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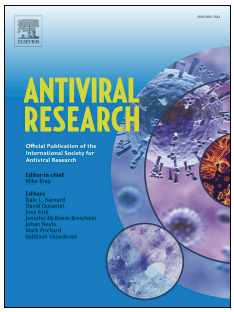

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Effective deploying of a novel DHODH inhibitor against herpes simplex type 1 and type 2 replication

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Running title: The new DHODH inhibitor MEDS433 blocks HSV-1 and HSV-2 infections

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Abbreviations: DHODH, Dihydroorotate dehydrogenase; ACV, Acyclovir; DPY, Dipyridamole.

Abstract

Emergence of drug resistance and adverse effects often affect the efficacy of nucleoside analogues in the therapy of Herpes simplex type 1 (HSV-1) and type 2 (HSV-2) infections. Host-targeting antivirals could therefore be considered as an alternative or complementary strategy in the management of HSV infections. To contribute to this advancement, here we report on the ability of a new generation inhibitor of a key cellular enzyme of *de novo* pyrimidine biosynthesis, the dihydroorotate dehydrogenase (DHODH), to inhibit HSV-1 and HSV-2 *in vitro* replication, with a potency comparable to that of the reference drug acyclovir. Analysis of the HSV replication cycle in MEDS433-treated cells revealed that it prevented the accumulation of viral genomes and reduced late gene expression, thus suggesting an impairment at a stage prior to viral DNA replication consistent with the ability of MEDS433 to inhibit DHODH activity. In fact, the anti-HSV activity of MEDS433 was abrogated by the addition of exogenous uridine or of the product of DHODH, the orotate, thus confirming DHODH as the MEDS433 specific target in HSV-infected cells. A combination of MEDS433 with dipyridamole (DPY), an inhibitor of the pyrimidine salvage pathway, was then observed to be effective in inhibiting HSV replication even in the presence of exogenous uridine, thus mimicking *in vivo* conditions. Finally, when combined with acyclovir and DPY in checkerboard experiments, MEDS433 exhibited highly synergistic antiviral activity. Taken together, these findings suggest that MEDS433 is a promising candidate as either single agent or in combination regimens with existing direct-acting anti-HSV drugs to develop new strategies for treatment of HSV infections.

Keywords: Herpes simplex virus type 1 and type 2; Antiviral activity; *de novo* pyrimidine biosynthesis; Dihydroorotate dehydrogenase; Salvage pathway; Combination treatment.

1. Introduction

The Herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2) causes lifelong infections characterized by periodic reactivations at the site of primary infection, and globally widespread among human populations with a seroprevalence of 50% to 90% in adults. HSV-1 is traditionally associated with orofacial lesions and encephalitis, while HSV-2 is associated with genital diseases, although both oral HSV-2 infections and genital herpes caused by HSV-1 are recognized with increasing frequency. Following primary infection, HSV establishes latent infections in the neurons of the sensory ganglia from where they may, or may not, reactivate, causing recurrent infections (Roizman et al., 2013).

HSV infections are associated to a wide range of clinical manifestations that diversify from asymptomatic infection or mild mucocutaneous lesions on the lips, cornea, genitals, or skin, to more severe diseases, such as encephalitis and recurrent keratitis, and up to even life-threatening systemic disseminate infections in immunocompromised patients or neonates after perinatal transmission (Roizman et al., 2013, Whitley, 2015; Samies and James, 2020).

Even if HSV infections are often subclinical, their incidence and the severity of diseases have increased over the past decades due mainly to the increasing number of transplant recipients. Concurrently, genital herpes has become one of the world's most prevalent sexually transmitted infections with evidence suggesting that HSV-2 infection increases the risk of acquiring HIV (Looker et al., 2017). Together the worldwide burden of HSV diseases is significant, as in 2016 3,7 billion of HSV-1 infections and about half a billion of HSV-2 infections have been estimated by WHO (James et al., 2020).

Treatment of symptomatic primary or recurrent HSV infections with nucleoside analogues, such as acyclovir (ACV), famciclovir (FAM), and valacyclovir (VCV) that act as viral DNA polymerase inhibitors, ensure an effective therapeutic management of most HSV diseases. However, to date, none of these drugs can eliminate an established latent

infection, and their extensive clinical use may lead to treatment failures due to the development of drug resistance, and to adverse effects mainly deriving from long-term toxicity (Whitley and Baines, 2018).

Taking into consideration both these limitations and the absence of efficacious vaccines, the treatment of HSV infections remains a high priority and highlights the need for the development of new antiviral agents directed against new targets. To this regard, host-targeted small molecules able to modulate virus-host interactions may be considered a compelling alternative to the *de novo* development of virally targeted agents, for which the typical timeline for approval can require more than 10 years.

Pyrimidines availability in infected cells is crucial for virus replication and thus compounds targeting the *de novo* pyrimidine biosynthetic pathway have the potential to be validated as host-acting antiviral (HTA) agents (Okesli et al., 2017). Furthermore, in addition to the advantage of overcoming viral drug resistance, inhibitors of pyrimidine biosynthesis, given the independence of the antiviral effects with respect to a specific virus replication strategy, may be broadly deployed against viruses belonging to different families, thus developed as broad-spectrum antivirals (BSAs) (Okesli et al., 2017). In the *de novo* pyrimidine biosynthesis pathway, the dihydroorotate dehydrogenase (DHODH) catalyzes the rate-limiting step of dehydrogenation of dihydroorotate (DHO) to orotate (ORO), essentially providing uridine and cytidine to fulfill cellular nucleotides demand (Reis et al., 2017; Loeffler et al., 2020). Therefore, due to its critical role in the biosynthetic pathway, DHODH is currently considered as a host target of choice for anti-infective drug development (Okesli et al., 2017; Boschi et al., 2019).

Recently, we have identified a novel class of human DHODH (*h*DHODH) inhibitors characterized by an unusual carboxylic group bioisostere 2-hydroxypyrazolo[1,5□a] pyridine (Sainas et al., 2018) that had been designed on the scaffold of brequinar, one of the most potent hDHODH inhibitors (Peters, 2018). These new *h*DHODH inhibitors

showed little, if any, toxicity for human normal cells, while they induced myeloid differentiation in Acute Myeloid Leukemia (AML) cell lines through *h*DHODH inhibition, and therefore they are further being developed for treatment of AML (Sainas et al., 2018; Sainas et al., 2020).

The aim of this study was to investigate the potential of deploying this new class of *h*DHODH inhibitors against HSV-1 and HSV-2. We report on the ability of one of these small molecules, named MEDS433, to potently inhibit the *in vitro* replication of HSV-1 and HSV-2 via a mechanism that stems from selective blocking the DHODH enzymatic activity. These results indicate MEDS433 as a promising candidate as either single agent or as combination regimens, to develop new strategies for treatment of HSV infections.

2. Materials and Methods

2.1 Compounds

MEDS433 and the small library of *h*DHODH inhibitors (Table 1) based on a hydroxyazole scaffold, were synthesized as described previously (Sainas et al., 2018; Sainas et al., 2021). Acyclovir (ACV), uridine (UR), orotic acid (ORO), dihydroorotic acid (DHO), and dipyridamole (DPY) were purchased from Sigma-Aldrich. All compounds were resuspended in DMSO.

2.2 Cells and viruses

African green monkey kidney cells (Vero) (ATCC CCL-81) and the human glioblastoma astrocytoma U-373 MG cell line (ATCC HTB-17) were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Euroclone) supplemented with 10% fetal bovine serum (FBS, Euroclone), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (P/S, both from Euroclone). Clinical isolates of HSV-1 (AS1 and AS2) and HSV-2 (AS3 and AS4) sensitive to ACV were a generous gift from V. Ghisetti, Amedeo di Savoia Hospital, Turin,

Italy. They were propagated and titrated by plaque assay on Vero cells, as previously described (Luganini et al., 2011; Terlizzi et al., 2016)

2.3 Citotoxicity assay

Vero cells were seeded in 96-well plates (15000 cells/well) and after 24 h the cells were exposed to increasing concentrations of compounds or vehicle (DMSO), as control. After 72 h of incubation, the number of viable cells was determined using the CellTiter-Glo Luminescent assay (Promega) according to the specifications of the manufacturer.

