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Three-dimensional analysis of combination effect of ellagitannins and acyclovir on herpes simplex virus types 1 and 2

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ABSTRACT

The effects of combinations of three nonahydroxyterphenoyl-bearing *C*-glucosidic ellagitannins (castalagin, vescalagin and grandinin) with acyclovir (ACV) on the replication of type-1 and type-2 *herpes simplex* viruses in MDBK cells were tested by the focus-forming units reduction test. Ellagitannins included in these combinations possess a high individual antiviral activity: selectivity index of castalagin and vescalagin versus HSV-1 was similar to that of ACV, and relatively lower against HSV-2. The three-dimensional analytical approach of Prichard and Shipman was used to evaluate the impact of drug–drug interactions. The combination effects of ellagitannins with acyclovir were markedly synergistic.

1. Introduction

Herpes simplex viruses of type 1 (HSV-1) and type 2 (HSV-2) are important pathogens for humans, especially in the case of immunocompromised patients. Moreover, HSV-2 infection has been reported to increase the risk of human immunodeficiency virus (HIV) transmission. HSV-1 and HSV-2 are the primary agents of recurrent facial and genital herpetic lesions (Roizman and Knipe, 2001), HSV-2 infection being classified as a sexually transmitted disease. For immunocompromised patients and neonates, this will often results in painful and disabilitating lesions, and in some cases death (Whitley, 1995; Andersen et al., 2003).

Acyclovir (ACV) was the first effective virus-specific antiherpes drug made available. Afterwards, other nucleoside analogues have been developed in the aim of exhibiting better bioavailability than that of ACV (Efstathiou et al., 1999; Crumpacker, 2001; De Clerq, 2001). These analogues have been used with some success in the treatment of mucocutaneous and ocular HSV infections (Cremonesi et al., 1994). Although there is at present no effective vaccine or drug capable of fully eradicating established HSV infections, these antiviral drugs are able to shorten the course and decrease severity of symptomatic episodes in both normal and immunocompromised patients (Malkin, 2002). As far as the clinical use of antiherpetic nucleoside analogues as ACV is concerned, their efficacy is often compromised by the appearance of drug-resistant HSV mutants in immunosuppressed patients, such as organ transplant recipients and patients with AIDS (Coen, 1994; Bacon et al., 2003). Therefore, it is of crucial importance to search for novel compounds with alternative mechanisms of antiviral action. At the same time, treatments based on the combination of different antiviral agents are considered as promising approaches to limit the manifestation of drug resistance (Freestone, 1985; Mucsi et al., 2001; Gong et al., 2004) and to increase the antiviral effect selectivity by decreasing the active drug concentration (Allen et al., 1982; Talarico et al., 2006).

Ellagitannins constitute a class of plant polyphenols composed of a central sugar core, typically β-D-glucose, which is acylated by galloyl units that are further connected through C–C biaryl and C–O diaryl ether bonds (Quideau and Feldman, 1996; Quideau, 2009; Quideau et al., 2010). Numerous members of this class of so-called hydrolyzable tannins have been identified as active principles in plant extracts used in traditional oriental medicines (Okuda et al., 1981, 1989; Haslam et al., 1989; Okuda, 1999). A significant number of these ellagitannins expressed antitumoral activities (Miyamoto et al., 1993; Yang et al., 1999; Ito et al., 2007), as well as antiviral activities, in particular against HIV infection (Nonaka et al., 1990; Nakashima et al., 1992; Martino et al., 2004; Notka et al., 2004). Moreover, some ellagitannins manifested inhibitory effects on replication of HSV-1 and/or HSV-2, as well as Epstein-Barr virus (EBV) (Takechi et al., 1985; Fukuchi et al., 1989; Corthout

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et al., 1991; Kurokawa et al., 1998, 2001; Liu et al., 1999; Cheng et al., 2002; Ito et al., 2007). In a previous work, Quideau et al. (2004) reported on the anti-herpesvirus activity of members of a particular subclass of *C*-glucosidic ellagitannins, composed of an open-chain glucose acylated by a galloyl-derived nonahydroxyterphenoyl unit (NHTP, also known as flavogalloyl group). Some of these substances (e.g. castalagin, vescalagin and grandinin) displayed a marked inhibitory effect on the replication of HSV-1 and HSV-2, including ACV-resistant strains. It was then of special interest to investigate the combination effects of these compounds with ACV against HSV-1 and HSV-2 strains, which is the topic of this communication.

2. Materials and methods

2.1. Cells

Monolayer cultures of Madin-Darbey bovine kidney (MDBK) cells (National Bank for Industrial Microorganisms and Cell Cultures, Sofia) were grown in DMEM medium containing 10% fetal bovine serum Gibco BRL, USA, supplemented with 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in CO_2 incubator (HERA cell 150, Heraeus, Germany) at 37 °C/5% CO_2.

2.2. Viruses

Herpes simplex virus type 1, Victoria strain (HSV-1) and herpes simplex virus type 2, strain Bja (HSV-2) was received from Prof. S. Dundarov, National Center of Infectious and Parasitic Diseases, Sofia. Viruses were replicated in monolayer MDBK cells in a maintenance solution DMEM Gibco BRL, Paisley, Scotland, UK, plus 0.5% fetal bovine serum Gibco BRL, Scotland, UK. Infectious titer of stock viruses was $10^{6.5}$ and $10^{6.75}$ $CCID_{50}$ (50% cell culture infectious doses) for HSV-1 and HSV-2 strains, respectively.

