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Research article

Comparative study of HIV-1 inhibition efficiency by carrageenans from red seaweeds family *gigartinaceae*, *Tichocarpaceae* and *Phyllophoraceae*

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ABSTRACT

The efficiency of human immunodeficiency virus-1 (HIV-1) inhibition by sulfated polysaccharides isolated from the various families of red algae of the Far East Pacific coast were studied. The anti-HIV-1 activity of kappa and lambda-carrageenans from *Chondrus armatus*, original highly sulfated X-carrageenan with low content of 3,6-anhydrogalactose from *Tichocarpus crinitus* and i/κ–carrageenan with hybrid structure isolated from *Ahnfeltiopsis flabelliformis* was found. The antiviral action of these polysaccharides and its low-weight oligosaccharide was compared with commercial κ-carrageenan. Here we used the HIV-1-based lentiviral particles and evaluated that these carrageenans in non-toxic concentrations significantly suppress the transduction potential of lentiviral particles pseudotyped with different envelope proteins, targeting cells of neuronal or T-cell origin. The antiviral action of these carrageenans was confirmed using the chimeric replication competent Mo-MuLV (Moloney murine leukemia retrovirus) encoding marker eGFP protein. We found that X-carrageenans from *T. crinitus* and its low weight derivative and λ-carrageenan from *C. armatus* effectively suppress the infection caused by retrovirus. The obtained data suggest that the differences in the suppressive effect of carrageenans on the transduction efficiency of HIV-1 based lentiviral particles may be related to the structural features of the studied polysaccharides.

1. Introduction

Marine algae are a good known source of sulfated polysaccharides (SPs). These compounds are used in various pharmacological applications [1,2]. Carrageenans (CRGs) are SPs isolated from red seaweed [3,4]. CRGs were found to have a variety of biological activities - immunomodulatory and anticoagulant activity, as well as antitumor and antiviral effects [5]. It is known that some CRGs are selective inhibitors of several viruses, including herpes simplex viruses [6–8], human papillomavirus (were the CRGs were studied

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as a potent vaginal microbicide) [9,10], human rhinoviruses and human coronovirus OC43 (*in vitro*/in vivo studies, nasal spray tests) [11–13]. In addition, CRGs selectively inhibit the HIV-1 reverse transcriptase (*in vitro* studies) [14], have strong anti-enterovirus activity (*in vitro* studies) [15], inhibit infection with dengue and yellow fever viruses (*in vitro*/in vivo studies) [16,17]. It was shown that CRGs can significantly inhibit the replication of swine flu virus SW731 [18,19] and rabies virus, in contrast to vesicular stomatitis virus [20]. Recent studies have shown the antiviral activity of CRGs against SARS-CoV-2 in Vero E6 cell culture [21].

The mechanism of anti-viral action of SPs is still not clearly understood. The primary non-specific interaction of the virus with the components of the cell surface called anchoring is the first step of viral infection. The interaction of viral envelope proteins (EP) with specific cell receptors and co-receptors results in viral entry. Sulfated heteropolysaccharides heparan sulfates (HS) are the components of the cell membrane. HS were shown to be responsible for primary non-specific anchoring of viruses (HIV-1, mouse leukemia virus, herpes simplex virus, hepatitis C virus, human cytomegalovirus, SARS-CoV-2, human papillomavirus) to cell surface [22–24]. The mechanism of viral anchoring to heparan sulfates is based on the electrostatic interaction between the anionic groups of heparan sulfates (mainly sulfates) and the basic amino acids of the viral glycoproteins [25–27]. Inhibition of this first step reduces the efficiency of virus entry and the spread of viral infection [26,28]. One of the possible mechanisms of antiviral activity of SPs is the generation of a stable polysaccharide-virion complex via the electrostatic interaction of the virus envelope glycoproteins with SP [29]. This leads to prevention of the primary anchoring of the viral particle at the cell surface followed by penetration of the virus into the host cell [28, 30].

CRGs belongs to a family of sulfated linear galactans which are the components of the cell wall of red algae (Rhodophyta). They consist of alternating 3-linked, β-D-galactopyranose (G-units) and 4-linked, α-D-galactopyranose (D-unis), forming the disaccharide repeating units of carrageenans. The 1,4-linked residues may commonly, present as the 3,6-anhydrogalactose (DA). Several types of CRG are identified based on the structure of the disaccharide repeating units, the number and location of sulfate groups, and the presence of 3,6-anhydrogalactose. For example, the most widely used in industry carrageenans kappa – (κ: G4S-DA), iota-(i: G4S-DA2S} and lambda – (λ: G2S-D2S,6S) CRGs} [31], characterized by the presence of one, two, and three ester sulfate groups per repeating disaccharide unit, have been elucidated in a number of classical works [31,32].

Natural carrageenans usually contain repeating disaccharide units of different types of CRGs which form hybrid structures with a predominance of one or another type. For example, κ/β-hybrids, κ/i-hybrids, κ/μ-hybrids or ν/i-hybrids [33–39]. Structural features of carrageenans are based on the type of algae and the phase of its life cycle [23,35,40].

It is suggested that the antiviral activity of CRGs is associated with the structure of the carbohydrate backbone, distribution of sulfate groups and degree of sulfation [38], conformation and their molecular weight [5]. Thus, it is possible to determine the main advantages of CRGs, namely, a wide variety and range of antiviral activity, good solubility, relatively low toxicity and cost of carrageenan extraction [35]. Thus, the study of the antiviral activity of CRGs of various structural types isolated from several sources is of considerable interest.

Earlier CRGs were shown to suppress the enveloped DNA or non-enveloped RNA viruses (Herpes simplex virus type 1 (HSV-1) and enterovirus (ECHO-1) respectively). It was demonstrated that CRGs containing 3,6 anhydrogalactose and non-sulfated β-carrabiose units significantly inhibit the binding of the virus with cell [41]. At the same time, unfractionated or total CRG, containing along with κ- and λ-CRGs, which lacks a 3,6-anhydro bridge, also showed high antiviral activity. Due to the fact that the total fraction of this CRG was used, the question of which structural features are responsible for the antiviral effect remains unclear. Here, for the first time, the antiviral action of the separate fractions of κ-CRG and λ-CRG from *C. armatus*, X-CRG with a high degree of sulfateion and a low content of 3,6-anhydrogalactose from *T. crinitus* and ι/κ-CRG by hybrid structure of *A. flabelliformis* against HIV-1(Human immunodeficiency virus-1) was studied. We compared the virus suppressing action of these polysaccharides and their low molecular weight oligosaccharide with commercial κ-CRG obtained from Sigma. Importantly, using the different types of CRGs which were isolated from the red algae *Gigartinaceae*, *Tichocarpaceae* and *Phyllohporaceae* we evaluated the possible structural features of CRGs, associated with anti-viral effect.

The antiviral action was studied using a set of HIV-1-based replication-defective lentiviral particles (LP). Earlier this system was used to study the antiviral activity of different drugs, including the potential inhibitors that prevent the interaction of retroviruses with primary cell surface receptors [39,42–44]. Here, the LP pseudotyped with different EP were used. That allowed us to evaluate that the type of cell receptor used for virus entry affect the anti-viral efficiency of carrageenans. Using the model system based on the chimeric Mo-MuLV retrovirus encoding marker GFP protein we confirmed that studied carrageenans may efficiently suppress the replication competent virus. Taken together, we identified novel original natural carrageenans possessing the pronounced anti-HIV-1 activity and their low molecular weight polysaccharides.

2. Materials and methods

2.1. Algal material

The algae *Chondrus Armatus* (Gigartinaceae), *Tichocarpus crinit*us (Tichocarpaceae) and *Ahnfeltiopsis flabelliformis* (Phyllophoraceae) were collected from Peter the Great Bay, Sea of Japan. According to the morphological and anatomical characteristics obtained using a transmission electron microscope (Professor E.A. and T.A. Titlyanov), *C. armatus* was represented by a male gametophyte and *A. flabelliformis* - by female gametophytes with cystocarps, while T. crinitus contained mature tetraspores. The algae were washed with water, freed from pigments with acetone (3 days), and dried in air in a dark place.

2.2. Extraction of carrageenans

Dried and crushed algae (50 g) were suspended in hot water (1.5 L) and the polysaccharides were isolated at 80 °C for 3 h. The extraction was repeated three times and the resulting extracts were combined and cleared of cell wall residues by centrifugation at 4000 rpm. Then, to separate low molecular weight components, the extracts were filtered through a Vivaflow200 membrane with a pore size of 100 kDa, (Sartorius, Germany) and concentrated on a rotary evaporator. Polysaccharides from the solution were precipitated with a triple volume of 96 % ethanol. The precipitates were purified by re-dissolving in water, concentrated, dialyzed and freeze-dried. The KCl solution was used for fractionation of polysaccharides. Polysaccharide from the algae C. armatus was fractionated with a 4 % KCl solution into gelling (insoluble in KCl) and non-gelling (soluble in KCl) fractions, as described previously [45]. The polysaccharide from tetraspore specimens of T. crinitus did not form a precipitate when exposed to a 4 % KCl solution. The *A. flabelliformis* polysaccharide extract was separated into gelling and nongelling fractions using 4 % CaCl2 as described [18]. The gelling polysaccharide fractions of CRG from *C. armatus* and *A. flabelliformi,* non-gelling fraction from *C. armatus* and unfractionated sample of polysaccharide from *T. crinitus* were used.

