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Pentacyclic triterpenes in birch bark extract inhibit early step of herpes simplex virus type 1 replication

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ABSTRACT

Antiviral agents frequently applied for treatment of herpesvirus infections include acyclovir and its derivatives. The antiviral effect of a triterpene extract of birch bark and its major pentacyclic triterpenes, i.e. betulin, lupeol and betulinic acid against acyclovir-sensitive and acyclovir-resistant HSV type 1 strains was examined. The cytotoxic effect of a phytochemically defined birch bark triterpene extract (TE) as well as different pentacyclic triterpenes was analyzed in cell culture, and revealed a moderate cytotoxicity on RC-37 cells. TE, betulin, lupeol and betulinic acid exhibited high levels of antiviral activity against HSV-1 in viral suspension tests with IC_{50} values ranging between 0.2 and 0.5 μg/ml. Infectivity of acyclovir-sensitive and clinical isolates of acyclovir-resistant HSV-1 strains was significantly reduced by all tested compounds and a direct concentration- and time-dependent antiherpetic activity could be demonstrated. In order to determine the mode of antiviral action, TE and the compounds were added at different times during the viral infection cycle. Addition of these drugs to uninfected cells prior to infection or to herpesvirus-infected cells during intracellular replication had low effect on virus multiplication. Minor virucidal activity of triterpenes was observed, however both TE and tested compounds exhibited high anti-herpetic activity when viruses were pretreated with these drugs prior to infection. Pentacyclic triterpenes inhibit acyclovir-sensitive and acyclovir-resistant clinical isolates of HSV-1 in the early phase of infection.

Introduction

Plants produce a variety of biochemical constituents with the potential to inhibit viral replication, compounds from natural sources are of interest as possible sources to control viral infection (Khan et al. 2005). Plant extracts have been widely used in traditional medicine to treat a variety of infectious diseases and represent an abundant source of new bioactive secondary metabolites. Triterpenes are biologically active secondary plant substances that display antimicrobial (Pavlova et al. 2003; Woldemichael et al. 2003; Aiken and Chen 2005), hepatoprotective (Shikov et al. 2011), anticancer (Cichewicz and Kouzi 2004) and anti-inflammatory (Fernandez et al. 2011) effects. Betulinic acid mediates a specific cytotoxicity and induces apoptosis in skin cancer cell lines by direct effects on mitochondria (Fulda et al. 1998). Cytotoxic effects for triterpenes were observed in cancer cell lines originating from breast, colon, lung and neuroblastoma as well as antitumor effects in mice (Strüh et al. 2013). In particular, the pentacyclic triterpenes betulin, lupeol and betulinic acid display anti-inflammatory activities which accompany immune modulation. Pentacyclic triterpenes are secondary plant metabolites widespread in fruit peel, leaves and stem bark. The outer bark of birches like *Betula pendula*, *B. pubescens* and *B. papyrifera* consists of cork layers that are rich in pentacyclic triterpenes of the oleanane and lupane types. Among these triterpenes, betulin, a lupane triterpene, predominates with up to 34% of dry weight (Laszczyk et al. 2006). Birch bark also contains lupeol, betulinic acid, erythrodiol and oleanolic acid and is a low-cost waste product in the veneer and paper industry that is usually burned.

Herpes simplex virus (HSV) is differentiated into two antigenic types of type 1 (HSV-1) and type 2 (HSV-2) and infects mucocutaneous membranes. HSV-1 is a wide spread human pathogen, which causes epidermal lesions in and around the mouth, whereas HSV-2 causes genital herpes (Wald et al. 1995). The symptoms caused by herpes infections are usually self-limiting in immunocompetent individuals, but can be extensive and prolonged in

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immunocompromised patients. Antiviral agents licensed currently for the treatment of herpesvirus infections include acyclovir and its derivatives, nucleoside analogues which function as DNA chain terminators, ultimately preventing elongation of viral DNA (De Clercq 2004). Some of these antiviral agents might produce toxic side-effects. In addition, the emergence of virus strains resistant to commonly used anti-herpesvirus drugs is a growing problem, particularly in immunocompromised patients (Chakrabarti et al. 2000; Chen et al. 2000).

Birch bark has been traditionally used as a diction, wash, or bath additive to treat small wounds and various skin diseases (Kim et al. 2008). Triterpenes are promising leading compounds for the development of new multi-targeting pharmaceutical agents and show anti-inflammatory, anti-microbial and wound-healing properties (Laszczyk 2009; Harish et al. 2008). A synergistic antiviral effect for betulin combined with acyclovir had been observed during intracellular replication (Gong et al. 2004). Oleanolic acid showed an antiviral effect against HSV during intracellular replication similar to acyclovir, as determined by PCR resulting in reduced viral load (Mukherjee et al. 2013). Glycyrrhizin, an oleanane-type triterpenoid, showed moderate *in vitro* anti-HSV-1 activity (Ikeda et al. 2005) and may attenuate inflammatory responses in HSV infection (Huang et al. 2012). Yu et al. (2013) demonstrated the inhibition of hepatitis C virus entry into cells by oleanolic acid. A previous report described disruption of HIV-1 fusion to cells in a post-binding step through interaction of betulinic acid with the viral glycoprotein gp41 as well as disruption of assembly and budding of HIV-1 particles (Cichewicz and Kouzi 2004). Betulinic acid was targeting the V3 loop of HIV gp120 thereby inhibiting viral entry (Lai et al. 2008).