2.3. Compounds tested

Nonahydroxyterphenoyl-containing *C*-glucosidic ellagitannins: castalagin, vescalagin and the lyxose-containing grandinin (Fig. 1), extracted from powdered pedunculate oak (*i.e.*, *Quercus robur*) heartwood and purified as previously described (Quideau et al., 2004), were first dissolved in distilled water to a concentration of 0.01 M and then diluted in DMEM to the required concentration. Acyclovir [9-(2-hydroxyethoxymethyl)-guanine] (ACV) was also dissolved in DMEM to the required concentration.

2.4. Cytotoxicity assays

The *in vitro* cytotoxicity on MDBK cell culture of ellagitannins and ACV was examined on both confluent monolayer and growing cells.

2.4.1. Cytotoxicity assays in resting cells

Confluent monolayer cell cultures in a 96-well plate were treated with culture medium containing either no antiviral agent or increased concentrations of ellagitannins and ACV. The viability of the cells after drug treatment was measured using three assays resulting in CC_{50} values evaluation: (a) neutral red uptake assay based on the initial protocol described by Borenfreund and Puerner (1984) using ELISA reader at $OD_{540\,nm}$ (measurement after incubation of 48 and 72 h); (b) lactate dehydrogenase (LDH) leakage assay (done at the 24th and 48th h after the treatment onset) used to determine the activity of LDH released in the medium using a commercially available kit from Sigma–Aldrich® (TOX7). The amount of LDH activity is an indicator of relative cell viability as well as a function of membrane integrity. This assay is based on the reduction of NAD by LDH to NADH, which is involved in the conversion of a tetrazolium dye to a red colored compound (formazan), the amount of which being measured spectrophotometrically at $OD_{490\,nm}$ (after incubation of 24 and 48 h); (c) MTT assay based on the protocol described for the first time by Mossmann (1983) and optimized for the MDBK cell line. At the end of the incubation time (on the 24th, 48th and 72nd h of treatment with the substance tested), a 1 mg/ml solution of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] was added (100 μl per well) to the culture maintenance solution and cells were incubated for 4 h. The yellow water soluble MTT dye is reduced by live cells to a water insoluble purple formazan. The amount of formazan, solubilized in isopropanol, is measured spectrophotometrically at $OD_{540\,nm}$. Each of the tests described above was done in triplicate to quadruplicate, with four cell culture wells per test sample.

2.4.2. Cytotoxicity assays in growing cells

The effect of substances tested on the growth curve of MDBK cells was examined using two techniques with evaluation of the 50% cell growth inhibitory concentration ($CGIC_{50}$) value: (a) by measurement of the cell count on the 24th, 48th and 72nd h after the seeding of 10^4 cells per well in 24-well plates (1 ml of a mixture 1:1 in the growing medium of 2×10^4 cells + substance tested at double concentration); (b) by neutral red uptake assay (as described in Section 2.4.1) in 96-well plates (200 μl 1:1 2×10^4

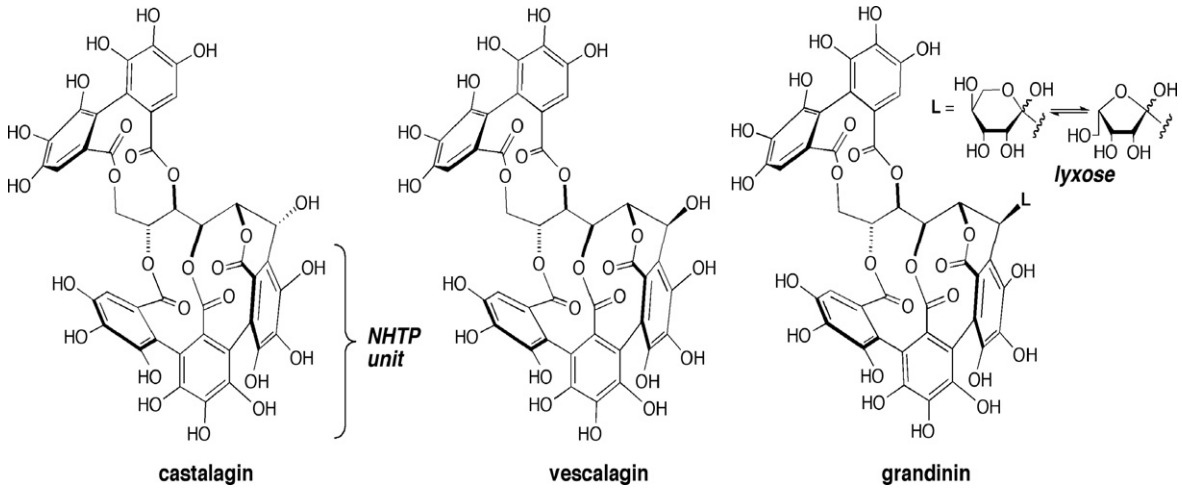

Fig. 1. Molecular structure of the ellagitannins.