As a reference we used commercial κ-carrageenan with predicted structure, which was purchased from Sigma-Aldrich (CRG S) (CAS Number: 11114-20-8; Lot #BCBR6980V).

2.3. Obtaining low molecular weight polysaccharides by mild acid hydrolysis

The polysaccharide (100 mg) was dissolved in 50 ml of 0.1 N HCl and heated at 37 °C (44 h). To stop hydrolysis, a few drops of a 0.1 N NH4OH solution were added to pH 8–9, and low molecular weight polysaccharides were precipitated with 5 vol of 96 % ethanol. The precipitate was obtained by centrifugation at 4000 rpm (30 min at 4 °C) and lyophilized (κ-CRG from *C. armatus*).

2.4. Analytical methods

The monosaccharide composition was determined by complete reductive hydrolysis. Monosaccharides as alditol acetate derivatives were identified by GLC using a 6850 chromatograph (Agilent, USA) equipped with an HP-5MS capillary column (30 m × 0.25 mm, 5 % phenylmethylsiloxane) and a flame ionization detector with a temperature gradient from 150 °C up to 230 °C at 3 °C/min.

The amount of sulfate ester was determined by the turbidimetric method [46] and the content of 3,6-anhydrogalactose by complete reducing hydrolysis according to [47].

2.5. Molecular weight estimation of CRGs

The molecular masses of the polysaccharides were calculated based on the Mark-Houwink equation: $[\eta] = K \times Ma$, where [η] is the intrinsic viscosity, and K and α are empirical constants for CRG at 25 °C in 0.1 M NaCl. , according to literature data for this polymer-solvent system [48]. The viscosity of polysaccharide solutions (1–2 mg/ml in 0.1 M NaCl) was measured at 25 °C in a modified Ubbellohde viscometer (Design Bureau Pushchino, Russia) with a capillary diameter of 0.3 mm. To determine the average molecular weight of low molecular weight polysaccharide derivatives, the reducing sugar method with ferricyanide was used [49].

2.6. FTIR and NMR spectroscopy of CRGs

FTIR spectra of the polysaccharides in the films were recorded by Invenio S and Equinox 55 Fourier transform spectrophotometers (Bruker) taking 120 scans with 4 cm^{-1} resolution. The OPUS/IR version 7.2, program set was used to measure the frequencies of absorption bands in IR spectra. The spectra were normalized by the absorption of the monosaccharide ring skeleton at ~1070 cm −1 (A1070 – 1.0) and spectra regions of 1900–700 cm−1 were used in analyses. 1H and 13C nuclear magnetic resonance (NMR) spectra were obtained at 50 °C on a DRX-500 spectrometer (125.75 MHz) (Bruker, Hamburg, Germany). Polysaccharides (3 mg) were deuterium exchanged twice with heavy water (0.6 ml, D2O, Sigma). Chemical shifts were described relative to the internal standard, acetone (δ_C 31.45, δ_H 2.25). NMR data were acquired and processed using XWIN-NMR 1.2 software (Bruker).

2.7. Cell cultures

In this study, we used HEK293 (human embryonic kidney cells), Lan-1 (human neuroblastoma), Jurkat JMP (human T-lymphoblastic leukemia 4 × 4 # 3 clone) and Sc-1 (mouse embryonic fibroblasts) cell cultures. Lan-1 and Jurkat JMP were cultured in RPMI-1640 medium with the addition of 100 μg/mL of streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate, 100U/mL of penicillin and 10 % FBS (fetal bovine serum. Sc-1 and HEK293 were cultured in a DMEM medium (Gibco/Invitrogen Life technologies) containing 10 % FBS, 100 μg/mL streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate and 100 U/mL penicillin. The cells were cultured at a temperature of 37 °C and in a humid atmosphere with 5 % CO_2.

2.8. Cytotoxicity analysis

To assess the cytotoxic effect of carrageenans within the concentration range of 0.01–100 μg/mL, the changes in the number of viable cells of Lan-1, Sc-1 and Jurkat JMP cell lines were determined by staining with a 0.4 % Trypan blue solution (Invitrogene Corp.,

Carlsbad, CA, USA). 72 h after the addition of carrageenans, cells Sc-1 and Lan-1 were removed by trypsin, resuspended in the medium and stained for 5 min. Jurkat (JMP) cells were also resuspended in the medium and treated with the staining dye for 5 min. The number of unstained (viable) cells in the population was counted in a Neubauer chamber and represented as a percentage of the total number of cells.

2.9. Obtaining of pseudotyped lentiviral vectors particles

HEK293 cells were used as packaging cells, seeded in 100 mm Petri dishes at a density of 3×10^6 cells per dish 12–14 h before transfection.

To obtain lentiviral particles (LPs) pseudotyped with different envelope proteins (EPs) we used the protocol, which was previously described [50–53]. For that LeGO lentiviral plasmid encoding the eGFP marker gene (10 μg per dish), gag-pol plasmid (10 μg per dish), Rev plasmid (5 μg per dish) and VSV-G, Gp160 or McERV envelope protein encoding plasmid (2 μg, 4 μg or 5 μg per dish, respectively) were introduced into the HEK293 cells using calcium phosphate transfection (ProFection ® Mammalian Transfection System, Promega). After 8 h the medium was replaced with fresh DMEM containing 20 mM HEPES, and after 24 h the supernatants containing pseudotyped particles were filtered through 0.22 mm filter (Millipore) and stored at −80 °C. The VSV-G or McERV pseudotyped particles titers were measured on Lan-1 cells seeded to 24-well plates 24 h before transduction. The VSV-G or GP160 pseudotyped particles were titrated on Jurkat JMP cells seeded to 24-well plates 24 h before transduction. GFP expression was measured by the number of fluorescent cells on a flow cytometer (BD Biosciences, Heidelberg, Germany Biosciences) 72 h after transduction. To determine the titer of lentiviral particles, the formula: $T = N*P/V$ was used, where T is the titer, V is the amount of added supernatant containing pseudotyped particles (ml), N is the number of seeded cells and P is the proportion of transduced cells in the population.

2.10. Obtaining Mo-MuLV particles

Retroviral vector containing the full length Mo-MuLV genome and the GFP marker gene was added to Sc-1 cells seeded on a 100 mm Petri dish at a density of 3×10^6 cells per dish 12–14 h before transfection, and replication competent retroviral particles were collected 72 h later. Next, the virus was titrated using Sc-1 cells seeded in a 24-well plate (density of 2×10^4 cells per well) 14–16 h before infection, as described above.

2.11. Antiviral activity assay

Antiviral activity of carrageenans was studied on the Jurkat JMP, Lan-1 and Sc-1 cell lines seeded in 24-well plates at densities of 5×10^4, 2×10^4, and 2×10^4, respectively. The oligo- and polysaccharides were added in concentrations of 0,1; 1,0; 5,0; 10,0; and 25 μg/mL per well to Lan-1 and Jurkat JMP 1 h before transduction with GP160/McERV/VSV-G pseudotyped lentiviral particles. The virus titers were 5×10^6 particles/mL for VSV-G; 5×10^5 particles/mL for GP160 and 5×10^5 particles/mL for McERV. Sc-1 cells were treated with the oligo- and polysaccharides taken at the same range of concentrations (mentioned above) 1 h before infection with the Mo-MuLV virus (titer 10^6 particles/mL). The flow cytometry (LSR Fortessa, BD Biosciences, Heidelberg, Germany) technique was used to determine the percentage of GFP-positive cells 72, 96, 144, 240 h after addition of the medium, containing Mo-MuLV viral particles. The FlowJoX software (True Star, 448 Oregon, USA) was used to analyze the results.

2.12. Statistical analysis

All available data are presented as mean ± SD. To determine the statistical significance of differences the Mann-Whitney non-parametric test was used, the difference was considered statistically significant at $p < 0.05$. The GraphPad Prism 8 software was used to process statistical data.

3. Results

3.1. Characteristics of CRGs

The polysaccharides isolated from the algae *C. armatus* and *A. flabelliformis* were fractionated using specific salts (KCl or CaCl2) into insoluble (gelling) and soluble (non-gelling) fractions, as described in the "Methods" section [45,54]. The polysaccharide isolated from *T.crinitus* (the tetrasporic plant) was represented only by the non-gelling fraction and designated by us as X-CRG [55]. All isolated polysaccharides contained only carrabious units, which indicates that they belong to carrageenans. According to chemical analysis the main components of the studied carrageenans were galactose (from 39 to 32 %) and 3,6 anhydrogalactose (27-15 %) and sulfate groups (32- 23 %). The polysaccharides contained 0.2–03 % K ion and 0.5–0.8 % Na (in terms of the dry weight of the polysaccharides); metals such as copper, zinc, magnesium, lead, cadmium, arsenic and boron were not detected. The protein content of the resulting carrageenans was less than 2 %. According to chemical analysis data, these polysaccharides varied in the degree of sulfation and presence or quantity of 3,6- anhydrogalactose (Table 1).

Based on Fourier transform infrared (FTIR) and NMR spectra polysaccharides were identified as carrageenans. The comparative analysis of the obtained spectra was carried out with the spectra of polysaccharides isolated from these algae species, as described in detail earlier [45,54].

Absorption bands in the IR spectra and chemical shifts in the NMR spectra were assigned by comparison with the signals of known carrageenan structures [56–60].

