig 1, *A*). The results were confirmed by using quantitative real-time PCR analysis of HSV-1 DNA. Inhibition of HSV-1 infection by SPME in this assay was weaker, with an IC_{50} of around

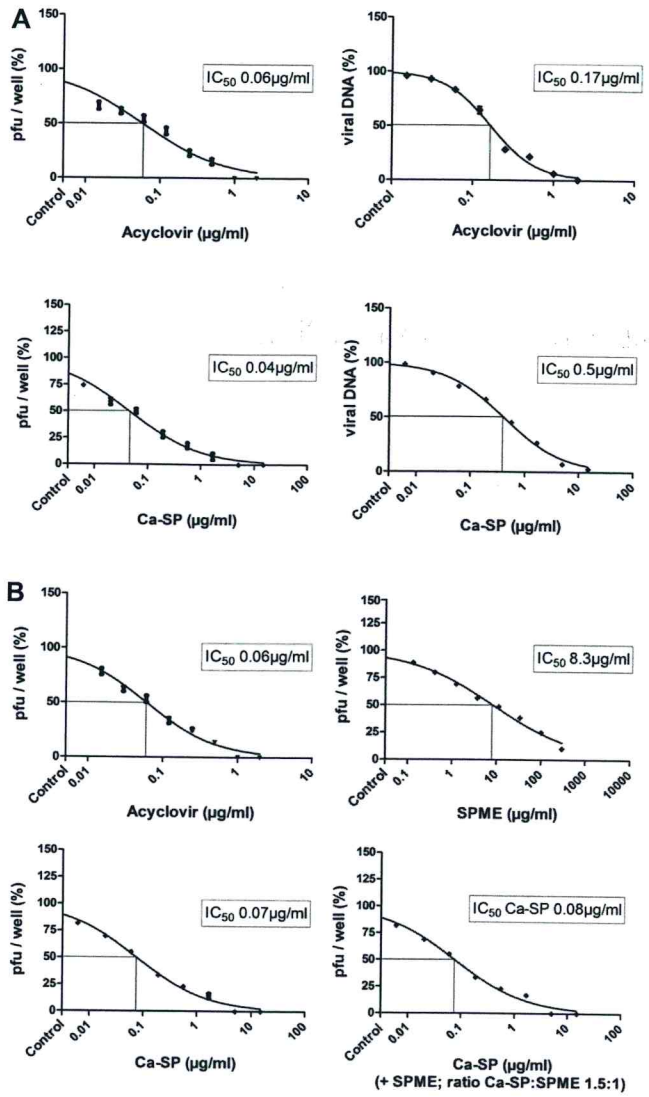

FIG 1. HSV-1 antiviral activity of Ca-SP and SPME compared with ACV. **A,** PRA *(left panels)* and quantitative real-time PCR analysis *(right panels)* in Vero cells show dose-dependent inhibitory effects of Ca-SP comparable with those of ACV. Plaque-forming units per well or viral DNA copy numbers are compared with those of untreated infected control cultures (100%). **B,** PRA in human keratinocytes (HaCaT cells) revealing a strong inhibitory potential of Ca-SP and ACV and a moderate effect of SPME. In the *lower right panel* SPME was added to Ca-SP at a ratio of 1:1.5. Each data point represents 3 independent experiments performed in triplicate. *Error bars* indicating SDs are smaller than the symbols.

30 µg/mL (not shown). Notably, there was little variation in the antiviral potential of Ca-SP as measured based on plaque reduction in Vero cells between batches collected several months apart (IC_{50} range, 0.04-0.2 µg/mL) or between presolved and lyophilized Ca-SP (data not shown), indicating stable bioactivity of the Ca-SP source used.

Vero cells are not from native hosts and might differ from human cells in the exact mechanisms involved in HSV infection, as well as in their sensitivity to HSV inhibitory substances.[16] Therefore we tested the antiviral effects of Ca-SP and SPME in human keratinocytes (HaCaT cells), a relevant cellular target of human HSV-1 infection. The inhibitory potency of Ca-SP with an IC_{50} of 0.07 µg/mL was similar to that observed in Vero cells and also similar to that of ACV in the same cell culture system (Fig 1, *B*). SPME showed a stronger antiviral effect in keratinocytes than in Vero cells (IC_{50}, 8.3 µg/mL). The addition of SPME to Ca-SP in a ratio similar to that contained in the topical formulation (1:1.5) had no synergistic inhibitory activity (Fig 1, *B*). Cell viability measured by using an MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) assay showed no toxic effect of the active substances and solvents used in the experiments (see Fig E1 and the Methods section in this article's Online Repository at www.jacionline.org).