2.4 Antiviral assays

To screen the mini-library of hDHODH inhibitors, plaque reduction assays (PRAs) were performed with Vero cells seeded in 24-well plate (50000 cells/well) and then treated with a single dose (0.5 μM) of the different compounds 1 h prior to and during infection with the clinical isolate of HSV-1 AS1 at 50 PFU/well. Following virus adsorption (2 h at 37 °C), viral inocula were removed, and cells were maintained in medium containing the corresponding compounds, 3% FBS, and 0.9% methylcellulose. After 48 h post-infection (p.i.) cell monolayers were fixed, stained with crystal violet, and viral plaques were microscopically counted; the mean plaque counts for each drug concentration were expressed as a percentage of the mean plaque counts of control virus (DMSO).

To evaluate the anti-HSV activity of selected compound, virus yield reduction assay (VRA) were performed on Vero cells, as previously described (Luganini et al., 2011; Terlizzi et al., 2016). For VRA, both untreated cells and those incubated with different concentrations of MEDS433 for 1 h before infection were infected with the clinical isolates HSV-1 and HSV-2 at a multiplicity of infection (MOI) of 0.01 or 1 PFU/cell. Following virus adsorption (2 h at 37°C), cultures were maintained in medium containing compounds and then incubated for 48 h p.i. until control cultures displayed extensive cytopathology. Thereafter, the cells and supernatants from the antiviral assay were harvested and disrupted by sonication. The extent of virus replication was then assessed by titrating the infectivity of supernatants of

cell suspensions on Vero cells. The mean plaque counts for compounds were expressed as a percentage of the mean plaque counts for the control virus (DMSO-treated), and the concentration that produced a 50% and 90% of reduction in plaque formation (EC_{50} and EC_{90}) was determined by GraphPad Prism software.

To evaluate the effect of uridine, DHO or ORO addition, PRAs were performed with Vero cells infected with HSV-1 AS1 or HSV-2 AS3 (50 PFU/well), and treated with increasing concentrations of uridine, ORO or DHO (10, or 100, or 1000 μM) in presence of 1 μM of MEDS433. After 48 h p.i., cell monolayers were fixed, stained with crystal violet, and plaques microscopically counted. To investigate the effect of blocking both the *de novo* biosynthesis and the salvage pathway of pyrimidines, Vero cells were infected with HSV-1 AS1 or HSV-2 AS3 (50 PFU/well) and treated throughout PRAs with different concentration of MEDS433 in combination with increasing concentrations of dipyridamole (0.5, 1.5 and 3 μM) in media supplemented with concentrations of uridine (20 μM) that exceed the physiological uridine plasma levels ranging from 2 to 6 μM (Pizzorno, et al. 2002*)*.

At 48 h p.i., cell monolayers were fixed, stained and plaques microscopically counted.

To assess the effects of the combination of MEDS433 and ACV or MEDS433, ACV and DPY on HSV-1 AS1 replication, PRAs were performed as described above. MEDS433 and ACV, as single agents or in combination, were added on Vero cells at equipotent ratio of 0.25 x, 0.5 x, 1 x, 2 x and 4 x the EC_{50} of each drug. For the three-drug combination, DPY (3 μM) was added to the MEDS433 and ACV concentrations used in the two-drug combination. The effect of the two- or three-drug combinations was then assessed using the Chou-Talalay method (Chou, 2006) based on the median-effect principle of the mass-action law computed in the CompuSyn software (http://www.combosyn.com)(Chou and Martin, 2005). With the Chou-Talalay method, a Combination Index (CI) = 1 represents an additive effect, a CI value > 1 means antagonism and a CI value < 1 indicates synergism.

2.5 Quantitative real-time PCR

Vero cells were seeded in a 6-well plate (600000 cells/well) and, after 24 h, treated with 5 μM MEDS433 1h prior to and during infection with the clinical isolate of HSV-1 AS1 at a multiplicity of infection (MOI) of 0.1 PFU/cell. At different times p.i., cells were harvested and DNA was extracted and purified using a DNA purification kit (Zymo Research). The levels of viral DNA were then evaluated by quantitative real-time PCR using a GENESIG standard kit (Primerdesign) to amplify a segment of the DNA polymerase UL30 gene. Briefly, 25 ng of DNA from each sample was amplified in triplicate using PrecisionPlus Low ROX qPCR master mix (Primerdesign), the oligonucleotide primers and the TaqMan UL30 probe, dually labeled (5′, fluorescein 6-carboxyfluorescein [FAM]; 3′, Iowa Black® dark quencher). After activation of Hot Start Taq DNA polymerase for 2 min at 95 °C, samples underwent 45 cycles of 10 s at 95 °C and 1 min at 60 °C in a QuantStudio3 real-time PCR (Applied Biosystem). For HSV genome copy number determination, a serially diluted UL30-encoding plasmid (GENESIG standard kit) was used to determine a standard curve for mean Ct values and to calculate HSV genome copy numbers.

2.6 Immunoblotting

Vero cells were seeded in 6-well plates (600000 cells/well) and, after 24 h, were treated with 5 μM of MEDS433 1 h prior to and during infection with HSV-1 AS1 at a MOI of 1 PFU/cell. Whole-cell extracts were prepared at different times p.i., as previously described (Luganini et al., 2008, Mercorelli et al., 2016). An equal amount of the cell extracts was fractionated by 8% SDS-PAGE and then transferred to PVDF membranes (BioRad). Filters were blocked for 2 h at 37 °C in 5% non-fat dry milk in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% Tween 20 and immunostained with the mouse anti-HSV-1 ICP27 mAb (clone H1113; Virusys) (diluted 1:500), anti-HSV-1 ICP5 mAb (clone 3B6; Virusys) (diluted 1:500), or with anti-tubulin mAb (Chemicon International) (diluted 1:2000) as a control for protein loading. Immunocomplexes were detected with a goat anti-mouse Ig Ab

conjugated to horseradish peroxidase (Life Technologies) and visualized by enhanced chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz).

2.8 Statistical Analysis

All statistical tests were performed using GraphPad Prism version 7.0. Antiviral assays data are presented as the means ± SDs of at least three experiments performed in triplicate. Differences were considered to be statistically significant for $p<0.05$.

3. Results

3.1 Inhibition of HSV-1 and HSV-2 replication by the DHODH inhibitor MEDS433

To generate new *h*DHODH inhibitors, we have recently applied a rational modulation to brequinar, one of the most potent *h*DHODH inhibitors so far discovered (Peters et al., 2018). A combination of structure-based and bioisosterism (Sainas et al, 2019) approaches resulted in a series of new 2-hydroxypyrazolo[1,5-a]pyridine derivatives of brequinar (Sainas et al., 2018; Sainas et al., 2021). Here, to investigate the feasibility of targeting DHODH activity to develop HTA aimed at inhibiting HSV replication, 9 of these new *h*DHODH inhibitors (Table 1) were selected using plaque reduction assays in which test compounds were present before, during and after virus infection. As shown in Fig. 1A, when tested at 0.5 μM, five compounds (**1**, **4**, **5**, **7**, and **MEDS433**) were able to decrease HSV-1 AS1 replication of more than 50%, while the remaining four compounds (**2**, **3**, **6** and **8**), reduced the virus replication of about 20-30%. Since MEDS433 was the most effective hDHODH inhibitor against HSV-1 replication (Fig. 1), it was selected for further analysis.

Virus yield reduction assays (VRAs) then confirmed a significant concentration-dependent inhibition of virus replication in Vero cells treated with MEDS433, and infected with the clinical isolates HSV-1 AS1 or HSV-2 AS3 at a multiplicity of infection (MOI) of 0.01 or 1 (Fig. 2). The EC_{50} and EC_{90} values against HSV-1 AS1 and HSV-2 AS3 (Table 2) also

indicated that the inhibitory effect of MEDS433 cannot be overcome by infecting cells at higher multiplicities of infection.

