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Artemisone demonstrates synergistic antiviral activity in combination with approved and experimental drugs active against human cytomegalovirus

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ABSTRACT

We have recently shown that the artemisinin derivative artemisone, which was screened against malaria in human clinical studies, is a potent inhibitor of human cytomegalovirus (HCMV). Here we evaluated the antiviral effect of artemisone when employed in 2-drug combinations with approved and experimental anti-HCMV agents. Using the Chou-Talalay method, we found that *in-vitro* combination of artemisone with cidofovir, brincidofovir, or with the HCMV UL97 inhibitor maribavir resulted in antiviral synergism and the combination of artemisone with ganciclovir or with the viral terminase inhibitors letermovir and BDCRB resulted in moderate synergism. Importantly, the combination of artemisone with maribavir demonstrated synergistic antiviral activity *ex-vivo*, in a clinically-relevant multicellular model of human placental tissues maintained in organ culture. Our findings provide the basis for the use of artemisone in synergistically acting drug combinations, to enhance viral control and reduce antiviral drug toxicities.

1. Introduction

Human cytomegalovirus (HCMV) is a major cause of disease in immunocompromised individuals, and the leading cause of congenital infection and neuro-sensorial disease (Griffiths et al., 2015).

All anti-HCMV drugs currently approved for systemic treatment of HCMV infection, including ganciclovir, valganciclovir, foscarnet, and cidofovir, target the viral DNA polymerase. Their use is limited by toxicities and drug resistance (Fisher et al., 2017; Lurain and Chou, 2010; Shmueli et al., 2014), creating a need for better-tolerated antiviral agents with alternative mechanisms of action.

Three new anti-HCMV drugs, namely, maribavir (targeting the HCMV UL97 kinase (Marty et al., 2011)), brincidofovir (a lipid formulation of cidofovir (Marty et al., 2013)), and letermovir (targeting the HCMV terminase-complex (Marty et al., 2017)) have reached phase-3 clinical trials. Brincidofovir and maribavir failed to meet the studies' primary endpoints (Marty and Boeckh, 2011; Marty et al., 2011, 2019). Letermovir prophylaxis was shown to reduce clinically-significant infection in stem cell transplant (SCT) recipients, and was consequently approved for use in adult CMV-seropositive SCT recipients (Marty et al., 2017). Phase-3 treatment trials of maribavir are currently ongoing (NCT02927067; NCT02931539).

In recent years, the anti-malarial artemisinin derivative artesunate, and subsequently dimeric and trimeric artemisinin derivatives as well as a synthetic ozonide, were shown to inhibit HCMV *in-vitro*. These compounds are believed to act via a unique mechanism, involving the inhibition of host-cell functions required for efficient virus replication (Arav-Boger et al., 2010; Chou et al., 2011; Efferth et al., 2002, 2008; Flobinus et al., 2014; He et al., 2011, 2012; Hutterer et al., 2015; Kaptein et al., 2006; Morere et al., 2015; Reiter et al., 2015; Roy et al., 2015; Schnepf et al., 2011; Wang et al., 2019). We and others have demonstrated variation in the antiviral efficacy of artesunate *in-vivo* (Germi et al., 2014; Lau et al., 2011; Shapira et al., 2008; Wolf et al., 2011). These observations, which could result from a limited *in-vivo* antiviral efficacy of artesunate at the given doses, and from its known instability (Oiknine-Djian et al., 2018), prompted our search for more potent anti-HCMV artemisinin derivatives. In line with this goal, we have recently shown that artemisone, a new artemisinin derivative that

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had been screened against malaria in human clinical studies with no adverse effects (Nagelschmitz et al., 2008), is a potent and non-cytotoxic inhibitor of HCMV, with antiviral activity superior to that of artesunate (Oiknine-Djian et al., 2018).

Beyond the avoidance of cross-resistance, antiviral agents with different modes of action may exhibit enhanced antiviral effect when employed in combination, and such combinations have become a standard strategy for treating HIV and hepatitis C virus infections (Alazard-Dany et al., 2019; Cihlar and Fordyce, 2016). There is a growing interest in the future development of combination-based therapy against HCMV (Britt and Prichard, 2018; Chou et al., 2018; Drouot et al., 2016; Evers et al., 2002; O'Brien et al., 2018; Wildum et al., 2015). Here we have evaluated the combinatory activity of artemisone with a range of currently approved and new experimental anti-HCMV drugs.

