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Antiviral Research 64 (2004) 127–130
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

The synergistic effects of betulin with acyclovir against herpes simplex viruses

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Received 14 January 2004; accepted 28 May 2004

Abstract

Betulin, a pentacyclic triterpenoid, was isolated from the bark of *Betula papyrifera*. The antiviral efficacies of betulin on herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) were evaluated using viral plaque reduction assays on Vero cells. The results indicate that betulin is active against both HSV-1 and HSV-2 infections with the 50% effective concentrations (EC_{50}) of 0.40 and 4.15 μg/ml, respectively. The cytotoxicity of betulin was examined on Vero cells using a neutral red uptake assay. The 50% cytotoxic concentration (CC_{50}) of betulin was 73.1 μg/ml. A synergistic antiviral effect between betulin and acyclovir (ACV) was determined by drug combination studies. Strong and moderate synergistic antiviral effects were observed for betulin and ACV against HSV-1 when the concentrations of ACV and betulin were higher than 0.068 and 0.4 μg/ml, respectively. At the concentrations lower than these, additive effect was found. Synergistic antiviral effects were also found against HSV-2 at higher concentrations than for HSV-1, i.e. 0.45 μg/ml of ACV combined with 8.4 μg/ml of betulin.

Keywords: Herpes simplex viruses; Betulin; Drug combination; Synergistic; Acyclovir.

Herpes simplex virus (HSV) infections continue to be a major public health problem. HSV-1 may cause genital infection, but more frequently is associated with herpes labialis, or 'cold sore', whereas HSV-2 is the most common cause of recurrent genital herpes (for reviews see Roizman and Knipe, 2001; Whitley, 2001). Chemotherapy (topical or systemic) for HSV infection has included the use of acyclovir, penciclovir, idoxuridine, trifluorothymidine, adenine arabinoside (ara-A), bromovinyl deoxyuridine, foscarnet, and other acyclic nucleoside analogues (for reviews see Crumpacker, 2001; De Clercq, 2001; Efstathiou et al., 1999). Among all the anti-HSV drugs, acyclovir was the first genuinely selective agent. It interferes with viral DNA polymerization through obligatory chain termination and competitive inhibition. The poor absorption rate and pharmacokinetics of ACV have been overcome to some extent by the use of prodrug valaciclovir in treating infected individuals. However, a major problem of ACV therapy is the development of HSV variants that are resistant to ACV (Kimberlin et al., 1995). Also, drugs such as ACV require the virus to be actively replicating and are not active when the virus is latent.

Betulin (lup-20(29)-ene-3.beta., 28-diol), a pentacyclic triterpenoid, was isolated from the bark of *Betula papyrifera* by Dr. P. Krasutsky of University of Minnesota in Duluth. Betulin, along with some of its derivatives, possesses biological

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doi:10.1016/j.antiviral.2004.05.006

activities. The University of Minnesota in Duluth investigated the antiviral efficacies of betulin on HSV-1 and HSV-2 (patents: US 5,750,578/1998, US 6,369,101/2002). Betulin derivatives are active against human immunodeficiency virus (HIV) (Kashiwada et al., 2001; Sun et al., 1998a,b), HSV-1 (Baltina et al., 2003; Pavlova et al., 2003) and can suppress ECHO 6 virus reproduction (Pavlova et al., 2003). In addition to the antiviral activities, betulin and betulinic acid have been described to possess anti-inflammatory activity. Both compounds can induce and modulate cytokine productions in human whole blood cell cultures (Zdzisinska et al., 2003). Betulin is a modest TNF-alpha inducer and also an enhancer of mitogen-induced TNF-alpha production (Zdzisinska et al., 2003). Betulinic acid is a derivative of betulin that has been effective as a selective inhibitor of human melanoma by induction of apoptosis (Pisha et al., 1995).

In this study, we report the antiviral activity and in vitro cytotoxicity of betulin. The synergistic antiviral effects of betulin in combination with ACV on HSV-1 and HSV-2 infections were also determined.

