Repurposing the clinically approved calcium antagonist manidipine dihydrochloride as a new early inhibitor of human cytomegalovirus targeting the Immediate-Early 2 (IE2) protein [Mercorelli B.*, Luganini A.* co-first author]

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

Beatrice Mercorelli[1,3], Anna Luganini[2,3], Marta Celegato[1], Giorgio Palù[1], Giorgio Gribaudo[1,4,*], Arianna Loregian[1,4,*]

[1]Department of Molecular Medicine, University of Padua, 35121 Padua, Italy.

[2]Department of Life Sciences and Systems Biology, University of Turin, 10123 Turin, Italy.

[3]Co-first author.

[4]Co-senior author.

*Correspondence: arianna.loregian@unipd.it; giorgio.gribaudo@unito.it.

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

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 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-car-boxamide) 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% CO2. 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-di-phenyl 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 goat anti-mouse antibodies conjugated to horseradish peroxidase (Life Technologies).

2.7 Plasmids

The pUL54-luciferase indicator plasmids pUL54–0.4, bearing the entire HCMV UL54 promoter, and pUL54–0.15, containing UL54 promoter sequence from −150 to +15 relative to the transcription start site, were previously reported (Gariano et al., 2012). The pSGIE72 (IE1 expression construct), the luciferase indicator construct phTS-243/+30 [that contains a portion of the promoter of the human thymidylate synthase gene (−243 and +30 relative to the AUG start codon)] were previously described (Gribaudo et al., 2002). pSG5 vector was purchased from Stratagene/Agilent Technologies and the pRL-TK vector expressing Renilla luciferase was from Promega.

2.8 Cell transfections and adenoviral transductions

HELF cells were grown on 24-well plates and cotransfected using calcium phosphate (Calcium Phosphate Transfection Kit, Sigma) with0.9 μg of pUL54–0.4 or pUL54–0.15 plasmids

along with 0.1 μg of pRL-TK plasmid as a control to assess transfection efficiency. At 24 h post-transfection, cells were transduced with either AdVIE2 or AdVLacZ (Mercorelli et al., 2014) at an MOI of 20 PFU/cell for 2 h at 37 °C and then treated with 80 μM MND, 30 μM NTZ, 5 μM fomivirsen, or 0.1% DMSO (v/v) as a control. At 48 h post-transduction, the cells were harvested and the activities of both firefly and Renilla luciferases were measured. To establish the specificity of the inhibition of IE2-dependent gene transactivation, HELF cells were co-transfected with calcium phosphate with 0.3 μg of phTS-243/+30 plasmid, 0.6 μg of pSGIE72, or empty pSG5 vector, and with 0.05 μg of pRL-TK plasmid. At 18 h post-transfection, cells were washed, maintained in low-serum medium (0.5% serum) and treated with 80 μM MND, 30 μM NTZ, or 0.1% DMSO (v/v) for 48 h before determination of firefly and Renilla luciferases activities. For all transfection experiments, the presented data were normalized by dividing the values obtained for firefly luciferase by the values obtained for Renilla luciferase and expressed as relative luciferase units (RLU).

2.9 Statistical analysis

All statistical tests were performed using GraphPad Prism version 5.0. Data are presented as the means ± standard deviations (SD) of at least two experiments in duplicate.

3. Results

3.1 MND inhibits HCMV replication in a dose-dependent manner

MND was selected in a cell-based screening aimed at identifying molecules able to interfere with the HCMV-mediated activation of a reporter construct driven by the promoter of a prototypic viral Early (E) gene, UL54 (Mercorelli et al., 2016). To characterize the anti-HCMV activity of MND, PRAs and cytotoxicity assays were performed in HCMV-infected HFFs and in uninfected cells, respectively. As reported in Fig. 1, the antiviral effect of MND against the replication of the

laboratory strain AD169 was concentration-dependent and the measured Effective Concentration (EC50) was 3.09 μM (Table 1). This value, combined with a determined Cytotoxic Concentration (CC50) of > 400 μM, resulted in a Selectivity Index (SI) of > 112 (Table 1), thus indicating that the antiviral activity of MND was not due to nonspecific cytotoxicity. To further sustain the anti-HCMV activity of MND, PRAs were performed with two additional HCMV strains, TB40-UL32-EGFP and the clinical isolate VR1814. As reported in Table 2, there were no significant differences between the EC50 values of MND against the laboratory AD169 strain and either TB40-UL32-EGFP or VR1814 strains. Altogether, these data indicate that MND exerted an inhibitory effect against the replication of HCMV that does not depend on viral strain.

