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Antiviral Activity of a Carrageenan from *Gigartina skottsbergii* against Intraperitoneal Murine *Herpes simplex* Virus Infection

Original Paper

Abstract

The partially cyclized μ/ν-carrageenan 1C3, isolated from the red seaweed *Gigartina skottsbergii*, was previously shown to be a potent inhibitor of the *in vitro* replication of *Herpes simplex* virus types 1 (HSV-1) and 2 (HSV-2). Here the protective effect of 1C3 in a murine model of intraperitoneal (*i.p.*) HSV-1 infection was evaluated. OF1 mice were *i.p.* infected with 5×10^5 PFU of HSV-1 KOS strain, and the effects of different treatments with 1C3 were studied. When 30 mg/kg of body weight of 1C3 was administered by the *i.p.* route immediately after HSV-1 infection, 87.5% survival of the animals was achieved ($p < 0.005$), associated with a delay in the mean day of death in 1C3-treated non-surviving mice. Animal survival was not improved when multiple doses of 1C3 were also given in the period 1 – 48 h post-infection, and no protection was afforded when treatment was started after 24 h of infection. When virus and compound were injected by different routes, *i.p.* and intravenous (*i.v.*), respectively, a still significant protection was achieved (40% survival, $p < 0.05$). No toxicity of 1C3 for the animals was recorded. The pharmacokinetic properties were analyzed after injection of 1C3 into the tail vein by monitoring of [^3H]-1C3 in plasma and organs and by a bioassay of the anti-HSV-1 activity remaining in serum after non-radioactive 1C3 inoculation. A very rapid disappearance of the compound from the blood was observed since only 5.9 – 0.9% of the radioactivity of the initially administered [^3H]-1C3 appeared in the plasma between 5 – 300 minutes after administration. A transient peak of radioactivity was detected in the kidney 15 minutes after inoculation. The bioassay confirms the presence of the compound circulating in a biologically active form up to 1 hour after injection.

Key words
antiviral agent · *Herpes simplex* virus · natural carrageenan · mouse intraperitoneal infection · pharmacokinetics · *Gigartina skottsbergii* · Gigartinaceae · Gigartinales

Introduction

Sulfated polysaccharides are potent *in vitro* inhibitors of a wide variety of enveloped viruses, such as *Herpes simplex* virus types 1 (HSV-1) and 2 (HSV-2), human immunodeficiency virus (HIV), human cytomegalovirus, dengue virus, respiratory syncytial virus and influenza A virus [1], [2].

In contrast with the successful effects obtained *in vitro* with polysulfates, their *in vivo* efficacy in animal and human systemic infections remains to be demonstrated. A series of undesirable drawbacks (poor absorption, toxic side effects, inability to reach target tissues, anticoagulant properties) have been determinants of the few data reported on *in vivo* antiviral activity of sulfated polysaccharides [3]. Furthermore, the outcome of systemic poly-

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sulfate treatment in animal models has been very variable. Studies performed with murine leukemia virus in mice proved the efficacy of sulphoevernan [4], and a crude seaweed extract [5]. Pentosan polysulfate and dextran sulfate also protected mice against the scrapie agent [6], whereas ι-carrageenan attained 100% survival in mice infected with murine cytomegalovirus [7]. Dextran sulfate was also active against HSV-2 in mice [8], but, on the other hand, it was not effective in cats infected with feline leukemia virus [9], and in ducklings infected with duck hepatitis B virus [10].

In recent years the efficacy of polysulfates for microbicidal topical use to prevent sexually transmitted diseases has been demonstrated. Commercial and natural sulfated polysaccharides with virucidal properties, such as λ-carrageenans, were found to be highly protective against intravaginal virus infection [11], [12], [13], [14].

In recent studies, we have identified diverse structural types of natural carrageenans, isolated from the red seaweed *Gigartina skottsbergii,* as selective inhibitors of HSV-1 and HSV-2, in Vero cells as well as in human and neural cells [15], [16]. Among these compounds, the partially cyclized μ/ν carrageenan 1C3 showed a very good relationship between antiviral efficacy and lack of cytotoxicity, with a high selectivity index and without anticoagulant action [15]. Mechanistic studies have shown that the main target of the antiviral action of 1C3 was virus adsorption [17], by interference with the initial interaction between the virion glycoprotein gC and the heparan sulfate residues of proteoglycans, the primary virus receptor on the cell surface [18]. Since 1C3 lacks virucidal activity [17], in the present study the *in vivo* antiviral properties of this carrageenan were studied in a murine model of systemic intraperitoneal HSV-1 infection.

