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ANTIVIRAL AGENTS

The Artemisinin Derivative Artemisone Is a Potent Inhibitor of Human Cytomegalovirus Replication

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ABSTRACT Human cytomegalovirus (HCMV) is a major cause of disease in immunocompromised individuals and the most common cause of congenital infection and neurosensorial disease. The expanding target populations for HCMV antiviral treatment along with the limitations of the currently available HCMV DNA polymerase inhibitors underscore the need for new antiviral agents with alternative modes of action. The antimalarial artemisinin derivative artesunate was shown to inhibit HCMV *in vitro* yet has demonstrated limited antiviral efficacy *in vivo*, prompting our search for more potent anti-HCMV artemisinin derivatives. Here we show that the innovative artemisinin derivative artemisone, which has been screened for its activity against malaria parasites in human clinical studies, is a potent and noncytotoxic inhibitor of HCMV. Artemisone exhibited an antiviral efficacy comparable to that of ganciclovir (50% effective concentration, 1.20 $\pm$ 0.46 μM) in human foreskin fibroblasts, with enhanced relative potency in lung fibroblasts and epithelial cells. Significantly, the antiviral efficacy of artemisone was consistently $\geq$10-fold superior to that of artesunate in all cells. Artemisone effectively inhibited both laboratory-adapted and low-passage-number clinical strains, as well as drug-resistant HCMV strains. By using quantitative viral kinetics and gene expression studies, we show that artemisone is a reversible inhibitor targeting an earlier phase of the viral replication cycle than ganciclovir. Importantly, artemisone most effectively inhibited HCMV infection *ex vivo* in a clinically relevant multicellular model of integral human placental tissues maintained in organ culture. Our promising findings encourage preclinical and clinical studies of artemisone as a new inhibitor against HCMV.

KEYWORDS HCMV, antiviral drugs, artemisinin derivatives, artemisone, human cytomegalovirus

Human cytomegalovirus (HCMV), a ubiquitous betaherpesvirus, is a major cause of disease in immunocompromised individuals, including hematopoietic stem cell (HSC) and solid organ transplant recipients and patients with AIDS, as well as critically ill patients in intensive care units (1–3). HCMV is also the most common cause of congenital infection, leading to neurodevelopmental disabilities and hearing loss (4).

All currently approved anti-HCMV drugs, including ganciclovir, valganciclovir, foscarnet, and cidofovir, used for the systemic treatment of HCMV infection target the viral DNA polymerase. While overall these drugs are effective, their use is limited by bone marrow and renal toxicity, low oral bioavailability (except for valganciclovir), and the development of antiviral drug resistance (2, 5–7). Hence, there is a need for effective and better-tolerated antiviral drugs with alternative modes of action.

In line with the growing interest in the development of new anti-HCMV agents, a few experimental anti-HCMV drugs, namely, maribavir (which targets the HCMV UL97

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kinase [8]), brincidofovir (a lipid formulation of cidofovir which inhibits the viral DNA polymerase [9]), and letermovir (an inhibitor of the HCMV terminase complex [10]), reached phase 3 clinical trials. Brincidofovir and maribavir eventually failed those phase 3 clinical trials, and consequently, their place in HCMV antiviral development remains uncertain. Letermovir has recently demonstrated significant efficacy in preventing clinically significant HCMV infection after HSC transplantation (10) and was approved by the FDA in November 2017 for the prophylaxis of HCMV infection and disease in adult cytomegalovirus (CMV)-seropositive recipients of an allogeneic HSC transplant.

All these compounds directly inhibit specific viral targets. The antimalarial artemisinin derivative artesunate (see Fig. S1 in the supplemental material) has been shown to inhibit CMV replication *in vitro* and in an experimental animal model, acting via a unique mechanism which involves the inhibition of host cell functions required for virus replication (11–18). Major examples of such HCMV-supportive cellular functions reported to be inhibited by artesunate *in vitro* include the HCMV-induced NF-κB and Sp1 activation pathways and the virus-modulated cell cycle progression state, both of which are known to support efficient HCMV replication (13, 14, 19, 20). We and others have further described the successful clinical use of artesunate in a few transplant recipients with drug-resistant HCMV infection (21, 22). However, the clinical antiviral efficacy of artesunate appeared to be divergent, resulting in both success and failure in different patients (21, 23, 24). While this may be consistent with the limited *in vivo* antiviral activity of the given regimens, it may also be due to the established instability of artesunate, wherein it reverts rapidly to the artemisinin derivative dihydroartemisinin (DHA; Fig. S1) (25): artesunate is a hemiester of the hemiacetal DHA that is susceptible to hydrolysis by virtue of the intrinsic chemistry of the ester acetal linkage. It has been shown that artesunate is susceptible to hydrolysis by gut wall, plasma, and tissue esterases (25) and is labile in aqueous solution even in the absence of esterases, being hydrolyzed to DHA merely by mixing with bicarbonate. Taken together, these findings provide an impetus for the development of more potent anti-HCMV artemisinin derivatives. In this regard, dimeric and trimeric artemisinin derivatives have been reported to exert enhanced anti-HCMV activity *in vitro* compared to artesunate (19, 20, 26–28).

Artemisone is a new artemisinin derivative (Fig. S1) that has been screened for its activity against malaria parasites in human clinical studies (29–32). Here we report for the first time that artemisone is a potent inhibitor of HCMV replication in cell cultures and in multicellular human placental tissues, with its antiviral activity being markedly superior to that of artesunate.

RESULTS

Artemisone is an effective and noncytotoxic inhibitor of HCMV replication in cell culture. (i) Artemisone effectively inhibits laboratory-adapted and low-passage-number clinical HCMV strains. To evaluate the susceptibility of HCMV to artemisone, we employed a classical plaque reduction (PR) assay: human foreskin fibroblasts (HFF) were infected with the HCMV laboratory strain AD169 or with the clinically related HCMV endothelium-tropic strain TB40/E-BAC4, and viral plaques were enumerated following incubation with serial drug concentrations (added to the cells 2 h after viral adsorption). The results obtained by this assay clearly revealed a consistent dose-dependent inhibition by artemisone (Fig. 1). In parallel experiments, the antiviral efficacy of artemisone was compared to the antiviral efficacies of the reference anti-HCMV drug ganciclovir (which, together with its oral prodrug, valganciclovir, is the agent that is the most frequently used for the prevention and treatment of HCMV infection and disease) and the artemisinin derivative artesunate. Representative dose-response curves are shown in Fig. 1, and the results of multiple independent *in vitro* susceptibility assays are summarized in Table 1. These results demonstrate that artemisone exhibited potent anti-HCMV activity in HFF, with a 50% effective concentration (EC_{50}; the drug concentration reducing 50% of viral plaques) of ~1 μM or lower (Table 1) and an EC_{90} of <10 μM (data not shown) for both viral strains. Similar results

