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Evaluation of the antiviral activity against Junin virus of macrocyclic trichothecenes produced by the hypocrealean epibiont of *Baccharis coridifolia*

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Abstract

Four macrocyclic trichothecenes, roridin A, roridin E, verrucarin A and verrucarin J, produced by the hypocrealean epibiont of *Baccharis coridifolia*, were evaluated for their inhibitory activity against the arenavirus Junin (JUNV), the etiological agent of Argentine hemorrhagic fever. The trichothecenes achieved a dose-dependent inhibition of JUNV multiplication at concentrations not affecting cell viability. The inhibitory concentration 50% (IC_{50}) values determined by a virus yield inhibition assay were in the range 1.2-4.9 ng/ml. The most active compound was verrucarin J which reduced JUNV yield more than 2 log units and had a similar effect against the arenavirus Tacaribe. The trichothecenes lacked virucidal effects on JUNV virions. From time of addition and removal experiments, it can be concluded that verrucarin J inhibited a late stage in the replicative cycle of JUNV, after 5 h of adsorption.

Keywords: trichothecenes, Junin virus, arenaviruses, antiviral activity, *Baccharis coridifolia,* Asteraceae

Introduction

Trichothecenes are a family of sesquiterpenoid compounds produced by various genera of fungi. There are two groups of trichothecenes: simple and macrocyclic, according to the presence or not of a cyclic linkage between C4 and C15. Although these trichothecenes have been associated to various biological properties, such as antibiotic, antimalarial and antileukemic activities and immunotoxic effects [1-4], very few studies on their antiviral activity have been reported. Both simple (nivalenol, deoxynivalenol, fusarenon, T-2 toxin and related compounds) and macrocyclic (satratoxin G, roridin A and baccharinoids B-4 and B-5) trichothecenes were found to inhibit the replication of Herpes Simplex virus [5-7].

A recent study reports the production of a series of macrocyclic trichothecenes, such as roridin A, roridin E, verrucarin A and verrucarin J from a new hypocrealean fungus associated to the meristem of *Baccharis coridifolia* DC (Asteraceae). This is a Southamerican shrub called "mio-mio" or "romerillo", toxic to mammalian herbivorous, because of the presence of macrocyclic trichothecenes in the plants. The production of roridins and verrucarins, by the associated epibiont, suggests that this fungus could be involved in the plant toxicity [8].

Junin virus (JUNV), a member of the *Arenaviridae* family, is the etiological agent of Argentine hemorrhagic fever (AHF), an endemoepidemic disease with hematological and neurological signs, which mainly affects male rural workers [9]. Although several compounds were found to be inhibitors of the *in vitro* replication of JUNV [10-13], no reliable drug therapy is presently available for the treatment of AHF, which consists of the early administration of standardized doses of convalescent plasma [14-16]. Thus, it was considered interesting to evaluate the inhibitory action against JUNV of the roridins and verrucarins produced by the hypocrealean epibiont of *B. coridifolia.*

Materials and methods

Trichothecenes

Four macrocyclic trichothecenes, roridin A, roridin E, verrucarin A and verrucarin J (Fig.1), were extracted from the hypocrealean fungal epibiont associated to the meristem of *Baccharis coridifolia* as previously described [8]. Stock solutions at a concentration of 2 mg/ml were prepared in dimethylsulfoxide (DMSO) and sterilized by centrifugation.

Cells and virus

Vero cells were grown as monolayers in Eagle`s minimum essential medium (MEM) (GIBCO, USA) supplemented with 5 % inactivated calf serum, 20mM HEPES, and 50 µg/ml gentamycin. Maintenance medium (MM) consisted of MEM supplemented with 1.5 % inactivated calf serum. The IV4454 strain of JUNV [17] and the TCRV 11573 strain of Tacaribe virus [18] were used. Virus stocks were prepared in Vero cell cultures and titrated by plaque formation. Cell cultures were always grown and maintained in a 4% CO_2 incubator.

Cytotoxicity assay

Cellular viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [19] in confluent cell cultures grown in 96-well plates and exposed during 48 h at 37°C to two-fold dilutions of the compounds, in the range 1.25-10 ng/ml, with three wells for each dilution. Control cultures without compound were incubated in the same conditions. Then 10 µl of MM containing MTT (final concentration 5 mg/ml) was added to each well. After 2 h of incubation, the

supernatant was decanted and 200 µl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader. Reduction in cell viability was calculated as [1 – (absorbance in treated cultures/absorbance in control cultures without compound)] x 100. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce the MTT signal by 50% compared with untreated control cultures.

