

Antiviral activity of *Arthrospira*-derived spirulan-like substances

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Abstract

Natural substances offer interesting pharmacological perspectives for antiviral drug development in regard to broad-spectrum antiviral properties and novel modes of action. In this study we analyzed polysaccharide fractions isolated from *Arthrospira platensis*. Fractions containing intracellular or extracellular spirulan-like molecules showed a pronounced antiviral activity in the absence of cytotoxic effects. Using specific assays for the quantification of viral replication *in vitro*, these substances exhibited strong inhibition of human cytomegalovirus, herpes simplex virus type 1, human herpesvirus type 6 and human immunodeficiency virus type 1, while only weak or no inhibition was noted for Epstein-Barr virus and influenza A virus. Considering herpesviruses, antiviral effects were most pronounced when the cells were preincubated with the substances prior to the addition of virus, indicating that antiviral action may be primarily targeted to virus entry. However, an inspection of the inhibition of human cytomegalovirus protein synthesis clearly demonstrated that intracellular steps also contributed to the antiviral effect. In the case of human immunodeficiency virus, inhibition occurred at a stage later than viral entry. Thus, spirulan-like substances possess a marked antiherpesviral and anti-HIV activity based on different modes of action. Further development of these substances might yield novel candidates of broad-spectrum antiviral drugs.

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1. Introduction

Recently the interest in identifying naturally occurring antiviral molecules largely intensified (Beutler et al., 2002; Jung et al., 2000; Lee et al., 2004; Yang et al., 2001). A huge chemical diversity can be found amongst natural products derived from plants, microalgae, bacteria, fungi and others. Importantly, a variety of natural substances is known possessing strong antiviral activity. Therefore, a number of laboratories focused their activities in antiviral research on substances derived from plants and microalgae including cyanobacteria (blue-green algae). Among other reasons, these organisms can be cultivated efficiently, possess a high potential to produce biologically active substances and eventually may be selected on the basis of their ethnomedical

use. In previous investigations, we optimized the use of phototrophic microorganisms as sources of biological active substances in photoautotrophic and mixotrophic cultivation modes by developing an adequate cultivation system, i.e. photobioreactor system *Medusa* (Walter et al., 2003). In this study, we analyzed the antiviral activity of intra- and extracellular substances from cyanobacterium *Arthrospira platensis* (formerly *Spirulina platensis*). Calcium spirulan, a sulfated polysaccharide isolated from *A. platensis* was described to possess antiviral activity (Hayashi et al., 1996a; Lee et al., 2001). It consists of two types of disaccharide repeating units, *O*-hexuronosyl-rhamnose and *O*-rhamnosyl-3-*O*-methylrhamnose, and trace amounts of a variety of other saccharidic constituents, i.e. xylose, glucuronic acid and galacturonic acid (Lee et al., 1998, 2000). The presence of sulfate in calcium spirulan was demonstrated by X-ray and flask combustion analysis (Lee et al., 1998). Further forms of sulfated polysaccharides are also produced by green algae and red algae. A broad spectrum of antiviral activity of calcium spirulan and spirulan-like substances was reported, including herpesviruses, paramyxoviruses, influenza viruses and human

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immunodeficiency virus type 1 (Hayashi et al., 1996a,b; Lee et al., 2001, 2004). In these studies, however, many indirect modes of measuring antiviral activity (e.g. measuring the reduction of virus-induced cytopathic effects) were applied. In the present study, we performed direct, virus-specific approaches to characterize antiviral activity of spirulan-like substances. For this, well-established classical methods as well as recently developed reporter-based virus replication assays were applied. As an important result, the susceptibility of selected human pathogenic viruses such as human cytomegalovirus (HCMV), herpes simplex virus (HSV-1), human herpesvirus type 6 (HHV-6) and human immunodeficiency virus type 1 (HIV-1) as well as the non-susceptibility of Epstein-Barr virus (EBV) and human influenza A virus (A/WSN/33) towards spirulan-like substances were observed. Potential determinants of the mode of action are discussed.

2. Materials and methods

2.1. Cell culture and viruses

Primary human foreskin fibroblasts (HFFs) were cultivated in MEM containing 5% (v/v) fetal calf serum (FCS). HCMV AD169 and AD169-GFP were propagated in HFFs and virus replication was quantified by indirect immunofluorescence (IE1-p72-positive cells) or automated GFP fluorometry, respectively (Marschall et al., 2000, 2001). Vero and 293T cells were cultivated in DMEM containing 10% FCS. A clinical isolate of HSV-1 was propagated in Vero cells and titers of viral stocks were determined by plaque assay. Recombinant B-lymphocytes, Raji R7-57, expressing HBsAg under control of the oriLyt of EBV (Marschall et al., 1993) were cultivated in RPMI 1640 with 10% FCS. B95-8 cells were used as EBV producer cell line and cultivated in RPMI 1640 containing 10% FCS. EBV production was induced chemically by the addition of 40 nM TPA (phorbol 12-myristate 13-acetate; Sigma) for 7 days and 3 mM butyric acid for 3 days. HHV-6A strain U1102 was propagated in HSB-2 cells and analyzed as described (Takemoto et al., 2004; Nitsche et al., 2001). Influenza A/WSN/33 virus was propagated in MDCK cells cultivated in DMEM medium containing 2% FCS at 33 °C. Recombinant T-lymphocytes, CEMx174-R5LTR-GFP_{luc}, carrying a green fluorescent protein (GFP) and luciferase expression module under the control of the HIV-1 LTR promoter (Gervaix et al., 1997) were cultivated in RPMI 1640 with 10% FCS. High-titer stocks of HIV-1 strain NL4-3 were produced in CEMx174 cells after the transfer of infectious supernatant from 293T cells transfected with a viral full-length cDNA clone.