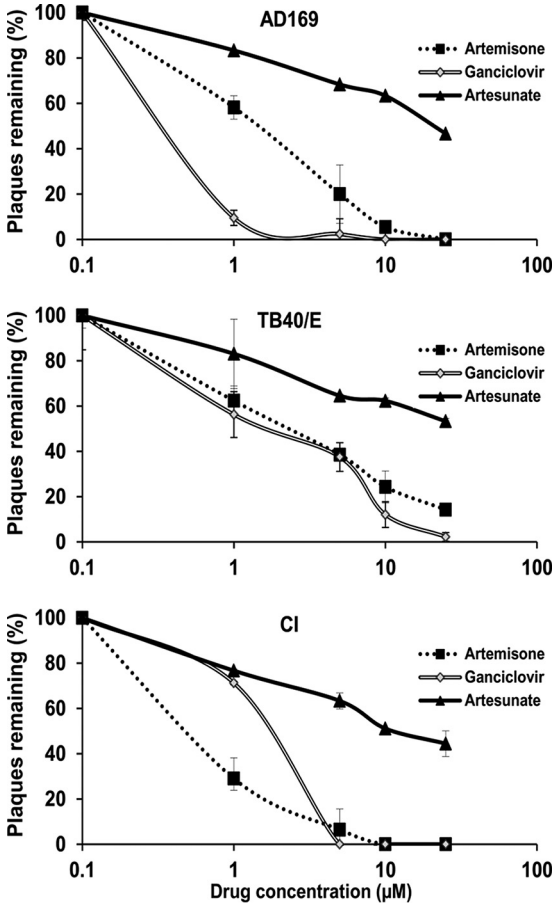

FIG 1 Artemisone, ganciclovir, and artesunate viral dose-response curves. The inhibition of plaque formation is shown for HCMV strains AD169 and TB40/E-BAC4 and a low-passage-number clinical isolate (CI). The results shown are representative of those from at least 3 independent experiments.

were obtained when artemisone was added to the cells at the time of viral adsorption. Importantly, the antiviral efficacy of artemisone was found to be comparable to that of ganciclovir and superior to that of artesunate, with its efficacy consistently surpassing that of artesunate by ≥10-fold (Fig. 1; Table 1). Artemisone was nontoxic to the cells, with no microscopically apparent cytotoxic effects at any of the artemisone concentrations used in the antiviral assays and with the drug concentration resulting in a 50%

TABLE 1 Susceptibility of HCMV strains to artemisone, artesunate, and ganciclovir in different cell cultures[b]

		Drug EC_{50} (μM)[a]		
Cell type	HCMV strain	Artemisone	Artesunate	Ganciclovir
HFF	AD169	1.20 ± 0.46	16.98 ± 8.75	1.36 ± 0.36
	TB40/E	0.68 ± 0.28	19.31 ± 2.04	0.63 ± 0.39
	CI 704	0.85 ± 0.18	10.79	1.79
	CI 943	0.25	ND	0.40
	CI 893	1.46	ND	1.66
MRC-5	TB40/E	0.22 ± 0.09	1.53 ± 0.42	1.67 ± 0.96
ARPE-19	TB40/E	0.49 ± 0.21	7.46 ± 2.14	2.08 ± 0.19

[a]EC_{50}s (the drug concentration resulting in a 50% reduction of viral plaques) were determined by the plaque reduction assay. The results represent those from at least 3 independent experiments and are expressed as means ± standard deviations.

[b]HFF, human foreskin fibroblasts; MRC-5, human fetal lung fibroblasts; ARPE, human retinal pigmented epithelial cells; CI, clinical isolate; ND, not done.

TABLE 2 EC_{50}, CC_{50}, and SI of artemisone, artesunate, and ganciclovir in different cell cultures[d]

Drug	Cell type	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI
Artemisone	HFF	0.68 ± 0.28	>164.83 ± 1.53[c]	>242.40[c]
	MRC-5	0.22 ± 0.09	18.67 ± 7.1	84.86
	ARPE-19	0.49 ± 0.21	>121.46 ± 5.83[c]	>247.88[c]
Artesunate	HFF	19.31 ± 2.04	53.56 ± 7.05	2.77
	MRC-5	1.53 ± 0.42	14.92 ± 3.1	9.75
	ARPE-19	7.46 ± 2.14	54.49 ± 7.81	7.30
Ganciclovir	HFF	0.63 ± 0.39	>640	>1,015.87
	MRC-5	1.67 ± 0.96	>640	>383.23
	ARPE-19	2.08 ± 0.19	>640	>307.70

[a]EC_{50}s for HCMV strain TB40/E were determined by the plaque reduction assay. The results represent those from at least 3 independent experiments and are expressed as means ± standard deviations.
[b]CC_{50} values (the drug concentration resulting in a 50% reduction of cell viability) were determined by the MTT assay. The results represent those from at least 3 independent experiments and are expressed as means ± standard deviations.
[c]As shown, in HFF and ARPE cells, the DMSO-only control demonstrated cellular toxicity. The four CC_{50} and SI values indicated relate to the artemisone concentrations for which the respective concentrations of the DMSO-only control demonstrated a 50% reduction of cell viability, thus limiting the ability to evaluate the effect of higher artemisone concentrations. The CC_{50} and SI of artemisone are therefore indicated to be greater than the value indicated for these cells.
[d]HFF, human foreskin fibroblasts; MRC-5, human fetal lung fibroblasts; ARPE, human retinal pigmented epithelial cells; SI, selectivity index. Artemisone was stored as a 10 mM stock solution in DMSO, and the respective concentrations of the DMSO-only control were tested in parallel in all assays.

reduction of cell viability (the 50% cytotoxic concentration [CC_{50}]) of >160 μM by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Table 2), thus demonstrating a high selectivity index (SI; CC_{50}/EC_{50}) of >240 (Table 2).

HCMV laboratory-adapted strains are known to differ from low-passage-number clinical strains, with the latter distinctively maintaining a predominant cell-to-cell mode of spread (as opposed to the cell-free mode of spread characteristic of laboratory-adapted strains) and a broader cellular tropism to epithelial, endothelial, and myeloid cells, which are characteristics that have been lost by laboratory-adapted strains during their continuous propagation in fibroblasts (33). We thus evaluated the antiviral activity of artemisone against clinical HCMV strains. As shown in Fig. 1 and Table 1, artemisone demonstrated comparable anti-HCMV activity against laboratory-adapted and clinical HCMV strains.

(ii) Artemisone effectively inhibits HCMV in different cell types. While HCMV drug susceptibility assays are traditionally performed in HFF, HCMV is known to infect diverse cell types, exploiting different entry pathways and modes of spread in epithelial/endothelial cells versus fibroblasts (33). Using the HCMV strain TB40/E (known to infect a wide range of cells [34]), we evaluated the antiviral activity of artemisone in epithelial (ARPE-19) and lung fibroblast (MRC-5) cells. Artemisone exhibited antiviral activity in ARPE-19 cells comparable to that in HFF and enhanced antiviral potency in MRC-5 cells compared to that in HFF (Tables 1 and 2). In ARPE-19 and MRC-5 cells, the artemisone EC_{50} was significantly lower than the ganciclovir EC_{50} ($P = 0.003$ and $P = 0.002$, respectively). Furthermore, the artemisone EC_{50} was consistently ≥10-fold lower than the artesunate EC_{50} (revealing a ≥10-fold superior efficacy) in all the cell types studied.

Employing a quantitative real-time PCR assay for HCMV DNA, we confirmed the susceptibility of HCMV to artemisone in the different cell types (EC_{50}s for strain TB40/E, 0.46 ± 0.24 and 0.13 ± 0.03 μM in HFF and MRC-5 cells, respectively) and further showed that artemisone reduced viral DNA accumulation (see Fig. S2A in the supplemental material).