3.2 MND is a specific inhibitor of HCMV

We then investigated whether MND could have antiviral activityagainst other viruses. To this end, antiviral activity assays were performed against a panel of DNA and RNA viruses. As reported in Table 3, MND did not show any significant inhibitory activity on the replication of different DNA viruses (HSV-1, HSV-2, and AdV), and RNA viruses (FluA, and VSV). These results determined that MND was a specific anti-HCMV agent.

3.3 MND is active against drug-resistant HCMV strains

Since the emergence of drug-resistant HCMV strains is a major problem in the management of HCMV-infected patients, we then evaluated the activity of MND against HCMV resistant to GCV, FOS, ACV, and CDV. As reported in Table 2, MND retained full antiviral activity against HCMV strains cross-resistant to GCV and CDV (GDGrP53) and FOS and ACV (PFArD100), thus indicating that the anti-HCMV activity of MND could be exploited for the treatment of HCMV infections sustained by drug-resistant or refractory HCMV strains.

3.4 MND blocks viral DNA synthesis

To gain further insight into the inhibitory activity of MND in HCMV-infected cells, we first evaluated by qPCR the kinetics of viral DNA synthesis in HCMV AD169-infected HFFs in the presence of MND, and of GCV used as a control. As reported in Fig. 2, viral DNA levels increased progressively in a time-dependent manner up to 96 h p.i. in infected, untreated cells. In contrast, a significant inhibitory effect was observed in HCMV-infected cells treated with MND at any of time point tested. A similar inhibition, as expected, was observed for GCV-treated, infected cells (Fig. 2). These results clearly indicated that MND affects a stage of the HCMV replication cycle that occurs prior to onset of viral DNA replication, and that this interference resulted in a significant impairment of viral DNA synthesis.

3.5 MND affects post-IE phases of HCMV replication cycle

To better define the phase of the HCMV replication cycle affected by MND, we evaluated by immunoblotting the accumulation of representative IE, E, and L proteins. To this end, HFFs were infected with HCMV AD169, treated with MND, or NTZ as a control for inhibition of E and L gene expression (Mercorelli et al., 2016), and then assayed for the content of representative IE1 and IE2 (IEA), UL44, and UL99, as controls for IE, E, and L proteins, respectively. As shown in Fig. 3, treatment of infected HFFs with MND had no significant effect on the accumulation of both IE1 and IE2 proteins at any time point analyzed. In contrast, MND reduced the expression of the Early UL44 protein by more than 5–fold at 48 h p.i. and of about 4–fold at 72 h p.i., as determined by densitometric analysis (data not shown). Also the protein content of the Late UL99 was decreased by MND by 3.5–fold at 48 h p.i. and of 6–fold at 72 h p.i., respectively (Fig. 3). A similar inhibitory effect on the accumulation of E and L proteins was observed in cells treated with NTZ, used as a positive control (Mercorelli et al., 2016). These results suggested that MND targets a phase in the virus cycle after IE gene expression, but prior to the expression of Early genes, in keeping with the observed block of viral DNA synthesis (Fig. 2).