Virus yield inhibition assay

Cells grown in 24-well plates (2 x 10^5 cell/ml) were infected at a multiplicity of infection (MOI) of 0.1 PFU/cell, and after 1 h adsorption cells were washed and refed with MM containing various concentrations of the compounds (from 1.25 to 5 ng/ml). Two replicates per dilution of each compound were tested. Ribavirin (Sigma-Aldrich) was used as a reference antiviral substance [10, 16] in the virus yield inhibition assay at concentrations ranging from 12 to 0.75 mg/ml. Positive virus controls without compounds were performed simultaneously. After 24 h of incubation at 37°C, supernatant cultures were harvested and extracellular virus yields were determined by plaque assay. The inhibitory concentration 50% (IC_{50}) was calculated as the drug concentration that reduced virus yield by 50% in the compound-treated cultures compared with the untreated ones.

Effect of cell pretreatment with the trichothecenes on virus yield

A virus yield inhibition assay was also performed by pretreatment of cell cultures with the compounds. Cells were grown in 24-well plates, were incubated during 3 h at 37ºC in the presence of the compounds (from 0.62 to 5 ng/ml) or without compound. Then, the compounds were removed, cells were washed with MM and infected with JUNV a

MOI of 0.1 PFU/cell. Virus yields were determined at 24 h post-infection as described previously and IC_{50} values was calculated.

Virucidal assay

A virus suspension of 10^6 PFU of JUNV was directly mixed with each compound diluted in MM to provide final concentrations ranging from 1 to 5 ng/ml, and after 1 h of incubation at 37°C the samples were diluted and titrated to determine residual infectivity.

Time of addition or removal experiments

Vero cells grown in 24-well plates were allowed to adsorb JUNV at a MOI of 0.1 PFU/ml for 1 h at 4°C. After removal of the inocula, the cells were washed twice with PBS and then MM containing 5 ng/ml of verrucarin J was added to infected cells at 0, 1, 3, 5, 7 and 9 h post-adsorption, duplicate wells for each time and further incubated at 37°C. Another set of infected cultures were incubated with MM containing compound immediately after adsorption, and the drug was removed by medium change at 1, 3, 5, 7 and 9 h post-adsorption, duplicate wells for each time. In all cases, extracellular virus yields were measured at 24 h post-infection. Results were expressed as % inhibition with respect to control infected cultures incubated for 24 h without compound.

Results and discussion

The toxicity of the four trichothecenes for Vero cells was first investigated by assessing their effects on cell viability. Vero cells were maintained in the presence of MM containing varying concentrations of each compound or were left untreated and then the MTT assay was performed. The concentrations required for 50% reduction in cell

viability (CC_{50}) were in the range 6.6-8.2 ng/ml (Table 1). Therefore, the antiviral activity against JUNV of the trichothecenes was examined at concentrations below 5 ng/ml by a virus yield inhibition assay. As can be seen in Fig.2, virus replication was inhibited by the four compounds in a concentration-dependent manner. The most active inhibitor in the range of noncytotoxic concentrations assayed was verrucarin J which reduced JUNV yield more than 2 log units. The IC_{50} values determined from these data are presented in Table 1 and confirmed that verrucarin J was the most effective JUNV inhibitor with IC_{50} and IC_{90} values of 1.2 and 2.5 ng/ml, respectively, and a selectivity index, defined as the CC_{50}/IC_{50} ratio, of 6.6. As seen in Table 1 roridins A and E exhibited an intermediate level of selectivity whereas verrucarin A inhibited JUNV replication only at concentrations near the CC_{50}. Ribavirin, a known compound active against arenaviruses, was inhibitor of JUNV at higher concentrations than trichothecenes, but with a greater selectivity index. Furthermore, also other arenavirus closely related to JUNV, the Tacaribe virus [20], was susceptible to verrucarin J showing an IC_{50} value of 2.5 ng/ml.

To test the possibility that the trichothecenes had virus-inactivating properties, a virucidal assay was performed. No differences in remaining infectivity titers between compound-treated and untreated virus suspensions were detected (data not shown), indicating that the trichothecenes did not exert a direct virucidal effect. The effect of the trichothecenes by cell pretreatment before infection was also studied. At concentrations below 5 ng/ml, only roridin E inhibited JUNV replication when cells were treated during 3 h before infection, with an IC_{50} of 0.86 ng/ml, lower than the IC_{50} value obtained when the treatment was performed after infection. The other three trichothecenes did not affect virus yield by cell pretreatment. These results suggest that

roridin E probably exerts the antiviral action through the induction of a cell refractory state to virus infection, an hypothesis that needs further investigation.

