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The Antifungal Drug Isavuconazole Inhibits the Replication of Human Cytomegalovirus (HCMV) and Acts Synergistically with Anti-HCMV Drugs

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Abbreviations: ICZ, isavuconazole; GCV, ganciclovir; PCZ, posaconazole; VCZ, voriconazole; hCYP51, human cytochrome P450 51; FOS, foscarnet; LMV, letermovir; CI, combination index; DRI, dose reduction index.

ABSTRACT

We recently reported that some clinically approved antifungal drugs are potent inhibitors of human cytomegalovirus (HCMV). Here, we report the broad-spectrum activity against HCMV of isavuconazole (ICZ), a new extended-spectrum triazolic antifungal drug. ICZ inhibited the replication of clinical isolates of HCMV as well as strains resistant to the currently available DNA polymerase inhibitors. The antiviral activity of ICZ against HCMV could be linked to the inhibition of human cytochrome P450 51 (hCYP51), an enzyme whose activity we previously demonstrated to be required for productive HCMV infection. Moreover, time-of-addition studies indicated that ICZ might have additional inhibitory effects during the first phase of HCMV replication. Importantly, ICZ showed synergistic antiviral activity *in vitro* when administered in combination with different approved anti-HCMV drugs at clinically relevant doses. Together, these results pave the way to possible future clinical studies aimed at evaluating the repurposing potential of ICZ in the treatment of HCMV-associated diseases.

KEYWORDS: HCMV, isavuconazole, drug repurposing, human cytochrome P450 51 (hCYP51), antiviral drug combination, synergism, ganciclovir, foscarnet, letermovir.

1. Introduction

Human cytomegalovirus (HCMV) is a β -herpesvirus which is a major cause of morbidity and mortality in immunocompromised population, particularly transplant recipients, immunosuppressed subjects, and AIDS patients (Griffiths et al., 2015). Moreover, HCMV is the viral leading cause of congenital defects in newborns (live at birth) when the infection is acquired during pregnancy (Rawlinson et al., 2017). Congenital HCMV is indeed associated with neurodevelopmental defects including hearing loss and mental deficits; very recently, also microcephaly has been linked to congenital HCMV infection (Messinger et al., 2020).

Currently, anti-HCMV therapy relies only on a few drugs and a vaccine is lacking (Britt & Prichard, 2018). All drugs have viral targets, i.e., the viral DNA polymerase (targeted by ganciclovir and its prodrug valganciclovir, acyclovir and its prodrug valacyclovir, foscarnet, and cidofovir) and the viral terminase complex (targeted by letermovir, the latest in order of license) (Britt & Prichard, 2018). The use of DNA polymerase inhibitors is however limited in some relevant clinical settings, including infections with drug-resistant or refractory HCMV strains, and congenital and pediatric infections (Meesing & Razonable, 2018). Moreover, the use of letermovir is restricted to adult HCMV-seropositive stem cell transplant recipients and has been associated to emergence of drug-resistant strains (Gerna et al., 2019; Douglas et al., 2018). Thus, there is still an urgent need for the development of alternative anti-HCMV therapeutic strategies.

Targeting host pathways relevant for virus replication and pathogenesis represents a promising approach that could overcome some issues such as viral resistance. Moreover, host-targeting drugs endowed with antiviral activity might be used in combination with

antiviral agents targeting the virus, exploiting the different mechanisms of action and the expected synergistic effect (Cai et al., 2014; Oiknine-Djian et al., 2019). Also repurposing existing drugs as antiviral agents and exploit them alone or in combination is a promising and convenient antiviral strategy for the development of new therapeutic protocols (Mercorelli et al., 2018a). The current Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) pandemic offers an example of how this approach could be beneficial in the treatment of viral infections (Valle et al., 2020; Saul & Einav, 2020).

Here, we describe for the first time the anti-HCMV activity of isavuconazole, a new, extended-spectrum, clinically approved antifungal drug (trade name Cresemba®) and its repurposing potential alone and in combination with available anti-HCMV drugs for the development of new anti-HCMV strategies.

2. Materials and methods

2.1 Compounds, cells, and viruses

Isavuconazole (ICZ), ganciclovir (GCV), foscarnet, posaconazole (PCZ), and voriconazole (VCZ) were from Sigma-Aldrich. Letermovir was from Selleckchem.

Human Foreskin Fibroblast (HFF) were from the American Type Culture Collection (ATCC) and 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).