3.6 MND inhibits the IE2-dependent transactivation of the UL54 promoter

Since we observed a significant reduction of E protein levels upon MND treatment, next we investigated whether the compound might inhibit the IE2-dependent transactivation of viral E genes. To this end, luciferase reporter plasmids bearing full-length or a minimal UL54 gene promoter (pUL54–0.4 and pUL54–0.15, respectively) as a prototypic HCMV E gene promoter transactivated by IE2, were employed in transfection-transduction experiments. First, the reporter plasmids were transfected into HELFs; then, transfected cells were transduced with the IE2-expressing adenoviral vector AdVIE2, or AdVLacZ, as a negative control of transcriptional activation. After 2 h, cells were treated with MND, NTZ or fomivirsen, as controls. The luciferase activity was assessed at 48 h post-transduction. As shown in Fig. 4, for both the UL54-based indicator plasmids, overexpression of IE2 significantly increased luciferase activity compared to cells transduced with AdVLacZ. Treatment with MND resulted in a significant reduction (approximately 8-fold decrease) in their transcriptional activity with respect to untreated AdVIE2-transduced cells. This reduction is comparable to that obtained in cells treated with NTZ or fomivirsen, in agreement with previous results (Mercorelli et al., 2014, 2016). Together, these data indicated that MND caused a marked interference with IE2-dependent transactivation of a representative Early gene promoter. Thus, the overall MND-mediated inhibition of HCMV replication likely stems from its ability to interfere with E gene expression required for viral DNA synthesis.

3.7. MND does not affect the transactivating activity of another HCMV IE transcription factor

To assess the specificity of the inhibition of IE2-mediated gene transactivation, we investigated the effects of MND on the transactivating activity of the Immediate-Early 1 (IE1) protein, another virus-encoded IE transcription factor. IE1 is, in fact, the other major protein product of the HCMV MIE locus that shares the first 85 amino acids with IE2 (Stinski and Petrik, 2008). We previously observed that the cellular human thymidylate synthase (TS) gene promoter is

transactivated by the IE1 protein in quiescent fibroblasts (Gribaudo et al., 2002). Thus, we investigated the effects of MND on the IE1-dependent transcriptional activation of TS promoter. As shown in Fig. 5, in transient transfection experiments performed in HELFs, the IE1-dependent transactivation of the human TS promoter was not affected by MND. NTZ, included as a IE2-specific inhibitor, did also not interfere with the IE1-mediated activation of cellular TS gene promoter, as previously observed (Mercorelli et al., 2016). Together, these results indicated that MND-mediated inhibition of the IE2-dependent transactivation (Fig. 4) was specific and not due to a general inhibitory effect on transcription.

4. Discussion

The viral transcription factor IE2 is an emerging virus-specific therapeutic target for developing alternative anti-HCMV intervention strategies. IE2 is in fact an essential transactivator of viral E gene expression, autoregulates its own expression, and modulates and hijacks several cellular pathways to promote HCMV replication. Indeed, in the last year we and others have reported the identification of a number of compounds endowed with early anti-HCMV activity, including some able to interfere with IE2-dependent activities (Arend et al., 2017; Beelontally et al., 2017; Majima et al., 2017; Mercorelli et al., 2016; Strang, 2017). In the present study, we have investigated the anti-HCMV properties and mechanism of antiviral activity of MND, a calcium antagonist approved for the treatment of vascular hypertension (McKeage and Scott, 2004). We report for the first time the repurposing potential of this anti-hypertensive drug as an anti-HCMV agent, since it efficiently inhibits in a dose-dependent manner the replication of different HCMV strains, including clinical isolates. Moreover, we demonstrated that MND does not affect the replication of other herpesviruses (HSV-1 and -2), DNA viruses (AdV), and RNA viruses (FluA and VSV), thus indicating that MND is, indeed, a specific inhibitor of HCMV. Most importantly, MND also prevents the replication of HCMV strains that are resistant or cross-resistant to the available anti-HCMV drugs that target the viral DNA polymerase. These findings support the view

