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Synergistic effects by combination of ganciclovir and tricin on human cytomegalovirus replication *in vitro*

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ABSTRACT

It has been demonstrated as the first report that combination treatment with ganciclovir (GCV) and tricin (4′,5,7-trihydroxy-3′,5′ -dimethoxyflavone), a derivative of *Sasa albo-marginata*, after human cytomegalovirus (HCMV) infection has synergistic effects on both infectious virus production and HCMV DNA synthesis in the human embryonic fibroblast cell line MRC-5. In this paper, we examined the anti-HCMV effects of GCV plus various concentrations of tricin, and tricin plus various concentrations of GCV in MRC-5 cells. We found that expression of the HCMV UL54 gene was significantly inhibited by combination of GCV with tricin when compared with GCV mono-treatment. These results suggest that tricin is a novel compound for combination therapy with GCV against HCMV replication. In addition, reduced-dose combination therapy may provide a direction for treatment in patients with HCMV infection while reducing drug toxicity.

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous infectious herpes-viruses known to infect humans. It is a widespread opportunistic pathogen in immunocompromised individuals, and remains the leading viral cause of birth defects, thereby causing severe morbidity and eventual mortality (Britt, 2008; Dollard et al., 2007; Sissons and Carmichael, 2002).

There are three systemic drugs approved for HCMV treatment: ganciclovir (GCV) and its prodrug valganciclovir; foscarnet (PFA); and cidofovir (CDV) (Biron, 2006). Symptomatic HCMV infection has been treated successfully with GCV, but the appearance of GCV-resistant viruses is a recurrent problem in the treatment of immunocompromised patients. The role of anti-viral inhibitors in HCMV therapy has not yet been clarified; however, the potential use of such agents as either mono-therapy or combination therapy with existing anti-HCMV agents may be justified as their mechanisms of action against HCMV replication become better understood. While combination therapy for some infectious diseases (Tuberculosis, HIV, hepatitis C) has become the standard of care, a similar approach for HCMV therapy is not common practice. To date, one *in vitro* study reported moderate synergism of GCV and PFA against a laboratory-adapted strain and several clinical isolates (Jabs, 1996; Manischewitz et al., 1990), and the effects of a combination of GCV and PFA have been reported in patients with HCMV retinitis or HCMV encephalitis (Tunkel et al., 2008; Velez et al., 2001). On the other hand, combinations of PFA with GCV are not always successful for GCV-resistant HCMV, as they ultimately target viral DNA polymerase (Desatnik et al., 1996; Rodriguez et al., 2007). Moreover, each of these drugs has the potential for significant toxicity, with GCV potentially causing bone marrow suppression (Noble and Faulds, 1998). Therefore, effective anti-HCMV agents and regimens need to be developed (McSharry et al., 2001; Murayama et al., 2006; Schroer and Shenk, 2008).

A recent study in our laboratory revealed that tricin, which is derived from the hot water extract of *Sasa albo-marginata*, has anti-

Abbreviations: HCMV, human cytomegalovirus; GCV, ganciclovir; PFA, foscarnet; CDV, cidofovir; DMEM, Dulbecco's modified Eagle's minimal essential medium; CI, combination index; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration.

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HCMV activity via inhibition of immediate early (IE) gene expression in a human embryonic fibroblast cell line (Akuzawa et al., 2011; Murayama et al., 2012; Sakai et al., 2008). As the development of drug resistance is a constant concern, the search for new antiviral agents from a variety of sources, including plants, has become more urgent (Jassim and Naji, 2003). In the present study, we demonstrate the effects of tricin and GCV both individually and in combination on *in vitro* replication in the human embryonic fibroblast cell line MRC-5.

2. Materials and methods

2.1. Cells and viruses

The human embryonic lung fibroblast cell line MRC-5 (Jacobs et al., 1970) was grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), as described previously (Akuzawa et al., 2011). The laboratory-adapted HCMV strain Towne was used as a standard strain throughout the experiment (Furukawa et al., 1973). This HCMV was propagated in MRC-5 cells. Viral infectivity was determined by plaque assay, as described previously (Wentworth and French, 1970).

2.2. Compounds

The tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone) compound used was synthesized as described previously (Akuzawa et al., 2011; Murayama et al., 2012), and was suspended in dimethyl sulfoxide (DMSO). We previously investigated the effective concentrations of tricin, and found that the EC_{50} for HCMV production was 0.51 μM (Sakai et al., 2008). Moreover, we previously reported on the cytotoxicity of tricin, finding that the IC_{50} of tricin for MRC-5 cells was 621 μM (Sakai et al., 2008). Therefore, the selectivity index of tricin, based on the ratio of IC_{50} to EC_{50}, was 1217.6.

GCV was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo), and was suspended in DMSO at the indicated concentrations.