2. Materials and methods

2.1. Cells, virus, and antiviral compounds

Human foreskin fibroblasts cells (HFF; locally derived; passages 10–25) and HCMV strain TB40/E (RV1164 (Weisblum et al., 2011)) were used for the antiviral assays.

Artemisone was synthesized as described (Oiknine-Djian et al., 2018). Ganciclovir, cidofovir (Sigma), brincidofovir, letermovir (MedChemExpress), maribavir (ViroPharma), and BDCRB (a kind gift from John Drach, University of Michigan) were stored as stock-solutions in DMSO (artemisone, maribavir, letermovir, brincidofovir, BDCRB) and H_2O (ganciclovir, cidofovir).

2.2. In-vitro evaluation of the effect of 2-drug combinations

The effect of 2-drug combinations was assessed using the Chou-Talalay method, based on the median-effect equation derived from the mass-action law as described (Chou, 2006). The EC_{50} (the drug concentration reducing the plaque number by 50%) of each drug was first determined using plaque-reduction assay in HFF as described (Oiknine-Djian et al., 2018). Next, serial 2-fold dilutions of the tested drugs - alone or in combination – in concentrations ranging from 4-fold to 0.25-fold the equipotent ratio (EC_{50} of $drug_1$/EC_{50} of $drug_2$; Table 1), along with no-drug control, were added in duplicate wells to HFF - infected at a multiplicity of infection of 0.001–0.002 PFU/cell. Viral plaques/well were enumerated at 10 days post infection (dpi).

The 2-drug combination effects were analyzed by the Chou-Talalay CalcuSyn software version 1.0 (Biosoft, Cambridge, UK) (Chou, 2006). The program extrapolated combination index (CI) values, representing the interaction between the two combined drugs at different specified levels of viral inhibition (derived from the percentages of inhibition induced by each drug alone and in combination; Table 1). The weighted average CI value was then calculated ($CI_{wt} = CI_{50} + 2xCI_{75} + 3xCI_{90} + 4xCI_{95})/10$) to define the overall effect of each combination. Drug combinatory effects were defined as: synergism for $CI_{wt} \leq 0.7$, moderate synergism for $CI_{wt} > 0.7$ and ≤ 0.9, addition for $CI_{wt} > 0.9$ and < 1.2, antagonism for $CI_{wt} \geq 1.2$ (Chou, 2006).

2.3. Ex-vivo evaluation of the effect of 2-drug combination in decidual organ cultures

Decidual organ cultures were prepared and infected as described (Oiknine-Djian et al., 2018; Weisblum et al., 2011, 2017). For the drug combination assay, we first defined the individual EC_{50}'s of artemisone and maribavir in the *ex-vivo* infected tissues as described (Oiknine-Djian et al., 2018): Briefly, artemisone or maribavir in parallel with no-drug control were added to the infected decidual cultures during viral adsorption and replaced (together with medium replacement) during further incubation. RNA samples purified from the infected (treated at different drug concentrations) and mock-infected tissues at 7dpi were subjected to quantitative real-time PCR of the late HCMV R160461 spliced mRNA as described (Oiknine-Djian et al., 2018). The EC_{50} was defined as the drug concentration reducing the viral late gene mRNA copy number (normalized by the cellular housekeeping gene βactin) by 50%. Serial equipotent 2-drug dilutions were then employed on newly-infected decidual cultures along with quantitative viral mRNA analyses, and the 2-drug combinatory effect was analyzed by the Chou-Talalay CalcuSyn software (see above). Each experimental condition for each tissue was tested in 5 parallel replicate wells. All comparative experiments were performed in parallel on tissues from the same donor.

2.4. Viability assay

Cell/tissue viability was assessed in parallel to the drug combination assays, under equivalent culture conditions and incubation times (7-10d), using the MTT assay as described (Weisblum et al., 2011). The assays were performed at least three times in quadruplicates employing the highest drug concentrations used in the combination assays.

Table 1
Analysis of the effects of 2-drug combinations against HCMV.

