Antiviral Research 91 (2011) 296–303

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Inhibitory effects of tricin derivative from *Sasa albo-marginata* on replication of human cytomegalovirus

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ARTICLE INFO

Article history:
Received 10 November 2010
Revised 14 June 2011
Accepted 23 June 2011
Available online 2 July 2011

Keywords:
Anti-cytomegalovirus activity
Tricin
HCMV
IE2
COX-2
Entry

ABSTRACT

The anti-human cytomegalovirus (HCMV) activity of tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone), a derivative from *Sasa albo-marginata*, was studied in the human embryonic fibroblast cell line MRC-5. In a plaque assay, tricin and ganciclovir (GCV) showed concentration-dependent inhibitory properties from 0.05 to 3.6 μM and 0.01 to 1.0 μM, respectively. Tricin had no virucidal effects on cell-free HCMV. Treatment with tricin 1 h before, or 1 h or 3 h after viral infection significantly suppressed HCMV replication. Moreover, tricin inhibited the expression of immediate early (IE) 2 mRNA and DNA polymerase (UL54) mRNA in HCMV-infected cells. Western blot analysis also demonstrated that tricin decreased the expression of IE antigen (especially IE2) and cyclooxygenase 2 (COX-2) expression in HCMV-infected cells. In the presence of tricin, prostaglandin E2 (PGE_2) accumulation by HCMV infection was completely inhibited. These results suggest that tricin is a novel compound with potential COX inhibitor-dependent anti-HCMV activity.

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous β-herpes virus known to infect humans. It is a widespread human pathogen that has a minor clinical impact on healthy individuals, but causes various organ diseases in immunosuppressed patients and neural damage in fetuses infected *in utero* (Mocarski et al., 2007). HCMV persists as a lifelong latent infection. However, HCMV is frequently activated in immunocompromised individuals, such as patients with AIDS or organ transplants, thereby causing severe morbidity and eventual mortality (Ho, 1977; Sissons and Carmichael, 2002; Zaia, 1993).

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 with HCMV infection. Although PFA and CDV have been used in combination with GCV for the treatment of GCV-resistant HCMV, these treatments are not always successful (Freitas et al., 1989), because all of them ultimately target the viral DNA polymerase. Moreover, each of these drugs has the potential for significant toxicity. GCV can cause bone marrow suppression (Noble and Faulds, 1998), whereas PFA (Deray et al., 1989) and CDV (Ho et al., 2000) are nephrotoxic. Therefore, effective anti-HCMV agents and regimens need to be developed (Buerger et al., 2001; McSharry et al., 2001; Schröer and Shenk, 2008; Yukawa et al., 1996). Maribavir, a benzimidazole riboside that inhibits the HCMV UL97 kinase, an enzyme that is involved in viral DNA synthesis and egress of viral capsids from cell nuclei, was under investigation in phase III clinical trials (Biron et al., 2002; Lischka and Zimmermann, 2008). However, it has been reported that maribavir failed in a recent pivotal phase III study of bone marrow transplant patients who were treated prophylactically. Moreover, since a parallel phase III trial in liver-transplanted patients was stopped, the future of this program is uncertain (Lischka et al., 2010). Several groups recently identified COX enzymes as a possible target for treatment of HCMV disease. Furthermore, COX inhibitors substantially block HCMV replication and direct cell-to-cell spread of HCMV in cultured fibroblasts (Schröer and Shenk, 2008; Speir et al., 1998; Zhu et al., 2002). A recent study in our laboratory revealed that tricin, which is derived from the hot water extract of *Sasa albo-marginata* (Chart 1), has anti-HCMV activity in a human embryonic fibroblast cell line (Sakai et al., 2008).

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doi:10.1016/j.antiviral.2011.06.014