In a quantitative time-lapse assay, pictures of live HCMV (strain TB40/E expressing IE2 to which enhanced yellow fluorescent protein [EYFP] was fused)-infected MRC-5 cells (untreated or treated with artemisone or ganciclovir) were taken every 3 h for 6

TABLE 3 Susceptibility of drug-resistant HCMV strains to artemisone[b]

HCMV strain	Resistance mutation	Resistance	EC_{50} (μM)[a]	
			Artemisone	Ganciclovir
TB40/E	None	None	0.46 ± 0.24	1.62 ± 0.19
CI SN3	UL54 F412S	Ganciclovir, cidofovir	0.40 ± 0.06	16.71 ± 0.08
CI TL8	UL97 L595S	Ganciclovir	0.17 ± 0.04	24.22 ± 0.11

[a]EC_{50}s in HFF were determined by a quantitative HCMV DNA assay. The results represent those from at least 3 independent experiments and are expressed as means ± standard deviations.
[b]CI, clinical isolate; UL54, HCMV DNA polymerase gene; UL97, HCMV UL97 kinase gene.

days postinfection (dpi), and the EYFP quantity per well was monitored over time (IncuCyte live cell imaging analysis software). Monitoring of the infected cultures revealed the accumulation of EYFP during the first cycle of infection (up to ~72 h postinfection [hpi]) and during further viral spread in untreated infected cells (Fig. S2B). While viral protein accumulation was similarly abrogated by high artemisone and ganciclovir concentrations (10 μM; Fig. S2B, left), at lower drug concentrations (1 μM; Fig. S2B, right), artemisone demonstrated an enhanced inhibition of HCMV replication compared to ganciclovir, in accordance with the EC_{50} data for MRC-5 cells.

(iii) Artemisone effectively inhibits HCMV in confluent and subconfluent HFF. The anti-HCMV activity of artesunate has been shown to be dependent on the confluence of HFF (20). We therefore compared viral inhibition by artemisone in confluent/resting versus subconfluent/growing HFF at the time of infection (Fig. S3A). Artemisone, like ganciclovir, significantly inhibited viral replication in both resting and growing HFF, as revealed by the significant reduction of viral mRNA accumulation (Fig. S3A; $P < 0.05$, Mann-Whitney test) and the complete inhibition of the viral yield (measured in the supernatant of infected cells; data not shown). Moreover, comparison of the degree of viral inhibition by artemisone (or ganciclovir) between the different cellular growth states (Fig. S3A) revealed no statistically significant differences ($P = 0.19$, Kruskal-Wallis test).

(iv) Artemisone effectively inhibits drug-resistant HCMV strains. The development of resistance to the currently available HCMV DNA polymerase inhibitors has been increasingly recognized (2, 5–7). We evaluated the antiviral activity of artemisone against representative drug-resistant HCMV clinical strains containing UL97 and DNA polymerase mutations (known to confer resistance to ganciclovir and cross-resistance to ganciclovir and cidofovir, respectively [6, 35]). By using a quantitative viral DNA susceptibility assay, we demonstrated that artemisone effectively inhibited drug-resistant HCMV strains in HFF (Table 3). This finding also implies that artemisone inhibits HCMV via a mechanism of action distinct from that of ganciclovir and other HCMV DNA polymerase inhibitors.

Reversibility of HCMV inhibition by artemisone. To learn more about the nature of HCMV inhibition by artemisone, we examined its potential reversibility, using a drug block-release experiment. HCMV-infected cells were incubated for 96 h with an inhibitory concentration of artemisone (drug block), followed by replacement of the drug-containing medium by drug-free medium (block release), with subsequent daily titrations of the progeny virus in the supernatants of the block-released cell cultures (Fig. 2). Parallel comparative experiments were performed using ganciclovir (as a reversible viral DNA polymerase inhibitor) and 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (BDCRB; an experimental *in vitro* inhibitor of the HCMV terminase; a kind gift from John Drach, University of Michigan), which (like letermovir) blocks the HCMV DNA cleavage-packaging step and has been shown by us to effectively and reversibly inhibit HCMV replication (36, 37). All three drugs effectively inhibited virus replication. Following removal of artemisone, progeny virus formation resumed by 24 to 48 h, further increasing by 96 h postrelease, thus demonstrating that the antiviral block of artemisone was reversible. The resumption of viral replication following artemisone release was faster than that in ganciclovir block-released cells (which demonstrated a

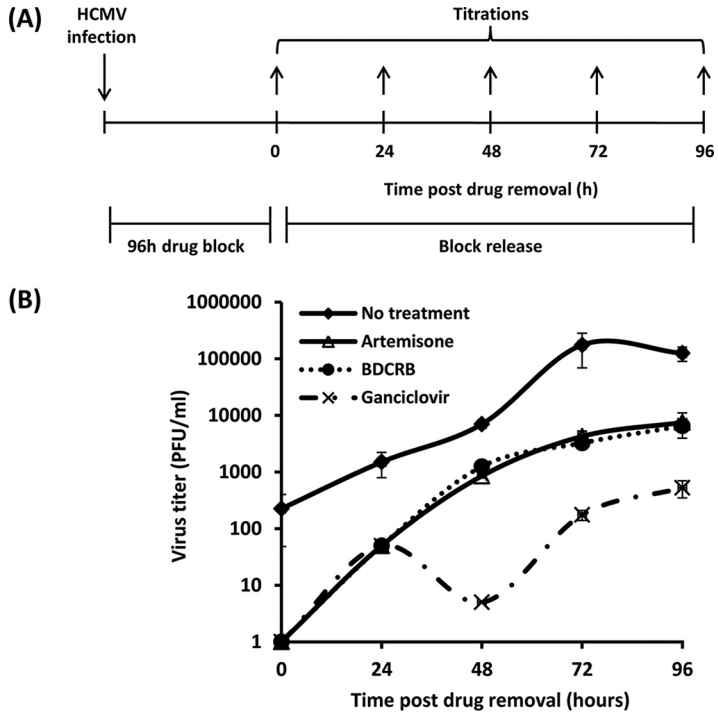

FIG 2 Reversibility of HCMV inhibition by artemisone by use of a drug block-release assay. (A) Schematic presentation of the experimental outline. HCMV (strain AD169)-infected HFF (MOI, 1) were treated for 96 h with the indicated inhibitor (drug block; 50 μM artemisone or ganciclovir or 20 μM BDCRB). At 96 h postinfection, the indicated drugs were removed from the culture medium (block release), and the titers of progeny virus in the cell culture supernatants, collected at the indicated times after block release, are shown (B). The results shown are representative of those from at least 3 independent experiments.

relatively slow reversibility, as previously described [36]) and comparable to that in BDCRB block-released cells. These kinetics could reflect either the different viral replication stages blocked by the different inhibitors or potential differences in the duration of the intracellular drug effect.

Artemisone inhibits an early phase of the HCMV replication cycle. (i) Time-of-addition studies. To trace the timing of the artemisone effect within the virus replication cycle, we employed drug addition studies, in which artemisone or ganciclovir was added to HCMV-infected cells at different times postinfection (p.i.) and the titer of the infectious progeny virus in the cell culture supernatants was determined at 100 hpi. As shown in Fig. 3, artemisone completely inhibited virus production even when added at 36 hpi, after which the inhibitory effect gradually decreased, a finding consistent with inhibition of an early phase of the HCMV replication cycle. In comparison, ganciclovir still exerted an inhibitory effect even when added later, by 48 hpi, implying that artemisone may target an earlier phase of the virus life cycle than ganciclovir.