To further strengthen these observations, a second pair of clinical isolates of HSV-1 and HSV-2, respectively HSV-1 AS2 and HSV-2 AS4, was examined by VRAs. As reported in Table 2, MEDS433 was effective also against HSV-1 AS2 and HSV-2 AS4, thus indicating that the anti-HSV activity of MEDS433 was independent from the viral strain used, and sustaining the view that indeed it targeted a host pathway in HSV-infected cells. Nevertheless, the antiviral activity of MEDS433 was not due to cytotoxicity of the target cells themselves, since the Cytotoxic Concentration (CC_{50}) determined in uninfected Vero cells was 234 ± 18.2 μM, with a favorable Selective Index (SI) on average greater than 2,100 and 3,000 for HSV-1 and HSV-2, respectively.

Noteworthy, MEDS433 showed an anti-HSV activity (Table 2) at least comparable to that of the reference drug ACV (EC_{50} 0.280 ± 0.080 and 0.120 ± 0.042 μM against HSV-1 AS1 and HSV-2 AS3, respectively), while it was more effective than the reference *h*DHODH inhibitor brequinar (EC_{50} 0.420 ± 0.124 μM against HSV-1 AS1 and 1.250 ± 0.276 μM against HSV-2 AS3, respectively).

Furthermore, in addition to being MOI-independent, the anti-HSV activity of MEDS433 was also cell-type independent, as it was observed in the human glioblastoma astrocytoma U-373 MG cell line permissive for HSV replication (Andrei et al., 1994). In these cells of neural origin, MEDS433 in fact inhibited HSV-1 AS1 replication with EC_{50} and EC_{90} values of 0.297 ± 0.080 and 1.881 ± 0.487 μM, respectively.

As a whole, these results indicate a potent anti-HSV activity of MEDS433 independent of the virus strain, the MOI, or the cell type used.

3.2 MEDS433 affects HSV DNA replication and Late gene expression

To get more insights into the nature of the antiviral activity of MEDS433, we investigated its effects on HSV-1 DNA synthesis at different times p.i.. As reported in Fig. 3, in untreated HSV-1-infected Vero cells, the qPCR analysis measured a progressive increase of viral DNA levels up to 48 h p.i.. Conversely, the MEDS433 treatment determined an impairment of HSV-1 DNA synthesis starting from 16 h p.i. and for the entire time frame that was evaluated, thus suggesting that it can target the HSV replication cycle at a stage prior to the onset of viral DNA replication.

To further supporting this hypothesis, total protein extracts were prepared at various times p.i. from HSV-1-infected Vero cells treated with MEDS433 and the content of ICP27 and ICP5 proteins was evaluated to monitor the levels of representative immediate-early (IE) and late (L) HSV protein expression, respectively. As seen in Fig. 4, compared to cells treated with the vehicle DMSO, MEDS433 treatment did not affect noticeably the expression of ICP27 protein accumulation at any time analyzed. In contrast, it determined a clear reduction of the late ICP5 protein levels at 12 and 24 h p.i..

Together, these results suggest that MEDS433 targets HSV replication cycle after the expression of IE proteins and likely interferes with the viral DNA synthesis, since the MEDS433-mediated inhibition at this stage (Fig. 3) reduces late gene expression (Fig. 4).

3.3 The pyrimidine biosynthesis pathway is involved in the anti-HSV activity of MEDS433

The above results suggest a mechanism of the anti-HSV activity of MEDS433 consistent with its ability to inhibit DHODH activity. To verify this hypothesis, we investigated whether the antiviral HSV activity of MEDS433 could be overcome by the addition of increasing concentrations of exogenous uridine. As shown in Fig. 5, the inhibitory effect of 1 μM MEDS443 on both HSV-1 (Fig. 5A) and HSV-2 (Fig. 5B) replication was significantly reversed by a 10-fold excess of uridine relative to MEDS433 concentration and further decreased by greater uridine concentrations that completely

restored HSV replication, thus indicating that the pyrimidine pathway was affected by MEDS433. Then, to confirm that DHODH inhibition by MEDS433 was responsible of its anti-HSV effect, cell medium was supplemented with increasing concentrations of the substrate dihydroorotic acid (DHO) or the DHODH product, orotic acid (ORO). In HSV-infected Vero cell cultures treated with MEDS433 (1 μM), the addition of orotic acid reversed significantly the inhibitory effect of MEDS433 on HSV-1 and HSV-2 replication already at a concentration 10-fold that of MEDS433 (Fig. 5, lower panels). However, the reverting effect of orotic acid to MEDS433 was almost complete at the highest concentration (1000 x the MEDS433 concentration) evaluated. In contrast, the addition of DHO even at 1 mM (1000 times more than MED433) did not affect the anti-HSV activity of MEDS433 (Fig.5, lower panels), thus indicating that MEDS433 inhibits a step in the *de novo* pyrimidine biosynthesis pathway downstream from DHO.

Together, these results confirm that MEDS433 selectively targets DHODH in HSV-infected cells and that the inhibition of DHODH is in charge of the overall anti-HSV activity of MEDS433.

3.4 The combination of MEDS433 with an inhibitor of the nucleoside salvage pathway enhances the antiviral activity

Despite useful in confirming the molecular target of MEDS433, the observation that uridine reversed the anti-HSV activity of MEDS433 may lead to hypothesize that, in the presence of physiological concentration of uridine as that occurring i*n vivo*, the pyrimidine salvage pathway may reduce the antiviral efficacy of a DHODH inhibitor by importing extracellular nucleosides. To tackle this problem, we explored the effects of a combination treatment of MEDS433 with an inhibitor of the nucleoside transport, such as dipyridamole (DPY). To this end, a checkerboard analysis of MEDS433-DPY combination was carried by plaque reduction assay in HSV-1-infected Vero cells in culture medium supplemented

even with 20 μM uridine to exceed physiological plasma conditions (Pizzorno, et al. 2002). As depicted in Fig. 6A, the presence of 20 μM exogenous uridine abrogated, as expected, the inhibitory activity of MEDS433 at concentrations reducing HSV replication by 65 - 90% when it was tested as single agent (Fig. 2). In contrast, when the same MEDS433 concentrations were examined in the presence of increasing amounts of dipyridamole, the combination of the two molecules successfully reduced HSV-1 replication despite the presence of exogenous uridine (Fig. 6A). At the used concentrations, DPY by itself did not exerted an inhibitory activity on HSV replication (data not shown), in accord to previous observations (Snoeck et al., 1994). Furthermore, the DPY-mediated enhancement of MEDS433 antiviral activity was not due to an aspecific cytotoxic effect, since none of the tested combinations affected Vero cell viability (Fig. 6B).

Taken together, these results sustain the feasibility of a combination of a DHODH inhibitor and a pyrimidine salvage inhibitor to determine an anti-HSV activity even in the presence of uridine concentrations that even exceed *in vivo* host conditions.

3.5 The combination of MEDS433, acyclovir and dipyridamole results in a synergistic effect on HSV-1 replication

Lastly, we investigated whether the antiviral activity of MEDS433 and the reference drug acyclovir would result in a synergistic, additive or antagonistic effect when tested in combination. To this end, the combined effects of 0.25-, 0.5-, 1-, 2-, or 4-fold of MEDS433 EC_{50} to ACV EC_{50} ratio were assessed by plaque reduction assays and then analyzed using the CompuSyn software. Results of MEDS433-ACV combinations are represented in Fig. 7A as a fractional effect analysis (Fa) plot in relation to the concentrations of drugs used, where Fa values closer to 1 indicate greater antiviral activity. The anti-HSV efficacy of ACV was clearly increased by the combination with MED433 as shown by the Fa values (blue triangles) closer to 1 than those of MEDS433 and ACV when used as single agent

(Fig. 7A). The EC_{50} of ACV (0.330 μM) was in fact decreased to 0.128 μM by the combination with MEDS433 (Fig. 7A). The calculated Combination Index (CI) values then indicated that combination of MEDS433 with ACV resulted in a synergistic effect at any of drug combinations tested, since all the CIs were < 0.9 (Table 3) (Chou, 2006).

