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Ribonucleotide reductase inhibitors hydroxyurea, didox, and trimidox inhibit human cytomegalovirus replication *in vitro* and synergize with ganciclovir

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ABSTRACT

Ganciclovir (GCV) is a deoxyguanosine analog that is effective in inhibiting human cytomegalovirus (HCMV) replication. In infected cells GCV is converted to GCV-triphosphate which competes with dGTP for incorporation into the growing DNA strand by the viral DNA polymerase. Incorporated GCV promotes chain termination as it is an inefficient substrate for elongation. Because viral DNA synthesis also relies on cellular ribonucleotide reductase (RR) to synthesize deoxynucleotides, RR inhibitors are predicted to inhibit HCMV replication. Moreover, as dGTP competes with GCV-triphosphate for incorporation, RR inhibitors may also synergize with GCV by reducing intracellular dGTP levels and there by promoting increased GCV-triphosphate utilization by DNA polymerase. To investigate potential of RR inhibitors as anti-HCMV agents both alone and in combination with GCV, HCMV-inhibitory activities of three RR inhibitors, hydroxyurea, didox, and trimidox, were determined. In both spread inhibition and yield reduction assays RR inhibitors had modest anti-HCMV activity with 50% inhibitory concentrations ranging from 36 ± 1.7 to 221 ± 52 μM. However, all three showed significant synergy with GCV at concentrations below their 50% inhibitory and 50% toxic concentrations. These results suggest that combining GCV with relatively low doses of RR inhibitors could significantly potentiate the anti-HCMV activity of GCV *in vivo* and could improve clinical response to therapy.

1. Introduction

Human cytomegalovirus (HCMV) causes a spectrum of diseases in immune compromised patients, including retinitis in HIV patients, pneumonitis in transplant patients, and serious birth defects characterized by sensorineural hearing loss and severe mental retardation when acquired during pregnancy. Presently there are three drugs licensed for the treatment of systemic HCMV infections: ganciclovir (and its prodrug valganciclovir), foscarnet and cidofovir. Ganciclovir (GCV) is the first drug found to be effective in treating established HCMV infections and continues to be the first-line treatment for HCMV infections in AIDS and organ transplant patients. GCV is a deoxyguanosine analog that is converted to the monophosphate form by the HCMV-encoded protein kinase pUL97, and subsequently, to its di and triphosphate form by host cell kinases. GCV-triphosphate inhibits synthesis of viral DNA by competing with dGTP for incorporation into the growing DNA strand by the viral DNA polymerase. Once inserted GCV provides an inefficient substrate and thereby impairs elongation.

Acyclovir (ACV) is a deoxyguanosine analog whose mechanism of action is similar to that of GCV. It is significantly less toxic and has demonstrated efficacy and safety for treating herpes simplex viruses type 1 and type 2 (HSV-1, HSV-2) infections during pregnancy (Kang et al., 2011). However, ACV has only weak activity against HCMV at clinically useful doses and is therefore not commonly used to treat HCMV infections.

Modest antiviral activity of existing drugs coupled with dose-limiting toxicities limits therapeutic effectiveness and often results in the development of resistance. Development of new antiviral therapies that have improved efficacy as well as reduced toxicity is needed. Here we explored the potential of "combination therapy" to augment the antiviral potency of GCV by co-administration with drugs that reduce intracellular deoxynucleotide pools by inhibiting ribonucleotide reductase (RR), the cellular enzyme that catalyzes the reductive conversion of ribonucleotides into deoxynucleotides. Three RR inhibitors were selected for study: hydroxyurea (HU), didox (DX), and trimidox (TX) (Fig. 1).

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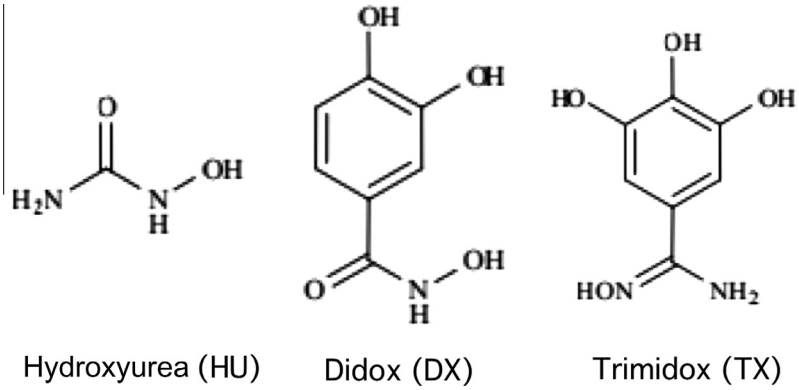

Fig. 1. Structures of HU, DX, and TX.