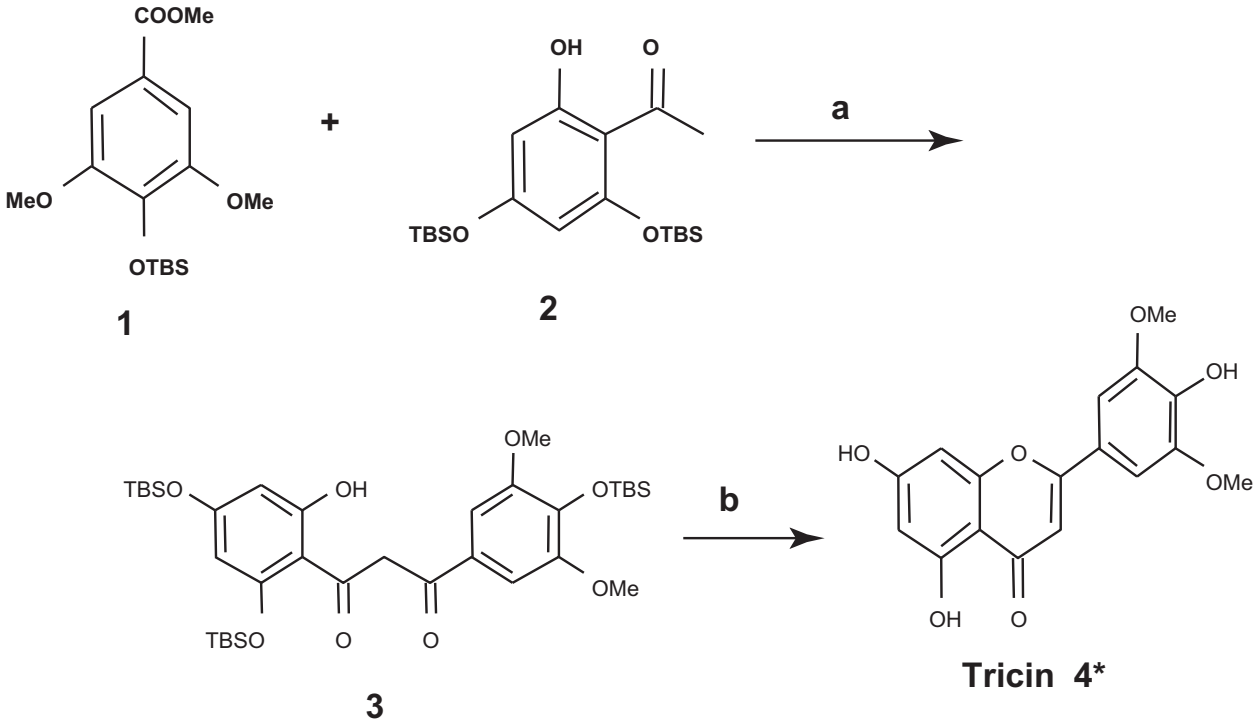

Chart 1. Synthesis of 4′,5,7-trihydroxy-3′,5′-dimethoxyflavone (tricin). *Reagents and conditions: (a) LiHMDS, THF, −78 °C → rt, 3 days; (b) 0.5% H_2SO_4 in AcOH, 100 °C, overnight.

Sasa albo-marginata is known as Kumazasa in Japan. Extracts of Kumazasa have been used as a traditional medicine and as wrapping material for foods throughout Asia. The water-soluble fraction of this plant has a number of biological activities, including anti-ulcerogenic and anti-inflammatory properties (Kuboyama et al., 1981; Shibata et al., 1975, 1976). With 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 show the anti-HCMV activity of tricin may be related to the inhibition of cyclooxygenase-2 (COX-2) induction in the human embryonic fibroblast cell line MRC-5.

2. Materials and methods

2.1. Cell 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) supplemented with 10% heat-inactivated fetal calf serum (FCS; Bocknek Ltd., Rexdale, Ontario, Canada), L-glutamine (0.3 mg/ml; Nacarai Tesque Inc., Kyoto, Japan), streptomycin (100 mg/ml; Nacarai Tesque Inc.) and penicillin (100 units/ml; Nacarai Tesque Inc.). All cell cultures were maintained in a humidified incubator at 37 °C in the presence of 5% CO_2.

The laboratory-adapted HCMV strain Towne was used as a standard strain throughout the experiment (Furukawa et al., 1973). This HCMV was propagated in HEL cells. Viral infectivity was determined by plaque assay (Wentworth and French, 1970). Briefly, confluent MRC-5 cells in 24-well plates (Falcon #3047; Becton Dickinson, Franklin Lakes, NJ, USA) were infected with HCMV. After virus adsorption at 37 °C for 1 h, each inoculum was removed, and the monolayer was overlaid with 2 ml of DMEM containing 2% FCS and 0.6% agar and incubated at 37 °C. After 6 days of culture, cells were fixed with 10% formaldehyde at room temperature for 30 min and stained with 0.03% crystal violet at room temperature for 60 min, and plaques were counted microscopically.

2.2. Compound

Tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone) compounds used was isolated from the hot water extract of dried *Sasa albo-marginata* leaves (Sakai et al., 2008) and synthetic tricin (Chart 1), and suspended in dimethyl sulfoxide (DMSO). We prepared tricin through a condensation reaction of methyl benzoate **1** with acetophenone **2**, followed by acid cyclodehydration. The required methyl 4-*O*-*tert*-butyldimethylsilyl-3,5-dimethoxybenzoate **1** and 2′,4′-*O*-bis(*tert*-butyldimethylsilyl)-6′-hydroxyacetophenone **2** were prepared by protection of suitable starting materials with *tert*-butyldimethylsilyl chloride in tetrahydrofuran (THF) in the presence of *N*,*N*-diisopropylethylamine. The condensation reaction of **1** with 8 equivalents of lithium bis(trimethylsilyl)amide (LiHMDS) and 1.5 equivalents of **2** in THF at −78 °C raised to room temperature over 3 days gave intermediate **3** as a mixture of tautomers. These were subjected to acid cyclodehydration and deprotection with 0.5% H_2SO_4 in acetic acid at 100 °C overnight. These reaction conditions afforded the corresponding tricin **4** at an overall yield of 68%. The purity of the tricin was checked using reverse-phase high-performance liquid chromatography (RP-HPLC) and confirmed to be 99%.

Ganciclovir was purchased from Wako Pure Chemical Industries, Ltd., and suspended in DMSO.

2.3. Viral production assays and infectious center assays

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 or without tricin for 3 or 6 days

after HCMV infection. Infectious virus production was titrated by plaque assay (Wentworth and French, 1970). Plaque reduction rate was calculated based on the mean plaque number in control cells incubated without extract compounds. The 50% effective concentration (EC_{50}) for viral replication (antiviral activity) was determined from a dose-response curve constructed using triplicate samples. The inhibitory concentration (IC_{50}) value for viable cell number (measure of cytotoxicity to MRC-5 cells) was determined from a dose-response curve constructed with triplicate samples using trypan blue dye exclusion test.