2.2. Antiviral substances

For the generation of spirulan-like substances, *A. platensis* was cultivated monoseptically in UTEX *Spirulina* medium in the 25 l-Medusa-type photobioreactor (Walter et al., 2003). Culture conditions were 35 °C, 3.25 l min⁻¹ air flow and constant illumination at a photon flux density of 200 μE m⁻² s⁻¹. For isolation of intracellular polysaccharides (TK-V2), the lyophilized

biomass was first extracted with CH₂Cl₂/MeOH (1 + 1, v/v). Subsequently, the dry biomass was extracted twice with ultrapure water for 1 h at 100 °C, according to Lee et al. (1998). The aqueous extract was centrifuged for 30 min at 10,000 × g, dialyzed (14,000 Da, Roth no. 0653.1) against deionized and ultrapure water and then lyophilized. Dry material was dissolved in 10% trichloroacetic acid and soluble material was dialyzed. Extracellular polysaccharides (TK-V3 and TK-V4) were isolated from culture supernatant by centrifugation of culture broth, lyophilization and dialysis (14,000 Da, Roth no. 0653.1) against deionized and ultrapure water. Substances contained in the primary extract (TK-V3) were further purified by precipitation with 80% ammonium sulfate and dialysis of the centrifuged supernatant (TK-V4). Carbohydrate content of samples TK-V2, TK-V3 and TK-V4 was determined by the anthron-H₂SO₄ assay (Analysis Committee of EBC, Analytica-EBC method 9.26, H. C. Geranke-Verlag, Nürnberg, 1998) with rhamnose as carbohydrate standard (Fluka 83650). Protein content of sample TK-V2, TK-V3 and TK-V4 was determined with biuret method (Merck 1.10307) using albumin fraction V from bovine serum as protein standard (Merck 1.12018). The molecular weight distribution of TK-V4 was estimated with gel permeation chromatography. The distinction between the batches a–c (e.g. TK-V3a, TK-V3b and TK-V3c) was made on the basis that different stock solutions were prepared from the same dry substance at several time points of analysis. Slight differences in biological activities between the batches might arise from minor variations in chemical homogeneity or solubility.

Reference drugs with known antiviral or cytotoxic activity were used as controls, such as ganciclovir (GCV, Cymeven; Syntex-Arzneimittel/Roche), artesunate (ART; Saokim Ltd., Hanoi, Vietnam), amantadine (AMA; Sigma), zidovudine (AZT, GlaxoSmithKline), indinavir (IDV, Merck Sharp & Dohme) and taxol (Paclitaxel; Calbiochem/Merck). Stocks were prepared in aqueous solution (GCV, AZT and IDV), in DMSO (AMA), in 50% DMSO (ART and taxol) or in PBS (TK-V2, TK-V3 and TK-V4) and aliquots were stored at –20 °C.

2.3. Cytotoxicity assay

HFFs or Vero cells were grown in 48-well plates to subconfluent layers and incubated with the antiviral substances in the culture media (without phenol red) for 7 days. Medium samples were taken and assayed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) to determine the lactate dehydrogenase (LDH) released from nonviable cells via colour substrate conversion. For this, cell debris was removed by centrifugation of the medium samples and 50 μl of each sample was incubated with 50 μl of the substrate mix for 30 min in the dark. Thereafter, 50 μl of stopping solution was added. The photometric determination was performed via ELISA Reader (OD 490 nm). In a second assay performed with HFFs in 48-wells, cytotoxicity was determined by the trypan blue dye exclusion assay. After an incubation of the antiviral substances for 7 days, media were removed and cell layers were stained with a 2% solution of trypan blue. The number of cytotoxic cells per field was determined by microscopic counting. Alternatively, cell proliferation

of MRC-5 cells was analyzed by the formazan dye reduction assay using WST-1 (Roche). Therefore, cells were cultured in 96-well plates in 100 μ l DMEM containing 5% FCS and antiviral substances for 1 day. To perform formazan reaction, 10 μ l of WST-1 reagent were added before signals were detected after 1 h by the use of an ELISA reader (OD 450 nm).

2.4. HCMV GFP-based antiviral assay

HFFs were cultivated in 12-well plates and used for infection with a recombinant green fluorescent protein (GFP)-expressing HCMV, AD169-GFP, at a multiplicity of GFP-TCID 0.25 (i.e. 25% GFP-forming tissue culture infectious dose 7 days post-infection). Antiviral substances were added either after virus adsorption (post), or preincubated on the cells for 1 h as well as during virus adsorption (pre), or preincubated plus remaining in the culture media throughout the experiment (pre–post). For preincubation of viral inoculum with the substances, aliquots of a stock of HCMV AD169-GFP (approximately 6×10^5 PFU/ml) were preincubated for 1 h at 37 °C under movement before virus particles were pelleted by centrifugation for 3 h at 25,000 $\times g$ at 10 °C and used for the infection of HFFs cultivated in 12-well plates. All infections were performed in duplicates. Seven days post-infection, cells were lysed and lysates from each well were divided into two samples for further processing and subjected to automated GFP quantification in a Victor 1420 Multilabel Counter (Perkin-Elmer Wallac GmbH, Freiburg, Germany) as described before (Marschall et al., 2000).

2.5. HSV-1 plaque reduction assay

Infection assays were performed with a clinical isolate of HSV-1 on Vero cells in 12-well plates at 90% confluence. Following virus adsorption at 37 °C for 90 min, inoculi were removed and 0.3% agarose overlays, optionally supplemented with antiviral substances, were added to the wells. The staining of viral plaques with 1% crystal violet was performed 4 days post-infection.

2.6. EBV reporter-based antiviral assay

R7-57 cells (a reporter B-lymphocyte clone which indicates lytic EBV replication by reporter protein expression driven by the viral oriLyt promoter; Marschall et al., 1993) were growth-stimulated by the addition of an equal volume of fresh culture medium one day before infection. Then, 5×10^6 cells were collected by centrifugation (2000 rpm, 5 min, 4 °C). In case of preincubation, antiviral substances were added to the cells in a medium volume of 500 μ l and incubated for 1 h. Thereafter, cells were inoculated with 1 ml of a stock of EBV strain B95-8 for 90 min, optionally supplemented with antiviral substances before adding 1 ml of fresh medium. Infected cells were cultivated in 6-well plates for 6 days at 37 °C. As R7-57 cells, lytically superinfected with EBV B95-8, express a secreted recombinant form of hepatitis B virus surface antigen as a reporter protein (HBsAg; Marschall et al., 1989), medium samples were taken and assayed with a diagnostic HBsAg detection kit (Abbott).

2.7. Influenza A GFP-based antiviral assay

293T cells were cultivated in 12-well plates and transfected with the plasmid pHH21-NP-UTRhi-eGFP, which is a reporter construct expressing GFP in specific response to influenza A virus RNA polymerase activity (Lutz et al., 2005). One day post-transfection, the cells were used for infection with influenza A/WSN/33 virus. In case of preincubation, antiviral substances were added to the cells in a medium volume of 500 μ l and incubated for 1 h. Following the addition of virus inoculum and incubation for 90 min for adsorption, cells were cultivated in fresh medium, optionally supplemented with antiviral substances, for further 24 h. Thereafter, the cells were harvested (5000 rpm, 3 min) and lysed before cell debris was removed by centrifugation (10,000 rpm, 5 min). Lysates from each well were then divided into two independent samples and subjected to automated GFP quantification.