Mechanism of HSV-1 inhibition by Ca-SP in human keratinocytes

Three independent approaches were used to better understand the mechanism involved in the HSV-1–inhibitory effects of Ca-SP in human keratinocytes. In a time-of-addition experiment inhibitory compounds (2× IC_{50}) were added at different time points relative to HSV-1 infection, and reduction of plaque formation was evaluated 72 hours after infection (Fig 2, *A*). Heparin was included as a substance known to interfere with the attachment of HSV-1 to human keratinocytes.[16] In contrast, ACV blocks the later phase of viral DNA synthesis through interference with the viral DNA polymerase. Ca-SP, SPME, heparin, and ACV clearly inhibited plaque formation when added 2 hours before HSV-1 infection. As expected, ACV retained a substantial antiviral activity when added up to 9 hours after infection (Fig 2, *A*), which is consistent with the onset of viral DNA synthesis around 7 hours after infection. In contrast, the antiviral activity of heparin, Ca-SP, and SPME sharply decreased when added more than 1 hour after infection, which is in line with the assumption that Ca-SP and SPME exert their inhibitory effects mainly during the early phase of viral entry.

Entry of HSV into host cells is a multistep process, including attachment (or adsorption) and penetration. A modified PRA was performed in which the attachment and postattachment phases were functionally separated to further dissect the effects of Ca-SP on these entry steps. HSV-1 was added to human keratinocytes at 4°C, and viral attachment was allowed to proceed. Cells were then incubated at 37°C to enable viral penetration and replication. Ca-SP, ACV, and heparin were added at different concentrations exclusively either before the attachment phase or during the postattachment phase, respectively. Ca-SP and heparin inhibited viral attachment (Fig 2, *B*) but had no effect when added during the postattachment phase (Fig 2, *C*). By contrast, ACV exhibited its full antiviral activity when added during the postattachment phase (Fig 2, *C*) but did not prevent attachment (Fig 2, *B*).

To investigate the inhibition of viral attachment by Ca-SP more directly, we analyzed the HSV-1 tegument protein VP16, which becomes detectable in infected cells as a consequence of viral penetration and release of capsid and tegument into the cytosol. Keratinocytes were exposed to saturating levels for infection of HSV-1 (multiplicity of infection [MOI] of 10 pfu per cell) at 4°C. CHX, a protein synthesis inhibitor, was added to ensure that only VP16 delivered on viral entry but not *de novo*–synthesized VP16 would be detected in infected cells. As shown in Fig 2, *D*, when added before viral attachment, high doses of Ca-SP and heparin completely inhibited the delivery of VP16 to cells, whereas ACV had no effect. Experiments were repeated with an MOI of 3 and flow cytometry for the analysis of the infected keratinocytes

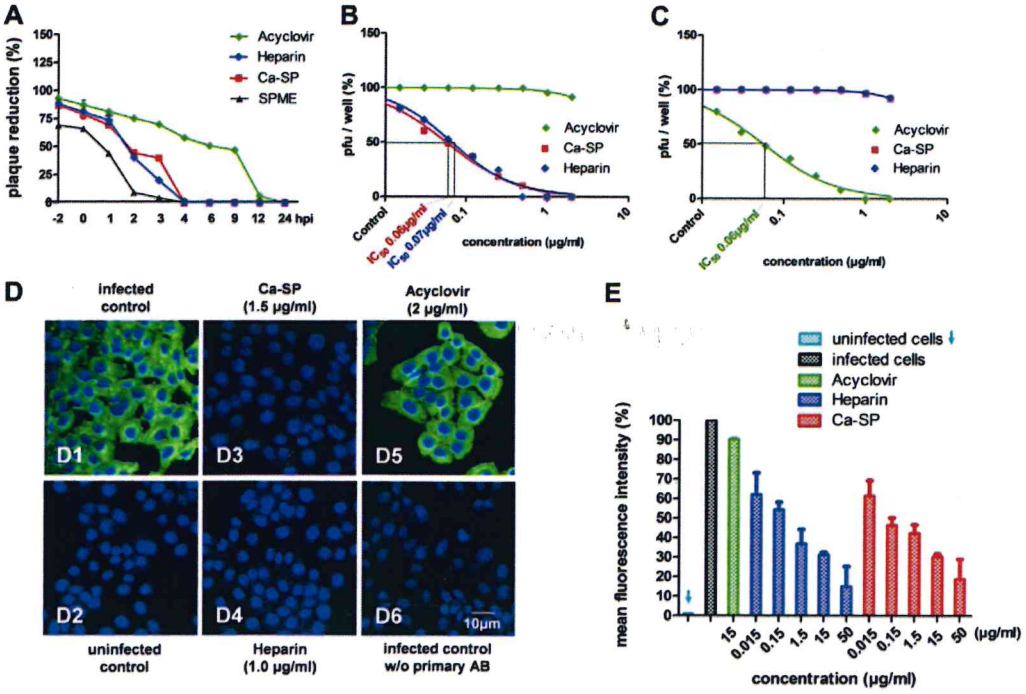

FIG 2. Mechanism of HSV-1 inhibition by Ca-SP in human keratinocytes. **A,** Time-of-addition assay shows that Ca-SP, SPME, and heparin, but not ACV, mainly inhibit plaque formation (plaque reduction [as a percentage] compared with infected untreated HaCaT cells) when added before or during infection. **B** and **C,** Preadsorption (Fig 2, *B*) and postadsorption (Fig 2, *C*) assays indicating inhibition of HSV-1 infection by Ca-SP and heparin when added before but not after viral attachment in contrast to ACV (for details, see the text). **D,** Immunofluorescence detection of the viral tegument protein VP16 (*green*) in infected *(D1)* but not uninfected *(D2)* HaCaT cells. High concentrations of Ca-SP *(D3)* and heparin *(D4)*, but not ACV *(D5)*, inhibit VP16 uptake are shown. Nuclei were counterstained with Draq5 (*blue*). *D6* shows staining without primary antibody. **E,** Dose-response analysis of VP16 uptake by using flow cytometry (mean fluorescence intensity of untreated HSV-1–infected cells set at 100%).

