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Isolation and partial characterization of an antiviral, RC-183, from the edible mushroom *Rozites caperata*

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Abstract

A protein of 10 425 Da was purified from the edible mushroom *Rozites caperata* and shown to inhibit herpes simplex virus types 1 and 2 replication with an IC_{50} value of ≤ 5 μM. The protein designated RC-183 also significantly reduced the severity of HSV-1 induced ocular disease in a murine model of keratitis, indicating in vivo efficacy. HSV mutants lacking ribonucleotide reductase and thymidine kinase were also inhibited, suggesting the mechanism does not involve these viral enzymes. Antiviral activity was also seen against varicella zoster virus, influenza A virus, and respiratory syncytial virus, but not against adenovirus type VI, coxsackie viruses A9 and B5, or human immunodeficiency virus. Characterization of RC-183 by mass spectroscopy, sequencing, and other methods suggests it is composed of a peptide (12 or 13 mer) coupled to ubiquitin via an isopeptide bond between the c-terminal glycine of ubiquitin and the epsilon amino group of a lysine residue in the peptide. The peptide sequence did not match any known sequence. Thus, RC-183 is a novel antiviral that may have clinical utility or serve as a lead compound for further development. Determining the mechanism of action may lead to identification of novel steps in viral replication.

Keywords: Antiviral; *Rozites caperata*; Protein; Herpes simplex virus

1. Introduction

Natural products have proven to be an important source of both new pharmaceuticals and lead compounds that can be modified for improved efficacy. Plants, in particular, have been a rich

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source of pharmaceuticals and approximately 25% of currently prescribed drugs have plant origins (Balick and Cox, 1996). Among the fungi, molds have been a significant source of anti-microbial and immunosuppressive compounds. Higher fungi have been tested for antibacterial activity but only recently has it been realized that some mushrooms also possess antiviral activity. For example, the basidiomycete *Fomes fomentarius* has antiviral activity against tobacco mosaic virus in plants (Aoki et al., 1993) and an extract of the edible Japanese mushroom *Lentinus edodes* has been found to inhibit the replication of herpes simplex virus (HSV), western equine encephalitis virus, poliovirus, measles virus, mumps virus (Sorimachi et al., 1990; Sarkar et al., 1993), and human immunodeficiency virus (Tochikura et al., 1988; Suzuki et al., 1990). Thus, screening of other mushroom species may lead to the identification of additional antiviral drugs.

Rozites caperata is an edible mycorrhizal mushroom that grows only in humus or sandy acidic soils in association with root systems of Jack Pine forests (Kibby, 1992). In a preliminary screen, we identified an antiviral activity in extracts of the mushroom and in this paper we describe the partial purification and characterization of an antiviral activity from *R. caperata*.

2. Materials and methods

2.1. Mushrooms

Specimens of *R. caperata* were identified and provided by Dr Martyn Dibben of the Milwaukee Public Museum (Milwaukee, WI) and Dr Dana Richter of the Michigan Technological University (Houghton, MI).

2.1.1. Preparation of mushroom extract

A total of 425 g of *R. caperata* mushrooms were homogenized in a blender with an equal weight of distilled water at room temperature. The homogenate was then heated at 60°C for 1.5 h and centrifuged at $2500 \times g$ for 60 min. The supernate was lyophilized and designated fraction 1. Fraction 1 was dissolved in water at a concentration of 100 mg/ml (dry weight). Ammonium sulfate was added slowly with constant stirring to a final concentration of 61%. The solution was then stirred at 4°C for 2 h and centrifuged at $1000 \times g$ for 15 min. The precipitate was resuspended in deionized water and both the supernate and precipitate were dialyzed against water for 2 days (six changes of water, 2 l each). Both the precipitate (fraction 2) and the supernate (fraction 3) were lyophilized and stored at −70°C. The dialysate was designated fraction 4. As shown in

Table 1
Purification of RC-183

Fraction	IC_{100}[a] (μg/ml)	Specific activity[b]	Yield[c](%)
1. Hot water extract	1200	833	4.25
2. 61% Ammonium sulfate precipitate	80	12 500	0.32
3. 61% Ammonium sulfate supernatant	Inactive	ND[d]	0.16
4. Ammonium sulfate dialysate	Inactive	ND	<0.1
5. Sephacryl 300 (fractions 20–40)	30	ND	0.06
6. Acetone precipitate[e]	30	33 000	0.06
7. Fraction 6 boiled 10 min	30	33 000	0.06

[a] Concentration resulting in 100% inhibition of cytopathic effect (μg/ml) against HSV-1 KOS. Buffalo Green cells were seeded into wells of a 96-well plate. When they were confluent, they were infected with HSV-1 KOS at 2 PFU per cell, serial dilutions of the samples were added and the wells scored at 2 days postinfection for the presence of cytopathic effect.

[b] IC_{100}/g dry weight of lyophilized material.