The infrared spectra of insoluble fractions from *C.armatus* and *A. flabelliformis* showed poorly resolved absorption band with maximum about at 1263 and 1214 cm^{-1} characteristic of sulfate groups (asymmetric stretching vibrations). The strong absorption of this band is represented by a polysaccharide from *A. flabelliformis*, which corresponds to a greater number of sulfate groups and is consistent with the results of chemical analysis (Fig. 1a and b). In these spectra, absorption bands are observed at 935 cm-1, (stretching vibration C(3′)-*O*-C(6′)) characteristic of 3,6-anhydrogalactose and stretching vibration of S–O(4) bonds 848-852 cm-1 characteristic of secondary axial sulfate at C-4 of 3-linked β-D-galactose, indicating k-carrageenan. In addition, in the IR spectrum of the insoluble fraction of *A. flabelliformis* (Fig. 1b) there is also a pronounced absorption band at 806 cm-1, belonging to the secondary axial sulfate group at C-2 of 4-linked 3,6-anhydro-α-D- galactose ι-disaccharide unit [61]. According to FTIR analysis, the KCl-insoluble polysaccharide from C. armatus was κ-CRG [45], while from A. flabelliformis it had a hybrid structure and was identified as ι/κ-CRG [54].

No absorption band corresponding to 3,6-anhydrogalactose was detected using the FTIR spectra of the soluble fraction from C. armatus (Fig. 1c–Table 1). But a broad asymmetric band was detected at 833 cm^{-1} and shoulder at about 820 cm^{-1} due to sulfate esters corresponding to the primary equatorial sulfate group at C-6 and the secondary equatorial sulfate group at C-2 of 4-linked α-D-galactose, which is characteristic of λ-carrageenan [58]. Analysis of this spectrum may also indicate the presence of ν- and μ-carrageenans (biosynthetic precursors of ι- and κ-carrageenan, respectively). According to partial reductive hydrolysis, soluble fraction of C. armatus consisted of only [→3)-β-D-Galp-(1 → 4)-α-D-Galp-(1→] disaccharide units (carrabiose) and designated by us as λ-CRG.

The infrared spectrum of polysaccharide from *T.crinitus* (X-CRG) showed a broad and strong absorbance at 1238 cm-1 due to the significant amount of sulfate groups (Fig. 1d). There was also weak absorption bands at 936 cm^{-1} for 3,6 anhydrogalactose and 811 and 854 cm^{-1} that were attributed to sulfated ester groups at C-2 or C-6 and C-4 positions, respectively [62]. The complete structure of this polysaccharide was established by methods of spectroscopy and mass spectrometry us earlier [55]. The application of the mass spectroscopy for the analysis of oligosaccharides obtained by mild acid hydrolysis of the polysaccharide allowed us to establish that the CRG from tetrasporic T.crinitus contains main blocks of 1,3-linked β-D-galactopynanosyl- 2,4 disulfates and 1,4- linked 3,6-anhydro-α-D-galactopyranosyl while 6-sulfated 4-linked galactopyranosyl restudies are randomly distributed along polysaccharide chain [55]. The composiotion of the oligosaccharide mixture of X- CRG (X-CRG-OS) was characterized with a negative-ion ESIMS (Supplementary Figs. S1–S2) NMR spectroscopy (^{1}H and ^{13}C NMR) confirmed FTIR spectroscopy data about structure of insoluble polysaccharides from *C. armatus* and *A. flabelliformis*. It was shown that polysaccharide from *C. armatus* was κ-CRG because there were anomeric signals at 103.1/4.60 ppm and 95.9/5.10 ppm which corresponded to C-1/H-1 of the 3-linked β-D-galactose (G4S) and the 4-linked 3,6-anhydro-α-D-galactose (DA), respectively (Table 2). The insoluble polysaccharide from *C. armatus* had a high viscosity and disordered macromolecular organization and therefore well-resolved NMR spectra were not obtained even at high temperatures.

The oligosaccharides ι/κ-CRG-OS and X-CRG-OS were obtained as a result of mild acid hydrolysis of the original polymers ι/κ-CRG and X-CRG, respectively, as described earlier [18]. According to the chemical analysis, galactose, 3,6-anhydrogalactose, and sulfate groups were the main components of ι/κ-CRG-OS as the ι/κ-CRG (Table 1).

The molar ratio of galactose to 3,6-anhydrogalactose, sulfation degree, the location of sulfate esters and regular or irregular structure of the polymer chain were the key differences in primary structure of carrageenans.

3.2. Cytotoxicity of CRGs

To evaluate the antiviral activity of CRGs sub-clone of human T-lymphoblastic leukemia Jurkat JMP cells positive for $CD4^{+}$ receptor [63], human neuroblastoma plasmolipin (PLLP) positive Lan-1 cells and mouse embryonic fibroblasts Sc-1 cells positive for CAT receptor were used. We evaluated that studied CRGs at concentrations of 10 μg/mL and up to 100 μg/mL had no significant cytotoxic effect on Jurkat JMP, Lan-1 (Fig. 2a and b) and Sc-1 cells (Fig. 5b). A slight cytotoxic effect of some CRGs was detected at the concentrations higher than 25 μg/mL. Therefore, to study their antiviral potential we used the polysaccharides at the concentrations not higher than 25 μg/mL.

Table 1
Characteristics of carrageenans from red algae family Gigartinaceae, Tichocarpaceae and Phyllophoraceae.

Sample of CRG/fraction	Seaweed source	Disaccharide repeating unit structure		Molar ratio	MW, kDa
		3-linked	4-linked	Gal/AnGal/SO_3N	
κ-CRG/insoluble	C. armatus.	G4S	DA	1.0:0.8:1.1	560
λ-CRG/soluble	C. armatus.	G2S	D2S,6S	1.0:0.02:1.8	185
X-CRG/soluble	T. crinitus.	G2S,4S	DA/6S	1.0:0.3:2.0	376
X-CRG -OS	T. crinitus. olig.			1.0: 0.2: 2.0	5.8
ι/κ-CRG/insoluble	A. flabelliformis cystocarps	G4S/G4S	DA2S/DA	1.0:0.6:1.5	330
ι/κ-CRG-OS	A. flabelliformis cystocarps	G4S/G4S	DA2S/DA	1.0:0.6:1.3	5.3

Remarks: G: 3-linked b-D-galactopyranose; D: 4-linked α- D -galactopyranose; DA 4-linked 3,6-anhydro-α-D-galactopyranose.

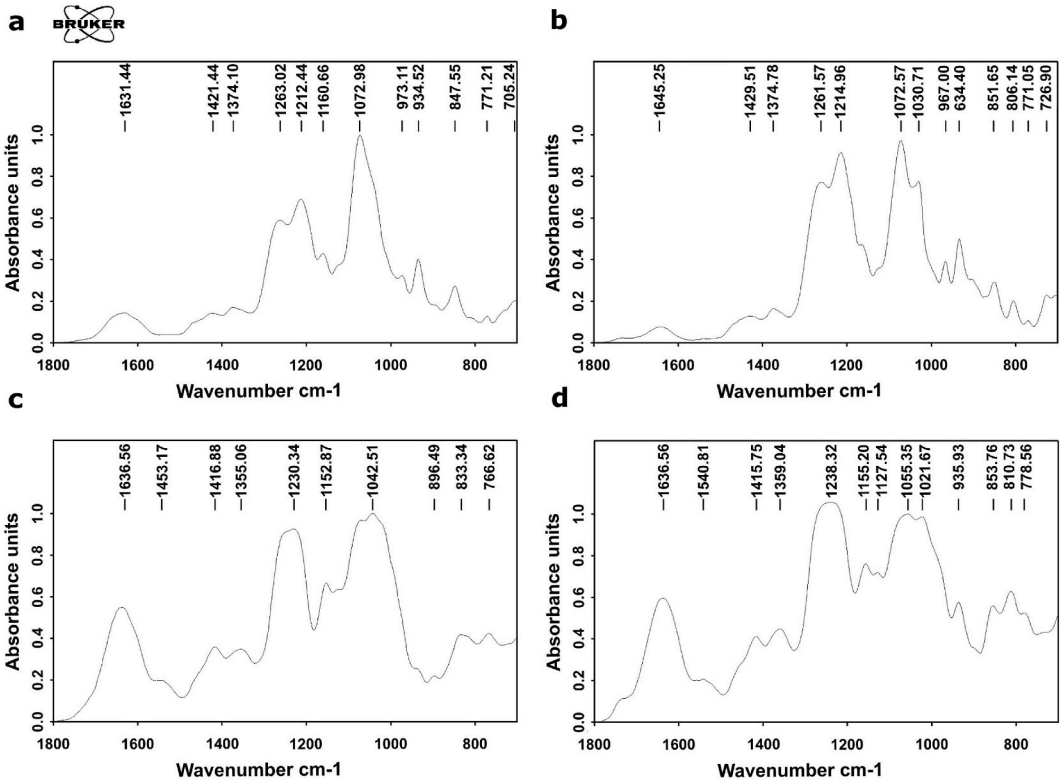


Fig. 1. FT-IR-spectra: **a.** κ-CRG from *C. armatus*; **b.** ι/κ-CRG from *A. flabelliformis;* **c**. the soluble fraction of λ –CRG from *C. armatus;* **d.** X-CRG from *T. crinitus*.

Table 2
NMR signals of insoluble polysaccharide from *C.armatus*.