Infectious center assays tested the adsorbed viral titers onto cells pre-treated with or without tricin. We used the infectious center assay method described by Gönczöl et al. (1984) with the following change. MRC-5 cells in 24-well plates were grown to light confluency and infected with HCMV as described above. After adsorption for 1 h at 4 °C, the inoculum was removed and the cells were washed three times with phosphate-buffered saline (PBS) to remove any residual virus, then cells were trypsinized, washed twice in PBS and counted. Cells (1×10^3) were incubated with a 1:4 dilution of pooled patient HCMV-immune sera (Murayama et al., 1992) (immunofluorescence titer 1:80). Samples were incubated for 45 min at room temperature. Cells were washed in PBS and resuspended in 1 ml of DMEM containing 2% FCS and plated onto subconfluent MRC-5 cells in 24-well plates. Cells were allowed to settle overnight and were overlaid with agarose as described (Gönczöl et al., 1984). Plaques were counted 10 days after infection as described above.

2.4. Western blot analysis

Proteins from HCMV-infected cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel. Proteins were then transferred to a PVDF membrane (Hybond-p; Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions using 20 mM Tris and 150 mM glycine (pH 8.3) in 20% methanol as the blotting buffer. Membranes were incubated for 1 h at room temperature with blocking reagent [5% skim milk, Tris-buffered saline–0.5% Tween-20, pH 7.6 (TBS–T)], followed by 1 h at room temperature with primary antibody [(mouse monoclonal antibody, MAB810, specific for an immediate early (IE1 and IE2) antigen of HCMV, (Chemicon International Inc., Temecula, CA); 0896, specific for a structural late protein of HCMV, (ViroStat, Portland, ME); C-20, specific for cyclooxygenase-2 (COX-2) antigen, (Santa Cruz Biotechnology Inc., Santa Cruz, CA); and C4, specific for β-actin, (Chemicon International Inc.)] diluted 1:2000 in TBS–T. β-actin was used to monitor actin levels as an internal control of protein induction. Membranes were washed three times in TBS–T and incubated with peroxidase-conjugated second antibody diluted 1:10,000 in TBS–T for 1 h at room temperature. After washing three times in TBS–T, immune complexes were detected using the ECL system (Amersham Pharmacia Biotech AB) according to the manufacturer's instructions.

2.5. Analysis of gene expression

Confluent monolayers of MRC-5 cells in 6-well plates (Falcon #3046; Becton Dickinson, Franklin Lakes, NJ) were treated in 2 ml of DMEM containing 2% FCS with or without the indicated concentrations of tricin. One hour after treatment, DMEM containing tricin was removed, and cell monolayers were washed with serum-free DMEM and infected with HCMV at an MOI of 1.0. One hour after infection, viral inocula were removed, and cells were cultured with fresh medium. RNA samples were collected at 1, 3 and 6 days post-infection (dpi).

Total RNA was extracted from mock- or HCMV-infected cells treated with or without tricin using the chaotropic Trizol method, followed by Isogen-chloroform extraction and isopropanol precipitation (Chomczynski, 1993). Prior to the reverse transcriptase (RT) reaction, potentially contaminating residual genomic DNA was eliminated with DNase I (Takara Shuzo, Otsu, Japan). RNA accumulation was monitored by quantitative real-time RT-PCR (qRT-PCR). RT-PCR analysis of HCMV IE and UL54 gene expression was carried out using 0.2 mg of total RNA per reaction. The RT reaction was performed with random primers (5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C) using the iScript cDNA Synthesis kit from Bio-Rad Laboratories, Inc. (Hercules, CA), according to the manufacturer's instructions. cDNA products were amplified for IE, UL54, COX-2 and β-actin gene expression via qRT-PCR with specific primers using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) for 34 cycles (10 s at 95 °C, 20 s at 55.5 °C, and 20 s at 72 °C) by Mini Opticon real-time PCR with Gene Expression Macro software (Bio-Rad Laboratories, Inc.). PCR primers were as follows: HCMV IE2 primers (forward: 5′-ATG AAC CAC CCT CCT CTT CC-3′, reverse: 5′-GAT ATT GCG CAC CTT CTC GT-3′) (Nishiwaki et al., 2006); 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); COX-2 primers (forward: 5′-GCTTCCATTGCCAGA GCAGGCA-3′, reverse: 5′-GAGCTCTGGATCTGGAACACTG-3′) (Zhou et al., 2005); 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 RNA levels.

2.6. PGE_2 assay

The amount of PGE_2 was measured using a commercially available enzyme-linked immunoabsorbance assay (ELISA) kit for PGE_2 (Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer's instructions.

2.7. Statistical analysis

Data were analyzed using Student's *t*-test.

3. Results

3.1. Inhibitory effects of tricin at different concentrations on HCMV production

The anti-HCMV activity of tricin was compared with that of GCV against the HCMV Towne strain. After adsorption for 1 h, cells were incubated in the presence of various concentrations of either tricin or GCV, followed by culture for 6 days. Tricin and GCV significantly inhibited the replication of the Towne strain at concentrations ranging from 0.14 to 3.6 μM and 0.01 to 1.0 μM, respectively ($P < 0.05$, Fig. 1). The 50% effective concentration (EC_{50}) of tricin and GCV against HCMV were 0.51 and 0.17 μM, respectively. However, viral replication of HCMV was inhibited more than 90% by 3.6 μM tricin treatment.

In the next experiment, the virucidal effects of tricin were examined after incubation of cell-free HCMV with concentrations of 0.14–1.2 μM tricin. However, the infectivity of cell-free HCMV was unchanged (Fig. 2A). The cytotoxic effects of tricin were then examined after incubation (1, and 6 days) of MRC-5 cells with concentrations of 0.14–3.6 μM tricin. However, no cytotoxic effects were observed, even at the highest tricin concentration (Fig. 2B).