(ii) Effect of artemisone on HCMV gene expression. Following viral entry into the cell, HCMV gene expression occurs in a cascade mode, with the orderly induction of immediate early (IE), early, and late viral genes. To delineate which phase of viral gene expression is inhibited by artemisone, we first assessed its effect on transcription of the main HCMV IE genes, IE1 and IE2. Using quantitative viral mRNA measurements, we found that artemisone (unlike ganciclovir) inhibited HCMV IE1 and IE2 mRNA at 6 hpi (Fig. 4A). This early inhibitory effect was dependent on the multiplicity of infection (MOI) and was detected only at a low MOI of <0.1 (Fig. 4A). In contrast, the inhibition of a late viral transcript was still shown at a higher MOI (Fig. 4A), and the overall inhibitory effect of artemisone on viral yield was still maintained at higher MOIs (MOIs of up to 5 PFU/ml were analyzed; Fig. S3B), suggesting that artemisone targets additional replication steps subsequent to IE gene expression.

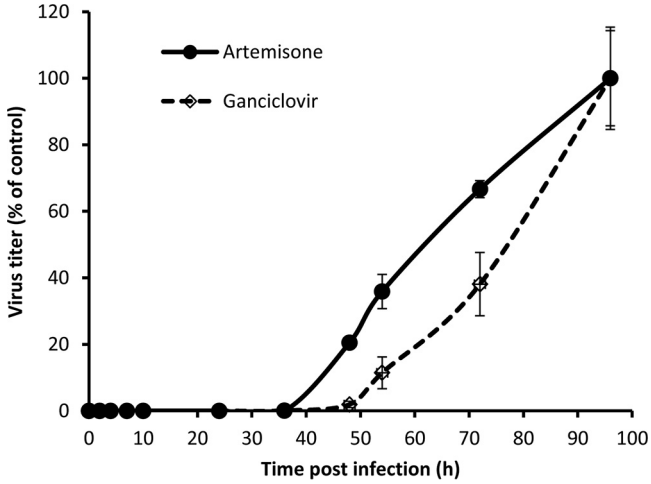

FIG 3 Effect of time of drug addition on HCMV yield. HFF were infected with HCMV strain AD169 (MOI, 1). Artemisone or ganciclovir (50 μM) was added at 0, 2, 4, 6, 10, 24, 36, 48, 54, 72, or 96 h postinfection (hpi). The progeny virus titers in cell culture supernatants collected at 100 hpi are presented as a percentage of the virus titer in the control untreated infected cells. The results shown are representative of those from at least 3 independent experiments.

Western blot analysis of representative IE, early, and late viral proteins at different times postinfection revealed some inhibition of viral IE proteins, with IE1 and mainly IE2 appearing at early times p.i. (36 hpi) and with moderate inhibition of early protein expression (UL44) and a complete abrogation of late protein expression (pp28) being seen (Fig. 4B).

The respective concentrations of the dimethyl sulfoxide (DMSO)-only control (0.5% DMSO as a control for 50 μM artemisone) were included as controls for the viral gene expression and viral yield experiments and did not affect any of the analyzed viral parameters.

***Ex vivo* antiviral activity of artemisone in human placental tissues.** Having shown that artemisone is a potent inhibitor of HCMV *in vitro*, we proceeded to study its antiviral activity in a clinically relevant *ex vivo* model of the human placenta. We employed our model of HCMV infection in human decidual tissues (representing the maternal aspect of the chimeric human placenta), maintained as multi-cell-type three-dimensional organ cultures (34, 38). Using fluorescence microscopy and quantitative viral mRNA analyses, we showed the consistent dose-dependent effect of artemisone on viral replication in the infected decidual tissues (Fig. 5). Furthermore, the artemisone EC_{50} was consistently lower than the ganciclovir EC_{50} (by 2- to 10-fold), when tested in parallel, in each of 4 independent comparative experiments performed in 4 tissue samples from different donors (see Fig. 5 for a representative result). Yet, it should be noted that we observed tissue-to-tissue variability in the drug EC_{50}s, which, according to our experience (34, 37, 38), is an inherent and expected feature of integral multi-cell-type human tissues (as opposed to conventional single-cell-type monolayer cell cultures). Overall, the mean EC_{50} of artemisone was 1.17 $\pm$ 1.71 μM (median, 0.5 μM), with no apparent tissue toxicity ($CC_{50} > 200$ μM, SI > 170), whereas the mean ganciclovir EC_{50} was 5.5 $\pm$ 3.78 μM (median, 5.9 μM) ($P = 0.04$, Mann-Whitney test). These findings corroborate the anti-HCMV activity of artemisone and demonstrate its potency in the authentic milieu of the human placenta. In view of the observed tissue-to-tissue variability, the results do not allow a definite conclusion regarding the activity of artemisone relative to that of ganciclovir in placental tissues.

DISCUSSION

The expanding target populations for HCMV antiviral treatment along with the limitations of current anti-HCMV drugs underscore the need for new antiviral agents

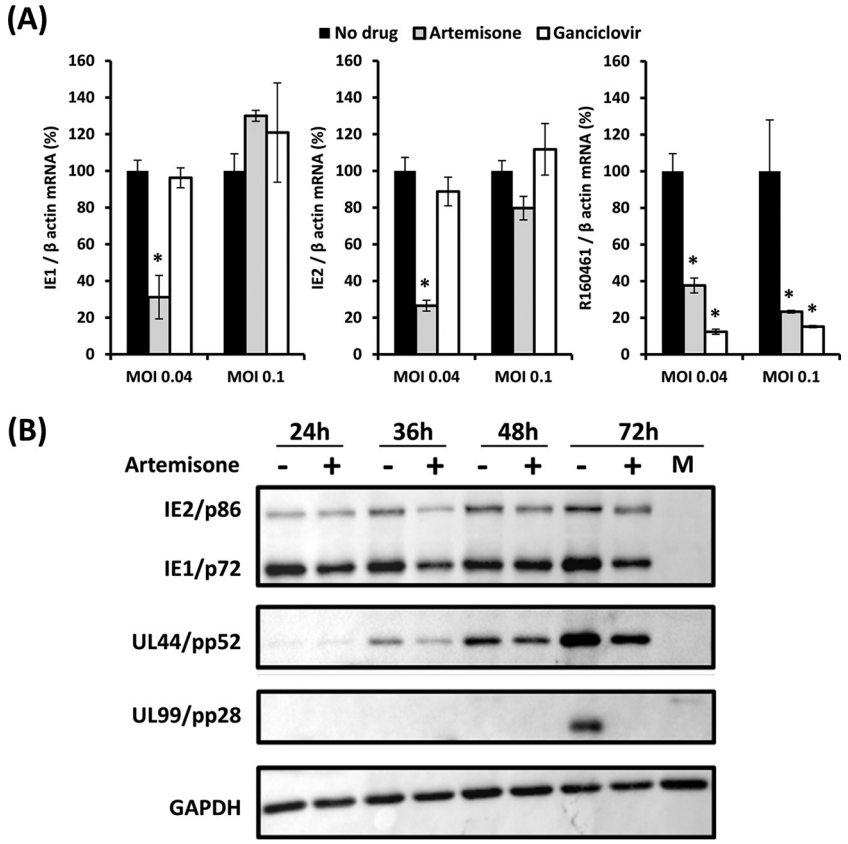

FIG 4 Effect of artemisone on HCMV gene expression. HFF were infected with HCMV strain TB40/E and treated with artemisone or ganciclovir (50 μM). (A) Cells were infected at the indicated MOIs, and the mRNA levels of the HCMV IE1 and IE2 genes (at 6 hpi) and the late (R160461) transcript (at 72 hpi) were analyzed by quantitative RT-PCR and normalized by the mRNA level of the housekeeping gene β-actin. The results are shown as a percentage of the level of normalized HCMV mRNA present in control untreated (no drug) infected cells. Significant changes ($P < 0.05$) of viral mRNA levels in artemisone- or ganciclovir-treated cells versus control untreated cells are indicated by an asterisk. (B) Expression of HCMV immediate early (IE2/p86 and IE1/p72), early (UL44/pp52), and late (UL99/pp28) proteins at the indicated times postinfection (MOI, 0.1) was detected by Western blotting. Cellular GAPDH was used as a loading control. Lane M, mock-infected cells.

with different mechanisms of action. The antimalarial artemisinin derivative artesunate was shown to inhibit HCMV *in vitro* yet has demonstrated limited antiviral efficacy *in vivo* (11, 14, 17, 21–24), prompting our search for more potent anti-HCMV artemisinin derivatives. Here we show that the innovative artemisinin derivative artemisone is a highly potent and noncytotoxic inhibitor of HCMV.