Type of CRG	unit	^{13}C /^{1}H chemical shift (ppm)					
		C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
κ-CRG	DA	95.9/5.10	70.5/4.14	79.8/4.52	79.1/4.62	77.1/4.66	69.5/4.20
	G4S	103.1/4.60	70.4/3.60	79.1/3.99	74.7/4.87	75.5/3.80	62.0/3.80

NMR analysis shown that insoluble polysaccharide from *A. flabelliformis* mainly consisted of ι- and κ-disaccharide units with ι:κ ratio of 2:1. The signals at 92.9/5.30 and 96.2/5.09 ppm were characteristic of C-1/H-1 of 4-linked 3,6-anhydro-α-D-galactose 2-sulfate (DA2S) and 3,6-anhydro-α-D-galactose of (DA) of ι- and κ-CRGs, respectively. There were poorly resolved signals at 102.9/4.64 and 103.1/4.64 ppm which corresponded to 3-linked β-D-galactose 4-sulfate of ι- (G4S′) and κ-CRGs (G4S), respectively. In addition, the weak signal at 5.50 ppm in ^{1}H NMR spectrum was assigned to *ν*-CRG (Table 3).

Table 3
NMR signals of insoluble polysaccharide fraction from *A. flabelliformis*.

Sample	Carrageenan type	MS residue	$^{13}C/^{1}H$ chemical shifts (ppm)					
			C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
PS	**iota**	G4S′	102.9/4.64	70.3/3.60	77.8/3.99	72.9/4.89	75.8/3.79	62.3/3.80–3.70
		DA2S	92.9/5.30	77.9/4.65	78.8/4.82	77.9/4.76	76.9/4.75	70.1/4.12–4.27
	kappa	G4S	103.1/4.64	70.0/3.60	79.0/3.99	74.3/4.87	75.8/3.80	62.3/3.80–3.70
		DA	96.2/5.09	70.3/4.14	79.7/4.52	78.7/4.62	77.3/4.66	70.1/4.20–3.62
OS	**iota**	G4S′	102.6/4.64	69.2/3.62	77.0/4.67	74.2/4.83	72.2/4.90	61.3/3.70–3.82
		DA2S	92.3/5.29	78.4/4.68	77.8/4.84	75.3/4.67	77.0/4.67	70.0/4.12–4.25
	kappa	G4S	103.1/4.61	69.2/3.59	78.9/3.95	74.1/4.81	74.8/3.70	61.3/3.70–3.80
		DA	95.6/5.10	69.9/4.14	79.2/4.50	78.3/4.62	76.8/4.60	69.8/4.10–4.21

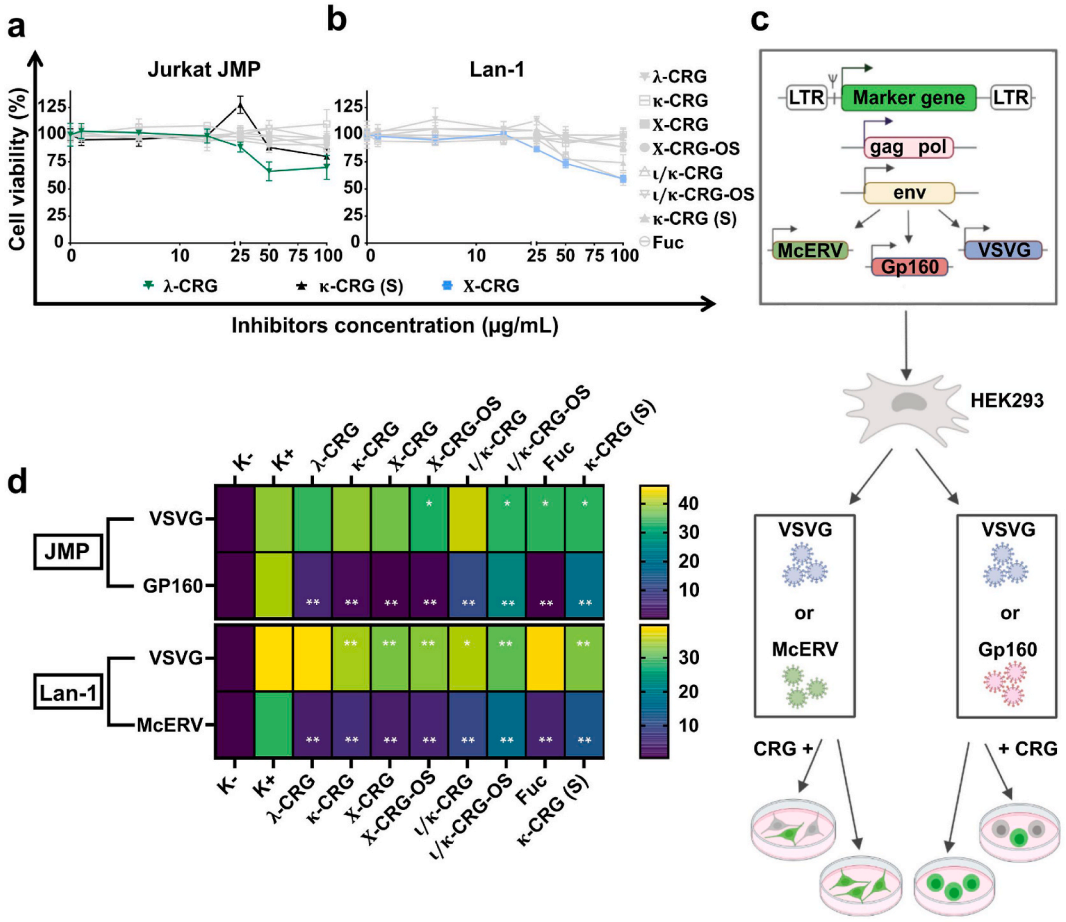


Fig. 2. Cytotoxicity and antiviral action of various CRGs. a. Viability of Jurkat JMP cells exposed to polysaccharides at concentrations of up 100 to 0,1 μg/mL represented as a % to non-treated control. **b.** Viability of Lan-1 cells exposed to polysaccharides at concentrations from 0,1–100 μg/mL represented as a % to non-treated control. **c.** Scheme of the experiment. Jurkat JMP or Lan-1 cells were treated or not treated with polysaccharides for 1h followed by addition of LPs encoding marker gene and pseudotyped with GP160 or McERV or VSV-G envelope proteins. The level of the transduced cells (green cells) was analyzed by flow cytometry. For generation of lentiviral vectors particles were used recombinant lentiviral vector plasmid encoding marker protein eGFP, plasmid encoding structural proteins (gag-pol) and plasmid encoding envelope protein (env). **d.** Heatmap represent the transduction efficiency of HIV-1 particles pseudotyped with GP160 or McERV or VSV-G EPs followed by treatment with CRGs, Fuc (25 μg/mL). The gradient represents the percentage of transduced cells (from the lowest transduction efficiency (0 %) - deep purple to highest transduction efficiency of 40 % - yellow). (K-) – Control sample of cells, which were not transduced or treated with CRGs cells. (K+) – Control sample of cells, which were transduced with viral particles but not-treated with polysaccharides $^{*}P < 0.05$ $^{**}P < 0.005$ as calculated by Mann-Whitney non parametric test.

3.3. *Antiviral activity*

3.3.1. *Generation of lentiviral particles pseudotyped with different envelope proteins*

To study antiviral activity of polysaccharides, a set of HIV-1 based replication defective HIV-1 lentiviral particles encoding marker fluorescent protein (eGFP) was used (Fig. 2c). Three different envelope proteins (EPs) were used for altering the tropism of these lentiviral particles to cells of different origin: HIV-1 GP160 protein to target CD4 positive lymphoid Jurkat JMP [63] cells and McERV EP of the *Mus caroli* endogenous retrovirus (McERV) [64] to target plasmolipin positive neuronal Lan-1 cells and VSV-G EP of vesicular stomatitis virus characterized by a broad tropism to different cell types. The transduction of the target cells by these lentiviruses leads to the synthesis of fluorescence protein which may be detected by FACS. Treatment of cells with antiviral drugs suppress the infection efficiency of lentiviral particles which results in decreased fluorescence of transduced cells and reduced percentage of GFP positive cells in the whole population of treated cells.

The cells JMP and Lan-1 were treated with PSs for 1h followed by addition of lentiviral particles. All polysaccharides, excluding λ-CRG and CRG S, were used at the concentration of 25 μg/mL to treat Jurkat JMP cells. λ-CRG and CRG S were used at the concentration of 10 μg/mL. In case of Lan-1 cells all polysaccharides, except X-CRG, were added at the concentration of 25 μg/mL. X-CRG was added at the concentration of 10 μg/mL. The volumes of medium containing of replication-defective particles was added to

achieve not more than 40 % of transduced cells in the whole population. The transduction efficiency of viral particles added to JMP or Lan-1 cells treated with polysaccharides was compared to transduction efficiency of viral particles added to cells not treated with polysaccharides (K+) (Fig. 2d). Cells not treated with polysaccharides and not transduced with lentiviral particles were used as negative control (K-). The transduction efficiency of LPs when studied polysaccharides were added was compared and represented as a