2. Materials and methods

2.1. Virus and cell culture

Human MRC-5 fibroblasts (ATCC CCL-171) were propagated in modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, and 10 mg/L streptomycin (Gibco-BRL) (MEM). Human ARPE-19 epithelial cells (ATCC CRL-2302) were propagated in high glucose Dulbecco's modified Eagle medium (Gibco-BRL) supplemented as above (DMEM). Viruses were propagated as described (Cui et al., 2012, 2008; Saccoccio et al., 2011). Virus BADrUL131-Y4 (a gift from Thomas Shenk and Dai Wang) is a variant of HCMV strain AD169 that contains a green fluorescent protein (GFP) expression cassette and in which a mutation in *UL131* has been repaired to permit replication in epithelial cells (Wang and Shenk, 2005). Virus RC2626 is a variant of HCMV strain Towne containing a luciferase expression cassette (McVoy and Mocarski, 1999).

2.2. Drugs

GCV and ACV were purchased from InvivoGen. HU was purchased from Sigma. DX and TX were gifts from Molecules for Health Inc., Richmond, VA. All drugs were solubilized in water and filter sterilized to produce stock solutions of 160 mM (GCV), 45 mM (ACV), 132 mM (HU), 117 mM (DX), or 22.6 mM (TX).

2.3. GFP-based spread inhibition assay

96-well plates containing confluent monolayers of MRC-5 or ARPE-19 cells were infected with virus BADrUL131-Y4 at an MOI of 0.015. One h post infection (hpi) 12 twofold serial dilutions of each drug in MEM (MRC-5s) or DMEM (ARPE-19s) were added. To ensure reproducibility each drug dilution, no-drug controls, and no-virus controls were assayed in triplicate on each plate. After 14 d relative fluorescent units (RFU) of GFP were measured for each well using a Biotek Synergy HT Multi-Mode Microplate Reader. Fifty-percent effective concentration (EC_{50}) values were determined using Prism 5 (GraphPad Software, Inc.) as the inflection points of four-parameter curves fitted to plots of GFP (mean RFUs from triplicate wells converted to % maximum) vs. log[drug] as described previously (Saccoccio et al., 2011).

2.4. Luciferase-based yield reduction assay

96-well plates containing confluent monolayers of MRC-5 fibroblast cells were infected with virus RC2626 at an MOI of 0.03. One hpi 12 twofold serial dilutions of each drug in MEM were added. Each drug dilution, no-drug controls, and no-virus controls were assayed in triplicate on each plate. After incubation for 5 d, 50 μl of supernatant from each well was transferred to corresponding wells in a black-walled, clear/flat-bottomed 96-well plate containing confluent MRC-5 monolayers. After 24 h 100 μl Steady-Glo luciferase assay reagent (Promega) was added and the luciferase activity was measured in relative light units (RLU) using a Biotek Synergy HT Multi-Mode Microplate Reader. EC_{50} values were determined as described in 2.3.

2.5. Evaluation of RR inhibitors for synergy with GCV

The luciferase-based assay described in 2.4 was modified to evaluate two-drug combinations of GCV-HU, GCV-DX, or GCV-TX. Rows contained twofold dilutions of RR inhibitors while columns contained twofold dilutions of GCV. Each plate included a dilution series of each drug alone, no-drug controls, and no-virus controls. RLU data were analyzed for synergy/antagonism using MacSynergy II software (Prichard and Shipman, 1990). This software uses inhibition data collected for each drug used alone to calculate predicted additive % inhibition values for each drug combination. It then subtracts the predicted additive inhibitions from the observed experimental values and for each drug combination and plots "% inhibition above additive predicted % inhibition" on a three-dimensional graph. Values above zero indicate synergy and negative values indicate antagonism.

2.6. Cytotoxicity

Black-walled, clear/flat-bottomed 96-well plates containing confluent cell monolayers were incubated with duplicate twofold serial dilutions of the drugs for 5 d (MRC-5) or 14 d (MRC-5 and ARPE-19); no-drug controls and no-cell controls were included in triplicate on each plate. After incubation, the drugs were removed by washing with PBS and 100 μL of fresh culture medium was added to each well. CellTiter-Glo assay reagent (100 μL; Promega) was added to each well and luciferase activity (RLU) was measured using a Biotek Synergy HT Multimode Microplate Reader. Fifty-percent cytotoxic dose (TD_{50}) values were determined as described in 2.3. For drug combinations, black-walled clear/flat-bottomed 96-well plates containing confluent MRC-5 cell monolayers were incubated with each drug combination in triplicate and RLU were measured after 5 days as described above. Percent toxicity was calculated as [(RLU (no-drug control) – RLU (drug combination))/RLU (no-drug control)] × 100.