2.8. HIV-1 GFP-based antiviral assay

CEMx174-R5LTR-GFPluc cells (kindly provided by Prof. Dr. N.R. Landau, La Jolla, USA; Gervaix et al., 1997) were cultivated in 12-well plates (5×10^5 cells) and used for infection with a high-titer stock of HIV-1 strain NL4-3. Antiviral substances were either preincubated for 1 h and remained on the cells or added after a 1 h-period of virus adsorption. Thereafter, cells were incubated in the presence of antiviral substances in the culture medium for 5 days. Then cells were lysed and lysates were inactivated at 65 °C for 40 min. Cell debris was removed by centrifugation (10,000 rpm, 3 min) before lysates were used for automated GFP quantification.

2.9. Indirect immunofluorescence

HCMV AD169 infection (MOI of 2) was performed with HFFs grown on coverslips in 6-well plates at 90% confluence. Virus adsorption took place either at 4 or 37 °C. At various time points after virus adsorption (0, 1, 4 or 8 h) cells were fixed with 4% paraformaldehyde (10 min, room temperature) and permeabilized using PBS/0.2% Triton X-100 (20 min, 4 °C). Indirect immunofluorescence analysis was performed as described (Marschall et al., 2005). In brief, fixed cells were incubated with primary antibodies (MAb-pp65 28-77, kindly provided by Prof. Dr. W. Britt, Univ. Birmingham, AL, USA; MAb810 IE1-p72/IE2-p86, Chemicon) for 90 min at 37 °C followed by secondary antibody incubation (anti-mouse-FITC, Dianova) for 45 min at 37 °C. Nuclear counterstaining was performed with DAPI Vectashield mounting medium (Vector Laboratories). Data for immunofluorescence were collected by the use of an Axiovert-135 microscope at magnifications of 400 \times (Zeiss). Images were recorded with a Cooled Spot Color Digital Camera (Diagnostic Instruments).

2.10. Western blot analysis

HFFs were grown in 6-well plates overnight to subconfluence and infected with HCMV AD169-GFP at GFP-TCID of 1

(i.e. a GFP-forming tissue culture infectious dose of 100% at 7 days post-infection). Antiviral substances were added either after virus adsorption (post) or 1 h prior and during the 1 h-period of virus adsorption before replaced by fresh culture medium lacking antiviral substances (pre). Alternatively, the cells were preincubated for 1 h and the antiviral substances remained in the culture media throughout the experiment (pre–post). All infections were performed in duplicates. At 20, 48 and 72 h post-infection, infected cells were lysed in 50 μ l RIPA buffer, incubated on ice for 10 min and centrifuged to remove cell debris. Thereafter, 50 μ l sample buffer was added to the lysates. After heating at 95 °C for 10 min, lysates were subjected to standard SDS-PAGE and Western blot detection procedures. Monoclonal antibodies were used as follows: MAb810 IE1-p72/IE2-p86 (Chemicon), MAb-UL44 BS 510 (kindly provided by Prof. Dr. B. Plachter, Univ. Mainz, Germany), MAb-MCP 28-4 (kindly provided by Prof. Dr. W. Britt, Univ. Birmingham, AL) and MAb- β -actin Ac-15 (Sigma).

3. Results and discussion

3.1. Chemical characterization of TK-V2, TK-V3 and TK-V4

Intracellular extract TK-V2 contained a high quantity of carbohydrates and no detectable proteins. Extracellular extract TK-V3 was composed of 41% carbohydrates and 57% proteins, as compared to carbohydrate and protein standards. TK-V4, which was the soluble part of precipitated TK-V3, contained the same amount of carbohydrates (45%); it was composed of at least four substances as indicated by gel permeation chromatography. Fractionation of the extracellular extract TK-V3 by ion exchange chromatography revealed the basic antiviral activity in the anionic polysaccharide fraction, but not in protein fractions.

3.2. Level of substance-induced cytotoxicity

Spirulan-like substances were analyzed for the induction of cytotoxic side-effects in primary human fibroblasts (HFFs) by the quantification of lactate dehydrogenase (LDH) released from nonviable cells. Substances TK-V2b, TK-V3a, TK-V3b, TK-V4a and TK-V4b did not produce any cytotoxic signals in the range of 1.1–90 μ g/ml. The concentrations of the substances resulting in 50% induction of cytotoxicity signals (CC_{50}) are summarized in Table 1. In parallel, LDH assays were also performed with Vero cells and in accordance to the findings for HFFs, no cytotoxicity was measured for either of the substances analyzed up to the concentration of 90 μ g/ml (data not shown). For HFFs, the results were confirmed by using the standard trypan blue dye exclusion assay (including TK-V3c in addition to the substances listed above). No or very low signals of cytotoxicity were counted on the single-cell level for concentrations up to 30 μ g/ml and some signs of cytotoxicity occurring at 90 μ g/ml were clearly less pronounced than those obtained with the positive control (taxol 1 μ M; data not shown). Additionally, human embryonic lung fibroblasts (MRC-5) were analyzed by the for-

Table 1

Antiviral activities of spirulan-like substances against HCMV and cytotoxicity in HFFs^a

Substance	CC_{50} [μ g/ml]	IC_{50} [μ g/ml]		Anti-HCMV index
		Post	Pre–post	
TK-V2b	535.8 \pm 59.0	93.3 \pm 0.1	31.0 \pm 5.8	17.3
TK-V3a	231.7 \pm 60.6	94.8 \pm 0.4	1.4 \pm 0.3	165.5
TK-V3b	460.2 \pm 71.9	92.6 \pm 0.6	2.2 \pm 0.1	209.2
TK-V4a	390.5 \pm 107.2	93.2 \pm 0.4	2.3 \pm 0.3	169.8
GCV	420.8 \pm 30.3	0.7 \pm 0.1 ^b	n.d.	601.1

CC_{50} , substance concentration resulting in 50% induction of cytotoxicity signals in the LDH assay; IC_{50} , substance concentration resulting in 50% inhibition of HCMV replication; post, substances added after virus adsorption; pre–post, incubation of antiviral substances before, during and after virus adsorption; therapeutic anti-HCMV index, CC_{50}/IC_{50} pre–post (post for GCV); n.d., not determined.

^a Values are expressed as means \pm standard deviations of two replicate assays and two sample preparations each.

^b Kaptein et al., 2006.

mazan dye reduction assay. In MRC-5 cells, substances TK-V2 and TK-V3 did not produce any effect when incubated for one day ($CC_{50} > 5$ mg/ml; data not shown).