Artemisone exhibited an antiviral efficacy comparable to that of ganciclovir in HFF with a favorable SI, broadly inhibiting both laboratory-adapted cell-free viral strains and cell-associated low-passage-number clinical isolates recovered from congenitally infected infants (Fig. 1; Tables 1 and 2). Moreover, expanding our analysis to include additional cell types, such as epithelial cells, representing an important cell target of HCMV *in vivo* (33), we showed that artemisone exhibited an enhanced potency (compared to that of ganciclovir) in epithelial cells and lung fibroblasts (Tables 1 and 2; see also Fig. S2 in the supplemental material). The augmented effect of artemisone in specific cell types (which was also observed for artesunate; Table 1) is reminiscent of the variable degree of inhibition reported for maribavir in different cells (39) and suggests host cell-dependent effects. It remains unknown whether the enhanced activity of artemisone in specific cell types will affect its antiviral activity *in vivo*.

Significantly, the antiviral efficacy of artemisone was consistently ≥10-fold superior to that of artesunate in all cells studied. When comparing artemisone to artesunate, one

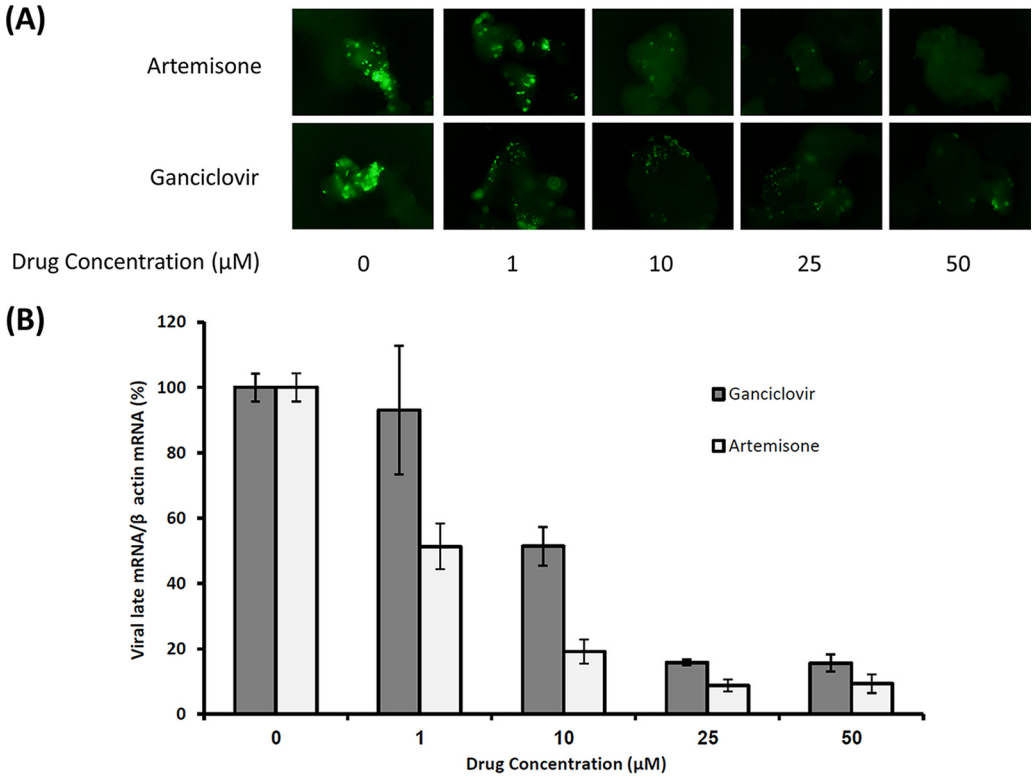

FIG 5 Effect of artemisone and ganciclovir on HCMV replication in human decidual tissues. Decidual organ tissue cultures were infected with HCMV strain TB40/E expressing UL83 to which GFP was fused and treated with the indicated drug concentrations. (A) Images of infected live tissues viewed by inverted fluorescence microscopy at 7 dpi. (B) Quantitative measurements of viral mRNA in infected drug-treated decidual tissues at 7 dpi. Viral late mRNA levels were analyzed by quantitative RT-PCR and normalized by the mRNA levels of the housekeeping gene β-actin. The data shown are representative of those from 4 independent experiments, each tested in 5 replicates.

should bear in mind that considerable variations of artesunate antiviral efficacy have been observed between different laboratories, with reported EC_{50}s ranging from ~1 to ~20 μM (11–14, 16, 26, 28). This variability may reflect the methodological differences between the various studies, i.e., the use of different antiviral assays, different timings of drug addition, and different host cell types and cell culture conditions. It also may reflect the intrinsic stability of artesunate and evident sensitivity to decomposition *in vivo* (25). It is thus important to note that all our comparative drug susceptibility assays demonstrating the superiority of artemisone over artesunate were performed in parallel under the same experimental conditions.

Our finding that artemisone effectively inhibited drug-resistant HCMV strains containing UL97 and DNA polymerase mutations is in accordance with the reported inhibition of drug-resistant HCMV strains by artesunate (11, 13, 14) and reveals the potential for a future treatment option for drug-resistant HCMV. This finding also argues for a mechanism of action different from that of ganciclovir and other viral DNA polymerase inhibitors. The mechanism of action of artemisone is currently unknown. The results of the drug block-release assay, together with those of the kinetic time-of-drug-addition studies and viral gene expression analysis, indicate that artemisone is a reversible viral inhibitor whose presence and activity are required during an early phase of the HCMV replication cycle, leading to the effective inhibition of viral early late protein expression and viral yield (Fig. 2 to 4 and S3B). Artemisone appeared to target an earlier viral replication stage than ganciclovir, as revealed by the earlier time of artemisone addition needed in order to inhibit infection and the (MOI-dependent) inhibition of viral IE gene expression, particularly by artemisone.

It is tempting to speculate that artemisone may exert its antiviral activity via a

mechanism(s) common to other artemisinin derivatives with anti-HCMV activity. While the specific target(s) mediating their antiviral activity is yet incompletely resolved, it is believed that artesunate and artesunate dimers inhibit HCMV by targeting virus-supportive cellular functions and cell cycle-related pathways (13, 14, 21, 22). Specifically, artesunate has been shown to inhibit the virus-induced activation of the cellular transcription factors NF-κB and Sp1, as well as the cellular signaling kinase phosphoinositide 3-kinase, thereby inhibiting their mediated activation of the HCMV major IE promoter and IE gene expression (13, 14). Artesunate has been further shown to interfere with NF-κB intracellular translocation and to covalently bind NF-κB subunit RelA/p65 (19). Additionally, artesunate and the artesunate dimer have been reported to exert their antiviral activity through cell cycle modulation, which is correlated with reduced levels of cyclin-dependent kinases and hypophosphorylation of the retinoblastoma protein (20).