Betulin (the purity: greater than 98%; FW: 442.73) was dissolved in DMSO (dimethyl sulfoxide, Sigma). HSV reference strains F (HSV-1) and G (HSV-2) (ATCC: American Type Culture Collection, Manassas) were used in the experiments. An HSV susceptible cell line, Vero cells (African green monkey kidney cells, ATCC), were used in the virus plaque reduction assays. The culture medium for Vero cells was MEM/5% FBS (minimum essential medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen). Acyclovir was purchased from Sigma.

To evaluate the antiviral efficacies of betulin on HSV-1 or HSV-2 infections, a standard procedure of plaque reduction assay was performed as previously described (Gong et al., 2002) according to the guidance of National Committee for Clinical Laboratory Standards (NCCLS). Basically, confluent Vero cells in a six-well plate were washed with PBS and subsequently infected with either HSV-1 or HSV-2 (200 pfu/well) for 1 h at 37 °C. After viral inoculum was removed, the infected cells were washed with PBS and overlaid with 0.5% methylcellulose in culture medium containing no or increasing concentrations of betulin for 2 days. The cells were then fixed with 10% formalin and stained with 0.5% crystal violet. ACV was included as a positive control and performed in parallel. The effect of a test compound at varying concentrations is expressed as a percentage of control (mean plaque counts in drug-treated wells/mean plaque counts in control wells without drug). The EC_{50}s (effective concentrations giving 50% of plaque reduction) were calculated by linear regression using a computer program Statview™ (SAS Institute Inc., Cary, North Carolina, USA) and summarized in Table 1. The results (Table 1) showed that betulin was active against both HSV-1 and HSV-2 infections. The EC_{50}s determined by the viral plaque reduction assay were 0.40 μg/ml (standard deviation: 0.40) for HSV-1 and 4.15 μg/ml (average value of two experiments) for HSV-2.

Table 1
The EC_{50}s of betulin against HSV-1 and HSV-2 determined by a viral plaque reduction assay

Compounds	Viruses	EC_{50}	Standard deviation
Betulin	HSV-1	0.40 μg/ml	0.40
	HSV-2	4.15 μg/ml*	
Acyclovir	HSV-1	0·28 μM	0·10
	HSV-2	0.88 μM	0.75

* Mean value of two sets of experiments.

The in vitro cytotoxicity profile of betulin was examined using Vero cells. Confluent cells in a 96-well plate were washed with PBS. Culture medium (100 μl) containing no or increased concentrations of betulin was added to each well. The final concentration of DMSO in the medium was less than 1%. The cells were incubated at 37 °C for 2 days. The viability of the cells after drug treatment was measured using a neutral red uptake assay (Schmidt and Korba, 2000) and an ELISA reader at OD_{550nm}. The results are shown in Fig. 1. The cytotoxic concentration giving 50% of cell death (CC_{50}) was 73.1 μg/ml. ACV was not toxic to Vero cells at the highest concentration tested (100 μM).

The drug combination studies between betulin and ACV were performed using HSV-infected Vero cells. Confluent cells were infected with either HSV-1 or HSV-2 at 37 °C for 1 h. Following removal of viral inoculum, the infected cells were washed with PBS and covered with 0.5% methylcellulose containing betulin alone (at various concentrations: 0, 0.25 × EC_{50}, 0.5 × EC_{50}, 1 × EC_{50}, 2 × EC_{50}, 4 × EC_{50}), ACV alone (at various concentrations: 0, 0.25 × EC_{50}, 0.5 × EC_{50}, 1 × EC_{50}, 2 × EC_{50}, 4 × EC_{50}), and the mixture of varying doses of betulin and various doses of ACV in a fixed ratio, i.e. 0.25 × EC_{50} of betulin combines with 0.25 × EC_{50} of ACV, for plaque assays. The viral plaques were counted and the antiviral effects were calculated based on the following formula:

$$\text{Antiviral effect} = \frac{P_{\text{control}} - P_{\text{test}}}{P_{\text{control}}}$$

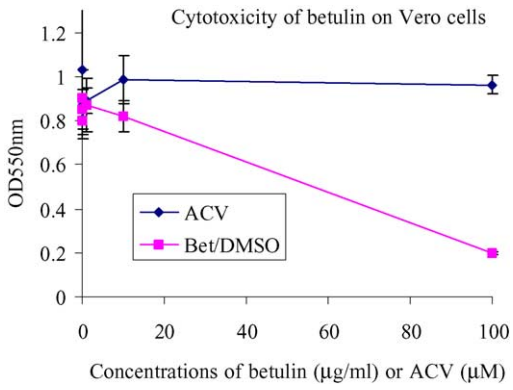

Fig. 1. The cytotoxicity of betulin on Vero cells measured by neutral red dye uptake assay.