to quantify dose-dependent effects of Ca-SP on viral entry. When allowed to block attachment, Ca-SP and heparin inhibited VP16 delivery to cells in a dose-dependent manner (Fig 2, *E*). By contrast, VP16 delivery was virtually unaltered, even in the presence of high concentrations of ACV.

In summary, these findings indicate that Ca-SP, similar to heparin, inhibits the infection of human keratinocytes by HSV-1 through interference with the attachment phase of viral entry.

Ca-SP inhibits KSHV/HHV-8 infection and attachment to human retinal pigment epithelial cells

Cutaneous Kaposi sarcoma is a malignant skin disease observed mainly in immunocompromised subjects and related to the infection of endothelial cells by KSHV/HHV-8. In a $TCID_{50}$ assay with a susceptible human cell line (RPE-1) cells, Ca-SP was found to exhibit potent dose-dependent KSHV/HHV-8 antiviral activity (Fig 3, *A*). Based on the reduction of KSHV/HHV-8 titers and viral DNA copy numbers in treated versus untreated infected cells, the IC_{50} of Ca-SP was 1.5 µg/mL, which is well below the IC_{50} of foscarnet (40-100 µg/mL; Fig 3, *B*), a drug used for intravenous treatment of KSHV/HHV-8 infection.

To test whether Ca-SP inhibits KSHV/HHV-8 infection through the same mechanism responsible for HSV-1 inhibition (ie, by blocking viral attachment), we performed an assay similar to the one used for HSV-1 in Fig 2, *E*. RPE-1 cells were infected with KSHV/HHV-8 in the presence of Ca-SP, heparin, or foscarnet, and uptake of the viral ORF45 tegument protein was measured by using flow cytometry. As shown in Fig 3, *C*, Ca-SP and heparin inhibited ORF45 uptake in a dose-dependent manner, whereas intracellular ORF45 levels remained virtually unaltered, even in the presence of high concentrations of foscarnet.

A topical formulation containing Ca-SP and SPME has prophylactic effects in a clinical model of herpes reactivation

Because the *in vitro* findings suggested an inhibition of HSV-1 attachment by Ca-SP, we aimed to investigate a potential prophylactic effect of topically applied Ca-SP against herpes labialis. A cream was developed containing Ca-SP and SPME in a concentration of 15 and 10 mg/g, respectively. Although *in vitro* experiments with Ca-SP and SPME in a ratio similar to those contained in the cream showed no synergistic HSV-1–inhibitory effect (Fig 1, *B*), SPME was included to capture antiviral activities not related to Ca-SP[19] and because of its regenerative and antibacterial properties (K. Reich and P. Günther, unpublished observation),[20] with bacterial infections being a known cofactor of herpes labialis.[21]

