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Anti-HSV activity of digitoxin and its possible mechanisms

Chun-Ting Su [a], John T.-A. Hsu [c,d], Hsing-Pang Hsieh [c], Pi-Han Lin [a], Ting-Chi Chen [a], Chuan-Liang Kao [a,b], Chun-Nan Lee [a,b], Sui-Yuan Chang [a,b,*]

[a] *Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei, Taiwan*
[b] *Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan*
[c] *Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Miaoli, Taiwan*
[d] *Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan*

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Abstract

Herpes simplex virus type 1 (HSV-1) can establish latent infection in the nervous system and usually leads to life-threatening diseases in immunocompromised individuals upon reactivation. Treatment with conventional nucleoside analogue such as acyclovir is effective in most cases, but drug-resistance may arise due to prolonged treatment in immunocompromised individuals. In this study, we identified an in-use medication, digitoxin, which actively inhibited HSV-1 replication with a 50% effective concentration (EC_{50}) of 0.05 μM. The 50% cytotoxicity concentration (CC_{50}) of digitoxin is 10.66 μM and the derived selective index is 213. Several structural analogues of digitoxin such as digoxin, ouabain octahydrate and G-strophanthin also showed anti-HSV activity. The inhibitory effects of digitoxin are likely to be introduced at the early stage of HSV-1 replication and the virus release stage. The observation that digitoxin can inhibit acyclovir-resistant viruses further implicates that digitoxin represents a novel drug class with distinct antiviral mechanisms from traditional drugs.

Keywords: Digitoxin; HSV; Cardiac glycoside

1. Introduction

Herpes simplex virus (HSV) is an enveloped DNA virus. During productive infection, HSV expresses its genes in a tightly regulated cascade, consisting of sequential expression of the immediate-early (IE), early (E) and late (L) genes (Honess and Roizman, 1974; Roizman and Knipe, 2001). In infected Vero cells, the IE mRNAs are first detected at 1–2 h post-infection (p.i.) and peak at 3 h p.i.; the transcription of E mRNAs is first detected at 2–3 h p.i. and peaks between 5 and 10 h p.i.; the L mRNAs are synthesized at increasing rates until at least 12 h p.i. (Honess and Roizman, 1974; Rezuchova et al., 2003; Spector et al., 1998). The IE genes, such as α4 and U_L54, encode *trans*-acting regulators of gene expression. The E genes, such as U_L52 and U_L23, are involved in viral DNA synthesis and nucleotide metabolism (Boehmer and Lehman, 1997; Roizman and Knipe, 2001). The L genes, mainly encoding structural proteins, such as glycoprotein B (gB) and glycoprotein D (gD), are further divided into γ_1 and γ_2 genes, according to their degree of dependence on DNA replication for their expression (Jones and Roizman, 1979).

Because of its wide distribution, efficient transmission and difficulties in developing prophylactic vaccines (Deshpande et al., 2000), development of chemotherapy is comparatively important in treating HSV infection (Kleymann, 2003). Nucleoside analogues such as acyclovir (ACV), pencyclovir and their orally bioavailable prodrugs such as valacyclovir and famciclovir are the most widely used drugs for treatment (Elion et al., 1977; Schaeffer et al., 1978). The selectivity of ACV and other analogues comes from the strict requirement of conversion to the triphosphate forms by both viral thymidine kinase (TK) and cellular kinase (Elion et al., 1977). While nucleoside-based therapeutics are effective for treatment of primary and recurrent mucocutaneous infections, delays in initiating treatment reduce their efficacy. In addition, long-term usage of nucleoside-based drugs in immunocompromised individuals may lead to the selection of naturally occurring resistant mutants (Danve-Szatanek et al., 2004; Morfin

* Corresponding author at: Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, No. 1 Chang-Te Street, Taipei, Taiwan. Tel.: +886 2 23123456x6908; fax: +886 2 23711574.
E-mail address: sychang@ntu.edu.tw (S.-Y. Chang).

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and Thouvenot, 2003). Another drug, foscarnet, which acts directly on HSV DNA polymerase (Reno et al., 1978), may induce mutations in DNA polymerase gene upon prolonged usage and the resulting mutants are often resistant to combination chemotherapy with existing compounds (Hwang et al., 1992; Sacks et al., 1989). Recently, one novel compound class against HSV, the helicase–primase inhibitor, was reported (Betz et al., 2002; Crumpacker and Schaffer, 2002; Crute et al., 2002). The helicase–primase complex, containing DNA helicase, RNA polymerase (primase) and ssDNA-stimulated ATPase activities, is essential for initiation of viral DNA replication (Crute and Lehman, 1991; Sherman et al., 1992; Zhu and Weller, 1992). The thiazolylphenyl compounds have been independently identified to specifically inhibit helicase–primase activity and reported to be superior to the traditional nucleoside analogues in different animal models (Betz et al., 2002; Crumpacker and Schaffer, 2002; Crute et al., 2002; Kleymann et al., 2002).