Materials and Methods

Compounds

Gigartina skottsbergii (Setchell & N.L. Gardner 1936) was collected in Bahía Camarones (Provincia de Chubut, Argentina), dried in the open and carefully sorted in the Instituto Nacional Patagónico (Puerto Madryn, Chubut). A voucher specimen (B.A. 26272) has been deposited in the herbarium of the Museo de Ciencias Naturales Bernardino Rivadavia (Buenos Aires, Argentina).

The compound 1C3 was obtained from the cystocarpic stage of *G. skottsbergii* as previously described [19]. Briefly, the milled seaweed was extracted with water at room temperature, and after centrifugation the polysaccharides were precipiatated from the supernatant with isopropyl alcohol. Then, polysaccharides were dissolved in water and KCl was added in small portions with constant mechanical stirring, so that the concentration was increased by 0.01 – 0.10 M each time, until an upper limit of 2.0 M KCl. The precipitates as well as the residual solution were dialyzed, concentrated and freeze-dried.

The compound 1C3 corresponded to the fraction soluble in 2.0 M KCl and its analysis showed that the galactose:3,6-anhydrogalactose:sulfate molar ratio was 1.00 : 0.37 : 1.14, its optical rotation was $[\alpha]$: + 55.1° in 0.1 M NaCl, and its molecular weight 198 kDa [15], [19]. The structural determination, carried out by methylation analysis and ^{1}H- and ^{13}C-NMR spectroscopy, indicated that it was a partially cyclized μ/ν-carrageenan [15], [20]. Its composition in structural units was: β-galactose 2-sulfate (4%), β-galactose 4-sulfate (44%), α-galactose 6-sulfate (3%), α-galactose 2,6-disulfate (11%) and 3,6-anhydro-α-galactose (38%). The stock solution of 1C3 (10 mg/mL) was prepared in phosphate-buffered saline (PBS).

For pharmacokinetic studies, 1C3 was labeled with tritium, using a reductive technique [21]. Briefly, 1C3 was dissolved in water and sodium borohydride was added; after 16 h the sample was dialyzed and freeze-dried. The reduced sample was dissolved in 5 mM sodium phosphate pH 8.0, and incubated for 90 min with 15 units of galactose oxidase (Sigma-Aldrich, USA). The mixture was then treated with sodium boro-[^{3}H]-hydride (NEN, USA, 50 μCi/μL), and after 2 h cold sodium borohydride was added and the sample was left overnight. The labeled polysaccharide was recovered after dialysis and freeze-drying.

A stock solution (10 mg/mL) of acyclovir (Filaxis, Argentina) was prepared in water (pH 10.5) to be used as a positive control.

Virus and cells

Vero cells were grown as monolayers in Eagle's minimum essential medium (MEM) (Gibco, USA) supplemented with 5% inactivated calf serum and 50 μg/mL gentamycin. The KOS strain of HSV-1 was propagated and titrated by plaque forming units (PFU) in Vero cells.

In vitro antiviral assay

Antiviral activity was evaluated by a plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 PFU of virus/well in the absence or presence of various concentrations of 1C3, two replicates per dilution. After 1 h adsorption, residual inoculum was replaced by MEM containing 0.7% methylcellulose and the corresponding dose of 1C3. After 2 days of incubation at 37 °C, virus plaques were counted. The 50% effective concentration (EC_{50}) was calculated as the concentration required to reduce plaque number by 50%.

In vivo antiviral assays

Adult male OF1 outbred mice (Iffa-Credo, Lyon, France) weighing 25 – 30 g were used. Mice were inoculated intraperitoneally (*i.p.*) with HSV-1 at 5×10^5 PFU per mouse in 0.1 mL of PBS and 1C3 (30 mg/kg of body weight for each dose, 0.1 mL) was administered following different protocols: a) 1C3 was *i.p.* administered immediately after HSV-1 inoculation (time 0); b) 1C3 was *i.p.* administered immediately after HSV-1 inoculation and further *i.p.* administered after 1 and 24 h, or after 1, 24 and 48 h post-infection; c) 1C3 was *i.p.* administered at 24, 48 and 72 h after infection; d) 1C3 was injected by the intravenous (*i.v.*) route immediately after HSV-1 inoculation. HSV-1 inoculated mice not treated with 1C3 received PBS as placebo. Control groups of mice were treated with acyclovir *i.p.* administered (30 mg/kg of body weight for each dose, 0.1 ml) immediately, 1 and 24 h after infection or during 10 consecutive days after HSV-1 inoculation. Toxicity controls of uninfected mice treated with 1C3 and acyclovir in the same way as described for each protocol were run in parallel. Animals were monitored daily for mortality during 21 days.