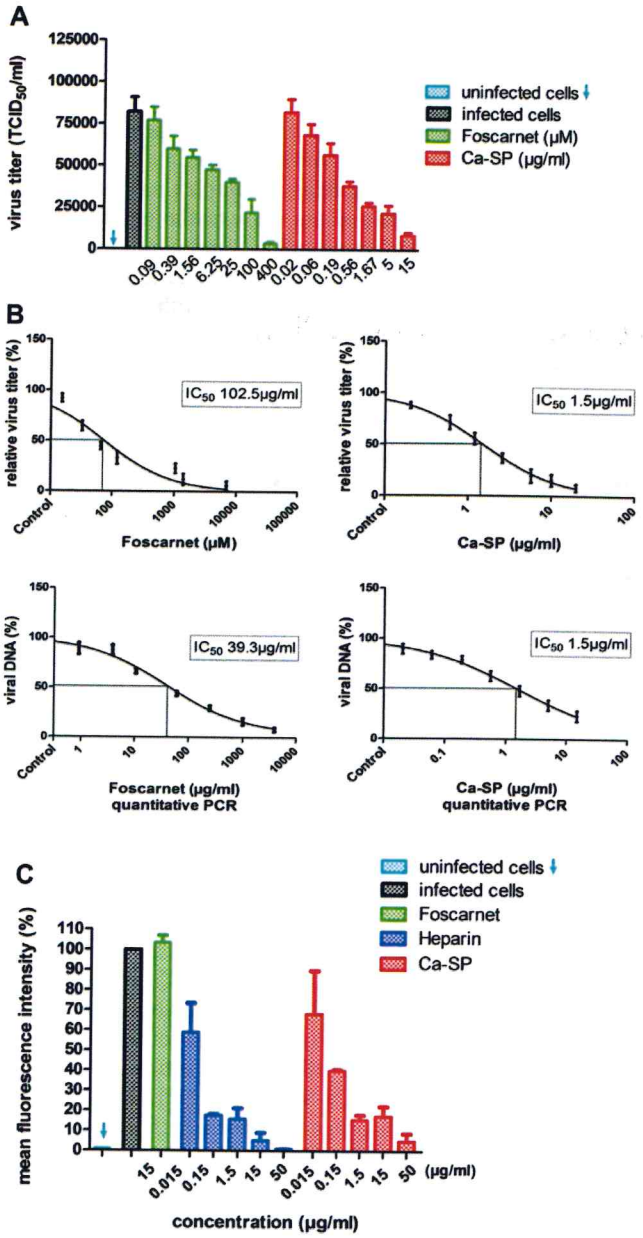

FIG 3. Antiviral activity of Ca-SP against KSHV/HHV-8. **A,** Reduction of viral infectivity (virus titer based on the median $TCID_{50}$) in susceptible human RPE-1 cells showing dose-dependent inhibition of KSHV/HHV-8 by Ca-SP and foscarnet compared with that seen in untreated infected control cultures. **B,** Inhibition of KSHV/HHV-8 by Ca-SP determined through $TCID_{50}$ and expressed as relative viral titer *(upper panels)* or viral DNA copies (by means of real-time quantitative PCR, *lower panels*) compared with untreated infected control cultures, confirming strong anti-HHV-8 activity of Ca-SP. **C,** Dose-response analysis of ORF45 uptake by using flow cytometry (mean fluorescence intensity of untreated KSHV/HHV-8–infected cells set at 100%) showing inhibition of viral entry by Ca-SP.

Two hundred sixty adult volunteers with previous herpes labialis undergoing PMU participated in the observational study. Full documentation was available from 202 subjects, of whom 4 were excluded because of the use of additional topical therapies. Of the remaining 198 subjects, 122 used the cream containing Ca-SP and SPME, 38 used topical ACV, and 38 used systemic ACV or valacyclovir for herpes prophylaxis (Table I). Fig 4, *A*, shows that the Ca-SP and SPME–containing cream was less potent than systemic antivirals but significantly more potent than topical ACV cream in preventing herpes labialis exacerbation. Moreover, if herpes occurred, crusts and dryness of the lips were reported less frequently by patients using the novel cream (Fig 4, *B* and *C*), whereas the duration of herpes labialis was similar in both topical treatment groups (5-8 days compared with 1-4 days with systemic therapy, data not shown). The cream was very well tolerated, and only a few subjects reported perception of an odor (n = 12) or yellowish color (n = 8).

TABLE I. Baseline characteristics of subjects included in the observational trial

	All therapies	SPME + Ca-SP	Topical ACV	Systemic ACV/ valacyclovir
No. of patients	198	122	38	38
Female sex (%)	100	100	100	100
Age (y), mean ± SD	45.4 ± 10.02	45.4 ± 10.53	45.5 ± 9.23	45.3 ± 9.46
Age (y), median (range)	45 (21-76)	45 (21-76)	46 (27-70)	45 (24-68)
Previous herpes labialis (%)	100	100	100	100
Herpes labialis reactivations/ last 5 y, mean	5.14	4.16	5.05	6.65
Herpes labialis reactivations/ last 5 y, median (range)	3.25 (1-50)	3 (1-20)	3.2 (1-35)	4 (1-50)
Previous PMU treatment (%)	100	100	100	100
Any herpes labialis prophylaxis with previous PMU treatment, no. (%)	107 (54.0)	63 (51.6)	18 (47.4)	26 (68.4)
History of atopic dermatitis, no. (%)	6 (3.03)	2 (1.64)	0	4 (10.5)

DISCUSSION

Marine environments contain a plethora of viruses,[22] and microorganisms, such as *S platensis*, have developed antiviral defense strategies during millions of years of evolution. In recent years, natural antiviral molecules have gained increasing attention as a potential source of new treatments for human diseases.[23] In particular, the study of antiviral properties of marine polysaccharides has led to the identification of a number of promising molecules.[24] However, there are several limitations to the available experimental data, including the lack of standardization of the natural sources and extraction processes used and the use of cell-culture or disease models with questionable relevance for human infections. Hence, until now, convincing strategies for the medical use of marine polysaccharides in the treatment of human viral infections are sparse. Here we confirm and extend previous findings regarding the antiviral activity of the sulfated polysaccharide Ca-SP contained in *S platensis* and present first data indicating a potential clinical use as topical prophylaxis against herpes labialis.

