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Mechanism of herpes simplex virus type 2 suppression by propolis extracts

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ABSTRACT

Genital herpes caused by herpes simplex virus type 2 (HSV-2) is a chronic, persistent infection spreading efficiently and silently as sexually transmitted disease through the population. Antiviral agents currently applied for the treatment of herpesvirus infections include acyclovir and derivatives. Aqueous and ethanolic extracts of propolis were phytochemically analysed, different polyphenols, flavonoids and phenylcarboxylic acids were identified as major constituents. The aqueous propolis extract revealed a relatively high amount of phenylcarboxylic acids and low concentrations flavonoids when compared to the ethanolic special extract GH 2002. The cytotoxic and antiherpetic effect of propolis extracts against HSV-2 was analysed in cell culture, and revealed a moderate cytotoxicity on RC-37 cells. The 50% inhibitory concentration (IC_{50}) of aqueous and ethanolic GH 2002 propolis extracts for HSV-2 plaque formation was determined at 0.0005% and 0.0004%, respectively. Both propolis extracts exhibited high levels of antiviral activity against HSV-2 in viral suspension tests, infectivity was significantly reduced by >99% and a direct concentration- and time-dependent antiherpetic activity could be demonstrated for both extracts. In order to determine the mode of virus suppression by propolis, the extracts 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 no effect on virus multiplication. However both propolis extracts exhibited high antiherpetic activity when viruses were pretreated with these drugs prior to infection. Selectivity indices were determined at 80 and 42.5 for the aqueous and ethanolic extract, respectively, thus propolis extracts might be suitable for topical therapy in recurrent herpetic infection.

Introduction

Propolis is a resinous hive product collected by honeybees from various plant sources. It has a long history of being used in folk medicine (Ghisalberti 1979) and also has been reported to possess a wide range of biological activities. The chemical components of propolis are qualitatively and quantitatively variable, depending on the geographic origin and regional plant ecology. Since propolis possesses a broad spectrum of biological activities, it is applied in popular folk medicine. It has also been used as a health drink in Asian, European and American countries (Banskota et al. 2001). Pharmacological properties of different propolis preparations have been reported as antihepatotoxic (Gonzales et al. 1995), anticarcinogenic, (El-khawaga et al. 2003), antioxidative (Russo et al. 2002), neuroprotective (Nakajima et al. 2007), anti-inflammatory (Borrelli et al. 2002), antimicrobial, (Takaisi-Kikuni and Schilcher, 1994; Kujumgiev et al. 1999; Scheller et al. 1999; Koo et al. 2000; Marcucci 1995; Abd El-Hadi and Hegazi, 2002; Kartal et al. 2003; Al-Waili 2005; Boyanova et al. 2005; Popova et al. 2005) and antiviral. A pharmacological activity against several viral infections has been demonstrated, e.g. influenza (Serkedjieva et al. 1992), HIV (Ito et al. 2001), adenovirus (Amoros et al. 1992a), and herpes simplex virus (Debiaggi et al. 1990; Amoros et al. 1994; Huleihel and Isanu 2002; Schnitzler et al. 2009). Therapeutic benefits have been reported for propolis extracts against respiratory tract infections in children (Cohen et al. 2004) and genital herpes (Vynograd et al. 2000). Propolis reveals a broad spectrum of biological activities, is used in food and folk medicine, thus there is a renewed interest in its antimicrobial and antiviral potential.

Herpes simplex virus (HSV) is differentiated into two antigenic types of type 1 (HSV-1) and type 2 (HSV-2) and infects mucocutaneous surfaces. In the acute stage of ganglionic infection, some sensory neurons undergo lytic virus infection and are destroyed, as are cells at the site of entry. After inoculation and limited replication at genital sites, HSV-2 ascends along neuronal axons to establish latent infection for life-time in the lumbosacral sensory ganglia. The latent virus is reactivated spontaneously or is

