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Antiviral activity of maribavir in combination with other drugs active against human cytomegalovirus

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ABSTRACT

The human cytomegalovirus (CMV) UL97 kinase inhibitor maribavir is in Phase III clinical trials as antiviral therapy, including use for infections refractory or resistant to standard therapy. To assess its activity in combination with approved and experimental CMV antivirals, and with the mTor inhibitor rapamycin (sirolimus), drug effects were tested by in vitro checkerboard assays and the data were analyzed using a three dimensional model based on an independent effects definition of additive interactions. Baseline virus and representative drug-resistant mutants were tested. According to the volume of synergy at 95% confidence, maribavir showed additive interactions with foscarnet, cidofovir, letermovir and GW275175X when tested against wild type and mutant viruses, strong antagonism with ganciclovir, and strong synergy with rapamycin, the latter suggesting a potentially useful therapeutic combination.

1. Introduction

Effective antiviral prophylaxis and therapy for opportunistic human cytomegalovirus (CMV) infection in transplant recipients and other immunocompromised hosts has long relied mainly on ganciclovir and its oral prodrug valganciclovir, with foscarnet and cidofovir in secondary roles. Use of these viral DNA polymerase inhibitors has improved clinical outcomes, particularly when used as prophylaxis or preventive therapy, but have well-known limitations of toxicity as well as cross-resistance due to the same antiviral drug target (Kotton et al., 2018). Recently, the CMV terminase inhibitor letermovir was approved for prophylaxis in stem cell transplant recipients (Marty et al., 2017). The separate drug target avoids issues of cross-resistance, and opens the possibility of combination therapy targeting multiple viral gene products, a strategy proven successful for HIV and hepatitis C virus. Combination studies of letermovir with existing CMV DNA polymerase inhibitors show an additive effect (Wildum et al., 2015).

Maribavir is a potent and specific inhibitor of the CMV UL97 kinase (Biron et al., 2002) that has been investigated in vitro and in clinical trials over an extended period. The UL97 kinase has important biological functions during CMV replication, including cell cycle modulation and nuclear egress of nascent viral particles (Marschall et al., 2011; Prichard, 2009). Genetic inactivation of the UL97 kinase results in severe viral growth impairment, thus offering a separate antiviral drug target distinct from the polymerase and terminase gene products. After promising early phase clinical trials, maribavir was unsuccessful in Phase III trials of low doses as CMV prophylaxis in stem cell and liver transplant recipients (Marty et al., 2011; Winston et al., 2012), although the drug was well tolerated. Subsequent trials at higher doses showed indications of success in treatment of CMV infections refractory or resistant to standard therapy (clinicaltrials.gov NCT01611974), or as treatment of asymptomatic infection, and Phase III treatment trials are ongoing (NCT02927067 and NCT02931539).

As more CMV antiviral targets are developed, a detailed assessment is needed of how drugs of different classes act when used in combination. Early studies on antiviral combinations were conducted before full appreciation of the potential for cell culture conditions to affect maribavir inhibitory concentrations by as much as 100-fold (Chou et al., 2006), presumably because of variable host cell metabolic compensation for the biological functions of the UL97 kinase. Initial in vitro combination studies with maribavir variously reported additive or synergistic interactions with CMV DNA polymerase inhibitors including ganciclovir, and with older experimental terminase inhibitors (Evers et al., 2002; Selleseth et al., 2003). Since the UL97 kinase mediates the initial phosphorylation of ganciclovir that is required for its conversion to the active ganciclovir triphosphate inhibitor of the CMV DNA polymerase, it can be predicted mechanistically that maribavir should antagonize the action of ganciclovir by interfering with its

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phosphorylation. Unlike earlier reports, antagonism was experimentally documented between maribavir and ganciclovir starting in 2006 (Chou and Marousek, 2006; Drouot et al., 2016). Because the measured antiviral effect of maribavir is greater in less metabolically active cells (e.g. incubated at lower temperatures (Williams et al., 2003) or in the presence of cyclin dependent kinase or mTor inhibitors (Chou et al., 2006)), there is the possibility of useful synergy with host-acting drugs that are used in CMV treatment populations with an acceptable safety profile. In particular, the possibility of synergy with mTor inhibitors such as rapamycin (sirolimus) is attractive because these inhibitors have anti-CMV activity both in cell culture (Chou et al., 2006) and clinically (Pascual et al., 2016), though insufficient for complete viral suppression.

Given some discordance of the published experimental data, the purpose of this study was to revisit the antiviral effects of maribavir in combination with standard DNA polymerase inhibitors (foscarnet, cidofovir, and ganciclovir), terminase inhibitors (letermovir and an older benzimidazole compound GW275175X) or the mTor inhibitor sirolimus. This was done using a standardized cell culture system, readout of viral growth based on a reporter gene, replicates of checkerboard assays analyzed according to the same three dimensional model used for several previous CMV antiviral combination studies, and CMV strains representing wild type and mutant sequences of the corresponding antiviral targets. Mutants resistant to one or the other of the drugs tested in combination were assessed for their effect on synergy determinations.