3.3. Anti-herpesviral activity of spirulan-like substances

Spirulan-like substances were analyzed for anti-herpesviral activities in comparison to GCV used as a reference drug (Fig. 1). For this, a quantitative determination of herpesviral replication was carried out using reporter-based antiviral assays for HCMV and EBV, and a plaque reduction assay for HSV-1. All substances analyzed showed a dose-dependent inhibitory effect on HCMV replication under the conditions of preincubation (pre–post). Hereby, especially TK-V3a, TK-V3b and TK-V4a revealed a strong inhibitory potential at 3.3 μ g/ml or higher. In parallel, incubation of the substances exclusively after virus adsorption did only produce a moderate antiviral effect towards HCMV. This indicates an antiviral action at the stage of virus adsorption and/or penetration. Notably, spirulan-like substances isolated either from intracellular (TK-V2b) or extracellular fractions (TK-V3a, TK-V3b and TK-V4a), both produced a clear anti-HCMV effect. The concentrations of the substances that resulted in 50% inhibition of HCMV replication (IC_{50}), as well as the anti-HCMV indices (CC_{50}/IC_{50}) are presented in Table 1. These data clearly illustrate the enhancing effect of preincubation of substances for the anti-HCMV activity. For example, the lowest IC_{50} value with preincubation (pre–post) is provided by TK-V3a, 1.4 \pm 0.3 μ g/ml, as compared to 94.8 \pm 0.4 μ g/ml without preincubation (post). This stands in contrast to the action of the known inhibitor of viral DNA synthesis, GCV, which produces a strong antiviral effect without preincubation (IC_{50} 0.7 \pm 0.1 μ g/ml) (Kaptein et al., 2006). Furthermore, the anti-HCMV index of TK-V3b, 209.2 (Table 1), which is highest among the spirulan-like substances tested, points out its strong potency as an inhibitor of HCMV replication *in vitro*. In addition, we investigated whether substance TK-V3c may bind to viral particles and alter infectivity. For this, aliquots of HCMV AD169-GFP were preincubated with various con-

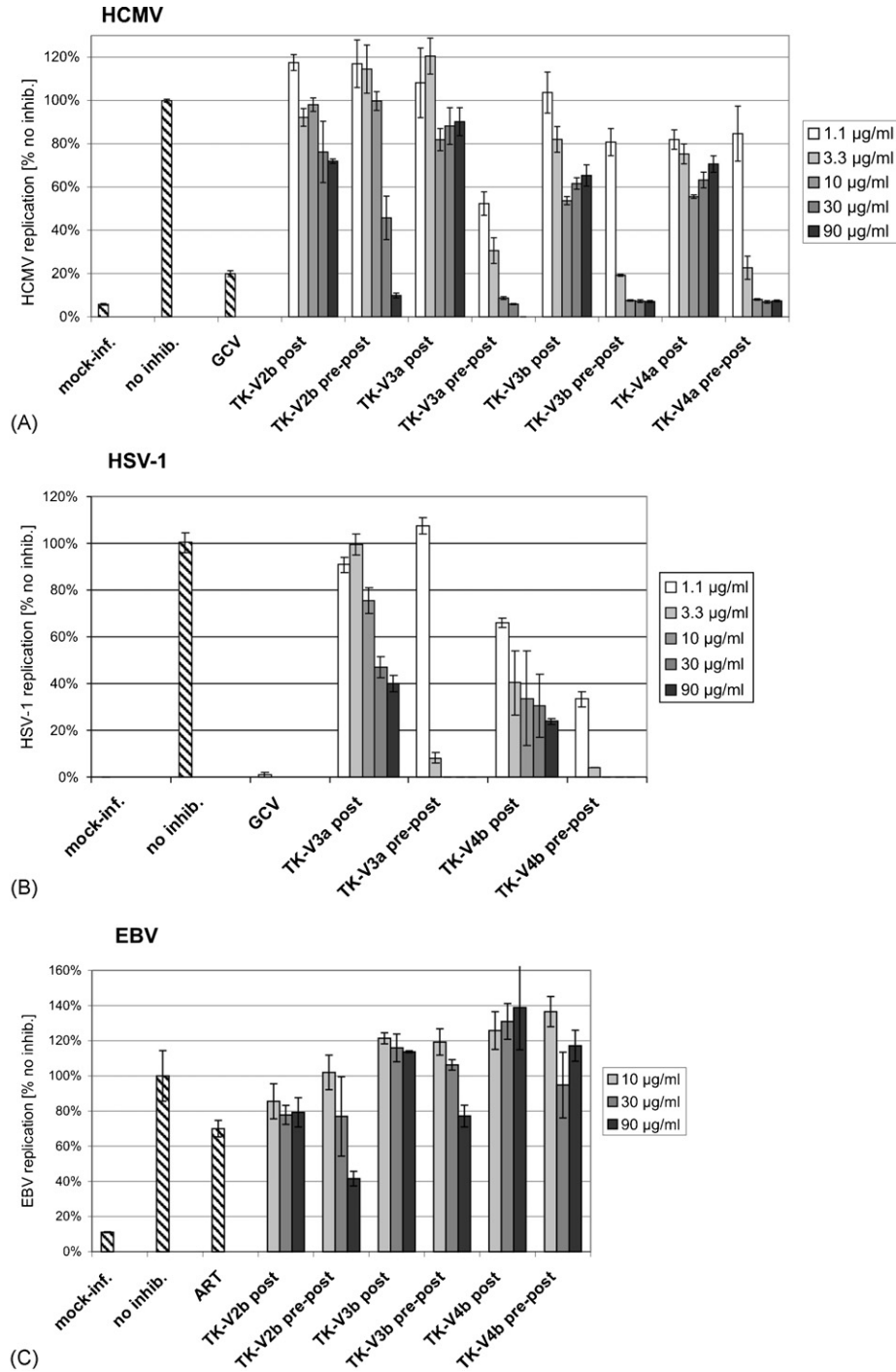


Fig. 1. Determination of antiviral activity towards three herpesviruses: HCMV, HSV-1 and EBV. (A) HFFs were cultivated in 12-well plates and infected with HCMV AD169-GFP. Antiviral substances were added either after virus adsorption (post) or 1 h before as well as during virus adsorption remaining in the culture media throughout the experiment (pre–post). Seven days post-infection, cell samples were subjected to quantification of viral replication by automated GFP fluorometry. A mean of four values is presented in each column (infection in duplicate and lysates processed in duplicate). Mock-infected, no infection; no inhibitor, infection without addition of a substance; GCV, ganciclovir (20 µM). (B) Vero cells were used for infection with a clinical isolate of HSV-1. Antiviral substances were added as described in (A). After virus adsorption, cells were overlaid with agarose medium. Four days post-infection, viral plaques were stained and counted under the microscope. A mean of four values is presented in each column (infection in duplicate and counting of plaques in duplicate). (C) R7-57 cells, stably carrying an EBV-based reporter construct, were used for infection with EBV strain B95-8. Antiviral substances were added as described in (A). Lytic EBV replication, as indicated by the presence of secreted reporter protein in culture media samples, was determined 6 days post-infection. A mean of three values is presented in each column. ART, artesunate (15 µM).