2.3. Viral production assays

The conditions used for viral production assay were essentially those described previously (Murayama et al., 2012; Wentworth and French, 1970). In brief, When MRC-5 cells in 24-well plates reached confluence, cells were inoculated with HCMV at a multiplicity of infection (MOI) of 1.0. After adsorption for 1 h, cells were incubated in 1 ml of DMEM containing 2% FCS with GCV and/or tricin at the indicated concentrations for 6 days after HCMV infection. Infectious virus production was titrated by plaque assay. Plaque reduction rate was calculated based on the mean plaque number in control cells incubated without compounds or in HCMV-uninfected control cells with compounds (compounds alone). The 50% effective concentration (EC_{50}) for viral replication (antiviral activity) was determined from a dose–response curve constructed using triplicate samples.

2.4. Analysis of gene expression

Confluent monolayers of MRC-5 cells in 6-well plates (Falcon #3046; Becton Dickinson, Franklin Lakes, NJ) were infected with HCMV at an MOI of 1.0. At 1 h after infection, viral inocula were removed, and cells were treated in 2 ml of DMEM containing 2% FCS with 0.0001 μM GCV (EC_{20} of GCV mono-treatment) and/or 0.002 μM tricin (EC_{20} of tricin mono-treatment). DNA samples were collected at 5 days post-infection.

Total DNA was extracted from mock- or HCMV-infected cells treated with or without GCV and/or tricin using a kit from Qiagen, in accordance with the manufacturer's instructions (DNeasy Blood & Tissue kit; Qiagen K.K. Japan). DNA accumulation was monitored by quantitative real-time PCR. The conditions used for analysis of gene expression were described previously (Akuzawa et al., 2011; Murayama et al., 2012). PCR primers were as follows; HCMV UL54 primers (forward: 5′-TTG CGG GTT CGG TGG TTA-3′, reverse: 5′-CGG CCA TAG TGT TGA GCT TAT AGT T-3′) (Petrik et al., 2006); and β-actin primers (forward: 5′-ATC ATG TTT GAG ACC TTC AAC-3′, reverse: 5′-CAG GAA GGA AGG CTG GAA GAG-3′) (Jassim and Naji, 2003). Results were normalized against β-actin DNA levels.

2.5. Methods for calculation of combination index (CI)

In order to conduct a statistical analysis on the median-effect principle for dose-effect and CI analysis, we used the median-effect method (Chou and Talalay, 1984). Briefly, the CI is calculated using the following numerical scheme. For each drug alone and for their combination, the linear portion of the dose-effect curves and the unweighted linear regression analysis are used to calculate: $CI = d1/D1 + d2/D2 + \alpha(d1d2/D1D2)$, where d1 and d2 are doses of agents 1 and 2 in a treatment with each drug alone, D1 and D2 are the doses of the agents 1 and 2 in combination treatment, $\alpha = 0$ for the mutually exclusive case and $\alpha = 1$ for the mutually non-exclusive agents. The interaction diagnosis in the CI method is based on a comparison of CI value; when $CI < 1$, synergism of effects is indicated, $CI > 1$ corresponds to antagonism, and $CI = 1$ indicates additive effects.

2.6. Statistical analysis

After means and SD were calculated, all data were analyzed using Student's *t*-test.

3. Results

3.1. Inhibitory effects GCV alone or tricin alone

In order to examine the inhibitory effects of tricin or GCV on HCMV replication, MRC-5 cells were infected with HCMV, and infected cells were treated with 1 ml of culture medium containing various concentrations of tricin or GCV, followed by culture for 6 days. Tricin and GCV mono-treatment inhibited the replication of HCMV in a dose-dependent manner, given as $y = -8.453\ln(X) + 28.78$ and $y = -6.228\ln(X) + 23.471$, respectively (Fig. 1). The EC_{50} of tricin and GCV against HCMV was 0.09 and 0.015 μM, respectively. Moreover, viral replication of HCMV was inhibited more than 90% by 10 μM tricin and GCV treatment.

3.2. Tricin enhanced GCV-induced inhibitory effects, and GCV enhanced tricin-induced inhibitory effects on HCMV replication

In combination therapy for HCMV replication, tricin was administrated with GCV. After adsorption for 1 h, cells were incubated in the presence of various concentrations of tricin or GCV in order to determine the optimal concentrations, followed by culture for 6 days. We examined the combination effects of tricin with GCV/or GCV with tricin as follows; first, HCMV-infected cells were treated with 0.0001 μM GCV (ED_{20} of GCV mono-treatment) with 0.00001–0.3 μM tricin. In the presence of tricin, virus production was most strongly inhibited in a dose-dependent manner ($y = -10.3\ln(X) - 14.384$) (Fig. 2A); viral replication of HCMV was inhibited more than 90% with 0.1 μM tricin treatment. Next, HCMV-