Experiments under way in our laboratory at the Hadassah Hebrew University Medical Center are aimed at identification of the molecular target(s) of artemisone. From a clinical standpoint and in view of results previously reported for artesunate (11, 12, 17, 28), it will be of interest to examine the potential synergistic effect of artemisone in combination with current anti-HCMV agents. It will also be important to establish the range of antiviral activities of artemisone; preliminary data suggest that the activity of artemisone is specific to HCMV and the closely related rhesus macaque CMV, with no consistent activity against murine CMV or herpes simplex virus 1 being found (data not shown). However, in view of the broad spectrum of activities reported for artesunate (14, 16), assessment of the antiviral activity of artemisone will be expanded to include other clinically important viruses.

The species specificity of HCMV (infecting only humans) and the differences between the various animal CMVs make it difficult to assess reliably the potential efficacy of anti-HCMV drugs in preclinical studies, although the SCID mouse HCMV infection model could be an option to test their efficacy. Importantly, by employing a clinically relevant model of HCMV infection in native human decidual tissues maintained as multi-cell-type organ cultures, we showed that artemisone most effectively inhibited HCMV replication *ex vivo*, despite an expected degree of tissue-to-tissue variability (Fig. 5). In previous studies, we have shown that the decidual infection model closely recapitulates authentic HCMV infection, mirroring both the natural diversity of HCMV-infected target cells and the characteristic cell-to-cell mode of HCMV spread observed *in vivo* (34). The antiviral potency of artemisone in the decidual tissue therefore implies potential efficacy in the clinical setting of tissue-invasive HCMV infection.

In summary, we have shown that artemisone, targeting an early step of the virus replication cycle, effectively inhibits a range of HCMV strains, including clinical isolates and drug-resistant strains, and has antiviral efficacy substantially superior to that of artesunate. Moreover, artemisone exhibits an antiviral potency comparable to or superior to that of ganciclovir in different cell types and in a clinically relevant model of the human placenta. Our findings indicate the considerable potential for the clinical use of artemisone as a new inhibitor against HCMV.

MATERIALS AND METHODS

Cells, viruses, and antiviral/virus-inhibitory compounds. HFF were used for HCMV propagation. The HCMV strains used were AD169 (obtained from the American Type Culture Collection [ATCC]), the TB40/E-BAC4 strain (generously provided by C. Sinzger, Germany), and TB40/E strains expressing UL83 to which green fluorescent protein (GFP) was fused (strain RV1305) or IE2 to which EYFP was fused (strain RV1164) (generously provided by M. Winkler, Germany) (34). These viral strains were maintained as cell-free viral stocks. In addition, we used the low-passage-number clinical isolates (CI) CI 704, CI 943, and CI 893, recovered at the Hadassah Clinical Virology Laboratory from the urine of congenitally infected newborns and propagated for 3 to 5 passages as cell-associated virus, and drug-resistant clinical strains SN3 and TL8 (generously provided by N. Lurain, USA). For determination of the viral titer, infected cell supernatants were collected, centrifuged to remove cellular debris, and stored at −80°C until assayed by a standard plaque assay on HFF.

ARPE-19 (human retinal pigmented epithelial) cells and MRC-5 cells (human fetal lung fibroblasts) were obtained from ATCC and maintained according to ATCC instructions.

Artemisone was synthesized as described previously (29) and stored as a 10 mM stock solution in DMSO. Artesunate and ganciclovir (Sigma) were stored as 10 mM stock solutions in DMSO and H_2O, respectively. The compounds were added to the infected cells after 2 h of viral adsorption, unless otherwise specified. Drug dilutions were made in the appropriate culture medium used for the cells/tissues analyzed.

Antiviral drug susceptibility assays. (i) PR assay. *In vitro* sensitivity phenotypes were determined by the PR assay as previously described (35). Briefly, cell-free virus (or virus-infected cells, in the case of cell-associated clinical isolates) was inoculated onto newly confluent cell monolayers in 24-well culture plates to yield 20 to 30 plaques/well, and the cell monolayers were cultured in the presence of a 0.3% low-melting-point (LMP) agarose overlay under a range of drug concentrations with a no-drug control. The drug concentration required to reduce the plaque number by 50% (EC_{50}) was determined by curve fitting. The reference antiviral drug ganciclovir was included for comparison in each assay.

(ii) Quantitative viral DNA/mRNA assays. Quantitative viral DNA or mRNA measurements were employed in specified experiments in cell culture and in all the drug susceptibility experiments in organ tissue cultures (see below). The EC_{50} was defined as the drug concentration required to reduce the viral DNA or viral late gene mRNA copy number (measured as described in "DNA and RNA purification and quantification" below) by 50%.

Cytotoxicity assay. Assessment of drug cytotoxicity was performed in parallel to the susceptibility assays under equivalent cell culture conditions and incubation times (7 to 10 days). Cells were monitored microscopically, and cells or decidual tissue viability was monitored by the mitochondrial dehydrogenase enzyme (MTT) assay as previously described (34). The assays were performed at least three times in quadruplicate, employing serial 2-fold drug dilutions. CC_{50} values (the drug concentration resulting in a 50% reduction of cell viability) were used to calculate the selectivity index (SI; CC_{50}/EC_{50}) for individual drugs.

Determination of antiviral drug susceptibility in decidual organ cultures. Decidual organ tissue cultures were prepared and infected as previously described (34) under approval by the Hadassah Medical Center Institutional Review Board (0138-08-HMO). For infection of the organ tissue cultures, decidual tissues were placed in 48-well plates and inoculated with the virus (5×10^4 PFU/well) for 12 h to allow effective viral adsorption. The tissues were further monitored for viral infection and spread for 7 days as described previously (34). For determination of the drug EC_{50}s in the tissues, artemisone or ganciclovir in parallel to a no-drug control was added to the infected decidual tissues during viral adsorption and replaced (together with medium replacement) during further incubation. The tissues were then subjected to RNA purification and quantification as described below. All comparative experiments were performed in parallel on tissues from the same donor.

DNA and RNA purification and quantification. Cell and organ tissue cultures were washed and stored at −80°C until assayed. RNA and DNA were extracted using a NucleoSpin RNA isolation kit and a NucleoSpin tissue kit, respectively (Macherey-Nagel). The purified DNA samples were subjected to a quantitative real-time PCR, using primers and probes derived from HCMV glycoprotein B (gB), as previously described (34). The purified RNA samples were subjected to reverse transcription (RT) using a GoScript RT system (Promega), followed by quantitative real-time PCR of the IE1, IE2, and late HCMV R160461 spliced mRNA as previously described (40). The viral DNA copy number was normalized by the copy number of the cellular single-copy gene RNase P (TaqMan RNase P kit; Applied Biosystems). The viral mRNA copy number was normalized by the copy number of the cellular housekeeping gene β-actin (37).

Protein purification and Western blotting. Cells were lysed using radioimmunoprecipitation assay buffer containing 1% protease inhibitors (Sigma). Lysates were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were used at the manufacturers' recommended concentrations for protein detection: anti-IE1, anti-IE2, anti-UL44 (pp52), and anti-UL99 (pp28) (Virusys Corporation) and anti-GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase; Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies (Promega) were used for detection.

Time-lapse studies of viral spread. MRC-5 cells infected with HCMV strain TB40/E expressing IE2 to which EYFP was fused (either drug treated or untreated in an LMP agarose overlay) were incubated in 24-well plates in an IncuCyte live cell analysis system (Essen BioScience) for 6 dpi, and pictures were acquired every 3 h. IncuCyte live cell imaging analysis software, which captures 96 images simultaneously from each well, was used to calculate the EYFP quantity per well.

