PHYTOTHERAPY RESEARCH
Phytother. Res. **23**, 707–712 (2009)
Published online 23 December 2008 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2723

Antiretroviral Activity of Fucoidans Extracted from the Brown Seaweed *Adenocystis utricularis*

Juan Trinchero[1], Nora M. A. Ponce[2,4], Osvaldo L. Córdoba[3], María L. Flores[2], Sandra Pampuro[1], Carlos A. Stortz[4], Horacio Salomón[1] and Gabriela Turk[1]*

[1]Centro Nacional de Referencia para el SIDA, Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, C1121ABG, Buenos Aires, Argentina
[2]Farmacognosia, CRIDECIT, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, km 4, 9000, Comodoro Rivadavia, Chubut, Argentina
[3]Química Biológica II, CRIDECIT, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, km 4, 9000, Comodoro Rivadavia, Chubut, Argentina
[4]Departamento de Química Orgánica, CIHIDECAR, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, 1428, Buenos Aires, Argentina

Treatment of human immunodeficiency virus type 1 (HIV-1, causative agent of AIDS) infection represents a major challenge in antiviral therapeutics. Many difficulties are associated with the treatment, including toxicity, resistance and high costs. Taking this into account, research for novel compounds able to overcome these limitations is needed. Sulfated polysaccharides appear to be interesting, given their abundance as components of seaweeds. Herein, a series of fractions obtained from the brown seaweed *Adenocystis utricularis* was analysed for *in vitro* anti-HIV-1 activity. These fractions, which have anti-herpes simplex virus activity, were determined previously to belong to the family of fucoidans, sulfated polysaccharides obtained from the cell walls of brown seaweeds. Assays in human PBMC primary cell culture demonstrated that two of the five fractions analysed had potent anti-HIV-1 activity both against WT and drug-resistant HIV-1 strains. For active fractions, it was also shown that the inhibitory effect was not due to an inactivating effect on the viral particle (i.e. no virucidal activity was detected) but rather to a blockade of early events of viral replication. Given these encouraging results, these seaweed-derived fractions appear as good candidates for further studies on their potential for *in vivo* therapy and/or prophylaxis of HIV-1 infection. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: HIV; brown seaweeds; fucoidans; antiviral activity.

INTRODUCTION

During the past two decades there has been a remarkable growth in the development of antiviral chemotherapies, mainly driven by the needs of immunocompromised individuals such as patients with AIDS and transplant recipients. However, the search for and/or development of new compounds should still continue since drugs currently in use are not always well tolerated, present several side effects and resistance rapidly emerges as a consequence of prolonged treatment.

In the particular case of AIDS, combination therapies of at least three antiviral drugs, also known as highly active antiretroviral therapy (HAART), is available nowadays. However, HAART is incapable of wiping out the causative virus (the human immunodeficiency virus, HIV), it has also been shown to be toxic (leading to poor adherence to treatment) and viral variants harboring drug-associated mutations are selected rapidly. Moreover, it has high costs and limited availability, depriving patients in developing countries of its benefits (Hoffmann *et al.*, 2006). These limitations, added to the ongoing growth of the AIDS epidemic, encourage the search for new antiviral agents.

Many investigators have explored the biodiversity of the plant kingdom as a new source to find novel and better antiviral agents with new mechanisms of action. Among this biodiversity, seaweeds are very abundant in most oceans and seas, and have started to receive consideration. It has been reported previously that products extracted from seaweeds have *in vitro* or *in vivo* activity against viruses such as herpes simplex viruses (HSV-1, HSV-2) and HIV, among other bioactivities (Balzarini and Van Damme, 2007; Cos *et al.*, 2004; Schaeffer and Krylov, 2000). In particular, brown seaweeds are known to produce different polysaccharides (namely alginates and fucoidans), anionic biopolymers constituting the cell wall, and laminaran, a neutral glucan which acts as a reserve biopolymer. Some fucoidans, such as those isolated from *Fucus vesiculosus* (Beress *et al.*, 1993; Moen and Clark, 1993) and *Sargassum horneri* (Hoshino *et al.*, 1998) have already demonstrated anti-HIV activity. Besides, other sulfated polysaccharides, such as dextran sulfate, have also been shown to inhibit HIV replication interfering with virus attachment to target cells (Callahan *et al.*, 1991; Harrop *et al.*, 1994).