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induced to reactivate by a variety of stimuli. During the reactivation process, the virus is transported through the nerve cells axons to the original peripheral infection site, where HSV replication occurs. Infectivity is highest in pimary infections and virus excretion can persist for many weeks beyond clinical healing. While both types of HSV produce first episode genital infection, most cases of symptomatic primary disease are due to HSV-2 (Sucato et al. 1998). HSV-2 prevalence is usually higher in women than men in populations with higher risk sexual behavior (Howard et al. 2003; Smith and Robinson 2002). Genital herpes is one of the most prevalent sexually transmitted disease worldwide and is the most common cause of genital ulcers. What makes HSV so difficult to control is that most sexual and perinatal transmissions occur during unrecognized or asymptomatic shedding (Koelle and Wald 2000). The impact of genital herpes as a public health threat is augmented because epidemiological studies clearly demonstrate a strong link to the HIV epidemic. Genital herpes is a chronic, persistent infection that, on any given day, causes subclinical reactivation in about 1 to 2% of infected persons (Roizman and Sears 1995; Wald et al. 1995). HSV-2 can spread efficiently and silently as sexually transmitted disease through the population. A dramatic increase in the prevalence of HSV-2 infection was observed in younger age cohorts (Fleming et al. 1997). Genital herpes continues to be a public health problem in both developed and developing countries.

In the current study we have investigated the virus suppression by an aqueous and a special ethanolic propolis extract named GH 2002 against herpes simplex virus type 2. Both extracts were prepared from propolis of Middle Europe, well characterized in respect to its botanical and geographical origin as well as chemical composition.

Materials and methods

Aqueous and ethanolic propolis extracts

Propolis, the bee glue of *Apis mellifera*, was collected at Moravia, Czech Republic, has a defined composition, quality and provenance and contains flavonoids and phenylcarboxylic acids. Aqueous propolis extract was prepared by extracting propolis with 15% (v/v) ethanol, resulting in a viscous gold brown to brown extract with an aromatic odour with a drug to dried extract ratio of 15:1; the dried extract corresponds to about 7% of the primary raw propolis material. For experiments, an aliquot of the viscous aqueous extract was dissolved in 15% (v/v) ethanol to obtain a 10% (v/v) stock solution (1:9 diluted native extract). This aqueous extract was further diluted in water for cell culture experiments, always resulting in an ethanol concentration below 1% final concentration which has no effect on cells and viruses as reported previously (Schnitzler et al. 2009). The ethanolic extract GH 2002 was prepared with a special procedure to remove the wax and resin components. Subsequently, propolis was extracted with 90% ethanol resulting in a viscous brown extract with a bitter flavour and a drug to dried extract ratio of 2:1; the dried extract corresponds to about 50% of the primary raw propolis material. For experiments, an aliquot of GH 2002 was dissolved in 90% (v/v) ethanol for preparation of a 10% (v/v) stock solution. For cell culture experiments, ethanolic propolis extract was further diluted resulting in a final ethanol concentration below 1% which is not toxic for cells and viruses.

Acyclovir

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

Phytochemical analysis of aqueous and ethanolic propolis extracts by HPLC

Analytical HPLC of the aqueous and ethanolic extracts of propolis was run on HPLC (Shimadzu LC 10) equipped with a diode array detector Shimadzu SPD-M 10 Avp. Separation was performed on a Purospher Star RP-18 column (250×4.6 mm), particle size of 5 μm, using a mobile phase of acidified water and acetonitril. The elution was carried out with a non-linear gradient and a flow rate of 1.0 ml/min, spectrophotometric detection was conducted at 220 nm. The components were identified by comparison of their retention times and UV-spectra with standards, acquired commercially. The content of phenols in the extracts was determined according to Pharm. Eur. 2.8.14.

Cell culture and viruses

RC-37 cells (African green monkey kidney cells) were grown in monolayer culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 u/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 propagated at 37 °C in an atmosphere of 5% CO_2 (Schnitzler et al. 2007). Herpes simplex virus type 2 (HSV-2) was used for experiments. Viruses were routinely grown on RC-37 cells as described previously (Koch et al. 2008).

Cytotoxicity and antiviral assay

For cytotoxicity assays, 6×10^3 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 propolis extracts were added onto subconfluent cells in eight replicates for each concentration of the drug. 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% (TC_{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 2×10^3 plaque forming units (pfu) were incubated with different concentrations of propolis extracts 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 macroscopically 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).

Mechanism of HSV-2 suppression

In order to determine the mechanism of virus suppression for propolis extracts, cells were pretreated with propolis extracts before viral infection, viruses were incubated with these drugs