In this report, we used an in vitro cell-based assay to screen 960 Food and Drug Administration (FDA)-approved compounds for their antiviral activity. Among them, digitoxin was chosen to determine its potential antiviral mechanisms.

2. Materials and methods

2.1. Compounds, cells and viruses

The compounds tested were mainly from The Genesis Plus Collection (MicroSource Discovery Systems, Inc., Gaylordsville, CT). The 960 compounds in this library are primarily Food and Drug Administration (FDA)-approved compounds. Digitoxin, acycloguanosine (acyclovir, ACV) (Sigma, St. Louis, MO), and ganciclovir (GCV) (Sigma), another nucleoside analogue used to treat cytomegalovirus infection, were dissolved in dimethyl sulfoxide (DMSO) or absolute ethanol, and then diluted with sterile deionized distilled water before use. The final concentration of DMSO was lower than 0.01%.

African green monkey kidney cells (Vero, ATCC CCL-81) were propagated in growth medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), penicillin G sodium 100 units/mL, streptomycin sulfate 100 μg/mL and amphotericin B 250 ng/mL (antibiotic–antimycotic; Gibco-BRL, Grand Island, NY). The components of maintenance medium are similar to growth medium except that they contain only 2% of FBS.

HSV-1F (ATCC VR-733) and HSV-2G (ATCC VR-734) stocks were propagated in Vero cells and stored at −80 °C before further analysis. The virus titer was determined by plaque assay (Russell, 1962). ACV-resistant HSV-1 was derived from serial passages of HSV-1F in the presence of ACV and isolated after 3 rounds of plaque purification. As reported for some ACV-resistant HSV (Sasadeusz et al., 1997), a frameshift induced by insertion of one guanosine nucleoside in the 7G stretch (nt. 433–439) of TK gene was identified on the isolated ACV-resistant viral DNA by sequence analysis (data not shown).

2.2. Screening for antiviral compounds

One day before infection, Vero cells were seeded onto a 96-well culture plate at a concentration of 10^4 cells per well. Next day, medium was removed and 10 plaque forming unit (pfu) HSV-1 suspension per well were added and incubated at 37 °C with 5% CO_2 for 1 h. The infected cell monolayer was then washed with phosphate buffered saline (PBS) and cultured in maintenance medium containing 1 μM of compounds. After 72 h of incubation at 37 °C, cell monolayer was fixed with 10% formalin and stained with 1% crystal violet. Compounds protecting more than 50% of cells from lysis by HSV infection were considered to possess antiviral activity and were further analyzed.

2.3. Assays for antiviral activity

Plaque assays were performed with monolayer cultures of Vero cells in 24-well culture plates. For plaque reduction assay, cell monolayer was infected with virus (50 pfu/well) and incubated at 37 °C with 5% CO_2 for 1 h. The infected cell monolayer was then washed three times with PBS and overlaid with overlapping solution (maintenance medium containing 1% methylcellulose and various concentrations of indicated compounds). After 72 h of incubation at 37 °C, cell monolayer was fixed with 10% formalin and stained with 1% crystal violet. Plaques were counted and the percentage of inhibition was calculated as $[100 - (V_D/V_C)] \times 100\%$, where V_D and V_C refer to the virus titer in the presence and absence of the compound, respectively. The minimal concentration of compounds required to reduce 50% of plaque numbers (EC_{50}) was calculated by regression analysis of the dose–response curves generated from plaque assays.

The cell pretreatment assay was performed with cells being treated with compounds at indicated concentrations for 3 h at 37 °C before infection for plaque assay (Gong et al., 2001). For virus pretreatment assay (Cheng et al., 2004), the virus suspension was pre-incubated with compounds at indicated concentrations for 3 h before adding to the cell culture for plaque assay.

The attachment assay was performed as described (Cheng et al., 2004; Wachsman et al., 2003). Briefly, cell monolayer was pre-chilled at 4 °C for 1 h. The cell monolayer was then infected with virus in the presence of compounds at indicated concentrations and incubated at 4 °C for another 80 min. After that, the infected cell monolayer was washed three times with cold PBS and treated as described for plaque assay.

For penetration assay (Cheng et al., 2004; Wachsman et al., 2003), the Vero cell monolayer was first pre-chilled at 4 °C for 1 h, then the cell monolayer was incubated with virus at 4 °C for another 2 h to allow attachment of virus. After 2 h of incubation, the compounds with indicated concentrations were added and the culture was incubated at 37 °C for 10 min to maximize penetration of virus. The infected cell monolayer was then treated with acidic PBS (pH 3) for 1 min to inactivate non-penetrated virus and subsequently treated as described for plaque assay.