HCMV (strain AD169) was purchased from the ATCC. HCMV TB40E-UL32-EGFP (kindly provided by C. Sinzger, University of Ulm, Germany) was previously described

(Sampaio et al., 2005) as well as HCMV VR1814 (kindly provided by G. Gerna, IRCCS Policlinico San Matteo, Pavia, Italy) recovered from a cervical swab from a pregnant woman (Revello et. al., 2001). HCMV 388438U clinical isolate was collected from a urine sample at the Microbiology and Virology Unity of Padua University Hospital (Italy) and was under passage 4 after primary isolation. 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).

2.2 Plaque reduction assays

Plaque reduction assays (PRAs) with HCMV were performed as previously described (Luganini et al., 2019). Briefly, 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 80 Plaque Forming Unit (PFU) per well of the different viruses in serum-free DMEM. At 2 h p.i., the inocula were removed, cells were washed, and media containing various concentrations of each compound, 2% FBS, and 0.6% methylcellulose were added. After 10 days of incubation at 37°C, cell monolayers were fixed and stained with crystal violet, and viral plaques were counted.

2.3 Cell viability assays

The effect on cell viability of test compounds was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) method as described previously (Loregian & Coen, 2006).

2.4 Virus yield reduction assays

Virus yield reduction assays with HCMV were performed as previously described (Loregian et al., 2010). Briefly, HFF cells were plated at 2×10^4 cells per well in 96-well plates, incubated overnight, and infected the next day with HCMV AD169 at MOI of 0.05 PFU/cell. After virus adsorption for 2 h at 37°C, cells were washed and incubated with 0.2 ml of fresh medium containing 5% FBS in the absence or in the presence of test compounds. Plates were incubated for 5 days at 37°C and then subjected to one cycle of freezing and thawing. Virus titres were determined by transferring 0.1 ml aliquots from each well to a fresh 96-well monolayer culture of HFF cells, followed by 1:5 serial dilutions across the plate. Cultures were incubated for 7 days and at the end of incubation were fixed and stained, and the numbers of viral plaques were counted.

2.5 Enzymatic assays *in vitro*

Recombinant human CYP51 and its redox partner NADPH-cytochrome P450 reductase (CPR) were expressed in *Escherichia coli* and purified as described previously (Hargrove et al., 2016). The standard reaction mixture contained 0.5 μ M hCYP51 and 1.0 μ M CPR, 100 μ M L- α -1,2-dilauroyl-*sng*lycerophosphocholine, 0.4 mg/ml isocitrate dehydrogenase, and 25 mM sodium isocitrate in 50 mM potassium phosphate buffer (pH 7.2) containing 10% glycerol (v/v). After addition of the radiolabeled substrate ([3-³H] lanosterol, ~4,000 dpm/nmol; dissolved in 45% aqueous 2-hydroxypropyl- β -cyclodextrin purchased from Sigma, w/v, final concentration 50 μ M) and inhibitors (concentration range 0.1 - 250 μ M), the mixture was preincubated for 30 s at 37 °C in a shaking water bath. The reaction was initiated by the addition of 100 μ M NADPH and stopped by extraction of the sterols with 5

ml of ethyl acetate. The extracted sterols were dried, dissolved in methanol, and analyzed by a reversed-phase HPLC system (Waters) equipped with a β -RAM detector (INUS Systems) using a NovaPak octadecylsilane (C_{18}) column and a linear gradient water/acetonitrile/methanol (1.0:4.5:4.5, v/v/v) (solvent A) to CH_3OH (solvent B), increasing from 0 to 100% B for 30 min at a flow rate of 1.0 ml/min. The IC_{50} values were calculated using GraphPad Prism 6, with the percentage of lanosterol converted being plotted against inhibitor concentration and the curves fitted with non-linear regression (log(inhibitor) vs. normalized response - variable slope).

2.7 Time-of-addition experiments

For time-of-addition studies, HFF were seeded at a density of 10^4 cells per well in 96-well plates, incubated overnight, and infected the next day with HCMV AD169 at a MOI of 0.1. After virus adsorption for 2 h at $37^\circ C$, the viral inocula were removed, and the end of viral adsorption was considered the zero point of the time course. At 0, 3, 6, 9, 22, 26, 29, 33, 46, 50, 53, 57, 70, 74, 77, and 80 h p.i., the cell medium was replaced with a medium containing 20 μM ICZ or 10 μM GCV as a control. The plates were incubated at $37^\circ C$ for a total of 100 h p.i.; at this point, cells were subjected to one cycle of freezing and thawing, and the amount of viral progeny in each well of the microtiter plates was determined by titration on fresh HFF cultures.