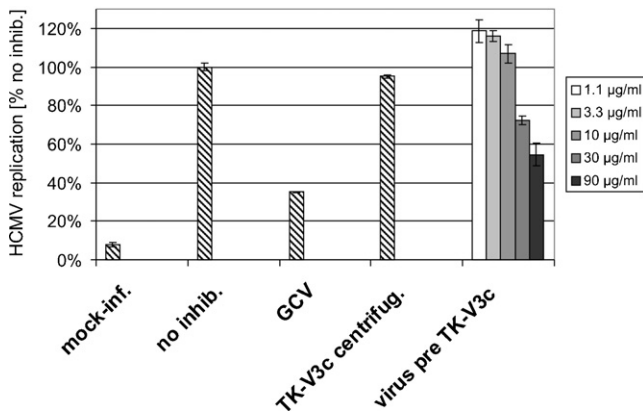


Fig. 2. Determination of anti-HCMV activity by preincubation of an antiviral substance with virus inoculum before infection. Aliquots of HCMV AD169-GFP were preincubated with the indicated concentrations of TK-V3c. Thereafter, virus particles were pelleted and used for the infection of HFFs. Seven days post-infection, viral replication was quantified by GFP fluorometry. A mean of four values is presented in each column (infection in duplicate and lysates processed in duplicate). Mock-infected, no infection; no inhibitor, infection without addition of a substance; GCV, ganciclovir (20 µM); TK-V3c centrifugation, an aliquot of the substance (90 µg/ml, in the absence of virus) was subjected to centrifugation, and the putative pellet was added together with virus inoculum to a control panel of infection.

centrations of TK-V3c before the substance was removed by centrifugation and viral particles were analyzed in an infection assay (Fig. 2). At high concentrations of the substance (30 and 90 µg/ml), partial inhibition of viral replication was measured. This antiviral effect, however, was clearly less pronounced than the effect detected for preincubation of cells with the substances described in Fig. 1A. Thus, direct interaction of the substances with viral particles may add to the overall antiviral effect but

does not explain their full activity. Importantly, this is the first description of antiviral activity of substances isolated from the extracellular fraction of *Arthrospira*.

The results for HSV-1 were very similar to those obtained for HCMV. When preincubated with cells (pre–post), particularly substances TK-V3a and TK-V4b showed a pronounced inhibitory effect on viral replication (Fig. 1B). Although antiviral activity was also detectable without preincubation, the degree of inhibition was considerably lower. This finding suggests similar modes of inhibition for the two herpesviruses HCMV and HSV-1.

Moreover, also HHV-6 showed sensitivity towards spirulan-like substances. The replication of HHV-6A strain U1102 in CCRF-HSB-2 T-lymphocytes was inhibited by substances TK-V2b and TK-V3b. Inhibition was demonstrated by the measurement of viral DNA synthesis one day post-infection (real-time PCR according to Nitsche et al., 2001). The concentration reducing viral load to 50% was less than 250 µg/ml for both substances. Preincubation of the substances (250 µg/ml) on cells 30 min before virus infection, followed by a continued incubation after infection (pre–post), did not increase the antiviral effect compared to an incubation of substances after infection (post) (data not shown). This may indicate differences in the mode of action between HHV-6 and HCMV.

Then, EBV replication was determined using a reporter assay with EBV-specific indicator cells (Fig. 1C) (Marschall et al., 1993). In contrast to the α- and β-herpesviruses described above, almost no or only very low inhibitory effects of the substances TK-V2b, TK-V3b and TK-V4b were measured for the γ-herpesvirus EBV. No more than marginal inhibitory effects were noted, e.g. for substance TK-V2b (pre–post) at 30–90 µg/ml. The drug ART which possesses a broad anti-herpesvirus activity (Kaptein et al., 2006) served as an inhibitor control and

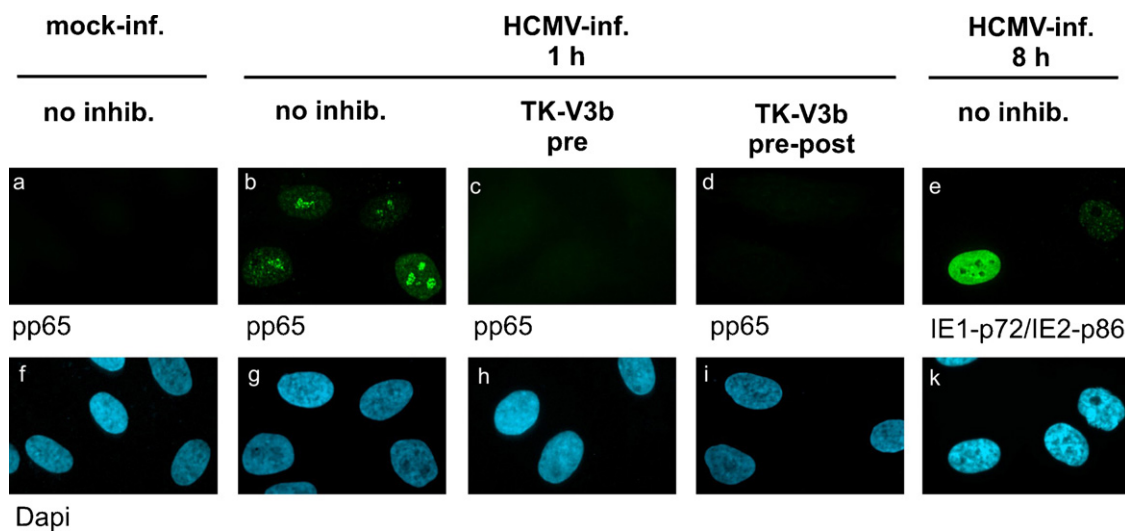


Fig. 3. (a–k) Indirect immunofluorescence analysis to detect the nuclear accumulation of HCMV pp65 directly after viral entry. HFFs were grown on coverslips in 6-well plates and used for infection with HCMV AD169 at a MOI of 2. Cells were preincubated with TK-V3b (10 µg/ml) for 1 h including virus adsorption in the presence of the substance, before the substance was removed and replaced by fresh medium (pre) or cells were preincubated followed by the continuous presence of the substance in the culture medium (pre–post). At the indicated time points, cells were fixed and subjected to an immunostaining of viral tegument protein pp65 (MAb-pp65 28-77) or viral IE proteins (MAb810 IE1-p72/IE2-p86). Cell nuclei were counterstained with DAPI. Mock-infected, no infection; HCMV-infected, HCMV infection with strain AD169; no inhibitor, HCMV infection without addition of a substance.

showed an intermediate level of EBV-specific inhibition. Thus, a strong anti-herpesvirus activity of spirulan-like substances was detected for HCMV and HSV-1, but not for EBV.