The time-of-addition assay was performed as described (Cheng et al., 2004; Wachsman et al., 2003) with minor modifications. Briefly, compound-containing maintenance medium was added into cell monolayer at different time points after virus infection. At 24 h post-infection, infected cultures were harvested and subjected to three cycles of freezing–thawing before titration of virus titer by plaque assay.

The time-of-removal assay was performed as described (Zhen et al., 2006). Briefly, compound-containing maintenance medium was added into cell monolayer immediately after virus infection. At different time points after virus infection, the medium was removed, and the well was washed three times before adding maintenance medium. At 24 h post-infection, the virus yield was determined by plaque assay.

2.4. Cytotoxicity assay

The cytotoxicity of tested compounds in Vero cell was determined by MTT assay. Briefly, Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells per well. Next day, medium was removed and maintenance medium containing different concentrations of compound was added. After 3 days of incubation, medium was removed and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) (0.1 mg/mL) was added. After incubating at 37 °C for 5 h, MTT reagent was removed and DMSO was added and incubated for another 10 min. The absorbance was then determined by ELISA reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA) at a wavelength of 550 nm. The percentage of inhibition was calculated using the following formula: inhibition % = $[100 - (A_t/A_s) \times 100]$%. A_t and A_s refer to the absorbance of test substances and solvent control, respectively. The 50% cytotoxicity concentration (CC_{50}) was defined as the concentration reducing 50% of cell viability.

2.5. Polymerase chain reaction (PCR)

Genomic DNA and total RNA of infected cells were respectively extracted using commercial kits (Blood & Tissue Genomic Mini and Total RNA Mini; Viogene, Sijhih Taipei County, Taiwan). DNase treatment was performed on the eluted RNA to avoid residual DNA contamination. One microgram of the eluted RNA was subjected to reverse transcription by the M-MLV Reverse Transcriptase RNase H Minus, Point Mutant (Promega, Madison, WI) at 40 °C for 60 min with oilgo $(dT)_{15}$ primer in a total volume of 25 μL. Aliquots of serially diluted genomic DNA or cDNA were subjected to PCR amplification. The PCR primer pairs for ICP4 and U_L13 were as described (Kleymann et al., 2002; Tal-Singer et al., 1997). The primer pairs for U_L52 were UL52F (5′ CAT CGA AAC CCA CTT TCC CGA ACA 3′) and UL52R (5′ GCT GTC GCA TTT GGC GGC AA 3′); for U_L30 were UL30F (5′ ATG GTG AAC ATC GAC ATC TAC GG 3′) and UL30R (5′ CCT CCC GTT CGT CCT CGT CCT CC 3′); and for β-actin were actin-F (5′ TCC TGT GGC ATC CAC GAA ACT 3′) and actin-R (5′ GAA GCA TTT GCG GTG GAC GAT 3′). The PCR reaction was carried out in a final volume of 50 μL containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.2 μM of each specific primer, 2.5 U of platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and serially diluted genomic DNA or cDNA. The PCR program for U_L52 consists of denaturation at 95 °C for 5 min and 35 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, and polymerization at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The PCR program for U_L30 consists of denaturation at 95 °C for 5 min and 35 cycles of denaturation at 96 °C for 1 min, annealing at 67 °C for 2 min, and polymerization at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The PCR program for β-actin consists of denaturation at 94 °C for 3 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and polymerization at 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. The expected sizes for U_L52, U_L30 and β-actin are 240, 469 and 314 bp, respectively. Five-microliter aliquots of the PCR products were resolved on a 1.5% agarose gel. The signal strength of PCR products was semi-quantitated by the ImageJ (http://rsb.info.nih.gov/ij/).

2.6. Immunofluorescence assay

A total of 1.5×10^5 Vero cells were seeded onto circular glass coverslips (12 mm, Assistant, Hecht, Sondheim, Germany) and maintained in the 24-well plate 1 day before infection. Next day, cells were infected with HSV-1 (MOI = 0.4) for 1 h, washed, and cultured in maintenance media with or without digitoxin. After 24 h, cells were washed with PBS, fixed with 4% paraformaldehyde, blocked with 1% bovine serum albumin (BSA) in PBS, and permeabilized with 0.2% Triton X-100. The coverslips were then incubated for 45 min at room temperature with FITC-conjugated anti-HSV-1 monoclonal antibodies (IMAGEN Herpes Simplex Virus, K6106, DAKO, Denmark). After that, cells were washed and stained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma) for another 20 min at room temperature. The coverslips were then mounted and analyzed using confocal microscope (Leica TCS SP2 Spectral Confocal System; X63 oil immersion lens, NA 1.32; Wetzler, Germany). The data was collected with fourfold averaging at a resolution of 512 × 512 pixels.