2.8 Drug combination studies

To evaluate the combined effects of ICZ and different anti-HCMV drugs on HCMV AD169 replication, plaque reduction assays were performed as described above using 0.125-, 0.25-,

0.5-, 1-, 2-fold their respective EC_{50} for each combination of ICZ and anti-HCMV drugs at equipotent ratio. The 2-drug combination effects were assessed using the Chou-Talalay method (Chou, 2006) according to mass-action law based dynamic theory computed in the CalcuSyn software version 2.0 (Biosoft, Cambridge, UK).

2.8 Statistical analysis

All statistical analyses were performed using GraphPad Prism (San Diego, CA).

3. Results

3.1 Isavuconazole is an inhibitor of HCMV replication.

In a drug repurposing campaign, we previously identified a series of molecules endowed with anti-HCMV activity (Mercorelli et al., 2016). Follow up studies revealed that some clinically approved imidazolic antifungal agents, i.e., miconazole, econazole, clotrimazole, ketoconazole, and a triazole, i.e., posaconazole, are broad-spectrum inhibitors of HCMV replication (Mercorelli et al., 2020). Here, we wished to investigate the possible anti-HCMV activity of a new, extended-spectrum antifungal, i.e., isavuconazole (ICZ).

To this aim, plaque reduction assays (PRA) in HFF cells infected with HCMV were performed with ICZ and other anti-HCMV drugs as a control (Table 1). As reported in Fig. 1A, ICZ showed a dose-dependent inhibitory effect on HCMV AD169 replication in HFF cells, similarly to what we previously reported for the other antifungals endowed with anti-HCMV activity (Mercorelli et al., 2016, 2020).

In parallel, to exclude that the antiviral activity of ICZ might be due to cytotoxicity, its effect on the viability of uninfected HFF cells was evaluated by MTT assays. As reported in Fig. 1A, we did not observe cytotoxicity at the same concentration range at which the antiviral activity was evident. Thus, the antiviral effect was not due to inhibition of cell viability. Cytotoxicity assays were then performed with higher concentrations of ICZ (up to 250 μM). We noticed an evident tendency of the ICZ to precipitate over time (at ≥ 125 μM at 5 days post-treatment and at ≥ 62.5 μM at 10 days post-treatment), a phenomenon also described for the pharmaceutical formulation of Cresemba®. Therefore, the cytotoxic concentration that inhibited *in vitro* cell viability by 50% (CC_{50}) at 5 days post-treatment resulted ~ 100 μM and a Selectivity Index (SI) of 14 was calculated (Table 1).

To further confirm the anti-HCMV activity, the effects of ICZ on virus yield were analyzed in a multi-cycle viral growth experiment. As reported in Fig. 1B, treatment of infected cells with ICZ resulted in a dose-dependent inhibition of the virus progeny production, with an EC_{50} value of 3.16 μM (Table 1).

3.2 Isavuconazole is a broad-spectrum inhibitor of HCMV, including drug-resistant viral strains.

To investigate the spectrum of anti-HCMV activity of ICZ, we repeated the antiviral assays with a panel of HCMV strains, including low passage-number clinical isolates (TB40, VR1814, and 388438U, Fig. 1A). As reported in Table 2, the anti-HCMV activity of ICZ was not dependent on the viral strain, since the EC_{50} values obtained with different HCMV strains were similar.

In addition, we tested ICZ against HCMV strains resistant to the available viral DNA polymerase inhibitors. Noteworthy, ICZ inhibited the replication of viruses resistant either to both GCV and cidofovir (GDG^rP53) or to both foscarnet and acyclovir (PFA^rD100) at levels comparable to those observed for drug-sensitive strains (Fig. 1C and Table 2). This result is of particular importance, since ICZ is clinically approved and the concentrations needed for antiviral activity fall within the therapeutic range as antifungal that is achieved in patients under therapy. Thus, ICZ may represent a potential therapeutic alternative in the case of infections with HCMV strains resistant or refractory to the available drugs.