	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		
Human foreskin fibroblasts cells	Artemisone + BDCRB (2:1)	1.15 ± 0.12	0.93 ± 0.11	0.81 ± 0.14	0.75 ± 0.16	0.84	*Moderate synergism*
	Artemisone + Letermovir (200:1)	1.20 ± 0.34	0.91 ± 0.12	0.76 ± 0.04	0.70 ± 0.12	0.81	*Moderate synergism*
	Artemisone + Ganciclovir (1:1)	1.22 ± 0.47	0.89 ± 0.25	0.68 ± 0.09	0.58 ± 0.04	0.73	*Moderate synergism*
	Artemisone + Cidofovir (2.5:1)	0.95 ± 0.16	0.71 ± 0.07	0.55 ± 0.05	0.47 ± 0.05	0.60	*Synergism*
	Artemisone + Brincidofovir (1000:1)	0.88 ± 0.31	0.50 ± 0.17	0.34 ± 0.26	0.27 ± 0.29	0.39	*Synergism*
	Artemisone + Maribavir (1:1)	0.48 ± 0.26	0.31 ± 0.17	0.21 ± 0.11	0.16 ± 0.08	0.27	*Synergism*
	Letermovir + Maribavir (1:200)	1.02 ± 0.18	0.71 ± 0.09	0.57 ± 0.09	0.51 ± 0.17	0.62	*Synergism*
	Ganciclovir + Maribavir (1:1)	2.11 ± 0.84	2.50 ± 0.42	3.77 ± 2.21	5.53 ± 4.15	4.02	*Antagonism*
Decidual tissue organ culture	Artemisone + Maribavir (2:1)	1.33 ± 0.79	0.74 ± 0.16	0.48 ± 0.14	0.38 ± 0.20	0.56	*Synergism*

[a] EC_{50} values for HCMV strain TB40/E were determined by the plaque reduction assay in human foreskin fibroblasts cells (HFF) and by quantitative viral mRNA analyses in *ex-vivo* infected placental organ cultures.

[b] EC_{50} of $drug_1$/EC_{50} of $drug_2$ yielding an equipotent concentration ratio between the two combined drugs.

[c] Combination index (CI) values extrapolated at the indicated % of virus inhibition by use of the CompuSyn Software. CI values represent the means ± standard deviations of at least three independent experiments.

[d] CI_{wt}, weighted average CI values were calculated as $(CI_{50} + 2 \times CI_{75} + 3 \times CI_{90} + 4 \times CI_{95})/10$.

[e] Drug combinatory effects were defined as: synergism for $CI_{wt} \leq 0.7$, moderate synergism for $CI_{wt} > 0.7$ and ≤ 0.9, addition for $CI_{wt} > 0.9$ and < 1.2, antagonism for $CI_{wt} \geq 1.2$ (Chou, 2006).

3. Results and discussion

3.1. Artemisone exhibits in-vitro synergism with approved and experimental anti-HCMV drugs

We examined whether the antiviral activity of artemisone would result in a synergistic, additive, or antagonistic effect, in 2-drug combinations with ganciclovir, cidofovir, brincidofovir, letermovir, BDCRB (an experimental *in-vitro* inhibitor of the HCMV terminase) and maribavir, employing the Chou-Talalay method. The obtained drug EC_{50}'s (Supplementary Table S1) were generally in agreement with the published values, except for the EC_{50} of maribavir (~1 μM), which was lower than that previously reported in HFF (Chou et al., 2006). It should be noted that the EC_{50} values of maribavir have been shown to vary in different cell types and culture conditions (Chou et al., 2006, 2018). Since maribavir was tested alone and in combination in each assay, under the same culture conditions, these differences should not influence its evaluated effects in the 2-drug combinations.

As shown in Table 1, the *in-vitro* combination of artemisone with any of the tested drugs resulted in antiviral synergism: Moderate synergism with ganciclovir, letermovir, and BDCRB, and synergism with cidofovir, brincidofovir, and maribavir. Further assessment of artemisone-maribavir and artemisone-ganciclovir combinations in different concentration ratios confirmed the consistency of the combinatory effects shown at equipotent ratios (Supplementary Table S2). For comparison, we also evaluated the combination of letermovir with maribavir, resulting in a synergistic effect. This finding was in agreement with previous studies showing the additive-to-synergistic effect of this combination (Chou et al., 2018; O'Brien et al., 2018). It is important to recognize that the amount of demonstrated synergy may depend on assay model and test conditions, complicating comparisons among different published synergy studies. As expected, the combination of maribavir with ganciclovir resulted in an antagonistic effect, in accordance with the known interference of maribavir with ganciclovir phosphorylation and antiviral activity (Chou and Marousek, 2006). No microscopically-apparent cellular toxicity was observed, and there was no decrease in cellular viability upon exposure to any of the drug/drug combinations when compared to control mock-treated cells (Supplementary Figure S1), indicating that the observed synergistic effect was not caused by cellular toxicity.