3.2. Anti-virus effects of tricin on HCMV replication

In order to delineate the most drug-sensitive phase of HCMV replication, tricin was used in time-of-addition experiments.

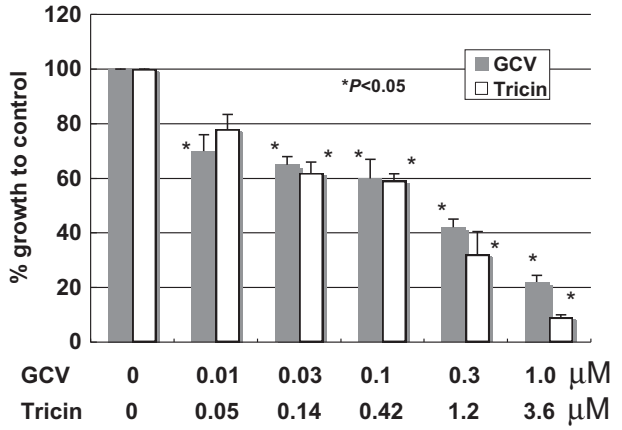

Fig. 1. Inhibitory effects of compounds on HCMV replication. MRC-5 cells were infected with HCMV and then incubated with the indicated concentrations of ganciclovir (GCV) or tricin. Viral titers in the culture supernatants were determined by plaque assay on day 6 after infection. Data are means ± SE of two independent experiments. $^{*}P < 0.05$ versus no compounds control by the Student's *t*-test.

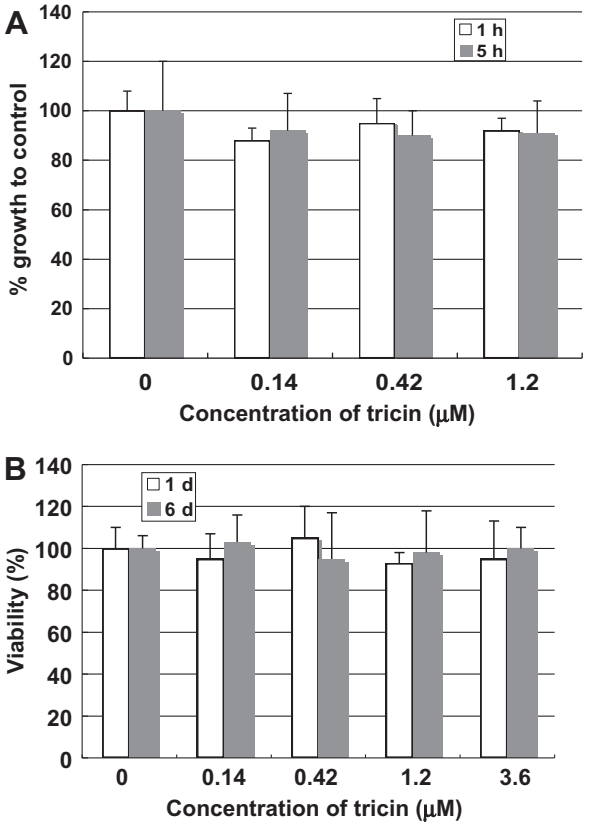

Fig. 2. Toxic effects of tricin. (A) Virucidal effect of tricin. Cell free-HCMV was incubated with the indicated concentrations of tricin at 37 °C. Virus titers were determined by plaque assay at 1 or 5 h after incubation. (B) Cytotoxic effect of tricin. MRC-5 cells were cultured in the absence or presence of various concentrations of tricin and incubated at 37 °C for the indicated times. Viable cell number was determined by dye exclusion test using trypan blue. Data are presented as means ± SE of two independent experiments.

MRC-5 cell monolayers were infected with HCMV at 1 MOI. Tricin was added 1 h before viral infection, immediately after infection and at 1, 3 or 8 h post-infection. At 6 dpi, culture supernatants were harvested and virus yield was determined by plaque assay. As shown in Fig. 3A, treatment with tricin at 8 h post-infection did not suppress HCMV replication, whereas tricin significantly