that MND could be repurposed as an alternative to the DNA polymerase inhibitors to treat or prevent HCMV infections sustained by drug-resistant viral strains, which currently represent a major problem in the HCMV management of transplant recipients and immunocompromised patients (Campos et al., 2016). We then investigated the effects of MND on viral DNA synthesis and on viral protein expression: treatment of infected cells with MND pre- vented both viral DNA synthesis and accumulation of viral E and L proteins, while leaving unaltered the expression of IE proteins. These results suggest that the IE2-dependent transactivation of viral E genes could be the target of MND, alike observed for the other anti-HCMV compounds we identified by a IE2-specific cell-based drug repurposing screening (Mercorelli et al., 2016). Indeed, when tested in transduction/transfection assays with a recombinant adenovirus expressing IE2, MND showed a significant inhibitory activity against the transactivation of two different versions of the prototypic IE2-dependent UL54 promoter (i.e., the full-length and the minimal IE2-responsive UL54 promoters) driven only by ectopically expressed IE2 protein, and therefore in the absence of other viral proteins. Importantly, the lack of effect of MND on the transactivating activity of another HCMV-encoded IE transcription factor, i.e., IE1, which shares 85 N-terminal residues with IE2 (Stinski and Petrik, 2008), strongly supports the specificity of MND inhibition toward IE2-dependent viral gene expression. Overall, these findings indicate that MND prevents HCMV replication by specifically interfering with the IE2-dependent transactivation of E genes, thereby preventing the progression of the virus cycle. MND has a well-established biological activity as a calcium antagonist by blocking the voltage-dependent calcium inward currents into smooth muscle cells (McKeage and Scott, 2004). This leads to systemic vasodilation and decrease of blood pressure. As a dihydropiridine, MND structurally differs from the other early-acting inhibitors of HCMV recently identified, which are mainly kinase inhibitors (Arend et al., 2017; Beelontally et al., 2017; Strang, 2017) or thyenilcarboxamide derivatives (Majima et al., 2017). It is therefore tempting to speculate that the specific effect shown by MND on IE2 transactivating properties may not be related to the mechanisms of action proposed for other IE2 inhibitors identified by us (such as the 6-

aminoquinolone WC5 and its analogs) (Loregian et al., 2010; Massari et al., 2013; Mercorelli et al., 2014) and others. Further studies are thus required to identify the precise molecular mechanism by which MND interferes with IE2-mediated transactivation of E promoters. However, one could envisage a scenario wherein Ca^{2+}-regulated host factors may play roles in the transactivating activity of IE2, or in its post-translational regulation, such as phosphorylation and SUMOylation (Barrasa et al., 2005; Berndt et al., 2009), or in its intracellular localization. Relevant to this hypothesis, HCMV infection can induce a very early rise in cytoplasmic Ca^{2+} due to increased Ca^{2+} influx from the extracellular environment and this effect can be prevented by L-type Ca^{2+}-channel blockers, such as nifedipine and verapamil (Nokta et al., 1987). It could therefore be possible that a calcium antagonist, such as MND, may interfere with some events occurring at the very early times of infection and that involve Ca^{2+} mobilization and/or influx. In this regard, it has been reported that compounds that affect the Ca^{2+} content of the endoplasmic reticulum (i.e., clotrimazole and thapsigargin) can block the production of HCMV infectious particles in treated cells (Isler et al., 2005). In keeping with this observation, in our cell-based screening we also identified clotrimazole as an inhibitor of HCMV replication (Mercorelli et al., 2016). In the same screening, other compounds that act as vasodilators, e.g., papaverine, were identified as inhibitors of HCMV as well. Thus, one could speculate that molecular pathways that lead to the relaxation of smooth muscle cells might be exploited by HCMV to modulate calcium homeostasis and cell signaling within the infected cells in favor of its replication. However, it might be also possible that the mechanism of viral inhibition by MND does not involve its calcium antagonistic effects, and that a completely new target/function in HCMV replication cycle is affected by this drug. Maximum plasma concentrations (Cmax) in human adults for an oral dose of 20 mg of MND were reported to be around 7 ng/ml (Rosillon et al., 1998), corresponding to ~10 μM. This is above the effective concentrations determined in this study to obtain 50%, and even 90% inhibition of viral replication (EC50 = 3.09 μM and EC90 = 6.68 μM, respectively; Table 1 and Fig. 1). Thus, MND could exert anti-HCMV activity in vivo within a range that is clinically achievable in humans. However, MND

use is contra-indicated during pregnancy, thus it is not suitable for repurposing in the case of HCMV congenital infection. Nonetheless, its anti-HCMV activity could be exploited for the treatment of those chronic or persistent HCMV infections that are associated to an increased risk of progression of a number of vascular diseases, such as atherosclerosis and restenosis (Streblow et al., 2008). Furthermore, in these diseases, there are increasing evidences that HCMV-induced secretion of both pro-inflammatory cytokines and enzymes could be a cofactor in hypertension and atherosclerosis (Zhang et al., 2011; Luganini et al., 2016). Thus, in this context, the repurposing of MND as a dual-acting drug able to reduce both hypertension and HCMV replication, could be of interest in designing new therapeutic strategies to ameliorate the outcomes of HCMV infections in individuals at risk to develop vascular diseases, such the transplant vascular sclerosis in solid organ transplant recipients.