Then, to evaluate the efficacy of the MEDS433-ACV combination in the context of the inhibition of the pyrimidine salvage pathway, as that determined by DPY, the anti-HSV activity of the combination of the different MEDS433 EC_{50} to ACV EC_{50} relative rates was measured in the presence of 3 μM DPY. As shown in Fig. 7A and Table 3, the antiviral strength of the MEDS433-ACV combination was further increased by the addition DPY, since Fa values of the triple combination (orange hexagons) were even more close to 1 than those of the MEDS433-ACV combination, and the EC_{50} of ACV further reduced to 0.089 μM. Furthermore, none of the MEDS433-ACV or MEDS433-ACV-DPY combinations exhibited significant cytotoxic effects against Vero cells (Fig. 7B), thus indicating that their synergistic anti-HSV activity was not the result of an increased cytotoxicity, which would have prevented the virus from replicating, but that it derived from the combined interference of different targets and mechanisms of action.

These results suggest that both combinations of MEDS433 and ACV and MEDS433, ACV and DPY might represent a new pharmacological strategy to be considered for the management of HSV infections.

4. Discussion

This study shows that the small molecule MEDS433 exerts a potent dose-dependent antiviral activity against clinical isolates of HSV-1 and HSV-2 with a mechanism that stems from the inhibition of the *de novo* synthesis of pyrimidines.

In a previous study, MEDS433 was shown to inhibit *h*DHODH activity with a potency similar to brequinar, while exerting a lower cell cytotoxicity and the ability to restores

myeloid differentiation in AML cell lines at concentrations one log digit lower than those of brequinar (Sainas et al., 2018). Crystallographic studies of MEDS433 in complex with *h*DHODH then showed that it has a binding mode similar to that of brequinar, inside the ubiquinone binding site of the enzyme (Sainas et al., 2018).

Here, we have observed that even when tested for anti-HSV activity, MEDS433 results more active than brequinar and all other derivatives examined (Fig. 1). Among the tested *h*DHODH inhibitors, MEDS433 is in fact characterized by the highest inhibitory potency against hDHODH (IC_{50} 1.2 nM) and a favorable lipophilicity with a $logD^{7.4}$ value of 2.35 (Table 1). To this regard, a $logD^{7.4}$ value of 2.50 has been suggested for optimal inhibition of mitochondrial DHODH, since inhibitors with lower $logD^{7.4}$ are disadvantaged in reaching the mitochondrial membrane, while higher $logD^{7.4}$ values may reduce their cellular adsorption (Gradl et al., 2019). It is therefore reasonable that MEDS433 was more active as anti-HSV agent than either the most polar (low logD values) brequinar, **2**, **6,** and **8** compounds, or the most lipophilic **5** and **7** derivatives ($logD^{7.4}$ 3.27 and 3.28, respectively).

Under normal physiological conditions, cells fulfill their requirement of pyrimidines mainly through the salvage pathways, therefore recycling pre-existing nucleosides from both degradation of intracellular nucleic acids and uptake of extracellular nucleosides. In contrast, in highly metabolically active virus-infected cells, the exceptional need for large pyrimidines pools associated to rapid viral replication cannot be supply sufficiently by nucleotide recycling. To keep up with the pyrimidine demand in infected cells, in fact, herpesviruses activate both the *de novo* pyrimidine biosynthetic gene expression and the metabolic flux to UTP (Gribaudo et al., 2002; Munger et al., 2008). It is therefore reasonable that *de novo* pyrimidine biosynthesis rather than its salvage pathway is more critical for herpesvirus replication, effectively making the rate-limiting DHODH enzyme activity crucial for maintaining high levels of viral DNA synthesis.

The observation that HSV replication was largely restricted when the DHODH activity was inhibited therefore confirms the dependence of HSV on the host *de novo* pyrimidine biosynthesis for efficient replication, albeit, given the premises summarized above, this finding is not unexpected. In addition to this, with regard to herpesviruses infections, it has already been observed that a DHODH inhibitor can potently block the replication of the Human Cytomegalovirus (HCMV) with an *in vitro* EC_{50} of 0.78 μM (Marschall et al., 2013). However, the present study is the first to report the antiviral efficacy of a low cell toxicity *h*DHODH inhibitor against HSV-1 and HSV-2 with a potency at least comparable to that of the reference drug acyclovir, thus suggesting that inhibition of DHODH activity by MEDS433 results in the reduction in the production of pyrimidines at an extent that restricts HSV replication but not cell growth.

However, it is worth noting that our study adds two new pieces of knowledge to the field of HTAs targeting the replenishment of nucleotides in virus-infected cells, that may be of interest to be considered to design new strategies for the management of HSV infections. The first consists in the observation of the efficacy of a combined treatment between an inhibitor of the *de novo* pyrimidine biosynthesis, MEDS433, and dipyridamole, a broad nucleoside transporter inhibitor that blocks the nucleoside salvage from extracellular environment (Fitzgerald, 1987). In fact, when DPY was combined with concentrations of MEDS433 no longer effective against HSV due to the presence of extracellular uridine which mimics the physiological conditions in the infected host, it restored the antiviral activity of the *h*DHODH inhibitor. This observation is relevant as there is experimental evidence of the absence of inhibitory effects of pyrimidine biosynthesis inhibitors on the growth of some RNA viruses, in both rodents and non-human primate models despite their high efficacy *in vitro*, and likely due to the systemic pyrimidine salvage pathway that by importing extracellular nucleosides into virus-infected cells, may compensate DHODH inhibition (Wang et al., 2011; Smee et al., 2012; Grandin et al., 2016).

While the combination of DPY with inhibitors of *de novo* pyrimidine biosynthesis has been investigated previously in both *in vitro* studies and clinical trials to increase the anticancer effects of the latter, the potential of this combination against virus infections has not yet been thoroughly investigated. To this regard, it has been reported very recently the efficacy of a combination of a *h*DHODH inhibitor, GSK983, with a pyrimidine salvage inhibitor such as the cyclopentenyl uracil (CPU) in suppressing the *in vitro* replication of dengue virus, even in the presence of physiological concentrations of uridine (Liu et al., 2020). CPU is a uridine analogue able to block the activity of uridine/cytidine kinase 2 (Cysyk et., 1995), a cellular enzyme that phosphorylates uridine to UMP in the pyrimidine ribonucleotide salvage pathway, and therefore acting at a stage downstream from that targeted by DPY (Fitzgerald, 1987). DPY in fact is a pyrimido-pyrimidine derivative widely used as an oral agent in the prophylaxis of thromboembolism in cardiovascular disease, because of its platelet antiaggregant and vasodilator activities due to the inhibition of the uptake of adenosine into platelets, endothelial cells and erythrocytes (Fitzgerald, 1987; Schaper, 2005). These cellular effects have been observed even after a single DPY dose at which the measured C_{max} was of 2.2 μg/ml, corresponding to 4.4 μM (Gregov et al., 1987). This value is greater than the highest DPY concentration observed to be effective in combination with either MEDS433 (Fig. 6A) or MEDS433 and ACV (Fig. 7A), that therefore it could be clinically achievable in patients treated with DPY. The wide clinical experience of DPY could thus allow its rapid repositioning against viruses to be clinically useful in combination with HTAs and/or direct acting antivirals.

The second finding which deserves a further comment is the observation that several combinations of MEDS433 and ACV interact in a synergistic manner, each reinforcing the other's antiviral activity against HSV-1. To our knowledge, this is the first observation of a synergistic effect between a direct acting antiviral agent used to treat infections caused by DNA viruses and a DHODH inhibitor. While, in the case of RNA viruses, as the arenavirus

Junín virus that causes the Argentine hemorrhagic fever, it has been reported that the combination of A771726, the active metabolite of the *h*DHODH inhibitor leflunomide approved by FDA for treatment of rheumatoid arthritis and autoimmune disorders (Sanders and Harisdangkul, 2002), and ribavirin showed a significantly more potent antiviral activity than each single drug treatment (Sepulveda et al., 2018).