3.3 Isavuconazole inhibits human cytochrome P450 51 enzymatic activity *in vitro*.

We previously linked the anti-HCMV activity of another azolic antifungal drug, PCZ, to its ability to inhibit the host enzyme human cytochrome P450 51 (hCYP51) (Mercorelli et al., 2020). In this context, we found that HCMV is able to induce the expression of hCYP51. Moreover, the pharmacological inhibition of hCYP51 resulted in an overall reduction of virus replication, production of infectious progeny, and infectivity of released viral particles (Mercorelli et al., 2020). We thus investigated the inhibitory activity of ICZ against purified hCYP51 *in vitro*, including PCZ and voriconazole (VCZ) as a positive and a negative control, respectively. As reported in Fig. 2, in enzymatic assays *in vitro*, both ICZ and PCZ inhibited the initial rate of lanosterol conversion catalyzed by purified hCYP51 in a dose-dependent manner, with IC₅₀ values of 50.5 and 8.3 μ M, respectively. This inhibitory effect was not observed with VCZ (IC₅₀ >250 μ M). In these experiments, ICZ resulted about 6-fold less potent than PCZ in inhibiting hCYP51 enzyme *in vitro*, while a slightly lower anti-HCMV activity in infected cells was observed for ICZ when compared with PCZ (EC₅₀ values of 3.9-7.4 μ M *versus* 2.6-4.8 μ M in PRAs with different HCMV strains, Tables 1 and

2 and Mercorelli et al., 2020), suggesting that ICZ might have other viral or cellular targets besides hCYP51.

3.4 Isavuconazole affects HCMV viral progeny production at different times during the replication cycle

To better characterize the mechanism of action of ICZ and to determine the phase of HCMV replication cycle that is affected by the drug we performed time-of-addition experiments, in which inhibitors are effective only if added prior to their target's engagement in HCMV replication cycle. In these experiments, 20 μ M ICZ, or 10 μ M GCV for a comparison, was added to HCMV-infected HFF cells at different time points p.i. from 0 to 80 h p.i. According to the mechanism of action of GCV, i.e., inhibition of viral DNA synthesis, it markedly reduced virus yield when it was added up to 50-57 h p.i. (Fig. 3), consistent with the inhibition of the viral DNA polymerase.

We previously reported that HCMV-induced overexpression of hCYP51, a host target of ICZ, was evident starting from 24-48 h p.i. (Mercorelli et al., 2020). Thus, we expected this to be also the time at which ICZ could start interfering with HCMV replication by inhibiting the host enzyme. In keeping with this, we measured an increase in HCMV titer when ICZ was added starting from 46 h p.i. (Fig. 3). Moreover, we noticed an additional reduction of the inhibitory effect of ICZ also when it was added within the first (0-22) h p.i. (Fig. 3). This result might support the hypothesis of the existence of other, early-acting target(s) of ICZ in HCMV infected cells.

3.5 Isavuconazole acts synergistically with approved antivirals against HCMV replication

We finally explored the possibility that ICZ may act synergistically with available approved anti-HCMV drugs (ganciclovir, foscarnet, and letermovir) against virus replication, given that the mechanisms of action should be different. To test this hypothesis, PRAs were performed with equipotent combinations of ICZ combined with either GCV, FOS, or LMV, and the antiviral effects were determined by applying the Chou & Talalay method (Chou, 2006). As reported in Table 3, a synergistic effect for ICZ and all the three drugs tested was observed, since the weighted Combination Index (CI_{wt}) resulted <0.7 (Chou, 2006). When the single drug combinations were analyzed, the effect ranged from *very strong synergism* (at 2 up to 0.25-fold the respective EC_{50}) up to *nearly additive* effect for the combination of the ICZ with GCV or LMV at doses equal to 0.125-fold their respective EC_{50} (Tables S1-3), when the effect is probably lost by the fact that the drug concentrations are too low to act synergistically against the different targets. We also observed that the combination of ICZ and LMV resulted less synergic than the combination of ICZ with DNA polymerase inhibitors, most likely because the timing of the respective targets' engagement might be similar for ICZ and LMV and the synergistic inhibitory effect is less evident.

The safety of the combinations at the highest dose (i.e., 4-, 2-, and 1-fold EC_{50}) was assessed in uninfected HFF cells at 10 days p.i., the same time course of the plaque reduction experiments. We observed a slight inhibitory effect on cell viability after 10 days when the 1- and 2-fold EC_{50} combinations of ICZ with the different drugs were tested, however not significant and very far from the CC_{50} (Fig. S1). Thus, the synergistic effect on the reduction of viral plaque numbers was most likely the result of combining two drugs with different targets. Only when the cells were treated with the drugs at 4-fold their respective EC_{50} , we

observed a significant effect on the cell viability (Fig. S1). Therefore, this combination was excluded from the synergism studies.