Monoterpenes and sesquiterpenes demonstrated anti-herpetic activities as recently described (Astani et al. 2010, 2011), an antiviral activity of triterpenes betulin, betulinic and betulonic acids has been demonstrated previously (Pavlova et al. 2003). However, the antiviral mechanism and the step, at which viral replication was interfered by triterpenes, has not been elucidated. In the present study we have analyzed the antiviral activity of a birch bark extract as well as pentacyclic triterpenes against herpes simplex virus type 1. The infectivity of HSV was significantly reduced *in vitro*, and the mode of antiviral action was analyzed at different steps in the viral infection cycle.

Materials and methods

Triterpene extract and pentacyclic triterpenes

The outer bark of birch contains pentacyclic triterpenes of the oleanane and lupane types. The raw material for the extract originated from Esthonian veneer industry, the birches belong to *Betula pendula* Roth, *Betula pubescens* Ehrh., hybrids of both species and other *Betula* species with white bark, all belonging to the family *Betulaceae*. A voucher specimen has been deposited. All Eurasian white-barked birches have the same triterpene content according to Krasutsky et al. (2006). The TE was obtained by a standardized, continuous extraction procedure with *n*-heptane including a clarification crystallization, the yield of extraction was 75%. No further purification was performed. The extract contained 75.4% betulin, 4.1% lupeol, 3.7% betulinic acid, 0.8% erythrodiol and 0.7% oleanolic acid. Other components in birch cork are betulinic aldehyde, betulonic aldehyde, betulonic acid, acetyloleanolic acid, ursolic acid, and sitosterol (Fig. 1). Quantification of silylated triterpenes within the extract (TE) was performed by GC-FID with external standard calibration in dependence on the method published by Laszczyk (2009). Reference standard material, i.e. betulin, lupeol and betulinic acid were provided by Birken company, structural formulas of these compounds are shown in Fig. 2. The purity of the compounds betulin, lupeol and betulinic acid was determined with HPLC and GC at >98%, >95% and >97%, respectively. TE and the pentacyclic triterpenes were dissolved in 99% ethanol for preparation of 10% stock solutions. For cell culture experiments, TE and triterpenes were further diluted resulting in a final ethanol concentration below 1% which is not toxic for cells and has no antiviral effect.

Acyclovir

Acyclovir was purchased from GlaxoSmithKline (Bad Oldesloe, Germany) and dissolved in sterile water.

Cell culture and viruses

RC-37 cells (African green monkey kidney cells) were grown in monolayer culture with Dulbecco' modified Eagle' medium (DMEM) supplemented with 5% foetal calf serum (FCS), 100 μg/ml penicillin and 100 μg/ml streptomycin. The monolayers were serially passaged whenever they became confluent, cells were plated into 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and incubated at 37 °C in an atmosphere of 5% CO_2 (Schnitzler et al. 2008). Acyclovir-sensitive herpes simplex virus type 1 strain KOS and two clinical, acyclovir-resistant isolates were used for experiments (Schnitzler et al. 2007). Viruses were routinely grown on RC-37 cells as described previously (Heidary Navid et al. 2013).

Cytotoxicity and plaque inhibition assay

For cytotoxicity assays, 5×10^4 cells were seeded into 96-well plates per well and incubated for 24 h at 37 °C. The medium was removed and fresh DMEM (Dulbecco's modified minimal essential medium) containing the appropriate dilution of TE or triterpenes was added onto subconfluent cells in eight replicates for each concentration of the drugs. Wells containing medium with 1% ethanol but no drug were also included on each plate as controls. After 3 days of incubation, the growth medium was removed and viability of the drug treated cells RC-37 was determined in a standard neutral red assay (Söderberg et al. 1996). The mean OD of the cell-control wells was arbitrarily assigned to 100%. The cytotoxic concentration of the drug which reduced viable cell number by 50% (CC_{50}) and the maximum noncytotoxic concentration of each drug were determined from dose–response curves. Inhibition of HSV replication was evaluated with plaque reduction assays. Usually 100 plaque forming units (pfu) were incubated with different concentrations of TE or selected compounds for 1 h at room temperature, afterwards treated viruses were allowed to adsorb to RC-37 cells for 1 h at 37 °C. The residual inoculum was then discarded and infected cells were overlaid with medium containing 0.5% methylcellulose. Each assay was performed in six replicates. After incubation for 3 days at 37 °C, monolayers were fixed with 10% formalin, stained with 1% crystal violet and subsequently clearly visible plaques were counted visually. By reference to the number of plaques observed in virus control monolayers without addition of drugs, the concentration of test compound which inhibited plaque numbers by 50% (IC_{50}) was determined from dose–response curves (Koch et al. 2008).

Mode of antiviral activity

Cells and viruses were incubated with drugs at different stages during viral infection cycle in order to trace the mode of antiviral action. Cells were pretreated with drugs prior to infection with HSV, or viruses were incubated with TE or triterpenes for 1 h at room temperature prior to infection, or the infected cells were incubated