Antiviral synergism is likely to be achieved by combining compounds with different modes of action. While the mechanism of action of artemisone is currently unknown, we have recently shown that it differs from that of the viral DNA polymerase inhibitors (Oiknine-Djian et al., 2018). It is conceivable that artemisone inhibits HCMV via mechanism/s similar to artesunate/artesunate dimers, which have been shown to inhibit virus-supportive cellular functions, including cellular transcription factors and cell-cycle related pathways which support productive infection (Efferth et al., 2002, 2008; Hutterer et al., 2015; Roy et al., 2015). A host-cell targeting mechanism could underlie the observed synergism of artemisone with direct viral-targeting agents. In this regard, previous studies showed a synergistic effect for artesunate with HCMV DNA polymerase inhibitors, letermovir, and maribavir (Chou et al., 2011; Drouot et al., 2016; He et al., 2012; Kaptein et al., 2006; Morere et al., 2015). Interestingly, maribavir, which appeared to demonstrate a relatively strong synergistic effect with artemisone (Table 1), was reported to exert a strong synergistic effect with the cellular mTor inhibitor rapamycin (Chou et al., 2018). This finding, along with the known enhanced antiviral effect of maribavir under reduced cellular metabolic/cell cycle conditions (Chou et al., 2018; Chou and Marousek, 2006), suggests that the synergy of maribavir with artemisone could indeed reflect the (yet unresolved) host-mediated functions of artemisone.

3.2. Dose reduction indexes of combined antiviral drugs

From a clinical standpoint, the use of synergistic anti-HCMV drug combinations could be advantageous in the transplantation setting - where a rapid control of viremia is associated with improved outcome, and in congenital infections - characterized by high viral loads. Additionally, it could allow the use of decreased drug doses (with reduced associated toxicity) while maintaining the antiviral efficacy. Employing a dose reduction index (DRI) simulation (Chou, 2006), we could show, for example, that the concentration of artemisone required to achieve 90% viral inhibition when employed in combination with ganciclovir, letermovir, and maribavir, may be reduced by ~ x7, x7, and x29 fold, respectively (compared with its concentration when employed alone; see Supplementary Table S3, which also exemplifies DRIs for additional levels of viral inhibition and additional artemisone combinations). While the true importance of the DRI is not clear, it suggests the potential that the drug combination would allow significant dose reductions (that is, DRI > 1) for a given effect (Chou, 2006).

3.3. Artemisone exhibits ex-vivo synergism with maribavir in human placental tissues

Finally, we tested the combinatory effect of artemisone and maribavir in our clinically-relevant *ex-vivo* model of HCMV infection in native human decidual tissues (representing the maternal aspect of the chimeric human placenta). First, we showed the consistent dose-dependent effect of artemisone (Oiknine-Djian et al., 2018) and maribavir on viral replication and determined their EC_{50}'s in the decidual tissues (tested in parallel in each of 4 independent experiments, performed in 4 tissues from different donors; Supplementary Table S1). Importantly, a clear synergistic effect of artemisone and maribavir was consistently measured in four newly-infected independent tissues (Table 1), with no tissue toxicity (Supplementary Figure S1). This finding in an authentic multi-cell-type human tissue model, which closely mirrors the *in-vivo* cellular-tropism and cell-to-cell mode of spread of HCMV (Weisblum et al., 2011), suggests the potential translation of the observed synergistic effect into the clinical setting.

3.4 In conclusion, our promising findings together with its apparent safety profile encourage clinical studies of artemisone as a new inhibitor against HCMV, and provide the basis for the use of artemisone in synergistically-acting drug combinations, to enhance viral control, and reduce antiviral drug toxicities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.antiviral.2019.104639.

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