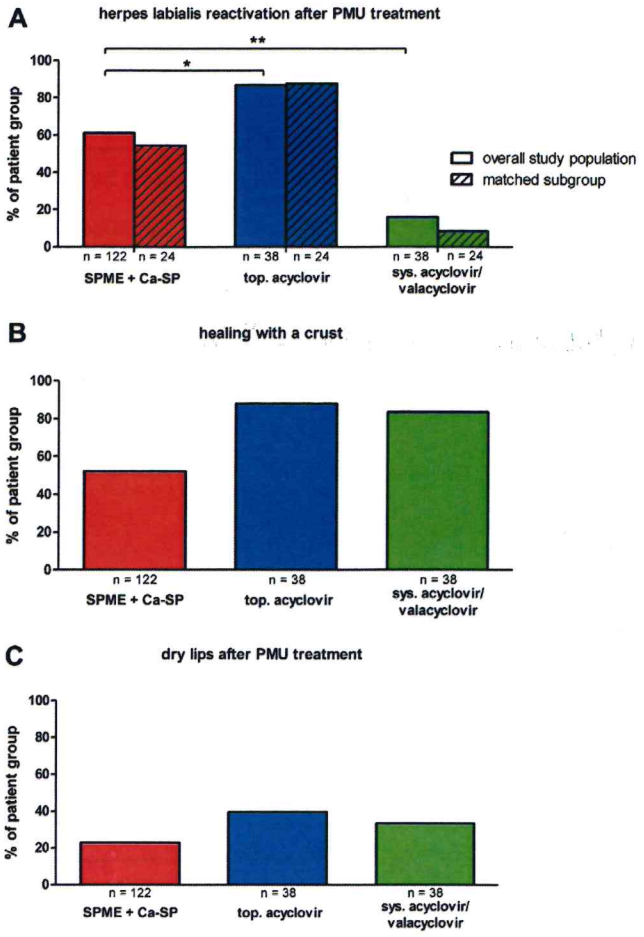

FIG 4. Clinical evaluation of herpes labialis reactivation in susceptible subjects undergoing PMU. **A,** Percentage of subjects with reactivation of herpes labialis after PMU treatment despite prophylactic treatment with a cream containing Ca-SP and SPME compared with topical and systemic antiviral prophylaxis in the overall study population *(solid bars)* and subgroups matched for the frequency of herpes infections in the last 5 years before PMU *(hatched bars).* **B** and **C,** Crust formation (Fig 4, *B*) and occurrence of dry lips (Fig 4, *C*) during the course of PMU-induced herpes labialis in the different treatment groups.

To overcome standardization problems with the extraction process, we developed a novel extruder-based method. Using *S platensis* from one source in Myanmar, this method yielded extracts with reliable antibacterial, antifungal, and antiviral bioactivity over time (K. Reich and P. Günther, unpublished observation). The HSV-1–inhibitory activity of this extract in Vero cells, as measured based on plaque reduction (IC_{50}, approximately 30 μg/mL), compared favorably with previously reported similar measures for hot water extracts (approximately 150-300 μg/mL).[9,25] Purified Ca-SP was obtained by using a modified 3-step protocol[12] with the same source. The original experiments showing inhibition of HSV-1 infection of Vero cells by Ca-SP[10] have been criticized for the indirect methods used[19] and, to the best of our knowledge, have not yet been replicated. Therefore we started by clearly establishing the antiviral potential of purified Ca-SP and the newly prepared SPME in an established standard test used to measure inhibition of HSV-1 infection[13] and confirmed the finding using quantitative assessment of HSV-1 DNA. The inhibitory potential of Ca-SP in these tests (IC_{50}, approximately 0.05-0.5 μg/mL) was at least in the range of similar measures generated earlier with Ca-SP or other *S platensis*–derived polysaccharides (approximately 0.8-2 μg/mL).[10,19] More importantly, the antiviral activity of Ca-SP and SPME could also be established in human keratinocytes, a natural cellular target of HSV-1 infection with specific properties regarding mechanisms of HSV infection and inhibition.[16,26,27]

Entry of HSV-1 into a host cell is a complex process involving attachment (or adsorption) of the virus to the cell surface and penetration into the cell. The simplified current model is that the viral glycoproteins gC, gB, or both mediate attachment of HSV to the cell surface through their interaction with heparan sulfate chains on cell-surface proteoglycans, whereas penetration occurring through fusion of the viral envelope with the plasma membrane requires the viral glycoproteins gB and gD and the hetero-oligomer gH/gL.[28,29] Heparin, a homologue of heparan sulfate, has been shown to inhibit HSV-1 infection of human keratinocytes by reducing viral attachment but not penetration.[16]

Although the exact mechanisms responsible for the inhibition of HSV-1 infection of human keratinocytes by Ca-SP remain to be fully explored, the results of the different experimental approaches presented here indicate that at least 1 major mechanism is the prevention of viral attachment to the cell surface similar to the effect of heparin. Our findings support the concept that the HSV-1–inhibitory effects of the *S platensis* extract depend predominantly on the presence of Ca-SP, but other polysaccharides contained therein might also be involved.[19]

The antiviral activity of Ca-SP and other substances contained in *Spirulina* species extracts might not be limited to HSV-1 because inhibitory effects have been demonstrated in cell-culture models of a variety of other infections by enveloped viruses, including human cytomegalovirus, measles virus, mumps virus, influenza A virus, and HIV-1.[10,19] In this study we tested the antiviral potential of Ca-SP against another human herpes virus, KSHV/HHV-8, because cutaneous Kaposi sarcoma might in principle be accessible to topical therapy, as evident from the successful phase III trial of topically applied alitretinoin gel.[30]

Using a susceptible human cell line, for the first time, we show potent antiviral effects of Ca-SP on KSHV/HHV-8 infection, which appeared to result primarily from the inhibition of viral attachment similar to our observations for HSV-1.