5. Conclusions

In conclusion, although further studies are obviously needed, our data suggest that MND has the potential as a novel candidate drug to treat HCMV infection either alternatively to available DNA polymerase inhibitors or in combination therapy. This latter could improve the current treatment standards in immunocompromised populations, since MND acts by a different mechanism. Moreover, the results reported in the present study further confirm the validity of a drug repurposing approach, not only by identifying a new potential use of an already approved drug, but also by providing new molecular tools that could be used to gain deeper insights into the mechanisms of HCMV-host cell interactions.

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Declaration of interest

All authors declare no conflicts of interest.

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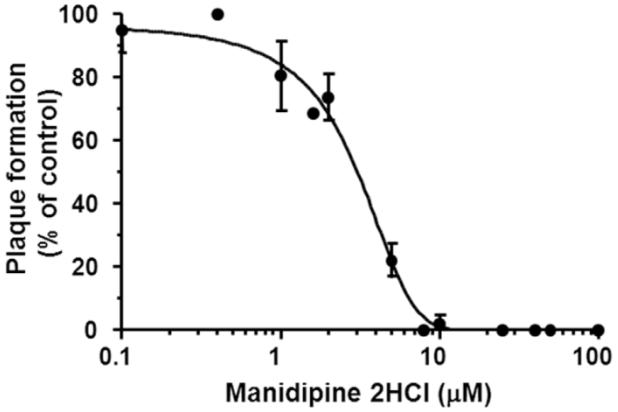

Fig. 1. Manidipine inhibits HCMV replication in a dose-dependent manner. EC50 curve was obtained by infecting HFF cells with HCMV AD169 and then treating them with different concentrations of MND. Data shown are the means ± SD of three experiments performed in duplicate.

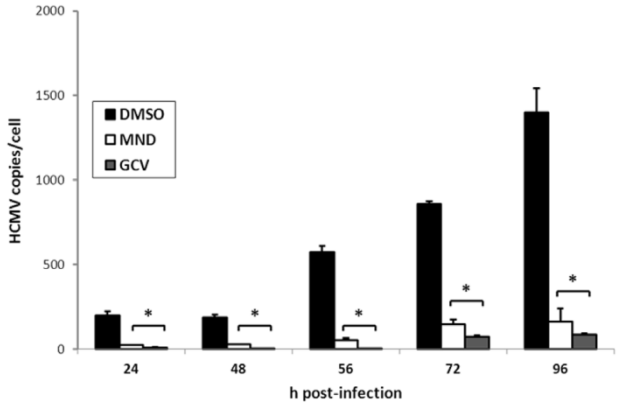

Fig. 2. Manidipine blocks viral DNA synthesis in HCMV-infected cells. HFFs were infected with HCMV AD169 and then treated with test compounds or DMSO as a control. At 24, 48, 56, 72, and 96 h p.i., total DNA was extracted and qPCR was performed with appropriate UL122 and β-

globin primers. HCMV genomic copies were normalized to the cellular β-globin gene copies. Data shown are the means ± SD of three independent experiments performed in duplicate. *$p < 0.05$; versus calibrator sample (HCMV-infected cells + DMSO) in a paired t-test.