Statistical analysis. All data (mean ± standard deviation) were analyzed using unpaired, two-tailed *t* tests for comparisons between two groups, unless otherwise specified; *P* values of <0.05 were considered significant. The nonparametric Mann-Whitney test was used when indicated for comparisons of small groups demonstrating higher variability. The nonparametric Kruskal-Wallis test was used for comparison of drug inhibition in the three different cell confluences.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00288-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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E.O.-D. designed and performed the study and analyzed the data. Y.W. helped perform the experiments and analyze the data, A.P. cosupervised the study and analyzed the data, H.N.W. prepared and analyzed artemisone stocks, R.K.H. provided artemisone stocks and analyzed the data, and D.G.W. designed and supervised the study, analyzed the data, and wrote the manuscript with E.O.-D.

D.G.W. and R.K.H. serve as scientific advisors for Artemis Therapeutics.

REFERENCES

1. Beam E, Dioverti V, Razonable RR. 2014. Emerging cytomegalovirus management strategies after solid organ transplantation: challenges and opportunities. Curr Infect Dis Rep 16:419. https://doi.org/10.1007/s11908-014-0419-8.
2. Boeckh M. 2011. Complications, diagnosis, management, and prevention of CMV infections: current and future. Hematology Am Soc Hematol Educ Program 2011:305–309. https://doi.org/10.1182/asheducation-2011.1.305.
3. Limaye AP, Stapleton RD, Peng L, Gunn SR, Kimball LE, Hyzy R, Exline MC, Files DC, Morris PE, Frankel SK, Mikkelsen ME, Hite D, Enfield KB, Steingrub J, O'Brien J, Parsons PE, Cuschieri J, Wunderink RG, Hotchkin DL, Chen YQ, Rubenfeld GD, Boeckh M. 2017. Effect of ganciclovir on IL-6 levels among cytomegalovirus-seropositive adults with critical illness: a randomized clinical trial. JAMA 318:731–740. https://doi.org/10.1001/jama.2017.10569.
4. Griffiths P, Plotkin S, Mocarski E, Pass R, Schleiss M, Krause P, Bialek S. 2013. Desirability and feasibility of a vaccine against cytomegalovirus. Vaccine 31(Suppl 2):B197–B203. https://doi.org/10.1016/j.vaccine.2012.10.074.
5. Fisher CE, Knudsen JL, Lease ED, Jerome KR, Rakita RM, Boeckh M, Limaye AP. 2017. Risk factors and outcomes of ganciclovir-resistant cytomegalovirus infection in solid organ transplant recipients. Clin Infect Dis 65:57–63. https://doi.org/10.1093/cid/cix259.
6. Lurain NS, Chou S. 2010. Antiviral drug resistance of human cytomegalovirus. Clin Microbiol Rev 23:689–712. https://doi.org/10.1128/CMR.00009-10.
7. Shmueli E, Or R, Shapira MY, Resnick IB, Caplan O, Bdolah-Abram T, Wolf DG. 2014. High rate of cytomegalovirus drug resistance among patients receiving preemptive antiviral treatment after haploidentical stem cell transplantation. J Infect Dis 209:557–561. https://doi.org/10.1093/infdis/jit475.
8. Marty FM, Ljungman P, Papanicolaou GA, Winston DJ, Chemaly RF, Strasfeld L, Young JA, Rodriguez T, Maertens J, Schmitt M, Einsele H, Ferrant A, Lipton JH, Villano SA, Chen H, Boeckh M, Maribavir 1263-300 Clinical Study Group. 2011. Maribavir prophylaxis for prevention of cytomegalovirus disease in recipients of allogeneic stem-cell transplants: a phase 3, double-blind, placebo-controlled, randomised trial. Lancet Infect Dis 11:284–292. https://doi.org/10.1016/S1473-3099(11)70024-X.
9. Marty FM, Winston DJ, Rowley SD, Vance E, Papanicolaou GA, Mullane KM, Brundage TM, Robertson AT, Godkin S, Mommeja-Marin H, Boeckh M, CMX001-201 Clinical Study Group. 2013. CMX001 to prevent cytomegalovirus disease in hematopoietic-cell transplantation. N Engl J Med 369:1227–1236. https://doi.org/10.1056/NEJMoa1303688.
10. Marty FM, Ljungman P, Chemaly RF, Maertens J, Dadwal SS, Duarte RF, Haider S, Ullmann AJ, Katayama Y, Brown J, Mullane KM, Boeckh M, Blumberg EA, Einsele H, Snydman DR, Kanda Y, DiNubile MJ, Teal VL, Wan H, Murata Y, Kartsonis NA, Leavitt RY, Badshah C. 2017. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. N Engl J Med 377:2433–2444. https://doi.org/10.1056/NEJMoa1706640.
11. Chou S, Marousek G, Auerochs S, Stamminger T, Milbradt J, Marschall M. 2011. The unique antiviral activity of artesunate is broadly effective against human cytomegaloviruses including therapy-resistant mutants. Antiviral Res 92:364–368. https://doi.org/10.1016/j.antiviral.2011.07.018.
12. Drouot E, Piret J, Boivin G. 2016. Artesunate demonstrates in vitro synergism with several antiviral agents against human cytomegalovirus. Antivir Ther 21:535–539. https://doi.org/10.3851/IMP3028.
13. Efferth T, Marschall M, Wang X, Huong SM, Hauber I, Olbrich A, Kronschnabl M, Stamminger T, Huang ES. 2002. Antiviral activity of artesunate towards wild-type, recombinant, and ganciclovir-resistant human cytomegaloviruses. J Mol Med (Berl) 80:233–242. https://doi.org/10.1007/s00109-001-0300-8.
14. Efferth T, Romero MR, Wolf DG, Stamminger T, Marin JJ, Marschall M. 2008. The antiviral activities of artemisinin and artesunate. Clin Infect Dis 47:804–811. https://doi.org/10.1086/591195.
15. Flobinus A, Taudon N, Desbordes M, Labrosse B, Simon F, Mazeron MC, Schnepf N. 2014. Stability and antiviral activity against human cytomegalovirus of artemisinin derivatives. J Antimicrob Chemother 69:34–40. https://doi.org/10.1093/jac/dkt346.
16. Kaptein SJ, Efferth T, Leis M, Rechter S, Auerochs S, Kalmer M, Bruggeman CA, Vink C, Stamminger T, Marschall M. 2006. The anti-malaria drug artesunate inhibits replication of cytomegalovirus in vitro and in vivo. Antiviral Res 69:60–69. https://doi.org/10.1016/j.antiviral.2005.10.003.
17. Morere L, Andouard D, Labrousse F, Saade F, Calliste CA, Cotin S, Aubard Y, Rawlinson WD, Esclaire F, Hantz S, Ploy MC, Alain S. 2015. Ex vivo model of congenital cytomegalovirus infection and new combination therapies. Placenta 36:41–47. https://doi.org/10.1016/j.placenta.2014.11.003.
18. Schnepf N, Corvo J, Pors MJ, Mazeron MC. 2011. Antiviral activity of ganciclovir and artesunate towards human cytomegalovirus in astrocytoma cells. Antiviral Res 89:186–188. https://doi.org/10.1016/j.antiviral.2010.12.002.
19. Hutterer C, Niemann I, Milbradt J, Frohlich T, Reiter C, Kadioglu O, Bahsi H, Zeittrager I, Wagner S, Einsiedel J, Gmeiner P, Vogel N, Wandinger S, Godl K, Stamminger T, Efferth T, Tsogoeva SB, Marschall M. 2015. The broad-spectrum antiinfective drug artesunate interferes with the canonical nuclear factor kappa B (NF-kappaB) pathway by targeting RelA/p65. Antiviral Res 124:101–109. https://doi.org/10.1016/j.antiviral.2015.10.003.
20. Roy S, He R, Kapoor A, Forman M, Mazzone JR, Posner GH, Arav-Boger R. 2015. Inhibition of human cytomegalovirus replication by artemisinins: effects mediated through cell cycle modulation. Antimicrob Agents Chemother 59:3870–3879. https://doi.org/10.1128/AAC.00262-15.
21. Germi R, Mariette C, Alain S, Lupo J, Thiebaut A, Brion JP, Epaulard O, Saint Raymond C, Malvezzi P, Morand P. 2014. Success and failure of