3.4. Mode of action of spirulan-like substances towards HCMV replication

Among the spirulan-like substances, TK-V3b was identified as the substance with the highest anti-HCMV index (Table 1). Therefore, an immunofluorescence staining experiment was performed in order to obtain information about the mode of action, in particular to visualize viral entry into human fibroblasts (Fig. 3). After infection with HCMV AD169 at MOI of 2, performed at 4 or at 37 °C, respectively, the cells were fixed at 1, 4 or 8 h post-virus adsorption.

When virus adsorption was performed at 37 °C, infected HFFs in the absence of inhibitor showed a typical dot-like nuclear distribution of pp65 at 1 h post-adsorption (Fig. 3b and g). In contrast, pp65 could not be detected in the presence of TK-V3b (Fig. 3c–d and h–i). Of note, preincubation (pre) of TK-V3b 1 h before and during virus adsorption (without incubation of the substance after virus adsorption) was sufficient to eliminate the pp65 staining signals almost completely. Compared to 1 h, cells analyzed at 4 h post-adsorption showed a slight nuclear accumulation of pp65 when exclusively preincubated with TK-V3b (pre) but were completely negative when continuously incubated with TK-V3b (pre–post) (data not shown). This might indicate that the inhibitory effect was not only directed to virus adsorption but also to virus penetration. The detection of newly synthesized IE proteins at 8 h post-adsorption served as a positive control for the onset of HCMV replication (Fig. 3e and k). For a quantification of inhibitory effects, cells showing the typical dot-like nuclear distribution of pp65 were counted and expressed as values in percent of total cells (Table 2). At 37 °C, 66.7% cells were positive in the absence of inhibitor, whereas only 3.6% cells were positive under preincubation with TK-V3b (pre) or 0.6% cells under continued incubation (pre–post), respectively. When virus adsorption was performed at 4 °C (followed by a shift to 37 °C post-adsorption) the percentage of positive cells was decreased compared to those with virus adsorption at 37 °C (Table 2). Hereby, at 4 °C a lower percentage of positive cells was detected under all conditions analyzed, i.e. in the absence (no inhibitor) or presence of inhibitor (TK-V3b pre, TK-V3b pre–post), and the relative levels of reduction were very similar. Since this procedure was chosen to induce a delayed and syn-

chronized mode of virus penetration, one would have expected no difference between the conditions of pre and pre–post, if inhibition was specifically directed to adsorption. However, as a difference was actually noted, the results suggest that both viral penetration and adsorption were involved in the inhibitory process. Combined, these findings underline that an important part of the complex activity of TK-V3b is directed to the stage of virus entry.

Next, a Western blot analysis was performed to gain deeper insight into the mode of action of TK-V3b. For this, a single round of HCMV infection of HFFs (duration of 3 days) was carried out under three different conditions of substance incubation, i.e. post, pre or pre–post with respect to the addition of virus. In each case HFFs were incubated with 10 µg/ml of the substance, infected with HCMV and the expression of distinct viral proteins was determined at 20, 48 and 72 h post-virus adsorption (Fig. 4). The addition of TK-V3b exclusively after virus adsorption (post) did not show any effect on the synthesis of viral proteins. Viral immediate early (IE), early (E) and late (L) proteins were expressed with normal kinetics during the viral replication cycle. In contrast, a clear decrease in all viral proteins was detected when TK-V3b was incubated 1 h before and during virus adsorption (pre). Hereby, already the synthesis of the IE proteins IE1-p72 and IE2-p86 was suppressed and an even more pronounced inhibitory effect was found for the E protein pUL44 and the L protein MCP. Importantly, pre–post-incubation of the substance (i.e. preincubation followed by the continuous presence of the substance during and after virus adsorption) almost completely prevented viral protein synthesis under these conditions. Thus, the continuous presence of the substance post-infection contributes to the efficiency of virus inhibition suggesting an entry-independent, intracellular effect. Taken together, this indicates a complex mode of HCMV-specific antiviral effects by substance TK-V3b. Primarily, a strong inhibition is directed to viral entry, but in addition

Table 2
Nuclear accumulation of HCMV pp65 (1 h post-virus adsorption)^a

	No inhibitor	TK-V3b pre	TK-V3b pre–post
Mock-infected	0.3 ± 0.5	n.d.	0.3 ± 0.5
HCMV-infected (adsorption at 37 °C)	66.7 ± 2.5	3.6 ± 1.7	0.6 ± 0.5
HCMV-infected (adsorption at 4 °C)	18.3 ± 3.3	1.4 ± 1.0	0.3 ± 0.5

n.d., not determined.

^a Values are expressed in percent of total cells as determined by three independent countings (means ± standard deviations).

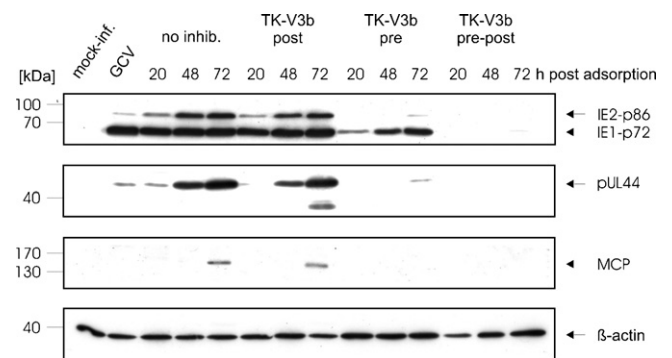


Fig. 4. Western blot analysis of the mode of action of TK-V3b towards HCMV. HFFs were cultivated in 6-well plates and used for infection with HCMV AD169-GFP at a MOI of 1 (GFP-TCID 100%). TK-V3b was added either after virus adsorption (post), or preincubated for 1 h as well as during virus adsorption (pre), or preincubated plus remaining in the culture media throughout the experiment (pre–post). At the indicated time points, cells were harvested and subjected to Western blot analysis for the detection of viral immediate early proteins IE1-p72 and IE2-p86, early protein pUL44 or late protein MCP (major capsid protein). Mock-infected, no infection; no inhibitor, HCMV infection without addition of a substance; GCV, infection followed by addition of ganciclovir (20 µM); β-actin, indicating equal amounts of loaded proteins.

a second inhibitory effect is directed to later intracellular steps of viral replication.