From this set of experiments, we also extrapolated the Dose Reduction Index (DRI), which gives an estimation of the extent to which the dose of one analyzed drug may be reduced when used in combination with the second drug in the case of synergism, compared to the dose of the same drug used alone to achieve the same effect (Chou, 2006). As an example, when GCV, FOS, or LMV are used in combination with ICZ *in vitro*, 95% inhibition of HCMV replication may be obtained by reducing ICZ dose by 7-, 5-, and 3-fold, respectively, compared to ICZ used alone (Tables S4-6).

4. Discussion

In the present study, we have identified for the first time the anti-HCMV properties of ICZ, an antifungal drug which acts by inhibiting fungal CYP51 (14- α lanosterol demethylase), an essential enzyme and an important antimicrobial target. ICZ demonstrated broad anti-HCMV activity being able to inhibit the replication of different HCMV strains, including clinical isolates and drug-resistant strains. We previously linked the anti-HCMV activity of another antifungal triazolic agent, posaconazole, to its ability to inhibit the human CYP51, which catalyzes a rate-limiting step in cholesterologenesis in mammals (Hargrove et al., 2016). ICZ also inhibited hCYP51 enzymatic activity *in vitro*, albeit less potently than PCZ. Thus, from this observation and taking into account the results of the time-of-addition experiments, we hypothesize that there might be additional target(s) of ICZ in HCMV-infected cells.

In this context, the antiviral activity of ICZ against Lassa virus and other arenaviruses was recently reported (Zhang et al., 2020) and it has been observed that ICZ binds to the stable signal peptide-GP2 subunit interface of Lassa virus glycoprotein, thus inhibiting pH-dependent viral entry. However, it seems unlikely that ICZ could act similarly against HCMV, a completely unrelated virus. Rather, since other azolic antifungal drugs show polypharmacology effects by interfering with different host pathways (Strating et al., 2015; Chen et al., 2016; Trinh et al., 2017; Meutiawati et al., 2018; Chen et al., 2016), we cannot exclude that ICZ might also interfere with known or yet-undefined host pathways relevant for HCMV replication and thus contributing to its overall antiviral activity.

PCZ, but to our knowledge not ICZ, is known to inhibit other host pathways including cholesterol intracellular trafficking (Trinh et al., 2017; Strating et al., 2015; Meutiawati et al., 2018) and Hedgehog signaling (Chen et al., 2016). Conversely, ICZ interactions with host proteins have not been equally well characterized yet. However, during preclinical and clinical development of ICZ, an off-target inhibitory effect of the drug on different human ion channels was observed, although it did not preclude its approval. In particular, voltage-dependent L-type calcium channels and potassium channels such as human ether-a-go-go (hERG) channel were the most sensitive to ICZ inhibition (unique among triazole antifungal agents) (Keirns et al., 2017 and <https://www.accessdata.fda.gov>). Intriguingly, different independent studies (including our drug repurposing studies) reported the early anti-HCMV activity of molecules that act by inhibiting these types of ion channel (Mercorelli et al., 2011; Mercorelli et al., 2016; Mercorelli et al., 2018; Albrecht et al., 1987; Nokta et al., 1988; Fons et al., 1991). Thus, a hypothesis, that remains to be tested, could be that in HCMV-infected cells at very early times p.i. ICZ affects a cellular or viral target that

contributes to virus replication and is sensitive to changes in ions homeostasis (particularly calcium). This hypothesis is in line with the inhibitory effects of the drug that we observed in the time-of-addition experiments. However, other viral or cellular targets cannot be excluded. Future studies will be aimed at identifying other possible targets of ICZ, if any.

Isavuconazole is an azole antifungal agent active against a broad range of clinically relevant fungi and was approved in 2015 for the treatment of invasive aspergillosis and mucormycosis (McCormack, 2015). Its prodrug isavuconazonium is rapidly converted by plasma esterases in the active form, isavuconazole. In healthy subjects, peak levels (C_{max}) of ICZ range from 7.5 up to 20 mg/L (corresponding to 17.1 and 45.6 μ M, respectively), depending on the daily dosage, which can range from 200 to 600 mg (McCormack, 2015). Within SECURE clinical trial, C_{min} was reported around 3.9 mg/L (corresponding to 8.9 μ M) (Maertens et al., 2016). In more recent studies, the $C_{through}$ range of ICZ was reported between 1.33-4.22 mg/L (corresponding to 3-9.6 μ M) in adult patients (Nannetti et al., 2018), and between 2.15-8.54 mg/L (corresponding to 4.9-19.5 μ M) in pediatric patients under clinical therapy (Decembrino et al., 2020). Thus, plasma levels of ICZ seem to be compatible with the dose required for the antiviral activity *in vitro*, since the EC_{50} s that we found against HCMV (Table 1 and 2) fall in the clinical range reached in humans. Noteworthy, although in our study ICZ exhibited slightly higher EC_{50} s than PCZ in antiviral cell-based assays, the clinically achievable levels of ICZ *in vivo* are much higher than those of PCZ. Indeed, peak levels for PCZ are reported around 1.5-2 μ g/ml (2.2-2.9 μ M) (Cornely et al., 2016 and 2017), concentrations which are below the EC_{50} against HCMV that was observed for PCZ (Mercorelli et al., 2020). ICZ bioavailability can be influenced by different factors, such as interactions with other drugs or genetic factors, thus it could be