Parameters used to evaluate the infection included death and mean days to death. Statistical significance of the differences in the number of survivors was assessed by means of Fisher's exact Chi-square test.

Pharmacokinetic studies

The pharmacokinetic behavior of 1C3 in mice was analyzed by two methods: a) monitoring of the radioactive compound in plasma and organs; b) bioassay of the antiviral activity remaining in serum samples after 1C3 inoculation.

To follow the clearance of radioactive 1C3 in plasma, mice were injected into the tail vein with 0.1 mL of [^3H]-1C3 (2×10^5 dpm, 5 mg/mL). At different times after injection (0 – 300 min), blood samples were taken from the tail vein from each mouse using heparinized needles (25 G × 5/8"), collected in tubes, and centrifuged at 2000 rpm for 5 min. The supernatant plasma samples were mixed with 3 volumes of isopropyl alcohol, kept 1 h at 0 °C to precipitate the polysaccharides and then filtered through Whatman paper. The precipitates were allowed to dry at room temperature and then radioactivity was counted in a liquid scintillation counter.

To analyze the permanence of the carrageenan in animal tissues, mice were injected with [^3H]-1C3 as above. At different times, the mice were sacrificed with ether and the liver and kidneys were removed and washed with PBS. The organs were cut in small pieces and mixed with 10 volumes of acetone. After 24 h, the insoluble material was removed, dried and rehydrated in PBS. The organs were then homogenized in a potter and centrifuged 10 min at 12 000 rpm. Three volumes of isopropyl alcohol were added to the supernatant. The mixture was kept 1 h at 0 °C, filtered through Whatman paper, and radioactivity was determined as above.

For the bioassay of antiviral activity, mice were injected with 0.1 mL of 1C3 into the tail vein. At different times, blood was collected from the tail vein and allowed to clot for 1 h at 37 °C, then the serum was collected by centrifugation. Serial two-fold dilutions of serum samples were made in PBS and the antiviral activity against HSV-1 was measured by a plaque reduction assay. Serum from untreated mice was used as control. All animal studies were performed in accordance to the "Manual sobre el cuidado y uso de los animales de experimentación" Vol. 1, 1998, Canadian Council on Animal Care (www.ccac.ca).

Results and Discussion

To assay the *in vivo* efficacy of 1C3, a model of intraperitoneal inoculation of OF1 mice with the KOS strain of HSV-1 was used. Since the antiviral activity of 1C3 against this strain has not been previously assayed, we first evaluated the *in vitro* inhibitory effect of 1C3 against the KOS strain. In a virus plaque reduction assay, 1C3 showed a strong dose-dependent inhibitory action (data not shown). The EC_{50} was calculated to be 0.7 μg/mL, comparable to previous values of EC_{50} obtained for this carrageenan against other reference strains and clinical isolates of HSV-1 [15].

Then, the effect of different treatment protocols with 1C3 on HSV-1 *i.p.* infection of OF1 mice was studied. As shown in Table **1** a variable level of protection was afforded according to the treatment conditions. When 1C3 was *i.p.* administered immediately after HSV-1, a highly significant animal survival was observed (87.5% survival, $p < 0.005$), associated with a delay in the mean day of death in non-surviving 1C3-treated mice in comparison with PBS-treated animals (10.5 ± 1.5 and 8.1 ± 2.1 days, respectively). When treatment was reinforced with further administration of 1C3 in the period 1 – 48 h post-infection, neither the percentage of survival nor the mean day of death were significantly modified in comparison with the administration of only one dose of 1C3. The treatment with acyclovir following a schedule similar to that used with 1C3 (immediately, 1 and 24 h after HSV-1 infection) failed to afford animal protection since only 10% survival was observed. The continuous administration of acyclovir during ten consecutive days was needed to achieve a significant protection of 90% ($p < 0.005$) with a mean day of death of 11.0 ± 0.5 days in the only died mouse. When the start of 1C3 treatment was delayed 24 h after infection, no protection against HSV-1 induced mortality was observed. Finally, when virus and compound were injected both at time 0 but by different routes, the virus

Table **1** Effect of 1C3 treatment on survival of mice infected with HSV-1

Route of 1C3 administration	*Treatment*[a]	*Survival: no. survivors/total (%)*	*Mean day of death ± SD*
i.p.	PBS (0)	0/11 (0)	8.1 ± 2.1
	1C3 (0)	14/16 (87.5)[b,c]	10.5 ± 1.5
	1C3 (0, 1, 24)	8/10 (80.0)[b,c]	10.5 ± 0.5
	1C3 (0, 1, 24, 48)	9/13 (69.0)[b,c]	11.0 ± 1.7
	1C3 (24, 48, 72)	0/13 (0)	7.8 ± 1.4
	ACV (0, 1, 24)	1/10 (10)	8.0 ± 1.0
	ACV (0, and daily for 10 days)	9/10 (90)[b]	11.0 ± 0.5
i.v.	PBS (0)	0/6 (0)	8.0 ± 1.8
	1C3 (0)	2/5 (40.0)[d]	9.7 ± 0.5

[a] OF1 mice were *i.p.* injected with 5×10^5 PFU of HSV-1, KOS strain. Doses of 1C3 (30 mg/kg of body weight) were given *i.p.* or *i.v.* immediately after virus inoculation (0) and/or at the indicated h post-infection. Acyclovir (ACV) used as a positive control was administered at a dose of 30 mg/kg of body weight at the indicated times. Animals were monitored daily for mortality.
[b] Significantly different from the PBS group, $p < 0.005$.
[c] Non-significant difference among the three groups, $p < 0.005$.
[d] Significantly different from the PBS group, $p < 0.05$.

via *i.p.* and the compound via *i.v.*, a lower but significant level of survival was obtained (40% survival, $p < 0.05$), and also the delay in time to death was diminished in comparison with the inoculation of virus and 1C3 by the same route (9.7 ± 0.5 days vs 10.5 ± 1.5 days). Furthermore, 1C3 was well tolerated by the mice, with all toxicity controls surviving and no weight loss (data not shown).

Experiments to characterize the pharmacokinetic properties of 1C3 were run in parallel with the antiviral assays. The plasma concentration-time profile after *i.v.* inoculation of [^3H]-1C3 is shown in Fig. **1**. A rapid disappearance of radioactivity from the animals' blood is apparent. In fact, we found that only 5.9 – 0.9% of the radioactivity of the initially administered [^3H]-1C3 appeared in the plasma between 5 – 300 min after administration. The rapid elimination of 1C3 from the circulation is in accordance with previous studies done with dextran sulfate which reported a half-life in blood for this compound of approximately 30 min in rats [22] and less than 30 min in mice [23]. The decrease in plasma concentration of the carrageenan was not associated with an accumulation of the compound in tissues. A transient peak of radioactivity was detected in kidney after 15 min after inoculation, but then it quickly disappeared (Fig. **1**). Furthermore, negligible amounts of radioactivity were detected in the liver (data not shown).

In addition to the outcome of [^3H]-1C3 in animals, a bioassay was also performed to determine the level of antiviral activity in serum after non-radioactive 1C3 inoculation. Anti-HSV-1 activity was detected in serum samples: the values of reciprocal dilutions of serum which achieved 50% inhibition of plaque formation were 564 and 189 for samples obtained 30 and 60 min after injection, respectively (Fig. **2**). These results confirm the presence of the compound circulating in the animals in a biologically active form. However, the rate of decline of bioactivity exceeded that of the radioactivity in the blood shown in Fig. **1**, since anti-HSV-1 inhibition was undetectable by 5 h after 1C3 injection (Fig. **2**). This may be explained by a partial degradation of the compound, with a consequent loss of molecular mass along

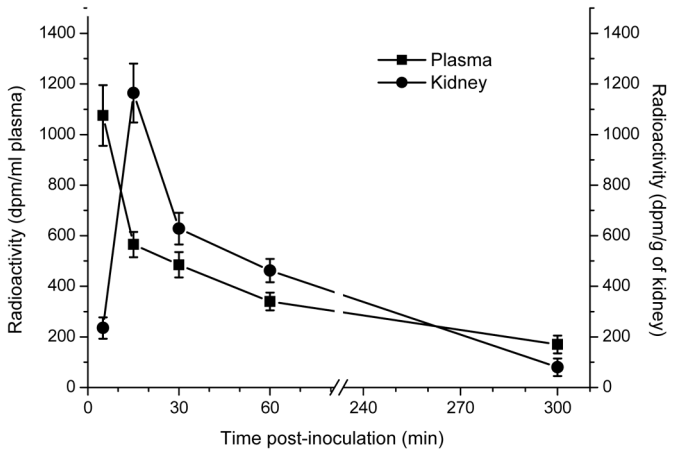

Fig. **1** Plasma and kidney distribution of [^3H]-1C3. OF1 mice were inoculated into the tail vein with 0.1 mL of [^3H]-1C3 (2×10^5 dpm). At the indicated times, radioactivity in the plasma and kidney was determined as described in Materials and Methods. Each value is the mean from 2 mice ± SD.

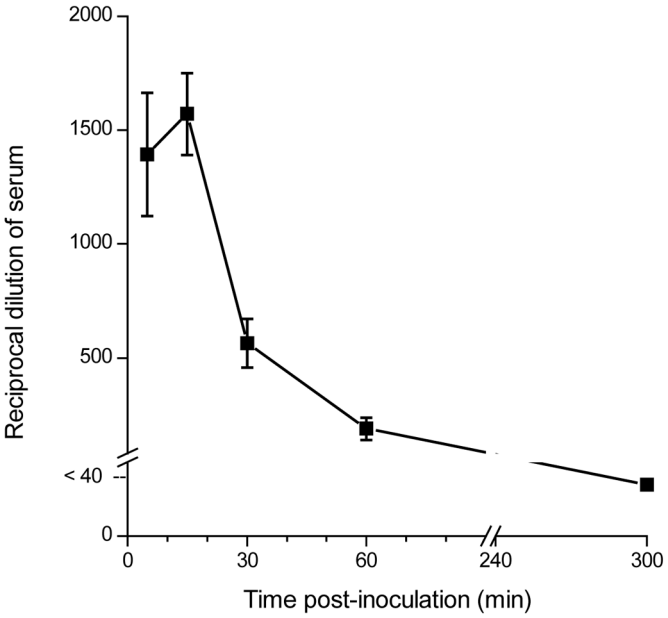

Fig. **2** Anti HSV-1 activity of serum from mice inoculated with 1C3. OF1 mice were inoculated into the tail vein with 30 mg/kg of 1C3. At different times post-inoculation, mice were bled by the tail vein and the anti HSV-1 activity in serum was evaluated by the plaque reduction assay. Each point represents the reciprocal serum dilution to achieve 50% inhibition of plaque formation. Data are mean values of 2 animals ± SD.

with the negatively charged groups involved, two factors known to affect the antiviral efficacy of polysulfates [24]. Thus, partially degraded metabolites should exhibit a reduced or lacking antiviral activity. This observation is in accordance with previous reports demonstrating that although there is evidence that sulfated polysaccharides from algae are not likely to be degraded by vertebrate enzymes *in vitro*, some degradation takes place *in vivo* [22], [25]. The partially degraded polysaccharide may be excreted in urine after a transient accumulation in kidney. No measurements of polysaccharides in urine were done by us, but it has been shown that polysaccharides of low molecular weight produced by degradation are eliminated via urine [25]. Another possibility is the unspecific binding of the compound, due to its polyanionic properties, to plasma proteins, and thus higher concentrations of the drug in blood should be required to inhibit the virus. This phenomenon was reported for dextran sulfate and other antiviral compounds [22], [23], [26].

In conclusion, the animal studies reported here indicate that the *in vitro* HSV-1 inhibition activity of 1C3 can also be seen *in vivo*, although efficacy was exhibited only when treatment began immediately after virus inoculation. This finding correlates with the *in vitro* timing studies of 1C3 in Vero cells [17] and the pharmacokinetic behavior of the compound shown above. The high antiherpetic activity of 1C3 was mainly ascribed to its interaction with the positively charged sites on the viral envelope glycoproteins necessary for virus attachment to cell surface, heparan sulfate [17]. For this reason, the best results in protection studies were obtained when the compound was administered to mice immediately after virus infection and by the same route as virus inoculation, and no protection was afforded when treatment was started once virus infection was well established. Moreover, the

quick clearance of active 1C3 from blood did not allow us to increase its efficacy by multiple inoculations of the compound at different times post-infection. However, the level of protection afforded by 1C3 in this systemic murine HSV-1 infection warrants further evaluation of this μ/ν carrageenan in a larger animal model when formulated in a clinically useful vehicle. The development of new drug delivery systems is a strategy gaining attention to improve the effectiveness of promising chemotherapeutic agents such as polysufates, polypeptides, proteins and oligonucleotides, for application in systemic viral infections.

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