[c] Percent of starting material.

[d] Not done.

[e] This material was designated RC-183.

Table 1 the antiviral activity was predominantly in fraction 2.

A column (73 × 2.5 cm) was packed with Sephacryl S300 in distilled water (Pharmacia Biotech, Piscataway, NJ), a 10-ml sample of fraction 2 (60 mg/ml) in distilled water was passed through the column at a rate of 14 ml/h at 4°C, and 6.2-ml fractions were collected. The absorbance of each fraction was measured at 280 nm. The column was calibrated with known molecular weight standards (Pharmacia Biotech, Piscataway, NJ). The fractions containing the antiviral activity were pooled and designated fraction 5. This material was then precipitated with 80% acetone, air dried and stored at −20°C. The antiviral activity in this fraction was designated RC-183.

2.2. Mass spectroscopy

Mass spectroscopy was carried out using matrix-assisted laser desorption/ionization (MALDI). A total of 1–10 pM of acetone precipitated RC-183 and 1–10 pM of calibrant (cytochrome C) were mixed with a 1000-fold excess of matrix (α-cyano-4-hydroxy cinnamic acid) and evaporated onto a stainless steel probe tip. The mass spectrograph was recorded using a Vestec Model VT2000 spectrometer. Desorption and ionization were accomplished using a Lumonics HY400 Nd:YAG laser (355-nm radiation, 10-ns pulse width, 10 Hz). The ions were accelerated to 30 Kev (source region) and detected with a 20 stage focused-mesh electron multiplier (Becton-Dickinson Model MM1-1SG) at the end of a 2-M drift tube.

2.3. Polyacrylamide gel electrophoresis, protein elution, and immunoblotting

Proteins were electrophoresed in 15% denaturing-reducing polyacrylamide gels as we have described previously (Balish et al., 1993; Visalli and Brandt, 1993). For elution, individual lanes were removed from the gel and cut in 0.5-cm pieces. Each piece was electrophoretically eluted from the gel using an LKB 2014 electrophor concentrator according to the manufacturer's instructions. Samples were lyophilized, resuspended in Dulbecco's Modified Eagles Medium (DMEM) and tested for antiviral activity. Immunoblotting was carried out as we have described previously (Visalli and Brandt, 1993) using commercially available anti-ubiquitin antibody (US379, Sigma, St. Louis, MO).

2.4. Protein sequence analysis

Acetone precipitated RC-183 was electrophoresed in polyacrylamide gels as described above and electrophoretically transferred to a PVDF membrane. The 10-kDa band containing the active material was cut from the membrane and sequenced from the amino terminus using the Edman degradation procedure (Edman, 1950; Dopheide et al., 1967).

2.5. Cell culture and virus

High titer virus stocks were grown in Vero cells as we have described previously (Grau et al., 1989). HSV-2 strain 333, HSV-1 strain KOS, HSV-1 3B (a thymidine kinase negative mutant provided by Dr James Smiley, McMaster University, Hamilton, Ont., Canada), and HSV-1 ICP6Δ (a ribonucleotide reductase null mutant), provided by Dr Sandra Weller (Goldstein and Weller, 1988), were used in these studies. Vero cells were grown in Dulbecco's Modified Eagles Medium (DMEM) with 5% serum, as we described previously (Grau et al., 1989). Virus stocks were prepared in cells fed with 2% serum.

2.6. In vitro HSV yield reduction and toxicity assays

Buffalo Green cells (African green monkey kidney) were seeded (2×10^4 cells/well) into wells of a 96-well plate and infected at a multiplicity of infection (MOI) of 2.0 with HSV-1 strain KOS, HSV-2 333, HSV-1 3B, or HSV-1 ICP6Δ. Following a 1-h adsorption period, media containing RC-183 at various concentrations was added. Each concentration was tested in triplicate. At 48 h post-infection (p.i.) the amount of infectious virus was determined by plaque assay. As a preliminary test of toxicity, replicate wells of uninfected cells were exposed to the same concentrations of RC-183 and tested for viability by trypan blue exclusion 48 h later.

2.7. In vivo antiviral activity

In vivo antiviral activity was tested using a mouse model of HSV-1 induced ocular disease as we have described previously (Brandt et al., 1992, 1996). Briefly, female BALB/c mice 5–6 weeks old obtained from Harlan Sprague-Dawley (Indianapolis, IN), were anesthetized with halothane and the cornea was scarified. A 5-μl drop of media containing 1×10^6 PFU of HSV-1 KOS was placed on the cornea and left for 30 s. Excess inoculum was removed with a cotton swab. A total of ten mice were used in each group. The mice were examined microscopically at various times post-infection (p.i.), and scored for ocular disease. Blepharitis was scored: 1+, noticeably puffy eyelids; 2+, puffy eyelids with moderate crusting; 3+, eye 50% swollen shut with severe crusting; and 4+, eye totally swollen and crusted shut. Vascularization was scored: 1+, <25% of the cornea involved; 2+, 25–50% involved; and 3+, >50% of the cornea involved. The placebo group was treated with vehicle alone (50 mM sodium acetate, 0.15 M sodium chloride) and the treatment group received 1% RC-183 in vehicle. For treatment, mice were anesthetized with halothane and a 5-μl drop of solution was placed on the cornea. Treatments were given six times per day over a 12-h period for the first 3 days and four times per day for the next 4 days. Treatment was begun 3–4 h p.i. The mean disease scores on each day were compared using a *t*-test.