possible that the concentration of the drug alone would result not sufficient to exert antiviral activity *in vivo* in some clinical settings. Importantly, our data suggest that much lower levels of ICZ could be required to inhibit HCMV when this drug is administered in combination with some approved anti-HCMV drugs. Thus, ICZ could be also exploited to potentiate the antiviral effect of GCV and FOS or LMV, although a drug-drug interaction with the latter cannot be excluded given that both drugs are CYP3A4 inhibitors. Clinical studies aimed at evaluating the efficacy of ICZ in controlling HCMV replication *in vivo*, both alone and in combination with anti-HCMV drugs used in clinical therapy, are needed to further validate the data we obtained in infected cells.

In conclusion, this study identified another FDA-approved drug already used in clinical practice in immunosuppressed patients who are at high risk of HCMV infections, endowed with potential of repurposing in the treatment of HCMV-associated infections. ICZ is well tolerated, and has a higher bioavailability than PCZ and a lower propensity to interact with other drugs compared to the other triazole antifungals (McCormack, 2015). Moreover, this study identified it as a broad-spectrum inhibitor of HCMV, including viral strains resistant to the currently available drugs. Thus, although a clinical validation of our observations is needed, ICZ may represent a valid alternative in the case of infections sustained by resistant or refractory HCMV strains or in stem cell transplant recipients.

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DECLARATION OF INTEREST

B.M., A. Luganini, G.G., and A. Loregian have filed a provisional patent on the use of isavuconazole alone and in combination with ganciclovir for the treatment of pathological conditions associated with cytomegalovirus infection.

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FIGURE LEGENDS

Fig. 1 Isavuconazole inhibits HCMV replication in a dose-dependent manner. (A) Plaque reduction assays were performed in HFF infected with the indicated HCMV strains and treated with different doses (from 0.1 to 25 μM) of ICZ. Cell viability curve in the presence of ICZ was obtained by MTT assays at 120 h. (B) Dose-dependent inhibition of virus progeny production by ICZ in HCMV-infected HFFs as determined by virus yield reduction assays. (C) Dose-dependent inhibition of the replication of HCMV strains resistant to currently available viral DNA polymerase inhibitors. In all panels, graphs report the mean \pm SD of $n \geq 3$ independent experiments in duplicate.

Fig. 2 Isavuconazole inhibits host hCYP51 enzymatic activity *in vitro*. Inhibition of enzymatic activity of purified hCYP51 by VCZ, ICZ, and PCZ (2-min reaction). Graph reports the mean \pm SD of $n = 3$ independent experiments in duplicate.

Fig. 3 Effects of addition of ICZ at different time-points after HCMV infection. HFF cells were infected with HCMV AD169 at an MOI of 0.1 and were then treated with ICZ or GCV at 0, 3, 6, 9, 22, 26, 29, 33, 46, 50, 53, 57, 70, 74, 77, and 80 h p.i. Virus titers derived from HCMV-infected cells treated with the drugs at each time point were determined by titration on fresh HFF monolayers. Graph reports the mean \pm SD of $n = 3$ independent experiments in duplicate.

Table 1

Activity of ICZ and other anti-HCMV drugs against HCMV AD169.

Compound	EC ₅₀ ^a (μM), CI ^b	EC ₅₀ ^c (μM), CI	CC ₅₀ ^d (μM)	SI ^e
ICZ	7.38 (4.95-10.87)	3.16 (1.69-5.54)	100 \pm 16	14
GCV	1.56 (1.09-2.22)	N.D.	>250	>160
FOS	28.4 (5.9-78.3)	N.D.	N.D.	N.D.
LMV	0.002 (0.0008-0.008)	N.D.	N.D.	N.D.