artesunate treatment in five transplant recipients with disease caused by drug-resistant cytomegalovirus. Antiviral Res 101:57–61. https://doi.org/10.1016/j.antiviral.2013.10.014.
22. Shapira MY, Resnick IB, Chou S, Neumann AU, Lurain NS, Stamminger T, Caplan O, Saleh N, Efferth T, Marschall M, Wolf DG. 2008. Artesunate as a potent antiviral agent in a patient with late drug-resistant cytomegalovirus infection after hematopoietic stem cell transplantation. Clin Infect Dis 46:1455–1457. https://doi.org/10.1086/587106.
23. Lau PK, Woods ML, Ratanjee SK, John GT. 2011. Artesunate is ineffective in controlling valganciclovir-resistant cytomegalovirus infection. Clin Infect Dis 52:279. https://doi.org/10.1093/cid/ciq050.
24. Wolf DG, Shimoni A, Resnick IB, Stamminger T, Neumann AU, Chou S, Efferth T, Caplan O, Rose J, Nagler A, Marschall M. 2011. Human cytomegalovirus kinetics following institution of artesunate after hematopoietic stem cell transplantation. Antiviral Res 90:183–186. https://doi.org/10.1016/j.antiviral.2011.03.184.
25. Batty KT, Iletr KE, Powell SM, Martin J, Davis TM. 2002. Relative bioavailability of artesunate and dihydroartemisinin: investigations in the isolated perfused rat liver and in healthy Caucasian volunteers. Am J Trop Med Hyg 66:130–136. https://doi.org/10.4269/ajtmh.2002.66.130.
26. Arav-Boger R, He R, Chiou CJ, Liu J, Woodard L, Rosenthal A, Jones-Brando L, Forman M, Posner G. 2010. Artemisinin-derived dimers have greatly improved anti-cytomegalovirus activity compared to artemisinin monomers. PLoS One 5:e10370. https://doi.org/10.1371/journal.pone.0010370.
27. He R, Forman M, Mott BT, Venkatadri R, Posner GH, Arav-Boger R. 2013. Unique and highly selective anticytomegalovirus activities of artemisinin-derived dimer diphenyl phosphate stem from combination of dimer unit and a diphenyl phosphate moiety. Antimicrob Agents Chemother 57:4208–4214. https://doi.org/10.1128/AAC.00893-13.
28. He R, Park K, Cai H, Kapoor A, Forman M, Mott B, Posner GH, Arav-Boger R. 2012. Artemisinin-derived dimer diphenyl phosphate is an irreversible inhibitor of human cytomegalovirus replication. Antimicrob Agents Chemother 56:3508–3515. https://doi.org/10.1128/AAC.00519-12.
29. Haynes RK, Fugmann B, Stetter J, Rieckmann K, Heilmann HD, Chan HW, Cheung MK, Lam WL, Wong HN, Croft SL, Vivas L, Rattray L, Stewart L, Peters W, Robinson BL, Edstein MD, Kotecka B, Kyle DE, Beckermann B, Gerisch M, Radtke M, Schmuck G, Steinke W, Wollborn U, Schmeer K, Romer A. 2006. Artemisone—a highly active antimalarial drug of the artemisinin class. Angew Chem Int Ed Engl 45:2082–2088. https://doi.org/10.1002/anie.200503071.
30. Nagelschmitz J, Voith B, Wensing G, Roemer A, Fugmann B, Haynes RK, Kotecka BM, Rieckmann KH, Edstein MD. 2008. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. Antimicrob Agents Chemother 52:3085–3091. https://doi.org/10.1128/AAC.01585-07.
31. Ramharter M, Burkhardt D, Nemeth J, Adegnika AA, Kremsner PG. 2006. In vitro activity of artemisone compared with artesunate against Plasmodium falciparum. Am J Trop Med Hyg 75:637–639.
32. Vivas L, Rattray L, Stewart LB, Robinson BL, Fugmann B, Haynes RK, Peters W, Croft SL. 2007. Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo. J Antimicrob Chemother 59:658–665. https://doi.org/10.1093/jac/dkl563.
33. Sinzger C, Digel M, Jahn G. 2008. Cytomegalovirus cell tropism. Curr Top Microbiol Immunol 325:63–83.
34. Weisblum Y, Panet A, Zakay-Rones Z, Haimov-Kochman R, Goldman-Wohl D, Ariel I, Falk H, Natanson-Yaron S, Goldberg MD, Gilad R, Lurain NS, Greenfield C, Yagel S, Wolf DG. 2011. Modeling of human cytomegalovirus maternal-fetal transmission in a novel decidual organ culture. J Virol 85:13204–13213. https://doi.org/10.1128/JVI.05749-11.
35. Wolf DG, Smith IL, Lee DJ, Freeman WR, Flores-Aguilar M, Spector SA. 1995. Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J Clin Invest 95:257–263. https://doi.org/10.1172/JCI117648.
36. Wolf DG, Courcelle CT, Prichard MN, Mocarski ES. 2001. Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. Proc Natl Acad Sci U S A 98:1895–1900. https://doi.org/10.1073/pnas.98.4.1895.
37. Weisblum Y, Oiknine-Djian E, Zakay-Rones Z, Vorontsov O, Haimov-Kochman R, Nevo Y, Stockheim D, Yagel S, Panet A, Wolf DG. 2017. APOBEC3A is upregulated by human cytomegalovirus (HCMV) in the maternal-fetal interface, acting as an innate anti-HCMV effector. J Virol 91:e01296-17. https://doi.org/10.1128/JVI.01296-17.
38. Weisblum Y, Oiknine-Djian E, Vorontsov OM, Haimov-Kochman R, Zakay-Rones Z, Meir K, Shveiky D, Elgavish S, Nevo Y, Roseman M, Bronstein M, Stockheim D, From I, Eisenberg I, Lewkowicz AA, Yagel S, Panet A, Wolf DG. 2017. Zika virus infects early- and midgestation human maternal decidual tissues, inducing distinct innate tissue responses in the maternal-fetal interface. J Virol 91:e01905-16. https://doi.org/10.1128/JVI.01905-16.
39. Chou S, Van Wechel LC, Marousek GI. 2006. Effect of cell culture conditions on the anticytomegalovirus activity of maribavir. Antimicrob Agents Chemother 50:2557–2559. https://doi.org/10.1128/AAC.00207-06.
40. White EA, Clark CL, Sanchez V, Spector DH. 2004. Small internal deletions in the human cytomegalovirus IE2 gene result in nonviable recombinant viruses with differential defects in viral gene expression. J Virol 78:1817–1830. https://doi.org/10.1128/JVI.78.4.1817-1830.2004.