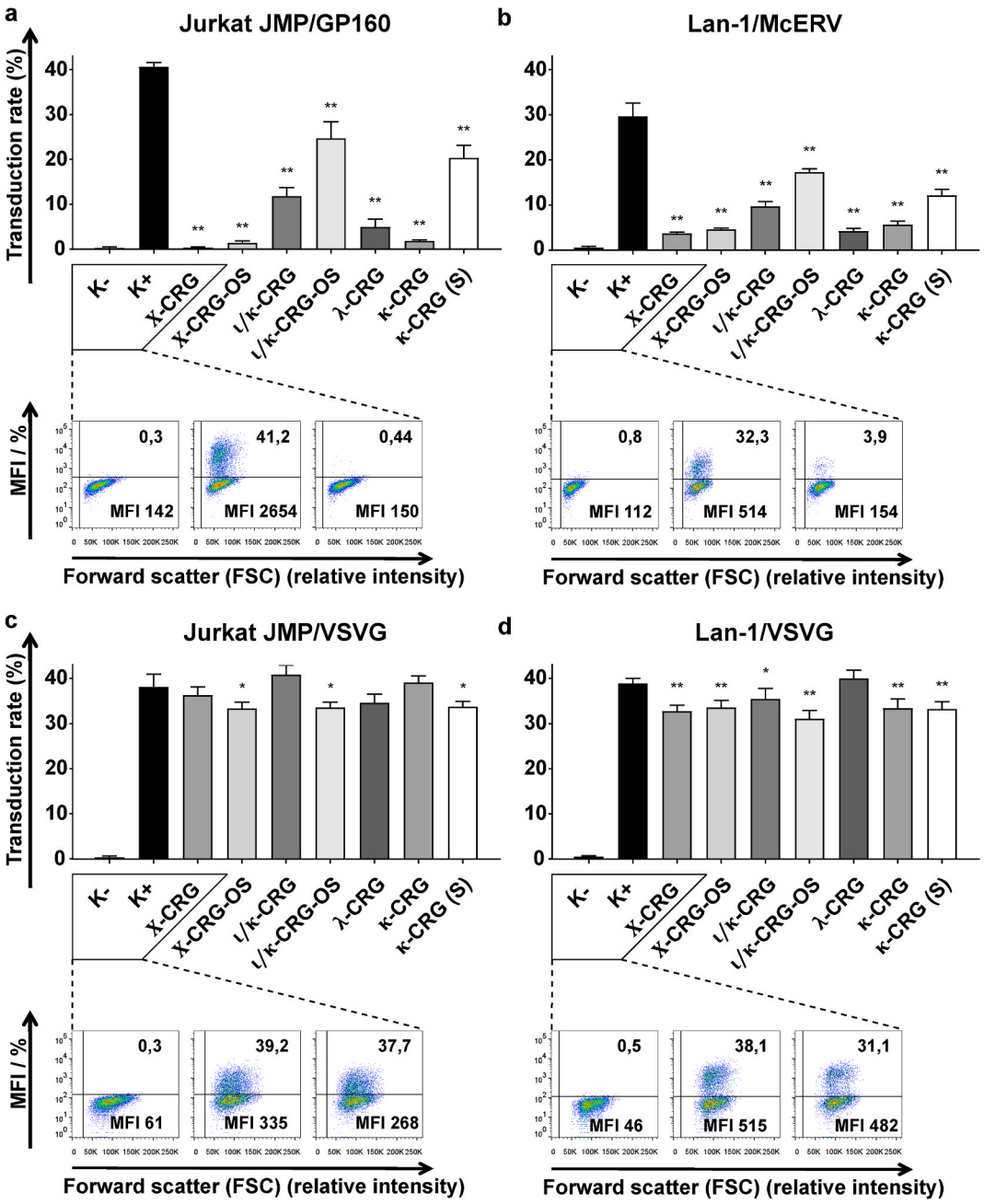
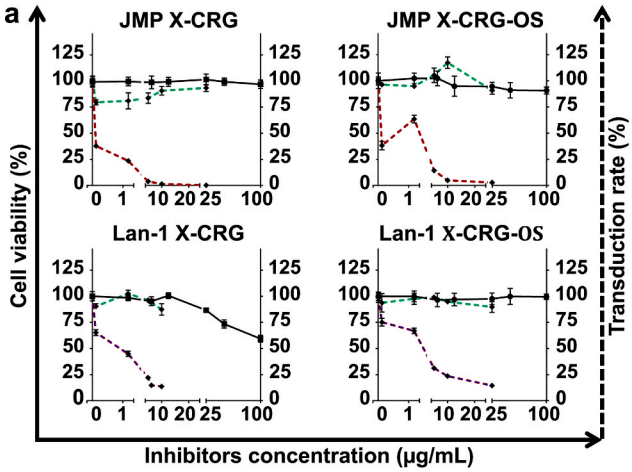
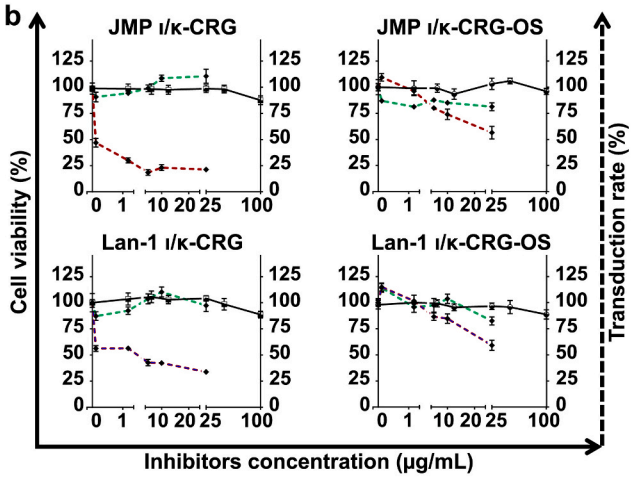


Fig. 3. Antiviral action of CRGs vs replication defective LPs pseudotyped with HIV-1 GP160, McERV or VSV-G EPs. a. CRGs suppress the transduction efficiency of LPs pseudotyped with HIV-1 GP160 EP when added to Jurkat JMP cells. Here and on **b-d** the transduction rate is compared to negative control sample (K-) – cells not treated with vLPs or CRGs. K + - control sample of cells which were treated with LPs but not CRGs. The vertical bars represent the mean values ± standard deviation (SD) from three independent measurements. Here and on the panels **b-d** the flow cytometry scatter plots, representing the gating logic and changes in % of GFP-positive infected cells and MFI (mean fluorescence intensity) for the first three underlined bars are represented. FSC (forward scatter intensity) measured in relative units. The scatter plots for these and other samples are represented at Supplementary Fig. S3 **b.** FACS analysis of Lan-1 cells transduced with LPs pseudotyped with McERV EP and treated with CRGs. **c.** FACS analysis of Jurkat JMP cells transduced with LPs pseudotyped with VSV-G EP and treated with CRGs. **d.** FACS analysis of Lan-1 cells transduced with LPs pseudotyped with VSV-G EP and treated with CRGs. $*P < 0.05$ $**P < 0.005$ as calculated by Mann-Whitney non-parametric test.



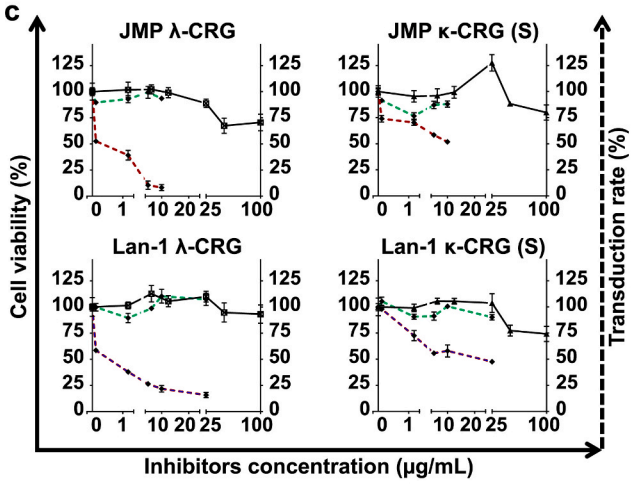
X-CRG		EC50	CC50	MNTC	SI	SI*
JMP	GP160	0,065 ± 0,02	>100	25	>1540	384
	VSVG	>100	>100	25	n/a	<1
Lan-1	McERV	0,44 ± 0,09	>100	10	>227	22,6
	VSVG	>100	>100	10	n/a	<1

X-CRG-OS		EC50	CC50	MNTC	SI	SI*
JMP	GP160	0,2 ± 0,129	>100	25	>500	125
	VSVG	>100	>100	25	n/a	<1
Lan-1	McERV	1,61 ± 0,37	>100	10	>62	15,5
	VSVG	>100	>100	10	n/a	<1



ı/κ-CRG		EC50	CC50	MNTC	SI	SI*
JMP	GP160	0,166 ± 0,06	>100	25	>602	151
	VSVG	>100	>100	25	n/a	<1
Lan-1	McERV	1,81 ± 0,07	>100	25	>55	13,8
	VSVG	>100	>100	25	n/a	<1

ı/κ-CRG-OS		EC50	CC50	MNTC	SI	SI*
JMP	GP160	>25	>100	25	n/a	<1
	VSVG	>100	>100	25	n/a	<1
Lan-1	McERV	>25	>100	25	n/a	<1
	VSVG	>100	>100	25	n/a	<1



λ-CRG-OS		EC50	CC50	MNTC	SI	SI*
JMP	GP160	0,192 ± 0,06	>100	10	>512	51,3
	VSVG	>100	>100	10	n/a	<1
Lan-1	McERV	0,35 ± 0,08	>100	25	>285	71,4
	VSVG	>100	>100	25	n/a	<1

κ-CRG (S)		EC50	CC50	MNTC	SI	SI*
JMP	GP160	>10	>100	10	n/a	<1
	VSVG	>100	>100	10	n/a	<1
Lan-1	McERV	15,26 ± 3,9	>100	25	>6	1,6
	VSVG	>100	>100	25	n/a	<1