^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 data derived from n = 3 independent experiments in duplicate.

^b CI, Confidence Interval (95% Profile likelihood, calculated with GraphPad Prism 8.0 software).

^c 50% Effective Concentration, the compound concentration that inhibits 50% of virus yield in HFF cells. Reported values represent data derived from n = 3 independent experiments in duplicate.
N.D., Not Determined.

Table 2

Activity of ICZ against different HCMV strains.

HCMV Strain	Drug resistance	ICZ EC ₅₀ ^a (μM), CI ^b	Control ^c EC ₅₀ (μM), CI
TB40	None	4.44 (2.47-7.63)	N.D.
VR1814	None	5.13 (2.48-9.89)	N.D.
388438U	None	3.88 (1.49-8.96)	N.D.
PFA ^d D100	FOS, ACV	5.21 (2.38-10.84)	278 (241-312)
GDG ^e P53	GCV, CDV	6.17 (3.14-11.56)	74.4 (42.4-145.5)

^a 50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs in HFF cells. Reported values represent data derived from n ≥ 3 independent experiments in duplicate.

^b CI, Confidence Interval (95% Profile likelihood, calculated with Graphpad Prism 8.0 software).

^c GCV was used for GDG^eP53 and FOS for PFA^dD100.

N.D., Not Determined.

Table 3

Analysis of the effect of the drugs combination on HCMV replication.

Drug Combination (EC ₅₀ ^a Ratio ^b)	CI values extrapolated at the indicated % of virus inhibition (mean ± SD) ^c				CI _{wt} ^d	Drug combination effect ^e
	50	75	90	95		
ICZ + GCV (3.5:1)	0.635±0.018	0.386±0.039	0.243±0.042	0.180±0.039	0.258	<i>Strong Synergism</i>
ICZ + FOS (1:4)	0.593±0.021	0.422±0.029	0.306±0.012	0.284±0.035	0.335	<i>Synergism</i>
ICZ + LMV (3,500:1)	0.750±0.031	0.624±0.022	0.522±0.037	0.464±0.019	0.542	<i>Synergism</i>

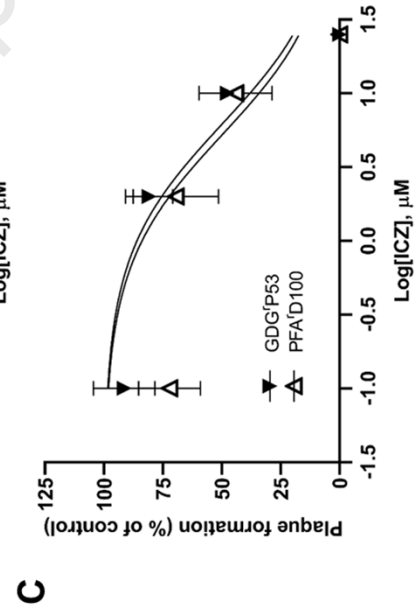
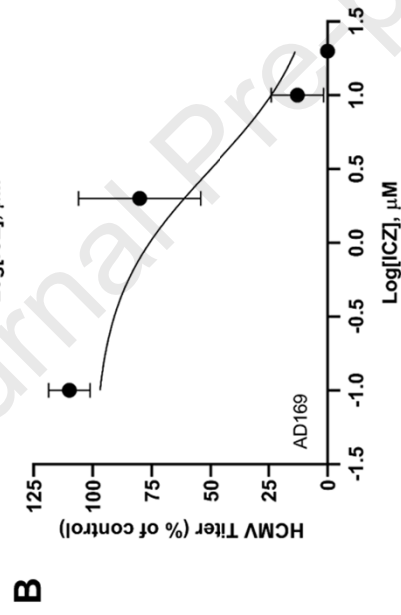
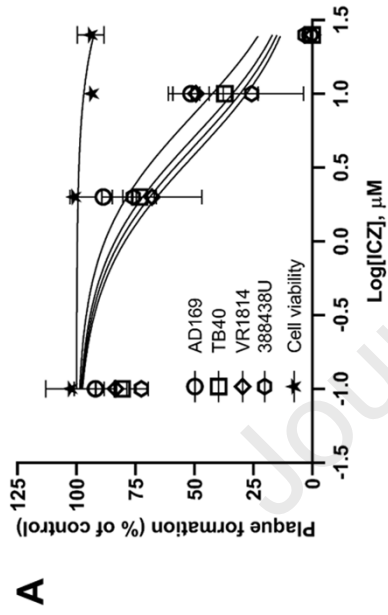
^a EC₅₀ values for HCMV strain AD169 considered for each drug (approximating values obtained in PRA and reported in Table 1).