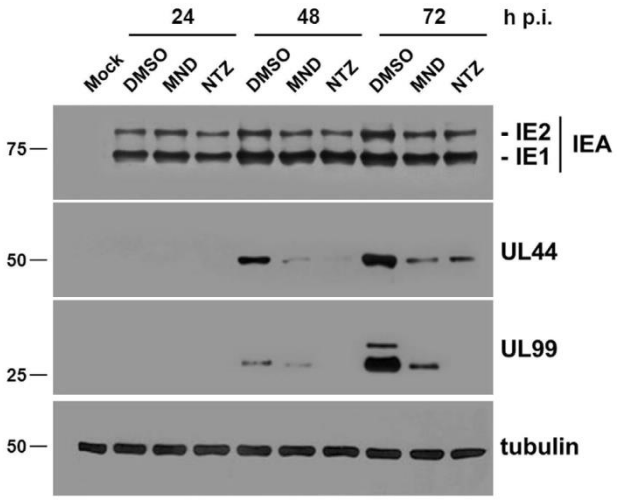

Fig. 3. Manidipine decreases the accumulation of HCMV Early and Late proteins. HFFs were infected with HCMV AD169 and then treated with test compounds or DMSO as a control. NTZ-treated samples were also included as a control for inhibition of E and L protein expression. At the indicated times p.i., total cell extracts were prepared and analyzed by WB with anti-IEA, anti-UL44, and anti-UL99 antibodies. Tubulin immunodetection served as a loading control. Images are representative of three independent experiments.

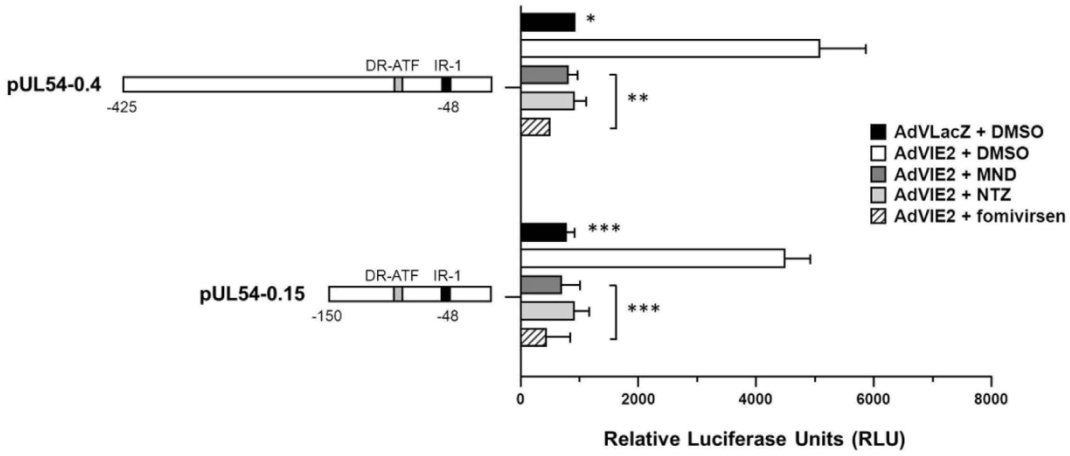

Fig. 4. IE2-dependent transactivation of HCMV early UL54 promoter is inhibited by manidipine. HELFs were transfected with luciferase reporter plasmids pUL54–0.4 or pUL54–0.15, and after 24 h were transduced with AdVIE2 or AdVLacZ. Cells were then cultured in the presence of MND, NTZ, fomivirsen, or DMSO as controls. At 48 h after transduction, luciferase expression was determined and the transcriptional activity was expressed as relative luciferase units (RLU). Data shown are the means ± SD of three experiments performed in duplicate. The presented results were analyzed with Bonferroni post-test correction for multiple comparisons. *p < 0.01, **p < 0.001, ***p < 0 .0001 versus calibrator sample (AdVIE2 + DMSO).