To determine the time in the JUNV replicative cycle at which verrucarin J, the most active inhibitor, caused its inhibitory action, we next examined the effect of the time of addition or removal of this compound, on virus yield. As shown in Fig.3 a similar level of inhibition was observed if 5 ng of verrucarin J was added immediately after adsorption (time 0) or as late as 9 h post-adsorption. In another set of experiments, the compound was added immediately after adsorption and then removed at various times by washing. When present only for a limited period of time after adsorption (either 1 or 3 h), verrucarin J was not effective to inhibit JUNV multiplication. At least, the presence of the compound during 5 h after adsorption was required to significantly reduce extracellular virus yields, suggesting that a late stage of JUNV replicate cycle is blocked.

Macrocyclic trichothecenes such as satratoxin G and roridin A have been previously reported active only against the multiplication of Herpes Simplex virus type 2, with IC_{50} values in the range 1-2 ng/ml [6]. Our results have shown an inhibitory effect of roridins and verrucarins against JUNV, with a comparable level of efficacy according to the IC_{50} values, suggesting that the trichothecene nucleus itself is responsible of the antiviral properties. The study of antioxidant and cytotoxic activity in extracts of *Baccharis coridifolia* has suggested the presence of compounds that interact with DNA [21]. In our studies, the target of trichothecene action in the virus life cycle is not presently elucidated. However it seems to be a stage independent of the virus genome since a similar level of effectiveness is observed against either JUNV, an ambisense single-stranded RNA virus, or Herpes Simplex virus, a double-stranded DNA virus.

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Legends to the Figures

Fig. 1. Structures of the trichothecenes mentioned in the text. **1**. Roridin A; **2**. Roridin **E**; **3**. Verrucarin A; **4**. Verrucarin J

Fig. 2. Dose-response curves of the effect of macrocyclic trichothecenes on JUNV multiplication. Vero cells were infected with JUNV (MOI:0.1 PFU/cell) and after 24 h of infection in the presence of different concentrations of roridin A (■), roridin E (▼), verrucarin A (●), and verrucarin J (▲) extracellular virus yields were determined. Virus yield in positive control = 1 x 10^3 PFU/ml. Results are expressed as percentage with respect to control cultures without compound treatment. Each point is the mean value of duplicate determinations ± standard deviation.

Fig. 3. Effect of the time of addition or removal of verrucarin J on JUNV replication. Vero cells were allowed to adsorb JUNV (MOI:0.1 PFU/cell) for 1 h at 4°C. After removal of the inocula, cells were washed with PBS and then MM containing 5 ng/ml of verrucarin J was added at 0, 1, 3, 5, 7 and 9 h post-adsorption and incubated further at 37°C (black bars). Other set of infected cultures were incubated with verrucarin J immediately after adsorption and then drug was removed at 1, 3, 5, 7 and 9 (grey bars). Extracellular virus yields were determined at 24 h p.i. in all cultures and results are expressed as % inhibition with respect to control infected cultures incubated for 24 h without compound. Virus titer in control cultures: 8.0x10^3 PFU/ml. Each value is the mean of duplicate determinations ± standard deviation.

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Table 1. Anti-JUNV activity and cytotoxicity of trichothecenes in Vero cells.

Trichothecenes	CC_{50}[a]	IC_{50}[b]	SI[c]
Verrucarin A	6.6±0.008	4.9±0.62	1.3
Verrucarin J	8.2±0.002	1.2±0.19	6.6
Roridin A	7.3±0.003	3.1±0.25	2.3
Roridin E	7.2±0.001	3.1±0.10	2.3
Ribavirin	> 100	4.5±0.7	>22.2

[a] Compound concentration required to reduce cell viability by 50%. Cytotoxicity was determined by the MTT assay. Results are presented as the mean from triplicate independent tests ± standard deviation. Optical density in control cell was 0.9.

[b] Compound concentration required to reduce virus yield by 50%. Antiviral activity was determined by virus yield inhibition assay. Results are presented as the mean from duplicate independent tests ± standard deviation. Virus yield in positive control = 1 x 10^3 PFU/ml.

[c] Selectivity index = CC_{50}/EC_{50}.

Values are expressed as ng/ml for trichothecenes and mg/ml for ribavirin.