Relevant to the possibility to exploit combinations between direct-acting antivirals and agents targeting the pyrimidine pathway against HSV infections, it is possible to have in view a regimen based on three different molecules: ACV, a DHODH inhibitor, and DPY. As we have observed in this study, indeed DPY not only restores the antiviral efficacy of the DHODH inhibitor that could be abolished by the uptake of exogenous pyrimidines from the bloodstream (Fig. 6A), but it also strengthens the synergism between ACV and MEDS433 against HSV replication (fig. 7A). Obviously, one could argue that the addition of DPY to an ACV-DHODH combination could reduce the synergistic effect *in vivo* due to the inhibition of nucleoside transport and thus impairing the uptake of ACV from bloodstream. However, this would not seem to be the case, as it has been observed that DPY does not compromise the transport of ACV into cells (Mahony et al., 1988), as well as that of others nucleoside analogs, such as AZT and ddC (Szebeni et al., 1989). Therefore, our findings along with the reported ability of DPY to inhibit the intracellular transport of thymidine and dCTP which could compete with ACV for viral kinase-mediated phosphorylation, suggest that DPY actually may potentiate the antiviral efficacy of ACV. Although future investigations in animal models of HSV infection will be required to validate the *in vitro* observations, our results suggest that combinations of ACV and MEDS433 or ACV, MEDS433 and DPY might represent a new therapeutic strategy for HSV infections.

In conclusion, the results of this study suggest MEDS433 as an attractive candidate as a novel HTA that might show advantageous features, such as an antiviral activity against a broad range of viruses since, in addition to HSV, we have observed MEDS433 effective

against respiratory viruses, such as Influenza virus, Respiratory Syncytial Virus, and SARS-CoV-2 (unpublished results), and the low risk of emergence of drug-resistant strains.

Moreover, MEDS433 could also be considered for combinatorial drug treatment with nucleoside analogues and other anti-pyrimidines, such as ACV and DPY, for the therapeutic management of HSV infection. In light of the fact that no new anti-HSV agents have been introduced in nearly three decades, the potent *in vitro* anti-HSV activity of MEDS433 calls for further studies to be performed to evaluate their efficacy and safety in animal models, in order to validate its development as novel agent for the treatment of HSV infection.

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Figure Legends

Figure 1. Identification of *h*DHODH inhibitors with anti-HSV activity. Vero cells were pretreated and treated with 0.5 µM of the different DHODH inhibitors (Table 1) 1 h prior to, during infection with HSV-1 AS1 (50 PFU/well), and throughout the experiment. At 48 h post-infection, viral plaques were stained and the mean plaque number in treated culture determined compared to that of DMSO-treated and mock-infected control monolayers. The data shown represent means ± SD (error bars) of three independent experiments performed in triplicate.

Figure 2. Antiviral activity of MEDS433 against HSV-1 and HSV-2. Vero cell monolayers were infected with the clinical isolates of HSV-1 AS1 or HSV-2 AS3 at MOIs or 0.01 or 1, and, where indicated, the cells were treated with increasing concentrations of MEDS433 1 h before as well as during virus adsorption. MEDS433 remained in the culture medium throughout the experiment, until an extensive viral cytopathic effect was observed in the untreated controls. HSV replication was then quantified by titrating the infectivity of

supernatants of Vero suspensions by standard plaque assay. The number of plaques was plotted as a function of MEDS433 and ACV concentrations, and the concentrations producing 50% and 90% reductions in plaque formation (EC_{50} and EC_{90}, respectively) were determined. The data shown represent means ± SD (error bars) of three independent experiments performed in triplicate.

Figure 3. MEDS433 inhibits HSV-1 viral DNA synthesis. Vero cell monolayers were infected with the clinical isolate of HSV-1 AS1 at an MOI of 0.1, and, where indicated, the cells were treated with 5 μM MEDS433, or 0.02% DMSO as a control. At 4, 8, 16, 24 and 48 h p.i., total DNA was purified and qPCR was performed with appropriate HSV primers. HSV-1 genomic copies were determined by interpolation from the standard curve, generated by a serially diluted UL30 plasmid and normalized to the number of cells. The data shown are the means ± SD of two independent experiments performed in triplicate and analyzed by unpaired t-test. ** ($p<0.0001$) versus calibrator sample (DMSO).

Figure 4. MEDS433 reduces the expression of HSV L proteins. Vero cells were infected with HSV-1 AS1 at an MOI of 1, or mock infected (mock) and, where indicated, the cells were treated prior to and during infection with 5 μM MEDS433, or 0.02% DMSO as a control. Total cell extracts were prepared at the indicated times p.i., fractionated by 8% SDS-PAGE and analyzed by immunoblotting with anti-HSV ICP27 and anti-HSV ICP5 mAbs. Tubulin immunodetection was used as internal control.

Figure 5. Inhibition of HSV replication by MEDS433 is reversed by uridine and orotic acid. Vero cell monolayers were treated with 1 μM of MEDS433 in the presence or absence of increasing concentrations of uridine (upper panels), dihydroorotic acid or orotic acid (lower panels) before and during infection with HSV-1 AS1 (**A**) or HSV-2 AS3 (**B**) (50 PFU/well). Following virus adsorption, cells were incubated in the presence of compounds and viral plaques were then stained and were microscopically counted at 48 h p.i.. The mean plaque counts for each drug concentration were expressed as a percent of the

mean count of the control cultures treated with the DMSO vehicle. The data shown represent means ± SD (error bars) of three independent experiments performed in triplicate and analyzed by a one-way ANOVA followed by Dunnett's multiple comparison test. ** ($p < 0.0001$) and * ($p < 0.05$) compared to the calibrator sample (MEDS433 alone).

Figure 6. A combination of modulators of pyrimidine metabolism inhibits HSV replication in the presence of exogenous uridine. A) Vero cell monolayers were treated with MEDS433 alone or in combination with different concentrations of DPY before and during HSV-1 AS1 infection (50 PFU/well). Following virus adsorption, cell monolayers were overlaid with 0.9% methylcellulose in the presence of compounds and viral plaques were then stained and counted at 48 h p.i.. In all the assays, culture medium was supplemented with 20 μM uridine. The data shown represent means ± SD (error bars) of three independent experiments performed in triplicate.

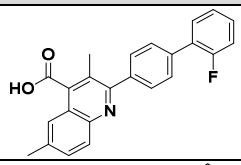
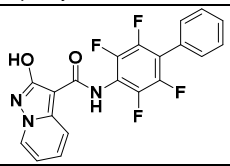
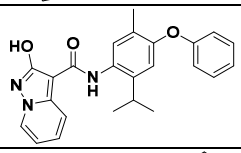
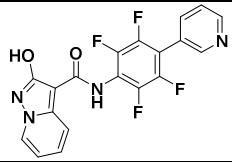
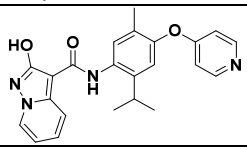
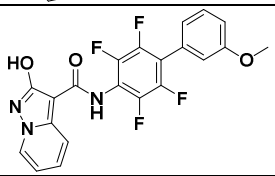
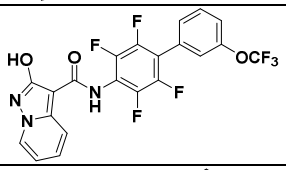
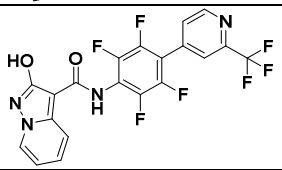
B) To determine cell viability, Vero cells were exposed to the different concentrations of MEDS433 alone or in combination with different amounts of DPY, or vehicle (DMSO), as control. After 72 h of incubation, the number of viable cells was determined by the CellTiter-Glo Luminescent assay. Results are shown as means ± SD (error bars) of three independent experiments performed in triplicate

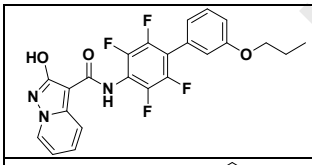
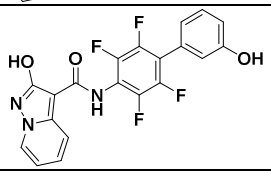
Figure 7. Effects of the combination of MEDS433, acyclovir and dipyridamole on HSV replication. A) Plaque reduction assays were performed on Vero cell monolayers treated with different concentrations of MEDS433 and ACV as single agents, or in combination in the absence or presence of 3 μM of DPY prior to and during HSV-1 AS1 infection (50 PFU/well). After virus adsorption, cells were incubated in the presence of compounds and at 48 h p.i., viral plaques were stained and counted. Plaque numbers were analyzed with the CompuSyn software. The effects on HSV-1 replication of MEDS433 or ACV used as single agents are depicted by red and black Fa curve, respectively. The effect of MEDS433-ACV or MEDS433-ACV-DPY combinations are

indicated by blue triangles and orange hexagons, respectively. The results are representative of three independent experiments performed in triplicate.

