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Repurposing the clinically approved calcium antagonist manidipine dihydrochloride as a new early inhibitor of human cytomegalovirus targeting the Immediate-Early 2 (IE2) protein

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ABSTRACT

Currently, there are no therapeutic alternatives to DNA polymerase inhibitors to treat human cytomegalovirus (HCMV) infections, a major threat for immunocompromised patients and pregnant women. Here, we explored the potential to repurpose manidipine dihydrochloride (MND), a calcium antagonist clinically approved to treat hypertension, as a new anti-HCMV agent. MND emerged in a previous drug repurposing screen to find early inhibitors of HCMV replication, and now we confirm that it inhibits in the low micromolar range the replication of different HCMV strains, including clinical isolates and viruses resistant to approved DNA polymerase inhibitors. The antiviral activity of MND is specific for HCMV over different both DNA and RNA viruses. Further experiments in HCMV-infected cells testing the effects of MND on viral DNA synthesis and viral proteins expression revealed that it halts the progression of the virus cycle prior to viral DNA replication and E genes expression, but after IE proteins expression. According to these results, we observed that the overall antiviral activity of MND involves a specific interference with the transactivating functions of the viral Immediate-Early 2 (IE-2) protein, an essential viral transcription factor required for the progression of HCMV replication. Given that the inhibitory concentration against HCMV is in the range of clinically relevant concentrations of MND in humans, and the mechanism of action differs from that of the other available therapeutics, this already approved drug is an attractive candidate for repurposing in alternative anti-HCMV therapeutic protocols.

1. Introduction

Human cytomegalovirus (HCMV) is a widespread herpesvirus that establishes a lifelong persistence in the host through both chronic and latent states of infection (Mocarski et al., 2013; Nogalski et al., 2014). Although HCMV infection is asymptomatic or mildly symptomatic in immunocompetent hosts, even in this setting it represents a risk factor for the development of immunosenescence and vascular diseases (Luganini et al., 2016; Mocarski et al., 2013; Nogalski et al., 2014). HCMV is also a major opportunistic pathogen in individuals with acquired or developmental deficiencies in innate and adaptive immunity, such as transplant recipients and the fetus, for which the virus is a major cause of morbidity and mortality (Britt, 2008; Mocarski et al., 2013). Congenital HCMV infections is in fact one of the main causes of birth defects and fetal death (Britt, 2008; Kenneson and Cannon, 2007; Mocarski et al., 2013).

Prevention and control of HCMV infections still pose major challenges. In fact, no vaccine is available despite the efforts made in last years (Fu et al., 2014), and only a limited number of drugs, all targeting the viral DNA polymerase, are licensed to manage HCMV diseases (Boeckh et al., 2015; Mercorelli et al., 2008). Moreover, the clinical utility of licensed anti-HCMV drugs is limited by several drawbacks, such as poor oral bioavailability, toxicity, and selection of drug-resistant viruses (Schreiber et al., 2009). Finally, none of the available drugs has been approved for the treatment of congenital infections (Manicklal et al., 2013). Given this, new therapeutic agents are needed

Abbreviations: IE-2, Immediate-Early 2; MND, manidipine dihydrochloride; NTZ, nitazoxanide

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to address these limitations, possibly endowed with novel mechanisms of action. The identification and validation of novel-acting anti-HCMV drugs may pave the way to alternative therapeutic strategies that could be exploited in clinical settings where the approved drugs are either not recommended (i.e., congenital infections and hematopoietic stem cell transplantations) or not effective (i.e., drug-resistant viral strains).

The multi-tasking Immediate-Early 2 (IE2) protein of HCMV represents an alternative target for the development of new anti-HCMV drugs, since it is an essential virus-specific protein that acts as a transcription factor for the transactivation of viral early (E) gene expression required for viral DNA replication (Mocarski et al., 2013). Moreover, IE2 induces a plethora of biochemical and functional modifications in HCMV-infected cells to create an intracellular environment favorable for the virus replication (Stinski and Petrik, 2008). The pharmacological inhibition of IE2 is thus an alternative anti-HCMV strategy that has already been successfully applied with the development of fomivirsen, an antisense oligodeoxynucleotide designed to silence *IE2* expression (Mercorelli et al., 2011). Recently, given the importance of IE2 in the HCMV replicative cycle, we and others have put much effort in identifying small molecules able to interfere with IE2 functions and/or its expression (Beelontally et al., 2017; Gardner et al., 2015; Loregian et al., 2010; Majima et al., 2017; Mercorelli et al., 2014, 2016; Strang, 2017). In this regard, we used a cell-based assay specific for the IE2-dependent transactivation of a reporter gene (Luganini et al., 2008) to screen a library of approved drugs or bioactive molecules. This drug repurposing approach resulted in the identification of 38 compounds capable of inhibiting the IE2-dependent transactivation of the viral E *UL54* gene promoter, as well as the replication of HCMV in infected cells (Mercorelli et al., 2016).