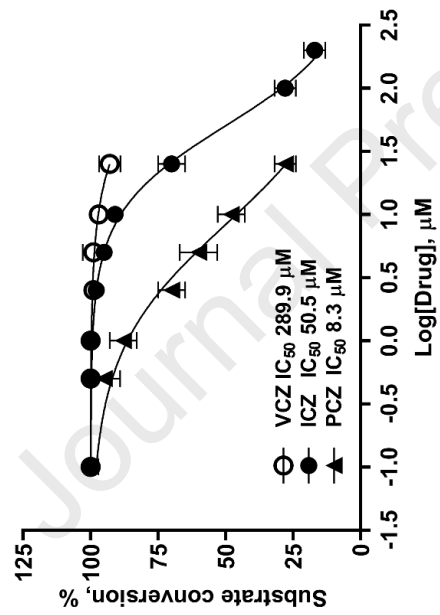
^b EC₅₀ of drug₁/EC₅₀ of drug₂ yielding an equipotent concentration ratio between the two combined drugs.

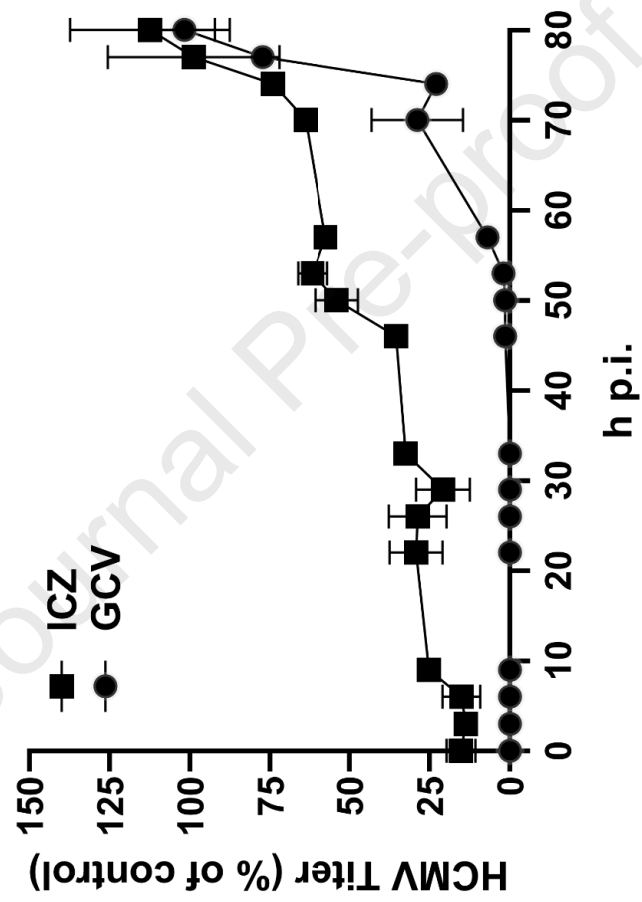
^c Combination index (CI) at the indicated % of virus inhibition values extrapolated by analysis with CalcuSyn Software. CI values represent the mean ± SD of n = 3 independent experiments in triplicate or duplicate.

^d Weighted average CI. Values were calculated as CI_{wt} = (CI₅₀ + 2 x CI₇₅ + 3 x CI₉₀ + 4 x CI₉₅)/10.

^e Drug combination effect defined as: strong synergism for 0.1 < CI_{wt} < 0.3 and synergism for 0.3 < CI_{wt} < 0.7, according to (Chou, 2006).







HIGHLIGHTS

1. Isavuconazole (ICZ) is a clinically approved antifungal that inhibits human cytomegalovirus (HCMV) replication.
2. ICZ is a broad-spectrum HCMV inhibitor, including clinical isolates and drug-resistant strains within its clinical range.
3. ICZ inhibits *in vitro* the activity of human CYP51 enzyme, required for productive HCMV infection.
4. ICZ acts synergistically with ganciclovir, foscarnet, and letermovir, indicating a repurposing potential in controlling HCMV.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Beatrice Mercorelli, Anna Luganini, Giorgio Gribaudo, and Arianna Loregian have filed a provisional patent on the use of isavuconazole alone and in combination with ganciclovir for the treatment of pathological conditions associated with cytomegalovirus infection.