(caption on next page)

Fig. 4. The antiviral action of CRGs compared to their cytotoxic activity. a. The antiviral action of X-CRGs compared to their cytotoxic activity. The antiviral effect of X-CRG and low weight polysaccharide X-CRG-OS added to the Jurkat JMP cells transduced with replication deficient HIV-1 pseudotyped with GP160 (red dotted line) or VSV-G (green dotted line) or Lan-1 cells transduced with replication deficient HIV-1 pseudotyped with McERV (blue dotted line) envelope proteins are represented. Here and on the panels **b** and **c** the solid lines on graphs represent the % of viable cells (left Y-axis) treated with CRGs at concentrations from 0,1 up to 100 μg/mL (X-axis). The quantity of living cells treated with CRGs was estimated and represented as a percentage to untreated cells. The dotted lines shows the percentage of transduced cells (right dotted transduction rate Y-axis). **b.** The antiviral action of ι/κ-CRGs and low weight polysacchride ι/κ-CRG-OS compared to their cytotoxic activity are represented. **c.** The antiviral effect of λ- and κ-CRG added to Jurkat JMP or Lan-1 cells transduced with replication deficient HIV-1 pseudotyped with GP160 (red dotted line), McERV (blue dotted line), VSV-G (green dotted line) are represented. The tables represents half maximal virus-inhibiting concentrations EC50 (μg/mL), cytotoxic concentrations CC50, maximal non-toxic concentrations of CRGs (MNTC) and selectivity indexes calculated with the use of CC50 (SI) or MNTC (SI*) are provided.

heat map (Fig. 2d). Green color represents less efficient suppression of transduction. The deep purple color more efficient suppression of transduction. It was evaluated that most of CRGs significantly reduce the transduction efficiency of HIV-1 based LPs pseudotyped with HIV-1 GP160 envelope protein or retro viral McERV envelope protein. The efficiency of studied CRGs was compared with commercial κ-CRG from Sigma-Aldrich (CRG S) and fucoidan SjGF (Fuc) whose anti-viral activity was previously described [63].The suppressive action of the most of the studied carrageenans was comparative to suppressive action of other polysaccharide fucoidan (Fuc) (Fig. 2d). Interestingly this primary screening assay did not revealed any significant anti-viral action of SPs against LPs pseudotyped with VSV-G EP.

3.3.2. CRGs efficiently suppress the transduction efficiency of LPs pseudotyped with HIV-1 GP160 envelope protein

Next, the detailed study of anti-viral action of CRGs against HIV-1 based LPs pseudotyped with HIV-1 GP160 or retroviral McERV envelope proteins was conducted. We evaluated that all the studied polysaccharides, demonstrated the significant ability to prevent transduction of the Jurkat JMP and Lan-1 cells with LPs pseudotyped with GP160 and McERV envelope proteins. We found that, that X-CRG from *T. crinitus* (X-CRG) and its oligosaccharide (X-CRG-OS) were significantly more efficient to suppress the transduction efficiency of HIV-1 based LPs pseudotyped with GP160 or McERV envelope protein compared to other polysaccharides. Treatment of cells with X-CRG at the maximum non-toxic concentration (25 μg/mL) led to 40-fold reduction of transcduction efficiency of HIV-1 based lentiviral particles pseudotyped with HIV-1 GP160 EP (Fig. 3a. Comparable, but less pronounced antiviral action of X-CRG-OS and λ-CRG from *C. armatus* was demonstrated when the cells Jurkat JMP were treated with these oligo- or polysaccharides followed by transduction with LPs pseudotyped with HIV-1 GP160 EP (Fig. 3a).

Importantly, X-CRG, X-CRG-OS and λ-CRG also demonstrated significant anti-viral action when added to Lan-1 cells followed by addition of LPs pseudotyped with McERV EP (Fig. 3b, Fig. S3). We evaluated that treatment of Lan-1 cells with these carrageenans led to the 8 fold decrease in the percentage of eGFP-positive cells treated with replication-defective LPs. We compared the antiviral efficiency of studied CRGs with commercial CRG S. Treatment of cells Jurkat JMP or Lan-1 with CRG S at the maximum non-toxic concentrations cause the significant reduction in infection efficiency of LPs pseudotyped with GP160 or McERV EPs, reducing the percentage of transduced cells up to 2 and 3 fold respectively which was comparable to antiviral action of ι/κ-CRG isolated from *A. flabelliformis* (ι/κ-CRG-OS) and its oligosaccharide ι/κ-CRG-OS but significantly less pronounced then X-CRG, X-CRG-OS or λ-CRG (Fig. 3 a,b).

3.3.3. Carrageenans do not cause pronounced suppressive antiviral action against LPs pseudotyped with VSV-G EP

Interestingly, only a slight suppressive effect was evaluated when polysaccharides were added prior the transduction with LPs pseudotyped with VSV-G EP. The percentage of cells transduced with this lentiviral particles decreased not more than 15 % and 25 % for Jurkat JMP (Fig. 3c) and Lan-1 (Fig. 3d) cells respectively compared to the control sample of cells treated with LPs, but not polysaccharides (control K+). The most pronounced anti-viral effect against VSV-G pseudotyped HIV-1 based LPs was achieved on Jurkat JMP cells treated with, X-CRG-OS, ι/κ-CRG-OS, λ-CRG and commercial CRG S, which caused up to 15 % reduction of transduction efficiency compared to the control sample (K+) (Fig. 3c). Mostly similar, very weak anti-viral action was demonstrated when Lan-1 cells were treated with carrageenans followed by transduction with LPs pseudotyped with VSV-G EP (Fig. 3d).

3.3.4. Carrageenans do not cause any cytotoxic action while suppress the transduction efficiency of LPs

Next we compared the antiviral action of several most efficient CRGs with its cytotoxic action. For that we used a set of concentrations and evaluated that dose-dependent antiviral action of CRGs is implemented in non-toxic concentrations. The half-maximal inhibitory concentrations of CRGs affecting transduction efficiency of HIV-1 based VPs (EC50) and cytotoxity of CRGs were calculated. The most of CRGs taken in relatively high concentrations (up to 100 μg/mL) do not cause suppressive effect on cell survival of Jurkat JMP (Fig. 4a) or Lan-1 cells (Fig. 3b). Only X-CRG can cause suppressive action on cell survival when added to cells Lan-1 in concentrations higher than 50 μg/mL (Fig. 4a). Importantly, CRGs obtained from *T. crinitus* (X-CRG and X-CRG-OS) significantly reduce transduction efficiency of LPs pseudotyped with HIV-1 GP160 and McERV EP in concentrations staring from 0.1 μg/mL. Expectedly antiviral action becomes more pronounced, when X-CRGs and X-CRG-OS were added at higher concentrations reaching the maximum at non-toxic concentration of 10 μg/mL (Fig. 3a). The strong antiviral action was also evaluated when CRGs from plant *A. flabelliformis* (ι/κ-CRG) or κ-CRG from *C. armatus* were added even in relatively low concentrations starting from 0.1 μg/mL. Interestingly ι/κ-CRG oligosaccharide from *A. flabelliformis* (ι/κ-CRG-OS) demonstrated significantly less pronounced antiviral action compared with its polysaccharide analog (Fig. 4b). Notably, the suppressive antiviral action of commercial κ-CRG (CRG S) was