B) To determine the cytotoxic effect of combinations, Vero cell monolayers were treated with the vehicle (DMSO), or with different concentrations of MEDS433 alone or in combination with different concentrations of ACV and in the absence or presence of 3 μM of DPY. At 72 h, Vero cell viability was assessed by the CellTiter-Glo Luminescent assay. Results are shown as means ± SD (error bars) of two independent experiments performed in triplicate.

Table 1. The *h*DHODH inhibitors investigated as anti-HSV agents in the study

Structure	Compound Code	*h*DHODH[a] IC_{50} ± SE (nM)	Log $D^{7.4}$ ± SDc	Reference
	**Brequinar**	1.8 ± 0.3	1.83 ± 0.02	(Peters, 2018)
	**MEDS433**	1.2 ± 0.2	2.35 ± 0.02	(Sainas et al., 2018)
	**1** (MEDS548)	10.5 ± 0.16	3.18 ±0.09	(unpublished results)
	**2** (MEDS604)	6.23 ± 0.63	0.98 ± 0.03	(Sainas et al., 2021)
	**3** (MEDS605)	30 ± 2.8	2.51 ± 0.07	(unpublished results)
	**4** (MEDS606)	2.75 ± 0.31	2.46 ± 0.04	(Sainas et al., 2021)
	**5** (MEDS608)	2.30 ± 0.33	3.27 ± 0.19	(Sainas et al., 2021)
	**6** (MEDS610**)**	150 ± 15	1.84 ± 0.06	(Sainas et al., 2021)

MOI of 0.01				
HSV	**EC_{50} (μM)[a]**	**EC_{90} (μM)[b]**	**CC_{50} (μM)[c]**	**SI[d]**
	**7** (MEDS613)	4.09 ± 0.62	3.28 ± 0.12	(Sainas et al., 2021)
	**8** (MEDS614**)**	2.78 ± 0.32	1.82 ± 0.09	(Sainas et al., 2021)

[a]The inhibitory effect of the compounds (expressed as IC_{50}) on hDHODH *in vitro* assay.

Table 2. Antiviral activity of MEDS433 against different HSV strains

HSV-1 AS1	0.085 ± 0.021	0.765 ± 0.167	234 ± 18.2	2752
HSV-1 AS2	0.116 ± 0.014	0.678 ± 0.127	234 ± 18.2	2017
HSV-2 AS3	0.061 ± 0.019	0.840 ± 0.178	234 ± 18.2	3836
HSV-2 AS4	0.095 ± 0.027	0.778 ± 0.168	234 ± 18.2	2463

MOI of 1

HSV	EC_{50} (μM)[a]	EC_{90} (μM)[b]	CC_{50} (μM)[c]	SI[d]
HSV-1 AS1	0.078 ± 0.024	0.702 ± 0.111	234 ± 18.2	3000
HSV-2 AS3	0.074 ± 0.023	0.731 ± 0.157	234 ± 18.2	3162

[a]EC_{50}, compound concentration that inhibits 50% of virus replication, as determined by VRAs against HSV-1 and HSV-2 in Vero cells. Reported values represent the means ± SD of data derived from three experiments in triplicate.

[b]EC_{90}, compound concentration that inhibits 90% of virus replication, as determined by VRAs

[c]CC_{50}, compound concentration that produces 50% of cytotoxicity, as determined by cell viability assays in Vero cells. Reported values represent the means ± SD of data derived from three experiments in triplicate.

[d]SI, selectivity index (determined as the ratio between CC_{50} and EC_{50}

Table 3. Analysis of the effects of the combination of MEDS433, ACV and DPY against HSV-1 replication

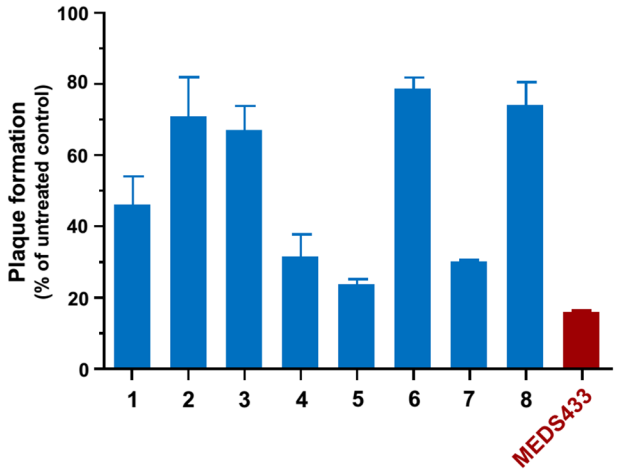
MEDS433/ACV combination at equipotent ratio (fold of EC_{50} [a])	MEDS433/ACV CI[b]	Drug combination effect of MEDS433/ACV[c]	MEDS433/ACV + 3 µM DPY CI[d]	Drug combination effect of MEDS433/ACV + 3 µM DPY[c]
4x	0.156 ±0.051	**Strong synergism**	0.156 ±0.096	**Strong synergism**
2x	0.082 ± 0.001	**Very strong synergism**	0.078 ± 0.033	**Very strong synergism**
1x	0.334 ± 0.039	**Synergism**	0.162 ± 0.043	**Strong synergism**
0.5x	0.419 ± 0.042	**Synergism**	0.299 ± 0.017	**Strong synergism**
0.25x	0.666 ± 0.152	**Moderate synergism**	0.306 ± 0.007	**Strong synergism**

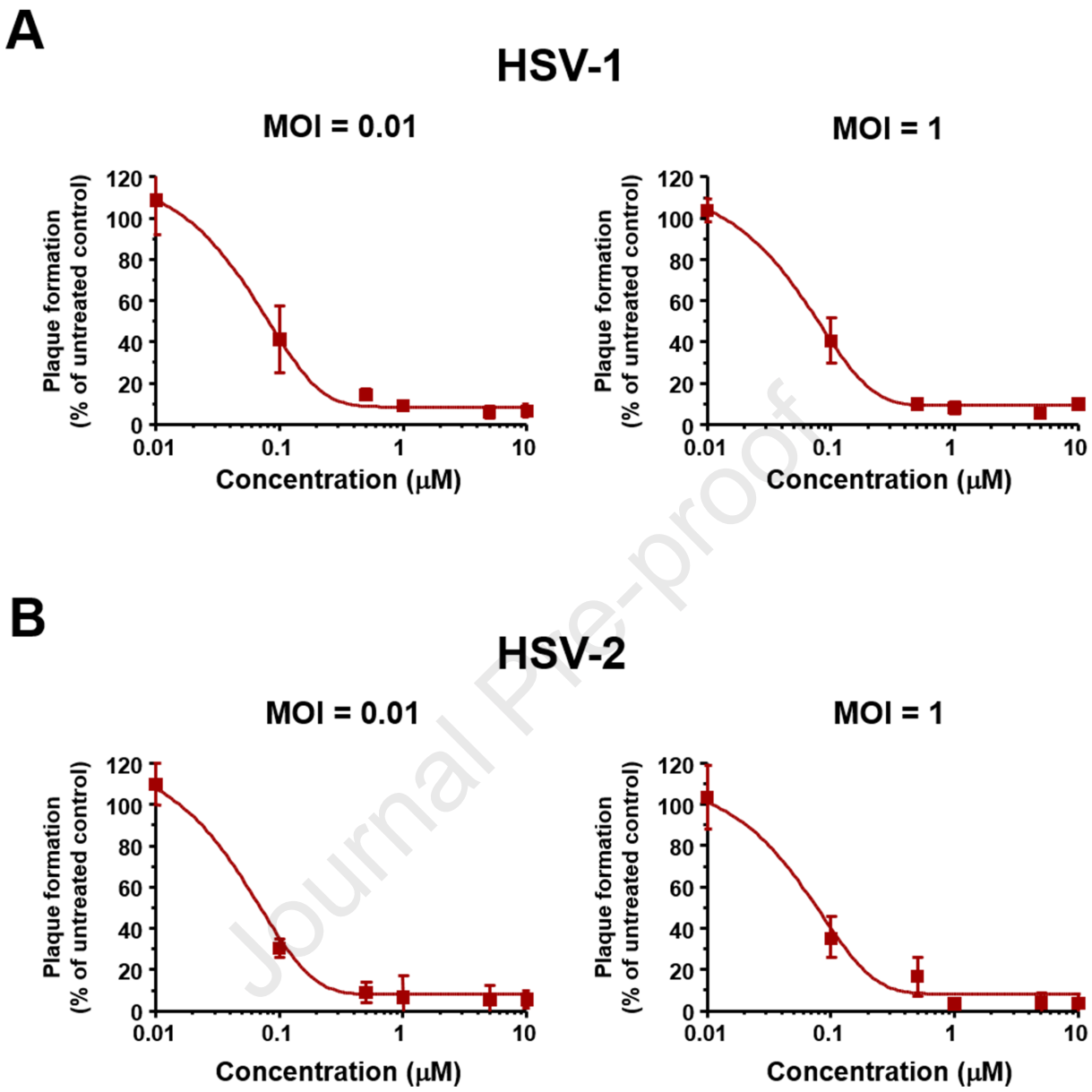
[a]Fold of EC_{50} MED/ EC_{50} ACV yielding an equipotent concentration ratio (approximately 1:0.31) between the two combined drugs. The EC_{50} values was determined by PRAs against HSV-1, as described in Material and Method. The ratio of the drugs was considered 1:0.31 from Fig.2.