2.8. Stability of RC-183

Equal volumes of RC-183 (10 mg/ml) in phosphate buffered saline (PBS) were mixed with potassium periodate (0.01 M), hexane, ether, or acetone (80%). The samples were mixed for 15 min and then incubated for 15 min at room temperature. For periodate treated samples, glycerol (1% final concentration) was added to neutralize excess periodate. For organic solvents, the aqueous layers were removed and the samples were air dried or lyophilized, resuspended at the original concentration (10 mg/ml) in DMEM and tested for antiviral activity. To determine the heat stability, RC-183 samples in PBS were boiled for 10 min, cooled to room temperature, and tested for antiviral activity in vitro.

To test for protease sensitivity, RC-183 (10 mg/ml) was incubated with trypsin (1.25%) overnight at 37°C. Phenylmethylsulfonylfluoride (PMSF, 10 mM) was added to inactivate the trypsin and the samples were tested for antiviral activity in vitro. The percent reduction in activity was determined by comparing the amount of RC-183 needed to reduce viral titers by 4-logs (untreated/treated × 100). Control cultures were exposed to solution containing PMSF with no RC-183 and it was determined that the PMSF had no effect (data not shown).

2.9. Spectrum of activity and cytotoxicity

Adenovirus type VI, coxsackie virus A9, and coxsackie virus B5 were obtained from the Franciscan Shared Laboratories, Virology Laboratory, Wauwatosa, WI. Influenza A/Shanghai/H3N2, and varicella zoster virus (VZV) were obtained from Dr G. Sedmak, City of Milwaukee Virology Laboratory, Milwaukee, WI. Cell lines appropriate for growth and testing each virus were obtained from the same sources. Respiratory syncytial virus strain long (RSV) was obtained from the American Type Culture Collection (ATCC VR-26). Cells were grown in MEM with 10% fetal bovine serum.

To assay activity, 2.5×10^5 cells were seeded into each well of a 24-well culture dish and were incubated overnight at 37°C with 5% CO_2. The next morning the cells were infected at a multiplicity of infection (MOI) of 4.0 for coxsackie B5 and A9 and influenza, 0.4 for adenovirus and VZV and 0.2 for RSV. Following a 1-h adsorption period, the cells were rinsed once with medium and refed with medium containing 10% serum and RC-183 at a final concentration of 3 μg/ml. Duplicate samples of virus infected, virus infected RC-183 treated, uninfected untreated cells, and uninfected cells treated with RC-183 were tested. The cultures were incubated for 3 days and then scored for cytopathic effect (cpe) as follows: 0, no cpe; 1+, <25% of cells with cpe; 2+, 25–50% of cells with cpe; 3+, 50–75% of the cells with cpe; 4+, 75–100% of cells with cpe.

The same scale based on cell morphology was used for scoring cytotoxicity in uninfected cells.

Activity against HIV was tested in the laboratory of Dr Louis Holland (IIT Research Institute, Chicago, IL) according to previously published methods (Holland et al., 1992). Briefly, CEM-T4 cells maintained in RPMI 1640 with 10% FBS were treated with polybrene (2 μg/ml) and 1×10^4 cells were seeded into each well of a 96-well culture dish. The RC-183 was added and the cells incubated at 37°C for 1 h. The IIIB strain of HIV was then added (MOI 0.5). Once maximum cpe was achieved (7–9 days), the MTT assay was used to determine the percent viable cells in the cultures. The percentage values were then converted to the scoring system described above for the other viruses.

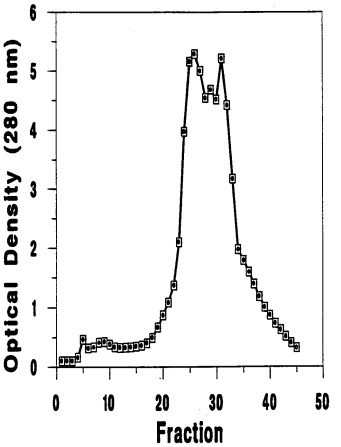

Fig. 1. Sephacryl S300 chromatography of ammonium precipitated material. The sample was loaded on a 75×2.5-cm column at a flow rate of 14 ml/h. Fractions of 6.2 ml were collected and the absorbance at 280 nm was measured.