2.7. Immunoblotting

A total of 1×10^6 Vero cells were seeded onto 10-cm plate 1 day before infection. Next day, cells were mock-infected or infected with HSV-1 (MOI = 0.1) for 1 h, and cultured in maintenance media with or without 0.1 μM digitoxin. Sixteen hours post-infection, cells were trypsinized and lysed with lysis buffer composed of 50 mM Tris–HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 2.5% deoxycholate, 1 mM PMSF, protease inhibitor (Sigma). After being denatured by boiling, equivalent amounts of protein (35 μg) were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bellerica, MA), and blocked with 5% non-fat milk in PBST (0.5% Tween 20 in PBS). All washing of membranes was performed using PBST. Mouse monoclonal antibody for HSV-1 glycoprotein D (catalog no. MAB8684; Chemicon, Temecula, CA) was

used at a 1:2500 dilution, and goat monoclonal antibody for β-actin (catalog no. sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:1000 dilution. The membrane was incubated with primary antibody at 37 °C for 1 h. After washing, the membrane was incubated with either goat anti-mouse antibody (catalog no. 81-6520; Invitrogen) or donkey anti-goat antibody (catalog no. sc-2020; Santa Cruz Biotechnology) conjugated with HRP at a 1:5000 dilution. The immunoblots were developed and detected using the enhanced chemiluminescence western blotting detection system (PerkinElmer, Waltham, MA).

2.8. Virus release assay

A Vero cell monolayer was infected with HSV-1 (MOI = 0.4) for 1 h. Drug-containing maintenance medium was added into wells after PBS wash. After 24 h of incubation at 37 °C, supernatant and cell pellet were collected, respectively. The cell pellet was frozen and thawed three times before titration by plaque assay. Virus titers of supernatant and cell pellet were determined by plaque assay and the percentage of inhibition was calculated as described.

3. Results

3.1. Screening for antiviral compounds

To identify compounds with anti-HSV activity, 960 FDA-approved drugs in the *Genesis Plus* library were screened by the 96-well cell-based screening assay. The screening was blind performed, such that the identity of these drugs was not revealed until screening was completed. Among the 960 compounds, 73 were found to possess antiviral activity (>50% protection of cells from HSV-induced cell lysis), and their antiviral activity was subsequently quantified by plaque reduction assay. Among them, 14 were confirmed to exhibit EC_{50} at or lower than 1 μM. One of these compounds, digitoxin, was chosen for further analysis because of its lower EC_{50} against HSV and the presence of two structural analogues, nerifolin and peruvoside, among the 14 compounds.

3.2. Assessment of anti-HSV activity and cytotoxicity of digitoxin

The effects of digitoxin on HSV replication and cell viability were first examined. As shown in Table 1, digitoxin inhibited both HSV-1 and HSV-2 with an EC_{50} of 0.05 μM. The EC_{50} for ACV and GCV were comparable to previous studies for HSV-1, with EC_{50} of 3.31 and 1.87 μM, respectively (Balzarini et al., 1998; Brand et al., 2001; Park et al., 2003). The CC_{50} of ACV and GCV were both higher than 500 μM and the CC_{50} of digitoxin was 10.66 μM. The selective index (SI) of digitoxin to HSV-1 and HSV-2 were both 213.2. The inhibitory effects of digitoxin on ACV-resistant viruses were also analyzed. The EC_{50} of digitoxin against ACV-resistant virus was the same as that for wild-type HSV viruses. This result implicated that the targets for anti-viral activities of digitoxin and ACV might be different. The anti-HSV effects of several structural analogues of digitoxin, including digoxin, gitoxigenin, ouabain octahydrate, G-strophanthin, strophanthidol, digoxigenin and digitoxigenin, were also analyzed. Among them, digoxin, ouabain octahydrate and G-strophanthin showed comparable anti-HSV activity (EC_{50} between <0.05 and 0.13 μM) and cytotoxicity (CC_{50} between 10.21 and 15.11 μM) (Table 1). The plaque size was significantly reduced in the presence of digitoxin, digoxin, ouabain octahydrate and G-strophanthin even upon suboptimal concentration (data not shown).

3.3. Effects of digitoxin on cells and virus particles

Some compounds can exert their antiviral activities through modifying membrane components of target cells (Kawamoto et al., 2003) or directly targeting virus (Cheng et al., 2004; Tome et al., 2005). To elucidate whether these are possible mechanisms for antiviral activity of digitoxin, cell and virus were pretreated with digitoxin before plaque assay. As shown in Fig. 1A, pre-