The present study was undertaken to further characterize the antiviral activity of one of these compounds, manidipine dihydrochloride (MND), a calcium antagonist that is already clinically approved as an anti-hypertensive drug (McKeage and Scott, 2004). We report that MND potently and specifically inhibits HCMV replication and virus cycle progression by interfering with the IE2-dependent activation of viral E genes. Taken together, these findings identify for the first time the calcium channel blocker MND as an attractive candidate for the development of novel antiviral regimens for the management of HCMV infections.

2. Materials & methods

2.1. Compounds

Manidipine (MND) was obtained from Selleck Chemicals. Nitazoxanide (NTZ), foscarnet (FOS), and ganciclovir (GCV) were from Sigma-Aldrich. Ribavirin (RBV; 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was purchased from Roche and cidofovir (CDV, Vistide) was from Gilead Sciences. Fomivirsen (ISIS 2922) was synthesized by Metabion International AG.

2.2. Cells and viruses

Human Foreskin Fibroblasts (HFF), Human Embryo Lung Fibroblasts (HELF), Madin-Darby Canine Kidney cells (MDCK), and L929 cell lines were cultured in Dulbecco modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate (P/S, both from Life Technologies) at 37 °C in a humidified atmosphere supplemented with 5% CO_2. HCMV (strain AD169) and herpes simplex virus type 1 (HSV-1, strain F) were purchased from the American Type Culture Collection (ATCC). HCMV TB40-UL32-EGFP (kindly provided by C. Sinzger, University of Ulm, Germany) was previously described (Sampaio et al., 2005). HCMV VR1814 (kindly provided by G. Gerna, IRCCS Policlinico San Matteo, Pavia, Italy) was recovered from a cervical swab from a pregnant woman (Revello et al., 2001). HCMV strains resistant to antiviral drugs were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and previously described (Mercorelli et al., 2009). Clinical isolates of HSV-2 and adenovirus (AdV) were kindly provided by V. Ghisetti, Amedeo di Savoia Hospital, Turin, Italy. Influenza A/PR/8/34 virus (H1N1, Cambridge lineage) was obtained from the Division of Virology's collection of influenza viruses (Department of Pathology, University of Cambridge, Cambridge, United Kingdom). Vesicular stomatitis virus (VSV), Indiana serotype, was obtained from ATCC (ATCC VR-1238). Recombinant adenoviral vectors expressing HCMV IE2 or *E. coli* β-galactosidase (LacZ) were previously described (Mercorelli et al., 2014).

2.3. Plaque reduction assays

For plaque reduction assays (PRA) with HCMV, HSV-1, HSV-2, and AdV, HFF cells were seeded at a density of 1.5×10^5 cells per well in 24-well plates. The next day, the cells were infected at 37 °C with 100 Plaque Forming Unit (PFU) per well of the different viruses in DMEM. At 2 h post-infection (p.i.), the inocula were removed, cells were washed, and media containing various concentrations of each compound, 5% FBS, and 0.6% methylcellulose were added. All compound concentrations were tested at least in duplicate. After a period of incubation at 37 °C that depends on the virus species, cell monolayers were fixed, stained with crystal violet, and viral plaques were counted. For PRAs with influenza A virus (FluA) and VSV, MDCK and L929 cells, respectively, were seeded at a density of 5×10^5 cells per well in 12-well plates. The next day, cells were infected at 37 °C with the different viruses at 30 PFU per well in DMEM. At 1 h post-infection (p.i.), media containing various concentrations of each compound in 50% Avicell were added. All compound concentrations were tested at least in duplicate. After incubation for 48 h at 37 °C, the cell monolayers were stained with toluidine blue and viral plaques were counted.

2.4. Cytotoxicity assays

The cytotoxicity of MND and reference compounds in the different cell lines was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) method as described previously (Loregian and Coen, 2006; Muratore et al., 2012).

2.5. Quantitative real-time PCR

To analyze the effects of MND on HCMV DNA synthesis, HFFs were seeded at a density of 1.5×10^5 per well in 24-well plates. The next day, cells were infected with HCMV AD169 at a multiplicity of infection (MOI) of 0.5 PFU/cell and, following virus adsorption (2 h at 37 °C), were incubated with 80 μM MND, or 25 μM GCV as a control. HCMV-infected and treated cells were then collected at 24, 48, 56, 72, and 96 h p.i., and total DNA was extracted. The levels of viral DNA were then determined by quantitative real-time PCR (qPCR), using the previously described probe and primers amplifying a segment of the *UL122* gene (Mercorelli et al., 2016). CMV DNA copy numbers were normalized to the amount of human *β-globin* gene as described (Loregian et al., 2010).

2.6. Western blot analysis

Subconfluent HFF cells in 6-well plates were infected with HCMV AD169 at an MOI of 0.5 PFU/cell and following virus adsorption cells were treated with 80 μM MND, 30 μM NTZ, or with DMSO (0.1% v/v) as a control. Whole-cell protein extracts were prepared at different times as previously described (Bronzini et al., 2012) and then analyzed by Western Blotting (WB) with monoclonal antibodies against IEA (IE1 and IE2, 1:400, clone E13, Argene Biosoft), UL44 (1:1000, clone CH16, Virusys), UL99 (1:2000, clone CH19, Virusys), and an anti-tubulin MAb (1:2000, clone TUB 2.1, Sigma). Immunocomplexes were detected with

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