A potential therapeutic use of Ca-SP and SPME is limited by problems associated with different routes of administration. A variety of beneficial effects have been described for dietary *S platensis*, mainly in rodent models of cell damage and, more recently, skin carcinogenesis,[8] but there are very few data documenting medically relevant effects in human subjects.[31,32] Intravenous application of Ca-SP appears to be complicated by its anticoagulant activity.[11] Because of our findings suggesting the blockade of HSV-1 attachment to keratinocytes, we developed a cream containing Ca-SP and SPME in a concentration that would ensure delivery of large enough amounts to the surface of infection-prone skin areas and tested the cream in a clinical model of mechanical herpes labialis reactivation. In this first observational proof-of-concept study the newly developed cream was more effective in preventing herpes labialis reactivation than topical ACV, a standard treatment for lip herpes.[33] The exact type of HSV infection, HSV-1 or HSV-2, was not determined during the study, and although it is likely that the majority of cases were due to HSV-1 reactivation, cases of herpes labialis caused by HSV-2 cannot be excluded. Noteworthy, the inhibitory effects of a *Spirulina* species extract on HSV-2 infection

of Vero cells have been documented.[25] Clearly, these results need to be validated in future randomized double-blind studies.

In summary, our data show inhibition of HSV-1 attachment to human keratinocytes by Ca-SP and possibly other substances contained in *S platensis* extracts. The herpes labialis prophylactic effects of a Ca-SP and SPME–containing cream in high-risk subjects stimulate further studies of the topical use of Ca-SP in the treatment of recurrent genital, oral, and ocular HSV infections, especially in light of recent findings that long-term prophylaxis with conventional antiviral substances predisposes to the development of drug resistance.[34] Further investigations are needed to clarify the potential clinical relevance of the effects of Ca-SP on KSHV/HHV-8 in the treatment of cutaneous Kaposi sarcoma.

Key messages

- **A *Spirulina* species microalgae–derived extract and a defined polysaccharide contained therein, Ca-SP, inhibit the attachment of HSV-1 to human keratinocytes.**
- **Topical application of a cream containing the *Spirulina* species extract and Ca-SP protect against herpes labialis in susceptible subjects, indicating potential clinical relevance.**
- **Ca-SP also inhibits KSHV/HHV-8 infection of susceptible human cells by blocking viral entry.**

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METHODS

Cell-culture and viability tests

All cells were cultivated in Dulbecco modified Eagle medium supplemented with FCS (10% for HaCaT and RPE-1 cells and 8% for Vero cells, respectively), 100 U/mL penicillin, and 100 μg/mL streptomycin (all from PAA Laboratories GmbH, Pasching, Austria) at 37°C in a 5% CO_2 atmosphere. Passaging of cells was done twice a week when confluency approached 80%.

For testing cell viability, Vero, HaCaT, or RPE-1 cells were grown in 96-well dishes and incubated for 48 hours in separate assays with the different concentrations of substances and solvents used in this article (see Fig E1). Cell viability was determined by using an MTS assay (CellTiter 96x AQueous One Solution Cell Proliferation Assay; Promega, Mannheim, Germany). Staurosporine (Sigma) dissolved in dimethyl sulfoxide (Sigma) served as a positive control. Cell viability was not influenced by any of the substances or solvents used.

Generation of SPME

SPME was generated by using a patented extruder-based method (Ocean Pharma). In principle, *S platensis* biomass (IGV, Potsdam, Germany) was converted into a powder and mixed with H_2O in an extruder machine and subjected to high pressure (up to 150 bar) and high temperature (up to 160°C) to increase the extraction of antimicrobial substances. Cells were disrupted by shearing stress and extracted with ethyl acetate to separate active ingredients from cell debris. Ethyl acetate was removed by means of evaporation, and the remaining extract was dissolved in ethanol and exposed to several purification steps, including a filter process with activated carbon. The final extract used in this study had a concentration of 268.7 mg/mL.

Cell-culture assays for antiviral effects

HSV-1 inhibition was assessed in Vero and HaCaT cells. Briefly, cell monolayers cultured in 6-well plates were infected with 50 pfu of HSV-1 per well. After 1 hour on a rocking platform, the inoculum was removed, and cells were incubated with medium containing 25 μg/mL human IgG (Sigma) to prevent secondary plaque formation. Serial dilutions of compounds (SPME, Ca-SP, and ACV) were added at various time points from 2 hours before to 1 hour after infection. All 3 compounds showed their maximum inhibitory effect when added 2 hours before infection, which is the time point given in the main article. Vero and HaCaT cells were fixed 48 and 72 hours after infection, respectively, and stained with 0.1% crystal violet in H_2O. Plaques were counted with an Axiovert 200M (Carl Zeiss, Jena, Germany) inverted microscope, and the inhibitory concentration required to reduce the plaque number by 50% (IC_{50}) in comparison with infected untreated control cells was calculated from dose-response curves of at least 3 independent experiments, each performed in triplicate with Prism 5.03 software (GraphPad Software, La Jolla, Calif). In some experiments Ca-SP and SPME were tested in combination at a ratio of 1.5:1, which is similar to the ratio in the newly developed cream (see the Methods section in the main article).