Table 1
Anti-HSV activity of digitoxin and its structural analogues

Compound	CC_{50}[a] (μM)	HSV-1		ACV-resistant HSV-1		HSV-2	
		EC_{50}[b] (μM)	SI[c]	EC_{50} (μM)	SI	EC_{50} (μM)	SI
Digitoxin*	10.66	0.05	213.20	0.05	213.20	0.05	213.20
Digoxin*	10.21	0.13	78.54	–	–	–	–
Ouabain octahydrate*	15.11	<0.05	>302.20	–	–	–	–
G-strophanthin*	14.50	0.08	181.25	–	–	–	–
Digitoxigenin	140.89	5.46	25.80	–	–	–	–
Digoxigenin	282.04	5.20	54.24	–	–	–	–
Gitoxigenin	>500	8.00	>62.5	–	–	–	–
Strophanthidol	>100	6.41	>15.60	–	–	–	–
ACV	>2000	3.31	>604.23	62.26	>32.12	3.67	>544.95
GCV	>500	1.87	>266.80	–	–	–	–

Values in this table represent the mean of three independent experiments.
[a] Cytotoxic effect was determined by MTT assay. CC_{50} was the concentration that showed 50% cytotoxic effects in Vero cells.
[b] Antiviral activity was determined by plaque assay (Russell, 1962). EC_{50} was the concentration that inhibited 50% of HSV replication in Vero cells.
[c] The selective index (SI) was calculated as CC_{50}/EC_{50}.
* The plaque size was significantly reduced in the presence of indicated compound.

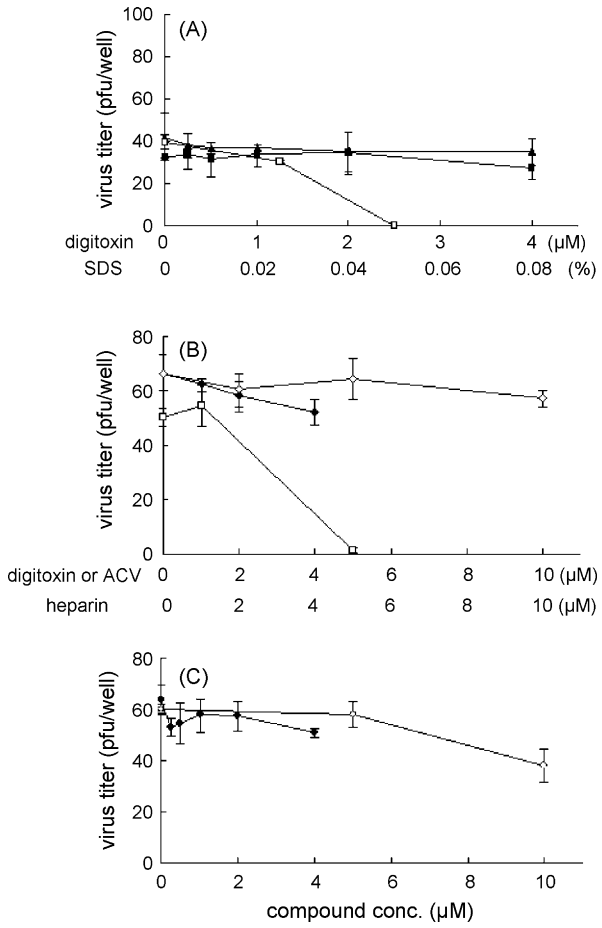

Fig. 1. Effects of digitoxin at different stages of HSV infection. (A) The effects of digitoxin on cell pretreatment (filled triangle) and virus pretreatment (filled square). SDS, which can inhibit virus infectivity upon pretreatment of virus (open square), was used as a positive control. (B) The effects of digitoxin (filled diamond) and ACV (open diamond) on virus attachment. Heparin, which can repress virus attachment (open square), was used as a positive control. (C) The effects of digitoxin (filled circle) and ACV (open circle) on virus penetration. The effects of compounds at different stages of virus replication were examined by plaque reduction assay. Each point represents the mean of three independent experiments with its respective standard deviation indicated.

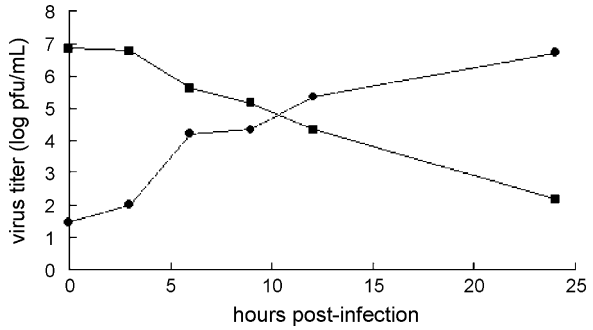

Fig. 2. Effects of delayed addition and early removal of digitoxin on virus yields. The Vero cell monolayer in 6-well plate was infected with HSV-1 (MOI = 1) at 37 °C for 1 h. For time-of-addition assay (filled circle), the drug-containing overlapping solution was added either immediately after infection (0 h) or at 3, 6, 9, 12 or 24 h post-infection. For time-of-removal assay (filled square), compound-containing maintenance medium was added into cell monolayer immediately after virus infection. At different time points post-infection, the medium was removed, and the well was washed three times before adding maintenance medium free of any compound. For both assays, at 24 h post-infection, the infected cultures were harvested and the virus yield was determined by plaque assay. The data shown here represents one representative experiment.