Table 2
CI for experimental values of betulin and ACV on HSV-1

ACV (μg/ml)	Betulin (μg/ml)	CI
0.034	0.2	0.949
0.068	0.4	0.552
0.135	0.8	0.751
0.27	1.6	0.166

where $P_{control}$ is the plaque numbers in the control wells (without drug) and P_{test} the plaque numbers in the test wells with a given concentration of a test compound.

Based on the values of antiviral effects, the synergistic or antigonistic antiviral effect between betulin and ACV was determined by calculating the combination index (CI) using median-effect mathematical model (Chou and Talalay, 1984) by a computer program Calcusyn (Biosoft, St. Louis). The results are summarized in Tables 2 and 3. For reference, the range of CI:

<0.1	Very strong synergism
0.1–0.3	Strong synergism
0.3–0.7	Synergism
0.7–0.85	Moderate synergism
0.9–1.1	Additive
>1.1	Antagonism

As shown in Table 2, strong and moderate synergistic antiviral effects were determined for betulin and ACV against HSV-1 when the concentrations of ACV and betulin were higher than 0.068 and 0.4 μg/ml, respectively. At the lower concentration, i.e. 0.034 μg/ml of ACV combined with 0.2 μg/ml of betulin, additive effect was detected.

For HSV-2 (Table 3), however, synergistic effect was found only at higher concentrations of the compounds tested, i.e. 0.45 μg/ml of ACV combined with 8.4 μg/ml of betulin. When in 1:1 ratio (0.225 μg/ml of ACV combined with 4.2 μg/ml of betulin), additive effect was seen. At lower concentrations of both ACV and betulin, moderate antagonistic effect was examined.

Betulin and derivatives exhibit antiviral activities against a number of viruses. Here we report the antiviral activities of betulin against HSV-1 and HSV-2. The results indicate that betulin is potent to both HSV-1 and HSV-2 infections with the EC_{50}s of 0.40 and 4.15 μg/ml, respectively. It appears that betulin is about 10-fold more potent against HSV-1 than HSV-2. The cytotoxicity profile measured on Vero cells using a neutral red dye uptake assay showed that the CC_{50} was approximately 73.1 μg/ml. Thus the therapeutic index (TI: the ratio of the median toxic dose to the median effective dose) of betulin is approximately 183 for HSV-1 and 18 for HSV-2. Betulin is inexpensive and available in abundant supply from common natural sources, notably the bark of white birch trees.

Table 3
CI for experimental values of betulin and ACV on HSV-2

ACV (μg/ml)	Betulin (μg/ml)	CI
0.056	1.05	1.441
0.113	2.1	1.277
0.225	4.2	0.952
0.45	8.4	0.354

The synergistic antiviral effects of betulin and ACV on HSV-1 and HSV-2 infections were initially observed by Keyel et al. (The National Conference on Undergraduate Research 2000, Missoula, Montana). We confirmed this observation by incubation of varying concentrations of betulin compound with various concentrations of ACV in fixed ratios using plaque reduction assays, and quantified by analysis with median-effect equation using a computer program.

The mechanism of antiviral synergism between ACV and betulin has not been determined. The mechanism of action of betulin against HSV is not yet clear. Betulin was shown to be a selective inhibitor of DNA topoisomerase II (Wada et al., 2001) and HIV reverse transcriptase (Akihisa et al., 2001). Dargan et al. (1988) and Dargan and Subak-Sharpe (1985, 1986) studied triterpenoid compounds and found that the drugs appear to be active throughout the HSV replication cycle. The synergistic antiviral effect of betulin and ACV provides a new option for the treatment of HSV infections.

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