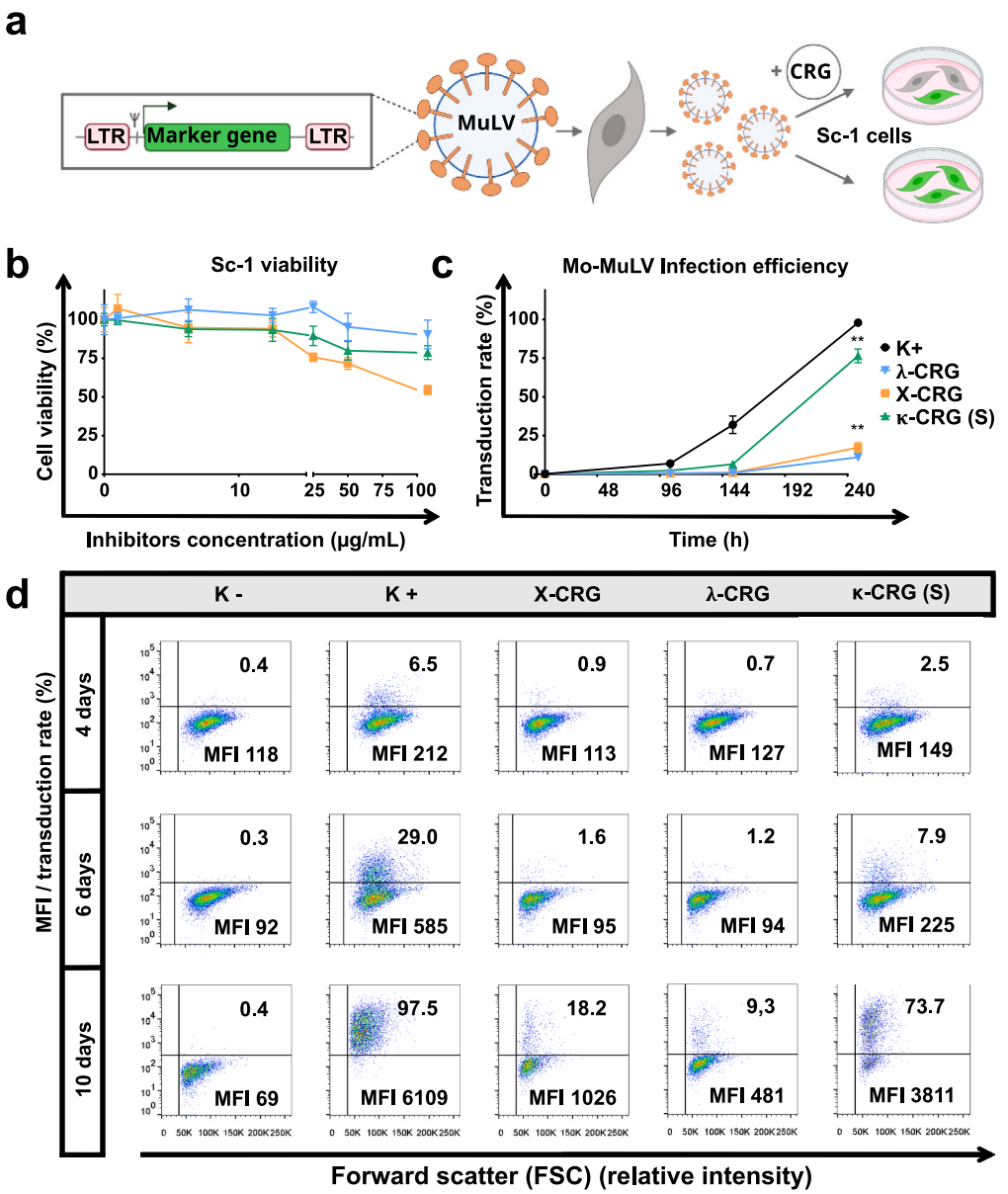


Fig. 5. Cytotoxicity and antiviral action of CRGs added to replication competent virus MoMuLV. a. Scheme of the experiment. Sc-1 murine fibroblast cells were treated with replication competent chimeric Mo-MuLV virus bearing a marker gene encoding eGFP followed by treatment with CRGs. The level of the transduced cells (green cells) was analyzed by flow cytometry. **b.** Viability of Sc-1 cells exposed to polysaccharides at concentrations from 0,1 up to 100 μg/mL represented as a percentage to non-treated control. **c.** The percentage of GFP positive infected cells measured every 2 days after virus addition within 10 days treated with CRGs (25 μg/mL) compared to non-treated with CRGs control cells infected by virus (K+). **d.** The flow cytometry scatter plots, representing the gating logic, changes in percentage of GFP-positive transduced cells and MFI (mean fluorescence intensity) after 4, 6 and 10 days post virus addition. FSC (forward scatter intensity) measured in relative units. K- - cells not treated with CRGs and not infected with virus; K+ cells not-treated with CRGs, but infected with virus; X-CRG and λ-CRG - samples of cells treated with virus and CRGs. $*P < 0.05$ $**P < 0.005$ as calculated by Mann-Whitney non parametric test.

significantly less pronounced, when compared with X-CRG and λ-CRG (Fig. 4c). Expectedly, all the studied CRGs did not provide any suppressive antiviral action when added prior transduction with LPs pseudotyped with VSV-G EP. To match the antiviral action of CRGs with cytotoxity of used CRGs the selectivity indexes (SI = EC50/CC50) were calculated. Therefore most of the studied CRGs do not affect cell survival even in the highest concentrations (up to 100 μg/mL) we used the maximal non-toxic concentrations (MNTC) to calculate the alternative Selective Indexes (SI* = EC50/MNTC) which are most representative (Fig. 4 tables). The highest SI* was obtained for X-CRG, its low weight polysaccharide X-CRG-OS and ι/κ-CRG when the LPs pseudotyped with HIV-1 GP160 EP were used.

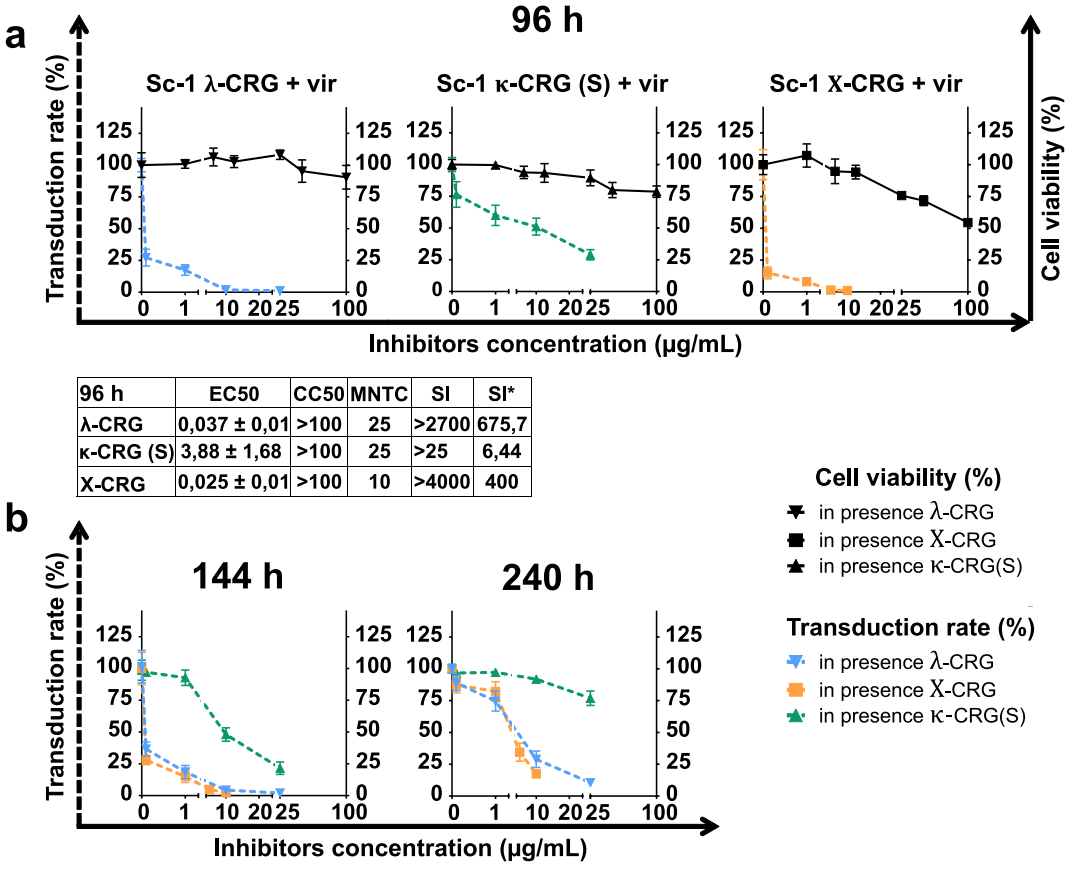
3.3.5. Carrageenans efficiently suppress the infection conducted by replication competent retrovirus Mo-MuLV

To verify whether the infection conducted by replication competent retroviruses may affected by CRGs we used a chimeric murine retrovirus Mo-MuLV, bearing the gene encoding marker eGFP [44]. The replication competent retroviral particles are generated by Sc-1 cells, which were previously transduced by the retroviral vector encoding the full length genome of the chimeric Mo-MuLV. Besides the marker gene the vector encodes the EP of ecotropic murine leukemia virus, targeting its specific receptor, murine cationic amino acid transporter (MCAT-1) exposed on the surface of murine Sc-1 cells. The medium obtained from the Sc-1 cells transduced with this virus may be used to infect the native Sc-1 cells, which became GFP positive over time while the virus spread among the cell population, resulting in the constitutive increase in percentage of GFP-positive cells in the whole population (Fig. 5a).

The λ-CRG from *C. armatus* and X-CRG from *T. crinitus* which showed the most pronounced antiviral action when the replication deficient HIV-1-based LPs were studied. The efficiency of these CRGs was compared with commercial carrageenan (CRG S) obtained from Sigma.

We evaluated, that all the tested polysaccharides do not cause cytotoxic action in concentrations up to 25 μg/mL. A slight suppressive action was determined when the X-CRG and CRG S were used in concentrations higher than 25 μg/mL (Fig. 5b). The λ-CRG from *C. armatus* did not cause any significant cytotoxic action even at the highest concentration of 100 μg/mL.