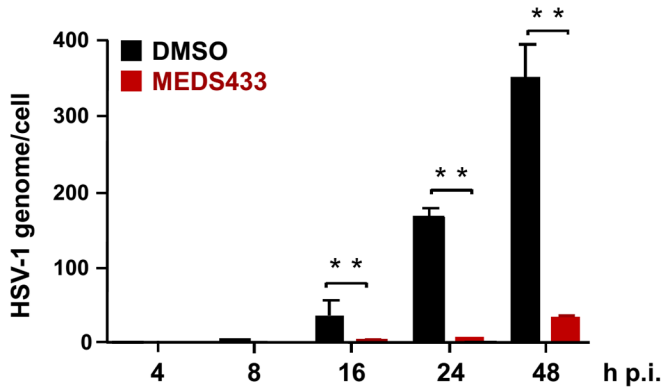
[b] Combination Index (CI), extrapolated by computational analysis with the CompuSyn software. Reported values represent means ± SD of data derived from n = 3 independent experiments in triplicate using MED/ACV concentration ratio showed in [a].

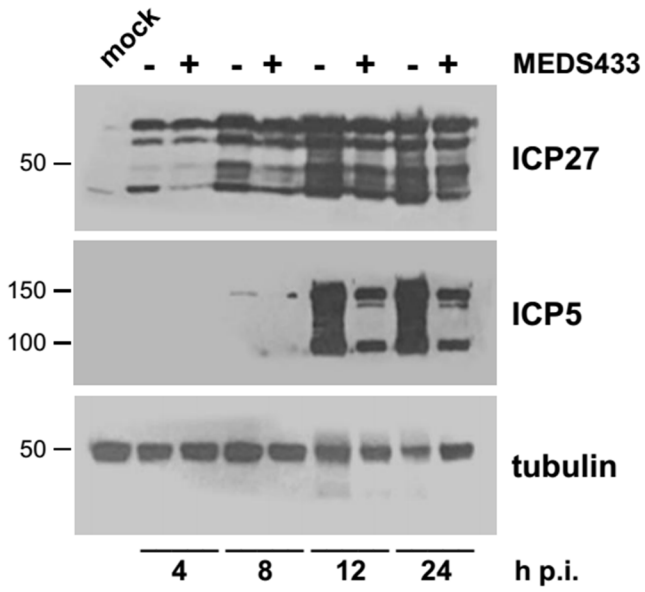
[c] Drug combination effect defined as: very strong synergism for CI<0.1; strong synergism for 0.1<CI<0.3; synergism for 0.3<CI<0.7; moderate synergism for 0.7<CI<0.85, according to Chou, 2006.

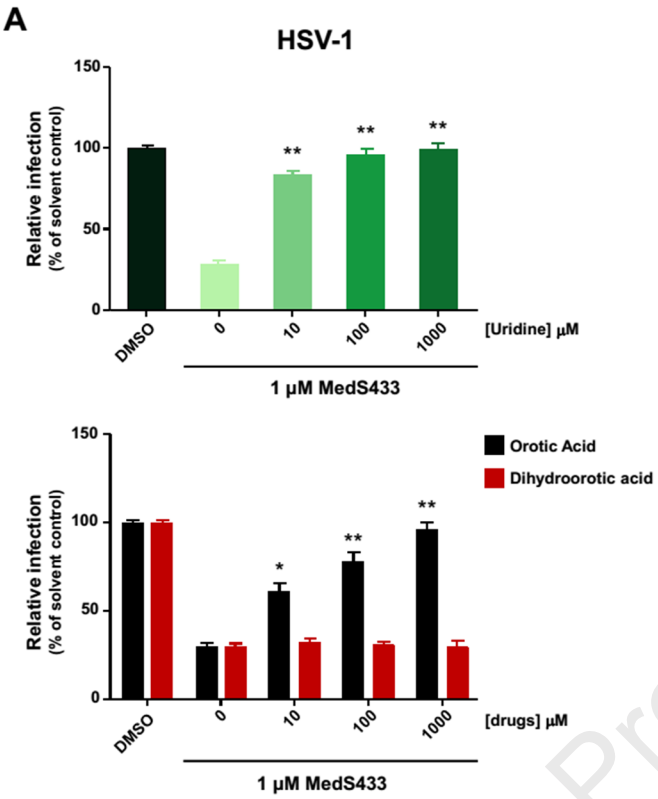
[d] Combination Index (CI), extrapolated by computational analysis with the CompuSyn software. Reported values represent means ± SD of data derived from n = 3 independent experiments in triplicate using MED/ACV concentration ratio showed in [a] added with 3 µM of DPY for each combination.