* Correspondence to: Dr Gabriela Turk, Centro Nacional de Referencia para el SIDA, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 Piso 11, C1121ABG - Buenos Aires, Argentina.
E-mail: gturk@fmed.uba.ar
Contract/grant sponsor: National Research Council of Argentina (CONICET); UBA; PNUD; UNPSJB.

Received 12 May 2008
Revised 25 July 2008
Accepted 22 September 2008

Studies of the polysaccharide system of *Adenocystis utricularis* have shown that this species biosynthesizes at least two different families of fucose-containing polymers. One of those families (galactofucans) is constituted only by fucose, galactose and sulfate ester (Ponce *et al.*, 2003). These products have been demonstrated to carry a high inhibitory action against herpes virus with neither anticoagulant nor antibacterial properties (Ponce, 2007). The other group of fractions (uronofucoidans) are more heterogeneous, have a small sulfate content and higher uronate proportions as well as a very slight inhibitory action against herpes virus (Ponce *et al.*, 2003).

In this paper, the antiviral activity of some galactofucan fractions from *A. utricularis* against wild type (WT) and drug-resistant HIV-1 strains are described. It was also determined that the inhibition of viral replication involves the early steps of the replication cycle (adsorption/entry) and that it is not due to a virucidal effect.

MATERIALS AND METHODS

Algal material. The brown seaweed *Adenocystis utricularis* was harvested in summer from the shores near Comodoro Rivadavia (Patagonia Region, Chubut Province, Argentina). The thalli were air-dried and milled to a fine powder.

Retrieval of the galactofucan fractions. The analytical procedures, extraction and fractionation were performed as reported previously (Ponce *et al.*, 2003). Briefly, the residue of the milled seaweed preextracted with aq EtOH was split into three equal fractions (ca. 100 g each) that were extracted separately with 800 mL of water, 2% $CaCl_2$ and HCl (diluted to pH 2). Each extraction was carried out at room temperature for 7 h giving EW1, EC1 and EA1, respectively, and then with the same solvents at 70 °C (giving EW2, EC2 and EA2, respectively). For the fractionation, 10% w/v aq. solution of hexadecyltrimethylammonium bromide (cetrimide, Sigma-Aldrich, USA) was added slowly to a solution of each extract (500 mg) in water (100 mL) with stirring, until no further formation of complex occurred. The precipitates were centrifuged, suspended in 0.5 M NaCl (60 mL), and stirring was continued overnight. The precipitate was centrifuged, and the supernatant was extracted with 1-pentanol, dialysed, concentrated and freeze-dried. The remaining precipitate was submitted to similar consecutive procedures with NaCl concentrations increased to 1, 2, 3, and 4 M, yielding fractions with acronyms -10, -20 and -30, respectively (4 M NaCl only dissolved negligible amounts of product). In this study, only fractions EW1-20, EC1-20, EA1-20, EC2-20 and EA2-20 (highly active against HSV) were evaluated for anti-HIV-1 activity.

Antiviral activity: cells and viruses. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Sweden) gradient centrifugation from peripheral blood of HIV-1 seronegative patients. PBMCs from at least three donors were pooled, stimulated with 0.1% phytohemagglutinin (PHA, Sigma-Aldrich) for 3 days and cultured at 37 °C in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Gibco BRL, USA), 100 U/mL penicillin (Gibco BRL), 100 mg/mL streptomycin (Gibco BRL), 10% fetal bovine serum (FBS, Gibco BRL, USA) and 10 U/mL interleukin-2 (IL-2).

The MT2 cell line was cultured at 37 °C in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FBS.

A stock of HTLV-IIIB strain of HIV-1 (titer: 1.58×10^6 $TCID_{50}$/mL) was derived from chronically infected H9 cells. Also, two drug-resistant HIV-1 strains (named 3788 and 4170; kindly donated by Dr Mark Wainberg, McGill University, Montreal, Canada) were isolated from HIV-1 infected patients experiencing treatment failure by co-culture with PBMCs from HIV negative donors. Direct nucleotide sequencing of the PCR-amplified *pol* gene from these strains revealed that both viruses have multiple mutations associated with resistance to most drugs often included in HAART regimens, including nucleoside and non-nucleoside analogs of reverse transcriptase and protease inhibitors.

Inhibition of HIV replication and cytotoxicity assays. PBMCs were infected at 5×10^5 $TCID_{50}/10^6$ cells for 2 h at 37 °C. After infection, the cells were washed and dispensed in a 96-well plate in the presence of various concentrations of galactofucan fractions (range 0.001–1 mg/mL). The experiments were performed in triplicate and the wells treated with AZT were also monitored as controls of antiviral activity. The culture medium was changed at day 4 maintaining the original concentration of galactofucan fraction. On day 7, the supernatant fluids were harvested and the production of p24 antigen was evaluated subsequently using a commercial enzyme linked immunosorbent assay (ELISA; Abbot Laboratories, USA). The absorbances were plotted against the extract concentration and the concentration required to inhibit 50% of p24 production (i.e. 50% inhibitory concentration, IC_{50}) was calculated.

Cytotoxicity studies were performed in parallel on uninfected PBMCs in order to determine the concentration of galactofucan that inhibited 50% of cell growth ($CCID_{50}$, cell culture inhibitory dose 50). Mock-infected cells were plated as described above and the cell viability was determined by trypan blue exclusion on day 7. The number of living cells was plotted against extract concentration and the $CCID_{50}$ was calculated. Once the IC_{50} and $CCID_{50}$ were obtained, the selectivity index ($SI = CCID_{50}/IC_{50}$) was determined.

In order to determine the antiviral activity against HIV strains isolated from patients experiencing treatment failure and harboring drug-resistance associated mutations, PBMCs were infected with strains 3788 or 4170. Assays were performed as described above. The IC_{50} obtained for each of these stocks were compared with that of the WT stock.

In every case, three independent experiments were performed, each in triplicate. The results are presented as mean ± standard deviation.

Virucidal activity. Vials containing 1 mL of WT viral stock were ultracentrifuged at 17 500 rpm for 1 h at 4 °C. Viral pellets were resuspended in 200 µL of selected fractions at a concentration of 0.5 mg/mL. Untreated vials (i.e. RPMI-1640 treated) were used as controls.

DOI: 10.1002/ptr

After 1 h at 37 °C, the vials were washed twice to remove algal fractions by ultracentrifugation and titrated in MT2 cells by end-point dilution. Briefly, 5×10^4 cells were incubated with 4-fold serial dilutions of the treated and untreated stocks. On day 4, the presence/absence of syncytia was observed and supernatant fluids were assayed for p24 antigen production by ELISA. The residual titer was calculated by the Spearman-Karber statistical method. This experiment was performed twice, each time in triplicate.

Inhibition of viral entry. Two vials containing 10^6 PBMCs each were pre-treated with culture medium containing a non-cytotoxic concentration of fraction EA1-20. After 1 h at 37 °C, the cells were washed twice, split in two vials each and infected in the presence or absence of fraction EA1-20 at 37 °C. Two hours later, the cells were washed twice and then cultured in complete culture medium. On day 4, the medium was replaced and, on day 7, the supernatant fluids were harvested and the production of p24 antigen was quantitated by ELISA. In parallel, mock-treated infected cells were cultured in the presence of a non-toxic concentration of EA1-20 as a control for viral replication inhibition. In this case, the galactofucan fraction was maintained in the culture medium throughout the assay.

This experiment was performed three times, each time in duplicate. The results are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The anti-HIV-1 activity of different polysaccharide fractions obtained from the brown seaweed *Adenocystis utricularis* by cetrimide-aided fractionation was evaluated in primary cell cultures of human PBMCs. Table 1 shows the inhibition of viral replication (measured as IC_{50}), cytotoxicity (measured as $CCID_{50}$) and antiviral activity (SI: cytotoxicity/viral inhibition) of the galactofucan fractions tested and those obtained for AZT.

The order of antiviral activity (selectivity index) obtained was as follows: EA1-20 > EC2-20 > AZT > EA2-20 > EW1-20 > EC1-20 (Table 1). The cytotoxicity obtained for all compounds did not differ significantly, all values being within the same order of magnitude (range 0.6–1 mg/mL). However, the fractions differed greatly regarding their IC_{50}. Fractions EW1-20 and EC1-20 showed a very low SI, mainly due to their high IC_{50} (0.07 and 0.04 mg/mL, respectively). In contrast, fractions EA1-20 and EC2-20 showed a strong *in vitro* antiviral activity (i.e. a high SI) mainly driven by their low IC_{50} (0.0006 and 0.0009 mg/mL, respectively). The activity of fraction EA2-20 was in the middle between the active and inactive algal fractions, showing one of the lowest $CCID_{50}$ but an intermediate IC_{50}. A survey in the literature indicated that other fucoidans studied previously had similar IC_{50} (within the same range observed here), although the different methodologies used to evaluate antiviral activity preclude the making of safe comparisons (reviewed in Schaeffer and Krylov, 2000). Given the results obtained for EA1-20 and EC2-20, these two fractions were used in subsequent assays.

The following step was to evaluate antiviral activity against viral isolates with drug resistance-associated mutation to antiretroviral compounds currently used in combination as part of HAART regimens. As expected, the AZT IC_{50} obtained when infecting cells with both 3788 and 4170 isolates were ten-fold higher than for the WT stock (Table 2). In contrast, no significant IC_{50} increments were observed when using EA1-20 or EC2-20. Even more, EA1-20 showed a lower IC_{50} when tested against isolate 4170. The results obtained indicate that these fractions are equally active against WT and *pol*-mutated viral stocks.

In order to test the hypothesis that the reduction of viral production may be due to a direct effect of these compounds on the viral particle, leading to its inactivation, a virucidal assay was performed. The WT viral stock was treated with 0.5 mg/mL of fractions EA1-20 and EC2-20 and subsequently washed and titrated on the lymphoblastoid T cell line MT2. No reduction of viral titer was observed after treatment with the algal fractions, indicating that these products have no virucidal activity. The concentration of the fucoidan fraction used to test the virucidal effect was >1000-fold higher than the IC_{50} obtained in the inhibition assays, thus indicating that the inhibitory effect detected in the latter is due to interference with some steps of the HIV-1 replication cycle rather than to a direct inactivating effect on viral particle.

Previous reports indicate that sulfated polysaccharides may inhibit HIV replication by blocking entry of the virus. Fraction EA1-20 was selected to investigate this hypothesis as it was the most active fraction tested in this study. In order to evaluate whether the galactofucan

Table 1. Cytotoxicity, *in vitro* anti-HIV activity and selectivity indexes of the studied galactofucan fractions, in PBMC

	$CCID_{50}$ (μg/mL)	IC_{50} (μg/mL)	SI
EW1-20	1000 ± 10	70 ± 6	14 ± 1
EC1-20	800 ± 60	40 ± 10	20 ± 6
EA1-20	600 ± 10	0.6 ± 0.1	1000 ± 192
EC2-20	700 ± 100	0.9 ± 0.01	778 ± 161
EA2-20	600 ± 100	3 ± 0.1	200 ± 72
	$CCID_{50}$ (μM)	IC_{50} (μM)	
AZT	10.00 ± 0.01	0.040 ± 0.001	250 ± 36

$CCID_{50}$, the concentration of drug that inhibits 50% of cell growth.
IC_{50}, the concentration of drug that inhibits 50% of viral production.
SI, selectivity index, defined as $CCID_{50}/IC_{50}$.
Results represent mean ± standard deviation of three independent experiments, each performed in triplicate.

Table 2. Antiviral activity against viral stocks isolated from patients experiencing treatment failure

	WT	3788	4170
		IC_{50} (μg/mL)	
EA1-20	200 ± 60	700 ± 60	700 ± 60
EC2-20	200 ± 50	600 ± 10	1000 ± 60
		IC_{50} (μM)	
AZT	0.30 ± 0.06	3.00 ± 0.01	4.00 ± 0.01

IC_{50}, the dose that inhibits 50% of viral production.
WT, wild type.
Results represent mean ± standard deviation of three independent experiments, each performed in triplicate.

DOI: 10.1002/ptr

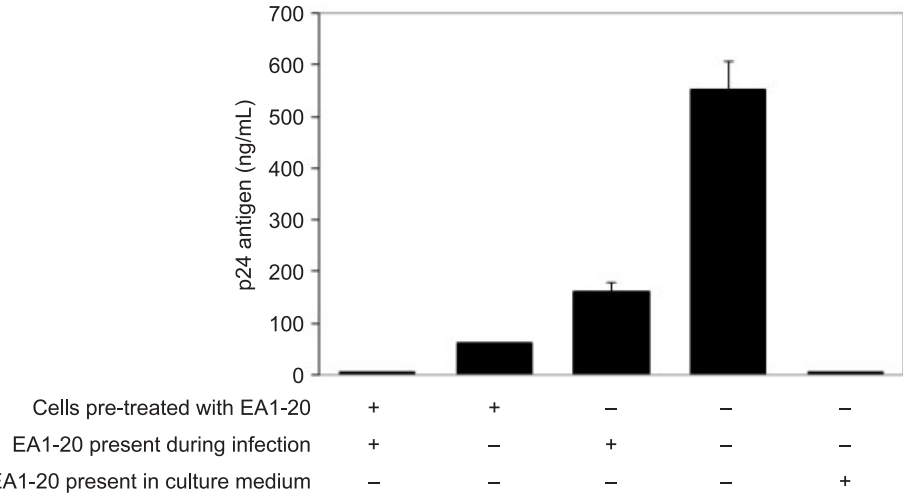

Figure 1. Fraction EA1-20 was evaluated in its capacity to inhibit early steps of viral replication. PBMCs were treated with or without the biopolymer prior to infection and then infected in the presence or absence of fraction EA1-20. After 7 days of culture, p24 antigen was quantitated in cell culture supernatants. Mock-treated infected cells were cultured in the presence of non-toxic concentration of EA1-20 as a control for viral replication inhibition. In this case, the polysaccharide was maintained in the culture medium throughout the assay. The results represent mean ± standard deviation of three independent experiments, each performed in duplicate.

fraction inhibited entry, PBMCs were treated with or without the biopolymer prior to infection. After 1 h, the cells were washed, split and infected in the presence or absence of fraction EA1-20 for 2 h. After 7 days of culture, the viral production was quantitated.

Viral production in cultures pre-treated with fraction EA1-20 (prior to and during virus adsorption) was severely compromised. Figure 1 shows that when the galactofucan fraction was present during cell pre-treatment and infection, the extent of inhibition was similar to that of the inhibition control (85-fold reduction in p24 antigen detected in supernatant, compared with the mock-treated infected cells) where the galactofucan was maintained throughout the 7-day culture. When the polysaccharide was present during pre-treatment but removed during infection, viral replication was evident (8.5-fold reduction). On the other hand, the presence of the polysaccharide only during infection but during cell pre-treatment was sufficient in order to get a 3.4-fold reduction in p24 antigen production, indicating that this galactofucan elicits a rapid and strong blockage of the cell surface avoiding viral entry. Of note, the extent of viral inhibition observed in the mock-treated infected cells, where the algal fraction is only present in culture medium after infection, indicates that there also exists a potent post-infection antiviral activity. This phenomenon denotes that the active compound also exerts its activity in subsequent rounds of infection, presumably not only inhibiting virus adsorption in successive cycles of replication but also inhibiting cell-to-cell transmission.

Research performed on purified products extracted from the plant kingdom led to the identification of a number of novel antiviral inhibitors, among them sulfated polysaccharides. These compounds are active against a broad-spectrum of enveloped viruses such as HIV-1, HIV-2 and members of the Herpetoviridae family (HSV-1, HSV-2 and human cytomegalovirus), which show low *in vitro* selection of viral resistance and inhibit early steps of viral replication (Schaeffer and Krylov, 2000). However, there exist several obstacles (mainly related to safety, tolerability and pharmacokinetic concerns) for these plant-derived molecules to pass through clinical trials (Cos *et al.*, 2004). Nevertheless, this kind of research must not be discouraged as these compounds might serve as templates for subsequent chemical modifications aimed at improving their therapeutic potential.

In a previous publication, fractionation with cetrimide of extracts obtained from the brown seaweed *Adenocystis utricularis* (abundant in the cold waters of the Southern Hemisphere) was reported (Ponce *et al.*, 2003). The procedure originated two major sets of fractions: some of them were redissolved with 0.5 M NaCl (rich in uronofucoidans, not analysed in this work), and the others were redissolved with 2 M NaCl. The last are typical galactofucans: they are almost devoid of monosaccharides, differing from fucose and galactose, carry high amounts of sulfate, and in most cases have molecular weights higher than 100 kD (Table 3). These products have a high inhibitory activity against herpes simplex virus, with no cytotoxic activity (Ponce *et al.*, 2003). The whole extracts from the room temperature extractions had a marked antiviral activity, which appears diminished in the extractions conducted at higher temperatures, as expected considering their low proportion of galactofucan. Furthermore, an increased antiherpetic activity is encountered in the purified galactofucan fractions from the room temperature extractions. A similar activity was found for the hot calcium extracted fraction EC2-20, which has analytical characteristics similar to those of the room temperature extracts. Previous works have shown that biological activities are concentrated in those galactofucan fractions (Duarte *et al.*, 2001; Nishino *et al.*, 1994; Zhuang *et al.*, 1995) since both large proportions of sulfate and high molecular weights are usually required for their action (Chevolot *et al.*, 1999; Nishino and Nagumo, 1991; Nishino and Nagumo, 1991, 1992; Venkateswaran *et al.*, 1989). Given the high anti-HSV activity of the galactofucan components of the fucoidans from *A. utricularis*, these fractions were chosen for anti-HIV activity studies. In this study, all fractions showed very low cytotoxicity in PBMC cultures but the main difference among the different fractions lay in their capacity to inhibit viral replication. As a result, different selectivity indexes were obtained for the different

DOI: 10.1002/ptr

Table 3. Analyses of the fractions obtained by cetrimide precipitation and redissolution of the fucoidans extracted from *Adenocystis utricularis* by different procedures (Ponce *et al.*, 2003)

Fraction[a]	Yield (%)	Carbohyd. (% anh.)	Uronate (%)	Sulfate (%SO_3Na)	Mol. wt. (kD)	Neutral sugars (mol/100 mol)					
						Rha	Fuc	Xyl	Man	Glc	Gal
EW1-20	25.8	59	6	24	>100	–	80	–	–	–	20
EC1-20	27.9	66	6	22	>100	–	82	–	–	–	18
EA1-20	33.0	57	4	23	>100	1	83	–	1	–	15
EC2-20	26.4	69	7	24	>100	1	82	–	–	–	17
EA2-20	13.9	76	6	21	33	1	75	1	1	1	21

[a] The acronym of the original fraction incorporates a number indicating the concentration of NaCl necessary to redissolve the fraction, in tenths of molarity (e.g. EA1-20 is the fraction of the EA1 product redissolved with 2 M NaCl).
This table was partially reproduced from Ponce *et al.,* 2003; with permission from Elsevier (License number 1995561426451).

fractions. The galactofucans originated in water and calcium chloride extracts at room temperature (EW1-20 and EC1-20) showed the lowest SI, whereas that from the hot acid extraction (EA2-20) showed an anti-HIV activity similar to that of AZT. On the other hand, the galactofucans from the hot calcium chloride extract (EC2-20) and cold acid extract (EA1-20) showed the highest SI values being four and five times more active than AZT, respectively. Fractions EA1-20 and EC2-20 were also tested against viral stocks resistant to HIV inhibitors used as components of HAART regimens as a consequence of mutations in the *pol* gene. Both fractions were equally active against these and the WT stock. This is a very important observation as these compounds (if proved to be safe for humans and active *in vivo*) could serve as components of salvage therapy, aimed for those patients experiencing repeated treatment failure to several combinations of HAART regimens.

Different authors have suggested that for seaweed-derived polysaccharides, the antiviral activity is determined by manifold structural factors, which mainly depend on the carbohydrate chain (molecular weight, linearity, flexibility and hydrophobic sites) and on the anionic groups present in the molecule (carboxylate and/or sulfate) as well as on the degree and distribution of sulfate ester (Baba *et al.*, 1990; Damonte *et al.*, 2004; Pauwels and De Clercq, 1996). A simple relationship indicates that antiviral activity increases with a higher molecular weight and degree of sulfation (Schaeffer and Krylov, 2000). Also, it has been reported that the activity might not depend upon the primary structure of the polysaccharide, since the chain can only be acting as a carrier of sulfate groups (Kolender *et al.*, 1997). This might not be true for fucoidans, since there is evidence of the role of fucose: fucoidans have a higher activity than similar polymers of galactose or glucose (Feldman *et al.*, 1999; Venkateswaran *et al.*, 1989). This might be due to the higher hydrophobic character of fucose (C-6 methyl group), and can be related to the fact that sulfated oligosaccharides glycosylated with hydrophobic long chain alcohols show a much higher activity than the original sulfated oligosaccharides (Nakashima *et al.*, 1995). The *A. utricularis* galactofucans showed a higher antiherpetic activity with an increase in sulfate content. At the same time, fractions with similar analytical characteristics showed similar activities (Ponce *et al.*, 2003). However, the galactofucans with no significant structural difference showed a considerable difference in anti-HIV activity. This indicates that although the degree of sulfation and the molecular weight are important factors for antiviral activity, other factors may be involved, as conformational factors and/or charge distribution (Witvrouw and De Clercq, 1997).