treatment of Vero cells with digitoxin did not significantly inhibit HSV-1 replication at a concentration of 4 μM, which is 80 times higher than its EC_{50} (0.05 μM). In addition, pretreatment of HSV-1 with 4 μM digitoxin did not profoundly repress HSV-1 replication, either (Fig. 1A). These data suggested that the anti-HSV activity of digitoxin was not exerted on cells or virus particles before viral entry.

3.4. Effect of digitoxin on virus entry

HSV entry involves complex ligand-receptor interactions and has been shown to be an ideal target for antiviral compounds (Bergefall et al., 2005; Pope et al., 1998). To determine whether digitoxin blocks HSV replication at the entry step, effects of digitoxin on viral attachment and penetration were individually investigated. As shown in Fig. 1B and C, the efficiency of both HSV-1 attachment and penetration was not significantly reduced at a concentration of 4 μM digitoxin. Therefore, the anti-HSV activity of digitoxin, like that of ACV, was not attributed to blockage of virus entry.

3.5. Effective time point of digitoxin on HSV replication

The time-of-addition and time-of-removal experiments were performed to determine the stage of HSV replication where digitoxin exerts its antiviral activity. As shown in Fig. 2, the inhibitory effect of digitoxin declined significantly when digitoxin was added at 6 h p.i. as compared to that when digitoxin was added at 3 h p.i. (2.19 Log_{10} decrease of virus yield, 4.19 vs. 2.0 Log pfu/mL). Concordantly, virus yields were profoundly affected when digitoxin was removed at 6 h p.i. compared with removal at 3 h p.i. in time-of-removal experiment (1.12 Log_{10} decrease of virus yield, 6.75 vs. 5.63 Log pfu/mL). These data suggested that digitoxin may affect HSV replication between 3 and 6 h post-infection.

3.6. Effect of digitoxin on HSV-1 gene expression

To explore the effect of digitoxin on HSV gene expression, we used RT-PCR to individually determine the RNA levels of HSV-1 IE, E and L genes in HSV-infected Vero cells at 2, 10 and 16 h post-infection. Using β-actin as an internal standard, the relative expression level of IE gene was not affected in the presence of digitoxin as compared to the control (Fig. 3A). Nevertheless, the relative expression levels of both E and L genes were inhibited in the presence of digitoxin. The observation that E gene expression was reduced with respect to IE gene correlates with the results from time-of-addition assay and supports that digitoxin exerts its effects at the early stage. The HSV-1 protein expression was also monitored by immunofluorescence staining and western blotting. As expected, HSV-1 antigen expression was obviously repressed in the presence of digitoxin (data not shown).

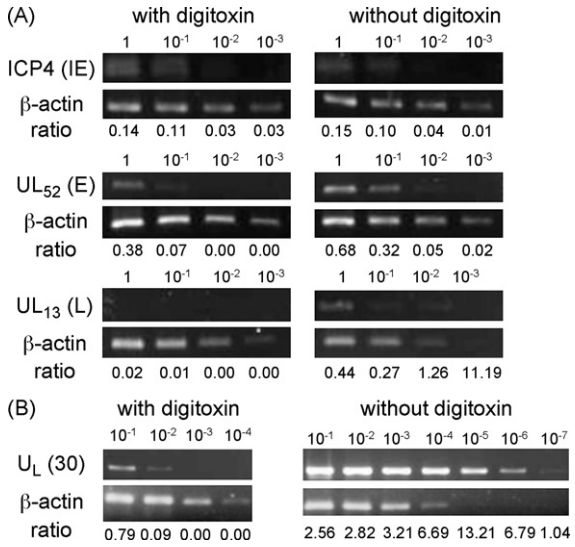

Fig. 3. Effects of digitoxin on (A) HSV gene expression and (B) HSV DNA synthesis. (A) The Vero cell monolayer in 6-well plate was infected with HSV-1 (MOI = 1) in the presence or absence of 0.5 μM digitoxin. The RNA of infected cells extracted at 2, 10, and 16 h post-infection was reverse transcribed to cDNA and the serially diluted cDNA was used for PCR-amplification for ICP4 (IE), U_L52 (E) and U_L13 (L), respectively. Five microliter aliquots of the PCR products were resolved on a 1.5% agarose gel. (B) The Vero cell monolayer in 6-well plate was infected with HSV-1 (MOI = 1) in the presence or absence of 0.5 μM digitoxin. The DNA of infected cells was extracted at 24 h post-infection. Aliquots of DNA were serially diluted (10^{-1} to 10^{-7}) and PCR-amplified for U_L30 and β-actin. Five microliter aliquots of the PCR products were resolved on a 1.5% agarose gel. The signal strength of PCR products was semi-quantitated by the ImageJ (http://rsb.info.nih.gov/ij/). The ratio of target gene signal strength to β-actin signal strength was indicated individually below the gel image for each dilution.