2. Materials and methods

2.1. Viral strains

The baseline bacterial artificial chromosome (BAC) clone BD1 (Chou, 2015) derived from standard laboratory strain AD169 was used to generate CMV strain T4175, with wild type sequence in all of the antiviral target genes: DNA polymerase UL54, terminase UL51, UL56, UL89 and kinase UL97. In addition, the BD series clones all contain a BAC vector, a secreted alkaline phosphatase (SEAP) reporter gene for viral growth quantitation located between US3 and US6, and a compensatory removal of the internal repeat of genes RL1 through RL13 to accommodate these inserted genetic elements (Chou, 2015). Mutant BAC clones containing known drug resistance mutations were generated from BD1 using previously described recombination techniques in genes UL54 (Chou, 2011), UL56 (Chou, 2015), UL89 (Chou, 2017a) and UL97 (Chou, 2010). Since the recombination method used a Frt-delimited upstream *Kan* selection marker and subsequent removal by Flp recombinase to leave a silent Frt motif, additional baseline strains containing the Frt motif upstream of UL54 (T4198), UL56 (T4190), UL97 (T4200) were generated and used as wild type controls as in previous studies.

2.2. Cell cultures

ARPEp cells were used at up to 45 passages from their original derivation and propagated in Dulbecco Minimal Essential Medium with 4.5 g/L glucose (DMEM) and 8% fetal bovine serum in the growth phase and 3% after viral inoculation (Chou et al., 2017). These are ARPE-19 cells made fully permissive for laboratory CMV strains by over-expression of the platelet derived growth factor receptor alpha receptor. Cells were trypsinized from confluent 75 cm^2 monolayers, seeded into 96-well plates at ~15,000 cells/well and incubated at 37 °C. Full confluency was reached within 3 days and the plates were used for antiviral assays 3 days after that. To mitigate uneven growth at the edges of plates, water-filled channels were designed around the margins of the plates. ARPEp cells in culture flasks were also used for routine propagation of viral strains and production of cell-free virus stock as used in the antiviral assays.

2.3. Antiviral compounds

Standard DNA polymerase inhibitors ganciclovir (Roche), foscarnet (Astra), cidofovir (Gilead), maribavir (Glaxo and Shire) and GW275175X (Underwood et al., 2004) (Shire) were obtained from their respective pharmaceutical sources. Letermovir was obtained from MedChemExpress (HY-15233). Rapamycin was obtained from LC Laboratories (RAP-5000). Ganciclovir and foscarnet were used as stock aqueous solutions of sodium salts of 125–200 mM. All other compounds were used as dimethyl sulfoxide (DMSO) stock solutions diluted into final working concentrations that included no more than 0.2% DMSO at the maximum drug concentration.

2.4. Cytotoxicity assay

Combinations of maribavir and other antiviral compounds were added to confluent uninfected ARPEp cell cultures in 96 wells at 2-fold increasing concentrations up to the maximum used for combination antiviral assays. One row of wells consisted of controls with no added drug. After 6 days of incubation in 100 µL of maintenance medium (DMEM with 3% fetal bovine serum), cytotoxicity was assessed by the MTT assay (Mosmann, 1983). Methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma M5655) was added to each well as 10 µL of a 5 mg/mL solution in phosphate buffered saline, mixed and incubated for 2 h at 37 °C, followed by the addition of 100 µL of a 1:1 mixture of isopropyl alcohol and DMSO to solubilize the formazan produced by live cells. After mixing, the formazan was assayed by light absorbance at 570 nm with subtraction of the background absorbance at 690 nm. The absorbance of wells containing drug was compared with that of control wells.

2.5. Checkerboard combination antiviral assays

An 8 by 8 matrix of drug combinations was prepared in a separate 96-well plate by loading one of the two drugs at one end of each axis at twice the maximum drug concentration and making serial two-fold dilutions in culture medium, leaving a final row or column without the drug. The resulting matrix had no drug in the left lower well, a single drug in rising 2-fold concentrations in the vertical and horizontal axes starting from that well, and the remaining wells with rising concentrations of drug mixtures reaching maximum concentrations of both drugs at the upper right well. After removal of culture media from 96-well cultures of ARPEp cells at 6 days after seeding, each well was inoculated with 50 µL of viral inoculum at a multiplicity of infection (MOI) of 0.02 and incubated for 90 min at 37 °C. After removal of the viral inocula, the 8 by 8 matrix of drug concentrations was transferred to the ARPEp culture plate at 150 µL per well and the plates were incubated at 37 °C under 6% CO_2. Additionally, two separate columns of 8 inoculated wells were maintained under culture medium with no added drug, as controls for viral growth across a sample of wells. At day 1, 20 µL aliquots were collected from one column of 8 control wells and assayed for SEAP activity compatible with the intended MOI (Chou et al., 2005). At day 6, 10 µL aliquots were collected from each well of the 8 by 8 matrix and the second column of 8 control wells with no added drug. Supernatant SEAP activity for viral growth quantitation was assayed using a dioxetane chemiluminescent substrate (Chou et al., 2005). A minimum of 5 replicates of checkerboard assays were performed for each drug combination and viral strain, over at least 3 setup dates and batches of cells.

2.6. Data analysis

The drug concentration required to reduce viral growth by 50% (EC_{50}) at 6 days as measured by culture supernatant SEAP activity (50% yield reduction) was calculated for each individual drug using the readings along the vertical and horizontal axis starting at the lower left