Given the strong indication that the antiviral activity is displayed by negatively charged polysaccharides, it seems unlikely that they could effectively enter the cells, although in principle this possibility has been demonstrated (Huleihel et al., 2002). It is more tempting to speculate that these substances may bind to the virus itself or to cellular surfaces. With respect to a possible virucidal activity of spirulan-like substances, a preincubation experiment was performed to investigate the direct effect of substance TK-V3c on the infectivity of HCMV. TK-V3c showed a certain inhibitory capacity when preincubated with the virus (Fig. 2). This effect, however, did not result in a complete block of viral replication and was quantitatively clearly lower than that measured for the preincubation of the cells with the substance (data not shown). This indicates that an even more effective antiviral mode of action might result from the binding of polysaccharides to cellular surfaces. Thereby, polysaccharides might interfere with viral entry and induce regulatory stimuli giving way to intracellular antiviral effects. The latter aspect might include signaling processes, changes in cell membrane properties or the production of potent antiviral agents such as interferons (IFNs). To address the question of IFN induction, substance-treated fibroblasts were investigated for type I IFN production using a commercially available quantitative IFN- α ELISA (IFN- α Module Set; Bender MedSystems GmbH, Vienna, Austria; performed with media samples collected after 7 days). Neither TK-V2b nor TK-V3b induced significant levels of IFN- α (<10 pg/ml) up to concentrations of 90 μ g/ml. Secondly, an IFN- β -neutralizing antibody (anti- β -IFN rabbit pAb no. 407299; Calbiochem) was coincubated in an experimental setting similar to that described for Fig. 1A. Substance TK-V3c was used at concentrations of 10, 30 and 90 μ g/ml (pre–post-incubation) and infected cells were cultivated in the absence or presence of 100 neutralizing units of anti-IFN- β . Thereby, anti-IFN- β had no effect on the inhibitory capacity of TK-V3c towards HCMV replication (data not shown). Moreover, in the experiments performed with HSV-1, an IFN-mediated antiviral effect can be excluded. For HSV-1 infections, Vero cells were used and this cell line possesses a general defect in the pathway responsible for IFN induction (Diaz et al., 1988). Concluding, antiherpesviral activity must have other reasons than IFN activity. It seems more likely that the intracellular antiviral mechanism induced by these substances might be linked with membrane properties and/or signaling processes.

3.5. Antiviral activity of spirulan-like substances towards other human viruses

As analyzed with an influenza A virus-specific GFP-based assay, almost no inhibition of influenza A virus replication was measured for substances TK-V2b and TK-V3b (Fig. 5A). Even pre–post-incubation of the substances only showed marginal effects. Amantadine, a clinical anti-influenza drug which was used as a reference substance, produced an antiviral effect in this assay. Thus, the spirulan-like substances analyzed show an anti-herpesviral but not an anti-influenza activity *in vitro*.

In contrast, a strong antiviral activity of spirulan-like substances was demonstrated towards HIV-1 (X4-tropic laboratory strain NL4-3). All analyzed substances (TK-V2b, TK-V3b and TK-V4b) were clearly inhibitory at the concentration of 90 μ g/ml (Fig. 5B). At least for the latter two substances, these effects were quantitatively comparable to the antiretroviral drugs AZT and IDV. Interestingly, the inhibitory potential of TK-V4b towards HIV-1 was identical for pre–post-incubation compared to an incubation post-virus adsorption alone (post) (Fig. 5C). This indicates that in case of HIV-1, in contrast to herpesviruses, viral entry may not be the primary antiviral mechanism. Thus, antiviral activity of the analyzed spirulan-like substances is not restricted to distinct herpesviruses but is also detectable for the nonrelated human immunodeficiency virus. This may indicate a broad-spectrum potential of these substances, possibly resulting from a complex mode of antiviral action.

3.6. Conclusion: spirulan-like substances are candidates for the development of broad-spectrum antiviral drugs with novel modes of action

Our analysis of a panel of substances isolated from *A. platensis* (spirulan-like substances) clearly demonstrated various antiviral activities (Table 3). Both substance types, i.e. substances isolated from intracellular (TK-V2b) as well as extracellular fractions (TK-V3a/b/c and TK-V4a/b), were active in the inhibition of viral replication *in vitro*. In particular the extracellularly derived substances, i.e. TK-V3a/b/c and TK-V4a/b, showed strong antiviral effects devoid of cytotoxicity in the relevant range of concentrations. Importantly, these substances possess a broad-spectrum antiviral activity which was characterized by strong inhibition of *in vitro* replication of human viruses such as HCMV, HSV-1, HHV-6 and HIV-1. This finding encourages a detailed characterization and development of these substances in the future. On the one hand, it will be interesting to see possibly further inhibitory effects towards additional human viruses which were not included in this study. On the other hand, further optimization of the isolation protocol of substances might yield substances with even enhanced antiviral activities. The ongoing biochemical analysis of these substance preparations indicates the presence of spirulan-like substances, in addition to a small group of uncharacterized other polysaccharides and possibly protein components. On this basis, it seems likely to separate specific antiviral activities from these substances by further fractionation. As described above, the intracellular compound TK-V2 was exclusively composed of polysaccharides, whereas TK-V3 and TK-V4 additionally contained a portion of proteins. An ion exchange chromatography of TK-V3 indicated that anionic polysaccharides are responsible for the antiviral activity against HCMV. Furthermore, after the identification of the chemical structures of individual constituents, a chemical modification of active substances might be a challenging long-term goal. Regarding the potential of sulfated polysaccharides as drug candidates, some problematic aspects described by related studies in the past have to be considered. Firstly, problems may arise from reproducibility of isolation and purification of sufficient amounts of pure bioactive sub-