3. Results

As shown in Table 1, the IC_{100} against HSV-1 in Buffalo Green cells for the crude extract was 1.2 mg/ml. The 61% ammonium sulfate precipitate was found to contain all of the antiviral activity (IC_{100}, 80 μg/ml). The active material was then chromatographed on a Sephacryl 300 column (S300, Pharmacia Biotech, Piscataway, NJ), and fractions were tested for antiviral activity. Fig. 1 shows the S300 column profile. The two peaks, composed of fractions 20–40 (fraction 5, Table 1), contained the antiviral activity. When pooled, fraction 5 had an IC_{100} of 30 μg/ml against HSV-1 KOS (Table 1). The pooled active material (fraction 5) was then precipitated with acetone (fraction 6) and denoted RC-183. Boiling of the RC-183 for 10 min did not reduce its antiviral activity (Table 1). Altogether, the protocol resulted in a 40-fold purification with a 0.06% yield.

The RC-183 was resuspended in distilled water at a concentration of 10 mg/ml, and treated with either lipid solvents, potassium periodate, or trypsin, to determine the nature of the antiviral activity. As shown in Table 2, neither ether nor hexane reduced the antiviral activity of RC183. Treatment with periodate partially reduced the activity whereas trypsin digestion resulted in a reduction in the activity of RC-183 to 25% after an overnight incubation. These results were consistent with RC-183 being a protein or having a proteinaceous component.

Analysis of RC-183 by SDS polyacrylamide gels (Fig. 2) indicated that the peak in the S300 column contained several bands ranging in molecular weight from 10 to 66 kDa. In order to determine which of the proteins contained the antiviral activity, a second, unstained, polyacrylamide gel was cut into small pieces, the proteins were recovered by electroelution from the gel, and the eluted material was tested for antiviral activity. Only gel pieces containing the 10-kDa band contained antiviral activity (data not shown). Note that as the intensity of the 10-kDa protein

Table 2
Stability of RC-183

Treatment	μM IC_{50}	Percent recovery[a]
None	24	100
Ether	28	86
Hexane	24	100
Acetone	28	86
Periodate	40	60
Trypsin	96	25

[a] μM IC_{50}sample/μM IC_{50}control × 100.

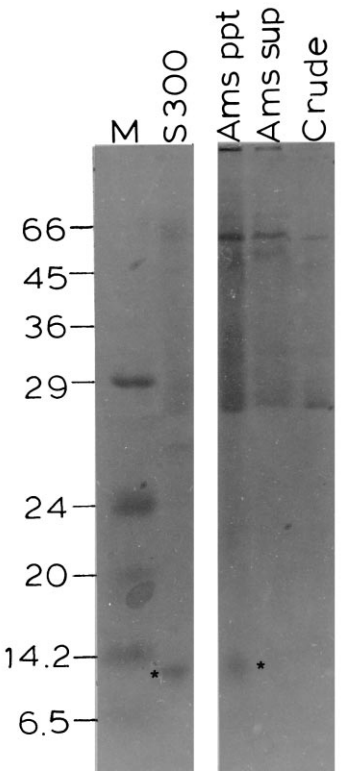

Fig. 2. Denaturing polyacrylamide gel electrophoresis of RC-183. The material loaded in each lane is denoted in the figure. The following amounts were loaded: S300, 15 μg; ammonium sulfate precipitate, 40 μg; ammonium sulfate supernatant, 250 μg; crude extract, 25 μg. Samples were electrophoresed in a 15% gel and stained with Coomasie blue. Unstained low range molecular weight markers were obtained from Sigma (St. Louis, MO). Only the material migrating at about 10 kDa (asterisks) had antiviral activity.

increased, the intensity of the higher molecular weight species decreased. Further analysis of the RC-183 by mass spectroscopy showed a single peak with a molecular mass of 10 425 Da (Fig. 3). The other higher molecular weight components contained within the RC-183 were not detected by mass spectroscopy even though the intensity of these bands relative to the 10-kDa band indicated that there should have been enough material to detect. The fact that only a single peak was seen suggests the higher molecular weight material is inactive aggregated RC-183. The mass spectroscopy peak was slightly broader than expected, which may be due to the fact that the material was resuspended in PBS and was not de-salted prior to mass spectroscopy. Note that the peak for the cytochrome C standard was also slightly broadened.

In an attempt to identify the 10-kDa protein with antiviral activity, S300 purified acetone precipitated RC-183 was electrophoresed in a 15% polyacrylamide gel and transferred to a polyvinylidenedifluoride (PVDF) membrane by electroblotting. A piece of the filter containing the 10-kDa protein was then cut out so as to eliminate the higher molecular weight inactive species and sequenced. Sequence analysis revealed two amino terminal sequences. A total of 21 amino acid residues of the first sequence were found to be a perfect match to the sequence of *Saccharomyces cerevesiae* ubiquitin. The second sequence consisted of an 11 amino acid peptide NH_2-Ala-Asn-Val-Val-Ala-Thr-Tyr-Pro-Ala-His-Ser-COOH, which did not match any known amino acid sequence. As additional confirmation that the 10-kDa band contained ubiquitin, RC-183 and purified *S. cerevesiae* ubiquitin were immunoblot-

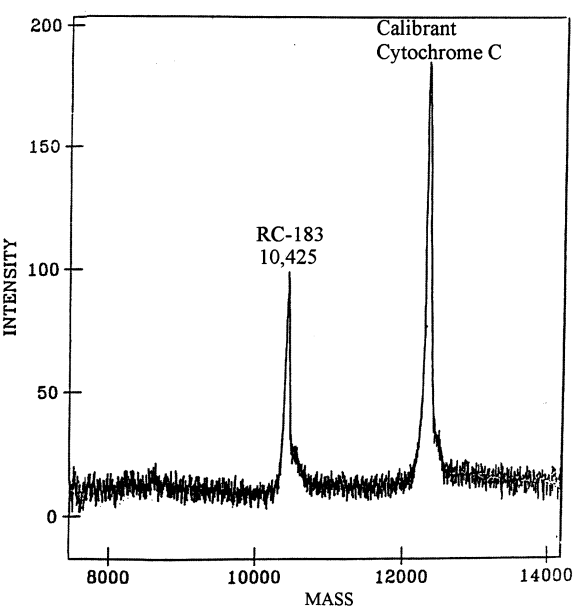

Fig. 3. MALDI analysis of Sephacryl S300 acetone precipitated active material from fractions 5 and 6.

ted using anti-ubiquitin antibody. A protein migrating at approximately 10 kDa was detected in the RC-183 sample (data not shown).

We next proceeded to test the antiviral activity using the S300-acetone precipitated material in yield reduction assays with HSV-1 strain KOS, a mutant (ICP6Δ) lacking the large subunit of ribonucleotide reductase (RR) (Goldstein and Weller, 1988), a thymidine kinase negative mutant (KOS 3B), and HSV-2 strain 333. The results are shown in Fig. 4A. The IC_{50} values ranged from 1.6 to 5.0 μM for all of the viruses tested and were comparable to the IC_{50} value of acyclovir (4.5 μM) in these types of assays (data not shown). These results indicated that the antiviral activity of RC-183 does not require the viral RR or TK enzymes and that a HSV-2 strain is inhibited as well as HSV-1. In a preliminary analysis using trypan blue staining, the RC-183 was not toxic to the Buffalo Green cells at concentrations up to 100 μM. Thus, the therapeutic index ranged from at least 20 (HSV-1 KOS) to 62 (ICP6Δ).

Since ubiquitin and a 11-mer peptide were identified as components of the material, we repeated the viral plaque reduction assays using either the purified RC-183, ubiquitin alone, or the synthetic 11-mer. The results are shown in Fig. 4B. The IC_{50} value for RC-183 was 0.8 μM compared to 90 μM for ubiquitin and 60 μM for the peptide. Thus, RC-183 was 112-fold more active than ubiquitin alone and 75-fold more active than the peptide. These results suggest that neither the ubiquitin or peptide alone constitutes the fully active material.

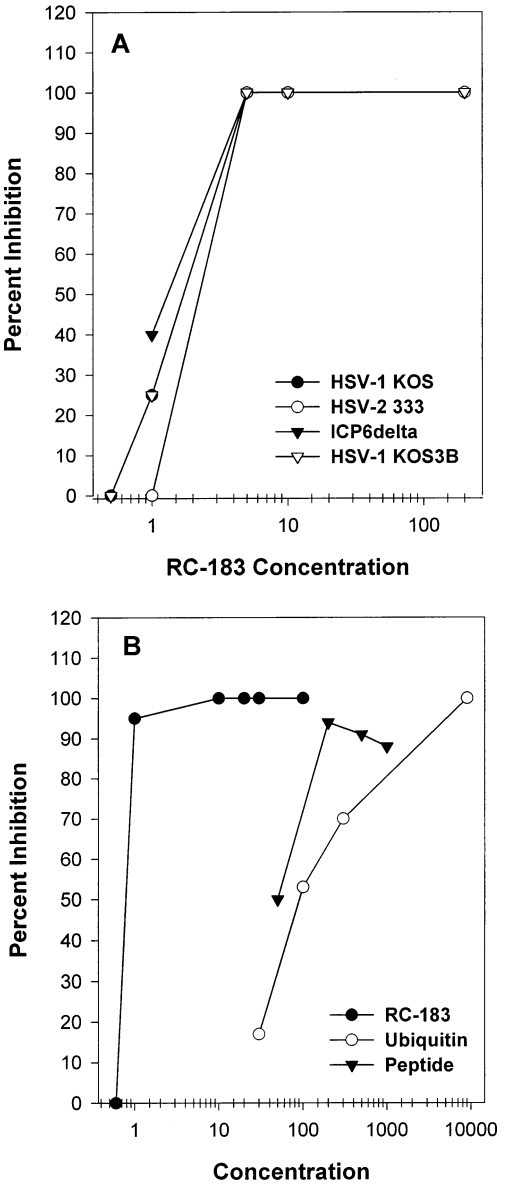

Fig. 4. Yield reduction assay for in vitro activity of RC-183. (A) Activity against viral mutants lacking ribonucleotide reductase (ICP6Δ), thymidine kinase (KOS3B) and a strain of HSV-2 (333). (B) A comparison of the activity of RC-183, ubiquitin, and the 11-mer peptide against HSV-1 KOS.

3.1. In vivo antiviral activity

To determine if RC-183 had in vivo activity against HSV-1, we used our previously described mouse model of HSV ocular infection (Brandt et al., 1992, 1996). Three parameters of ocular disease, blepharitis, corneal neovascularization and stromal keratitis were scored. The results are shown in Fig. 5. On day 4 p.i., the blepharitis score in the group treated with RC-183 was 1.8 (Fig. 5A). On subsequent days, the severity of blepharitis decreased. In the group treated with buffer only, the blepharitis score was 0.9 on day 4, then increased to a score of 2.2 on day 6. It remained between 1.8 and 2.2 from day 8 to 11, but decreased after that. Statistical

analysis of the results using Student's t-test showed that only on days 11 and 13 were the differences significant ($P < 0.006$).

The effect of treatment on the development of corneal neovascularization is shown in Fig. 5B. In the group given buffer only, vascularization was first noticed on day 8 and continued to increase in severity. Vascularization in the RC-183 treated group was also first detected on day 8, then increased slightly on day 11 and continued to increase, but at a slower rate than the vehicle group (B). Statistical analysis of the results using Student's t-test, revealed that the differences were significant ($P < 0.05$) only on days 11 through 15.

Stromal keratitis was noticeable on day 4 in the vehicle group (Fig. 5C). Corneal clouding then continued to increase until day 15. A slight amount of corneal clouding was seen in the RC-183 treated group on days 4–6. On day 8, the severity of corneal clouding increased to 0.88 in the RC-183 group and it remained near this level through the end of the experiment. Analysis of the results on all days using the Student's t-test, revealed that the differences were highly significant ($P < 0.002$) only on days 11–15.

3.2. Spectrum of activity

Table 3 shows the results of tests of RC-183 antiviral activity against different viruses. We found that RC-183 was inhibitory to influenza A, VZV, and RSV, in addition to HSV-1 and HSV-2 (Table 3). RC-183 was not active against adenovirus, coxsackie virus, or human immunodeficiency virus. RC-183 was also not toxic at the doses used to any of the cell lines used based on cell morphology.

4. Discussion

In this study, we describe the isolation, partial structural characterization, and in vitro and in vivo activity of an antiviral substance from the edible mushroom *R. caperata*. We found the active material most likely consists of a water soluble, heat stable protein that is partially sensitive to trypsin digestion. Analysis using mass spectroscopy and gel electrophoresis suggests the active material has a molecular weight of 10 425 Da and sequencing indicated the presence of two

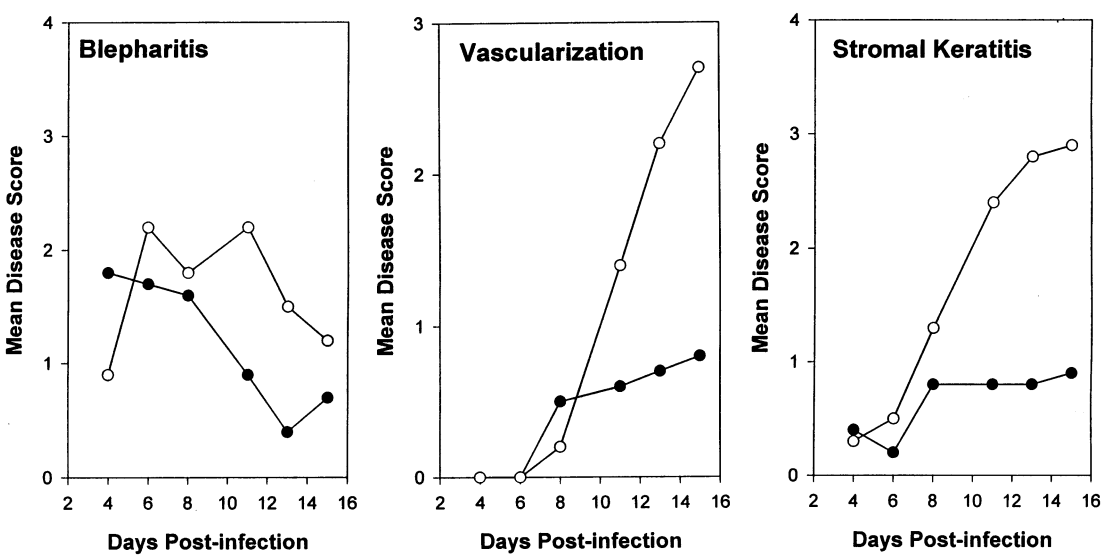

Fig. 5. In vivo activity of RC-183 in a murine model of HSV-1 ocular disease. The blepharitis scores were significantly different on days 11 and 13 ($P < 0.006$), the neovascularization scores were significantly different on days 11–15 ($P < 0.05$), and the keratitis scores were significantly different on days 11–15 ($P < 0.002$). ●, RC-183-treated; ○, placebo-treated. A total of ten mice were tested per group and all points represent the mean score on each day.

Table 3
RC-183 spectrum of activity and cytotoxicity

Virus	Cell line	CPE score		
		Infected	RC-183 treated[a]	Cells only
Adenovirus type VI	Vero	4+	4+	0
Coxsackie B5	Hep-2	4+	4+	0
Coxsackie A9	MRC-5	4+	4+	0
Varicella zoster	549	4+	0	0
Influenza A/Shanghai/(H3N2)	MDCK	4+	0	0
Respiratory syncytial virus	Hep-2	4+	0	0
Human immunodeficiency virus	CEM	4+	4+	0

[a] 3.0 μg/ml for all tests.

amino terminal peptides. One was identified as ubiquitin and the other (an 11-mer) peptide failed to match any sequences in database searches. The presence of ubiquitin in the active material was not surprising since the purification scheme is similar to protocols used for purification of ubiquitin (Haas and Wilkinson, 1985).

Tests of antiviral activity using a yield reduction assay, showed that RC-183 had IC_{50} values of 5.0 μM or less against HSV-1 KOS and 3.5 μM against HSV-2 strain 333, which compare favorably with acyclovir (4–5 μM) in similar assays (Liuzzi et al., 1994). We also found that RC-183 inhibited the growth of a TK mutant (KOS 3B) and a ribonucleotide reductase mutant (ICP6Δ) with IC_{50} values of 4.2 and 1.6 μM, respectively. Tests of in vivo efficacy revealed that 1% RC-183 significantly reduced the severity of HSV-1 induced ocular disease. In addition to its activity against HSV we also found that RC-183 had activity against influenza virus, VZV, and RSV. Interestingly, with the exception of HIV, RC-183 inhibited the growth of all enveloped viruses tested but had no activity against non-enveloped viruses.

Based on our results and the known function of ubiquitin in marking proteins for turn-over (Hochstrasser, 1996), we propose that RC-183 is composed of a conjugate of ubiquitin and a peptide linked by an isopeptide bond between the carboxy terminal glycine of ubiquitin and a lysine residue in the peptide (Fig. 6). The prediction of the lysine residue in the peptide, although not identified by sequencing, is based on the mechanism of attachment of ubiquitin to proteins and the additional amino acid denoted x is based on the difference between the predicted molecular weight and that determined by mass spectroscopy. This hypothesis is supported by several observations. (i) The molecular weight of 10 425 is consistent with a structure that consists of ubiquitin conjugated to a peptide of 12–13 amino acids. (ii) Mass spectroscopy gave a single peak of 10 425 Da. Pure ubiquitin has an apparent molecular weight of 8600 Da (Goldstein et al., 1975) and we were unable to detect any protein migrating at that size in the mass spectroscopy analysis or on polyacrylamide gels. (iii) Ubiquitin is heat stable as is RC-183. (iv) Both ubiquitin and the peptide alone were at least 50-fold less active than RC-183.

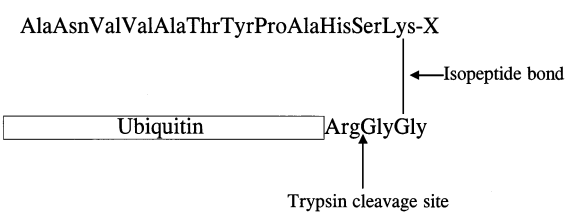

Fig. 6. Proposed structure of RC-183. Ubiquitin is coupled to a peptide (13 mer) via an isopeptide bond between the c-terminal glycine of ubiquitin and a lysine residue in the peptide. The x in the peptide denotes an additional amino acid residue predicted to exist based on the discrepancy between the molecular weights obtained by mass spectroscopy and calculated from the molecular weights of the components. The only trypsin cleavage site in native ubiquitin is also denoted in the figure.