Next, we measured weather the polysaccharides taken in maximal non-toxic concentrations may suppress the infection efficiency of Mo-MuLV. For that the Sc-1 cells were incubated with CRGs taken in concentration of 25 μg/mL for 1 h followed by addition of



96 h	EC50	CC50	MNTC	SI	SI*
λ-CRG	0,037 ± 0,01	>100	25	>2700	675,7
κ-CRG (S)	3,88 ± 1,68	>100	25	>25	6,44
X-CRG	0,025 ± 0,01	>100	10	>4000	400

144 h	EC50	CC50	MNTC	SI	SI*
λ-CRG	0,06 ± 0,02	>100	25	>1665	416,6
κ-CRG (S)	8,95 ± 1,58	>100	25	>11	2,8
X-CRG	0,04 ± 0,01	>100	10	>2631	263,1

240 h	EC50	CC50	MNTC	SI	SI*
λ-CRG	3,12 ± 0,65	>100	25	>32	8
κ-CRG (S)	>25	>100	25	n/a	n/a
X-CRG	3± 0,52	>100	10	>33	3,3

Fig. 6. The anti-Mo-MuLV action of CRGs compared to their cytotoxic activity. a. The antiviral action of CRGs compared to their cytotoxic activity after 96h post virus addition. The antiviral effect of λ - CRG, X-CRG or Sigma κ-CRG (S) added to the Sc-1 cells infected with replication competent chimeric Mo-MuLV (dotted lines). The solid lines on graphs represent the percentage of viable cells (left Y-axis) treated with CRGs at concentrations of up 100 to 0,1 μg/mL (X-axis). The quantity of living cells treated with CRGs was estimated and represented as a percentage to untreated cells. The dotted lines represent the % of transduced cells (left dotted transduction rate Y-axis). **b.** The antiviral action of CRGs after 6 and 10 days post virus addition. The tables representing half maximal virus-inhibiting concentrations EC50 (μg/mL), cytotoxic concentrations CC50, maximal non-toxic concentrations of CRGs (MNTC) and selectivity indexes calculated with the use of CC50 (SI) or MNTC (SI*) are provided.

chimeric Mo-MuLV containing medium. The volume of medium containing viral particles was taken to achieve about 5–7% of infected (GFP-positive) cells 96 h post addition of viral particles in control sample not treated with polysaccharides. The percentage of GFP-positive cells was measured 4 days, 6 days and 10 days post addition of viral particles. We evaluated that addition of medium, containing chimeric Mo-MuLV leads to fast spreading of infection among the population of Sc-1 control cells not treated with polysaccharides resulting in 30 % of GFP-positive infected cells of all population 6 days post virus addition. Finally, 10 days post infection the percentage of GFP-positive cells in the whole population was about 98 % demonstrating the significant replicative efficiency of chimeric retrovirus when no inhibitor was added (Fig. 5c). We evaluated that treatment of cells with X-CRG and λ-CRG leads to pronounced suppression of retroviral infection performed by chimeric Mo-MuLV. This effect was administrated 4, 6 and 10 days post infection (Fig. 5d). The less pronounced effect and significantly less stable in time was detected when commercial κ-CRG (S) was used.

We compared the antiviral action of studied polysaccharides with its cytotoxic action. The strong antiviral action was detected 4 days post infection (96h) when the X-CRG and λ-CRG were taken in relatively low concentration of 0,1 μg/mL (Fig. 6a).

Expectedly, less pronounced action was detected than commercial CRG S was used. Importantly, the antiviral action of X-CRG and λ-CRG taken in non-toxic concentrations of 10 μg/mL was detected even 6 and 10 days post infection, demonstrating that X-CRG and λ-CRG cause a prolongated suppressive effect on virus (Fig. 6b). Although CRG S also demonstrated the pronounced antiviral action, but less stable than it was shown for X-CRG and λ-CRG. Ten days post infection we did not found any significant antiviral action of CRG S against Mo-MuLV and the percentage of GFP-positive cells were mostly identical to control samples of cells not treated with polysaccharides.

4. Discussion

The antiviral activity is one of the most important biological activities of sulfated polysaccharides. The diverse primary structure of these polysaccharides is closely related to their biological activity. Due to their pronounced polyanionic properties, sulfated polysaccharides can interact with viruses or host cell surface proteins through electrostatic interaction, preventing the adsorption of the virus onto the cell or blocking the binding sites of the virus with the host cell receptor [65]. The structural diversity of carrageenans may result in different mechanisms of activity for these polysaccharides, but such structure-activity relationships among these sulfated polycharides are not fully understood.

The most relevant features of CRGs which can be associated with its activity are structural aspects and composition: (i) type of carrageenan and the content of α-D-galactose 2,6-disulfate residues, the molar ratio of galactose to 3,6-anhydrogalactose; (ii) the sulfation degree and the position of the sulfate group, and (iii) the molecular weight [66].

To clarify the possible mechanism of action, we studied the antiviral effect and toxicity of different types of CRGs and low molecular weight polysaccharides (OS-CRG) isolated from the red algae of Far East Pacific coast.

The presented in this work CRGs were isolated from various algae species. They are characterizing by the presence of 3,6-anhydro-D-galactose (κ-, ι/κ-, X- CRG) or absence (λ-CRG). These polysaccharides differ in the composition of disaccharide repeating units, the number and position of the sulfate groups or hybrid structure (ι/κ-CRG) (Table 1).

Using a set of replication deficient HIV-1 based LPs we identified that these CRGs in non-toxic concentrations significantly suppress transduction efficiency of LPs pseudotyped with envelope proteins (McERV or HIV-1 GP160) specific to different cell receptors targeting cells of neuronal or T-cell origin. The relatively low efficiency of suppressive action of CRGs against LPs pseudotyped with VSV-G EP compared to its action against LPs pseudotyped with HIV-1 GP160 EP may be explained by different mechanisms of viruses (VSV and HIV-1) penetration into cells. It is known that, heparan sulfate acts as the primary cellular receptor for the binding of HIV-1 virus, while VSV penetrates into the cell by the contact with the membrane phospholipids. Since used lentiviral particles are basically identical and differ only in envelope proteins, this may be explained that carrageenans, most likely, act as penetration inhibitors, blocking primary non-specific binding of HIV-1 virus with heparan sulfate. The exact mechanism of McERV penetration into cell is not still known. But therefore both carrageenans and fucoidan efficiently suppress the transduction of LPs pseudotyped with McERV EP this may point, that heparan sulfate or other similar mechanism play a significant role in penetration of McERV virus into the cell.

Our results showed that, depending on the structural features, carrageenans exhibit more or less pronounced antiviral effect. The virus suppressive activity of ι/κ-CRG isolated from *A. flabelliformis* was slightly greater than that of commercial κ-CRGs when Jurkat JMP or Lan-1 cells were transduced with LPs pseudotyped with GP160 or McERV EPs.

Both of these polysaccharides contain 3,6-anhydro-D-galactos and κ-disaccharide units. However, ι/κ-CRG has a hybrid structure consisting of kappa and iota units and contains more sulfate groups. This may explain its relatively high antiviral activity.

The treatment of the cells with low concentrations of X-CRG or λ-CRG (0.1 μg/mL) leads to pronounced suppression of retroviral infection caused by chimeric Mo-MuLV. This effect is probably due to the high content of sulfate groups in these carrageenans. It has been suggested that the activity of λ-CRG may be related to the sulfate content [20,67]. Early it was demonstrated that λ-CRG may suppress influenza A and B viruses in mice followed by intranasal administration. The efficiency of its antiviral activity was associated with high sulfation degree [68].

Previously, the effectiveness of different types of CRGs in inhibiting HIV-1 reverse transcriptase was studied *in vitro*. As the results showed, the most active were carrageenans with a high content of sulfated groups: λ-CRG of carrageenan, ι-CRG and κ-CRG [14]. λ-CRG has been shown to be a potent and selective inhibitor of viral infection targeting viral adsorption and internalization due to structural similarity to heparan sulfate on the surface of mammalian cells [68]. However, not only the degree of sulfation can play a major (determining) role in the manifestation of antiviral activity, but also the conformation of the polysaccharide. λ-CRG polymer chains do not contain 3.6 anhydrogalactose and have a random coil conformation, which ensures their flexibility. It can be assumed that this allows λ-CRG, when directly exposed to the virus, to interact more effectively with some glycoproteins of the viral envelope

proteins. This is confirmed by the results of Yamada et al., who found different anti-HIV activities of λ-CRG and ι-CRG, although they did not differ much in the number of sulfate groups [69,70]. This indicates that structural features also strongly influence anti-HIV activity.

In our study the presence of α-D-galactose 2,6-disulfate residues in λ-CRG is high, while X-CRG contains main blocks of β-D-galactose 2,4 disulfates residues and has a high molecular weight. It is known that CRGs with relatively high molecular weight may significantly suppress the replication of viruses targeting, the initial attachment stages of virus life cycle [19]. It is known that the number of sulfate groups, as well as the molecular weight of the biopolymer, play an important role in the anti-HIV activity of sulfated carrageenans. However, as the results show, not only the number of sulfates, but also their location and the density of sulfate groups in the sugar chains can be important factors in the manifestation of activity [68,71].

Here we showed that the X-CRG from red alga *T. crinitus* and its oligosaccharide (X-CRG-OS) caused the most pronounced antiviral action against lentiviral particles pseudotyped with HIV-1 or GP160 and the replication competent retrovirus Mo-MuLV compared to other polysaccharides. The high activity of X-CRG could be explained by the unique chemical structure of this polysaccharide, which has a high sulfation degree and also contains 3,6 – anhydrogalactose in the polymer chain, that adopt the $^{1}C_{4}$-chair conformation. This results in the generation of the helical structure [72] which may form a grid on the cell surface, that possibly inhibit the anchoring of the virus to cell surface and suppress the efficiency of infection. Taking together, the differences in antiviral activities of studied CRGs may be partially associated with the features of their primary structure. These may include the content of 3,6-anhydrogalactose, the molar ratio of monosaccharides and sulfate groups the quantity and the position of the sulfate groups in the D-galactose residues.

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CRediT authorship contribution statement

Andrey Shulgin: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Pavel Spirin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Timofey Lebedev:** Writing – review & editing, Validation, Software, Resources, Methodology, Funding acquisition, Conceptualization. **Anna Kravchenko:** Resources, Methodology, Investigation. **Valery Glasunov:** Visualization, Validation, Resources, Methodology, Investigation. **Irina Yermak:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Vladimir Prassolov:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33407.

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