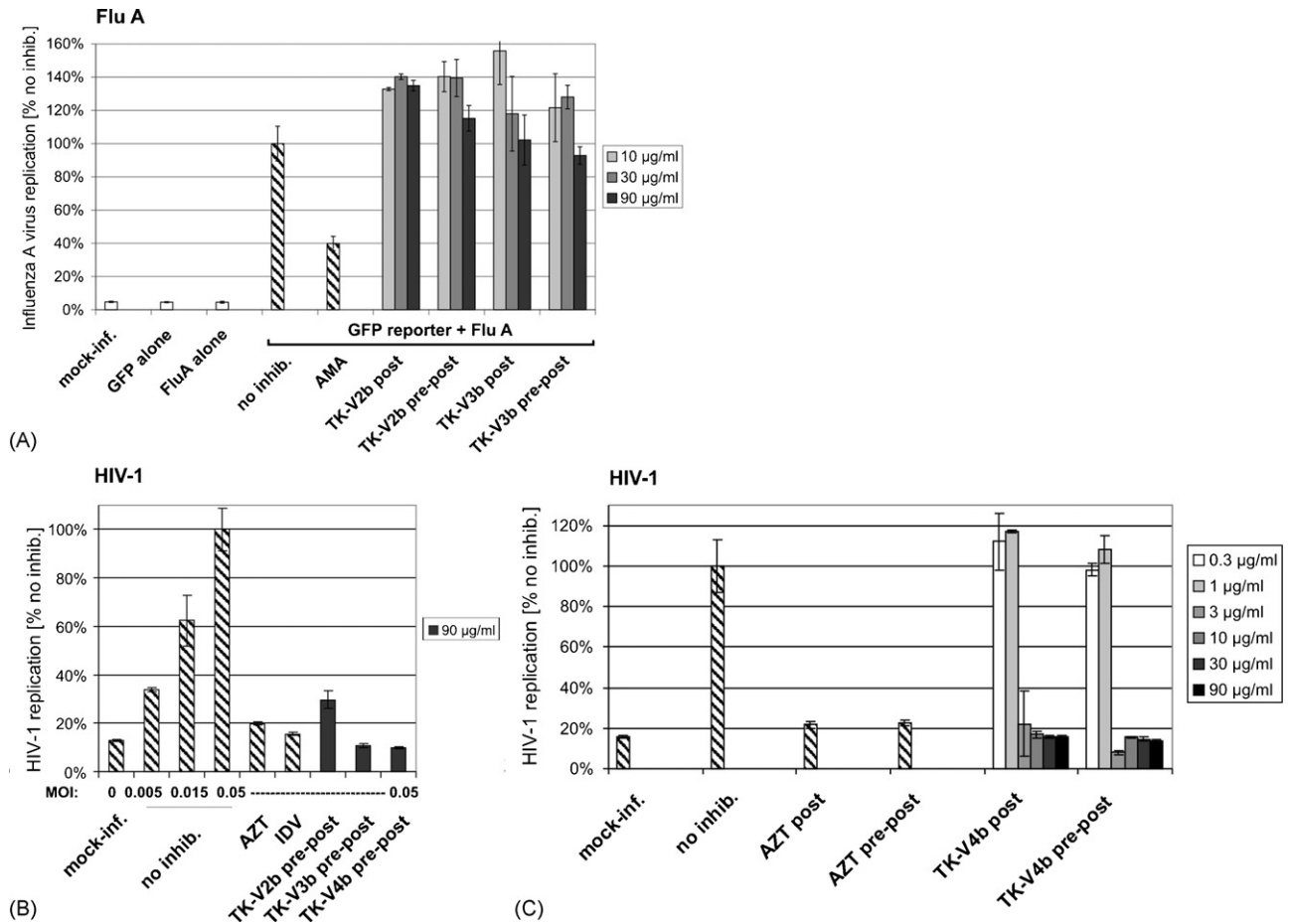


Fig. 5. Determination of antiviral activity against influenza A virus and human immunodeficiency virus. (A) 293T cells were cultivated in 12-well plates and transfected with the influenza-specific GFP reporter plasmid as indicated. One day post-transfection, infection was performed with the strain WSN/33 of influenza A virus. Antiviral substances were added either after virus adsorption (post) or 1 h before as well as during virus adsorption remaining in the culture media throughout the experiment (pre–post). After 24 h, cell lysates were assayed by automated GFP fluorometry. A mean of four values is presented in each column (transfection/infection in duplicate and processing of lysates in duplicate). Mock-infected, no infection; GFP alone, pHH21-NP-UTRhi-eGFP reporter transfection without virus infection; Flu A alone, influenza virus infection without reporter transfection; no inhibitor, transfection/infection without addition of a substance; AMA, amantadine (4 µM). (B and C) CEMx174-R5LTR-GFP_{Luc} cells were infected with HIV-1 strain NL4-3 and incubated with antiviral substances at the indicated concentrations. Five days post-infection, cell lysates were prepared and used for measurement of viral replication by automated GFP fluorometry. A mean of four values is presented in each column (infection in duplicate and processing of lysates in duplicate). Mock-infected, no infection; no inhibitor, HIV-1 infection without addition of a substance; AZT, zidovudine (5 µM); IDV, indinavir (5 µM).

stances (Wagner and Kraus, 2000). In our analysis, however, a clear advantage is provided by the fact that polysaccharides have not to be isolated from plants but can be produced in cyanobacteria using the optimized technology of thermally sterilizable photobioreactors (Walter et al., 2003). Secondly, it was reported that sulfated polysaccharides isolated from seaweeds are poorly absorbed *in vivo* with a bioavailability of less than 1% when given by the oral route (Damonte et al., 2004). This

obstacle is mainly due to the polymeric structure and high molecular weight of seaweed-derived polysaccharides. The molecular weight, however, is lower in the case of polysaccharides produced by *Arthrospira*. Thirdly, as the stability of substances in the plasma is a crucial point, it should be mentioned that the half-life of calcium spirulan in murine blood is much longer than that of other sulfated polysaccharides such as dextran sulfate (Hayashi et al., 1996b).

Table 3
Substances TK-V3^a and TK-V4^b, possessing variable degrees of antiviral activity against selected human viruses

	IC ₅₀ [µg/ml]				
	HCMV	HSV-1	EBV	Flu A	HIV-1
TK-V3 ^a pre–post	2.2 ± 0.1	2.4 ± 0.01	93.5 ± 0.5	94.6 ± 0.3	3.2 ± 0.6
TK-V4 ^b pre–post	2.3 ± 0.3	0.8 ± 0.04	95.7 ± 0.3	n.d.	2.1 ± 0.05

^a Batch b was analyzed for HCMV, EBV, Flu A and HIV-1; batch a for HSV-1.

^b Batch b for HSV-1, EBV and HIV-1; batch a for HCMV.

Thus, the presented strategy seems promising and might also facilitate to clarify the modes of action of these substances in detail. As shown in the present study, more than one mechanism seems to be responsible for antiviral activity, particularly in the case of HCMV. For HCMV it could be demonstrated that an important part of the activity of TK-V3b is directed to viral entry and another part to intracellular regulation of viral replication. Further activities of these substances, such as virucidal effects, might play additional roles in the antiviral action of these substances. This combined effect of several modes of antiviral activity might be highly useful in the development of novel antiviral drugs. Such substances might have advantages in terms of lacking an induction of drug resistance. The targeting of two or more regulatory steps in the viral replication cycle renders the quick occurrence of resistance-conferring mutations in the viral genome unlikely. Combined, the identified spirulan-like substances possess promising antiviral properties regarding a broad spectrum of activity as well as a complex mode of action and therefore represent interesting candidates for further drug development.

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