The behavior of RC-183 in trypsin digestion is particularly instructive with regard to the proposed structure. Treatment with trypsin reduced the activity of RC-183 by 75% but did not completely eliminate antiviral activity which is consistent with previous work on the susceptibility of ubiquitin to trypsin digestion (Cox et al., 1986). Although ubiquitin has a number of potential trypsin cleavage sites, trypsin cleaves ubiquitin only between the last arginine residue and the two c-terminal glycines in its native conformation in aqueous solution (Schlesinger et al., 1975; Busch and Goldknopt, 1981; Wilkinson and Audhya, 1981), and the trypsin cleavage site in native ubiquitin is also partially resistant to trypsin cleavage. Trypsin digestion thus mimics the activity of isopeptidases that specifically cleave peptides from ubiquitin and reduces the antiviral activity by removing the peptide from RC-183. Although these results suggest that ubiquitin is a component of RC-183, as a conjugate with a peptide, further studies are needed. For example, we need to confirm the presence of the isopeptide linkage and identify any additional amino acids present at the carboxy-terminus of the peptide component. Purification of additional material for these structural studies is underway.

The presence of ubiquitin in the RC-183 also explains the higher molecular weight bands in the SDS–PAGE, since purified ubiquitin electrophoresed under the same conditions results in the formation of higher molecular weight aggregates (unpublished data). It is unlikely that these represent contaminants that affected the structural determination, since the mass spectroscopy analysis revealed only one peak at 10 425 Da and we should have seen the contaminants during mass spectroscopy. If the antiviral activity were due to a contaminant, it would have to have an IC_{50} value lower than the 1–5-μm range which is highly unlikely. In addition, the protein sequencing was done on acetone-precipitated material that had been subjected to the additional step of denaturing polyacrylamide gel electrophoresis and this same material contained antiviral activity when eluted from a gel. Note also that no bands migrating at the size of ubiquitin were seen in the protein gels or in the mass spectroscopy analysis.

At present we do not know the mechanism of action of RC-183. The fact that RC-183 inhibited HSV TK and RR mutants to the same extent as HSV-1 KOS or HSV-2 333 indicates that the activity is not dependent on either of these viral enzymes. These results also suggest, but do not prove, that RC-183 does not act on the HSV polymerase. Our assay for antiviral activity relied on inhibition of cytopathic effect. For HSV, at least, this suggests that RC-183 acts on some step prior to DNA replication. We do not know if RC-183 acts at the cell surface or enters the cells. The assay for HSV was also carried out at a MOI of 2.0 where a high percentage of cells would be infected. Thus, the inhibition observed under these conditions suggests that RC-183 is not acting by inhibiting cell to cell spread. RC-183 also displayed activity against several enveloped viruses with RNA or DNA genomes, but was inactive against non-enveloped viruses. The only exception was HIV. Although these results raise the possibility that some process or processes common to several enveloped viruses are inhibited by RC-183, the existence of separate mechanisms of inhibition specific for each virus cannot be ruled out.

A number of proteins with antiviral activity have been described. The first to be described was interferon (Isaacs and Lindeman, 1957), which acts by inducing several proteins, including 2′-5′ oligo A synthetase, P1 kinase, RNAase L and MxA (Vilček and Sen, 1996). Interferon also induces synthesis of a soluble form of the low density lipoprotein receptor which inhibits vesicular stomatitis virus (Fischer et al., 1993). Three different proteins with similar antiviral activity have been isolated from pokeweed (*Phytalacca americana*) (Irvin and Uckun, 1992). The pokeweed antiviral proteins (PAPs) act by inhibiting ribosomes and at least one PAP is structurally similar to ricin (Endo et al., 1988; Hartley et al., 1991; Irvin and Uckun, 1992). Lactoferrin, an iron binding protein in milk and tears, inhibits HSV replication in vitro and blocks HSV-1 ocular disease in vivo (Fujihara and Hayashi, 1995). A proteinaceous material with broad spectrum antiviral activity has been isolated from the cactus *Opuntia streptocantha* (Ahmad et al., 1996) and

lectins are known to have activity against several viruses (Ito and Barron, 1974; Ito et al., 1978; Khelita and Menezes, 1982; Lifson et al., 1986). *Trichosanthes kirilowii* contains a 29-kDa material that inhibits HIV (Lee-Huang et al., 1991). It is unlikely that RC-183 is related to any of these previously reported proteins since the amino acid sequences we obtained did not match proteins with known antiviral activity, some of the proteins have different spectra of activity, and the *O. streptocantha* activity is heat labile. Thus, it is likely that RC-183 represents a novel structure.

In summary, we have isolated and partially characterized a novel structure with antiviral activity from the edible mushroom *R. caperata*. The antiviral, RC-183, has a favorable IC_{50} value of 1–5 μM, is active against a number of enveloped viruses, and significantly reduces the severity of HSV-1 ocular disease in mice. Confirming the structure and determining the mechanism or mechanisms of action of RC-183 may lead to development of new antivirals and identification of novel features of the viral life cycle.

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