A $TCID_{50}$ reduction assay was used to determine the antiviral effects of Ca-SP on KSHV/HHV-8 infection. RPE-1 cell monolayers cultured in 96-well plates were infected with serial dilutions of KSHV/HHV-8 (1:10 steps) in the presence of different concentrations of Ca-SP and foscarnet. Cell cultures were centrifuged (for 30 minutes at 37°C and 500*g*) to enhance infection and monitored daily with the Inverted Microscope Axiovert 200M (Carl Zeiss) until green fluorescent foci became detectable. Around 9 days after infection, foci were counted microscopically, and $TCID_{50}$ values were calculated by using the Spearman-Kärber formula. IC_{50} values were calculated as described for the PRA.

For HSV-1 or KSHV/HHV-8 quantitative PCR, cells were harvested and DNA was extracted by using an innuPREP DNA Mini Kit (Analytik Jena, Jena, Germany), according to the manufacturer's instructions. Viral genome copies relative to controls and standards were determined by using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany), as described previously.[E1] The following primers (Invitrogen, Darmstadt, Germany) were used for quantification of HSV-1 *UL27*, KSHV *ORF25*, and human β-actin *(ACTB)* gene copy numbers: *UL27* (5′-GGTAGGTCTTCGGGATGTAAAG-3′ and 5′-CTAAACCTGACTACGGCATCTC-3′), *ORF25* (5′-GGTCCACCCCTTCTTTGATT-3′ and 5′-GCGAGCGGTTGTGGTATATT-3′), and *ACTB* (5′-GCTGAGGCCCAGTTCTAAAT-3′ and 5′-TTCAAGTCCCATCCCAGAAAG-3′).

Cell-culture assays for mechanisms of HSV-1 and KSHV/HHV-8 inhibition

For the time-of-addition assays, Vero and HaCaT cells were infected with HSV-1, as described for the PRAs. Ca-SP, SPME, ACV, and heparin were added to the cells at defined concentrations (2× IC_{50}) at different time points (2 hours before infection, 0 hours/during infection, and 1, 2, 3, 4, 6, 9, 12, and 24 hours after infection). Cells were analyzed by using PRA, as previously described.

For the modified preadsorption and postadsorption assays, HaCaT cells were first preincubated with different concentrations of Ca-SP, ACV, and heparin at 37°C for 2 hours. Cell cultures were cooled to 4°C for at least 30 minutes before precooled HSV-1 was added (allowing for attachment if not blocked by pretreatment with inhibitors). After 1 hour, cells were washed 3 times with PBS, and medium preheated to 37°C was added. For testing effects occurring after adsorption, precooled cells were infected with HSV-1 for 1 hour at 4°C in the absence of inhibitors (allowing attachment). Cells were washed 3 times with PBS, and inhibitors were added in medium preheated to 37°C (allowing for inhibition of postadsorption steps, including penetration). In both cases cells were evaluated for plaque formation, as previously described.

HaCaT cells were treated essentially as in the preadsorption experiment to more directly analyze inhibition of viral attachment by Ca-SP. VP16 uptake was determined by using immunofluorescence and flow cytometry. Briefly, HaCaT cells were grown on cover slips; incubated with Ca-SP, ACV, and heparin for 1.5 hours at 37°C; prechilled on ice; and infected with HSV-1 (MOI of 10) at 4°C in the presence of CHX. After 90 minutes, cells were washed 3 times with PBS, and medium preheated to 37°C was added. Infection proceeded for 3.5 hours in the presence of CHX. Cells were fixed 5 hours after infection with 4% paraformaldehyde (Carl Roth GmbH, Karlsruhe, Germany) in PBS. Cover slips were treated with NH_4Cl (Merck, Darmstadt, Germany) in PBS for 15 minutes, permeabilized with 0.3% Triton X-100 (Carl Roth GmbH) for 10 minutes, and blocked with 0.2% gelatin. Cells were washed with PBS between every incubation step. VP16 was visualized by means of immunofluorescence with a rabbit polyclonal anti-VP16 antibody (1:100; Sigma) and an Alexa Fluor 488–coupled anti-rabbit antibody (1:500; Invitrogen). Nuclei were counterstained with DRAQ5 (1:1000; BioStatus, Leicestershire, United Kingdom). Cover slips were washed with PBS and mounted with Aqua Poly/Mount (Polysciences, Eppelheim, Germany), and sections were analyzed with a confocal laser scanning microscope (LSM-510 Meta, Carl Zeiss). For flow cytometric analysis, HaCaT cells, (3×10^6/10-cm dish) were preincubated, prechilled, and infected (MOI of 3) as described for immunofluorescence labeling. Subsequently, cells were washed and further incubated at 37°C. At 3.5 hours after infection, cells were washed 3 times with PBS and harvested by means of trypsinization. Cells were fixed with 2% paraformaldehyde in PBS (40 minutes at 4°C), permeabilized with 0.1% saponin (Sigma) in PBS (30 minutes at 4°C), and maintained in saponin buffer throughout the staining procedure. VP16 was stained with the same antibodies, and cells were washed as described for immunofluorescence. Flow cytometric analysis was performed with a FACSCanto I flow cytometer and FACSDiva Version 5.0.3 software (BD Biosciences, San Jose, Calif). RPE-1 cells were treated essentially as described for the HSV-1 flow cytometric experiment to further analyze the inhibitory effect of Ca-SP on KSHV/HHV-8 entry. Briefly, RPE-1 cells (6×10^5 per 6 wells) were preincubated with serial dilutions of Ca-SP and heparin for 1.5 hours at 37°C. Concentrations of test substances were similar to those in the HSV-1 flow cytometric assay, but foscarnet was used instead of ACV. Briefly, RPE-1 cells were prechilled and infected with KSHV/HHV-8 at 4°C in the presence of CHX. Cells were washed and further incubated at 37°C. At 3 hours after infection, cells were washed 3 times with PBS and harvested by means of trypsinization. Cells were fixed with 2% paraformaldehyde in PBS (40 minutes at 4°C), permeabilized with 0.1% saponin in PBS (30 minutes at 4°C), and maintained in saponin buffer throughout the staining