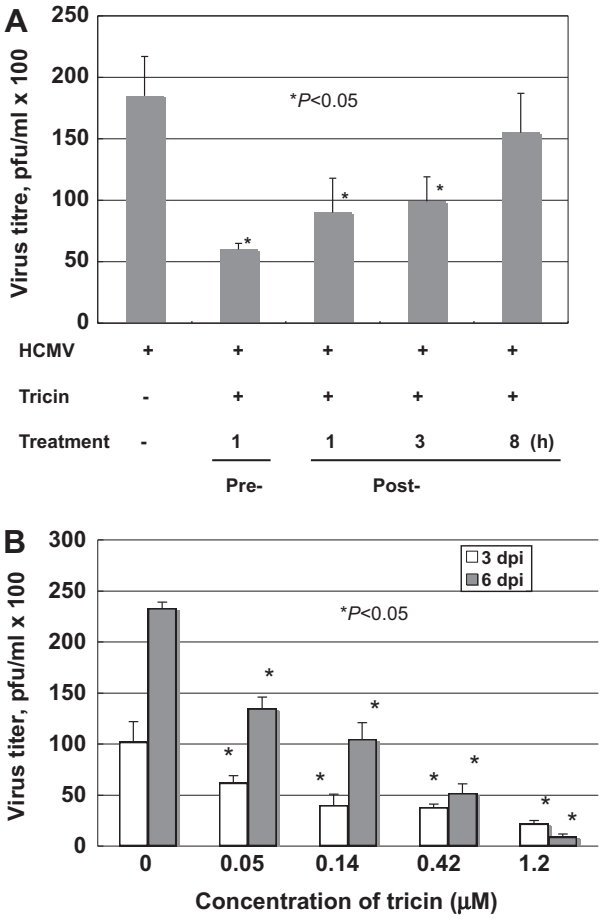

Fig. 3. Effect of tricin on HCMV replication. (A) Time-of-addition effects of tricin on HCMV replication. Tricin (0.42 μM) was added to culture medium of HCMV-uninfected cells and incubated for 1 h at 37 °C, and after wash with PBS, tricin treated cells were infected with HCMV. Tricin was also added to HCMV-infected cells at the indicated treatment times and incubated at 37 °C. (B) Effect of tricin treatment to before infected cells with HCMV. MRC-5 ells were incubated with indicated concentration of tricin for 1 h at 37 °C, and after washing, were infected with HCMV. The viral titers in the culture supernatants were determined by plaque assay on day 3 or 6 after HCMV infection. Data are means ± SE for two independent experiments. $^{*}P < 0.05$ versus no tricin control by the Student's *t*-test.

suppressed HCMV replication when added 1 h before, or 1 h or 3 h after viral infection. It is time when the most-sensitive phase of tricin to anti-HCMV effects was added before viral infection. To confirm that tricin inhibits HCMV replication, a monolayer culture of MRC-5 cells was treated with the indicated concentration of tricin. One hour after treatment, culture medium containing tricin was removed, and cell monolayer were washed with PBS and infected with HCMV. After adsorption for 1 h, viral inocula were removed, and infected cells were cultured with fresh medium at 3 or 6 days. In the HCMV-infected cells, the virus titer increased until 6 days after infection in the untreated control. However, in the treatment before virus infection with tricin, virus production was significantly inhibited in a dose-dependent manner (Fig. 3B). Therefore, tricin is thought to interfere with virus adsorption and/or penetration, or to suppress HCMV production, when added after of viral infection and throughout subsequent incubation.

The effects of tricin on viral adsorption onto host cells were evaluated by infectious center assay and adsorption inhibitory assay of HCMV particles, which determined the number of cells binding the virus particles at low temperature (4 °C) in the presence of different concentrations of tricin. As shown in Fig. 4, tricin suppressed the adsorption of HCMV onto host cells surface in a

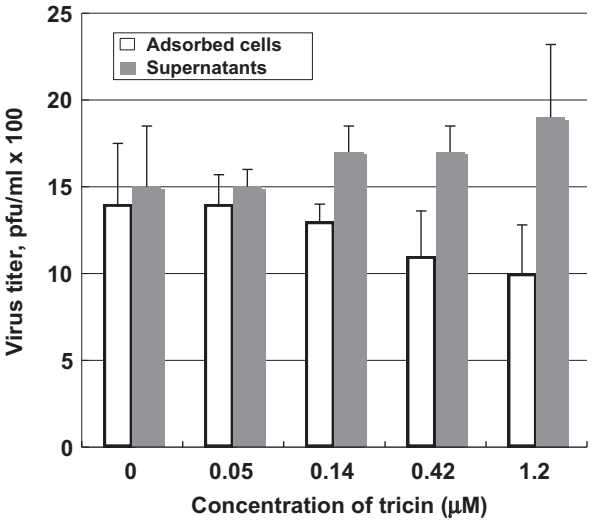

Fig. 4. Inhibition of HCMV adsorption onto cells pre-treated with tricin. MRC-5 cells were incubated with the indicated concentrations of tricin for 1 h at 37 °C, and after washing, were subjected to adsorption by HCMV for 1 h at 4 °C. The non-adsorbed viral titers in the culture supernatants were determined by plaque assay (■), while adsorbed viral titers were determined by infectious center assay (□). Data are means ± SE for three independent experiments.

dose-dependent manner. Adsorption of HCMV on the host cell surface was suppressed by about 40% by treatment with 1.2 μM tricin before HCMV infection.

3.3. Detection by Western blot analysis

In order to determine the expression of IE and late viral proteins, as well as COX-2 expressed in tricin-treated cells, we examined their synthesis in MRC-5 cells at the indicated time intervals by Western blot analysis. As shown in Fig. 5, HCMV IE and late protein were synthesized in HCMV-infected, tricin-untreated cells at every time interval tested. In contrast, IE protein synthesis (especially IE2) was strongly inhibited by treatment of HCMV-infected cells with 0.05 μM tricin at 3 dpi (Fig. 5A and B, lane 3). Moreover, late antigen synthesis was also inhibited by treatment with 0.05–0.42 μM tricin (Fig. 5A and B). In addition, COX-2 was synthesized in HCMV-infected MRC-5 cells at least 5–12 h after infection (Fig. 6A, control). However, COX-2 synthesis was concentration-dependently reduced by treatment of infected cells with tricin (0.05–0.42 μM) (Fig. 6B).

3.4. Detection of viral genes by RT-PCR analysis

Tricin suppressed expression of IE2 or COX-2 mRNA at 3 dpi and UL54 mRNA at 6 dpi in a dose-dependent manner, based on the protein expression data. This compound at 0.05 μM inhibited expression of IE2 mRNA by 20%, while at 0.42 μM, expression of both IE2 and UL54 mRNA was inhibited by 50% (Fig. 7A). In addition, tricin inhibited the expression of COX-2 mRNA in HCMV-infected cells at concentrations ranging from 0.14 to 1.2 μM (Fig. 7B).