3.7. Effect of digitoxin on HSV-1 DNA synthesis

Since viral DNA replication at the early stage represents an essential step in virus infection, experiment was performed to determine whether digitoxin could inhibit HSV DNA synthesis. PCR reaction was conducted to amplify HSV-1 U_L30 to determine the viral genomic DNA abundance in infected Vero cells in the presence or absence of digitoxin. As shown in Fig. 3B, the viral genomic DNA was significantly reduced (10^4 to 10^5 times) in the presence of digitoxin. This result suggested that digitoxin could fundamentally reduce HSV-1 DNA synthesis in Vero cells.

3.8. Effect of digitoxin on virus release

Some antiviral agents have been shown to inhibit release of HSV particles (Palamara et al., 1995; Wachsman et al., 2003). To determine whether digitoxin could have any effect on virus release, the intracellular and extracellular virus titers in the presence or absence of digitoxin were compared. In the presence of 0.025 μM digitoxin, the titer of extracellular virus was moderately inhibited (2.2×10^6 pfu/well vs. 3.5×10^6 pfu/well, 38% inhibition), but the titer of intracellular virus was not inhibited (1.8×10^5 pfu/well vs. 1.6×10^5 pfu/well) (Fig. 4A). When the drug concentration increased to 0.05 μM, the titer of extracellular virus was almost completely inhibited (1.5×10^5 pfu/well vs. 3.5×10^6 pfu/well, 96% inhibition), but the titer of intracellular virus was only 69% inhibited (5.0×10^5 pfu/well vs. 1.6×10^5 pfu/well). On the contrary, there was no significant difference between intracellular and extracellular virus titers in the presence of different concentration of ACV, which is known to affect the E stage of virus replication and has no effects on virus release (Fig. 4B). The percentage of virus release at different concentration of digitoxin was also calculated. The percentage of virus release was gradually decreased when the concentration of digitoxin increased (Fig. 4C), while the percentage of virus release remained relatively constant at different concentrations of ACV (Fig. 4D). Based on these data, digitoxin may also inhibit HSV release from host cells.

4. Discussion

In the present study, anti-HSV activity of digitoxin and some of its structural analogues was described. The antiviral effects of digitoxin are likely to be introduced at the early stage of viral replication and the virus release stage. First, according to the time-of-addition and time-of-removal assays, delayed addition or early removal of digitoxin between 3 and 6 h post-infection had significant influences on virus yields. The 3–6 h post-infection period coincides with the expression of early genes during HSV infection (Honess and Roizman, 1974; Rezuchova et al., 2003; Spector et al., 1998). The interference of early gene expression by digitoxin was further supported by the decreased mRNA levels of one early gene U_L52 and the reduced viral DNA synthesis. Second, based on the virus release assay, the extracellular virus titer was significantly reduced as compared to the intracellular virus titer in the presence of digitoxin and there was a dose-dependent effect on inhibition of virus release by digitoxin. These results implicate that digitoxin has effects on both viral early gene expression and virus release, distinct from what is known for the widely used drug, ACV.

Digitoxin is a steroidal glycoside extracted from the leaves of digitalis purpurea and has been widely used as cardiac drugs (Belz et al., 2001; Haux, 1999). The well known pharmacological effect of digitoxin is through inhibition of Na^+/K^+-ATPase (NKA), which promotes muscle contraction and cardiac contractile force. The NKA belongs to the P-type ATPase family, a group of integral membrane proteins containing specific conserved sequences related to their ATP hydrolytic function (Horisberger, 2004). The NKA maintains the concentration gradient of Na^+ and K^+ ions across the surface membrane by exchanging of three intracellular Na^+ with two extracellular K^+ at the expense of hydrolysis one ATP (Lindenmayer et al., 1974). Previous study revealed that blockage of sodium channels could significantly increase HSV replication in primary neuronal cultures (Zhang et al., 2005) and HSV infection itself can induce internalization of sodium channels in ganglia neurons (Storey et al., 2002). Thus, the concentration gradient of cations across cell membrane may be important for HSV replication and may play a role in anti-herpes activity of digitoxin.