In spite of great international efforts, no anti-HIV vaccine is available nowadays to help stop the epidemic spread. Vaccine clinical trials conducted so far supported disappointing results and many years will pass until, hopefully, a safe and efficacious vaccine will be available. In the meantime, focus on the development of efficient, widely available and low cost microbicides should be given. Among other ideal properties, these microbicides should preferably possess broad activity against sexually transmitted pathogens other than HIV and direct microbicidal activity (Balzarini and Van Damme, 2007). Although the more active *A. utricularis* galactofucan fractions tested here (EA1-20 and EC2-20) have no inactivating effect on the viral particle, investigation of the putative mechanism of the antiviral action revealed that the anti-HIV action is produced early and rapidly by blocking viral adsorption/penetration. These findings agree with those reported for other sulfated polysaccharides, whose inhibitory action was attributed to inhibition of the viral adsorption and not to virucidal effects (Hoshino *et al.*, 1998; Renn, 1993; Schaeffer and Krylov, 2000). These results, added to their poor cytotoxicity and a previous work demonstrating their activity against other sexually transmitted viruses (HSV-1 and HSV-2 (Ponce *et al.*, 2003)), make these fractions interesting for further investigation of their potential as microbicidals. One product (Carraguard) derived from the red seaweed *Gigartina skottsbergii*, consisting of galactose-linked polysaccharides (named carrageenans) and shown to block HIV infection *in vitro*, has already passed phase I and II clinical trials proving to be safe (Fernandez-Romero *et al.*, 2007; Kilmarx *et al.*, 2006; Whitehead *et al.*, 2006). Currently, Carraguard has moved to a phase III HIV-prevention trial (Hart and Evans-Strickfaden, 2007).

In summary, the present work showed that two of five galactofucan fractions obtained from the brown seaweed *Adenocystis utricularis* (EA1-20 and EC2-20) have the capacity to inhibit HIV-1 replication *in vitro* with low cytotoxicity, thus resulting in high selectivity indexes. These results were obtained using either WT virus or two HIV-1 primary isolates harboring drug resistance-associated mutations. Further assays suggested that the mechanism of action involves blocking of viral entry and revealed no virucidal activity. As already mentioned,

DOI: 10.1002/ptr

the chemical structure of these polysaccharides has been described previously. Moreover, these fractions have shown potent anti-HSV-1 and HSV-2 activity but neither anticoagulant nor antibacterial properties (Ponce, 2007). The latest observations stress the specificity of the findings on antiviral activity and also reduce the possibility of side effects associated with them.

Although other publications have studied the antiviral activity of fucoidans extracted from diverse algae species, this work represents the first report on anti-HIV activity of purified fractions from a member of the Adenocystaceae family.

Acknowledgements

G.T., S.P., C.A.S. and H.S. are sponsored by the National Research Council of Argentina (CONICET). The authors are indebted to CONICET, UBA, PNUD, ARG and UNPSJB for granting this project. The authors thank Mr Sergio Mazzini for assistance during the preparation of the manuscript.

REFERENCES

Baba M, Schols D, De Clercq E *et al.* 1990. Novel sulfated polymers as highly potent and selective inhibitors of human immunodeficiency virus replication and giant cell formation. *Antimicrob Agents Chemother* **34**: 134–138.

Balzarini J, Van Damme L. 2007. Microbicide drug candidates to prevent HIV infection. *Lancet* **369**: 787–797.

Beress A, Wassermann O, Tahhan S *et al.* 1993. A new procedure for the isolation of anti-HIV compounds (polysaccharides and polyphenols) from the marine alga *Fucus vesiculosus*. *J Nat Prod* **56**: 478–488.

Callahan LN, Phelan M, Mallinson M, Norcross MA. 1991. Dextran sulfate blocks antibody binding to the principal neutralizing domain of human immunodeficiency virus type 1 without interfering with gp120-CD4 interactions. *J Virol* **65**: 1543–1550.

Chevolot L, Foucault A, Chaubet F *et al.* 1999. Further data on the structure of brown seaweed fucans: relationships with anticoagulant activity. *Carbohydr Res* **319**: 154–165.

Cos P, Maes L, Vanden Berghe D, Hermans N, Pieters L, Vlietinck A. 2004. Plant substances as anti-HIV agents selected according to their putative mechanism of action. *J Nat Prod* **67**: 284–293.

Damonte EB, Pujol CA, Coto CE. 2004. Prospects for the therapy and prevention of dengue virus infections. *Adv Virus Res* **63**: 239–285.

Duarte ME, Cardoso MA, Noseda MD, Cerezo AS. 2001. Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. *Carbohydr Res* **333**: 281–293.

Feldman SC, Reynaldi S, Stortz CA, Cerezo AS, Damonte EB. 1999. Antiviral properties of fucoidan fractions from *Leathesia difformis*. *Phytomedicine* **6**: 335–340.

Fernandez-Romero JA, Thorn M, Turville SG *et al.* 2007. Carrageenan/MIV-150 (PC-815), a combination microbicide. *Sex Transm Dis* **34**: 9–14.