3.5. Detection of PGE_2 accumulation by enzyme immuno-assay

As COX-2 synthesis was inhibited in tricin-treated cells, we examined whether tricin suppressed PGE_2 accumulation after HCMV infection. HCMV-infected cells were cultured in the absence or presence of tricin (0.42 μM), and PGE_2 in the culture supernatants was measured by ELISA. HCMV-infected cells secreted PGE_2 within 5 h of infection, and the PGE_2 accumulated until 72 h after infection in a time-dependent manner in the absence of tricin. However, in the presence of tricin, PGE_2 accumulation was completely inhibited (Fig. 8).

4. Discussion

We previously investigated the effective concentrations of tricin (Sakai et al., 2008), and found that the EC_{50} for HCMV production was 0.51 μM (0.17 μg/ml). Zhen et al. (2006) reported that GCV also effectively inhibited HCMV plaque formation with an IC_{50} value of 1.03 μg/ml. Tricin was first examined for its inhibitory effects on HCMV replication in MRC-5 cells, and its anti-HCMV activity was further demonstrated through plaque and gene expression assays. This low concentration is sufficient to inhibit HCMV replication *in vitro*. Although it was thought that inhibitory effects on the growth of host cells might reduce the production of HCMV virions, no decrease in the number of MRC-5 cells was observed during incubation for 6 days in the presence of tricin, as compared with controls (Fig. 2B). Moreover, we previously reported about cytotoxicity of tricin that the IC_{50} of tricin for MRC-5 cells was 205 μg/ml (621 μM) (Sakai et al., 2008). The EC_{50} of tricin for HCMV production was 0.17 μg/ml (0.51 μM). Therefore, the selectivity index of tricin, based on the ratio of IC_{50} to EC_{50}, was 1205.8 (205/0.17).

Studies on action mechanisms are consistent with tricin mainly affecting the immediate early events of viral replication, including viral adsorption and penetration, as indicated by time-of-addition and infectious center assay experiments (Figs. 3 and 4). Adsorption of HCMV onto the host cell surface is mediated by the envelope glycoprotein gB–gH, which binds to a poorly characterized receptor present on the surface of host cells (Compton et al., 1993; Patrone et al., 2007; Taylor and Cooper, 1990). Virus penetration following adsorption onto cells is mediated by fusion of the viral envelope and the cell surface in a pH-independent manner (Compton et al., 1992). Tricin interferes about 40% with adsorption of HCMV onto cell surface, and to a lesser extent, delays the internalization of virus, although, at present, the sites of viral and cellular components with which the compound interacts remain unknown.

IE protein expression following tricin treatment was investigated and the results showed that tricin inhibited IE (particularly IE2) protein synthesis in the viral replication cycle, thus suggesting that tricin inhibits IE gene expression. Recently, we demonstrated similar effects with crude extracts isolated from *Sasa albo-marginata*, which inhibited HCMV replication via inhibition IE2 gene expression (Sakai et al., 2008). The inhibitory effects of tricin on HCMV replication after viral entry could thus be attributed to a reduction in IE protein synthesis, as IE proteins play an important role in HCMV pathogenesis (Murayama et al., 1998; Scholz et al., 2001; Taylor and Bresnahan, 2006).

In a previous report, we demonstrated that IE gene expression might be sufficient to activate AP-1 and NF-κB, resulting in interleukin-8 gene expression (Murayama et al., 2000). Based on the results of this study, tricin appears to interact with IE protein formation. Viral DNA inhibition by tricin was thus indirectly due to reduced IE gene expression (Hwang et al., 2009; Marchini et al., 2001). The anti-HCMV activities of tricin may be attributed both to inhibition of viral adsorption/penetration and to the reduction of IE molecule expression.

Several groups recently identified COX enzymes as a possible target for treatment of HCMV disease (Schröer and Shenk, 2008; Speir et al., 1998; Zhu et al., 2002). We demonstrated that the PGE_2 accumulation and induction of COX-2 synthesis by HCMV infection were inhibited by tricin treatment. Thus, tricin may also