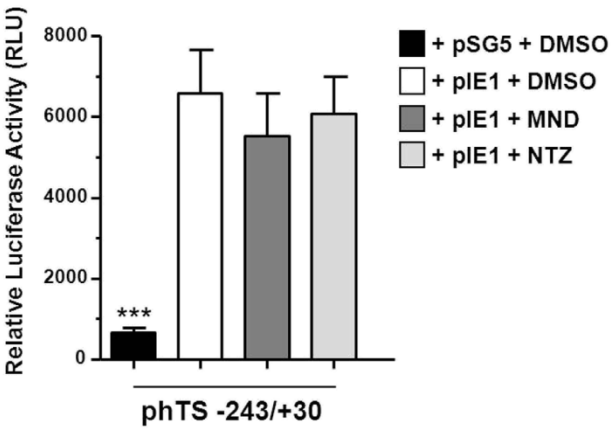

Fig. 5. Manidipine does not interfere with the IE1-dependent transactivation of the cellular thymidylate synthase gene promoter. HELFs were co-transfected with a luciferase reporter plasmid containing a portion of the human thymidylate synthase promoter (phTS-243/+30) and a plasmid expressing HCMV IE1 (pSGIE72), or the pSG5 empty vector. Transfected cells were then treated with MND, NTZ, or DMSO as a control, and maintained in low-serum medium. At 48 h post-transfection, the luciferase activity was determined. Data shown are the means ± SD of two independent experiments performed in duplicate. The presented results were analyzed in a paired t-test. ***p < 0 .0001 versus calibrator sample (phTS-243/+30 + pIE1 + DMSO).

Table 1

Antiviral activity and cytotoxicity of MND against HCMV AD169.

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
MND	3.57 ± 0.90	> 400	> 112
GCV	3.85 ± 0.91	> 1000	> 260

[a] 50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs against HCMV AD169 in HFF cells. Reported values represent the means ± the SD of data derived from five independent experiments in duplicate.

[b] 50% Cytotoxic Concentration, the compound concentration that results in 50% of cytotoxicity, as determined by MTT assays in HFF cells. Reported values represent the means ± the SD of data derived from three independent experiments performed in quadruplicate.

[c] SI, Selectivity Index (determined as CC50/EC50).

Table 2

Activity of MND against different HCMV strains.

HCMV Strain	Drug resistance	MND EC_{50}[a] (μM)	Control[b] EC_{50} (μM)
TB40-UL32-EGFP	None	3.47 ± 0.32	N.D.
VR1814	None	3.30 ± 0.71	N.D.
$PFA^{r}D100$	FOS, ACV	3.15 ± 0.92	280 ± 20
$GDG^{r}P53$	GCV, CDV	2.65 ± 0.35	72 ± 15

N.D., not determined.

[a] 50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs in HFF cells. Reported values represent the means ± the SD of data derived from at least three independent experiments in duplicate. Statistical analysis of the MND EC50 values showed that there was no significant difference among the considered HCMV strains ($p > 0.05$).

[b] GCV was used for GDGrP53 and FOS for PFArD100.

Table 3

Antiviral activity and cytotoxicity of MND against a panel of DNA and RNA viruses.

Virus (Strain)	Family	Genome	Antiviral activity (EC_{50}[a], μM)		Cytotoxicity (CC_{50}[b], μM)	
			MND	Control[c]	MND	Control
HSV-1 (F)	*Herpesviridae*	dsDNA	> 25	1.5 ± 1.8	> 400	> 1000
HSV-2 (Clinical isolate)	*Herpesviridae*	dsDNA	> 25	0.8 ± 0.5	> 400	> 1000
AdV (Clinical isolate)	*Adenoviridae*	dsDNA	> 25	ND	> 400	> 1000
FluA (PR/8/34)	*Orthomixoviridae*	(−)ssRNA	> 25	12 ± 8	N.D.	N.D.
VSV (Indiana)	*Rhabdoviridae*	(−)ssRNA	> 25	15.1 ± 3.7	> 400	> 500

N.D., not determined.

[a] 50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs. PRAs for HSV-1 and -2 and AdV were performed in HFFs

cells; PRAs for FluA were performed in MDCK cells; PRAs with VSV were performed in L929 cells. Reported values represent the means ± the SD of data derived from at least three

independent experiments in duplicate.

[b] 50% Cytotoxic Concentration, the compound concentration that results in 50% of cytotoxicity, as determined by MTT assays. Reported values represent the means ± the SD of data

derived from at least three independent experiments performed in quadruplicate.

[c] GCV was used as a control in PRAs with HSV-1 and -2; CDV was used as a control in PRAs with AdV; ribavirin was used as control in PRAs with FluA and VSV.