Harrop HA, Coombe DR, Rider CC. 1994. Heparin specifically inhibits binding of V3 loop antibodies to HIV-1 gp120, an effect potentiated by CD4 binding. *AIDS* **8**: 183–192.

Hart CA, Evans-Strickfaden T. 2007. HIV entry inhibitors as microbicides. In *Entry Inhibitors in HIV Therapy*, Reeves JD (ed.). Birkhauser: Basel, 99–117.

Hoffmann C, Rockstroh JK, Kamps BD. 2006. *HIV Medicine 2006.* Flying Publisher: Paris, 818.

Hoshino T, Hayashi T, Hayashi K, Hamada J, Lee JB, Sankawa U. 1998. An antivirally active sulfated polysaccharide from *Sargassum horneri* (Turner) C. Agardh. *Biol Pharm Bull* **21**: 730–734.

Kilmarx PH, van de Wijgert JH, Chaikummao S *et al.* 2006. Safety and acceptability of the candidate microbicide Carraguard in Thai women: findings from a phase II clinical trial. *J Acquir Immune Defic Syndr* **43**: 327–334.

Kolender AA, Pujol CA, Damonte EB, Matulewicz MC, Cerezo AS. 1997. The system of sulfated alpha-(1–>3)-linked D-mannans from the red seaweed *Nothogenia fastigiata:* structures, antiherpetic and anticoagulant properties. *Carbohydr Res* **304**: 53–60.

Moen LK, Clark GF. 1993. A novel reverse transcriptase inhibitor *Fucus vesiculosus*. *Int Conf AIDS* **9**: 145–161.

Nakashima H, Inazawa K, Ichiyama M *et al.* 1995. Sulfated alkyl oligosaccharides inhibit human immunodeficiency virus in vitro and provide sustained drug levels in mammals. *Antiviral Chem Chemother* **6**: 271–280.

Nishino T, Nagumo T. 1991. The sulfate-content dependence of the anticoagulant activity of a fucan sulfate from the brown seaweed *Ecklonia kurome*. *Carbohydr Res* **214**: 193–197.

Nishino T, Nagumo T. 1992. Anticoagulant and antithrombin activities of oversulfated fucans. *Carbohydr Res* **229**: 355–362.

Nishino T, Nagumo T, Kiyohara H, Yamada H. 1991. Structural characterization of a new anticoagulant fucan sulfate from the brown seaweed *Ecklonia kurome*. *Carbohydr Res* **211**: 77–90.

Nishino T, Nishioka C, Ura H, Nagumo T. 1994. Isolation and partial characterization of a novel amino sugar-containing fucan sulfate from commercial *Fucus vesiculosus* fucoidan. *Carbohydr Res* **255**: 213–224.

Pauwels R, De Clercq E. 1996. Development of vaginal microbicides for the prevention of heterosexual transmission of HIV. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**: 211–221.

Ponce NMA. 2007. Estudio del sistema de polisacáridos de *Adenocystis utricularis*, un alga parda de las costas patagónicas argentinas de posible utilización farmacológica y/o industrial. PhD Thesis. Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Comodoro Rivadavia.

Ponce NM, Pujol CA, Damonte EB, Flores ML, Stortz CA. 2003. Fucoidans from the brown seaweed *Adenocystis utricularis*: extraction methods, antiviral activity and structural studies. *Carbohydr Res* **338**: 153–165.

Renn DW. 1993. Medical and biotechnological applications of marine macroalgal polysaccharides. In *Marine Biotechnology*, Vol. 1, Attaway DH, Oskar R (eds). Plenum Press: New York, 191–196.

Schaeffer DJ, Krylov VS. 2000. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicol Environ Saf* **45**: 208–227.

Venkateswaran PS, Millman I, Blumberg BS. 1989. Interaction of fucoidan from *Pelvetia fastigiata* with surface antigens of hepatitis B and woodchuck hepatitis viruses. *Planta Med* **55**: 265–270.

Whitehead SJ, Kilmarx PH, Blanchard K *et al.* 2006. Acceptability of Carraguard vaginal gel use among Thai couples. *AIDS* **20**: 2141–2148.

Witvrouw M, De Clercq E. 1997. Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *Gen Pharmacol* **29**: 497–511.

Zhuang C, Itoh H, Mizuno T, Ito H. 1995. Antitumor active fucoidan from the brown seaweed, umitoranoo (*Sargassum thunbergii*). *Biosci Biotechnol Biochem* **59**: 563–567.