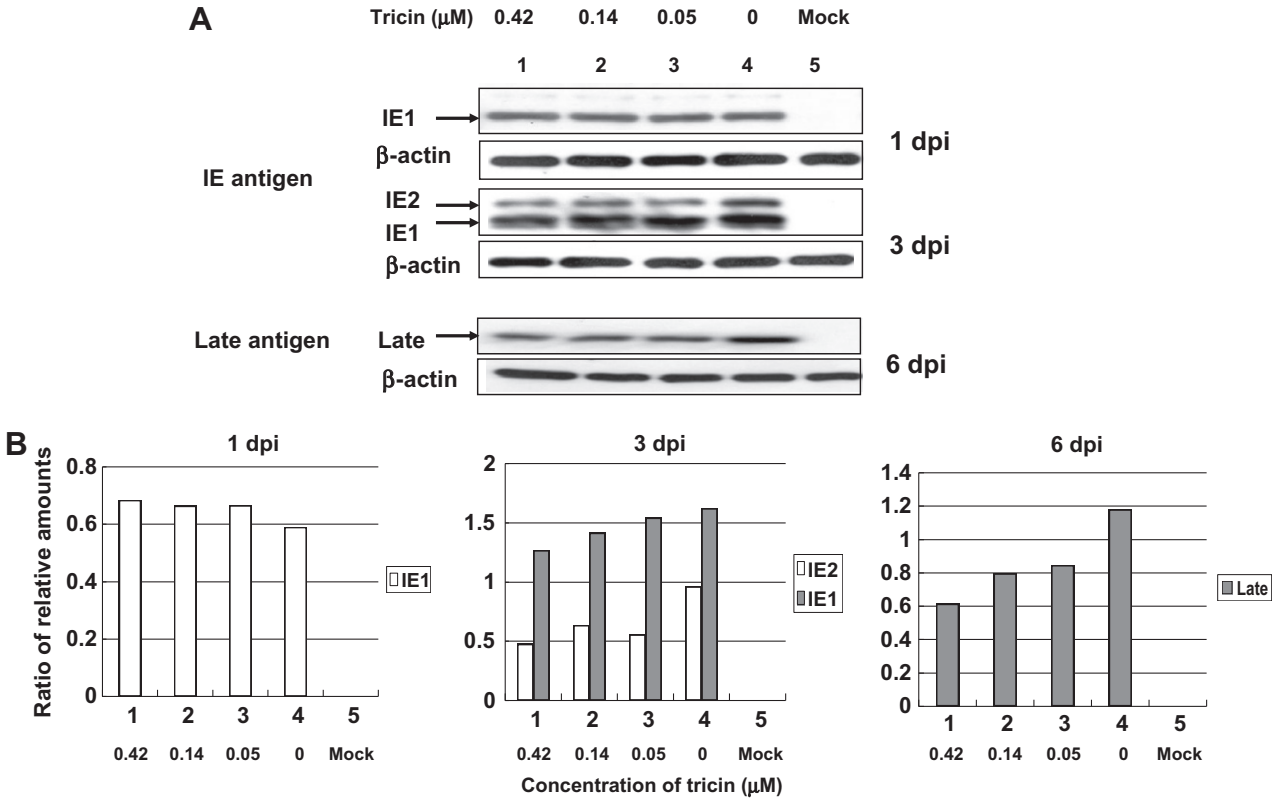

Fig. 5. Regulation of protein synthesis by tricin in HCMV-infected cells. MRC-5 cells were incubated with or without the indicated concentrations of tricin for 1 h before infection with HCMV. Proteins were prepared on day 1, day 3 and day 6 after infection. (A) Western blot analysis was preformed using antibodies against the immediate early (IE) antigen, late antigen or β-actin. After electorophoresis using SDS–PAGE, proteins were transferred to polyvinylidine fluoride (PVDF) membranes. Immunoblotting was detected by the ECL system. (B) Relative amounts of proteins were estimated by densitometric analysis with NIH Image software and the data are represented as the ratio of relative amounts of IE1 (1 dpi), IE1 and IE2 (3 dpi), late (6 dpi) to β-actin, respectively. dpi, days post-infection.

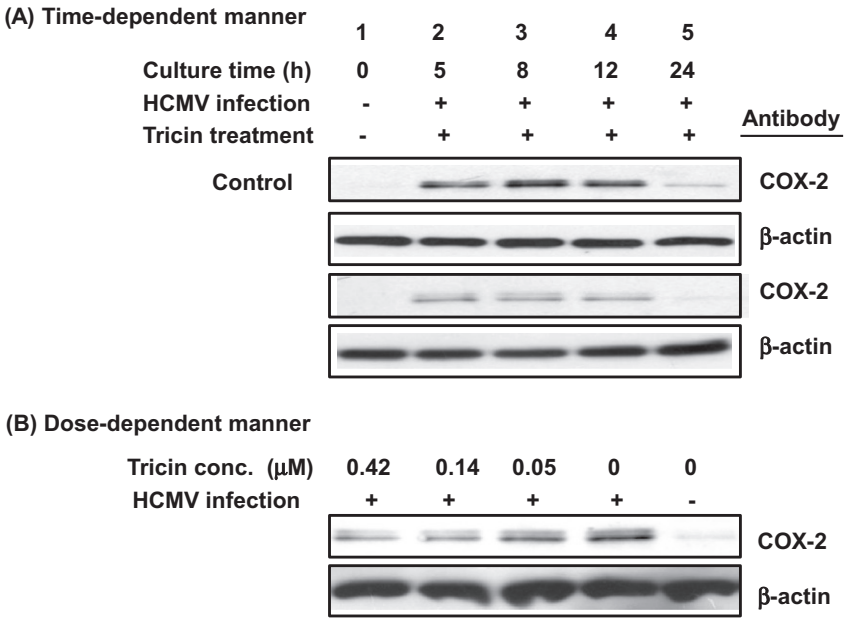

Fig. 6. Regulation of COX-2 enzymes synthesis by tricin in HCMV-infected cells. (A) MRC-5 cells were incubated with tricin (0.42 μM) for 1 h and then infected with HCMV. Proteins were prepared at the indicated times after HCMV infection. (B) MRC-5 cells were incubated with the indicated concentrations of tricin for 1 h before HCMV infection. Proteins were prepared at 8 h after HCMV infection. Control; not treated with tricin.

be a novel COX inhibitor. Schröer and Shenk reported that HCMV has been shown to induce COX-2 RNA accumulation, as well as protein and enzyme activity. Furthermore, COX inhibitors substantially block HCMV replication and cell-to-cell spread of HCMV in cultured fibroblasts (Schröer and Shenk, 2008; Zhu et al., 2002). In HCMV-infected cells, US28 contributed to the viral induction