There are three types of ion-pumping ATPases, and P-type pumps are distinguished from the V-type and F-type enzymes by their functional and structural properties (Pedersen and Carafoli,

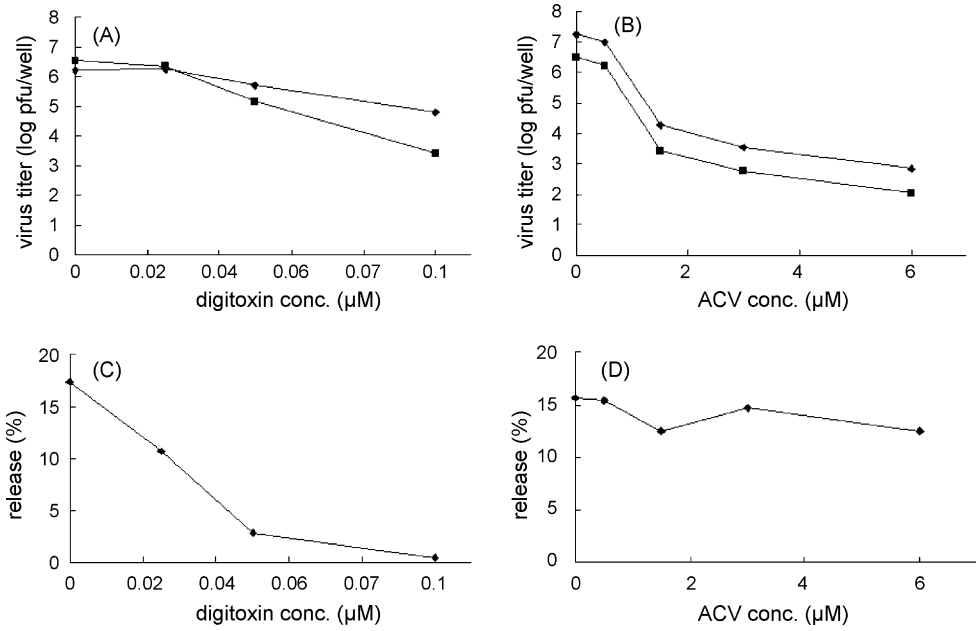

Fig. 4. Virus release assay. The Vero cell monolayer in 24-well plate was infected with HSV-1 (MOI = 0.4) for 1 h. The infected cell monolayer was then washed and drug-containing maintenance medium was added. After 24 h incubation at 37 °C, supernatant and cell pellet were separately collected, and the virus titer was determined by plaque assay. Virus titer of extracellular (filled square) and intracellular (filled diamond) fractions in the presence of digitoxin (A) and ACV (B) was shown in terms of logarithmic values. The effect of digitoxin (C) and ACV (D) on virus release was defined using the formula: release % = 100 $V_{ex}/(V_{ex} + V_{in})$ %, where V_{ex} and V_{in} represent the extracellular and intracellular virus titer, respectively.

1987). Some V-type ATPase-specific inhibitors, such as concanamycin A and SS33410, have been reported to inhibit virus entry or viral glycoprotein transportation (Irurzun and Carrasco, 2001; Seog, 2003). Concanamycin A was also shown to inhibit HSV-1 replication, likely through inhibition of translocation and maturation of glycoproteins and/or virus penetration (Hayashi et al., 2001). Similarly, one F-type ATPase-specific inhibitor, leucinostatin A, was demonstrated to inhibit virus production and cytopathic effect of virus-infected cells (Muroi et al., 1996). To date, although different applications for digitoxin have been proposed, such as a potential anticancer agent for several types of cancers (Belz et al., 2001; Haux, 1999; Haux et al., 2001), the anti-viral activity of P-type ATPase-specific inhibitor has not been reported. Our study revealed that digitoxin can inhibit HSV replication through inhibition of viral early gene expression and virus release. Unlike other anti-viral ATPase inhibitors, digitoxin has no effects on HSV entry. Whether digitoxin can repress glycoprotein translocation and maturation requires subsequent analysis. In addition, when we analyzed the anti-HSV activity of digitoxin and its analogues, we noted that those who exhibited anti-virus activity contain glycone in their structures. The importance and potential role of the glycone in anti-HSV activity of cardiac glycosides necessitate further analysis.

One of the major concerns about the clinical usage of digitoxin is its toxicity upon uptake orally. Because the therapeutic window of digitoxin in treating cardiac diseases is extremely narrow (Haustein and Winkler, 1989), monitoring the concentration in patients on therapy is important. Therefore, the disadvantage coming from the side effects of digitoxin may limit its future usage in treating HSV infection, compared with the wide safety profile of ACV. However, the effectiveness of digitoxin in inhibiting ACV-resistant viruses and there is no antagonism between digitoxin and ACV (data not shown) open the possibility of its usage as ACV supplement in the future. Further studies will be required to determine the potency and therapeutic window of digitoxin for treating HSV infection.

In conclusion, the present study described that digitoxin possesses an anti-HSV activity, which is likely through inhibition of early stage of HSV replication and blockage of virus release. Further studies will be required to explore the detailed antiviral mechanism of digitoxin.

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