procedure. ORF45 was detected with a mouse anti-ORF45 mAb (Santa Cruz Biotechnology), followed by an Alexa Fluor 488–coupled anti-mouse antibody (Invitrogen). Flow cytometric analysis was performed as described above.

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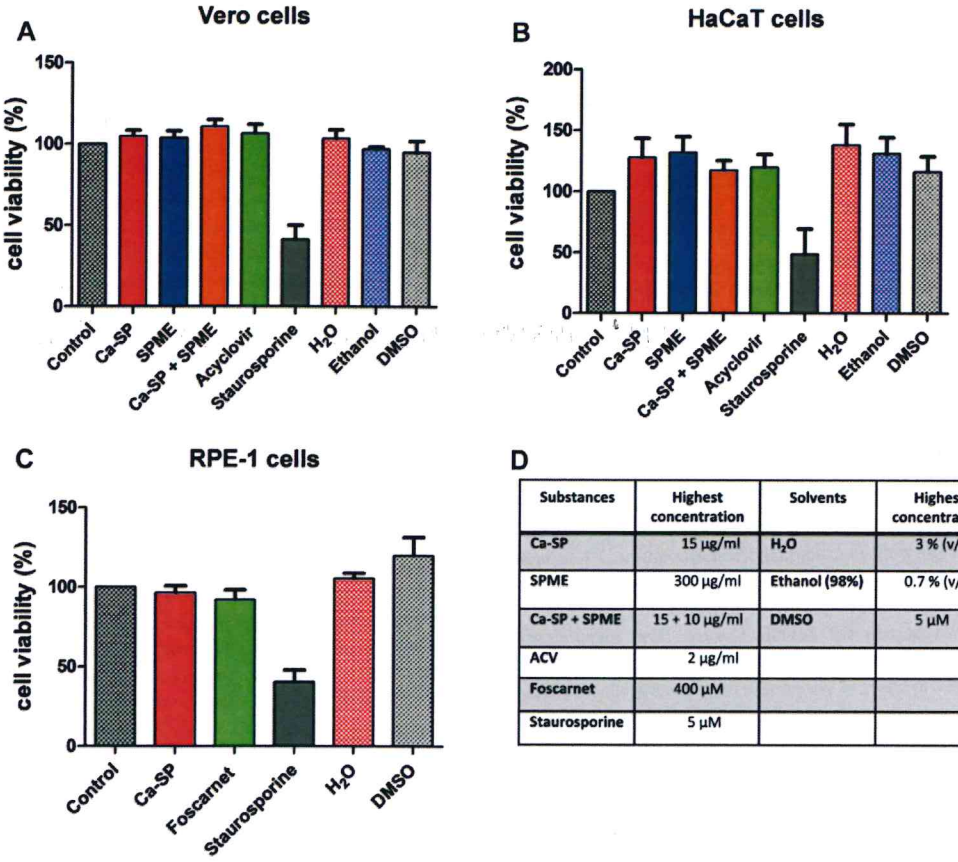

D

Substances	Highest concentration	Solvents	Highest concentration
Ca-SP	15 µg/ml	H_2O	3 % (v/v)
SPME	300 µg/ml	Ethanol (98%)	0.7 % (v/v)
Ca-SP + SPME	15 + 10 µg/ml	DMSO	5 µM
ACV	2 µg/ml		
Foscarnet	400 µM		
Staurosporine	5 µM		

FIG E1. Cell viability test of inhibitory compounds and solvents. **A-C,** Cell viability (in percentages of untreated control cultures) of Vero cells (Fig E1, *A*), HaCaT cells (Fig E1, *B*), and RPE-1 cells (Fig E1, *C*), as measured by using the MTS assay 48 hours after treatment with inhibitory substances (Ca-SP, SPME, combination of Ca-SP and SPME, ACV, and foscarnet) and solvents (H_2O, ethanol, and dimethyl sulfoxide *[DMSO]*). **D,** Results for the highest concentrations used in the experiments described in this article. Staurosporine solved in DMSO served as a positive control. Data represent mean values of 3 independent experiments, each performed in triplicate.