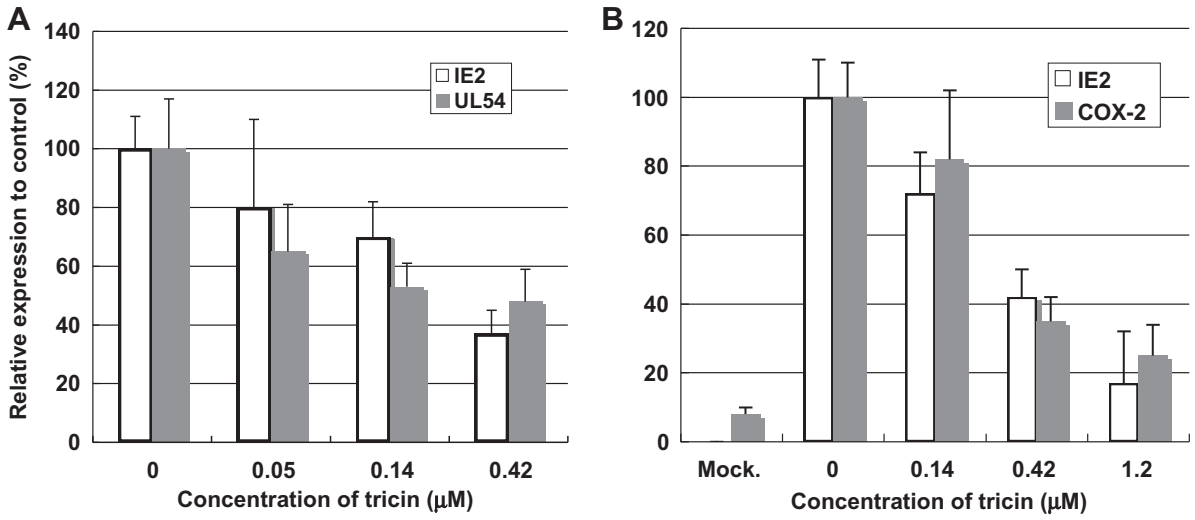

Fig. 7. Effects of tricin on mRNA expression in HCMV-infected cells. MRC-5 cells were incubated with the indicated concentrations of tricin for 1 h before HCMV infection. (A) Total RNA was prepared on day 3 for IE2 and day 6 for UL54 after HCMV infection, and reverse transcribed. Real-time RT-PCR analysis was performed using IE2, UL54 and β-actin primers. (B) Total RNA was prepared on day 3 for IE2 and COX-2 after HCMV infection, and reverse transcribed. Real-time RT-PCR analysis was performed using IE2, COX-2 and β-actin primers. mRNA levels were normalized against the expression of β-actin. Data are means ± SE for three independent experiments. Mock.; Mock infection and without tricin.

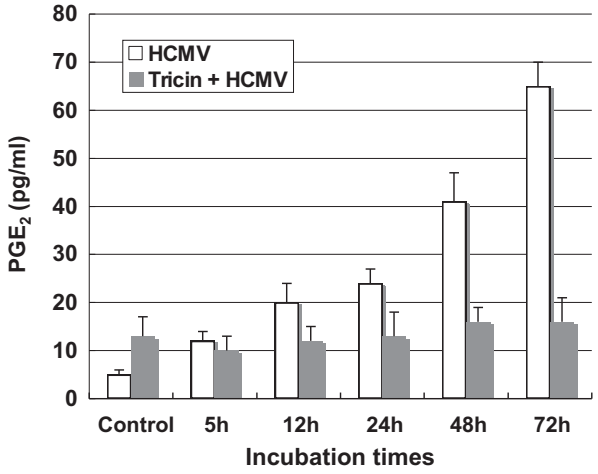

Fig. 8. Influence of tricin on PGE_2 accumulation in cultures of HCMV-infected cells. HEL cells were incubated with 0.42 μM tricin for 1 h, and were then infected with HCMV. PGE_2 accumulation in the culture supernatants were determined by enzyme immuno-assay at the indicated times after infection. Data are means ± SE for two independent experiments. Control; uninfected with HCMV.

of COX-2. Also, COX-2 was highly up-regulated upon US28 expression (Maussang et al., 2009). However, little is known to data about the pathways of cellular signaling networks of HCMV infection leading to COX-2 expression. Yi et al. (2009) reported that HCMV major immediate early genes are not needed for COX-2 expression. Also, epidermal growth factor receptor kinase inhibitors (EGFRK), C-raf, mitogen-activated protein kinases 1/2 inhibitors (MEK 1/2) and extracellular signal-regulated kinases (ERK 1/2) pathway may participate in the COX-2 mediated inflammatory response to HCMV infection (Yi et al., 2009). Although the effects of COX inhibitors on HCMV disease have not yet been tested, there are indications that this class of drugs will help control herpes simplex virus; COX-2 inhibitors have been shown to suppress herpes simplex virus reactivation in a mouse model of latency (Gebhardt et al., 2005). In addition, in human cases, oral administration of indomethacin or ibuprofen reduced the frequency of reactivation of herpes simplex virus infections (Wachsman et al., 1990).

In conclusion, our results predict that tricin is a significant candidate for the control of HCMV infection through the inhibition of COX-2, and might be useful as potential therapeutic agent against HCMV in immunocompromised hosts.

Acknowledgements

This work was supported in part by the Specific Research Fund of Hokuriku University.

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