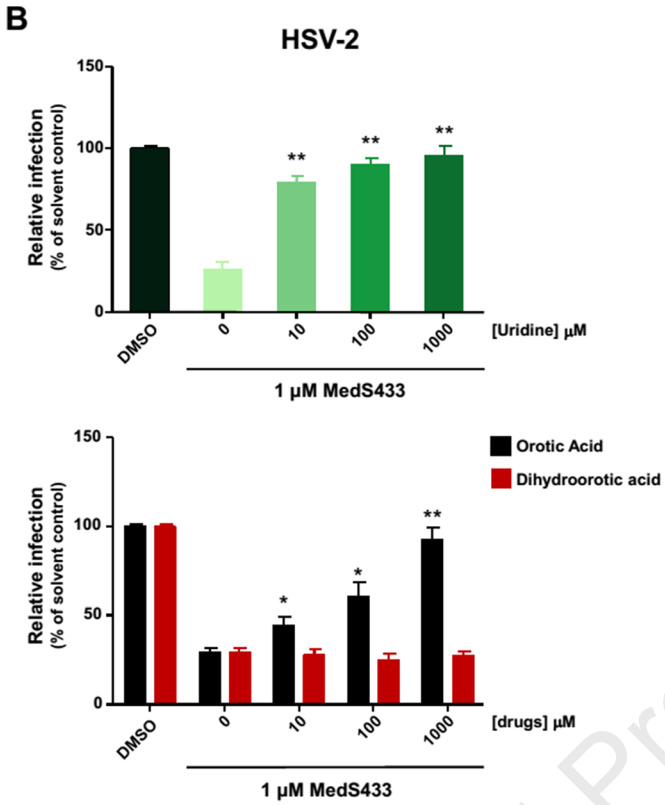


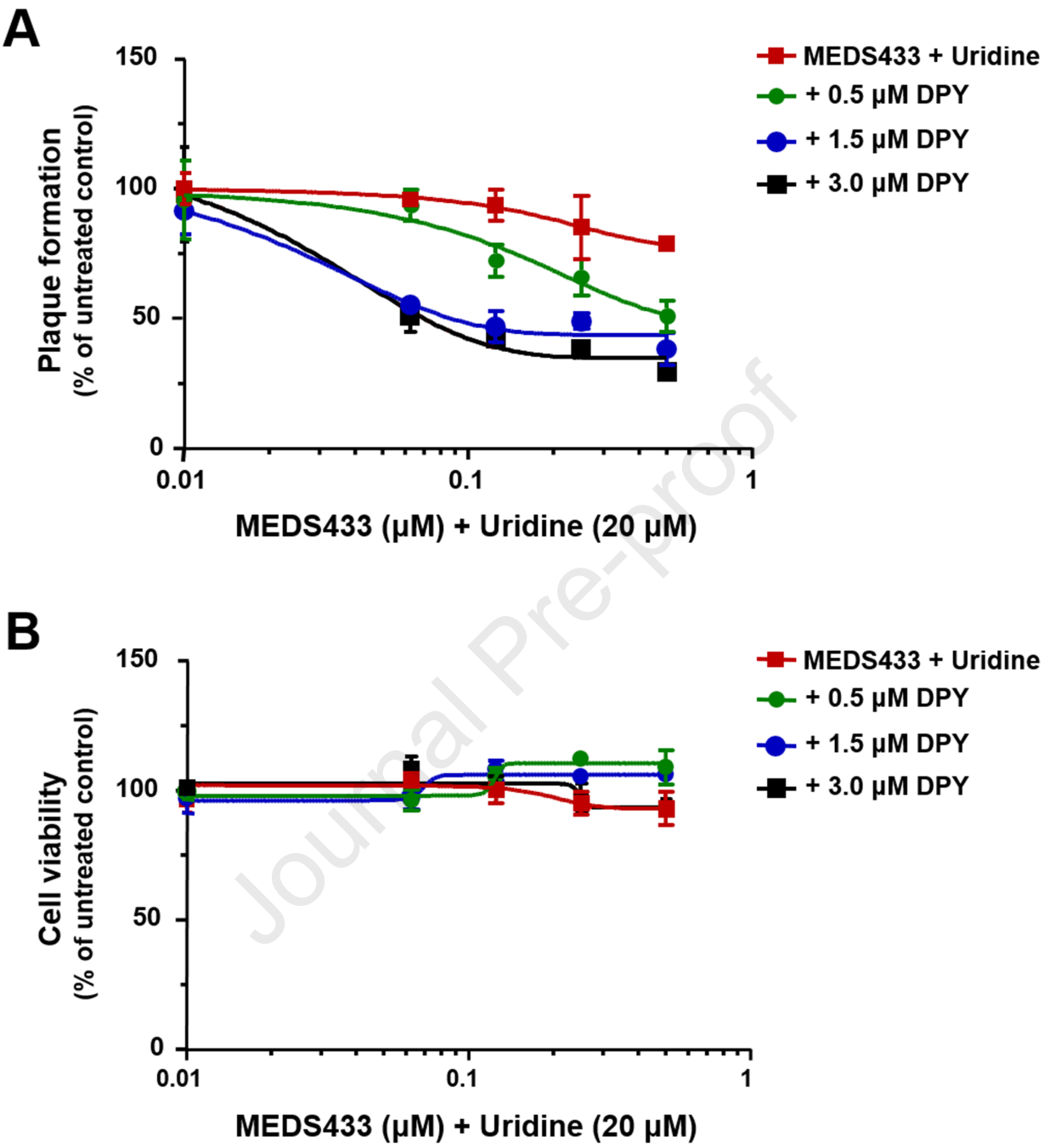


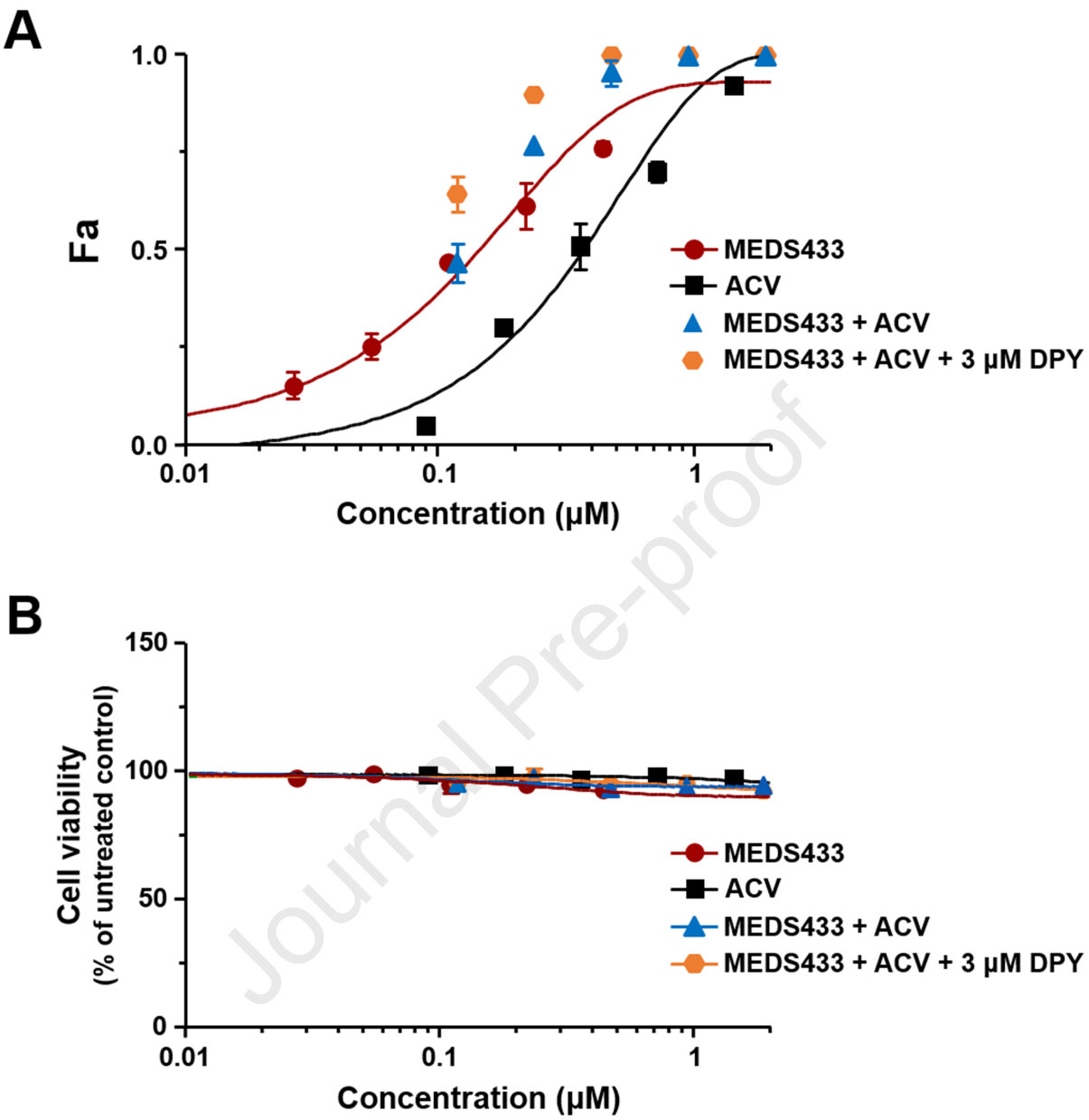












Effective deploying of a novel DHODH inhibitor against herpes simplex type 1 and type 2 replication

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Highlights

- We characterize the anti-HSV activity of the new DHODH inhibitor MEDS433 that targets the *de novo* pyrimidine biosynthesis
- MEDS433 impairs the *in vitro* replication of HSV-1 and HSV-2 in the nanomolar range
- The anti-HSV activity of MEDS433 is reversed by uracil and orotic acid confirming that it derives from inhibition of DHODH
- The simultaneous treatment with MEDS433 and acyclovir potentiates the anti-HSV activity of the combination
- MEDS433 differs from current anti-HSV drugs, and may represents a promising candidate for new antiviral strategies

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: